Coder, Brandon D., Thymic involution perturbs negative selection and leads to chronic inflammation. Doctor of Philosophy (Biomedical Sciences), July, 2015, 152pp., 4 tables, 29 illustrations, references: 240 titles

The ubiquitous presence of chronic low-level pro-inflammatory factors in elderly individuals (termed inflammaging) is a significant risk factor for morbidity and mortality. The etiology of inflammaging is largely unknown. Recent evidence has identified the persistent activation of immune cells, thought to arise from latent viral infections, as key contributors towards the development of a chronic inflammatory environment. However, the contribution of autoreactive T cells towards the development of inflammaging has yet to be investigated. Another pervasive feature of the aging process is the age-related involution of the thymus gland, which has been linked with a predisposition toward developing autoimmunity. In the present study, we determined how age-related thymic involution leads to the persistent release and activation of autoreactive T cells capable of inducing inflammaging. We utilized a *FoxN1* conditional knock-out (*FoxN1*-cKO) mouse model that mimics thymic involution while maintaining a young periphery and naturally aged C57Bl/6 mice. We found that thymic involution leads to T cell activation shortly after thymic egress, which is accompanied by cellular infiltration into non-lymphoid tissues, elevated serum IL-6, and enhanced production of TNFα. Additionally, activated autoreactive T cell clones were detected in the periphery of *FoxN1*-cKO mice. We determined that a failure of negative selection, facilitated by decreased AIRE expression rather than impaired regulatory T cell (Treg) generation, and led to autoreactive

T cell activation in the periphery. Furthermore, we have demonstrated that the young environment can reverse the age-related accumulation of Tregs but not inflammatory infiltration. Together, these findings identify thymic involution and the persistent activation of autoreactive T cells as a source of chronic age-related inflammation (inflammaging).

THYMIC INVOLUTION PERTURBS NEGATIVE SELECTION AND LEADS TO CHRONIC INFLAMMATION

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

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Fort Worth, Texas

July 2015

ACKNOWLEDGEMENTS

I would like thank my family, friends, and fellow classmates for their support during my time as a graduate student. I would also like to thank my major professor, Dr. Dong-Ming Su, my committee, Dr. Rance Berg, Dr. Hriday Das, Dr. Harlan Jones, Dr. Mark Mummert, and Dr. Eric Gonzales for their mentorship and guidance. I would like to thank Dr. Mark Anderson (UCSF) for providing his expertise and reagents for the IRBP immunization experiments and Dr. Xiangle Sun for her expertise in flow cytometry and cell sorting. Finally, I would like to thank all of my lab mates, and especially Lili Cheng for teaching me lab techniques.

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CHAPTER I

INTRODUCTION

Aging is the foremost risk factor for the development and severity of chronic age related diseases including Rheumatoid Arthritis, cardiovascular disease, Alzheimer's Disease, Parkinson's Disease, various cancers, and overall frailty [1]. The current U.S. population of persons 65 years or older is 39.6 million and expected to increase to 72 million over the next 25 years [2]. Importantly, the most frequent causes of death in the elderly are overwhelmingly dominated by non-communicable chronic diseases [3]. There exists an immense economic burden associated with chronic age-related disease. With regards to cardiovascular disease alone, the U.S. spent an estimated $450 Billion dollars in 2010 [4]. Complicating the issue further, over 2 million elderly individuals are completely homebound [5], which is reflected in Medicare spending for the elderly accounting for 14% of the federal budget, ~ $555 billion in 2011 [4]. Medicare spending is expected to double by 2030 as the elderly population doubles. It is universally understood that chronic inflammation is a pervasive feature of aging that significantly exacerbates virtually all chronic age-related diseases. The term immunosenescence describes the multi-faceted process by which the immune system undergoes profound age-related transformations that ultimately lead to global immune dysfunction [6]. Understanding the mechanisms driving immunosenescence will uncover new avenues for adjunctive therapeutic treatments for chronic diseases. In this dissertation, I aim to elucidate how aging of the thymus gland contributes to systemic inflammation

and to reveal novel therapeutic targets for the treatment of chronic inflammation, and thereby identify potential treatments for virtually all chronic age-related diseases.

The Thymus Gland: Structure and Function

The thymus gland is a specialized bi-lobed lymphoid organ of the central immune system that is located above the heart and responsible for the differentiation of hematopoietic progenitor cells, termed thymocytes, into naïve T lymphocytes (T cells), a process known as thymopoiesis. Unlike other hematopoietic lineage cell types, T cells development occurs outside of the bone marrow in the thymus gland [7, 8]. Thymic development of naïve T cells consists of a compartmentalized multi-step process that take place in distinct niches of the thymic epithelial environment. These steps include entry of hematopoietic stem cells into the thymus at the corticomedullary junction, early thymic progenitor (ETP) migration and differentiation into $CD4^{-}CD8^{-}$ Double Negative cells (DN1-4) in the cortex, $CD4^{+}CD8^{+}$ Double Positive (DP) selection in the cortex, $CD4^{+}$ or $CD8^{+}$ Single Positive (SP) selection in the medulla, and thymic egress of SP thymocytes through the high endothelial venules located in the corticomedullary junction.

The distinct niches of the thymic epithelial environment are comprised of keratinocytes that are similar to the epithelial layers of the skin. The thymic microenvironment exists as an interconnected meshwork of epithelial cells, with medullary thymic epithelial cells (mTECs) forming the inner regions. The outer region is formed of cortical thymic epithelial cells (cTECs) surrounded by the subcapsular epithelium, and supporting cells like dendritic cells, macrophages, and fibroblasts, which migrate throughout the epithelial meshwork and provide additional developmental signals to immature thymocytes. cTECs and mTECs can be distinguished from one

another through differential expression of various keratins, epithelial adhesins, MHC and MHC processing subunits. Firstly, all TECs can be distinguished from hematopoietic cells through the absence of the hematopoietic marker CD45. Going further, cTECs can generally be defined by a few unique characteristics: the expression of keratins K8 and K18, exclusive expression of the β5t thymoproteosome subunit (for MHC-I peptide processing and CD8 development), and expression of the epithelial cell adhesion molecule (EpCAM) and the glycoprotein Ly51 [9], [10]. mTECs can largely be identified by their characteristic expression of K5, K14, the lectin ulex europaeus agglutinin lectin 1 (UEA1), and the absence of Ly51 [11]. Furthermore, mature cTEC and mTEC subsets can be distinguished from their immature counterparts by expressing high levels of MHC-II, high levels of β5t for cTECs, and high levels of the autoimmune regulator gene AIRE for mTECs [11]. Mature TECs are the subset most responsible for directing the differentiation of developing thymocytes.

Thymocytes encounter various developmental signals from the distinct epithelial niches as they migrate through the thymic epithelial environment (Figure 1.1). First, hematopoietic stem cells (HSCs) must enter into the thymus near the corticomedullary junction. Both mTEC and cTEC work together to recruit hematopoietic progenitors into the thymus via chemotaxis. mTECs chemoattract HSCs by producing CCL19 and CCL21 [12, 13], which are the ligands for CCR7 [7] expressed on HSCs. Additionally, cTECs chemoattract HSCs by producing CCL25 [14], which is the ligand for CCR9 expressed on the surface of HSCs [7]. Interestingly, HSCs that have migrated into the thymus have not yet committed to the T cell fate, but retain the potential to become B cells or even myeloid cells. Commitment of early T cell progenitors (ETPs) toward the T cell fate requires the up-regulation of the myeloid inhibitor HES1 via Notch1 signaling via the concerted effort of mTECs and cTECs at the corticomedullary junction [15-17]. ETPs entering through the

corticomedullary junction migrate through the cortex and receive differentiation signals based on their location in the distinct epithelial niches. Differentiation of ETPs is characterized by temporally varying expression of surface proteins: CD4, CD8, CD44, and CD25. Once arriving into the thymus, ETPs lack their T cell receptor (TCR), CD4 and CD8, and are termed double negative (DN). DN cells migrate through the cortex towards the subcapsular zone progressing from DN1 ($CD44^{+}CD25^{-}$), DN2 ($CD44^{+}CD25^{+}$), and once reaching the subcapsular zone they become DN3 ($CD44^{-}CD25^{+}$) [1, 18]. Rearrangement of α, β, γ and δ TCRs and commitment towards either αβ or γδ T cells occurs at the DN3-DN4 stage. Correct assembly and joining of the pre-TCRα and TCRβ or γδ /CD3 complex commits thymocytes to the αβ or γδ lineage. At the DN3 stage, the non-rearranged pre-TCRα pairs with the rearranged TCRβ [19, 20]. TCR rearrangement results from stochastic somatic DNA, rearrangement that requires expression of the recombination-activating gene (*RAG*) at two points: TCRβ rearrangement and TCRα rearrangement [21]. TCR rearrangement allows for the extremely diverse TCR repertoire capable of recognizing more than 10^{8} epitopes [22, 23]. Loss of the *RAG* gene prevents assembly of the TCR and results in severe combined immune deficiency characterized by the absence of both T and B cells [24]. During the late DN3 and the DN4 stage rearrangement of the TCRα occurs and the pre-TCRα is lost. Next, thymocytes begin to express low levels of CD8 followed by low levels of CD4, and then they enter into the $CD4^{+}CD8^{+}$ double positive (DP) stage [25].

During the transition from the DP stage in the cortex to the SP stage in the medulla , thymocyte selection events unfold that discard the unwanted progeny of a stochastically generated TCR by removing thymocytes that are useless(death by neglect), keeping thymocytes that are useful (positive selection), and deleting thymocytes that are potentially harmful (negative selection). These selection processes involve TCR interactions with self-peptide loaded onto MHC-I and self-

peptide onto loaded MHC-II. 90% of DP thymocytes possess TCRs unable to recognize self MHC expressed by cTECs making them functionally useless. These thymocytes are unable to receive the intracellular survival signal that results from TCR: self-peptide-MHC binding and they undergo death by neglect [26], [25]. Positive selection results in the survival of thymocytes with TCRs that possess a high enough affinity and strong enough avidity to interact with self-peptide loaded onto MHC, which is followed by commitment toward just one single positive (SP) lineage: CD4 or CD8 [26]. After commitment to the CD4 or CD8 lineage, SP thymocytes migrate into the medulla where they once again interact with self-peptide-MHC complexes expressed by both mTECs and medullary dendritic cells [27, 28]. Thymocyte TCRs that react too strongly to self-peptide-MHC are potentially specific for self-peptides and are given signals to undergo apoptosis through the process known as negative selection, discussed further in section 1.3. Following positive and negative selection, the remaining surviving thymocytes migrate back towards the corticomedullary junction and exit into the bloodstream through high endothelial venules, where they will eventually settle into secondary lymphoid tissues including the spleen and lymph nodes [1].

Development of the Thymus Gland: The Role of *FoxN1*

The thymic epithelial environment supports the immensely intricate development of thymocytes through the compartmentalized and joint efforts of the morphologically and functionally distinct mTEC and cTEC subsets. The development of the thymus gland and the differentiation of the TEC subsets is a complicated multi-genic process, but the actions of one gene

are of the utmost importance. The master transcription factor, *FoxN1* (forkhead box N1), controls the differentiation of keratinocytes, including cTECs and mTECs, during proliferative conditions [29, 30]. *FoxN1* located on chromosome 11 in rodents and *FOXN1* located on chromosome 17 in humans are highly conserved in sequence and function, and predominately expressed in thymic epithelium, specific keratinocyte populations in the epidermis, and hair follicles [31-35]. Thymic organogenesis occurs in two genetic stages: *FoxN1*-independent outgrowth and *FoxN1*-dependent maturation (Figure 1.2). The first stage involves the *FoxN1*-independent budding of the thymic anlage from the third pharyngeal pouch, controlled by various genes including *Eya1* and *Six* [36], *Hoxa3* [37], and *Tbx1* [38, 39]. The second step involves the *FoxN1*-dependent differentiation of immature epithelial cells into cortical thymic epithelial cells (cTECs) and medullary thymic epithelial cells (mTECs) [40]. However, exactly how *FoxN1* regulates the differentiation of mTEC and cTEC is largely unknown. Much of what is known about prenatal *FoxN1* driven thymic development comes from the inborn null mutation in *FoxN1* (also known as the "nude" mutation). The nude mutation results in TEC differentiation failure, which suspends thymic development at the rudimentary stage [41]. The nude thymus rudiment is still present, however thymopoiesis is completely blocked, [42, 43] thereby causing an alymphoid thymus and severe primary T cell immunodeficiency in both nude mice and humans [34, 44, 45]. Using the K14Cre driven conditional knockout of *FoxN1* mouse model (*FoxN1*-K14Cre), it was determined that *FoxN1* is required for the morphogenesis and maintenance of the three-dimensional structure of the thymic medulla [46]. A Two-dimensional monolayer of stromal cells cannot support T cell development [47], because a three-dimensional architecture is required to maintain the delta-like ligand expression that is necessary for thymopoiesis [48].

The importance of *FoxN1* in TEC development is not necessarily equal in the prenatal and postnatal life. In fact, the postnatal role of *FoxN1* may be more significant. Using *FoxN1*-K14Cre mice, Guo et al. demonstrated that homozygous *FoxN1*$^{flox/flox}$ mice lacking the *Cre* gene are still able to display *FoxN1*^{flox} deletion when their mother expresses the *Cre* gene [46]. This deletion comes from a historic Cre-mediated *FoxN1*^{flox} deletion and only happens in their prenatal life while inside the mother's uterus. Interestingly, these mice have a completely normal thymus and skin phenotype, suggesting prenatal expression of *FoxN1* is not critical. However, homozygous *FoxN1*$^{flox/flox}$ mice carrying their own *Cre* gene have a *FoxN1*^{flox} deletion that results in the loss of thymic three-dimensional architecture, stalls TEC maturation, and reduces overall thymopoiesis [46]. In this system, *FoxN1*^{flox} deletion occurs in both the prenatal and postnatal life. This finding demonstrated that *FoxN1* mutant phenotypes associated with the nude mutation are associated with postnatal defects that emerge because of the absence of *FoxN1*.

TEC subsets may not be equally dependent on *FoxN1* in the postnatal thymus. TECs can be divided into two distinct regions: the cortex and the medulla. In the fetal stage, cTECs and mTECs arise form a bi-potential TEC progenitor, and their differentiation is equally dependent on *FoxN1* [43] [49, 50]. However, in the postnatal thymus mTECs, identified by keratin type K5 and K14, which are similar to epithelial stem cell markers with high progenitor activity [51-53], are extremely sensitive to the loss of *FoxN1* [54]. On the other hand, cTECs expressing K8 and K18, which are similar to terminally differentiated epithelial cells in the apical layer of the stratified squamous epithelium of the skin [51-53], are more resistant to the loss of *FoxN1* [54]. These findings suggest that *FoxN1* may not be required for K8/K18 cTEC maturation, in the adult thymus, however *FoxN1* is required for K5/K14 mTEC maturation. Interestingly, recent reports demonstrated that thymopoiesis is dependent on *FoxN1*-positive TECs [55]. Although *FoxN1*-

negative TECs descend from *FoxN1*-positive TECs, they do not contribute to thymopoiesis in the adult thymus [55]. Thus, *FoxN1* is paramount to the proper development of thymocytes, and studying *FoxN1* mutations may provide key insight into defects related to thymopoeisis.

Thymus Involvement in Immune Tolerance

In addition to fostering the development and maturation of thymocytes toward mature T lymphocytes, the thymus is also tasked with preventing autoimmune disease by generating tolerance toward self-tissue. The process of T cell quality assurance is predicated on two phases of immune tolerance: Negative Selection and the Generation of Natural Regulatory T cells.

Negative Selection

Developing thymocytes undergo V(D)J recombination, where the random and improper joining of variable, diverse, and joining gene segments generates junctional diversity and leads to an incredibly diverse T cell Receptor (TCR) repertoire capable of recognizing a myriad of antigens, ranging from bacteria and virus to tumor cells [26, 56]. The thymic epithelium ensures a functional TCR repertoire that is self-MHC restricted, but tolerant to self-antigen [1]. Self-MHC restriction occurs at the DP stage and tolerance is selected at the SP stage [1, 57, 58]. While cTECs control self-MHC restriction and thymocyte survival through positive selection, mTECs are the predominate epithelium for promoting tolerance by eliminating self-reactive TCRs through the process of negative selection [1, 59]. The fate of developing thymocytes is primarily contingent

upon one key function: the binding of the TCR to the self-peptide-MHC complex. Whether thymocytes are given the signals to survive (positive selection) or the signals for death (negative selection) rests on how strongly the TCR binds to the self-peptide-MHC complex. Although the mechanisms driving thymocyte selection have not been fully understood, the differential avidity model best describes the delicate balance between positive and negative selection. When the avidity of the TCR-MHC interaction is low, there is only partial phosphorylation of the TCR-associated protein linker for activation of T cells (LAT), resulting in low-level extracellular signal-related kinase (ERK) activation, which ultimately results in survival and positive selection [1]. However, when the avidity of the TCR-MHC interaction is high, downstream signaling cascade member LAT [60] is fully phosphorylated, which recruits the TCR associated adaptor protein complex Grb2/SOS1, which lead to transient ERK activation alongside JNK and p38 activation, and ultimately the thymocyte is induced to undergo clonal deletion through apoptosis [61, 62]. The activation of JNK and p38 MAP kinases lead to the activation of the Bcl-2 apoptosis pathway mediated by the pro-apoptotic protein BIM and the caspase 9/ apoptosis protease activation factor-1 (APAF-1) pathway of cell death [1, 63, 64].

The fate of thymocytes during selection depends on the strength of binding, or avidity. The strength of the avidity is predicated on the affinity of the ligands being presented to the developing thymocytes. Low-affinity TCR interaction results in the activation of the guanine nucleotide exchange factors Ras via RasGRP and produces low level ERK activation, resulting in positive selection and survival [1, 65]. High-affinity interactions result in strong Grb2/SOS1-mediated ERK activation, ultimately leading to JNK and p38 activation and clonal deletion [1]. Low-affinity ligands are required for thymocyte survival and positive selection, but interestingly, thymocytes

lose their sensitivity to these low-affinity ligands [66, 67]. However, thymocytes that survive high-affinity interactions retain their sensitivity for these high-affinity ligands [66, 68].

What are these low and high-affinity ligands, and where do they originate? Low-affinity ligands are presented by cTECs, and their unique low-affinity properties result from the unique antigen processing machinery of the cTECs. In the case of MHC-I presentation, cTECs express a unique subunit of the proteasome known as β5t, and proteasomes composed of the β5t subunit are referred to as thymoproteasomes [69, 70]. In terms of MHC-II presentation, cTECs express the unique lysosomal proteases cathepsin L, and thymus-specific serine protease (TSSP) [71, 72]. These unique antigen processing machinery yield low-affinity ligands that induce weak binding reactions. The β5t thymoproteosome inefficiently cleaves substrates adjacent to hydrophobic amino acids. This inadequately cleaved peptide results in "wobbly" binding to the TCR, which leads to weak signaling events, thereby promoting positive selection [73, 74].

On the other hand, high-affinity ligands drive negative selection and induce T cell tolerance. These ligands, encoded by genes that are expressed by only one tissue-specific cell lineage, are termed tissue-specific antigens (TSAs) [73]. Thus, TSAs that would normally only be found in specific peripheral tissues, are presented to developing thymocytes in order to induce tolerance via negative selection. TSAs exist in the thymus because of the unique gene expression system of mTECs known as promiscuous gene expression. Promiscuous gene expression describes the ectopic expression of TSAs in the thymus by mTECs [73]. Promiscuously expressed TSAs are not governed by the same biological rules enforced in most cases of protein expression. Instead, TSAs bypass tissue specificity, developmental switches, and sex specificity [73]. The distinct rule-set governing TSA expression is possible because the unique protein expressed in mature mTECs, known as the autoimmune regulator (AIRE), regulates promiscuous gene expression [75].

However, AIRE does not function like a classical transcription factor. Instead, AIRE forms a complex with activating transcription factor 7 interacting protein (ATF7ip) and methyl CpG binding protein 1 (MBD1), and is directed to silent TSA genes [76]. AIRE then turns on the silent TSA genes by preferentially binding to non-methylated histone 3 lysine 4 (H3K4), which is associated with gene repression, and allows for the binding of multi-protein complexes that facilitate transcription by generating double-strand breaks and relieving stalled RNA polymerase [77-80]. Fascinatingly, although mTECs express the transcripts for all TSAs, any given TSA is only expressed by 1-3% of mTECs at any given time [81]. TSAs are expressed in a somewhat stochastic nature, with fluctuating gene expression forming a mosaic of linked TSA lineage groupings. These co-expression groups range from approximately 100 to 300 TSA groupings [82, 83]. Nevertheless, developing thymocytes are tolerized to self-tissue.

Importantly, mTECs are not solely responsible for the presentation of TSAs to developing thymocytes to induce negative selection. Thymic dendritic cells (tDCs) contribute approximately 0.5% of total thymic cellularity and are professional antigen-presenting cells tasked with presenting TSAs to developing thymocytes [73]. tDCs are decidedly more abundant in the medulla than in the cortex, signifying their importance in negative selection. tDCs migrate into the medulla through the chemokine XC-chemokine ligand 1 (XCL1) [84], which is exclusively produced by mTECs in an AIRE-dependent fashion [73, 84]. Although tDCs induce negative selection by presenting TSAs on MHC-I and MHC-II to developing thymocytes, tDCs are not able to generate TSAs themselves. Instead, tDCs acquire TSAs through the unidirectional AIRE-dependent transfer of antigen from mTECs (Figure 1.3) [85, 86]. Even though both mTEC and tDC can induce tolerance via presentation of TSAs to developing thymocytes, mTECs are the driving cell type of negative selection. The pivotal role of mTECs in tolerance induction has been demonstrated by

experiments that induce the ablation of mTECs. The loss of mTECs precludes any capabilities of tDCs to induce negative selection [85].

AIRE does not account for all promiscuous gene expression of ectopic TSAs [87]. AIRE-dependent and AIRE-independent mechanisms for gene expression result in differing and non-redundant TSA expression [88]. Interestingly, AIRE-independent TSAs are enriched in ovary and testis specific genes [88], implying that AIRE-independent mechanisms likely govern ectopic expression of germ cells, whereas AIRE-dependent mechanisms govern ectopic somatic cell expression in the thymus. However, the molecular mechanisms controlling AIRE-independent gene expression are elusive, as AIRE is the only molecular regulator of promiscuous gene expression that has been identified [73].

Even though AIRE cannot account for the presentation of all TSAs in the thymus, the importance of AIRE in maintaining immune tolerance cannot be understated. In fact, AIRE was first identified through the condition autoimmune polyglandular syndrome type 1 (APS1), also known as autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED), which is a monogenic autoimmune disease that presents with multi-organ endocrinopathy, chronic mucocutaneous candidiasis, and ectodermal dystrophies [89, 90]. Through sequence analysis, researchers were able to detect mutations of one particular gene located in the nuclear speckles of APS1 patients responsible for the widespread autoimmunity, hence the namesake Autoimmune Regulator or AIRE [75, 91]. Furthermore, AIRE-deficient mice strains display APS1-like disease with multi-organ autoimmunity, lymphoid infiltration into non-lymphoid tissues, and serum autoantibodies [92, 93]. Strikingly, the absence of even one single AIRE-dependent TSA will lead to autoimmunity in the organ that naturally expresses the antigen. Investigation into retinal autoimmunity in C57Bl/6 mice identified interphotoreceptor retinoid-binding protein (IRBP) as

the targeted antigen in AIRE-deficiency induced eye inflammation or uveitis [94]. IRBP is expressed within the thymus in an AIRE-dependent fashion, and targeted deletion of IRBP from the thymus leads to IRBP-targeted uveitis [94]. Therefore, individual AIRE-dependent TSAs are pivotal in generating immune tolerance towards self-tissue [95]. It turns out that AIRE-deficiency induced autoimmunity is highly strain dependent [96], similar to the heterogeneity observed between individuals with APS1 [89]. On the C57Bl/6 background, AIRE-deficiency leads primarily to autoimmunity against the salivary gland and the retina, and there is an age-dependent increase in inflammatory infiltration [93]. The role of AIRE in the development of age-related autoimmunity requires further investigation. The dysregulation of AIRE-dependent salivary antigens in the thymus [93] may play a role in the development of Sjögren's syndrome, an age-related autoimmune disease characterized by serum autoantibodies and lymphoinfiltration into the salivary and lacrimal glands [97]. Interestingly, recent whole exome sequencing has correlated the development of Sjögren's syndrome with genetic mutations associated with AIRE [98]. While the mechanisms of AIRE in thymic derived tolerance induction have been well characterized, the mechanisms leading to defective AIRE and AIRE-controlled autoimmunity are not well understood. Further understanding of the regulation of AIRE could lead to widespread treatments for multi-organ autoimmunity. Additionally, AIRE's role in tolerance induction is not limited to negative selection, but extends into peripheral tolerance. The discovery of extrathymic AIRE-expressing cells (eTACs) [99] and the role of AIRE in the generation of suppressive regulatory T cells reveal AIRE as a dominant regulator of immune tolerance.

Regulatory T cells (Tregs)

Not all thymocytes receiving a strong TCR signal will undergo clonal deletion. Thymocytes receiving an intermediate/strong TCR signal in conjunction with the appropriate cytokine environment (IL-2) may differentiate into a specialized subset of immunomodulatory T cells known as regulatory T cells (Tregs) (Fig. 1.5) [100]. Tregs are immunosuppressive $CD4^+$ T helper cells that express the master transcription factor forkhead box P3 (FoxP3) and high levels of surface IL-2 receptor, denoted by the IL-2R α-chain (CD25) [101]. Tregs are highly immunosuppressive, and were first discovered through experiments that identified a subset of T cells that could prevent the development of autoimmunity in thymectomized mice [102]. Furthermore, depletion of Tregs in wild type animals or transfer of bulk T cells, minus Tregs, into immunodeficient scid mice results in widespread autoimmunity including colitis, wasting disease, ulcerations, and mononuclear infiltration into the lungs, liver, thyroid, stomach, colon, and pancreas [103] [104, 105] [106]. Although there is a small subset of peripherally induced Tregs (iTregs) that stem from transforming growth factor beta (TGFβ) receptor signaling in naïve $CD4^+$ T cells, it is now understood that these iTregs likely arise from and regulate responses to the foreign antigens of the gastrointestinal associated lymphoid tissue, e.g., food, commensal microbiota, and allergens [100, 107, 108]. The vast majority Tregs found in the periphery originate from the thymus and are termed natural regulatory T cells (nTregs) [100, 109, 110]. Furthermore, the TCR repertoires and ligand specificities between iTregs and nTregs are distinct [111]. As nTregs develop from a divergent pathway during negative selection, they are the Treg subset responsible for maintaining tolerance against self-tissues.

During the developmental process of thymocyte negative selection, differential TCR signaling strength determines the fate of immature thymocyte; low binding strength for survival or high binding strength for clonal deletion. However, there is a third fate for developing thymocytes that receive strong TCR signaling in the form of a divergent pathway towards nTreg differentiation. Evidence supporting the divergent pathway following strong TCR signaling emerged from studies that showed the simultaneous generation of Tregs alongside autoreactive clonal deletion when mice expressing the neo-self antigen OVA were crossed with TCR transgenic mice engineered to recognize OVA [112, 113]. However, when effector T cells ($Foxp3^-$) are transfected with Treg TCRs, they can only mount a weak response to syngeneic APCs compared to the robust response against foreign ligands [114]. These data suggest that effector T cell response to foreign antigen occur at a much higher binding strength than the range of avidity for Treg TCRs for self-peptide-MHC complexes [100]. Furthermore, when negative selection is partially impaired due to reduced MHC-II expression in mTECs, nTreg frequency is increased [59]. Moreover, autoreactive T cells found in Foxp3-deficient mice, which lack nTregs, express TCRs that are found in the TCR repertoire of Tregs from Foxp3-sufficient mice, suggesting autoreactive T cells and nTregs share overlapping self-reactive TCRs [115]. Additionally, it has been demonstrated that the same exact high affinity self-peptide will lead to both negative selection and nTreg differentiation, suggesting nTregs emerge from thymocytes escaping clonal deletion [116]. The culmination of these data suggests that nTregs likely exhibit intermediate TCR-MHC binding strength, which allows for their escape from negative selection [100]. In addition to variances in TCR signal strength, the cytokine environment plays a pivotal role in determining negative selection versus nTreg differentiation. The cytokine signals essential for nTreg differentiation include IL-2 at the forefront, and IL-7 and IL-15 serving supplemental roles [117,

118]. Although the precise mechanisms of cytokines in nTreg differentiation are unclear, a two-step model for nTreg development has been proposed that suggests intermediate/high TCR-MHC upregulates CD25, and stimulation by IL-2, IL-7, and IL-15 induce expression of Foxp3 and nTreg lineage commitment (Figure 1.4) [119]. Additionally, TGF-β has been shown to be required in nTreg development [120], either by inducing activation of Foxp3 [121] or promoting survival by inhibiting Bim-dependent apoptosis [120]. With the process of nTreg development so closely linked to negative selection, a role for AIRE is highly anticipated. However, there is evidence to support both AIRE-dependent [122, 123] and AIRE-independent [124] nTreg development, yet how AIRE dysfunction effects the generation of nTregs and their suppressive function is not fully understood.

The breadth of nTreg suppressive mechanisms are as immense as the array of cell types nTregs are capable of regulating (Figure 1.5). Additionally, nTregs are capable of both non-specific and targeted suppression, as well as immunomodulation via direct cell-cell contact or through the release of secreted factors. The classical method of nTreg suppression of an immune response is a non-specific inhibitory mechanism that involves the original Treg cell marker, CD25. The constitutively high-level of IL-2R expression on nTregs deprives effector T cells of the available IL-2 and inhibits their proliferation [125]. *In Vitro* studies have shown that nTregs are much less effective at suppressing T effector responses when separated by a semi-permeable membrane, suggesting a role for cell contact mediated suppression [126]. Following direct contact, nTregs can induce cyclic adenosine monophosphate (cAMP) in effector T cells, leading to the inhibition of IL-2 synthesis and proliferation [127]. Additionally, after initiating targeted direct cell contact, nTregs are capable of releasing granzyme and perforin, resulting in the induction of apoptosis and eventual cytolysis of target effector T cells or antigen presenting B cells [128, 129].

Furthermore, nTregs are capable of down-regulating CD80 and CD86 co-receptor expression on APCs by binding these co-receptors with cytotoxic T lymphocyte antigen and inducing trans-endocytosis, which ultimate blocks CD28 co-stimulation [130]. The more long distance methods of Treg suppression include the secretion of anti-inflammatory factors like TGF-β, IL-10, IL-35, and IL-9 [131]. Recently, nTregs have even been shown to inhibit effector T cell responses by secreting exosomes containing inhibitory microRNA [132]. Additionally, nTreg control of the immune response can become extremely specialized. In addition to Foxp3, Tregs can co-express the transcription factors associated with distinct effector T cell subsets (*T-bet*:Th1, IRF4*/Gata3*:Th2, STAT3/*RORγt*:Th17), which allows the nTreg to migrate, proliferate, and specifically inhibit the effector T cell subset at the site of the response [133, 134], [135, 136].

Peculiarly, there is an age-related accumulation of Tregs, in both mouse and human, which mirrors the observed increase in autoimmunity and inflammation in the elderly [137, 138]. Because nTreg generation and negative selection are tightly joined, it is plausible that the age-related increase in both nTregs and autoimmunity arise from a common link: the thymus. However, there are conflicting reports regarding the accumulation of nTregs in the periphery. Some studies have demonstrated that Tregs accumulate due to increased survival in the periphery, which has been attributed to decreased levels of the pro-apoptotic gene *Bcl2* family member *Bim* [139-141]. Conversely, others have shown that defective negative selection, brought about by diminished MHC-II expression in mTECs, increases the frequency of nTregs [59]. Furthermore, whether aging affects the suppressive functionality of Tregs is unclear. While many labs have shown that aged Tregs maintain their suppressive function [137, 142-144], others have reported a functional decline from Tregs emigrated from an aged thymus [145, 146]. Therefore, in order to understand the role aged Tregs have on the emergence of autoimmunity and inflammation in the elderly, it is

imperative to investigate the mechanisms governing age-related Treg accumulation and whether advanced age alters Treg suppressive function.

