Uncovering Mechanisms Regulating Thymus Regeneration: Damage Induced Interleukin-18 Triggers NK Cells to Suppress Thymus Repair

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A dissertation

submitted in partial fulfillment of the

requirements for the degree of

Doctor of Philosophy

University of Washington

2024

Reading Committee:

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Program Authorized to Offer Degree:

Molecular and Cellular Biology

University of Washington

Abstract

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The thymus is the primary lymphoid organ responsible for T cell production. While the thymus is highly sensitive to acute stressors, in particular, treatments for malignancy such as chemotherapies and the myeloablative conditioning received pre-hematopoietic cell transplantation (HCT), it also has a tremendous capacity for regeneration following acute damages. However, the organ declines in its T cell production and reparative capacity with age in a natural process known as age-related thymus involution. This leaves patients experiencing thymus damage, particularly HCT-recipients, vulnerable to leading causes or post-transplant mortality (relapse of primary malignancy, graft-versus-host disease and opportunistic infection) during a prolonged period of T cell lymphopenia. Though the phenomeneon of endogenous thymus regeneration following its injury was known even before the organ's immunological function was understood, the mechanisms underpinning it remain incompletely understood. Recent works have begun to uncover these regenerative mechanisms. Yet, there remains no clinically approved treatment for T cell lymphopenia following thymus damage.

In this dissertation, I identify a novel pathway regulating thymus recovery following acute injury in which damage-induced Interleukin-18 (IL-18) suppresses regenerative processes via the activation of organ-resident cytotoxic natural killer (NK) cells. We show that several different models of acute damage led to an acute rise in the cleavage of Caspase-1 which mediates an immunogenic form of cell death known as pyroptosis during which mature IL-18 and IL-1β are released from dying cells. While IL-1β had no effect on thymus recovery, mice deficient in IL-18 signaling showed improved thymus reconstitution and pharmacologic abrogation of IL-18 improved thymus reconstitution in clinically relevant modeling of hematopoietic cell transplantation. We identify organ resident dendritic cells (DCs) and rare stromal populations including capsular fibroblasts and mesothelial cells as sources of functional IL-18 following injury. Our studies demonstrate that IL-18 mediates its effects via the stimulation of damage-resistant cytotoxic NK cells that suppress thymic recovery by targeting thymic epithelial cells (TECs), which serve as master regulators of organ function and regeneration. Finally, we show that injury induced genotoxic stress in TECs, and particularly medullary TECs (mTECs), results in their increased vulnerability to NK cytotoxicity via the downregulation of NKG2D inhibitory ligand MHC-I and upregulation of NKG2D stimulatory ligand Rae-1.

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ACKNOWLEDGEMENTS

I would like to begin by thanking Jarrod for his careful mentorship over the last four years. My time as a PhD student has been among the most challenging and rewarding of my life and it would not have been possible without Jarrod. I was the first graduate student in the lab joining during a time of turnover and sudden change brought on by the pandemic. Upon reflection, I realize that this created a unique set of circumstances through which Jarrod, and I worked and grew together. It is through these times of “figuring things out as we go” that we learned how the other thinks and operates and were able to foster a wonderful mentor: mentee relationship. Jarrod always held me to a high standard and had such confidence in me that I was able to confront my own self-doubt and find success in the lab. He sought out opportunities for me to elevate myself always supporting my ambitions. From Jarrod, I’ve learned countless lessons about thinking as a scientist, leadership, and importantly, prioritization when balancing work and family. I am deeply indebted to him for these last several years and the indelible mark he’s had on my training.

I must also thank my labmates Kirsten Cooper, Dante Acenas, Emma Lederer, Paul deRoos, Lorenzo Iovino and Vanessa Hernandes for making coming into lab such an enjoyable experience. I am honored to have been a part of this team. I would be remiss to not specifically highlight the contribution of Kirsten Cooper to this work. Kirsten has been with me throughout every step of my PhD process: in early 2020 when the pandemic was beginning, it was Kirsten who masked up to show me lab protocols, she managed our entire mouse colony for years nearly single-handedly, and she was instrumental in her scientific advice and technical contribution to countless experiments. A special thank you to Kirsten Cooper for everything she has done for not only me, but the entire Dudakov lab these many years.