Age-related Thymic Involution

The thymus gland, in all of its complexity, serves its purpose and establishes an adaptive immune system capable of staving off foreign invaders, while being selective enough to distinguish between self and non-self. However, the fully functional and healthy thymus gland is short lived. The thymus, naturally and inevitably, progresses through age-dependent atrophy through a process known as thymic involution. Thymic involution begins at adolescence, and shrinks in volume by ~ 3% per year through middle age [147]. By age 50, humans will have approximately 15% of their original thymus remaining [147], [148]. The thymus will then proceed to shrink by ~1% per year through the rest of life, with complete loss of all thymopoietic tissue estimated to occur approximately at age 105 [149]. Thymic involution is characterized by a severe decrease in cTECs and mTECs, an increase in the proportion of fibroblasts and cyst formation [150], and a very strong accumulation of adipose tissue [151]. Additionally, the thymic stromal environment goes through various phenotypic changes, including a decrease in keratin and cortical and medullary markers, and a decrease in MHC-II [152]. Nevertheless, thymic involution is an evolutionary conserved process, and occurs in almost all vertebrates [153]. Despite being evolutionarily conserved, thymic involution is considered to be detrimental to immune function [154]. Thymic involution is associated with diminished T cell output and thus decreased TCR repertoire diversity, increased susceptibility to emerging pathogens, decreased vaccine efficacy, and increased susceptibility to autoimmune diseases including: Sjögren's Syndrome, Rheumatoid

Arthritis, Giant-cell Arteritis, Myasthenia Gravis, and Multiple Sclerosis [155-157]. The mechanisms by which thymic involution contributes to these immune dysfunctions and diseases are not fully understood nor agreed upon. Decreased thymic output likely reduces the TCR repertoire, which in turn decreases the T cells capable of recognizing new foreign antigens, and skews the T cell pool towards autoreactivity [158]. Even though thymopoiesis is diminished in the involuted thymus, T cell output does not cease, and newly generated T cells can be observed in the elderly [159]. Therefore, involution-associated changes, e.g. decreased MHC-II expression [152], which may affect the generation of tolerance, are likely to result in increased autoimmunity in the elderly.

The mechanisms driving age-related thymic involution are not fully understood. Potential explanations for why the thymus involutes have included the lack of TCR rearrangements [160], the loss of selecting peptides [161], diminished hematopoietic stem cell progenitors [162], loss of thymic cytokines (IL-2, IL-9, IL-7) [155], sex hormones [163], and adipose tissue associated inflammation [164], and inflammasome activation [165]. Treating many of these aforementioned potential causes have shown promise in reducing thymic involution, e.g. castration or caloric restriction [166] [163]. However, there is a lack of evidence to support a fat deposition or sex hormones as initiators of thymic involution [167]. However, there is strong evidence to support the loss of *FoxN1* as one of the initial driving forces behind age-related thymic involution. *FoxN1* is not expressed in all adult TECs, however all *FoxN1*-negative TECs are derived from young *FoxN1*-positive TECs [55]. This conversion of young *FoxN1*-positive TECs into *FoxN1*-negative adult TECs implies that there is an age-related decline in *FoxN1*. In fact, *FoxN1* is progressively lost with age, with a 37-fold reduction in *FoxN1* observed by 18 months in C56Bl/7 mice [168]. On its own, the loss of *FoxN1* is sufficient to cause severe thymic atrophy, mTEC patterning

disruption, loss of MHC-II, decreased thymopoiesis, and a deficient cytokine environment [145, 169]. The loss of *FoxN1* leads to the dysregulation of TEC homeostasis. The TEC environment is maintained by a p63-*FoxN1* regulatory axis, where the ΔNp63 isoform leads to TEC proliferation and the TAp63 isoform drives senescence [170]. The loss of *FoxN1* directly leads to an increase in TAp63, which then leads to the senescence and eventual apoptosis of TECs, ultimately driving involution [170]. However, the biological effects of the loss of *FoxN1*-dependent thymic involution on the development of T cells including: tolerance induction via negative selection and the generation of Tregs, TCR repertoire diversity, and the susceptibility to autoimmunity are largely unknown.

Inflammaging

Chronic inflammation is a ubiquitous feature of the aging process and implicated in virtually every age-related disease [171, 172]. The term "inflammaging" describes the low-level, chronic, and systemic pro-inflammatory state that accompanies advanced age in the absence of infection [171]. Even though clinical manifestations are not obvious, the presence of pro-inflammatory factors such as interleukin-6 (IL-6), tumor necrosis factor alpha (TNFα), interleukin-1 (IL-1), and C-reactive protein (CRP) are associated with the severity, incidence, and mortality of cardio vascular diseases such as atherosclerosis and myocardial infarction [173], neurodegenerative diseases such as Parkinson's Disease [174] and Alzheimer's Disease [171], and late-life cancers including colitis-associated colon cancer and hepatocellular carcinoma [175]. IL-6 and TNFα are the most predictive inflammatory biomarkers and are highly correlated with "all-cause" morbidity and mortality in the elderly [176]. The mechanisms by which pro-inflammatory

cytokines exacerbate disease pathology are varied, and generally unique to the diseases being affected. Soluble TNF has been implicated in Parkinson's Disease pathogenesis and was shown to induce ceramide accumulation in dopaminergic neurons leading to endoplasmic reticulum stress, loss of mitochondrial membrane potential, and caspase-3 activation [174]. These effects could be attenuated with inhibitors of TNF [174]. Additionally, IL-6 primarily exacerbates colitis-associated colon cancer by promoting the differentiation of colitogenic T cells [177] and neutrophil recruitment [178], while suppressing Tregs [179].

The etiology of inflammaging is not fully understood, and most likely is the result of a wide variety of age-related processes. Nevertheless, inflammaging has been primarily attributed to the combination of two pro-inflammatory mechanisms: cellular senescence induced senescence-associated secretory phenotype (SASP) and the persistent activation of immune cells [171, 172, 180]. The molecular mechanisms leading to the activation of the SASP signaling pathway are not clear, but it has been proposed that a three-way signaling pathway involving the activation of the DNA damage response (DDR), p38 mitogen-activated protein kinase (p38 MAPK), and mechanistic target of rapamycin (mTOR) trigger nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) and CCAAT/enhancer binding protein (C/EBP) and induce pro-inflammatory cytokine transcription [180-182]. Importantly, activating NF-kB signaling is a major inducer in triggering IL-6 production via SASP in senescent cells [183, 184].

The persistent activation of immune cells is hypothesized to result from two main pathways: chronic cell death and self-debris serving as damage-associated molecular patterns (DAMPs) leading to innate immune cell activation [171, 185] or repeated life-long exposure to latent infections like CMV [186, 187]. The inflammasome has been implicated in both pathways because they can sense both cellular damage and pathogens through DAMPS and pathogen

associated molecular patterns (PAMPS) to induce inflammatory signals [188]. The NLRP3 inflammasome is the best known inflammasome complex, and it has been shown to induce IL-1 family member cytokines in an age-dependent manner [189], and exacerbate Alzheimer's Disease [190] and colitis [191]. However, the inflammasome is only a potent activator of IL-1 family member cytokines, and is therefore not the likely cause of the more damaging elevation of IL-6 and TNFα that is associated with inflammaging. Still, other means of chronic immune cell activation remain largely unknown. Even though thymic involution is a process that leads to immune dysregulation and is tightly linked with aging, investigation into the involvement of thymic involution in the emergence of inflammaging has largely been ignored. Because inflammaging is a systemic aging condition, the combination of many age-dependent defects are likely involved. The identification of further causal sources will lead to additional therapeutic targets for the treatment of chronic inflammation.

Significance

The purpose of this study is to improve scientific knowledge in the fields of thymus development and immune system aging by demonstrating a novel mechanism by which thymus involution contributes to the development of inflammaging. Both thymic involution and the emergence of chronic inflammation are ubiquitous features of the aging process. However, a link between thymic involution associated immune dysfunction and an increase in systemic inflammation has yet to be established. Thymic involution begins at adolescence and progresses to the point where the thymus gland is virtually nonexistent in the elderly, through a process involving the progressive loss of *FoxN1*. This decrease in thymic function is associated with increased susceptibility to T cell mediated autoimmune disorders including: Sjögren's Syndrome,

Rheumatoid Arthritis, Multiple Sclerosis, and Giant Cell Arteritis [157, 192]. Meanwhile, inflammaging is associated with the chronic activation of immune cells leading to the persistent production of pro-inflammatory cytokines. However, it is not known if thymic involution can lead to the persistent activation of T cells that are capable of inducing inflammaging. Even though thymic involution has been correlated with increased susceptibility to autoimmunity, the mechanisms driving thymic involution-associated autoimmune disease are not well defined. Importantly, understanding how the age-related loss of *FoxN1* may impact the functional ability of the thymus to generate immune tolerance, through negative selection and the generation of nTregs, may lead to novel therapeutic treatments. This could become especially important as it may offer a single genetic target capable of treating inflammaging, a major risk factor associated with poor prognosis in nearly all age-related diseases [157, 171, 172]. Immune aging is exceedingly complex and involves the total body aging of many different systems. Of particular importance, our lab has generated a *FoxN1* conditional knockout mouse model that allows us to study the effects of thymic involution without all of the confounding variables associated with total body aging. The current proposal utilizes a loxp-*FoxN1*-uCreER^{T} conditional knockout (*FoxN1*-cKO), that induces accelerated thymic involution of a fully matured thymus while maintaining a young periphery [54], and naturally aged C57BL/6 mouse models (Figure 1.6). The *FoxN1*-cKO mouse model allows for the inducible deletion of *FoxN1* after the thymus has fully matured, either by administering tamoxifen or the slow-leakage of uCreER^{T}, resulting in accelerated epithelial driven thymic atrophy that is comparable with thymic epithelium dysfunction observed in naturally aged C57BL/6 mice [54, 169]. Although the slow leakage of uCreER^{T} results in weak deletion of genomic *FoxN1* at ~1 month of age [169], observable biological effects including the loss of *FoxN1* expression, thymic involution, mTEC disruption,

and thymic dysfunction do not become apparent until ~3-9 months of age [169] or until induced with the administration of tamoxifen [54]. In the current study, I initiated studies to identify the role of *FoxN1* in the generation of immune tolerance towards self-tissues and the role of loss of *FoxN1*-induced thymic involution in the development of age-related inflammation (inflammaging).

Hypothesis

Here, I hypothesized that the progressive age-related loss of *FoxN1* and subsequent thymic involution perturbs immune tolerance leading to the activation of autoreactive T cells which help to generate a chronic low-grade pro-inflammatory state.

The hypothesis was evaluated through three specific aims.

Specific Aim 1

To determine if the progressive loss of *FoxN1* and subsequent thymic involution impairs negative selection

Specific Aim 2

To determine if thymic involution affects the generation of natural regulatory T cells

Specific Aim 3

To determine if the progressive loss of *FoxN1* and subsequent thymic involution directly leads to inflammaging

Figure 1. 1: Thymocyte Development. Once arriving into the thymus, ETPs lack a T cell receptor (TCR), CD4, and CD8 and are termed double negative (DN). DN cells migrate through the cortex towards the subcapsular zone progressing from DN1 ($CD44^+CD25^-$), DN2 ($CD44^+CD25^+$), and once reaching the subcapsular zone DN3 ($CD44^-CD25^+$). *RAG* mediated rearrangement of the β TCR occurs at the DN3 stage, followed by *RAG* mediated TCRα rearrangement at the DN4 stage. $CD4^+CD8^+$ Double Positive (DP) selection in the cortex, $CD4^+$ or $CD8^+$ Single Positive (SP) selection in the medulla, and thymic egress of SP thymocytes through the high endothelial venules located in the corticomedullary junction. Adapted from Starr, *Annu Rev Immunol*, 2013 [1].

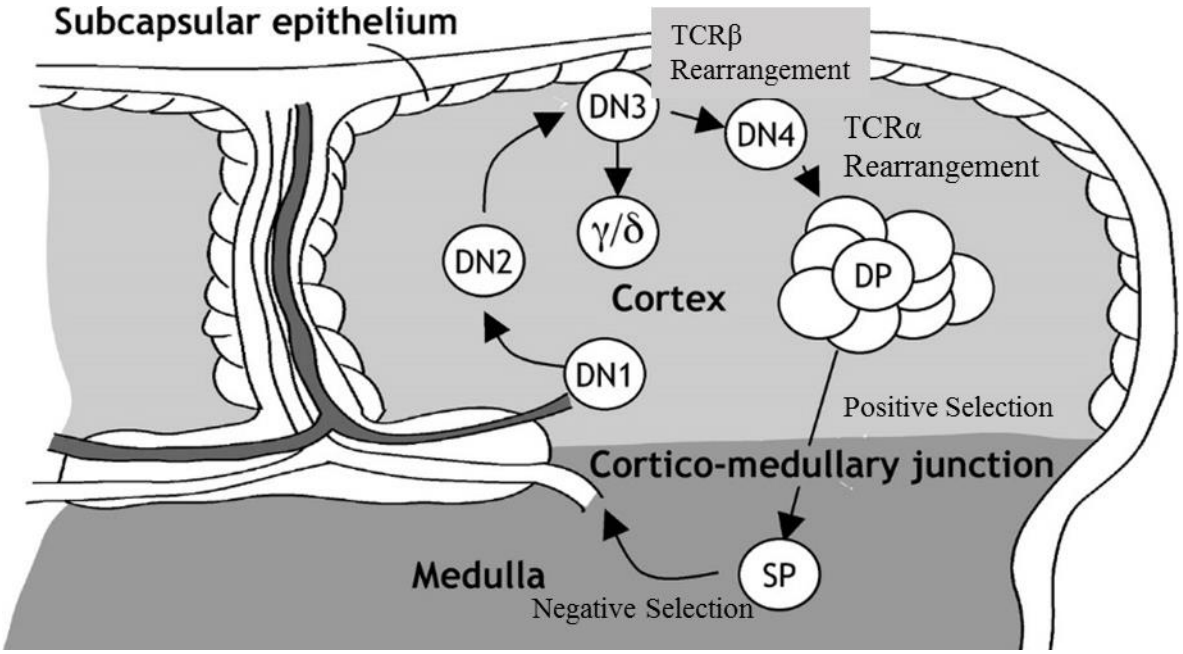


Figure 1. 2: *FoxN1*-dependent differentiation of TECs. Thymic organogenesis occurs in two genetic stages: *FoxN1*-independent outgrowth and *FoxN1*-dependent maturation. The first stage involves the *FoxN1*-independent budding of the thymic anlage from the third pharyngeal pouch, controlled by various genes including *Eya1* and *Six*, Dll4, Hoxa3 and *Tbx1*. The second step involves the *FoxN1*-dependent differentiation of immature epithelial cells into cortical thymic epithelial cells (cTECs) and medullary thymic epithelial cells (mTECs) that are capable of supporting thymocyte maturation. Adapted from Romano, *Front Immunol,* 2013 [193].

|*FoxN1*-independent Budding| |*FoxN1*-dependent TEC Differentiation| | TEC-Thymocyte Environment|

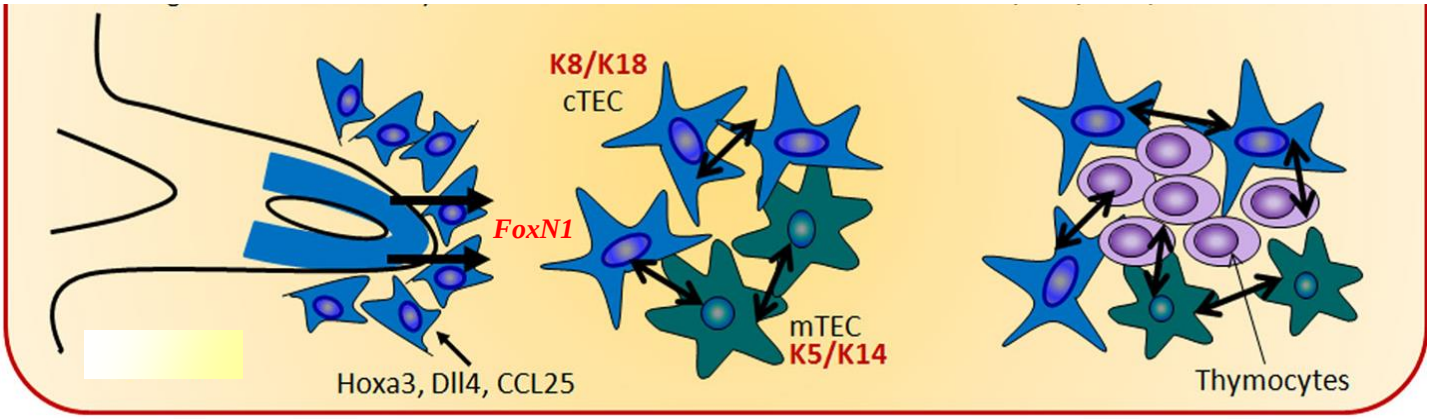


Figure 1. 3: AIRE-dependent presentation of TSA to developing thymocytes. Left panel shows the direct presentation of AIRE-dependent tissue-specific antigens (TSAs) to developing thymocytes. AIRE allows for the promiscuous gene expression of TSAs by mTECs which will be presented to developing thymocytes via MHC-I and MHC-II. Right panel shows the AIRE-dependent transfer of TSAs to thymic dendritic cells. tDCs are then able to present TSAs to developing thymocytes to facilitate negative selection. Adapted from Klein, *Nature Rev Immunol,* 2014 [73].

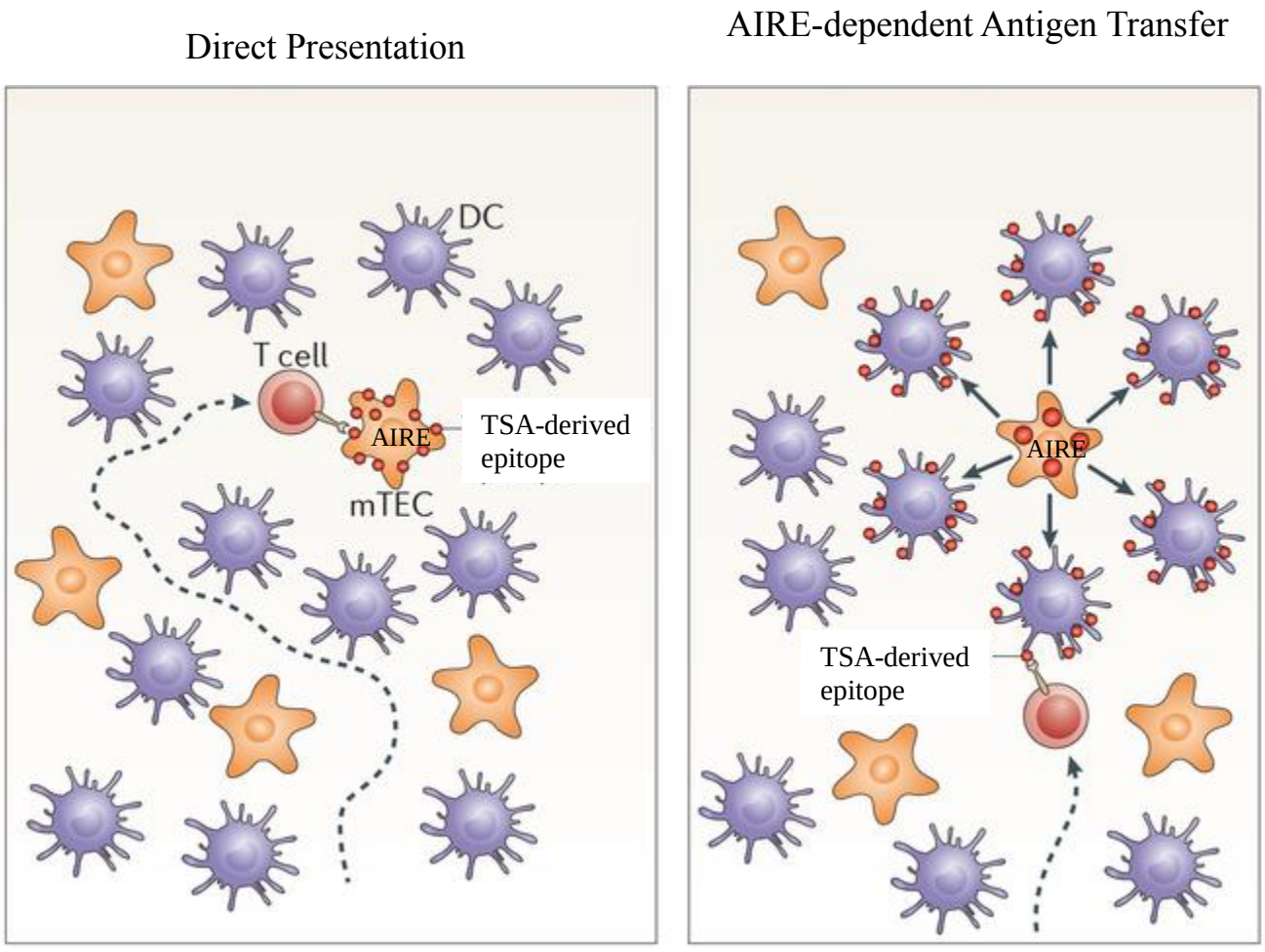


Figure 1. 4: TCR signal strength determines thymocyte fate. Developing SP thymocytes receive differential TCR signals based on the affinity for the selecting ligand. The combination of TCR signal strength and the cytokine environment determines if the developing thymocyte will exit the thymus as naïve T cells, differentiate into nTregs, or proceed through clonal deletion. Low TCR signal strengths favors naïve T cell development, intermediate TCR signal strength favors nTreg differentiation, and high TCR signal strength favors the clonal deletion via apoptosis. The weight of the arrow indicates the probability of the indicated T cell fate. Adapted from Josefowicz, Annu Rev Immunol, 2012 [100].

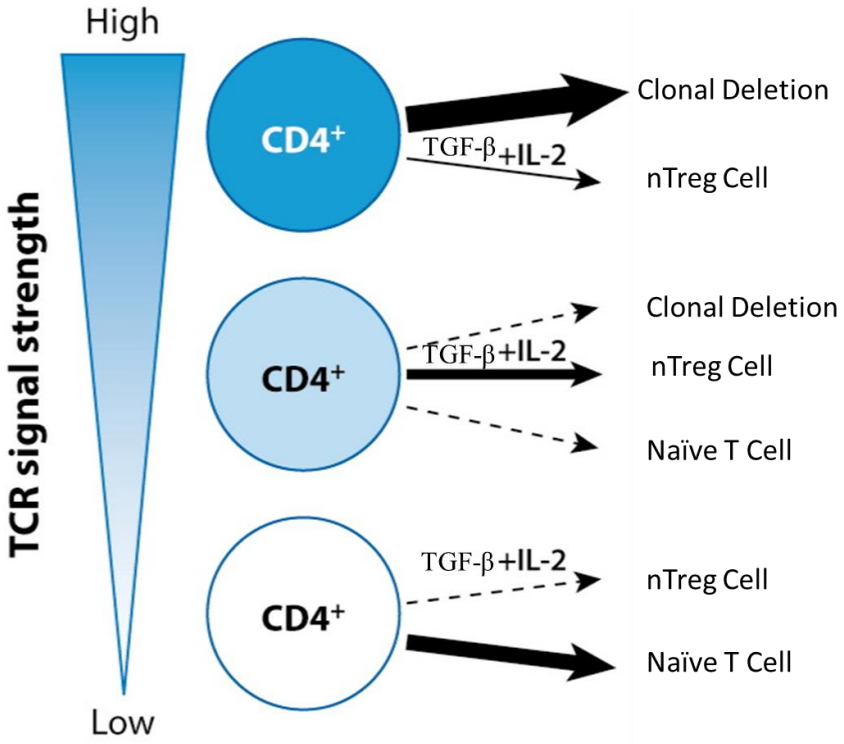


Figure 1. 5: Methods of Treg suppression of an immune response. **(A)** Treg suppression includes the release of anti-inflammatory factors like TGF-B, IL-10, IL-35, and IL-9. Recently, nTregs have even been shown to inhibit effector T cell responses by secreting exosomes containing inhibitory microRNA. **(B)** After initiating targeted direct cell contact, nTregs are capable of releasing granzyme and perforin, resulting in the induction of apoptosis and eventual cytolysis of target effector T cells or antigen presenting B cells. **(C)** Tregs can disrupt effector T cell metabolism through cytokine depravation via high levels of the IL-2R (CD25) or by inhibiting IL-2 synthesis by inducing cAMP. **(D)** nTregs are capable of down-regulating CD80 and CD86 co-receptor expression on APCs by binding these co-receptors with cytotoxic T lymphocyte antigen and inducing trans-endocytosis, which ultimate blocks CD28 co-stimulation. Adapted from Vignali, Nat Rev Immunol, 2008 [194].

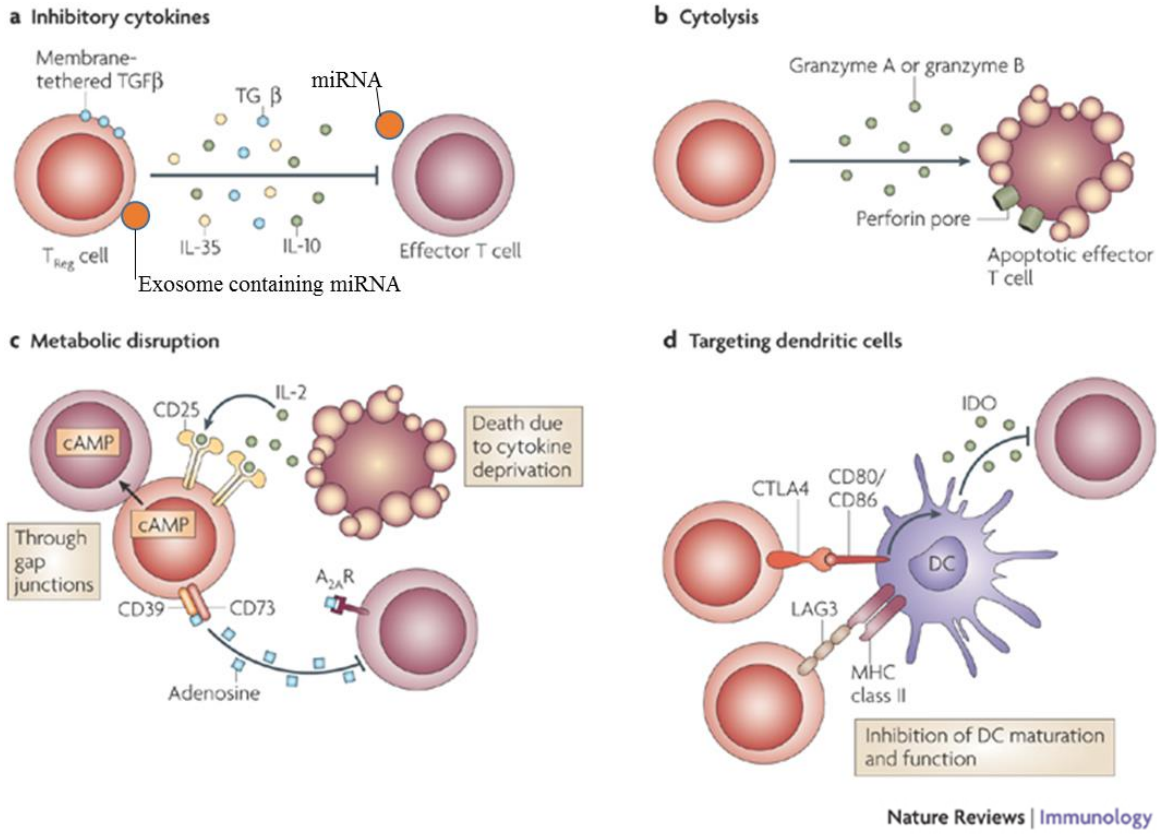
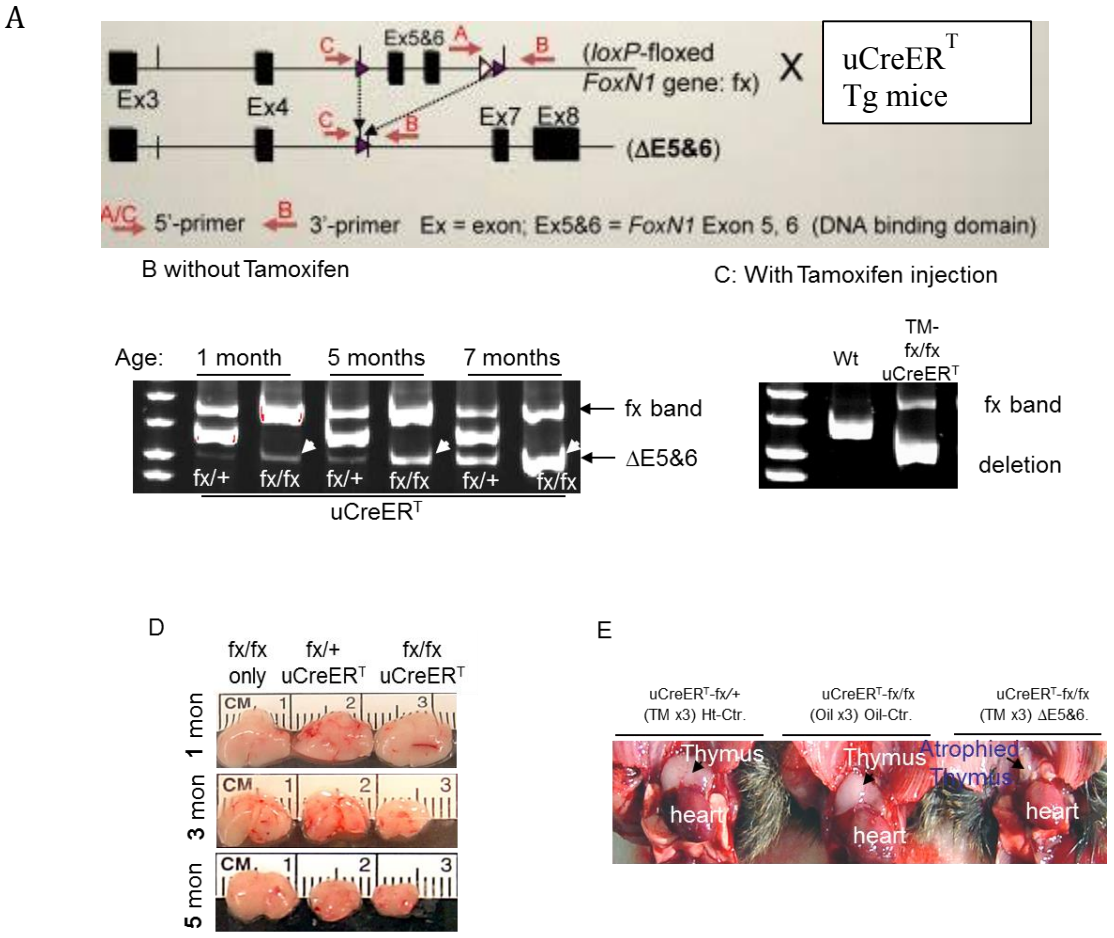


Figure 1.6: u**CreER^{T}-mediated *loxP*-deletion *FoxN1* conditional gene knockout system**: **(A)** The *FoxN1* functional domain, exons 5 and 6, are flanked by two *loxP* sites, termed floxed or "fx". After introduction of the uCreER^{T} transgene (Tg) into these mice by crossbreeding (termed as "X"), and induction with tamoxifen (TM), or waiting for the slow-leakage of uCreER^{T,} the *loxP*-flanked exons 5 and 6 are excised (termed "ΔE5&6"), and the *FoxN1* gene loses its function (conditional knockout). **(B)** Genotyping results showing slow-leakage of *cre*. **(C)** Genotyping results showing tamoxifen induced *FoxN1* knockout. **(D)** Image showing thymic involution through the injection of tamoxifen. **(E)** Thymic involution from the slow-leakage of *cre*. Adapted from Zhang and Coder et al., *Int J Biol Sci.*, 2012 [195] , Sun et al., *Aging Cell*, 2010 [169], Cheng et al., *JBC.*, 2010 [54] .



METHODS

Mice, Crossbreeding, and animal care

All animal experiments were in compliance with protocols approved by the Institutional Animal Care and Use Committee of the University of North Texas Health Science Center, in accordance with guidelines of the National Institutes of Health. All mice colonies are on the C57BL/6 background and their crossbreeding schemes are detailed in Table-S1. The colonies are the following: *FoxN1* conditional knockout (cKO) (fx/fx-uCreER^{T} mice with induced *FoxN1* deletion via tamoxifen treatment: TM, termed "*FoxN1*-cKO") [54]; fx/fx-only (without uCreER^{T}, phenotypically identical to wild-type "WT" mice, including wild-type levels of *FoxN1* expression, termed "FF-Ctr"[54]; *Rag2*-GFP (green fluorescent protein) Recent Thymic Emigrant (RTE) reporter mice; Rag2$^{-/-}$ knockout; RIP-mOVA (Ovalbumin) transgenic (Tg); OT-II T cell receptor (TCR) transgenic; and autoimmunity regulator gene (*AIRE*)$^{-/-}$ knockout mice. With regards to the characteristics of ~1-2 month old *FoxN1*-cKO mice, they display a very faint deletion of *FoxN1* at exons 5 and 6 as detected by PCR, but do not differ from FF-Ctrl mice in *FoxN1* expression, mTEC maturation, thymic size, etc [169]. Following induced *FoxN1* deletion via tamoxifen, ~1-2 month *FoxN1*-cKO mice exhibit strong deletion of *FoxN1* at exons 5 and 6 and undergo accelerated thymic involution [54]. Ages of all animals are indicated in each figure legend; young are defined, as 1 - 2 months old and aged are defined 18 - 22 months old. Aged wild-type C57BL/6 mice were purchased from the National Institute on Aging.

Table 1*:* List of mouse colonies and significance

Colony	Targeted gene and generation	Significance	Jackson Lab #
FoxN1-cKO (fx/fx-uCreER^{T})	Two loxp tags were inserted into the *FoxN1* gene as described in previous publication. [54]	Accelerated Thymic involution in a young mouse	#012941 crossed to #004682
FF-Ctr (fx/fx-only)	Same as above but no Cre gene	Use as littermate controls for *FoxN1*-cKO mice	#012941
RAG-GFP	Green fluorescent protein (GFP)$^{+}$ reporter gene is driven by *Rag2* gene	Used to identity T and B cells that have recently undergone RAG recombination; used here as marker of recent thymic emigrants (RTE)	#005688
fx/fx-uCreER^{T} or fx/fx-only carrying *Rag*-GFP	Crossbreeding fx/fx-uCreER^{T} with *Rag*-GFP mice	Tracking of RTEs derived from an involuted thymus	(#012941 crossed to #004682) crossed to #005688
Rag2$^{-/-}$	*Rag2* gene knockout	Used as adoptive transplantation hosts due to the absence of T and B lymphocytes	#002216
RIP-mOVA (Ovalbumin)	Chicken OVA driven by the RAT Insulin Promoter	AIRE dependent mOVA expressed in mTEC as neo-self antigen.	#005431
OT-II	Transgenic TcrαTcrβ recognizing chicken OVA in the context of I-A^{b}	TCR transgenic producing CD4 SP thymocytes with high affinity OVA:MHC and strong TCR signal strength	#004194
AIRE$^{-/-}$	*AIRE* gene knockout	Used as positive controls of autoimmunity in the IRBP model	#004743

Adoptive transfer

Donor erythrocyte-depleted spleen cells from young (6 weeks) and aged (>18 months) wild-type mice, or young (6 weeks) *FoxN1*-cKO mice, respectively, were intravenously (i.v.) injected through the retro-orbital route into young $Rag2^{-/-}$ host mice (2.5×10^7 cells per recipient mouse). Sixty days after adoptive transfer, the representative tissues (salivary gland and serum) of host $Rag2^{-/-}$ mice were collected for analysis of inflammatory cell infiltration.

Thymic lobe kidney capsule transplantation

The surgical operation of the kidney capsule transplantation was performed as previously described [196]. Intact newborn mouse thymic lobes of equal size from fx/fx-uCreER^T (*FoxN1*-cKO) and fx/fx-only (FF-Ctrl), both with and without the RIP-mOVA-Tg were directly transplanted into young host OT-II$^+$ TCR-Tg mice. Tails from newborns were collected for genotyping to confirm the presence of the OT-II TCR, fx/fx-uCreER^T, and fx/fx-only transgenes. Detailed survival surgical procedure is outlined as below: step-1), host mouse was anesthetized with Ketamine (100 mg/kg) plus Xylazine (10 mg/kg) administered via intraperitoneal injection; step-2), the anesthetized host mouse was shaved on one side of the ventral skin between front and rear legs, and the surgical area was prepared with 70% ethanol and betadine alternated 3 times; step-3), 1cm long of incision was made between the front and rear legs; step-4), the kidney was located, and individual thymic lobes were placed under the kidney capsule; step-5), the incision was closed by sterile suture: Prolene (polypropylene) sutures accompanied with bio-gel (Tissumend II) seal. Surgery was performed under sterile conditions. Post-surgery, the incision line was applied with 1-2 drops of Marcaine for post-operative analgesia to reduce post-surgery pain. Additionally, the mice were given a subcutaneous injection of burpenorphine-SR .05-

1mg/kg. 3 days post thymic graft, the host mice were intraperitoneally (i.p.) injected with tamoxifen (TM; 1mg/10g body weight/day) for 3 consecutive days to induce deletion of the *FoxN1* gene. Two weeks after the last TM injection, the grafted thymi were isolated for FACS analysis of CD4 and CD8, as well as the TCR-Tg (Vα2Vβ5) marker, staining following flow cytometry assay mentioned previously.

Detection and amplification of a self-reactive T cell clone: (IRBP) P2 immunization and P2-tetramer enrichment of IRBP specific T cells

The fx/fx-uCreER^{T} (*FoxN1*-cKO) or fx/fx-only (FF-Ctr) mice (6 weeks old) were given 3x TM intraperitoneal (i.p.) injections to induce deletion of the *FoxN1* gene. 4 weeks after the last TM injection, mice were immunized by subcutaneous injection of 100ug interphotoreceptor retinoid protein IRBP P2 peptide suspended in PBS and emulsified in 100ul of complete Freund's adjuvant (CFA). 10 days following immunization, cells from lymph nodes and spleen of the mice were harvested for IRBP-P2-IAb-tetramer (APC labeled) enrichment according to published protocols [197]. In short, single cell suspensions are stained with 1:100 dilution of IRBP-P2-IAb tetramer (APC labeled) blocked in 200µl Tetramer Block (20ml FACS buffer, 400ul FBS, 400ul Rat/mouse serum, 400µl 2.4G2) for 1 hour in the dark at room temperature. Cells were washed and incubated with anti-APC microbeads and MACS LS columns (Miltenyi Biotech) with a MidiMacs™ following Miltenyi Biotech MACS protocol (cat# 130-042-401) to collect the positive fraction. Positively-selected cells were counted and then stained with anti-CD4-APC/Cy7, anti-CD8-PE, anti-CD44-FITC, and anti-CD3-PerCP for flow cytometry. P2-I-Ab tetramer was generated by the NIH Tetramer Core Facility and kindly provided by Dr. Mark Anderson (UCSF).

Table 2: IRBP peptide & IRBP tetramer sequence

Peptide Name	Tetramer Name	Protein	Sequence	Sequence Position
	Mouse Interstitial retinol binding protein 3: 294-306	MHC Class II I-A(b)	QTWEGSGVLPCVG	IRBP: 294-306
P2			PLGGGGQTWE GSGVLPCVGT, > 80% purity	IRBP: 271-290

Flow cytometry assay

Single cell suspensions were prepared from the thymus, lymph node, and spleen of mice using a 70μm cell strainer in FACS buffer (PBS, 10% FBS, .1% NaN3 sodium azide). Spleen cells were erythrocyte-depleted with RBC lysing buffer (Sigma, Cat# R7757) and washed with FACS staining buffer. Samples were then treated with Fc receptor blocking antibody 2.4G2. Samples were then stained with specific antibody of cell surface CD markers and or fixed with 2% PFA and permeabilized with Triton X100, as previously reported [170], followed by intracellular staining for Bim(cell signaling #2819s), Ki-67, Foxp3(ebioscience kit #12-5773-82) and AIRE(ebioscience #50-5934-80). TECs were digested following previously published methods [170], and then stained with surface and intracellular antibodies. Fluorochrome conjugated antibodies (α:clone) CD4(GK1.5), CD8(53-6.7), CD44(IM7), Ki67(16A8), TNFα(MP6-XT22), CD25(PC61), TCR Vα2(B20.1), TCR Vβ5(MR9-4), CD45(30-F11), MHC-II(M5/114.15.2), Ly51(6C3), EpCAM(G8.8), and GITR(YGITR 765) were purchased from BioLegend. Flow cytometry was performed using a LSRII flow cytometer (BD Biosciences) and data were analyzed using Flow Jo software.

Table 3: Antibodies used for flow cytometry staining

Antibody	Catalog #	Supplier	Staining
CD4	100412	BioLegend	Fig. 1
CD44	103036		
Ki67	652404		
CD4	100406		Fig. 2
CD8	100710		
TNFα	506305		
CD4	100406		Fig. 3
CD8	100712		
CD25	102010		
FoxP3	12-5773-82	ebioscience	
CD4 and CD8	Same as above		Fig. 4
TCR Vα2	127809		
TCR Vβ5	139505		
CD4, CD25, Foxp3	Same as Fig. 3		Fig. 5
CD4, CD25, Foxp3	Same as Fig. 3		Fig. 6
Bim	2819s	Cell signaling technology	
CD45	103129		Fig. 3
MHC-II	107628		
Ly51	108308		
EpCAM	118208		
AIRE	50-5934-80		
CD4, CD25, Foxp3	Same as Fig. 3		Supp. Fig. S2
GITR	120222		
CD4, CD25, Foxp3	Same as Fig. 3		Supp. Fig. S3

Thymic epithelial cell (TEC) digestion

Whole thymus was torn gently with forceps and shaken several times in cold PBS to remove thymocytes. Then the thymus was placed in MEM medium with continuous agitation for at least 1 h at 4°C. Thymic tissue was further enzymatically digested by 1 mg ml^{-1} of collagenase/dispase (Roche, Indianapolis, IN, USA) plus 5 U ml^{-1} DNase I (Sigma–Aldrich) and incubated at 37°C for 10 min with intermittent shaking.

Real-time RT-PCR

Total RNA was isolated using TRIzol reagent (Invitrogen) from thymic epithelial cells post-digestion. RNA was reverse transcribed with the SuperScriptIII cDNA kit (Invitrogen). Real-time PCR was performed using a Step-One-Plus real-time PCR system (Applied Biosystems), with TaqMan-probes for *AIRE*, *Spt1*, and *Chrna1* (Applied Biosystems qPCR Cat# Mm00477461_m1, Mm00839568_m1, and Mm00431627_m1, respectively) or SYBR-green reagents [*Insulin I*, *Insulin II*, and *I-FABP* primers previously published [198] :

Table 4: AIRE-dependent gene primers

Gene	Forward (5 → 3)	Reverse (5 → 3)
Insulin I	GGCTTCTTCTACACACCCAAG	CCACAATGCCACGCTTCT
Insulin II	GAAGTGGAGGACCCACAAG	GATCTACAATGCCACGCTTC
I-FABP	TCCGAGAGGTTTCTGGTAATG	GTGCTGATAGGATGACGAATGAG

Samples were normalized to 18S RNA or GAPDH for internal control. The results were analyzed by the relative quantitative (RQ) gene expression $\Delta\Delta C_T$ method, setting the value for average controls as 1.0.

Lymphocyte isolation from non-lymphoid organs and flow cytometry based Treg analysis

Lymphocytes from non-lymphoid organs (liver, lung, and salivary gland) were isolated by two-layer density gradient centrifuge on Lympholyte-M (Cedarlane Labs #CL5031, Canada) at 2000 rpm for 15 min. Lymphocytes (middle layer) were collected for Treg cell staining. Liver cells were erythrocyte-depleted with RBC lysing buffer and single cell suspensions were stained with fluorochrome-conjugated anti-mouse CD antibodies (BioLegend,) Intracellular detection of FoxP3 with PE-conjugated anti-FoxP3 (clone FJK-16s, e-Bioscience), and detection of Bim with anti-Bim followed by secondary APC-conjugated anti-rabbit was performed on fixed and permeabilized cells via Cytofix/Cytoperm (e-Bioscience). Data were acquired using a LSRII flow cytometer (BD Biosciences) and data were analyzed using Flow Jo software.

Treg suppressive function assay

FoxN1-cKO and FF-Ctr mice (3-4 months old) were treated with TM i.p., 2-3 weeks following the final injection, spleens were harvested and passed through a 70µm cell strainer to obtain a single cell suspension. The samples were erythrocyte-depleted and washed, and then stained with anti-CD4 and anti-CD25. Cells were sorted on BD Influx Cell Sorter and collected into two groups: Treg ($CD4^+CD25^+$) or T effector (Teff, $CD4^+CD25^-$). 5 $x10^4$ Teff: $2.5x10^4$ Treg were co-cultured in 96-well U-bottom plate with: 5 $x10^4$ irradiated APCs (splenocytes from C57Bl/6 mice), 1ug/ml anti-CD3ε, 2ug/ml anti-CD28, and RPMI-1640 medium totaling 100ul per well, for 72 hours. Proliferation of cells was determined using CellTiter 96 Aqueous One Solution Reagent (Progema, Cat#TB245) following the Progema protocol: adding 20ul solution per well for the last 2 hours of culture, and absorbance was measured at 490nm using an ELISA 96-well plate reader (BioTek ELx800).

Enzyme-linked immunosorbent assay

Anti-Nuclear Antibody concentration was determined using an ELISA kit (Alpha Diagnostic International, #5210), following the manufacturer's instructions. In brief, sera was diluted 1:100 and incubated in an ANA antigen coated well. Secondary antibody was HRP-conjugated anti-mouse IgG (H/L), and the substrate was TMB (3, 3′, 5, 5′-tetramethylbenzidine). The absorbance was measured at 450 nm with the BioTek ELx800 ELISA plate reader. Standard curve was generated using purified immunoglobulin isotypes provided in the Alpha Diagnostic ANA kit. Concentration (μg/ml) of Anti-Nuclear Antibody in mouse serum samples were extrapolated against this standard curve. Samples were run in duplicate and the data represent the mean.

Pro-inflammatory cytokines were enumerated using an ELISA kit (BioLegend #431302), following the manufacturer's instructions. In brief, undiluted sera was incubated in an IL-6 capture antibody coated well. The detection antibody is biotinylated and detected following incubation with Avidin-HRP and then TMB substrate. The absorbance was measured at 450 nm with the BioTek ELx800 ELISA plate reader. The standard curve was generated using the BioLegend supplied standards, and concentration of IL-6 (pg/ml) was extrapolated against this standard curve. Samples were run in duplicate and the data represent the mean.

H&E staining for visualizing lympho-infiltration

Organs of interest were collected and fixed in solution for at least 48 hours. Following adoptive transfer into young $Rag2^{-/-}$ mice, salivary glands were harvested and fixed, cut into 5 μm-thick sections, and stained with hematoxylin and eosin (H&E).

Immunofluorescence Staining

To detect serum autoantibody, 6 μm cryosections from OCT frozen lacrimal gland, salivary gland, adrenal gland, stomach, pancreas, ovary, prostate, intestine, testicle, and eye were prepared from young $Rag2^{-/-}$ and wild-type mice. After fixation in -20C cold acetone for 5 min, sections were incubated with 0.1% NP-40/TBS for 5 min and blocked with SuperBlock (Thermo scientific #37516) for 15 min at room temperature. The slides were then blocked a second time with Affinipure Fab Fragment Donkey Anti-Mouse IgG (H+L) (1:10) (Jackson ImmunoResearch Laboratories, #715-007-003) for 1hr at 37°C. Sections were divided into two groups: 1) incubated with serum from age-matched *FoxN1*-cKO; 2) incubated with serun from FF-Ctr mice (1:100 dilutions) at 4°C over night, then stained with Cy3-conjugated donkey anti–mouse IgG (Jackson ImmunoResearch Laboratories, #715-166-151) for 30 min at room temperature. Immunofluorescence staining of the thymus environment was performed via cryosections (6 μm) that were fixed in -20C cold acetone and blocked in 10% donkey serum/TBS. Primary antibodies were rabbit anti-mouse Keratin-5 (K5, Covance), rabbit anti-mouse Claudin-3,4 (Cld3,4, Invitrogen, #34–1700 and #36–4800), rat anti-mouse K8 (Troma-1 supernatant), and rabbit anti-AIRE (Santa Cruz, #M-300) and rabbit anti-mouse CD11c. Secondary reagents included Cy3-conjugated donkey anti-rabbit IgG, FITC-conjugated donkey anti-rat IgG (Jackson ImmunoResearch Laboratories) or Alexa-Fluor-488-conjugated anti-rabbit IgG (Invitrogen).

Statistics

For evaluation of group differences, the unpaired two-tailed Student's *t*-test was used assuming equal variance. F-test was used to test the null hypothesis that two groups have equal variance. In instances that the two groups do not display equal variance, the Welch's *t*-test is used in place of

the standard Student's *t*-test. Differences were considered statistically significant at values of * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. Animal numbers are indicated with *N*, and all data are expressed as mean ± SEM. Data are pooled from three independent experiments (each symbol represents an animal) as indicated. In the cases of microscopy, data are representative of at least three independent experiments.

CHAPTER III

THE PROGRESSIVE LOSS OF *FOXN1* AND SUBSEQUENT THYMIC INVOLUTION PERTURBS NEGATIVE SELECTION

The elderly population has a high susceptibility toward developing autoimmunity. Not only are the elderly more likely to develop autoimmunity, but many autoimmune diseases almost exclusively emerge in aged individuals, including Rheumatoid Arthritis, Sjögren's Syndrome, and Myasthenia Gravis in males [199]. Autoimmunity requires a break-down of immune tolerance check-points, and many of these check points are influenced by immunosenescence [199]. The thymus maintains immune tolerance towards self-tissues by inducing the clonal deletion of autoreactive thymocytes through a process known as negative selection. Clonal deletion of an autoreactive thymocyte is initiated when the TCR has a high affinity for the self-peptide-MHC complex expressed by resident thymus APCs, leading to a strong TCR signaling pathway response and the initiation of the caspase-9 apoptosis pathway [63, 64]. However, natural immune aging results in the severe involution of the thymus, which has been correlated with the development of various autoimmune diseases. This study aims to determine if thymic involution alone is sufficient to disrupt immune tolerance by impairing negative selection.

Specific Aim 1

To determine if the progressive loss of *FoxN1* and subsequent thymic involution impairs negative selection.

Rationale

Thymic involution results in the reduction of MHC complexes that would normally be initiating negative selection, suggesting thymic involution impairs the clonal deletion of autoreactive thymocytes. Although thymic involution is accompanied by many genetic and phenotypic changes, the age-related loss of *FoxN1* has been identified as a dominant trigger of thymic involution. Furthermore, the progressive loss of *FoxN1* directly leads to decreased MHC-II expression in mTECs [54, 200]. Although both TEC subsets are capable of driving negative selection, cTECs are not very efficient, present ligands other than self-antigen, and are not in close proximity to tDCs [73, 201]. mTECs on the other hand are in close proximity to tDCs and are potent initiators of negative selection. Furthermore, the transcriptional regulator AIRE is primarily responsible for initiating clonal deletion through the production and transfer of TSAs to tDCs [85, 86]. AIRE is expressed exclusively by mTECs, and allows for the presentation of TSAs on mTEC MHC-II complexes [85, 86]. Therefore, any disruption of the mTEC compartment is likely to disrupt the machinery that drives the TSA presentation that is necessary for negative selection. Importantly, the loss of *FoxN1* more severely deteriorates the mTEC compartment resulting in a reversed cTEC/mTEC ratio, mTEC cyst formation, decreased mature mTECs, diminished MHC-II expression, and mTEC apoptosis [54, 200]. Thus, it is likely that the involuted thymus lacks the

epithelial environment to support thymocyte development. These reports imply that the progressive loss of *FoxN1* impairs negative selection by disrupting the mTEC compartment.

Results

The progressive loss of FoxN1 impairs thymocyte development

To investigate whether the age-related loss of *FoxN1* directly impairs the development of thymocytes we utilized the loxp-*FoxN1*-uCreER^{T} conditional knockout model (*FoxN1*-cKO) of accelerated thymic involution. The *FoxN1*-cKO mouse model allows for the inducible deletion of *FoxN1* after the thymus has fully matured, either by administering Tamoxifen (TM) or the slow leakage of uCreER^{T}, resulting in accelerated epithelial driven thymic atrophy that is comparable with thymic epithelium dysfunction observed in naturally aged C57BL/6 mice [54, 169]. Furthermore, the loss of *FoxN1* is only observable in experimental *FoxN1*-cKO mice, thus littermate floxed FF-Ctr mice serve as controls because they lack the *cre* gene and therefore cannot delete *FoxN1* after TM treatment. Importantly, only the thymus mimics aging; the peripheral environment remains consistent with the age of the mouse at the time of administration (6 weeks of age). In order to determine how the progressive loss of *FoxN1* impacts the development of thymocytes, I determined CD4 and CD8 expression on thymocytes in *FoxN1*-cKO mice using flow cytometry based analysis. I observed a reduction in the frequency and total number of DP thymocytes in *FoxN1*-cKO mice compared to FF-Ctr mice (Fig 3.1 A). TCR specificity is generated through random V (D) J recombination [26, 56] and, because of this stochastic nature, the frequency of high affinity TCRs and clonal deletion should remain constant. Therefore, a decrease in the DP population should result in a proportional decrease in the single positive

populations. However, I observed an increase in the frequency of CD4 SP and CD8 SP thymocytes in *FoxN1*-cKO mice compared to the FF-Ctr control (Figure 3.1 A). Even though the total thymocyte number is decreased approximately 5-fold comparing *FoxN1*-cKO to FF-Ctr mice, there are no significant differences in the total numbers of either CD4 SP or CD8 SP thymocytes (Figure 3.1 B). These data imply that the progressive loss of *FoxN1* results in the survival of a higher percentage of SP thymocytes, or in the diminished clonal deletion of SP thymocytes.

The progressive loss of FoxN1 directly impairs clonal deletion

In order to definitively determine if the progressive loss of *FoxN1* impairs the clonal deletion of self-antigen recognizing thymocytes, we utilized the OT-II-RIP-mOVA model of AIRE dependent negative selection. RIP-mOVA$^+$ mTECs express ovalbumin as a neo-self antigen under the control of the Rat Insulin Promoter (RIP) by an AIRE-dependent mechanism [85, 95]. OT-II bone marrow progenitors seed the grafted thymic lobes and gives rise to thymocytes with TCRs that are specific for ovalbumin, MHC-II restricted (CD4), and bind MHC: OVA complexes with a strong avidity. Therefore, thymocytes possessing OT-II TCRs that bind to MHC: OVA complexes with too strong of an avidity are signaled to undergo clonal deletion. RIP-mOVA mice have been crossbred with *FoxN1*-cKO mice yielding three genotypes: *FoxN1*-cKO-mOVA, FF-mOVA, and FF-Ctr. Fetal thymic lobes of equal size were harvested from *FoxN1*-cKO-mOVA, FF-mOVA, and FF-Ctr newborns, and then surgically transplanted under the kidney capsule of adult OT-II host mice. Three days post transplantation surgery, the knockout of *FoxN1* was induced by administering intraperitoneal TM injections into OT-II host mice for three consecutive days. Two weeks after the final TM injection, the grafted thymic lobes were isolated for flow cytometry analysis. As expected, OT-II hosts grafted with newborn thymic lobes from FF-Ctr mice

that lack the mOVA transgene contained a normal proportion of OT-II CD4 SP thymocytes ($CD4^+CD8^-V\alpha2V\beta5^+$) (Figure 3.2 B top panel and 3.2 C filled circles); when the OT-II progenitors seeded into the grafted FF-mOVA thymus that carries the mOVA transgene, then strong clonal deletion was observed in OT-II CD4 SP thymocytes (Figure 3.2 B middle panel and 3.2 C filled squares). However, when the OT-II progenitors seeded into the grafted *FoxN1*-cKO-mOVA thymus, which carries the mOVA transgene and *FoxN1*-cKO induced thymic involution, the proportion of $CD4^+CD8^-V\alpha2V\beta5^+$ cells was increased (Figure 3.2 B bottom panel and 4 C open squares) compared to FF-Ctr thymus, indicating that the OT-II CD4 SP thymocytes developing in the *FoxN1*-cKO-mOVA thymus escape cell death. These results provide direct evidence that the loss of *FoxN1* and subsequent thymic involution impairs clonal deletion leading to the survival of SP thymocytes that are potentially autoreactive.

AIRE and AIRE-dependent tissue-specific antigens are decreased in the FoxN1-cKO Thymus.

In order to understand the mechanisms impairing clonal deletion in the *FoxN1*-cKO mice, I decided to focus on the autoimmune regulator (AIRE), which regulates expression of AIRE-dependent TSAs in order to induce thymocyte negative selection. First, thymus cryosections from middle-aged 9-month-old *FoxN1*-cKO and age-matched FF-Ctr mice (slow leakage of *cre*) were immunofluorescently stained for the expression of AIRE and TECs (K8). AIRE expression in TECs was significantly decreased following the loss of *FoxN1* (Figure 3.3 A), and the ratio of AIRE/K8 TECs was significantly reduced in the *FoxN1*-cKO group compared to the FF-Ctr (Figure 3.3 B). Furthermore, flow cytometry analysis revealed that in the young (6 wk.-old) *FoxN1*-cKO group (TM induced), AIRE was lost specifically in mTECs (Figure 3.4 A and B), and AIRE expression was reduced on a per cell basis, measured by mean fluorescence intensity (MFI)

(Figure 3.4 C). I next wanted to determine if the loss of AIRE in *FoxN1*-cKO mice results in a decrease in the expression of TSAs. RT-PCR showed a decrease in AIRE-dependent self-antigen genes, including the genes responsible for presenting salivary, insulin, and intestinal self-antigens (Figure 3.5 A). This disruption in the expression of AIRE in mTECs following accelerated thymic involution elucidates a potential mechanism driving the impaired clonal deletion of *FoxN1*-cKO thymocytes.

Summary

Here, I report that thymic involution perturbs negative selection by partially impairing the AIRE-dependent clonal deletion of autoreactive thymocytes. By utilizing the *FoxN1*-cKO mouse model, I was able to demonstrate that thymic involution alone is sufficient to disrupt the process of negative selection. The observed defects in negative selection result from the progressive loss of the transcription factor *FoxN1* from the already mature thymus. Thymic involution, induced by the loss of *FoxN1*, impaired the overall development of thymocytes from the DN→DP→ SP stages. The loss of *FoxN1* in the mature thymus leads to severe thymic involution, an overall decline in thymic cellularity, and more importantly, alters the distribution of thymocyte subpopulations. The frequency of DP thymocytes is almost cut in half and the total number is reduced over 5-fold following thymic involution (Figure 3.1). Interestingly, SP thymocyte numbers are not reduced. In fact, the frequencies of CD4 and CD8 SP thymocytes have doubled in the *FoxN1*-cKO mice. This implies that thymic involution does not impair the capacity of the thymus to generate SP thymocytes. Additionally, there is not a significant difference between the total numbers of SP thymocytes between the *FoxN1*-cKO and FF-Ctr groups, suggesting SP thymocytes have increased survival following thymic involution. Increased survival of SP

thymocytes can occur in two ways: increased positive selection (survival), or decreased negative selection (clonal deletion).

In order to determine if negative selection was impaired in *FoxN1*-cKO mice, I utilized a RIP-mOVA-OT-II model of AIRE-dependent negative selection. When the FF-Ctr. mice were crossed with RIP-mOVA mice (FF-Ctr-mOVA) to enable the expression of the neo self-antigen mOVA by mTECs, then OT-II thymocytes that recognized mOVA were clonally deleted. However, over twice as many autoreactive OT-II thymocytes survived in the *FoxN1*-cKO-mOVA thymus (Figure 3.2). These data provide very strong evidence that the *FoxN1*-cKO involuted thymus cannot efficiently induce the clonal deletion of thymocytes that recognize TSAs.

Next, I wanted to understand the mechanisms leading to the impaired clonal deletion observed in the *FoxN1*-cKO thymus. I focused on the transcriptional regulator AIRE, because the RIP-mOVA-OT-II model of negative selection is dependent on AIRE-driven promiscuous gene expression of the Rat Insulin Promoter (RIP). mTECs are the only cell type in the thymus that can express AIRE, and they are severely depleted in the *FoxN1*-cKO thymus. Furthermore, the total expression of AIRE is decreased (Figure 3.3), and the expression of AIRE in mTECs on a per cell basis is diminished in the *FoxN1*-cKO thymus (Figure 3.4). The functional consequence of decreased AIRE expression in the *FoxN1*-cKO thymus is that promiscuous gene expression of AIRE-dependent self-antigen genes is severely compromised (Figure 3.5). In particular, the genes coding the TSAs associated with the salivary gland, the pancreas, the intestines, and muscle were decreased. These genes are all associated with age-related autoimmunity: salivary (Sjögren's Syndrome), pancreas (diabetes), intestine (colitis), muscle (Myasthenia Gravis).

In summary, thymic involution, induced by the loss of *FoxN1*, impairs negative selection by decreasing the promiscuous gene expression of AIRE-dependent TSAs. Therefore, the availability of TSAs to be presented to developing thymocytes is reduced. The diminished expression of TSA genes results in thymocytes that never encounter their negative selection ligand. Thusly, thymocytes with autoreactive TCRs are able to survive, because the components that initiate clonal deletion are missing. Importantly, the defects in clonal deletion associated with thymic involution resulted from the loss of one single gene, *FoxN1*. Therefore, these data identify *FoxN1* as a potential therapeutic target for the treatment of age-related autoimmunity and autoimmune diseases including but not limited to, Sjögren's Syndrome, inflammatory bowel disease, and Myasthenia Gravis.

Figure 3. 1: Clonal deletion of SP thymocytes was impaired in the *FoxN1*-cKO atrophied thymus. Five days after inducing *FoxN1*$^{fx/fx}$ deletion with TM in 6-week-old FF-Ctr and *FoxN1*-cKO mice, thymocytes were freshly isolated for cell surface staining of CD4 vs. CD8. **(A)** Representative flow cytometry plots show CD4^{+} and CD8^{+} SP gates of *FoxN1*-cKO (right panel) and FF-Ctr control (left panel) mice. **(B)** Summarized results of absolute cell numbers per thymus (left panel) and % of each subpopulation in total thymocytes (right panel). Open bars represent FF-Ctr control mice, filled bars represent *FoxN1*-cKO mice. Each group contains seven animals.