I am incredibly appreciative for my supervisory committee members Drs. Stanley Riddell, Jakob von Moltke, Marie Bleakley, Andrea Wills and Marshall Horwitz. Learning from you has been a privilege and I can recall instances when each of you has challenged me and contributed

to my thinking as a scientist. Your feedback was always welcomed, and your generosity to take time out of your busy schedules to support trainees humbles me. A special thank you to Drs. Stanley Riddell and Jakob von Moltke for serving on my reading committee. And another special thank you to Marshall Horwitz whose phone call in Spring 2018 to inform me of my acceptance into the UW MSTP, an opportunity that has changed my life, I vividly remember. I am appreciative of the UW MSTP and MCB graduate program, and the administrators behind them Sara Carlson and Maia Low, for providing me the structure to pursue my goals.

I would also like to thank my former mentor Dr. Ivan Maillard and the previous members of their lab Drs. Jooho Chung and Eric Perkey for fostering in me a love for the lab and for their examples as physician scientists dedicated to improving patient care.

I also want to acknowledge the many staff members that quietly provide daily support. Thank you to the Fred Hutch flow cytometry core staff and most especially Rebecca Reeves who was unfailingly kind to a perpetually behind schedule, stressed out PhD student. Thank you to the Fred Hutch animal husbandry staff led by Jennifer Kiesel and Amber Carte and specifically to Lynsey Curulla, Trevon Covington, and Justin Ruiz for maintaining our mouse colonies. I also want to thank our custodial staff Montales Joseph, Dinh Luong, and Xuan Tran for their tireless contributions to making the Hutch a better environment and for their kind presence during many late-night experiments.

I want to thank my wonderful partner Olivia Do for sharing with me the many highs and lows of my PhD over these years. Hard days were made easier by your presence, and milestones and successes were better when I could share them with you. Witnessing your journey as a physician motivates me and reminds me of the people our research ultimately aims to help. I am so deeply thankful to have had you with me throughout my PhD.

Finally, and most of all, I want to thank my parents Robert and Roberta Granadier. My parents raised me to believe the cliché that I could accomplish anything to which I set my mind

and supported me in all my ambitions. Anything that I've accomplished stems from the unconditional love and support that they have given me. My father, a neuro-ophthalmologist, gave me my love for medicine and instilled in me a strong ethical code built around helping others. He spent his career teaching resident physicians, and I am inspired by the impact he's had on dozens of trainees who admire him as I do. My mother, an accomplished lawyer, is an absolute force and my role model in so many ways. My mom gave me my work ethic and the ability to communicate ideas thoughtfully and effectively. Despite their busy careers, there was not a ceremony or a game that they missed. Their love and support mean the world to me.

Chapter 1. INTRODUCTION

Parts of this chapter were adapted from the following publication:
Granadier, D., Iovino, L., Kinsella, S. & Dudakov, J.A. Dynamics of thymus function and T cell receptor repertoire breadth in health and disease. *Semin Immunopathol* **43**, 119-134 (2021).

The adaptive immune system relies on a T cell repertoire that is self-tolerant while also allowing for the recognition of an untold number of pathogen antigens and the surveillance of potentially cancerous self-mutations. The integrity of the thymus, the primary lymphoid organ responsible for T cell production, is therefore essential to these functions. This point is most dramatically demonstrated by the T cell immunodeficiency in patients with DiGeorge syndrome (DGS) in which a 22q11 deletion results in defective third and fourth pharyngeal pouch development, and thus severe hypoplasia of the thymus. Fascinatingly, the thymus was possibly the last organ to be physiologically described; its immunological role was discovered by Jacques Miller in the 1950's [1]. Unearthing the function of this mysterious organ along with the discoveries of somatic recombination and MHC-restriction helped answer the long-standing question of how the formation of a broad, yet selective T cell repertoire capable of cell mediated immunity was accomplished. The first subchapters will describe the organogenesis of the thymus and overview the process by which it carries out T cell production.