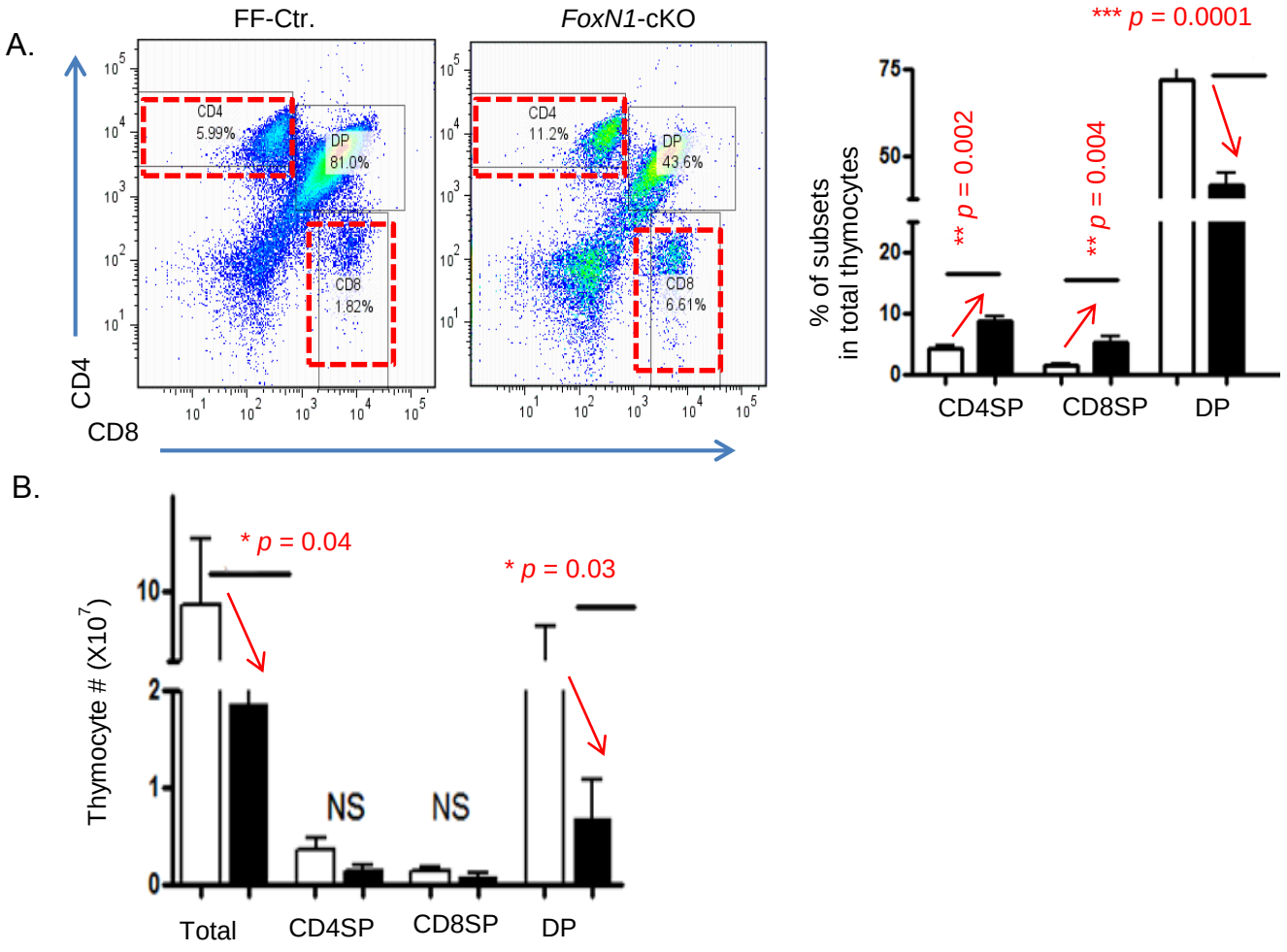
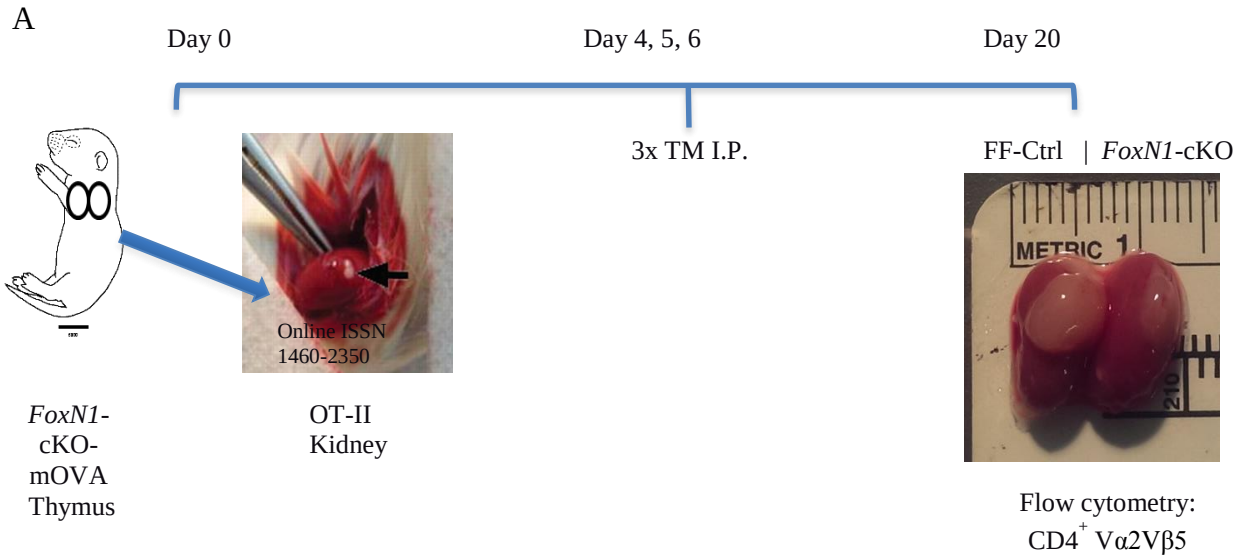
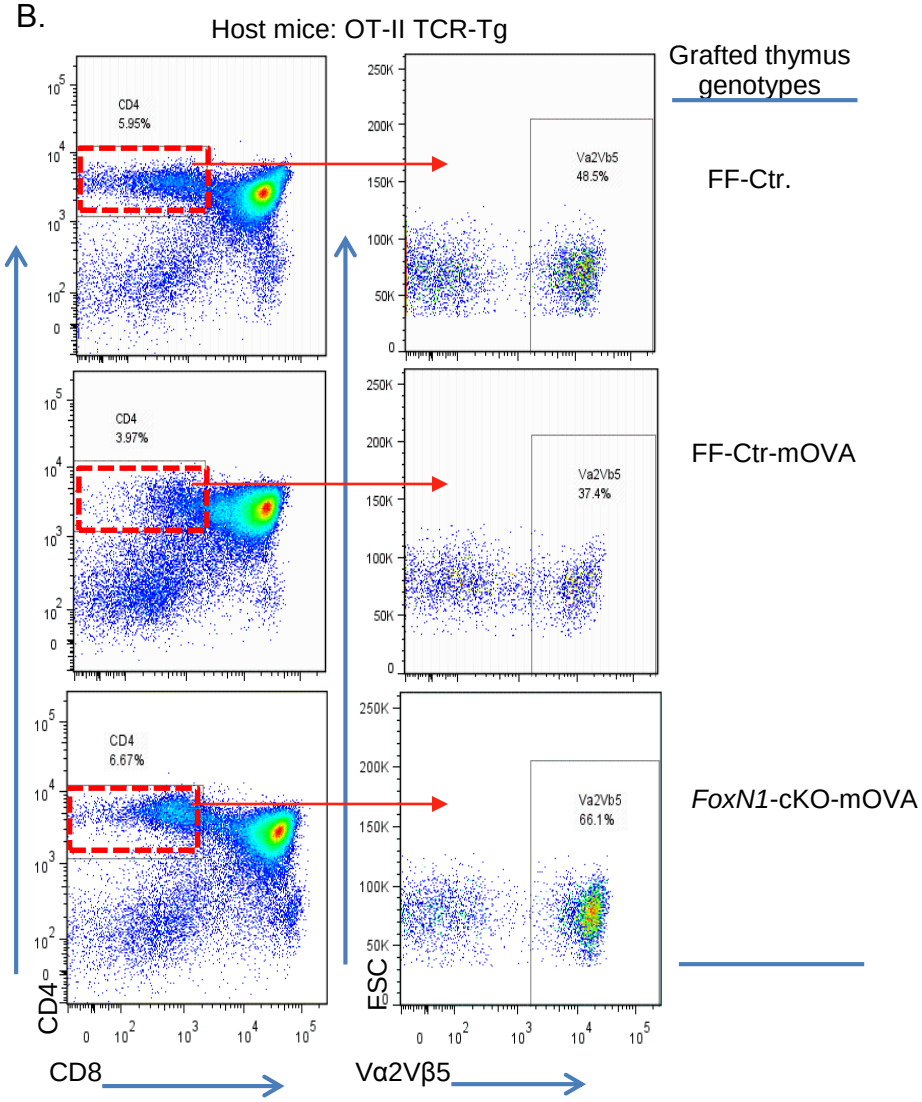


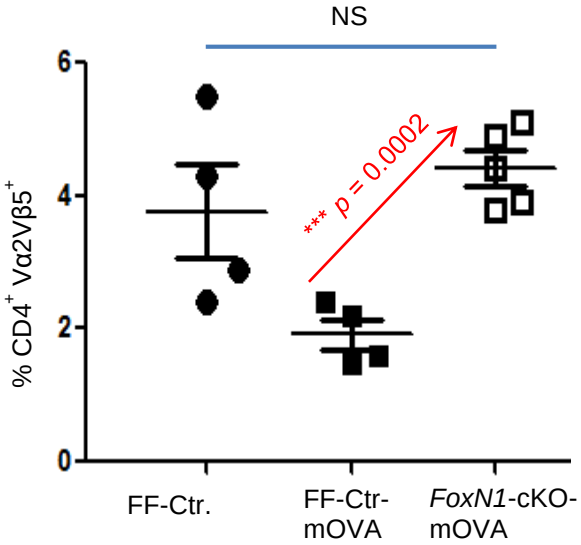
Figure 3. 2: mOVA expressing *FoxN1*-cKO thymi do not efficiently delete OT-II TCR transgenic thymocytes. mOVA expressing mice were crossed with *FoxN1*-cKO and FF-Ctr to generate *FoxN1*-cKO-mOVA, FF-Ctr-mOVA, or FF-Ctr (without mOVA expression) genotypes. Newborn thymi of equal size from each genotype were grafted separately under the kidney capsule of OT-II host mice, which were then injected i.p. with TM to induce *FoxN1* deletion in the grafted thymi. 2-weeks following kidney capsule transplantation, grafted thymi were harvested and stained with CD4, CD8, Vα2, and Vβ5 antibodies. **(A)** Schematic of kidney capsule surgery. **(B)** Representative dot plots of thymocytes from the grafted thymi gated on CD4 SP thymocytes (Left panels) and OT-II TCR$^+$ (Vα2$^+$Vβ5$^+$) population (right panels) **(C)** Summarized results of the % of CD4SP-Vα2$^+$Vβ5$^+$ thymocytes of FF-Ctr (closed circle), FF-Ctr-mOVA (closed square) or *FoxN1*-cKO-mOVA (open square) grafted thymi. **(D)** Image of grafted thymus under OT-II kidney capsule two weeks post-surgery. A Student t-test was used to determine statistical significance between groups. All data are expressed as mean ± SEM. Data are pooled from three independent experiments (each symbol represents one animal).



B.


Host mice: OT-II TCR-Tg
Grafted thymus genotypes
FF-Ctr.
FF-Ctr-mOVA
FoxN1-cKO-mOVA
CD4
5.95%
Va2Vb5
48.5%
CD4
3.97%
Va2Vb5
37.4%
CD4
6.67%
Va2Vb5
66.1%
CD4
CD8
FSC
Vα2Vβ5

C.


NS
*** p = 0.0002
% CD4+ Vα2Vβ5+
FF-Ctr.
FF-Ctr-mOVA
FoxN1-cKO-mOVA

D.

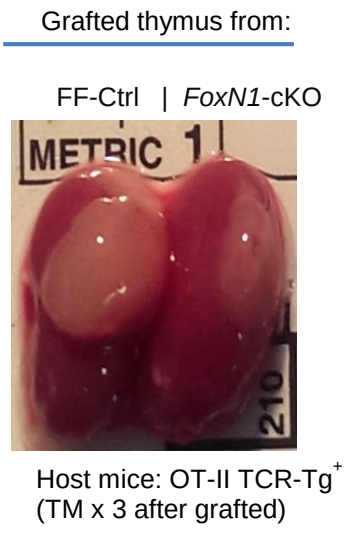

Grafted thymus from:
FF-Ctrl | FoxN1-cKO
METRIC 1
210
Host mice: OT-II TCR-Tg+
(TM x 3 after grafted)

Figure 3. 3: AIRE expression is decreased in *FoxN1*-cKO TECs. **(A)** Representative immunofluorescence staining of thymic cryosections, AIRE (red) versus K8 TECs (green), shows decreased AIRE expression in middle-aged *FoxN1*-cKO mice. **(B)** Ratio of AIRE expression measured by % AIRE^{+}TECs / % K8^{+} TECs, analysis by NIH Image-J software based on immunofluorescence staining in (A). n = animal numbers.

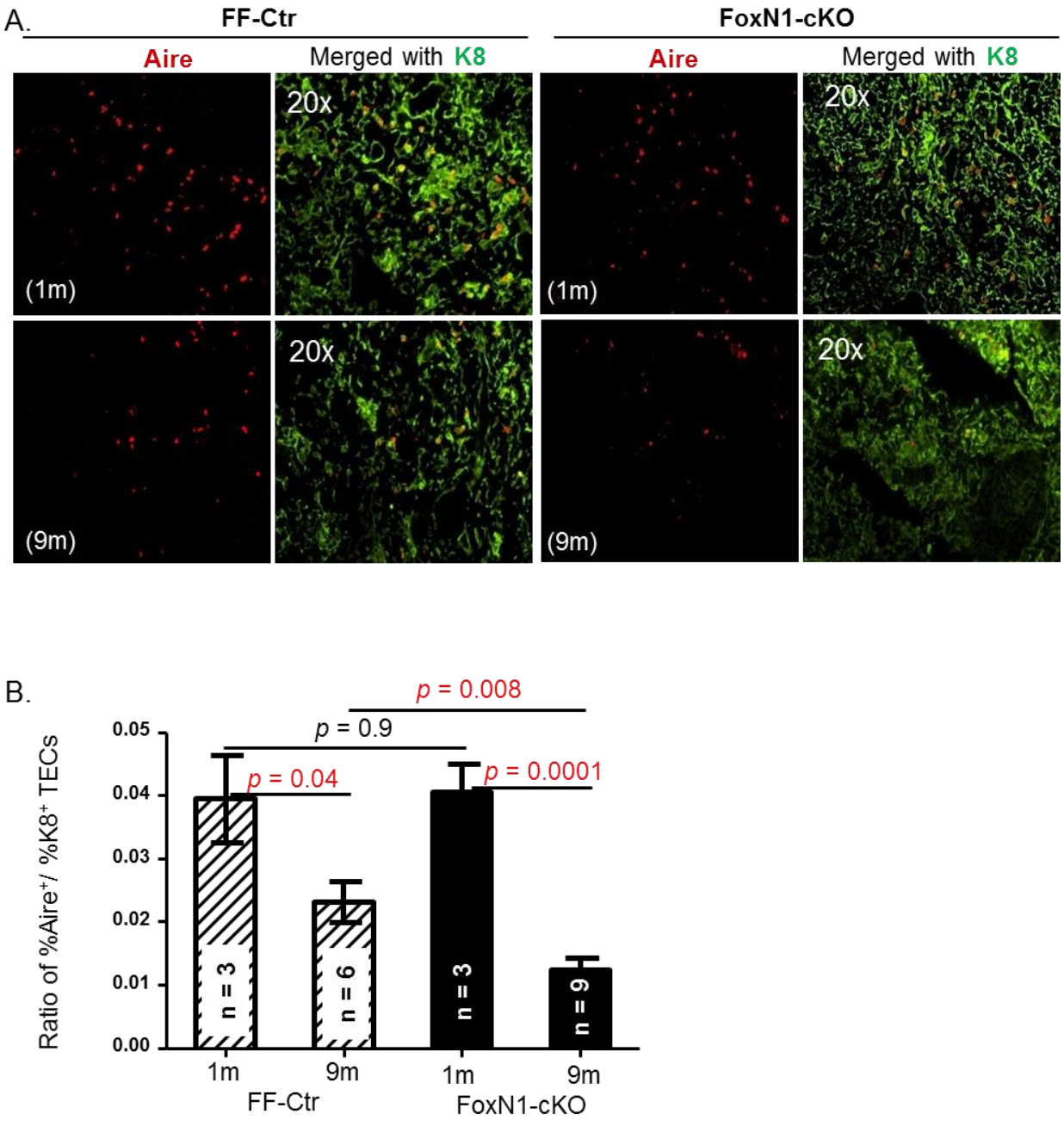
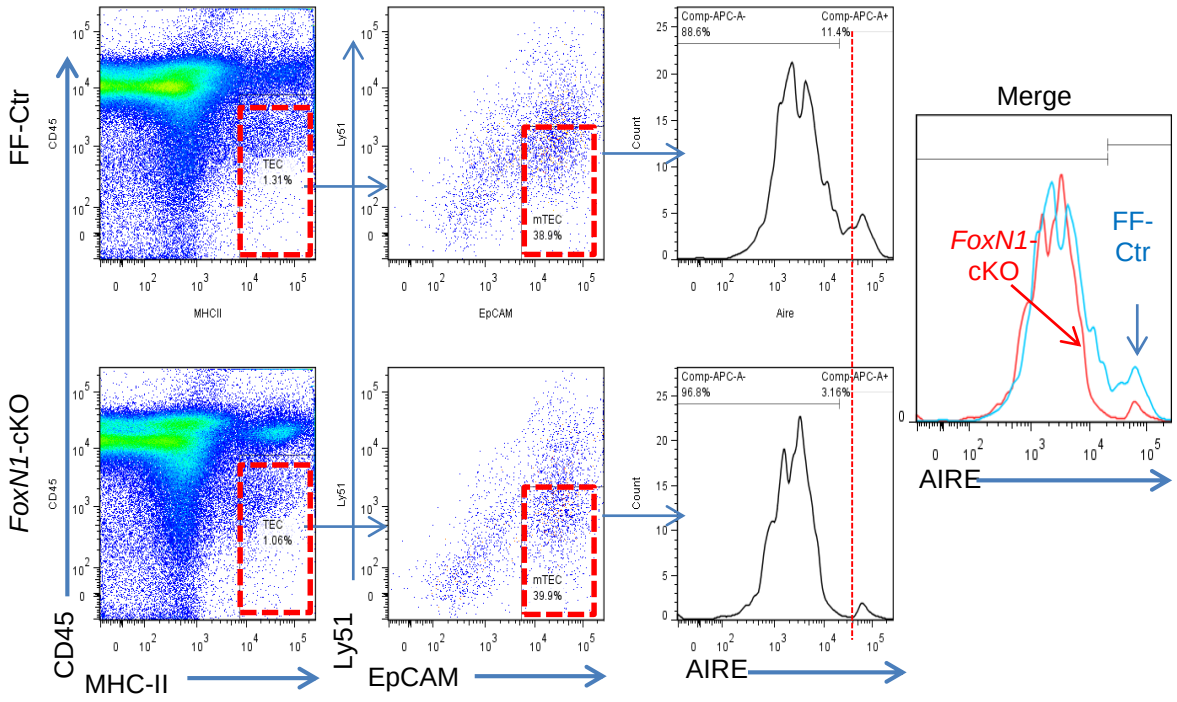
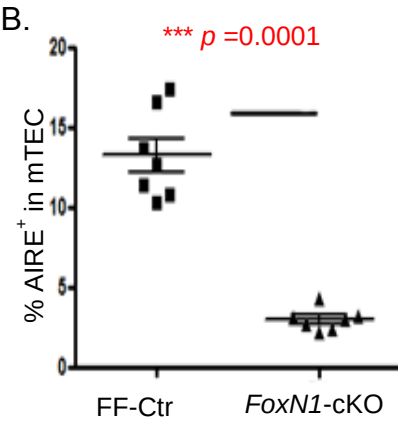


Figure 3. 4: AIRE expression is decreased in mTECs of *FoxN1*-cKO mice. Thymic epithelial cells were enzymatically digested from *FoxN1*-cKO and FF-Ctr thymi and intracellularly stained with AIRE antibody. **(A)** Flow cytometry was used to gate on mTECs ($CD45^-$, $MHC\text{-}II^+$, $EpCAM^+$, $Ly51^-$), **(B)** Summarized results of the percentage of $AIRE^+$ mTECs, and **(C)** the mean fluorescent intensity of $AIRE^+$ mTECs. A Student t-test was used to determine statistical significance between groups. All data are expressed as mean ± SEM. Data are pooled from at least three independent experiments with a total of n = 7 animals per group.

A.


FF-Ctr
FoxN1-cKO
CD45
MHCII
TEC
1.31%
TEC
1.06%
Ly51
EpCAM
mTEC
38.9%
mTEC
39.9%
Count
Aire
Comp-APC-A-
88.6%
Comp-APC-A+
11.4%
Comp-APC-A-
96.8%
Comp-APC-A+
3.16%
AIRE
Merge
FoxN1-cKO
FF-Ctr
MHC-II
EpCAM
AIRE

B.


*** p =0.0001
% AIRE+ in mTEC
FF-Ctr
FoxN1-cKO

C.

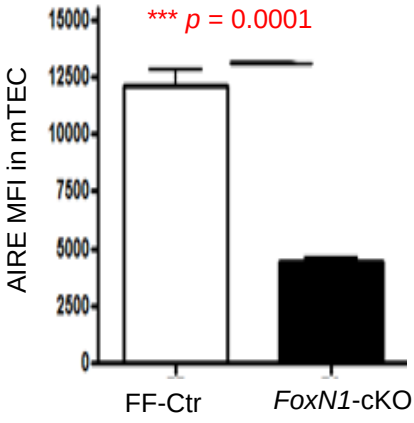
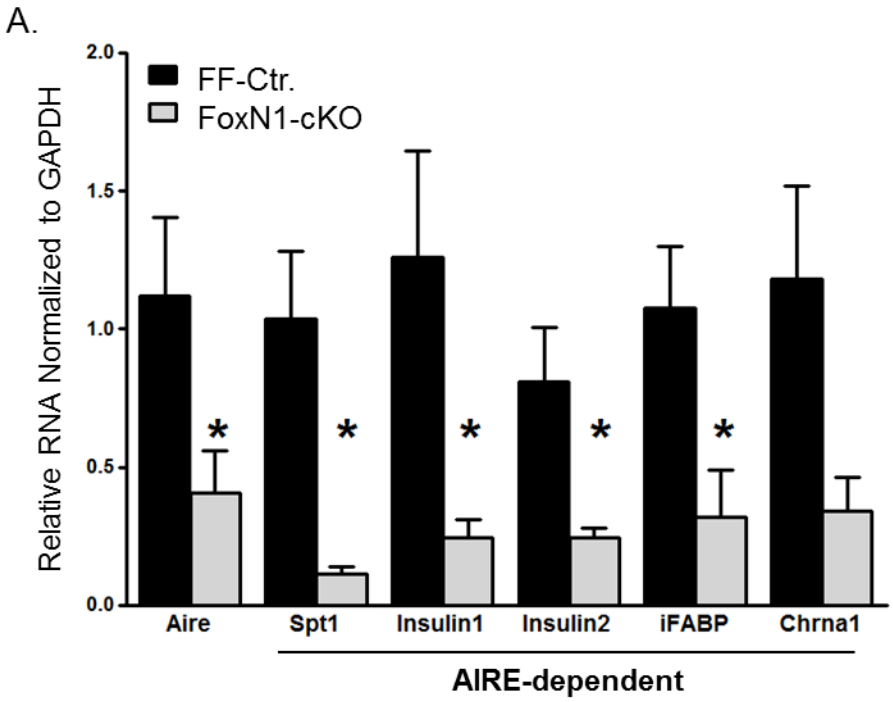

*** p = 0.0001
AIRE MFI in mTEC
FF-Ctr
FoxN1-cKO

Figure 3. 5: AIRE-dependent self-antigens are decreased in the *FoxN1*-cKO thymus. Expression AIRE, and AIRE-dependent tissue-specific self-antigens in 9-month-old middle-aged FF-Ctr and *FoxN1*-cKO mice measured by real-time RT-PCR. Spt1 = salivary protein 1; iFABP = intestinal fatty acid binding protein; Chrna1 = Cholinergic Receptor, Nicotinic, Alpha Polypeptide 1. Bar graph shows summarized relative fold-change of mRNA analyzed. Each group includes 4 animals and each sample was run in duplicate. Statistical significance was analyzed by unpaired two-tailed Student's t-test. Data show Mean ± SEM. "*" = "p" value less than 0.05 (significant).



CHAPTER IV

THE PERIPHERAL ENVIRONMENT, RATHER THAN THYMIC INVOLUTION, LEADS TO THE AGE-RELATED ACCUMULATION OF TREGS

The thymus gland generates immune tolerance by removing autoreactive TCRs from the effector T cell repertoire. This is accomplished during negative selection; not only through the clonal deletion of thymocytes that recognize self-antigen, but also by diverting the differentiation of a subset of these autoreactive thymocytes into immunosuppressive nTregs. Even though the elderly have a higher frequency of autoimmunity and are plagued by systemic inflammation, clinical signs of many full blown autoimmune disease, like systemic lupus erythematosus (SLE), are often much rarer in this group [202], [203]. The decreased incidence of overt autoimmune disease in the elderly is likely driven by the age-related accumulation of Tregs observed in elderly individuals [203]. However, the age-related accumulation of Tregs likely comes with unwanted consequences. Immunological aging is characterized by an immune system that struggles to fight invading pathogens. Additionally, vaccine efficacy in the elderly is severely diminished compared to young people, and often offers no protective immunity at all [192]. Therefore, it is imperative to understand the mechanisms of immunological aging in order to treat age-related defects and promote healthy aging of the immune system.

Understanding the effects aging has on the generation and function of Tregs will allow targeted therapies to treat the underlying immunological defects associated with advanced age, in particular, autoimmunity, chronic inflammation, susceptibility to infections, and vaccine efficacy. The vast majority of Tregs originate from the thymus gland [100], however the epithelial meshwork that facilitates nTreg development atrophies with age [145, 169]. How thymic involution effects the generation and function of nTregs is largely unknown. Thus, this aim focuses on the impact thymic involution has on the generation of Tregs.

Specific Aim 2

To determine if thymic involution affects the generation of natural regulatory T cells

Rationale

Tregs accumulate in the periphery of aged individuals, however the mechanisms driving their enhanced frequency are not fully understood. In fact, there are conflicting reports regarding the mechanism of age-related Treg accumulation. Several reports have suggested that the age-related accumulation of Tregs in the periphery of aged individuals results from increased survival facilitated by decreased expression of the pro-apoptotic gene *Bcl2* family member *Bim* [139-141]. On the other hand, others have reported that defects in negative selection mediated by the loss of MHC-II on mTECs enhances the differentiation of Tregs. Conversely, others have demonstrated that the disruption of thymic medullary microstructure and a reduction in medullary size leads to the generation of fewer Tregs [204]. Additional controversy surrounds Treg functional ability with advanced age. Several reports show that Treg suppressive function is maintained with advanced

age [142], while others show age-related defects and thymic epithelial dysfunction can decrease Treg suppression [145], [146]. In specific aim 1, I showed that the loss of *FoxN1* and subsequent thymic involution impairs negative selection (Chapter III). Furthermore, AIRE, which has been shown to facilitate the development of nTregs, is decreased in the *FoxN1*-cKO mice. Thus, the *FoxN1*-cKO mouse model (involuted thymus, but young periphery) will be an invaluable tool used to differentiate the roles of impaired negative selection, resulting from thymic involution, and peripheral aging on the accumulation and suppressive capabilities of Tregs.

Results

The progressive loss of FoxN1 and subsequent thymic involution increases the proportion of nTregs in the thymus

In order to assess the effects of thymic involution on the generation of Tregs, I utilized our *FoxN1*-cKO mouse model that displays accelerated thymic involution and defective negative selection, but still maintains a young peripheral environment. Following induced thymic involution, there was an approximate 3-fold increase in the frequency $CD4^{+}Foxp3^{+}$ nTreg cells located in the thymus of *FoxN1*-cKO mice, assessed by flow cytometry (Figure 4.1 A and B). Furthermore, even though total thymic cellularity is decreased (Chapter III, Figure 3.1 B), the total number of nTregs in the thymus of *FoxN1*-cKO mice is not significantly different from the FF-Ctr group. In fact, the total numbers of nTregs in the thymus of *FoxN1*-cKO mice are slightly elevated (not significant) (Figure 4.1 C). Taken together, these results indicate that the loss of *FoxN1* and thymic involution does not impair the ability of the thymus to produce nTregs, and may even facilitate nTreg differentiation.

The peripheral environment, rather than the involuted thymus, determines the age-related accumulation of peripheral Tregs

The increased frequency of nTregs in the thymus of *FoxN1*-cKO mice implied that thymic involution enhanced the generation of nTregs. Next, I wanted to determine if thymic involution in the *FoxN1*-cKO mice resulted in the peripheral accumulation of nTregs consistent with aged individuals. Flow cytometry analysis of $CD4^+CD25^+FoxP3^+$ Tregs in the spleen of *FoxN1*-cKO mice (Figure 4.2 A) revealed no significant difference in the frequency of peripherally located Tregs that had developed in the involuted thymus (Figure 4.2 B). In order to determine if the lack of Treg accumulation in the spleen of *FoxN1*-cKO mice was due to their migration into non-lymphoid tissues, I stained for $CD4^+CD25^+FoxP3^+$ Tregs in the salivary gland, lung, and liver (Figure 4.3 A). There was no observable difference in the frequency of non-lymphoid Tregs derived from the *FoxN1*-involuted thymus and FF-Ctr mice (Figure 4.3 B).

To better assess whether thymic involution imparts any intrinsic changes onto nTregs that could lead to their peripheral accumulation, I performed an adoptive transplantation by infusing a pool of wild-type naturally aged (>18 months) T cells into young *Rag2*$^{-/-}$ host mice, which lack T and B cells; therefore all Tregs observed in the host mice are derived from the transferred splenocytes (Figure 4.4 A). Before adoptive transfer, peripheral aged Tregs occur at a frequency of up to 3-fold higher than their young counterparts (Figure 4.4 A&B). However, two months following adoptive transfer of aged Tregs into the young *Rag2*$^{-/-}$ host mice, the accumulation of aged Tregs was reversed, and frequency of peripheral Tregs derived from either young or old donors was no longer significantly different (Figure 4.4 A&B). Furthermore, the relative ratio of CD4+Foxp3+ Tregs derived from young and old donors becomes equalized after adoptive transfer into the young peripheral environment (Figure 4.4 C). Additionally, we confirmed that the

proportion of aged wild-type mice expressing the pro-apoptotic protein Bim is decreased prior to transfer [139-141], signifying an increased survival of these Tregs (Figure 4.5 A). However, following transfer of aged Tregs into the young *Rag2* $^{-/-}$ hosts, the frequency of Bim^{+} Tregs is increased, implying a decrease in the survival of aged Tregs (Figure 4. 5 A). Furthermore, the expression of Bim on a per cell basis follows the same trend as the frequency of Bim^{+} Tregs, where Bim expression is absent in aged wild-type Tregs before adoptive transfer, and Bim expression is restored in aged wild-type Tregs after transfer into young *Rag2* $^{-/-}$ hosts (Figure 4. 5 B). Together, these findings suggest that the accumulation of Tregs with age is predicated on the peripheral environment rather than intrinsic changes to Tregs derived from an involuted thymus. Furthermore, the young peripheral microenvironment can reverse age-related survival and accumulation of the peripheral Treg cell pool.

Thymic involution does not impair nTreg function

Tregs help maintain immune tolerance by actively suppressing aberrant self-reactive T cell responses. Irrespective to their numbers, Tregs must retain adequate suppressive capabilities in order to sustain immune tolerance. In order to determine if *FoxN1*-cKO-induced thymic atrophy impairs nTreg function, I tested the suppressive capability of peripheral Tregs derived from the *FoxN1*-cKO atrophied thymus to suppress the proliferation of effector T cells (Teff). Fluorescence Activated Cell Sorting (FACS) was used to isolate Tregs ($CD4^{+}CD25^{+}$) and Teffs ($CD4^{+}CD25^{-}$) from the spleens of either *FoxN1*-cKO or FF-Ctr mice (Figure 4. 6 A). Treg and Teff cells were co-cultured in a 2:1 ratio (Teff: Treg) in the presence of irradiated antigen presenting cells, CD3ε and αCD28 antibodies, and metabolism of the compound CellTitre96 (formazan) was used to assess proliferation (Figure 4.6 B). There were no observable differences in the suppressive ability

of Tregs coming from either *FoxN1*-cKO or FF-Ctr mice to inhibit the proliferation of Teff cells (Fig. 4. 6 B left panel). Both *FoxN1*-cKO and FF-Ctr Tregs suppressed Teff proliferation compared to the Teff group cultured without Tregs (Figure 4.6 B middle panel), and *FoxN1*-cKO and FF-Ctr Tregs themselves proliferated equally (Figure 4.6 B right panel). Additionally, there were no measureable differences in the Treg functional marker/co-stimulatory molecule GITR (glucocorticoid-induced TNF receptor family-related gene) [205, 206] between *FoxN1*-cKO and FF-Ctr Tregs (Figure 4. 7 A & B). These data suggest that the progressive loss of *FoxN1* and subsequent thymic involution does not impair the suppressive capacity of Tregs.

Summary

Although the elderly have increased autoimmunity, they are less likely to develop many overt autoimmune diseases [202, 203]. It is thought that the accumulation of Tregs in the periphery of the elderly suppress autoimmune reactions [202],[203]. However, the factors leading to the age-related accumulation of Tregs are not fully understood. In this study, I demonstrate that the peripheral environment, rather than thymic involution, drives the accumulation of Tregs with age. By using both the *FoxN1*-cKO model of thymic involution and naturally aged C57BL/6 wild-type mice, I was able to differentiate the effects of the thymus and the periphery on Treg accumulation. First, the ability of the involuted thymus to produce nTregs was assessed in the *FoxN1*-cKO model. There was not a significant difference in the total numbers of nTregs in the thymus of *FoxN1*-cKO and FF-Ctr mice. This suggested that thymic involution did not impair the ability of the thymus to generate nTregs. In fact, the frequency of nTregs in the *FoxN1*-cKO thymus was over 3-fold higher than the FF-Ctr mice, implying that thymic involution may even enhance nTreg generation (Figure 4.1). The notion that thymic involution may enhance the production of nTregs is consistent with

previous studies that demonstrate enhanced nTreg generation following defective negative selection [59]. Negative selection was shown to be defective in the *FoxN1*-cKO involuted thymus in specific aim 1.

However, the elevated frequency of nTregs in the thymus of *FoxN1*-cKO mice did not persist into the periphery. The frequency of nTregs in the spleens of *FoxN1*-cKO and FF-Ctr was not significantly different (Figure 4.2). Furthermore, the proportion of nTregs in the salivary gland, lung, and liver was unchanged between *FoxN1*-cKO and FF-Ctr mice, eliminating the possibility of enhanced nTreg migration and accumulation into non-lymphoid tissues (Figure 4.3). Although nTregs were accumulated in the involuted thymus of *FoxN1*-cKO mice, these data suggest that the age-related accumulation of Tregs in the periphery of elderly individuals is not related to intrinsic properties imprinted on developing nTregs.

Next, I addressed whether age-related accumulation resulted from extrinsic factors related to the peripheral environment. By adoptively transferring naturally aged wild-type Tregs into young *Rag2*$^{-/-}$ mice I was able to test the effects of extrinsic peripheral factors on the maintenance of aged Tregs. Fascinatingly, Treg accumulation was extinguished in the young *Rag2*$^{-/-}$ mice. Before transfer, wild-type splenocytes had a 2-fold increased frequency of Tregs compared to young wild-type mice. However, transfer into the young peripheral environment reduced the frequency of aged Tregs to become equal with young Treg levels (Figure 4.4).

Recent studies suggest that Treg accumulation in the elderly results from heightened survival rather than increased proliferation. Reports suggest that aged Tregs lack the pro-apoptotic gene *Bcl2* family member *Bim*, and therefore do not readily undergo apoptosis [139-141]. Therefore, I assessed Bim expression in aged wild-type Tregs before and after adoptive transfer into the young periphery of *Rag2*$^{-/-}$ mice. Compellingly, the young *Rag2*$^{-/-}$ peripheral environment

was able to reverse the age-related decline in Treg Bim expression. This finding provided convincing evidence that age-related Treg accumulation results from peripherally induced survival signals.

Regardless of whether Tregs accumulate in the elderly, aged Tregs must maintain the ability to suppress an immune response in order to sustain immune tolerance and prevent autoimmunity. While many labs have shown that aged Tregs retain their suppressive function [137, 142-144], others have reported a functional decline from thymic involution derived Tregs [145, 146]. In order to determine if thymic involution impairs Treg function, I tested the ability of peripheral Tregs derived from the *FoxN1*-cKO involuted thymus to suppress T cell proliferation. I demonstrated that Tregs derived from either the *FoxN1*-cKO involuted thymus or the healthy FF-Ctr thymus were equally capable of suppressing the proliferation of effector T cells (Figure 4.6) and possess equal functionality (Figure 4.7). These data further suggest that thymic involution does not affect nTregs.

In summary, Treg accumulation with age is not related to thymic involution. Even though thymic involution impairs negative selection (specific aim 1), and enhances the proportion of nTregs in the involuted thymus, this does not result in the buildup of Tregs in the periphery. The accrual of peripheral Tregs results from enhanced survival, which is dictated by the loss of the pro-apoptotic protein Bim. Therefore, age-related Treg accumulation is not an intrinsic phenomenon, but rather a phenomenon controlled by extrinsic factors related to the periphery. Intriguingly, the loss of Bim and enhanced survival of aged Tregs is not permanent; the young environment can reverse it. This identifies the peripheral Treg compartment as a region of the aging immune system that can be therapeutically modulated in order to address age-related diseases.

Figure 4. 1: The progressive loss of *FoxN1* and subsequent thymic involution enhances the generation of nTregs. Two – three weeks after inducing *FoxN1*$^{fx/fx}$ deletion with TM in *FoxN1*-cKO or FF-Ctr mice thymocytes were freshly isolated for cell surface staining of CD4, CD8, CD25 and intracellular staining of FoxP3. **(A)** Representative dot plots show nTregs ($CD4^+CD25^+FoxP3^+$) in the thymi of *FoxN1*-cKO and FF-Ctr control mice. Summarized results show the percentage **(B)**, and absolute number **(C)** of thymic nTregs. A Student *t*-test was used to determine statistical significance between groups. All data are expressed as mean ± SEM. Data are pooled from three independent experiments with an *n*= 6.

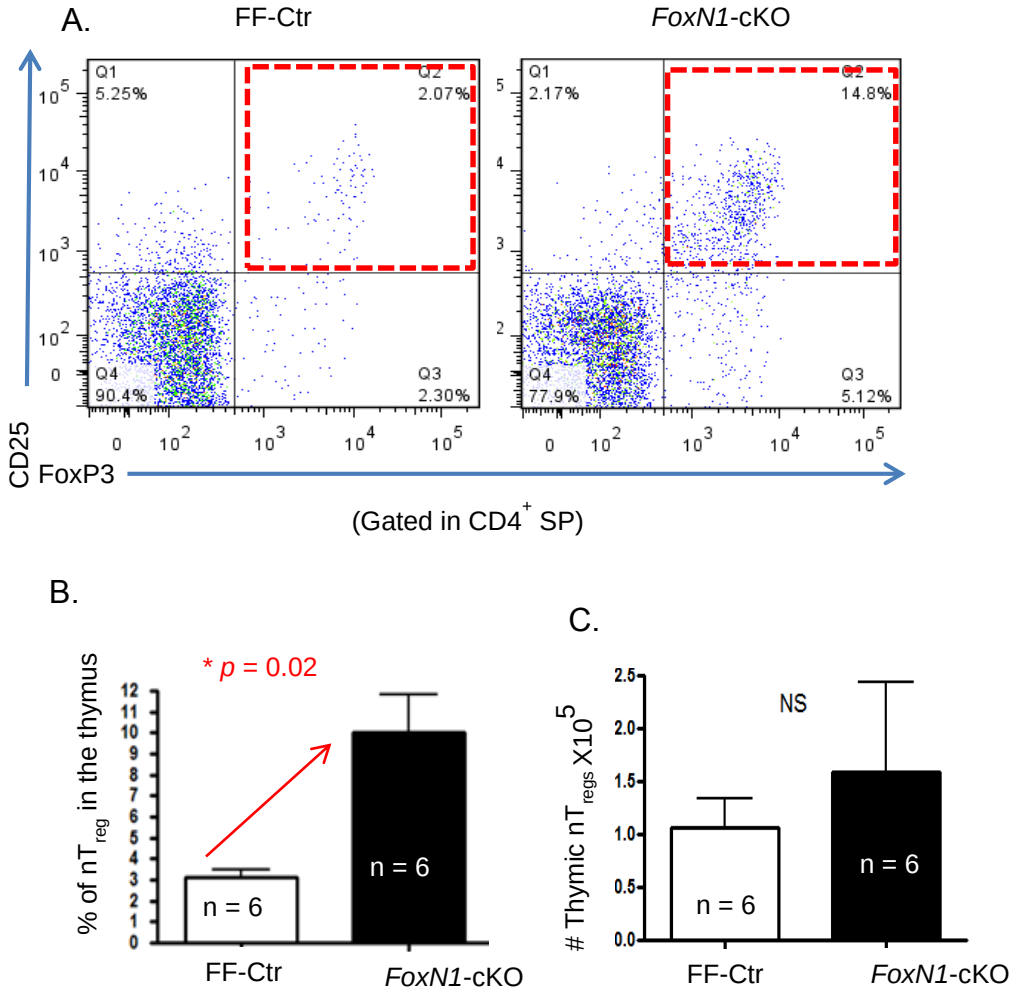


Figure 4. 2: Tregs do not accumulate in the periphery of *FoxN1*-cKO mice. Five days after inducing *FoxN1*$^{fx/fx}$ deletion with TM in 6-week-old FF-Ctr and *FoxN1*-cKO mice, splenocytes were freshly isolated for cell surface staining of CD4 and CD25, and intracellular staining of FoxP3. **(A)** Representative dot plots show the percentage of peripheral Tregs ($CD4^+CD25^+FoxP3^+$) from the spleens of FF-Ctr and *FoxN1*-cKO mice. **(B)** Summarized results of % peripheral Tregs in FF-Ctr and *FoxN1*-cKO mice. A Student *t*-test was used to determine statistical significance between groups. All data are expressed as mean ± SEM. There is an (n = 9 per group). Data are pooled from at least three independent experiments.

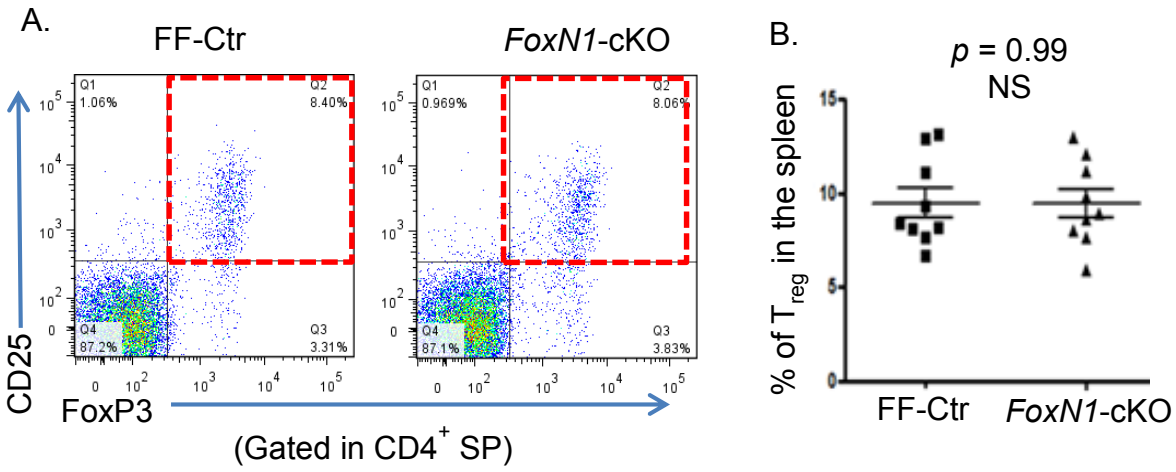
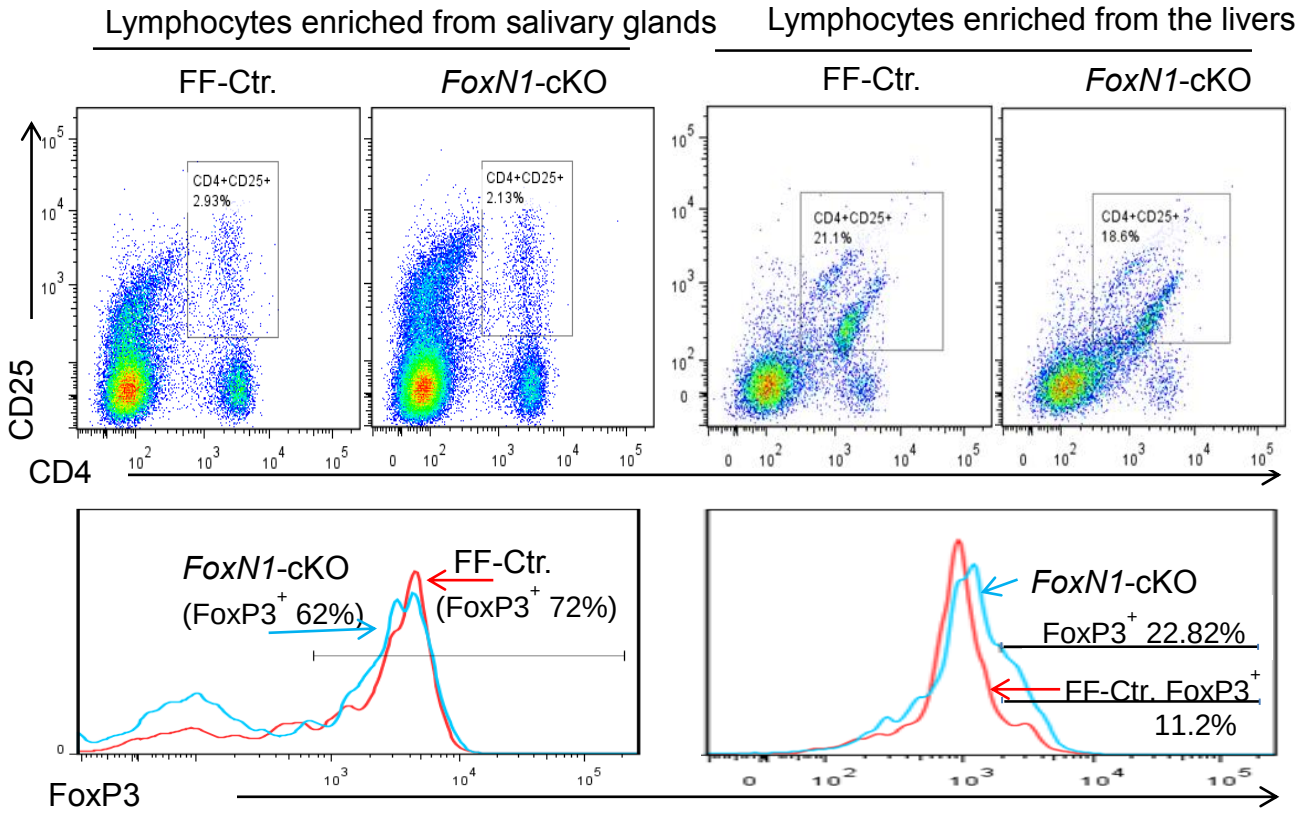


Figure 4. 3: Tregs from *FoxN1*-cKO mice do not accumulate in non-lymphoid organs. **(A)** Representative flow cytometry results from salivary gland and liver lymphocytes enriched through two-layer density gradient centrifuge, showing $CD4^+CD25^+$ and $FoxP3^+$ gates. **(B)** Summarized results of % T_{reg} cells ($CD4^+CD25^+FoxP3^+$) in the liver, lung, and salivary gland lymphocytes. (n = animal numbers, NS = not significant). A Student *t*-test was used to determine statistical significance between groups. All data are expressed as mean ± SEM.

A.



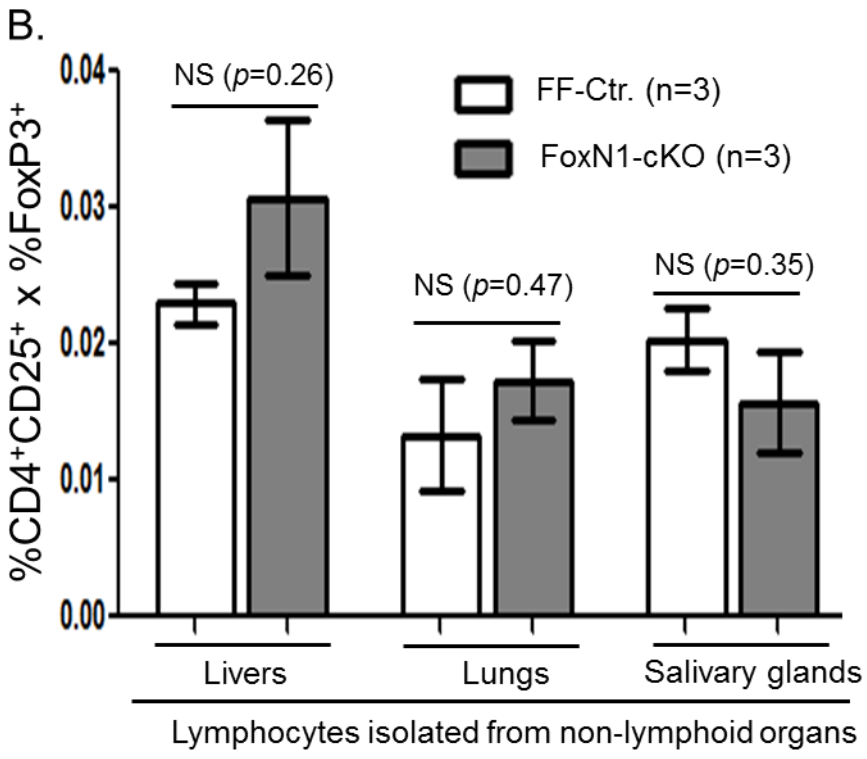

B.
0.04
0.03
0.02
0.01
0.00
%CD4+CD25+ x %FoxP3+
NS (p=0.26)
NS (p=0.47)
NS (p=0.35)
FF-Ctr. (n=3)
FoxN1-cKO (n=3)
Livers
Lungs
Salivary glands
Lymphocytes isolated from non-lymphoid organs

Figure 4. 4: The peripheral environment rather than the atrophied thymus determines the age-related accumulation of peripheral Tregs. Young or aged erythrocyte-depleted spleen cells from wild-type mice were adoptively transferred into young *Rag2*$^{-/-}$ mice. The peripheral Treg cells were analyzed before and 8 weeks after the transplantation via flow cytometry. **(A)** Representative dot plots show Treg gates before (Top panels) and after (Bottom panels) the transfer. **(B)** Shows % FoxP3^{+} cells in CD4^{+} splenocytes; **(C)** shows relative ratio of FoxP3^{+} cells in CD4^{+} splenocytes. A Student *t*-test was used to determine statistical significance between groups. All data are expressed as mean ± SEM. There is an (n = 7-11/group). Data are pooled from at least three independent experiments.

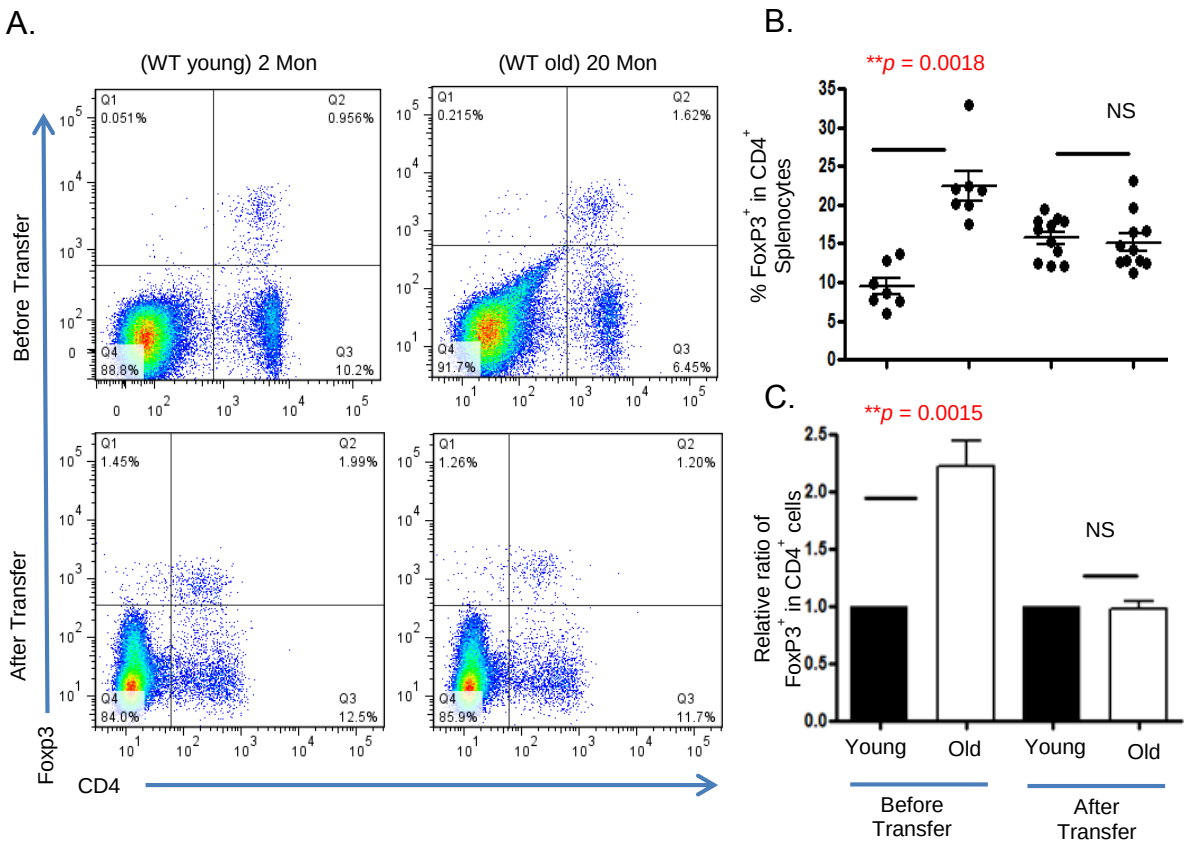


Figure 4. 5: Aged Tregs turn on expression of pro-apoptotic Bim after transfer into young environment. Young or aged erythrocyte-depleted splenocytes from wild-type mice were adoptively transferred into young *Rag2*$^{-/-}$ mice. The peripheral Treg cells were analyzed before and 8 weeks after the transplantation via flow cytometry. **(A)** Representative histogram of Bim^{+} Tregs in young and aged mice before and after the transfer. **(B)** Relative mean fluorescent intensity (MFI) of Bim within Treg cells before and after the transfer. A Student *t*-test was used to determine statistical significance between groups. All data are expressed as mean ± SEM. There is an (n = 5/group). Data are pooled from at least three independent experiments.

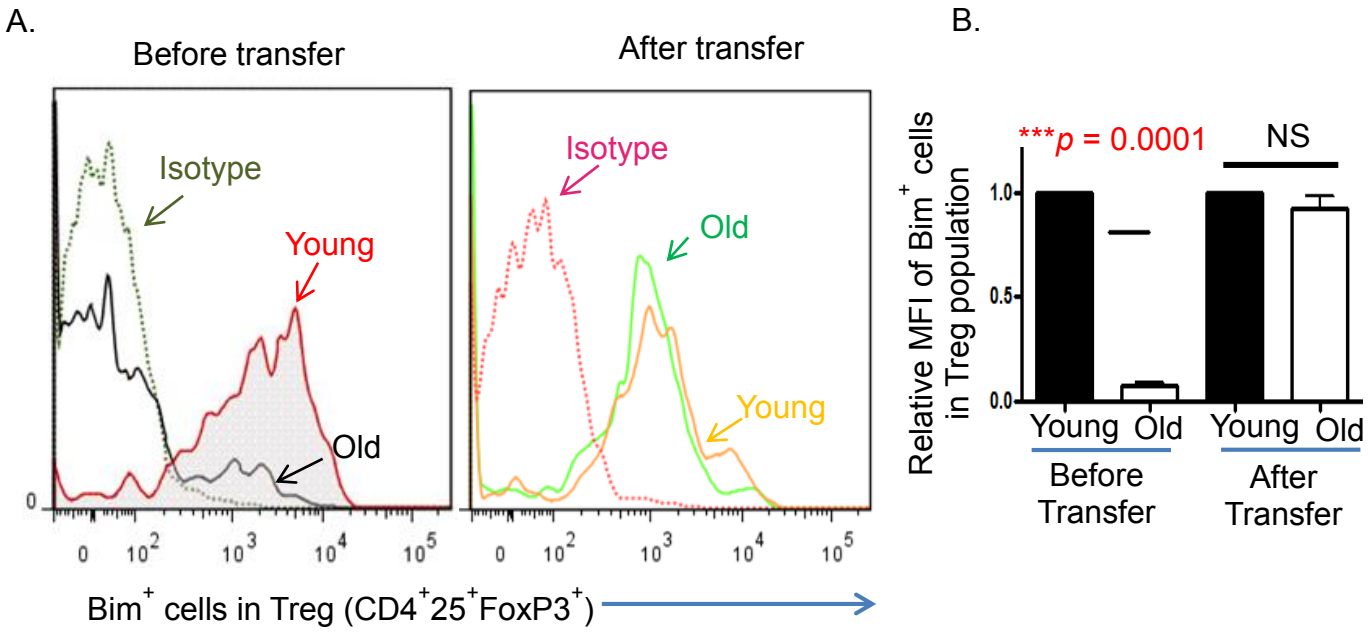
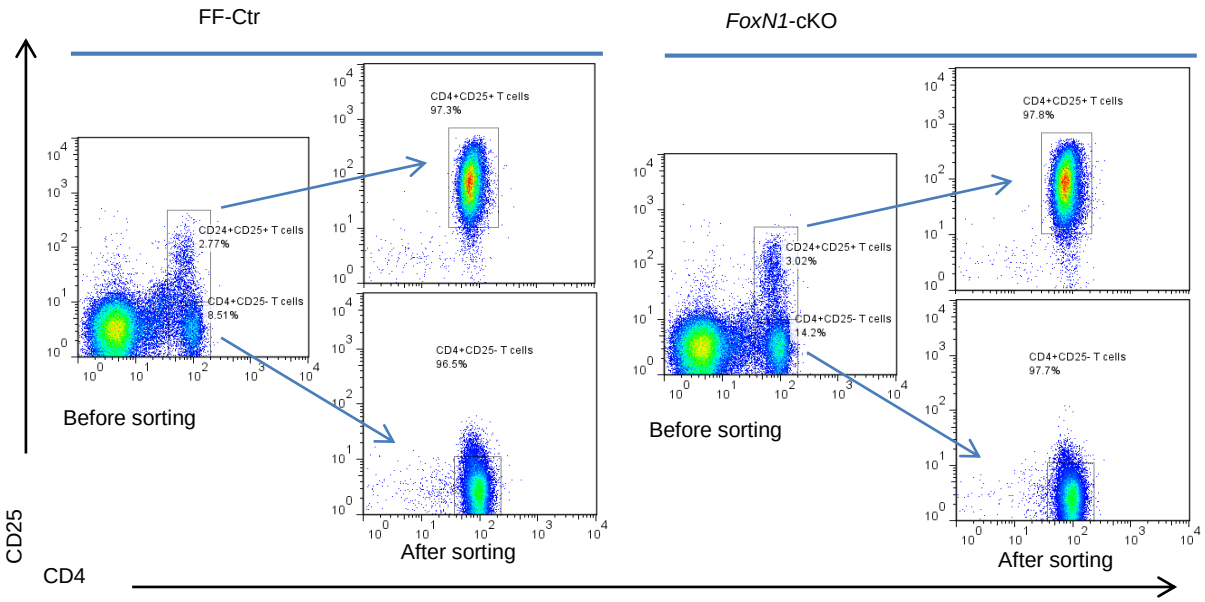


Figure 4. 6: Thymic involution does not impair the function of nTregs. (A) freshly isolated erythrocyte-depleted spleen cells were sorted for Treg-containing ($CD4^+CD25^+$) cells and T effector cells ($CD4^+CD25^-$ Teffs) and then co-cultured in a 2:1 (Teff: Treg) ratio, in the presence of irradiated APCs, anti-CD3, and anti-CD28 to determine the suppressive function of Tregs on Teffs. **(B)** Summarized results show suppressive function of Treg cells from *FoxN1*-cKO (dark bar) and FF-Ctr control mice (light bar), and proliferation of Teff cells from *FoxN1*-cKO (dark striped bar) and FF-Ctr control mice (light striped bar). A Student *t*-test was used to determine statistical significance between groups. All data are expressed as mean ± SEM. Data are pooled from three independent experiments with 3 animals/group.

A.


FF-Ctr
FoxN1-cKO
CD24+CD25+ T cells
2.77%
CD4+CD25- T cells
8.51%
CD4+CD25+ T cells
97.3%
CD4+CD25- T cells
96.5%
Before sorting
After sorting
CD24+CD25+ T cells
3.02%
CD4+CD25- T cells
14.2%
CD4+CD25+ T cells
97.8%
CD4+CD25- T cells
97.7%
Before sorting
After sorting
CD25
CD4

B.

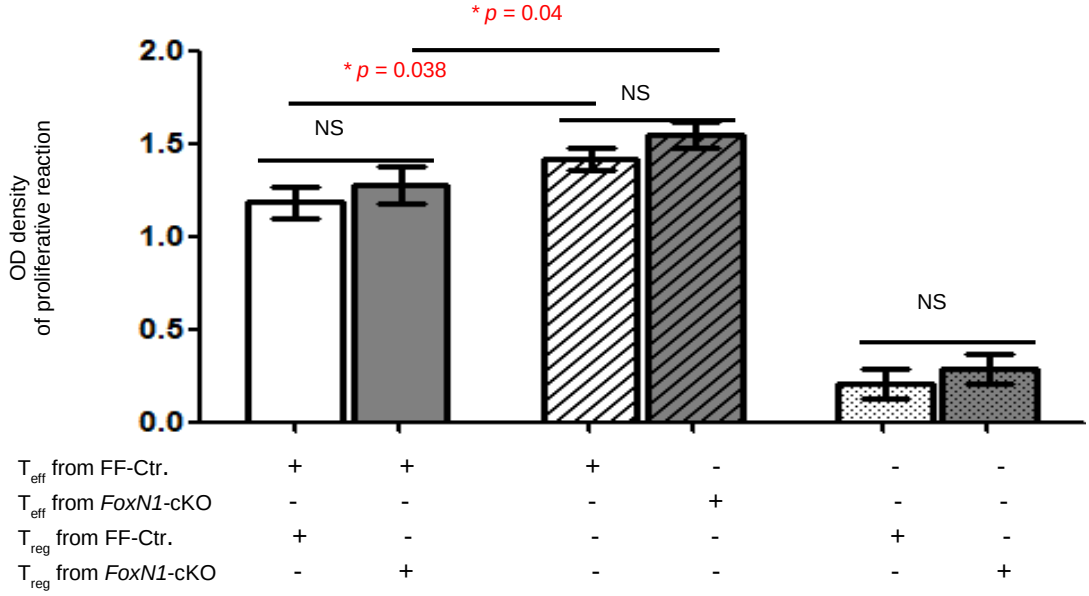
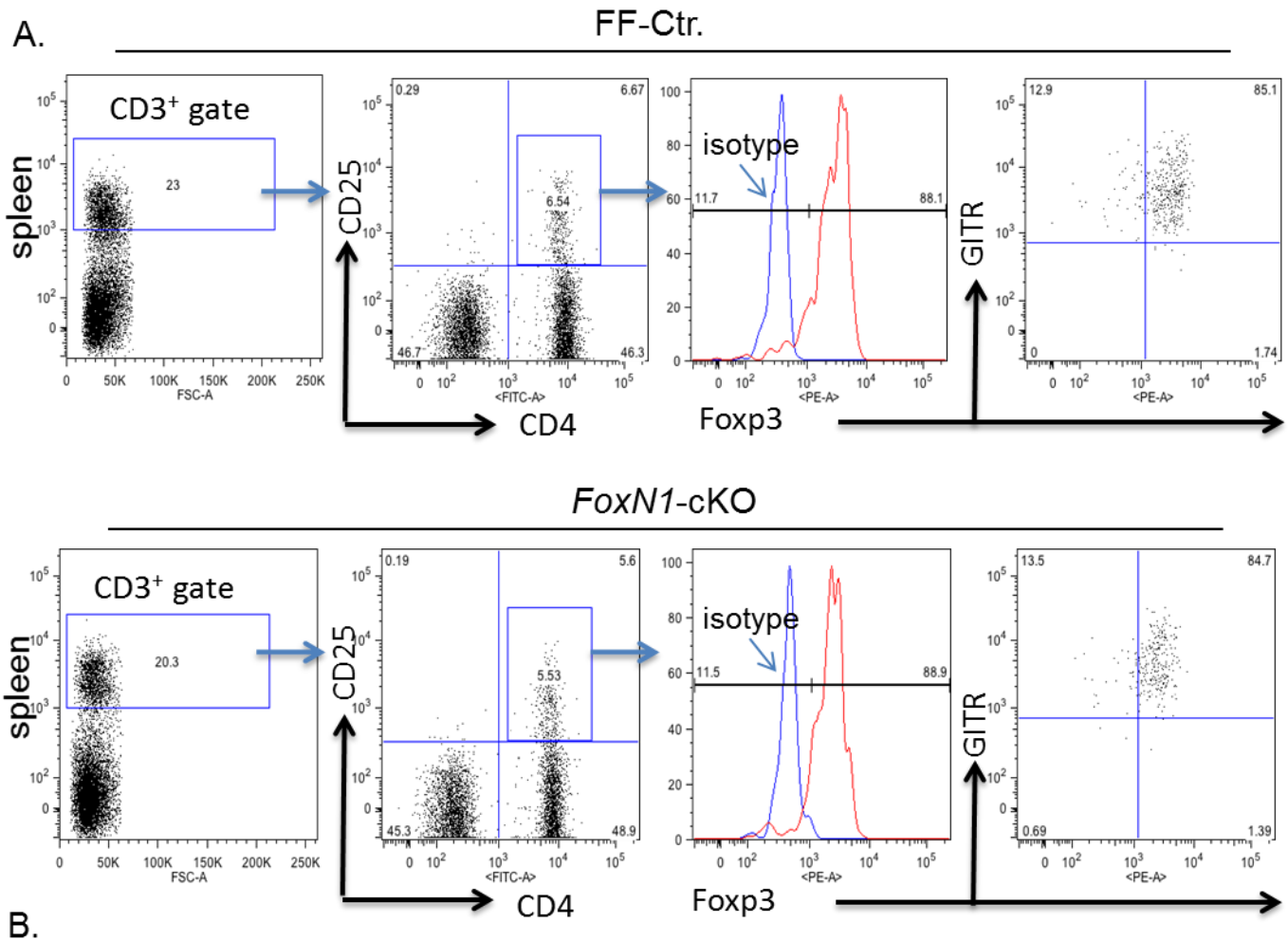
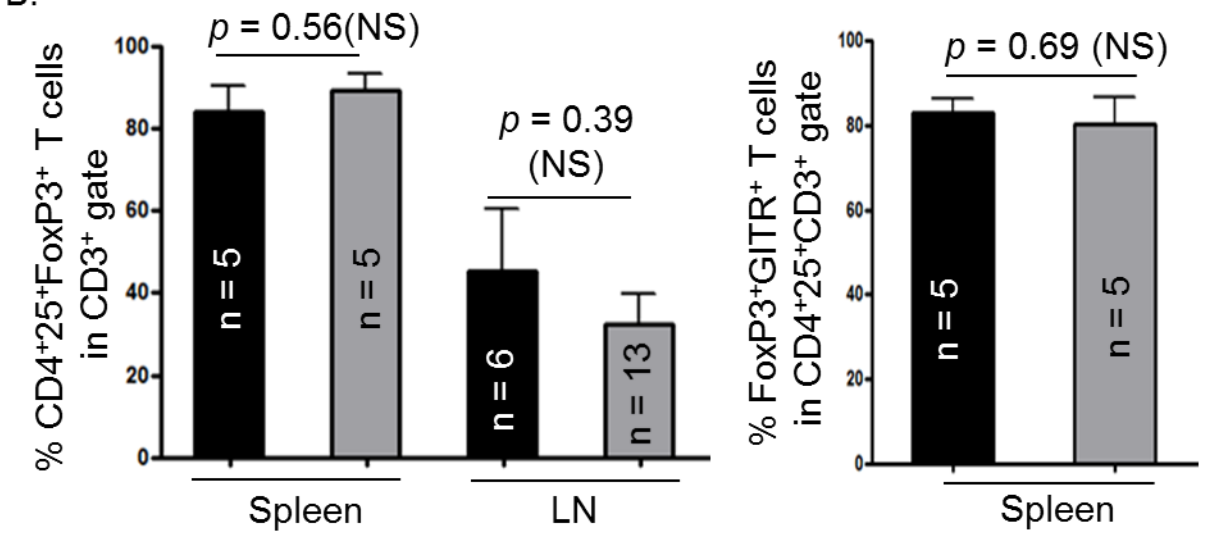

* p = 0.04
* p = 0.038
NS
NS
NS
OD density
of proliferative reaction
2.0
1.5
1.0
0.5
0.0
Teff from FF-Ctr. + + + - - -
Teff from FoxN1-cKO - - - + - -
Treg from FF-Ctr. + - - - + -
Treg from FoxN1-cKO - + - - - +

Figure 4. 7: **Functional markers for $CD4^+$ regulatory T cells in the periphery of FoxN1-cKO mice were not changed. (A)** Representative flow cytometry plots of spleen cells show gating strategy of Treg cells from spleen of *FoxN1*-cKO and FF-Ctr control mice. **(B)** Summarized results of the % $CD4^+CD25^+FoxP3^+$ (left panel), and % $CD4^+FoxP3^+GITR^+$ (right panel) functional Treg cells in the spleen and lymph nodes (LN) from *FoxN1*-cKO (grey bars) and age-matched FF-Ctr control mice (black bars) mice (n = animal numbers, NS = not significant). A Student *t*-test was used to determine statistical significance between groups. All data are expressed as mean ± SEM.