Thymic function is a dynamic process, and it is severely impacted by negative stimuli. These include acute injuries such as everyday insults (i.e., stress and infection), but also more profound injuries such as those aimed at targeting malignancy (chemotherapy and the cytoreductive conditioning received prior to hematopoietic cell transplantation). There are also chronic forms of organ damage, most notable of which is the natural process of age-related

thymic involution. This intro will review these different injuries to the thymus and their influence in disease pathology.

Despite the high sensitivity of the thymus, it also holds a remarkable capacity for self-regeneration. In fact, the phenomenon of thymus regeneration was recognized decades before the organ's immunological function was understood[2]. Children who have undergone partial thymectomy (common in reparative surgeries for cardiac abnormalities) exhibit significant rejuvenation of the remaining thymic tissue[3]. Endogenous thymic regeneration is thus a critical process to restoring immune competence following thymus injury. However, thymus repair is a prolonged process that also diminishes with age making it a clinical hurdle, particularly for adult patients receiving cytoreductive chemotherapies and myeloablative HCT. Though its restorative potential has long been appreciated, the underlying pathways responsible for regeneration of thymic function are only beginning to be understood. This introduction will overview the known cellular and molecular pathways controlling endogenous thymus regeneration, describe the limitations to thymus repair, and the clinical implications of altered thymus function.

1.1 THYMUS DEVELOPMENT AND THYMIC EPITHELIAL CELL REVIEW

To begin, this introduction will overview thymus organogenesis including the differentiation and heterogeneity of thymic epithelial cells (TECs) – the definitive functional unit of the organ. The thymus is derived from the third pharyngeal pouch of the of the embryonic gut tube[4]. On either side of the tube, a single layer of the pharyngeal pouch's endoderm "buds off" from the structure and migrates to form both the parathyroid glands and the two lobes of the thymus, which will meet to medially to form the bilobed structure within the mediastinum superior and ventral of the heart[4,5]. Neural crest cells of the pharyngeal pouch adjacent to the third and fourth branchial arches will go on to form the mesenchyme of the thymus including pericytes, fibroblasts and organ lining mesothelium[6].

Foxn1 is the defining gene of Thymic Epithelial Cells. However, *Foxn1* expression is not actually required for the specification of TEC lineage, and is rather necessary for later TEC maturation, function, and maintenance[7-13]. The gene(s) that 'switch on' the differentiation of progenitors into TECs are not presently defined. A likely hypothesis is that an axis between thymic differentiation favoring *Bmp4* (bone morphogenic protein 4), and parathyroid differentiation favoring *Shh* (sonic-hedgehog) exists within the primordial pharyngeal endoderm[14,15]. TECs will differentiate into either Cortical (cTECs) or Medullary (mTECs) which will define the structure of the thymus and establish the stages of T cell development. Historical models proposed distinct progenitor cell type between cTECs and mTECs, though, this theory has been largely abandoned in favor of the existence of an embryonic bipotent TEC progenitor (TECP) that gives rise to both cTECs and mTECs[4,9,16-18]. The existence of a bipotent TEC progenitor that gives rise to cTECs and mTECs in the *postnatal* thymus remains controversial, though. Because of this controversy and due to the confusing nature of the question of TECPs even when well reviewed[19,20], I will take this opportunity to summarize several works that clarify the origin of TECs and whether a population with medullary/cortical plasticity exists in the postnatal thymus **(Table 1.1, Fig. 1.1)**.

TABLE 1.1: Thymic Epithelial Progenitor Cell (TEPC): A Twisting Tale of (Likely) Temporal Biopotency