A.
FF-Ctr.
CD3+ gate
spleen
23
FSC-A
CD25
CD4
6.54
0.29
6.67
46.7
46.3
isotype
11.7
88.1
Foxp3
GITR
12.9
85.1
1.74
FoxN1-cKO
20.3
5.53
0.19
5.6
45.3
48.9
11.5
88.9
13.5
84.7
0.69
1.39


B.
p = 0.56(NS)
p = 0.39 (NS)
% CD4+25+FoxP3+ T cells in CD3+ gate
n = 5
n = 5
n = 6
n = 13
Spleen
LN
p = 0.69 (NS)
% FoxP3+GITR+ T cells in CD4+25+CD3+ gate
n = 5
n = 5
Spleen

CHAPTER V

THE PROGRESSIVE LOSS OF *FOXN1* AND SUBSEQUENT THYMIC INVOLUTION DIRECTLY LEADS TO AGE-RELATED CHRONIC INFLAMMATION (INFLAMMAGING)

Chronic inflammation associated with advanced age, termed inflammaging is implicated in virtually every age-related disease [171, 172]. Even in the absence of infection, the elderly have a sustained and measureable, albeit low-level and above the baseline, increase in soluble pro-inflammatory factors [171]. The chronic pro-inflammatory state exacerbates all chronic age-related diseases, and is often considered to be a co-morbidity with neurological conditions, cardiac conditions, and many age-related cancers [173], [174], [171], [175]. Although there is a systemic increase in a myriad of pro-inflammatory cytokines, IL-6 and TNFα are considered to be the most predictive inflammatory biomarkers in determining a poor prognosis, and are associated with "all-cause" morbidity and mortality in the elderly [176]. However, the causes of inflammaging are not fully understood. Because inflammaging is the result of the aging process, there are likely to be a number of processes driving the development of systemic and chronic inflammation. One such process indicated in the development of inflammaging is the persistent activation of immune cells by life-long infections [186, 187]. However, other sources of immune cell activation have not been thoroughly investigated for their role in the development of inflammaging. The focus of this aim

is to determine if thymic involution can lead to the release and persistent activation of T cells that are capable of inducing inflammaging.

Rationale

Persistent immune cell activation has been identified as a key contributor to the emergence of inflammaging. However, the causes of chronic immune cell activation with age have not been clearly defined. Persistent infections and cellular senescence via DAMPS have been hypothesized to chronically stimulate the immune system in the elderly. However, whether the chronic state of age-related autoimmunity contributes to inflammaging has not been investigated. T cell associated autoimmune diseases, like Rheumatoid Arthritis, Psoriasis, and Multiple Sclerosis, cause the production of many of the same pro-inflammatory cytokines associated with inflammaging, including IL-1, IL-6, and TNFα [207-209]. Additionally, thymic involution has been correlated with the increased frequency of autoimmunity observed in the elderly. Furthermore, in specific aim 1, I have demonstrated that thymic involution, induced by the loss of the transcription factor *FoxN1* (*FoxN1*-cKO model), impairs negative selection and leads to the survival of autoreactive thymocytes. If these thymocytes are released to the periphery, then they have the potential to become activated and directly secrete or induce the secretion of pro-inflammatory cytokines in other cell types. Moreover, specific aim 1 identified the decreased expression of AIRE in *FoxN1*-cKO mice. The loss of AIRE in both mice and humans (APS1 syndrome) has been linked to systemic autoimmunity, chronic hepatitis, and multi-organ inflammation. Not only is thymic involution associated with autoimmune inflammation, but the process of thymic involution itself is characterized by chronic IL-1 inflammation related to adipose accumulation [165]. Thus, it is important to investigate the role of thymic involution in the emergence of age-related

inflammation. However, whether thymic involution alone, without further stimuli, is capable of generating a pro-inflammatory environment is not known. By using the *FoxN1*-cKO mouse model, I can elucidate the role of thymic involution, without additional stimuli, on the development of inflammaging. The focus of this aim is to determine if thymic involution alone is sufficient to induce an autoimmune phenotype that is capable of driving inflammaging.

Specific Aim 3

To determine if the progressive loss of *FoxN1* and subsequent thymic involution directly leads to inflammaging

Results

Newly released T cells from the a*trophied thymus acquire an activated immune cell phenotype*

One potential cause for inflammaging is the persistent activation of immune cells in the absence of overt infection. Thymic involution has been hypothesized to increase the susceptibility to autoimmune diseases that are characterized by the activation of autoreactive T cells [158, 192, 199]. To investigate the potential for the progressive loss of *FoxN1* and thymic involution to directly lead to an activated T cell phenotype in the absence of additional stimuli, markers for activation (CD44) and proliferation (Ki67) were assessed on T cells that have recently exited the involuted thymus. In order to differentiate T cells that have egressed from the involuted thymus versus those that had egressed prior to thymic involution, we crossed our *FoxN1*-cKO mice with *Rag2*-promoter driven GFP expressing mice, which identifies newly generated T cells, or recent thymic emigrants (RTEs) [210-212]. The GFP signal is turned on in thymocytes undergoing

recombination-activating gene (*RAG*) dependent TCR recombination, and persists for up to two weeks following thymic egress [211].

Two weeks following the induction of thymic involution in *FoxN1*-cKO-RagGFP mice, splenocytes were harvested for analysis by flow cytometry. As to be expected, using tamoxifen to conditionally knockout the *FoxN1* gene in young *FoxN1*-cKO mice to induce accelerated thymic atrophy [54] resulted in fewer $CD4^+$ and $CD8^+$ RTEs in the spleen due to decreased thymic output (Figure 5. 1A, box in top-left panel, and Figure 5.2 A.). However, within this reduced population of *FoxN1*-cKO CD4 RTEs ($CD4^+GFP^+$) and CD8 RTEs ($CD8^+GFP^{+)}$ there was an elevated proportion of $CD44^{hi}$ cells (Figure 5.1 B-C & E and 5.2 B-C & E), which likely represent antigen-experienced T cells [213], and these *FoxN1*-cKO RTEs were more proliferative, as determined by intracellular Ki67 staining (Figure 5.1 D and 5.2 D). We further established that the RTEs undergoing proliferation ($Ki67^+$) were $CD44^{hi}$RTEs (Figs. 5.1 B, red boxes, and Fig. 5.2 B, red boxes). These data demonstrate that RTEs that have undergone thymocyte development in an involuted thymus quickly adopt an activated immune cell phenotype shortly after interacting with the peripheral environment.

Thymic involution releases autoreactive T cells into the periphery

Seeing as *FoxN1*-cKO mice are not noticeably infected with any pathogens, the most likely situation for the activation of RTEs upon encountering the peripheral environment is a response to self-peptide. To determine if the involuted thymus releases T cells that respond to peripheral self-antigens, we employed an IRBP (interstitial retinol-binding protein) immunization model to detect and amplify autoreactive T cell clones within a polyclonal T cell repertoire. IRBP is an eye protein

expressed in mTECs as a tissue-specific antigen (TSA) under the control of the autoimmune regulator gene *AIRE* [197, 214-216]. Autoreactive T cells can be detected in the spleen and lymph nodes of AIRE-deficient mice following specific tetramer enrichment [94]. In order to expand and detect autoreactive T cells, I first induced thymic involution with the TM-induced deletion of *FoxN1* in the *FoxN1*-cKO mice. One month following induction of thymic involution, *FoxN1*-cKO, FF-Ctr, and $AIRE^{-/-}$ (positive control) mice were immunized with an IRBP peptide-2 epitope (P2) and 10 days later lymph nodes and spleen were pooled to enumerate $CD4^+$ T cells reactive to the P2 peptide by P2-I-A^b tetramer enrichment (Figure 5.3 A). A significant expansion of activated $CD4^+CD44^+$ P2-I-A^{b+} autoreactive T cells were detected in the periphery of *FoxN1*-cKO mice, almost as strongly as the $AIRE^{-/-}$ positive control (Figure 5.3 B and C). However, these autoreactive clones were mostly undetectable in littermate FF-Ctr controls (Figure 5.3 B and C). This suggests that the source of activated RTEs in our *FoxN1*-cKO thymic involution model are autoreactive T cell clones responding to peripheral self-antigens.

The FoxN1-cKO involuted thymus leads to autoimmunity

The detection of IRBP specific T cells in the periphery of *FoxN1*-cKO mice confirms that the loss of *FoxN1* and subsequent thymic involution releases T cells that recognize self-tissue. However, in order to detect autoreactive T cells, the IRBP model requires a 100ug peripheral immunization with the self-peptide and a strong stimuli in the form of Complete Freund's Adjuvant (Figure 5.3). To determine if thymic involution induced by the loss of *FoxN1* is sufficient to drive autoimmunity without the need for additional stimuli, middle-aged *FoxN1*-cKO mice were analyzed for inflammatory infiltration into non-lymphoid organs. Paraffin sections of liver, lung,

and salivary organs were stained with H&E. Inflammatory infiltrates were detected in the lung (38% positive), liver (57% positive), and salivary glands (71% positive) of *FoxN1*-cKo mice, but were mostly absent in the FF-Ctr group (Figure 5. 4 A-liver, B-lung, C-salivary gland).

One hallmark of age-related autoimmunity is the circulation of autoantibodies [217, 218]. Furthermore, AIRE-deficient mice and APS1-patients are characterized by increased serum autoantibodies [92, 93]. Because *FoxN1*-cKO mice have an AIRE deficiency, it is likely that they also produce serum autoantibody. Sera from 9-month-old middle-aged *FoxN1*-cKO (slow leakage of *cre*) and age-matched FF-Ctr mice were analyzed for the detection of anti-nuclear antibody (ANA). There was a significant increase in the concentration of ANA was in the serum of *FoxN1*-cKO mice (Figure 5. 5). In order to establish the organs affected by the ANAs, frozen sections of non-lymphoid organs (lacrimal gland, salivary gland, pancreas, prostate, retina, ovary, and testicle) from *Rag2*$^{-/-}$ mice (lack B cells and thus lack their own antibodies) were incubated with ANAs from 9-month-old *FoxN1*-cKO or age-matched FF-Ctr mice. ANAs deposited mostly in the salivary and lacrimal glands, the prostate, the ovaries, and the testicles (Figure 5. 6). These data indicate that thymic involution leads to autoimmunity and inflammatory infiltration.

The data shown in Figures 5.1-.6 establish the emergence of autoimmunity following thymic involution induced by the loss of *FoxN1*. However, the data presented in specific aim 2 indicated that the increased frequency of thymic nTregs does not persist into the periphery of *FoxN1*-cKO mice, and the age-related accumulation of peripheral Tregs can be reversed by the young microenvironment. Therefore, whether inflammatory infiltration is an intrinsic defect among T cells derived from an involuted thymus or dependent on age-related changes to the peripheral environment is unclear. In order to determine if inflammatory infiltration can be reversed by the young microenvironment, I performed an adoptive transfer of aged (18-22 month)

C57BL/6 and young (6 week) C57BL/6 into young (6 week) *Rag2* $^{-/-}$ mice. Histological sections of the salivary gland show that *Rag2*$^{-/-}$ hosts receiving either young C57BL/6 appeared generally healthy, however, *Rag2*$^{-/-}$ hosts receiving splenocytes from aged C57Bl/6 donors showed inflammatory foci around the blood vessels (Figure 5.7 A left panel) The severity of inflammatory infiltration progressively increased with the increasing age of the C57BL/6 donors. The 18 month donor was more likely to present with a single inflammatory foci, and hosts given donor cells from mice >22 month old were likely to display multi-foci infiltration (Figure 5.7 A middle-18 month, right - >22 month).

To eliminate the effect of peripheral age-related factors (thymus independent) on inflammatory infiltration, a pool of T cells from young *FoxN1*-cKO (TM-induced) or FF-Ctr splenocytes were adoptively transferred into young *Rag2*$^{-/-}$ mice. Although *FoxN1*-cKO mice lack any peripheral age-related modifications not directly resulting from thymic involution, we found inflammatory cell infiltration in the salivary glands of the young *Rag2*$^{-/-}$ host mice infused with *FoxN1*-cKO splenocytes, but infiltration was not observed in hosts infused with FF-Ctr splenocytes (Figure 5.7 B). Taken together, these data suggest that inflammatory infiltration is an intrinsic defect in T cells imprinted during development in the involuted thymus. Unlike peripherally controlled age-related accumulation of Tregs (specific aim 2), the young peripheral environment cannot reverse age-related inflammatory infiltration into the salivary gland.

Low-grade pro-inflammatory state following thymic involution

In addition to increased autoimmunity, natural aging is accompanied by an increase in inflammation, known as inflammaging. Inflammaging is characterized by a state of systemic, low-grade, and chronic pro-inflammatory mediators. Age-related inflammation partly arises from the persistent activation of immune cells [171, 172, 180]. Additionally, we have previously shown that

thymic involution produces T cells with an activated immune cell phenotype (Figure 5.1). In order to determine if thymic involution leads to an age-related pro-inflammatory state, I measured the most predictive cytokines of poor prognosis: IL-6 and TNFα [176]. Serum IL-6 concentrations were measured with ELISA, and splenic $CD4^{+}$TNFα and $CD8^{+I}$FNγ producing T cells were quantified with intracellular staining after *ex vivo* culture following stimulation with αCD3 and αCD28. One month following induction of thymic involution in *FoxN1*-cKO mice, the percentages of $CD4^{+}$TNFα and $CD8^{+}$IFNγ producing T cells were found to be approximately 1.5-fold higher in *FoxN1*-cKO mice compared to FF-Ctr mice (Figure 5.8 A & B). Additionally, a nearly two-fold increase in IL-6 concentration was observed (Figure 5.8 C), implicating thymic involution in both local and systemic inflammation. This low-grade (slightly above baseline; but significant) inflammatory state found in the periphery of *FoxN1*-cKO mice is consistent with the conditions described in inflammaging.

Summary

Inflammaging is a permeating feature of the overall aging process, with an etiology that has yet to be fully elucidated. In this study, I have identified thymic involution as a contributing source of the pro-inflammatory environment associated with advanced age. By using the *FoxN1*-cKO mouse model, I was able to determine the effects of thymic involution on the emergence of inflammaging separately from the myriad of changes that occur during the total aging process. Immune cell activation has been identified as one likely cause of inflammaging, although the forces driving immune cell activation have not been clearly defined. Here, I demonstrated that thymic involution alone, induced by the loss of *FoxN1*, leads to an activated immune cell phenotype. Both CD4 and CD8 RTEs that have egressed from the atrophied thymus adopt an

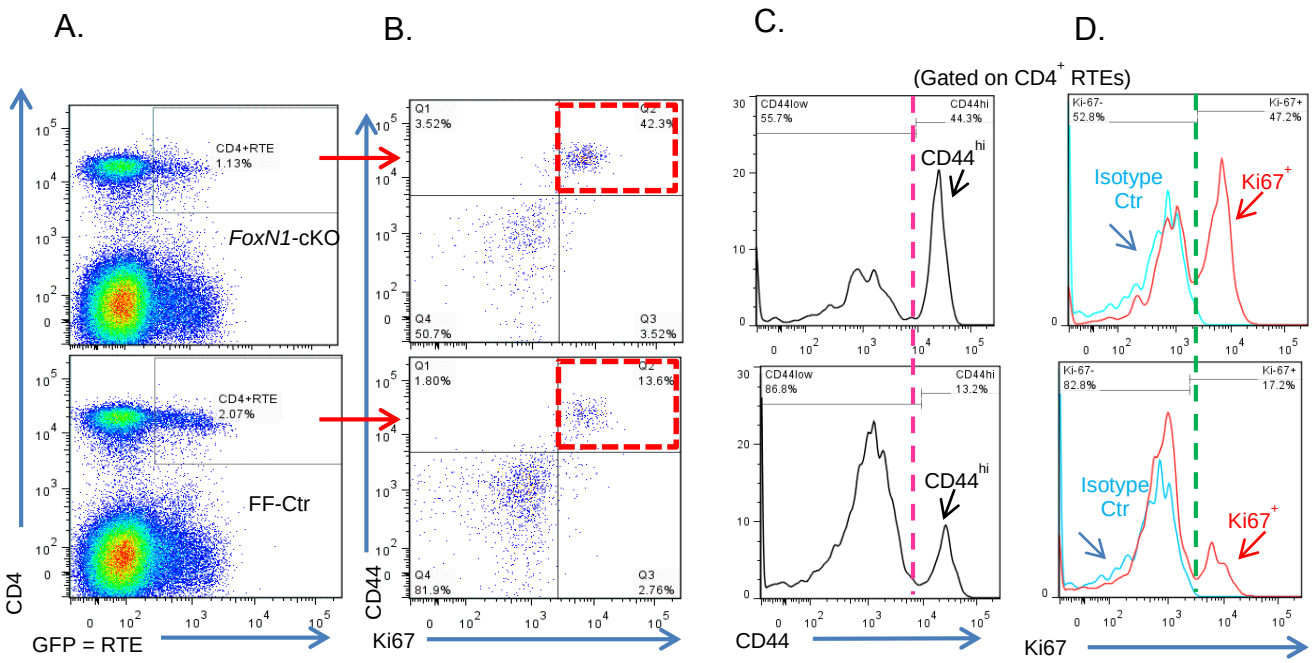
activated phenotype once they encounter the periphery. More of these *FoxN1*-cKO RTEs display the $CD44^{hi}$ surface marker, which usually denotes that the T cell has come into contact with its cognate antigen, and more undergo proliferation at an increased rate compared to FF-Ctr mice (Figure 5.1 and 5.2). Immune cell activation requires TCR stimulation via MHC loaded with the cognate antigen. Because the *FoxN1*-cKO mice are housed in a clean environment and devoid of any foreign pathogens, self-antigens were the mostly likely source of TCR stimulation. By immunizing *FoxN1*-cKO mice with the mouse eye antigen IRBP, I demonstrated that autoreactive T cells escape the involuted thymus and survive in the periphery. FF-Ctr mice harbor very few, often zero, IRBP-specific T cells. However, *FoxN1*-cKO mice contain activated IRBP-specific T cells in their lymph nodes and spleen following immunization. The numbers of IRBP-specific T cells enumerated in the *FoxN1*-cKO mice following IRBP immunization are comparable to the $AIRE^{-/-}$ positive control (Figure 5.3). These data, taken together with the AIRE-dependent negative selection defects observed in specific aim 1, indicate that thymic involution, driven by the loss of *FoxN1,* perturbs clonal deletion and leads to autoimmunity.

Next, I sought to determine the extent of autoimmunity in the *FoxN1*-cKO mice. Without the need for any stimulation, *FoxN1*-cKO mice naturally develop inflammatory infiltration into the liver, lung, and salivary gland (Figure 5.4). The salivary gland is the organ most affected, with 71% of *FoxN1*-cKO mice developing infiltration compared to 0% of the FF-Ctr mice. This correlates with the age-related development of Sjögren's Syndrome that is often seen in the elderly [97]. Another hallmark of age-related autoimmunity is the prevalence of antinuclear antibodies in the serum of elderly individuals. *FoxN1*-cKO mice displayed an increased concentration of ANAs in their serum (Figure 5.5). These ANAs were reactive to various non-lymphoid organs, including the salivary and lacrimal gland, further indicating a role for thymic involution in the development

of Sjögren's Syndrome and other age-related autoimmune diseases (Figure 5.6). Because age-related Treg accumulation was found to be extrinsically controlled in specific aim 2, I next wanted to determine if inflammatory infiltration was reversible by the young peripheral environment. Adoptive transfer of aged wild-type splenocytes into the young *Rag2*$^{-/-}$ periphery resulted in inflammatory infiltration into the salivary gland. Interestingly, the inflammatory infiltration progressively worsened with increasing donor age (Figure 5.7). Additionally, adoptive transfer of *FoxN1*-cKO splenocytes into young *Rag2*$^{-/-}$ mice results in inflammatory infiltration, while FF-Ctr mice remained healthy. These data suggest that inflammatory infiltration results from intrinsic defects related to development in the involuted thymus. The extrinsic factors associated with the young peripheral environment cannot reverse the inflammatory infiltration. Therefore, thymic involution alone is sufficient to induce autoimmunity.

Finally, I wanted to determine if the autoimmunity associated with thymic involution was sufficient to induce the pro-inflammatory cytokine environment associated with inflammaging. T cell production of TNFα and IFNγ were elevated in *FoxN1*-cKO mice, and the concentration of IL-6 in the serum of *FoxN1*-cKO mice was elevated. These data demonstrate that thymic involution alone is sufficient to induce the heightened production of pro-inflammatory cytokines that are a hallmark of inflammaging. Furthermore, these data implicate the loss of *FoxN1* in the emergence of age-related autoimmunity and inflammaging. Thus, *FoxN1* has been identified as a potential therapeutic target for the treatment of systemic age-related autoimmunity and chronic inflammation.

Figure 5. 1: Newly released CD4$^+$ T cells from the atrophied thymus acquired an activated immune cell phenotype. *FoxN1*-cKO and FF-Ctr mice were crossed with *Rag2*-GFP mice and then *FoxN1*$^{fx/fx}$ deletion was induced in 6-week-old adult mice with i.p. injected tamoxifen (TM). 14 days later, peripheral splenocytes were freshly isolated and stained with CD4, CD44, and Ki67 antibodies, and CD4$^+$GFP$^+$ cells were defined as CD4$^+$ RTEs. **(A)** Representative dot plots show CD4+GFP+ RTEs **(B)** CD44^{hi}Ki67$^+$ cell gates (red boxes) in CD4$^+$ RTEs from *FoxN1*-cKO (top panels) and FF-Ctr control (bottom panels) mice. **(C)** Representative histograms of CD44^{hi} (left and **(D)** Ki67$^+$ in CD4$^+$ RTEs from *FoxN1*-cKO (top panels) and FF-Ctr control (bottom panels) mice. **(E)** Summarized results of % CD44^{hi}Ki67$^+$, CD44^{hi}, and Ki67$^+$ cells (from left to right panels). A Student *t*-test was used to determine statistical significance between groups. All data are expressed as mean ± SEM. Data are pooled from at least three independent experiments (n = animal numbers).


A.
B.
C.
D.
CD4+RTE
1.13%
FoxN1-cKO
CD4+RTE
2.07%
FF-Ctr
CD4
GFP = RTE
Q1
3.52%
Q2
42.3%
Q4
50.7%
Q3
3.52%
Q1
1.80%
Q2
13.6%
Q4
81.9%
Q3
2.76%
CD44
Ki67
(Gated on CD4+ RTEs)
CD44low
55.7%
CD44hi
44.3%
CD44hi
CD44low
86.8%
CD44hi
13.2%
CD44hi
CD44
Ki-67-
52.8%
Ki-67+
47.2%
Isotype
Ctr
Ki67+
Ki-67-
82.8%
Ki-67+
17.2%
Isotype
Ctr
Ki67+
Ki67

E.

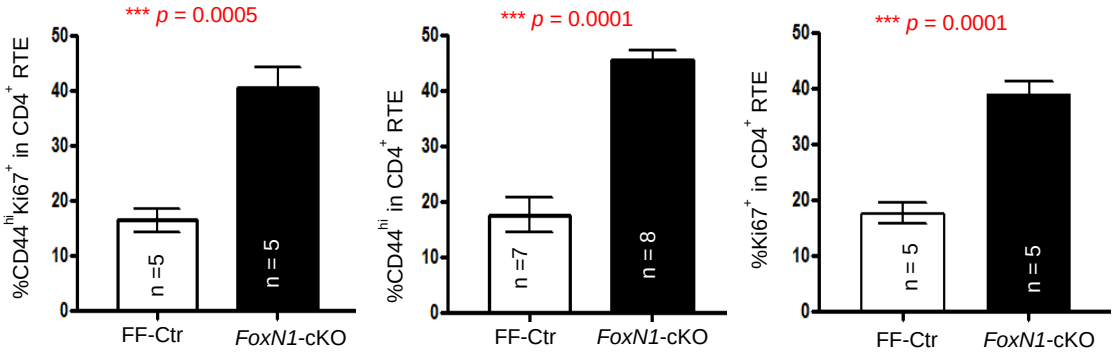
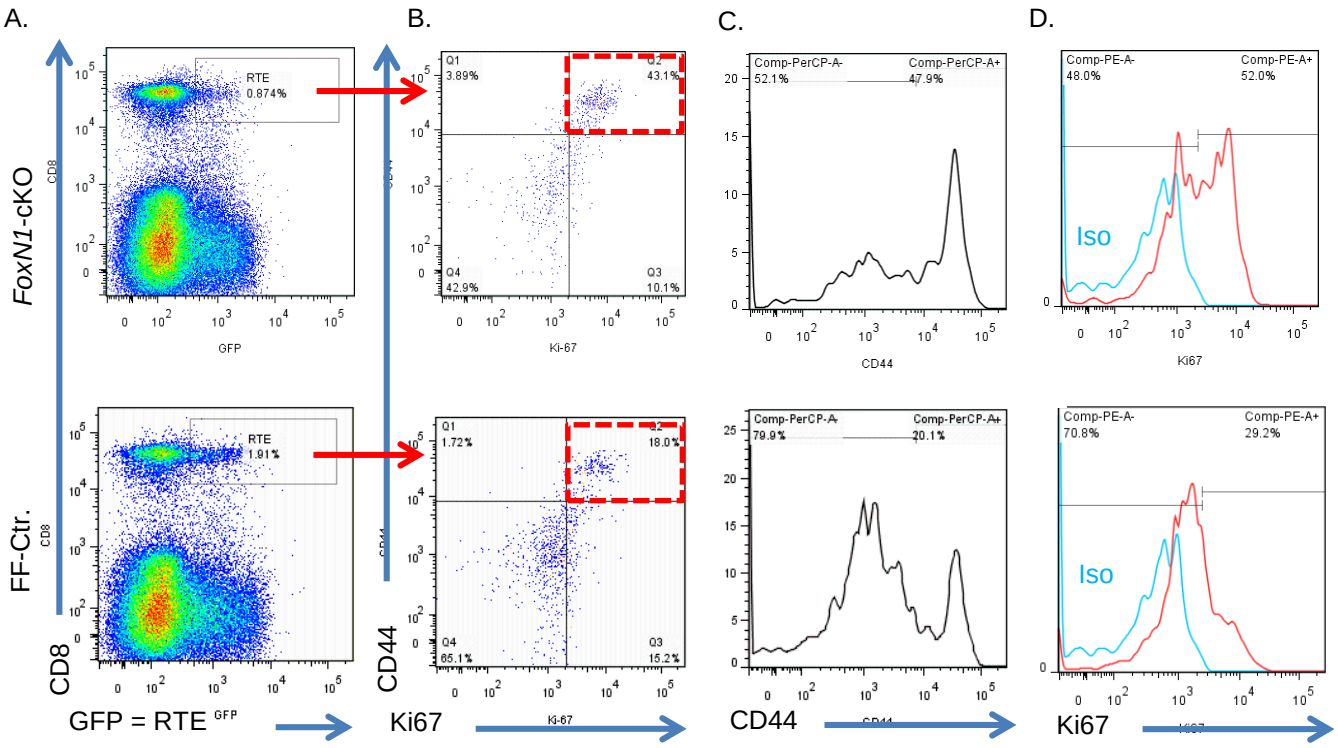

*** p = 0.0005
%CD44hiKi67+ in CD4+ RTE
n =5
n = 5
FF-Ctr
FoxN1-cKO
*** p = 0.0001
%CD44hi in CD4+ RTE
n =7
n = 8
FF-Ctr
FoxN1-cKO
*** p = 0.0001
%Ki67+ in CD4+ RTE
n = 5
n = 5
FF-Ctr
FoxN1-cKO

Figure 5. 2: Newly released CD8$^+$ T cells from the atrophied thymus acquired an activated immune cell phenotype. *FoxN1*-cKO and FF-Ctr mice were crossed with *Rag2*-GFP mice and then *FoxN1*$^{fx/fx}$ deletion was induced in 6-week-old adult mice with i.p. injected tamoxifen (TM). 14 days later, peripheral splenocytes were freshly isolated and stained with CD8, CD44, and Ki67 antibodies, and CD8$^+$GFP$^+$ cells were defined as CD8$^+$ RTEs. **(A)** Representative dot plots show CD8+GFP+ RTEs **(B)** CD44^{hi}Ki67$^+$ cell gates (red boxes) in CD8$^+$ RTEs from *FoxN1*-cKO (top panels) and FF-Ctr control (bottom panels) mice. **(C)** Representative histograms of CD44^{hi} (left and **(D)** Ki67$^+$ in CD8$^+$ RTEs from *FoxN1*-cKO (top panels) and FF-Ctr control (bottom panels) mice. **(E)** Summarized results of % CD44^{hi}Ki67$^+$, CD44^{hi}, and Ki67$^+$ cells (from left to right panels). A Student *t*-test was used to determine statistical significance between groups. All data are expressed as mean ± SEM. Data are pooled from at least three independent experiments (n = 4 per group).


A.
B.
C.
D.
FoxN1-cKO
FF-Ctr.
CD8
GFP
RTE
0.874%
RTE
1.91%
GFP = RTE GFP
CD44
Ki-67
Q1
3.89%
Q2
43.1%
Q4
42.9%
Q3
10.1%
Q1
1.72%
Q2
18.0%
Q4
65.1%
Q3
15.2%
Ki67
Comp-PerCP-A-
52.1%
Comp-PerCP-A+
47.9%
Comp-PerCP-A-
79.9%
Comp-PerCP-A+
20.1%
CD44
Comp-PE-A-
48.0%
Comp-PE-A+
52.0%
Comp-PE-A-
70.8%
Comp-PE-A+
29.2%
Iso
Iso
Ki67

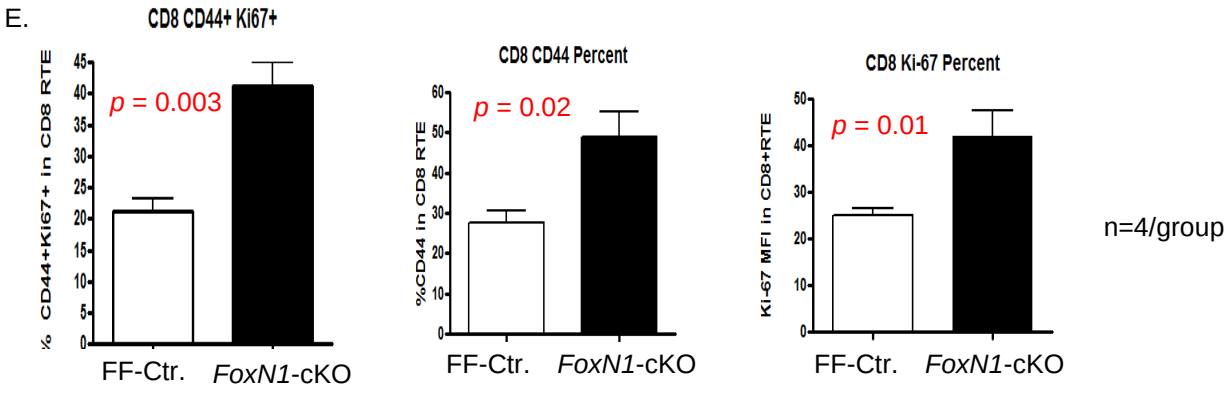
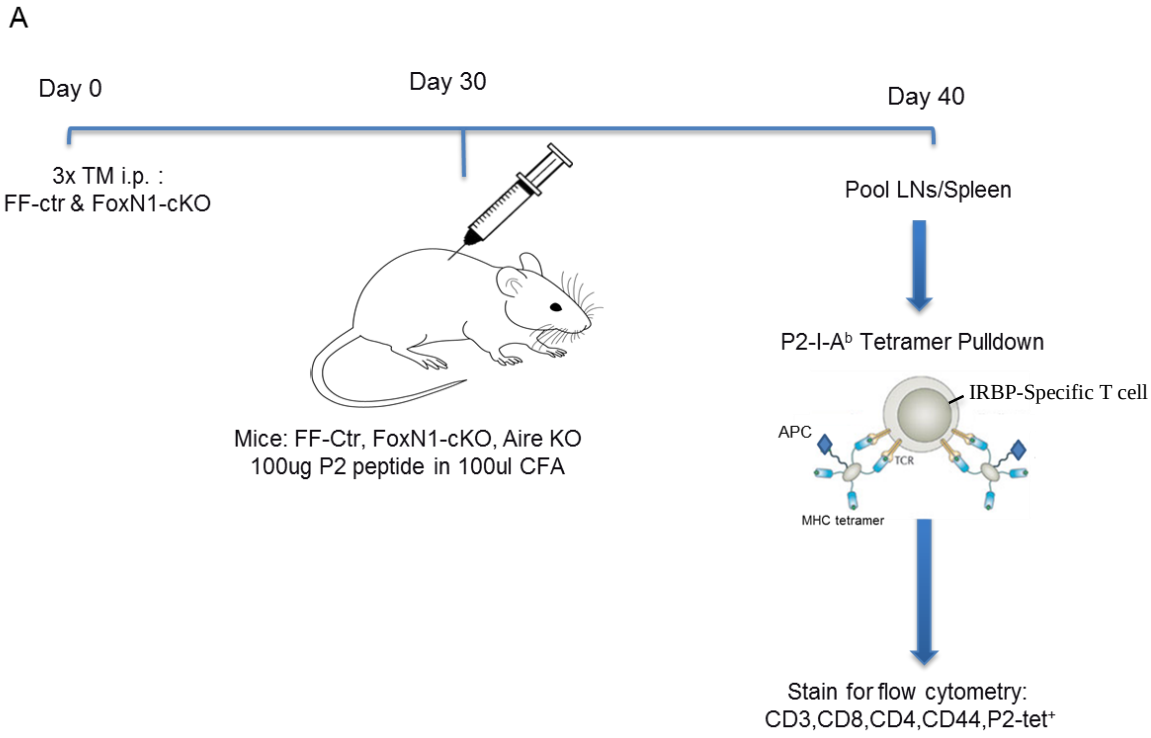

E.
CD8 CD44+ Ki67+
% CD44+Ki67+ in CD8 RTE
p = 0.003
FF-Ctr.
FoxN1-cKO
CD8 CD44 Percent
%CD44 in CD8 RTE
p = 0.02
FF-Ctr.
FoxN1-cKO
CD8 Ki-67 Percent
KI-67 MFI in CD8+RTE
p = 0.01
FF-Ctr.
FoxN1-cKO
n=4/group

Figure 5. 3: Autoreactive T cells are released following *FoxN1*-cKO induced thymic involution. **(A)** Schematic illustration details experimental design: One month following TM treatment for the conditional knockout of *FoxN1*, *FoxN1*-cKO, FF-Ctr, and *AIRE*$^{-/-}$ (positive control) mice were immunized with IRBP P2 peptide and lymphocytes were harvested 10 days later for flow cytometry following P2 tetramer-pulldown enrichment of specific T cells. **(B)** Plots are pre-gated on CD3^{+}CD4^{+} events, and the CD44^{hi}P2-tetramer^{+} population is displayed in red boxes. **(C)** Calculated absolute number of P2 specific CD3^{+}CD4^{+}CD44^{hi} peripheral T cells is shown in a bar format. A Student *t*-test was used to determine statistical significance between groups. All data are expressed as mean ± SEM. Data are pooled from three independent experiments (each symbol represents an individual animal).



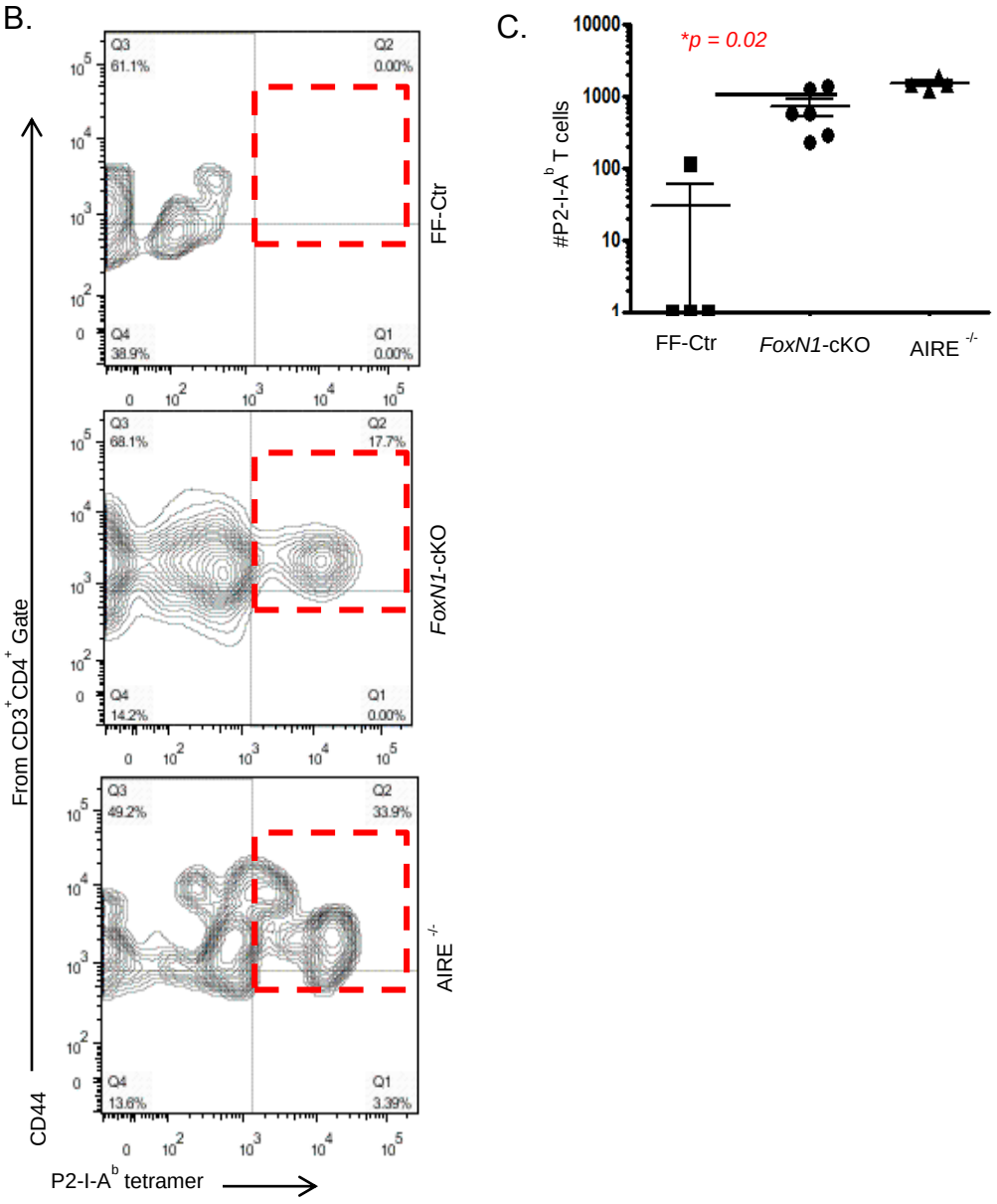
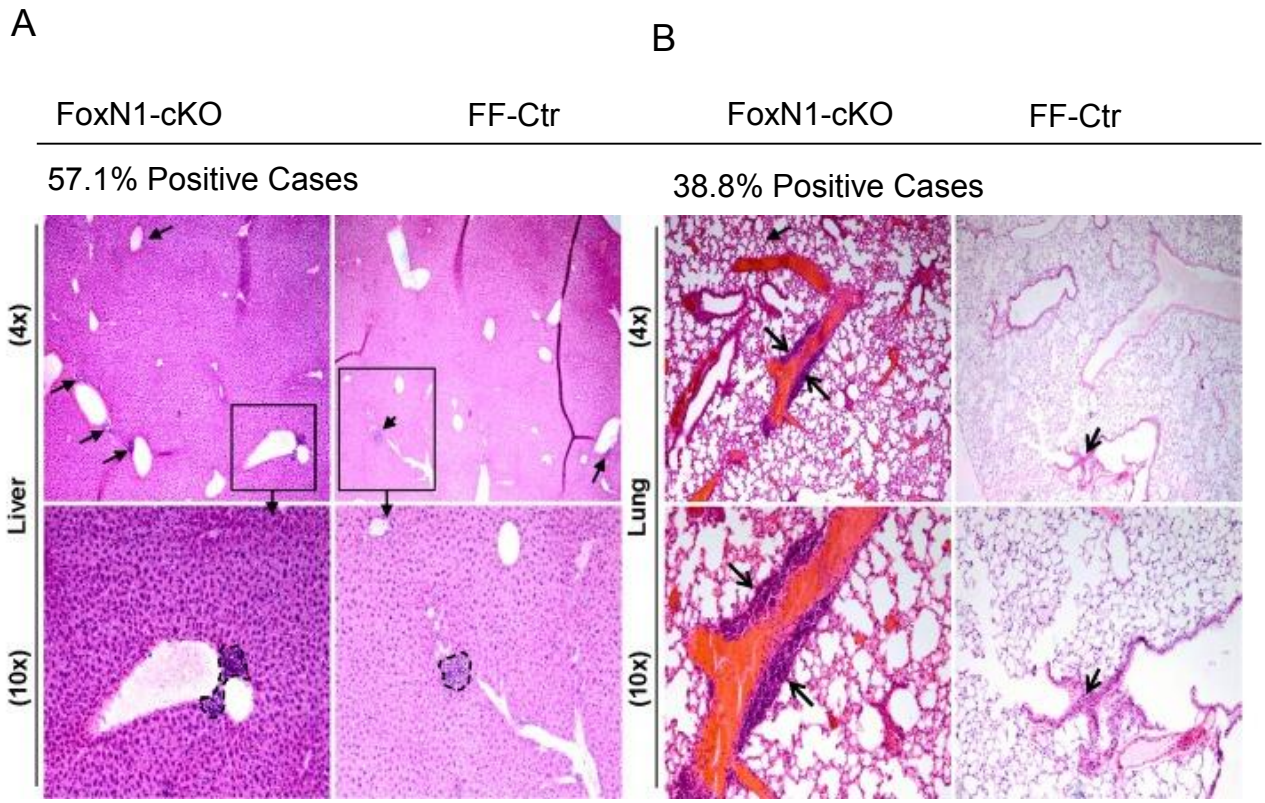

B.
Q3
61.1%
Q2
0.00%
Q4
38.9%
Q1
0.00%
FF-Ctr
Q3
68.1%
Q2
17.7%
Q4
14.2%
Q1
0.00%
FoxN1-cKO
Q3
49.2%
Q2
33.9%
Q4
13.6%
Q1
3.39%
AIRE -/-
From CD3+CD4+ Gate
CD44
P2-I-Ab tetramer
C.
*p = 0.02
#P2-I-Ab T cells
10000
1000
100
10
1
FF-Ctr
FoxN1-cKO
AIRE -/-

Figure 5. 4: Multiple organ inflammatory infiltration in middle-aged *FoxN1*-cKO mice. **(A)** Representative H&E staining shows inflammatory cell infiltration in the livers of middle-aged 9-month-old *FoxN1*-cKO (left panel) and FF-Ctr mice (right panel), at different magnifications. Arrows indicate the inflammatory infiltrated cell clusters. **(B)** Representative H&E staining shows inflammatory cell infiltration in the lungs of middle-aged 9-month-old *FoxN1*-cKO (left panel) and FF-Ctr mice (right panel). Arrows indicate the infiltrated area. **(C)** Representative H&E staining shows inflammatory cell infiltration in the salivary glands of middle-aged 9-month-old *FoxN1*-cKO (left panel) and FF-Ctr mice (right panel). Arrows indicate the inflammatory infiltrated cell clusters. For all groups, mice that displayed infiltration are denoted as "positive cases."

A

B


FoxN1-cKO
FF-Ctr
FoxN1-cKO
FF-Ctr
57.1% Positive Cases
38.8% Positive Cases
(4x)
Liver
(10x)
(4x)
Lung
(10x)

C

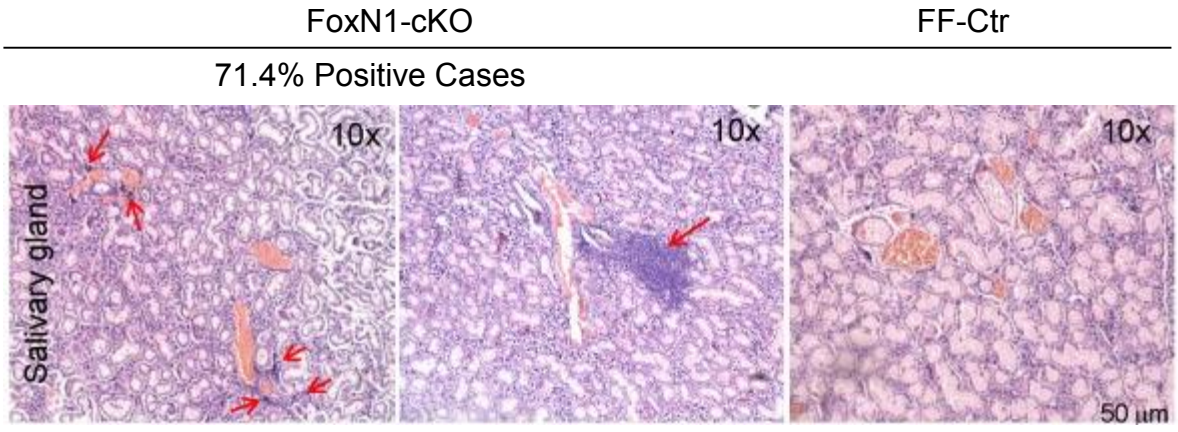

FoxN1-cKO
FF-Ctr
71.4% Positive Cases
10x
10x
10x
Salivary gland
50 µm

Figure 5. 5: Antinuclear antibodies were significantly increased in the serum of *FoxN1*-cKO mice. Antinuclear antibody (ANA) levels in 9-month-old middle-aged *FoxN1*-cKO (open circles) and age-matched FF-Ctr mice (black squares) measured by ELISA. Each symbol represents one animal. Statistical significance ($p < 0.05$) was analyzed by unpaired two-tailed Student's *t*-test between the two groups. Data show Mean ± SEM.

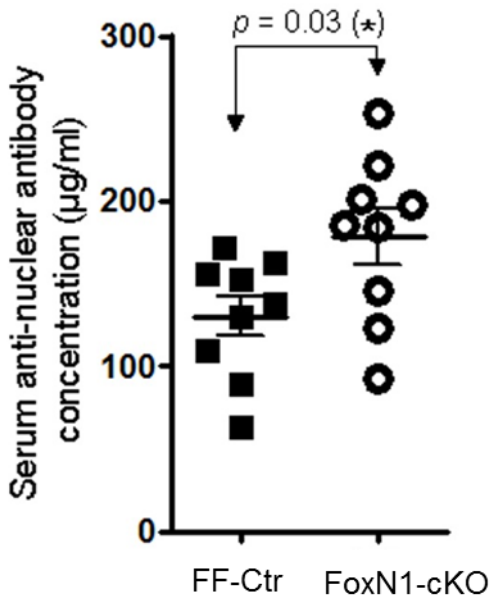


Figure 5. 6: Autoantibodies deposit onto multiple non-lymphoid organs in *FoxN1*-cKO mice. Representative immunofluorescence staining depicts sera (1:100 dilutions) from early middle-aged (9-month-old) *FoxN1*-cKO and age-matched FF-Ctr mice incubated with different organ tissues from young *Rag2*$^{-/-}$ mice. Red = positive for autoantibody deposition; blue = counterstained with DAPI. Scale = 50 μm. Data is representative of 2-3 independent replicates per group, with essentially identical results.

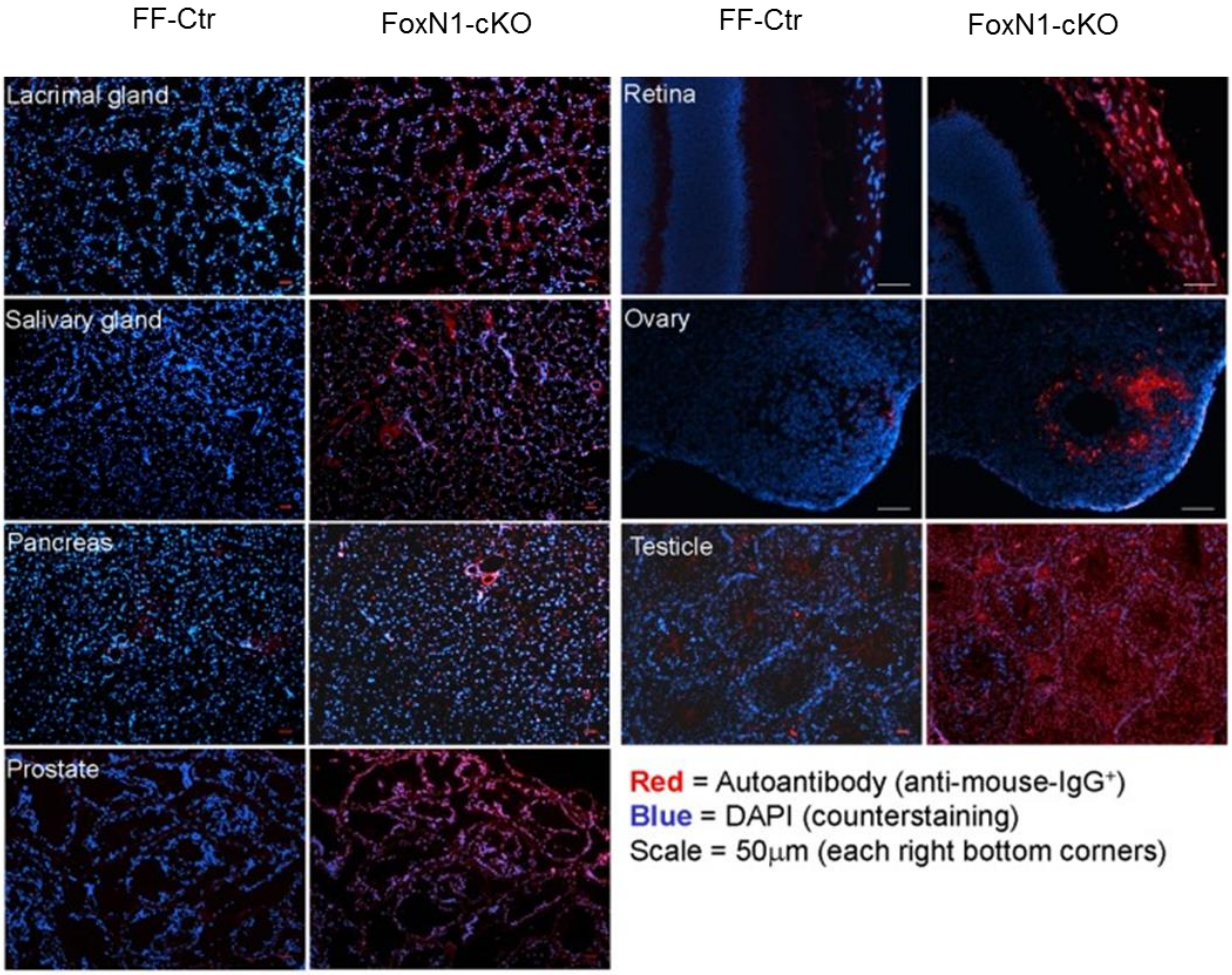
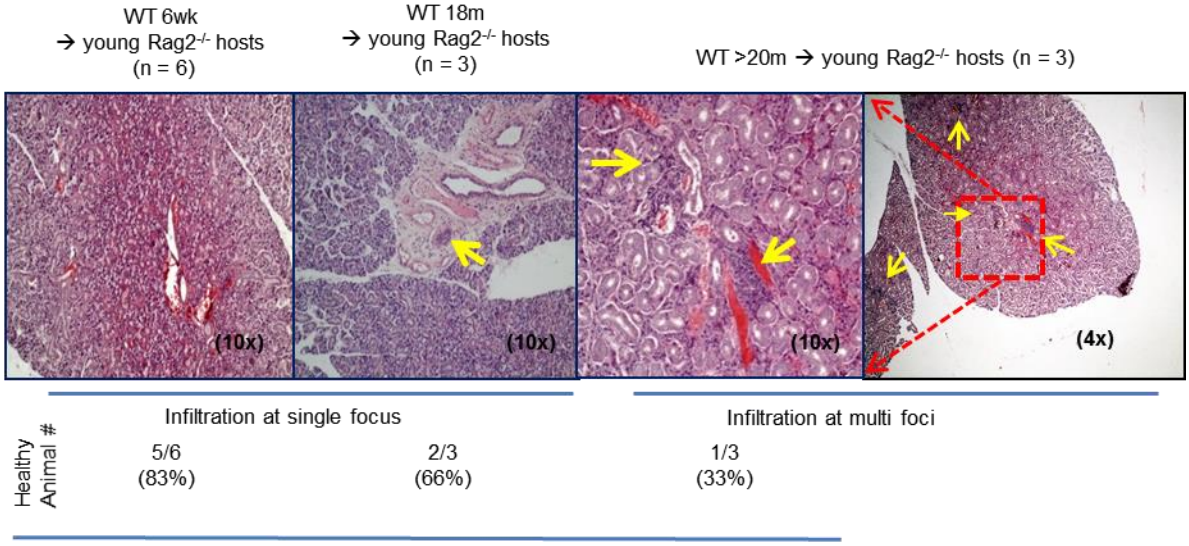


Figure 5. 7: Young microenvironment does not reverse age-induced inflammatory infiltration. (A) Representative images of H&E stained salivary tissue slides from young *Rag2*$^{-/-}$ mice, which were transplanted with splenocytes from donor young (6-week-old) and aged (18- or 20-month-old) C57Bl/6 wild-type mice for 8 weeks. Healthy refers to the absence of inflammatory infiltration. Single focus refers to one inflammatory infiltrating cluster being found in one tissue slide; Multi foci indicates that there were more than one inflammatory infiltrating cluster found in one tissue slide. Yellow arrows point out infiltrating clusters. n = animal numbers at the indicated age. **(B)** Representative images of H&E stained salivary tissue slides from young *Rag2*$^{-/-}$ mice, which were transplanted with splenocytes from *FoxN1*-cKO (middle and right panels) and FF-Ctr control (left panel) mice for 8 weeks. Yellow arrows point out inflammatory infiltrating clusters. There were at least 5 animals in each group producing consistent results and data are representative of at least three independent experiments.

A.


WT 6wk
→ young Rag2-/- hosts
(n = 6)
WT 18m
→ young Rag2-/- hosts
(n = 3)
WT >20m → young Rag2-/- hosts (n = 3)
(10x)
(10x)
(10x)
(4x)
Infiltration at single focus
Infiltration at multi foci
Healthy Animal #
5/6
(83%)
2/3
(66%)
1/3
(33%)

B.

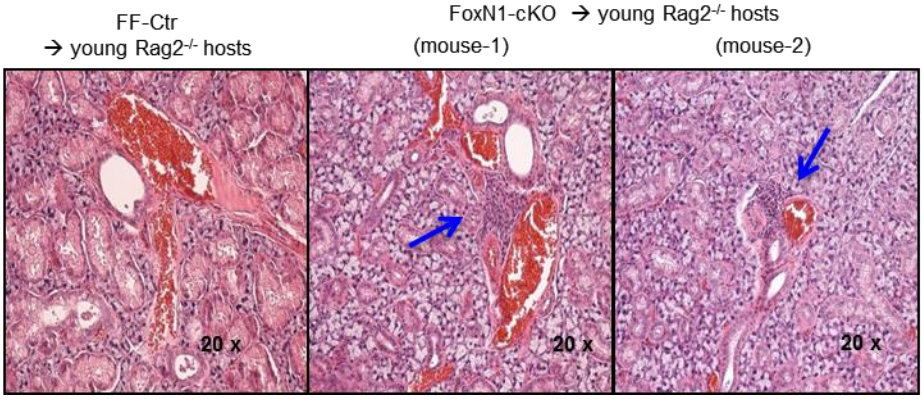
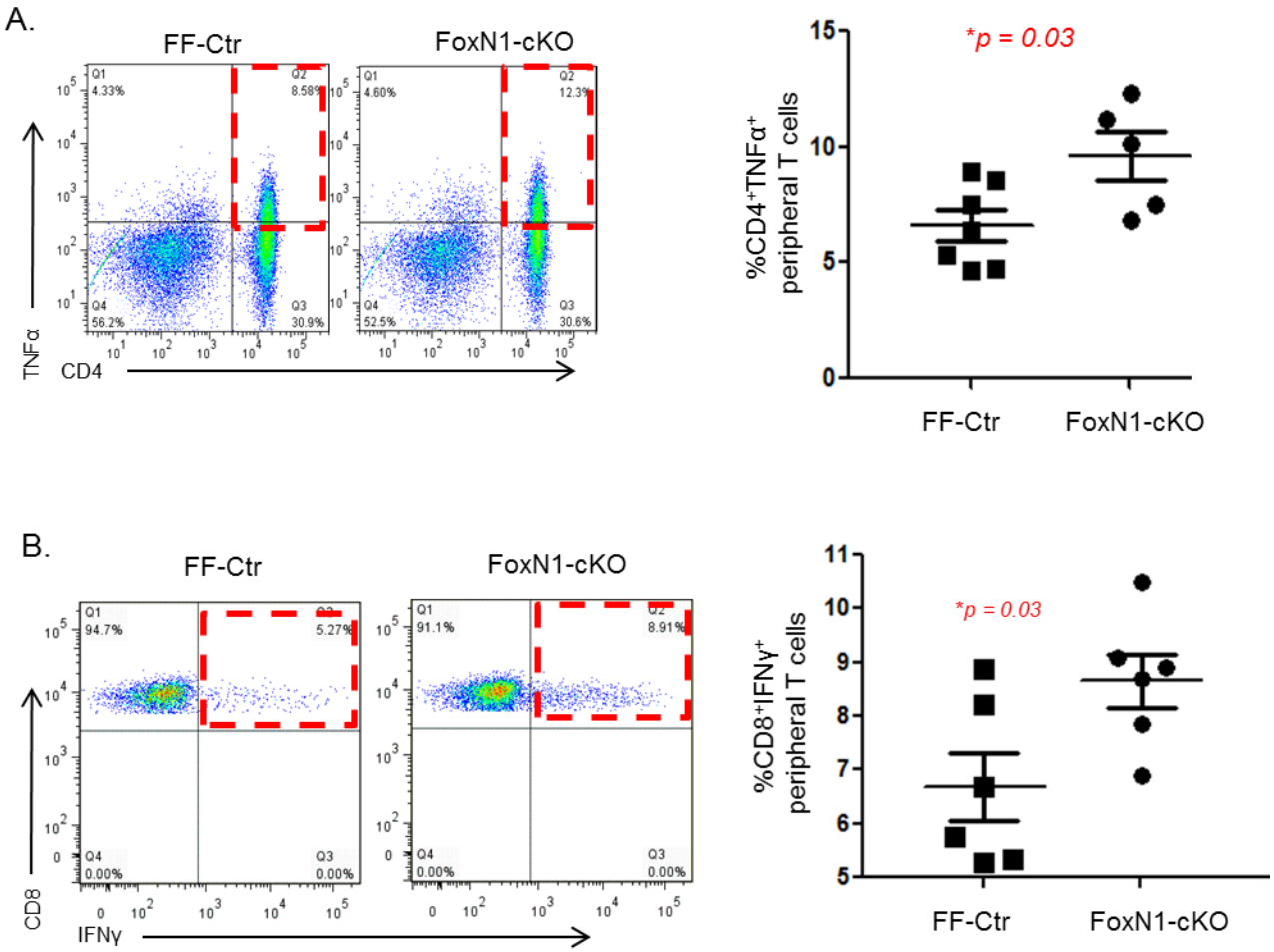

FF-Ctr
→ young Rag2-/- hosts
FoxN1-cKO → young Rag2-/- hosts
(mouse-1)
(mouse-2)
20 x
20 x
20 x

Figure 5. 8: Chronic low-grade inflammation following thymic involution induced by the loss of *FoxN1*. One month following TM treatment for the conditional knockout of *FoxN1,* freshly isolated splenocytes from FF-Ctr and *FoxN1*-cKO mice co-stimulated with anti-CD28 & anti-CDε in culture for 4.5 hrs., and then **(A)** CD4 TNFα and **(B)** CD8 IFNγ production were assessed by intracellular cytokine staining. **(C)** Sera from FF-Ctr and *FoxN1*-cKO mice were collected and IL-6 was measured by ELISA. A Student *t*-test was used to determine statistical significance between groups. All data are expressed as mean ± SEM. Data are pooled from three independent experiments (each symbol represents an animal).


A.
FF-Ctr
FoxN1-cKO
Q1 4.33%
Q2 8.58%
Q4 56.2%
Q3 30.9%
Q1 4.60%
Q2 12.3%
Q4 52.5%
Q3 30.6%
TNFα
CD4
*p = 0.03
%CD4+TNFα+ peripheral T cells
FF-Ctr
FoxN1-cKO
B.
FF-Ctr
FoxN1-cKO
Q1 94.7%
Q2 5.27%
Q4 0.00%
Q3 0.00%
Q1 91.1%
Q2 8.91%
Q4 0.00%
Q3 0.00%
CD8
IFNγ
*p = 0.03
%CD8+IFNγ+ peripheral T cells
FF-Ctr
FoxN1-cKO
C.
**p = 0.002
(IL-6 conc: pg / ml)
FF-Ctr
FoxN1-cKO

CHAPTER VI

CONCLUSION, DISCUSSION, AND FUTURE DIRECTIONS

The United States is considered to be an aging nation, which is primarily due to baby boomers entering old age [219]. Currently, approximately 13 percent of U.S. residents are age 65 and over, compared to only 9.8 percent in 1970. The percentage of elderly residents in the U.S. is expected to grow to more than 20 percent by the year 2030. Even though the elderly are only 13 percent of the total U.S. population, they are far more likely to have poor physical health, and therefore consume 36 percent of the total U.S. personal health care expenses [219], [220]. Consequently, there is a strong demand to reduce the financial burden and improve the quality of life for this growing U.S. demographic by promoting healthy aging. Sadly, healthy aging is a bit of a misnomer. Natural aging is accompanied by the breakdown of many biological systems, with the immune system included among these. Immune aging, termed immunosenescence, is considered to be the primary reason for many age-related health complications, including low vaccine efficacy, autoimmunity, and systemic low-grade inflammation [6], [192]. Therefore, understanding and preventing immunosenescence is the key to promoting healthy aging in the growing elderly population [192].

The studies presented in this dissertation advance our knowledge about the consequences of age-related thymic involution and its role in the development of autoimmunity and chronic inflammation with advanced age. First, I have demonstrated that there is a breakdown in negative selection following thymic involution induced by the progressive loss of the *FoxN1* gene. Second, I have shown that the peripheral environment regulates regulatory T cell (Tregs) maintenance and thymic involution does not impair Treg generation or function. Finally, I have demonstrated that thymic involution alone is sufficient to induce autoimmunity and leads to the development of inflammaging.

Immunosenescence is a multi-faceted process that includes degeneration of both the innate and adaptive immune systems. However, the T cell is routinely implicated in a plethora of age-related maladies, including poor vaccine responses, autoimmune disease, neurodegenerative disease, cardiac disease, certain cancers, and frailty. Therefore, the thymus gland, the organ responsible for T cell development, is a suitable candidate for studying the mechanisms driving immunosenescence. Furthermore, the thymus gland itself is naturally affected by age and undergoes severe age-related involution. However, thymic involution being an age-related phenomenon complicates elucidating thymic aging defects separate from defects resulting from the collective total body aging process. Therefore, I have utilized our lab's *FoxN1* conditional knockout mouse model (*FoxN1*-cKO) to answer questions related to the impact of thymic involution on the development of immunosenescence. Importantly, the *FoxN1*-cKO mouse model allows for the temporal induction of accelerated thymic involution in an otherwise young animal. Therefore, implications from thymic involution can be studied separately from total body aging.

The healthy thymus protects against autoimmunity by inducing tolerance towards self-tissues in developing thymocytes. Because thymic involution results in the deterioration of the

supporting epithelial meshwork and a loss of MHC-II [145, 169], I suspected thymocyte development would be impaired. Data presented in Chapter III demonstrated that thymic involution impairs normal thymocyte development via perturbing negative selection. Although thymic involution results in decreased total thymic cellularity, the diminished cell numbers are disparately proportioned throughout the different thymocytes subsets. The frequency of the $CD4^+CD8^+$ double positive (DP) subset in the *FoxN1*-cKO involuted thymus is severely diminished, but the single positive (SP) subsets are not reduced. Because TCR diversity is based on stochastic V (D) J rearrangement [26, 56], the reduced frequency and total number of DP thymocytes should carry over into the SP subpopulation, but this is not the case. Suspicions of defective negative selection following thymic involution were confirmed when the *FoxN1*-cKO thymus failed to clonally delete OT-II TCR transgenic thymocytes that developed in a thymus that expressed mOVA as a neo self-antigen. Furthermore, mOVA expression in mTECs is under the control of the rat insulin promoter and dependent on AIRE [85, 95]. Complete knockout of the *AIRE* gene ($AIRE^{-/-}$) impairs negative selection and leads to systemic autoimmune disease. Notably, even the partial loss of the *AIRE* (heterozygous $AIRE^{+/-}$) causes a drastic increase in islet-reactive T cells and progression towards diabetes in an insulin TCR transgenic mouse model [221]. Here, I demonstrate that the percentage of $AIRE^+$ mTECs is reduced in *FoxN1*-cKO mice, and the AIRE expression in mTECs is decreased on a per cell basis. This implies that the observed negative selection impairment associated with thymic involution may result from the diminished expression of AIRE in mTECs. This was further corroborated by a reduction in AIRE-dependent TSA genes in the *FoxN1*-cKO thymus. These data suggest the involuted thymus cannot efficiently express the TSAs that are needed for negative selection. However, whether a reduction in promiscuous gene expression will lead to a reduction in TSAs presented to developing thymocytes remains unclear.

Recent reports indicate that any one TSA is only expressed by 1-3% of mTECs at any given time, despite mTECs expressing the transcripts for all TSAs [81]. Interestingly, mTECs expressing a particular TSA co-express distinct sets of genes, and these mTECs are capable of shifting through distinct gene pools in order to represent the entire TSA repertoire [83]. How the loss of *FoxN1* and subsequent thymic involution influences the expression pattern of TSAs is unclear. Therefore, understanding the protein and gene expression patterns of mTECs following thymic involution, and their ability to shift towards different subsets, would provide additional insight into the mechanisms impairing negative selection in the *FoxN1*-cKO model. Furthermore, the details of the *FoxN1*-AIRE regulatory axis are not fully understood. There is sufficient evidence, reported here and by others [222], to support AIRE being downstream of *FoxN1*. However, it remains unclear if *FoxN1* directly controls AIRE expression. The recent discovery of AIRE in peripheral *FoxN1*-negative bone marrow-derived extrathymic AIRE-expressing cells (eTACs) suggests that AIRE does not require *FoxN1* for its expression [223]. Therefore, the regulatory axis of *FoxN1* and AIRE, including their binding and indirect/direct control mechanisms, needs to be investigated further.

Although AIRE drives the expression of many TSAs, there are many other TSAs that are produced independent of AIRE. AIRE-independent TSAs are enriched in ovary and testis specific genes [88], which implies that AIRE-independent gene expression likely drives ectopic expression of germ cell peptides. However, data presented in Figure 5.6 demonstrates that autoantibodies from *FoxN1*-cKO mice are reactive to ovaries and testes, and the AIRE-independent C-reactive protein (CRP) gene is slightly decreased (not significant) in the *FoxN1*-cKO thymus (Supplemental Figure 1). These data suggest AIRE-independent TSA expression is likely impaired following thymic involution induced by the loss of *FoxN1*. Nevertheless, how the loss of *FoxN1*

and thymic involution affects AIRE-independent TSA expression requires further investigation. Furthermore, mTECs are not the only cell type capable of initiating negative selection. Thymic dendritic cells (tDCs) have been shown to play a pivotal role in delivering self-peptides to developing thymocytes [73]. Interestingly, tDCs accumulation shifts from mTEC to cTEC regions in the *FoxN1*-cKO involuted thymus and the naturally aged wild-type thymus, which corresponds to decreased mRNA expression of the chemokine XCL1 (Supplemental Figure 2). However, further work needs to be done to determine if the loss of *FoxN1* and thymic atrophy influences the functional ability of tDCs to facilitate negative selection. Lastly, the enhanced survival of SP thymocytes in the *FoxN1*-cKO thymus could also be explained by increased positive selection or circulation of mature peripheral T cells back into the thymus. Preliminary data suggest that positive selection may be enhanced in the *FoxN1*-cKO thymus (Supplemental Figure 3). Additionally, mature T cells have been known to migrate back into the thymus [224], especially under times of stress and inflammation [225], which are hallmarks of thymic involution [165]. Further characterization of the SP cells in the *FoxN1*-cKO thymus (RTE-GFP^{+}) vs (Mature-GFP^{-}) and the strength of positive selection could enhance the understanding of the thymocyte's developmental defects associated with the loss of *FoxN1* and subsequent thymic involution.

In addition to negative selection, the thymus maintains immune tolerance by generating natural regulatory T cells (nTregs). Others have reported that premature thymic involution, induced by a hypomorphic mutation in the *FoxN1* gene (*FoxN1*$^{\Delta/\Delta}$ mice with a germline mutation), weakens the ability of peripheral Tregs to suppress an immune response [145]. However, this is contrary to the observations in Chapter IV, which show Tregs derived from the *FoxN1*-cKO thymus can suppress the proliferation of FF-Ctr effector T cells just as efficiently as Tregs derived from the normal FF-Ctr thymus. The discrepancy between Treg function, when comparing the

hypomorphic germline mutation model and our *FoxN1*-cKO model, is likely explained by the developmental stage of the thymus. The hypomorphic *FoxN1* mutation occurs in the prenatal thymus, where TECs have not had the chance to develop. However, the loss of *FoxN1* in the *FoxN1*-cKO model is induced after the thymus has fully matured (3-4 months-old), and TECs have had the opportunity to fully mature [54]. The loss of *FoxN1* after the thymus has matured is more similar to natural aging, where *FoxN1*-negative TECs are derived from *FoxN1*-positive TECs [55]. One caveat to the functionality experiment performed in Chapter IV comes from defining Tregs as $CD4^+CD25^+$. Although $CD4^+CD25^+$ cells can possibly include effector T cells, and these markers may not define all Tregs, our results are in agreement with others, which show aged Foxp3-GFP Tregs retain their suppressive abilities equally to young Tregs [137]. Additionally, recent evidence suggests that age-related autoimmunity is partially compensated by an age-related accumulation of peripheral Tregs with increased suppressive function [142]. Age-related peripheral Treg accumulation has been proposed to result from the decreased expression of the pro-apoptotic gene *Bcl2* family member *Bim* [139-141]. Here, we have demonstrated that peripheral Treg accumulation and diminished *Bim* expression in naturally aged wild-type mice can be normalized by the young peripheral microenvironment. Therefore, age-related Treg accumulation is not due to an intrinsic defect associated with thymic involution, but is potentially caused by an extrinsic abnormality in the aged peripheral microenvironment. Recently, circulating soluble factors in the serum have been linked with many age-related diseases. By utilizing heterochronic parabiosis, where the circulatory systems of two mice (one young and one aged) are joined, it was demonstrated that the old systemic environment impaired cognitive function in young mice [226]. However, the reverse was also true, and young blood was able to reverse age-related neurocognitive disorders and strengthen skeletal muscle in aged mice [227] [228].

Although there are many age-related alterations to the peripheral environment, including changes to circulating soluble factors, the stromal compartment, the hematopoietic compartment, and microbiota; the exact mechanisms signaling an age-related decrease in *Bim* and subsequent accumulation of Tregs remain unclear. Based on these findings, thymic involution does not significantly contribute to Treg accumulation and function.

Even though the loss of *FoxN1* and subsequent thymic involution did not result in any changes to the peripheral Treg pool, Treg frequency was increased in the involuted thymus of *FoxN1*-cKO mice. Although, there is evidence to support both AIRE-dependent [122, 123] and AIRE-independent nTreg development [124], we did not observe any defects in the ability of the involuted thymus to generate nTregs, despite the diminished expression of AIRE in *FoxN1*-cKO thymi. It is possible that the low level of AIRE present in the *FoxN1*-cKO thymus is sufficient to support nTreg development, but is not sufficient enough to support the strong avidity necessary for the clonal deletion of autoreactive thymocytes [100]. It is unclear whether the loss of *FoxN1* affects TCR avidity for self-peptide-MHC complexes. The decreased negative selection with enhanced (at least unimpaired) generation of Tregs in the *FoxN1*-cKO thymic involution model is probably better explained by an "avidity window" between negative selection (strong TCR signal strength) and deviation into the Treg lineage (moderate TCR signal strength) [59]. In this scenario, diminishing the avidity of TCR for self-peptide-MHC complexes should reduce the clonal deletion of negative selection and favor the production of nTregs.

Additionally, it is possible that nTreg generation is maintained in the *FoxN1*-cKO thymus by AIRE-independent thymic dendritic cells (tDC). Further investigations into the TCR repertoire and binding strength of Tregs developing in the *FoxN1*-cKO thymus would help to clarify how thymic involution affects Treg generation. Interestingly, the increased proportion of Tregs in the

FoxN1-cKO thymus is consistent with reports that defects in negative selection skew thymocyte development towards the diverted nTreg pathway [59]. However, I found that the increase in thymic Tregs do not result in an increase in peripheral Tregs. It is possible that Tregs are recirculating from the periphery back into the *FoxN1*-cKO thymus. Recent reports have demonstrated that mature Tregs migrate from the periphery back into the thymus during involution and suppress the development of new Tregs [229]. Use of the Rag2-GFP model to differentiate between new and mature T cells would greatly aid in determining the source of Tregs in the *FoxN1*-cKO thymus. Furthermore, because the vast majority of peripheral Tregs originate from the thymus as nTregs, I have assumed that the Tregs that have accumulated with age are nTregs [100, 109, 110]. However, it is possible that aging results in the expansion of the induced Treg (iTreg) pool. Analyzing the TCR repertoire between young and aged Tregs would be useful in identifying the exact cohort of Tregs that accumulate with age. Lastly, because Treg accumulation naturally occurs with aging, it is possible that Tregs increase in order to compensate for the increased susceptibility to autoimmunity that results from defective negative selection. Whether autoreactive T cells, pro-inflammatory cytokines, or systemic age-related factors influence *Bim* and the survival of aged Tregs needs to be investigated further.

Finally, the propensity for thymic involution to induce autoimmunity and inflammaging was evaluated in the *FoxN1*-cKO model. Current knowledge of the etiology of inflammaging identify a role for SASP expressing senescent tissue cells [230] and the persistent activation of immune cells that likely arise from persistent latent viral infection [171]. However, the prevalence of age-related autoimmunity in the elderly and the contribution of autoreactive T cells in generating a systemic inflammatory environment have been widely overlooked in the prevention and treatment of inflammaging. In Chapter V, I demonstrated that thymic involution, resulting from

the loss of *FoxN1*, is an additional source of activated immune cells (autoreactive T cells) that lead to the development of low-grade immunopathology and systemic inflammation. Recent thymic emigrants (RTEs) that have just egressed the *FoxN1*-cKO involuted thymus become activated and begin to proliferate shortly after entering the periphery, despite having never encountered foreign antigens. Because the *FoxN1*-cKO mice are housed in a clean and sterile environment, I suspected that the RTEs were being activated by self-tissue antigens. I previously showed that the *FoxN1*-cKO involuted thymus could not efficiently delete autoreactive T cells in the thymus. By immunizing *FoxN1*-cKO mice with the peripheral self-tissue antigen IRBP, I demonstrated that autoreactive T cells escape the *FoxN1*-cKO involuted thymus and respond to self-antigens in the periphery, while the FF-Ctr mice do not. Importantly, the promiscuous gene expression of IRBP in the thymus is dependent on AIRE and mTECs. Thus, the lack of AIRE and viable mTECs in the *FoxN1*-cKO thymus is the likely mechanism driving the release of autoreactive IRBP-specific T cells. However, further analysis of IRBP expression in the *FoxN1*-cKO thymus is required to definitively determine the mechanism.

The detection of IRBP specific T cells in the periphery of *FoxN1*-cKO mice confirms that the loss of *FoxN1* and subsequent thymic involution releases T cells that recognize self-tissue. However, in order to detect autoreactive T cells, the IRBP model requires a 100µg peripheral immunization with the self-peptide and a strong stimulus in the form of Complete Freund's Adjuvant (Figure 5.3). In order to determine if thymic involution induced by the loss of *FoxN1* is sufficient to drive autoimmunity and inflammaging, without the need for additional stimuli, the periphery of unimmunized *FoxN1*-cKO mice were analyzed for signs of immunopathology. Using *FoxN1*-cKO mice, thymic involution alone was demonstrated to be sufficient to lead to increased inflammation. Importantly, TNFα and IL-6, the most indicative pro-inflammatory factors of poor

prognosis [176], were slightly (although significantly) elevated in the *FoxN1*-cKO mice, consistent with the low-grade phenotype associated with inflammaging. Furthermore, peripheral CD4 and CD8 T cells producing pro-inflammatory cytokines were increased in unimmunized *FoxN1*-cKO mice, indicating a role for activated T cells in the development of inflammaging. However, because both CD4 and CD8 T cells play a large role in the activation of other cell types, including macrophages and natural killer cells [231], the involvement of innate cells in the emergence of inflammaging in *FoxN1*-cKO mice requires further investigation.

Additionally, inflammatory infiltration was detected in the salivary gland, liver, and lung of *FoxN1*-cKO mice. Moreover, antinuclear antibodies (ANAs) that reacted strongly with the lacrimal and salivary glands were elevated in the *FoxN1*-cKO mouse serum. These data are in agreement with the characteristics of age-related autoimmunity. Importantly, AIRE-deficiency, in both mice and APS-1 patients, have been linked with increased ANAs and autoimmunity against the salivary gland [93]. Furthermore, Sjögren's Syndrome, perhaps the most common age-related autoimmunity, is characterized by serum ANAs and lymphoinfiltration into the salivary and lacrimal glands [97]. Interestingly, AIRE-independent TSAs are enriched in ovary and testis specific genes [88], and ANAs from the *FoxN1*-cKO serum react to ovary and testes tissue. This finding suggests that defects in AIRE-independent negative selection are likely occurring in the *FoxN1*-cKO thymus. AIRE-independent negative selection in the *FoxN1*-cKO thymus, including tDCs, requires further investigation.

Although the young peripheral environment was able to reverse age-related Treg accumulation (Chapter IV), inflammatory infiltration associated with aged wild-type splenocytes cannot be reversed by the young environment. Additionally, *FoxN1*-cKO splenocytes transferred into young *Rag2*$^{-/-}$ mice led to salivary infiltration, indicating that autoimmunity is an intrinsic defect related

to development in the involuted thymus. Interestingly, salivary infiltration progressively worsened with increasing age of the donor, and >20-month wild-type splenocytes caused more severe infiltration than *FoxN1*-cKO splenocytes. These findings suggest that perhaps circulating soluble age-related factors or aging of other cell types (not thymus related) significantly contribute to the severity of autoimmunity. Certainly, soluble factors in the aged blood have been shown to exacerbate disease [226], and further investigation into the combination of these effects with thymic involution should be performed. Interestingly, *FoxN1*-cKO mice do not develop overt autoimmune disease that would account for the elevated inflammatory milieu, but rather present with general and relatively mild infiltration in multiple non-lymphoid organs. Why the breakdown in immune tolerance associated with *FoxN1*-cKO thymic involution does not lead to autoimmune disease is unknown. Perhaps overt autoimmune disease requires additional genetic and environmental stimuli. Certainly, many infections have been associated with triggering autoimmune disease [232]. Ascertaining if and how *FoxN1*-cKO mice can be triggered to progress into overt autoimmune disease requires further investigation.

One important limitation to this study is the frequent use of our *FoxN1*-conditional knockout mouse model of thymic involution in lieu of a true natural age-related atrophied thymus from >18 month wild-type mice. This limitation is common to all accelerated aging models. Progressive thymic involution is an age-related alteration and its causes are almost certainly not due to only the loss of one gene. This complex and multifaceted nature of thymic involution is evidenced by the various processes implicated in age-related thymic atrophy including the involvement of other genes like *ink4a* [233], hormones [234], and adipocyte expansion [166, 235, 236]. However, because thymic involution occurs alongside total body aging, separating the effects of thymic age from systemic aging, which includes the circulation of soluble factors, T cells, and dendritic cells

back into the thymus, is extremely difficult-- if not impossible, in an 18 month wild-type mouse. Further complicating the issue, thymic aging and peripheral aging likely interact with each other through cell-cell interactions, secreted factors, and hormones. The strength of our *FoxN1*-conditional knockout model lies in the ability to induce the involution of a mature thymus while avoiding the effects of systemic aging, thereby delineating the age-related effects of thymic involution from total bodying aging. In fact, our model undergoes thymic involution including a loss of $FoxN1^{+}$ TECs [54, 169], a decline in mature mTEC [54, 169, 237], thymic dendritic cell distribution [237], increased senescent clusters, and increased $TAp63^{+}$ TECs [170] similar to 18 month C57BL/6 mice. Additionally, unlike the pre-natal $FoxN1^{\Delta\Delta}$ mutation that blocks TEC maturation and reduces Treg function [145], our inducible *FoxN1*-cKO model of mature thymic atrophy maintains Treg suppressive capacity consistent with naturally aged Tregs [142]. However, our *FoxN1*-cKO model undergoes an extremely accelerated thymic involution that is dissimilar to the 1-3% shrinkage per year observed in the naturally involuted thymus [158]. Despite the accelerated involution, the *FoxN1*-cKO model does not exhibit thymic regrowth that is seen in acute thymic involution following infection and pregnancy [236]. *FoxN1*-cKO induced involution is permanent, which is similar to natural age-related thymic involution. However, the *FoxN1*-cKO model still does not fully recapitulate the physiology of the chronic and extremely gradual nature of natural age-related thymic involution. Therefore, the effects of rapid, as opposed to gradual, involution on defective negative selection and the emergence of chronic inflammation cannot be eliminated. Although the induced conditional knockout of *FoxN1* and subsequent accelerated thymic involution in the fully mature thymus may not exactly mimic the naturally aged thymus, there are many molecular, morphological, and functional characteristics shared between the two.

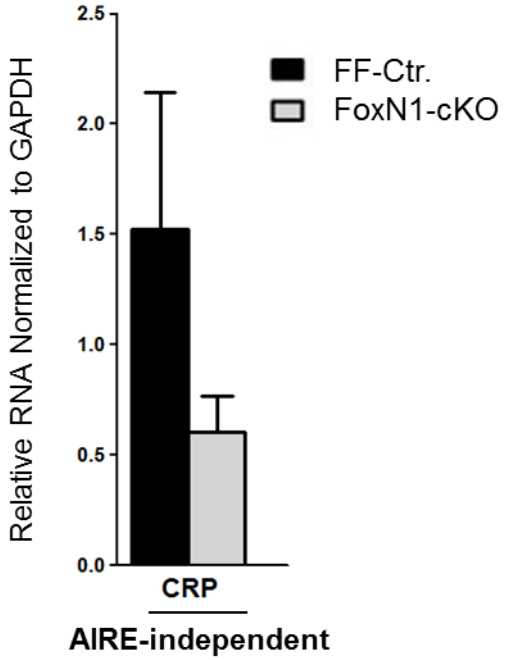
Therefore, the *FoxN1*-cKO model is useful to study thymic involution and is applicable for age-related thymic involution with careful consideration for the limitations of the animal model.

Going forward, plans for the therapeutic targeting of *FoxN1* for the treatment of immunosenescence, including age-related autoimmunity and inflammaging, should emerge from this work. Although direct anti-inflammatory interventions, such as the use of low dose aspirin or statins, to suppress, prevent, and alter the state of chronic inflammation hold great promise for treating multiple age-related diseases [171], targeting the sources of chronic inflammation will be the key to enhancing the prognosis of chronic age-related disease. Here, we have shown that in addition to developing a strategy for the elimination of senescent cells to suppress SASP [238], reduction or elimination of autoreactive T cells emigrated from the atrophied thymus is particularly promising to dampen age-related inflammation. While vaccination against persistent infections is a possible strategy to manage the persistent activation of immune cells related to chronic infection, it does not address autoreactive immune cell activation. The work presented here demonstrates that the involuted thymus should be a key target for treating immunosenescence. Whether thymectomy or *FoxN1* gene therapy would be the best approach requires further investigation. It is important to note that because *FoxN1* has been directly linked with thymic involution, many approaches have been used to target *FoxN1* as a therapeutic agent, and with varying degrees of success. Injecting a plasmid containing *FoxN1* cDNA increases the proliferation associated with ΔNp63, decreases thymic involution, and leads to the partial restoration of peripheral T cells and IL-2 [169, 170]. However, close attention must be paid to the dosage of *FoxN1* therapy. Low-level overexpression of *FoxN1* attenuated age-related thymic involution in aged mice [239], however a mouse model systemically overexpressing high levels *FoxN1* exhibited defective thymopoiesis

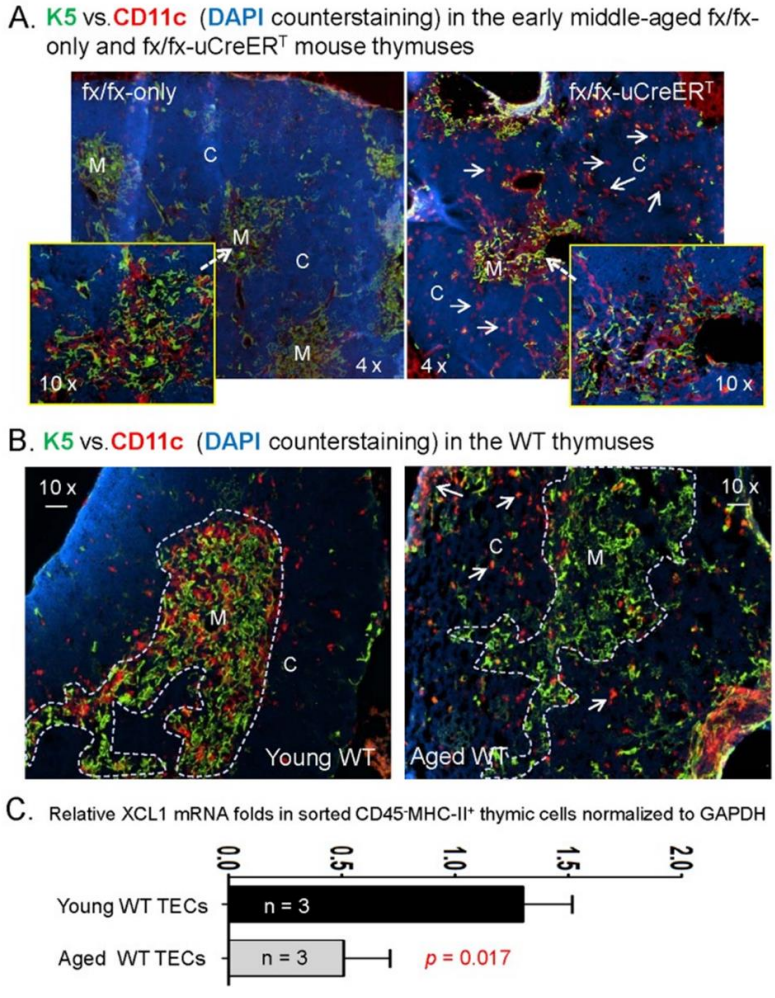
and thymic cellularity [240]. Therefore, future experiments targeting *FoxN1* therapeutically must be cautious with respect to dosage, time, and target cell type.

In conclusion, the study presented here has demonstrated that thymic involution releases autoreactive T cells that cause tissue-specific inflammation and contribute towards the development of low-grade chronic inflammation, known as inflammaging (Supplemental Figure 4). The observed inflammaging is the consequence of a T cell predisposition towards autoimmunity that arises from the progressive loss of *FoxN1* expression leading to the disruption of the steady-state thymic medullary compartment [168, 169, 237] and subsequent thymic involution. Thymic involution results in the release of autoreactive T cells that become activated shortly after reaching the periphery and produce low levels of inflammatory cytokines without causing overt autoimmune disease. The autoreactive T cells result from intrinsic defects in negative selection, rather than changes to the extrinsically maintained Treg pool. Our studies shed new light on the importance of targeting thymic involution in addition to improving the peripheral immune microenvironment as a potential treatment for eliminating age-related autoimmunity and inflammaging. It may be possible to promote healthy aging by directly targeting *FoxN1* and thymic involution, and thereby reduce the overall morbidity and mortality in chronic age-related disease.

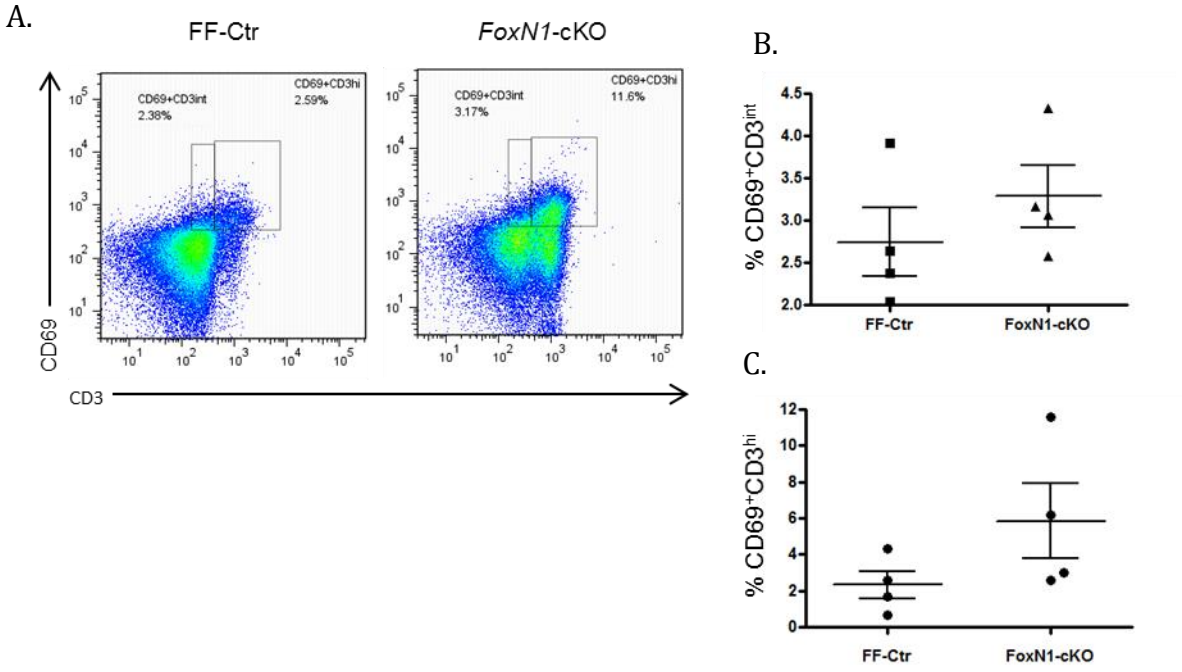
Supplemental Figure 1: AIRE-independent TSA gene is slightly decreased in the *FoxN1*-cKO thymus. Expression of AIRE-independent tissue-specific self-antigen: CRP in 9-month-old middle-aged FF-Ctr and *FoxN1*-cKO mice measured by real-time RT-PCR. CRP = C-reactive protein. The difference is not significant, however C-reactive protein (*CRP)* expression is slightly decreased in the *FoxN1*-cKO group. Each group includes 4 animals and each sample was run in duplicate. Statistical significance was analyzed by unpaired two-tailed Student's *t*-test.



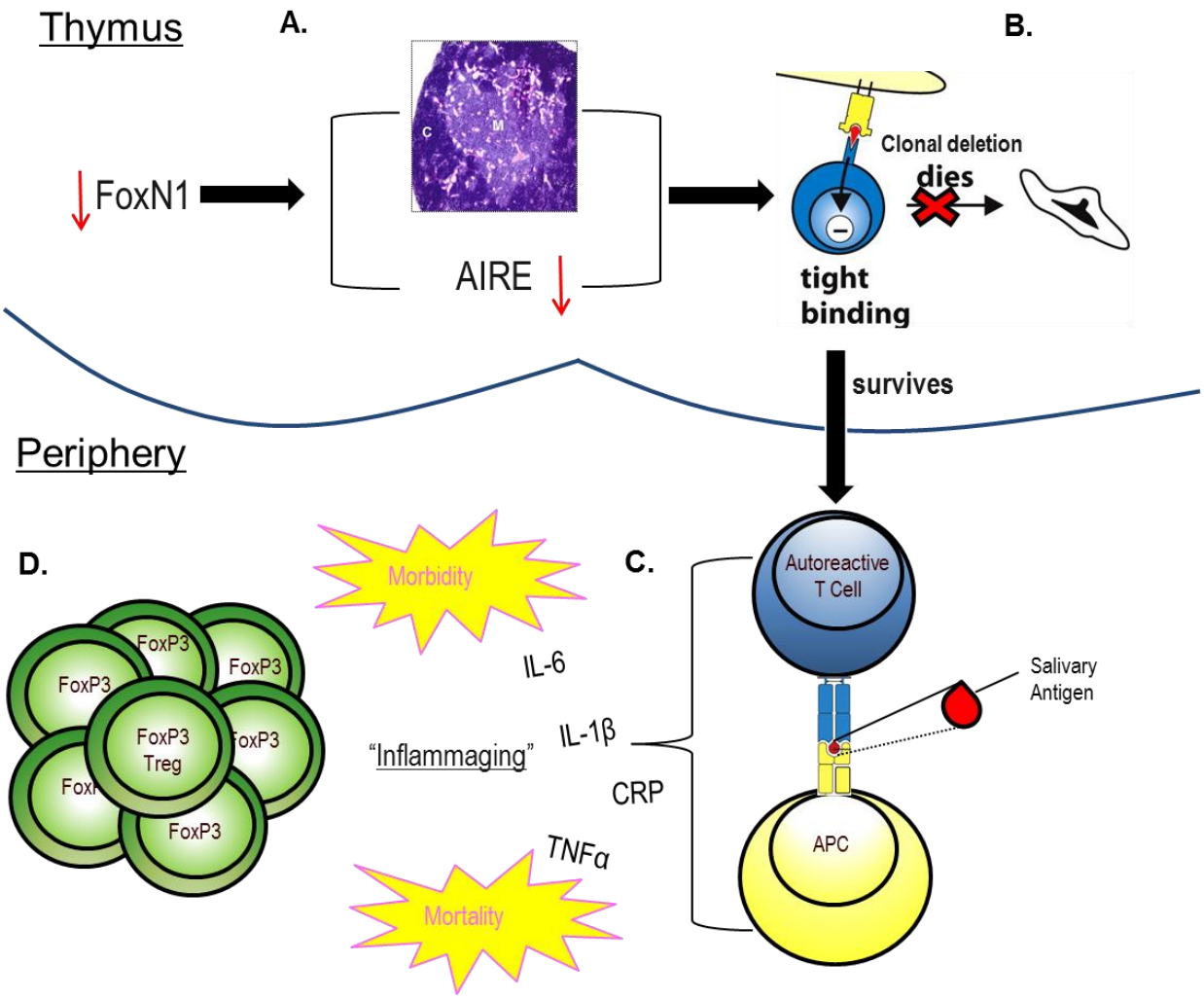
Supplemental Figure 2: tDCs accumulate in the cortex of the involuted thymus. **(A)** Distribution of tDCs in the early middle-aged fx/fx-only (FF-Ctr, left panel) and fx/fx-uCreER^{T} (*FoxN1*-cKO, right panel) mouse thymi with immunofluorescence staining of thymic mTECs (K5, green) and tDCs (CD11c, red). **(B)** Immunofluorescence staining shows thymic mTECs (K5, green) and tDCs (CD11c, red) in young wild-type mice (left panel) and naturally aged (21-month-old) WT mice (right panel). Arrows indicate representative tDCs in the cortex. Scale in each image represents 50 μm. Images are representative of 3 independent replicates per group, with essentially identical results. **(C)** Real-time RT-PCR results of XCL1 expression in FACS-sorted CD45^{-}MHC-II^{+} TECs from young (2 month) and naturally aged (20 month) wild-type mice (n = animal numbers).



Supplemental Figure 3: Positive Selection may be increased in the *FoxN1*-cKO thymus. (A) Flow cytometry analysis was performed on thymocytes excluding DN cells isolated from *FoxN1*-cKO and FF-Ctr mice. Representative plots of CD3 and CD69 are shown, with left gate showing $CD69^{+}CD3^{int}$, and right gate showing $CD69^{+}CD3^{hi}$. During positive selection, thymocytes upregulate CD3 and CD69 expression. First, thymocytes sequentially express the $CD69^{+}CD3^{int}$ phenotype and then transition into the $CD69^{+}CD3^{hi}$ phenotype. **(B)** Shows that the $CD69^{+}CD3^{int}$ population is slightly elevated (not significant) in *FoxN1*-cKO mice and **(C)** shows that the $CD69^{+}CD3^{hi}$ phenotype is slightly elevated in *FoxN1*-cKO mice. Data are representative of two independent experiments with 4 animals per group, and shown as mean ± SEM.



Supplemental Figure 4: Thymic involution perturbs immune tolerance and leads to inflammaging. Diagram summarizing the results in the study—T cell immunosenescence. **(A)** *FoxN1* naturally declines with age, resulting in thymic involution and loss of the transcriptional regulator AIRE. **(B)** Thymic involution and the loss of AIRE perturbs negative selection by impairing the clonal deletion of thymocytes with autoreactive TCRs. Therefore, autoreactive thymocytes survive and egress into the periphery as autoreactive T cells. **(C)** Shortly after entering the peripherally, autoreactive RTEs respond to APCs presenting self-antigens (e.g. salivary gland peptides) on MHC. The autoreactive RTEs become activated and can directly secrete pro-inflammatory cytokines like TNFα and IFNγ, or they can help other cell types secrete pro-inflammatory cytokines. These pro-inflammatory cytokines systemically and chronically accumulate and lead to inflammaging, which increases the morbidity and mortality of chronic age-related disease. **(D)** Meanwhile, aging drives the peripheral accumulation of $Foxp3^+$ regulatory T cells. Tregs likely accumulate in the periphery of aged individuals in order to compensate for the enhanced autoimmunity associated with age.


Thymus
A.
B.
FoxN1
AIRE
C
M
Clonal deletion
dies
tight
binding
survives
Periphery
D.
C.
Morbidity
IL-6
"Inflammaging"
IL-1β
CRP
TNFα
Mortality
FoxP3
FoxP3
FoxP3
FoxP3
FoxP3
FoxP3
Treg
Autoreactive
T Cell
Salivary
Antigen
APC

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