Publication	Methodology and Finding	Takeaway	Comments
Establishing Existence of Embryonic Bipotent Progenitor			
Bennet, A., et al., 2002 Identification and characterization of thymic epithelial progenitor cells[16] *Gill, J., et al., 2002* Generation of a complete thymic microenvironment by MTS24$^+$ epithelial cells[17]	MTS24$^+$ epithelium purified from E12.5 or E15.5 thymus primordium, respectively, used to establish Reaggregate Thymic Organ Cultures (RTOC) that when transplanted under kidney capsule of nude athymic mouse can differentiate into mTECs and cTECs	Among first evidence of embryonic bipotent progenitor.	MTS24$^+$ is no longer accepted as a marker for TECPs[21] RTOCs established by coculture of hundreds of embryonic thymic primordium epithelial cells so the bipotent differentiation is dependent on the selection of a highly pure population, which MTS24$^+$ cells are not. Furthermore, RTOC studies cannot show *in situ* bipotent potential
Rossi, S., et al., 2006 Clonal analysis reveals a common progenitor for thymic cortical and medullary epithelium[18]	Single eYFP$^+$ EPCAM1$^+$ cells from E12 thymus rudiment were then injected into a non-fluorescent thymus rudiment of a WT mouse which was then retransplanted into	Showed definitive evidence of bipotency of single cell thymic	This and the study below are the earliest most convincing evidence of embryonic bipotent TEPC, however this study utilizes thymic rudiment transplantation under the

	the kidney capsule of eYFP mcie; 4 weeks later, lineage tracing eYFP$^+$ cells found both cortical and medullary TEC progeny	epithelial progenitors	kidney capsule rather than direct thymus *in situ* lineage tracing.
Ohigashi, I. et al., 2013 Aire-expressing thymic medullary epithelial cells originate from β5t-expressing progenitor cells[22]	Generated *β5t-Cre x LoxP-EGFP* mice which expressed GFP under control of gene expressed in mature cTECs and not mTECs (β5 is component of thymoproteosome) and identify GFP$^+$ mature cTECs and mTECs	Progenitor cells expressing component of cTECs could give rise to mTECs. Defines broadly accepted 'cTEC footbprint' (PLET1$^+$ CD205$^+$ β5t$^+$ and IL-7 producing).	These lineage tracing studies implicate a progenitor population that expresses a "cTEC footprint" that is capable of bipotency between downstream mTEC and cTEC differentiation.
Balik, S. et al., 2013 Generation of both cortical and Aire$^+$ medullary thymic epithelial compartments from CD205$^+$ progenitors[23]	Use *Foxn1-EGFP* to show that a CD205$^+$ FoxN1+ population can give rise to mature mTECs. CD205 is expressed by mature cTECs and not TECs		
Proposing Existence of Postnatal Bipotent TEPC Progenitor			
Bleul, C., et al., 2006 Formation of a functional thymus initiated by a postnatal epithelial progenitor cell[9]	*In vivo* lineage tracing using *hK14*::Cre-ERT2;*Rosa26R*-eYFP mice propose single cell gave rise to mTECs and cTECs. Crossed this mouse to one with a null allele for *Foxn1* which can be reverted back to WT to show that bipotent progenitors are present in the neonate thymus.	*Foxn1*$^+$ embryonic bipotent TECPs exist + give rise to islets of differentiated unipotent mTECs and cTECs and first evidence of postnatal biopotency	Demosntrates TEPC capability to differentiate into either unipotent mTECs or cTECs. However, its suggestion of a postnatal TEC is dependent on reactivation of a null *Foxn1* allele and not proof that a WT postnatal thymus contains bipotent TEC progenitors. *Note though, embryonic bipotent TECPs since identified as *Foxn1*$^-$
Ulyanchenko, S., et al., 2016 Identification of a bipotent epithelial progenitor population in the adult thymus[24]	Adolescent mouse corticomedullary junction GFP$^+$ Ly51$^+$ PLET1$^+$ TECs isolated for RTOCs transplanted under kidney capsule gave rise to cTEC and mTECs and had progeny 9 months later	Established capacity for isolated TEC populations from the adult thymus to give rise to long-lived cTECs and mTECs	Only showed potential of these adolescent mouse derived TECs to differentiate in an RTOC transplant system. Strengthened argument for cTEC "footprint" of a bipotent progenitor described above.
Wong, K., et al., 2014 Multilineage potential and self-renewal define an epithelial progenitor cell population in the adult thymus[25]	Identifies low turnover, stem-like MHCII^{lo} α6-integrin^{hi} Sca-1^{hi} corticomedullary junction located TECs in adolescent mice that could give rise to cTEC and mTEC lineages in 3D culture and RTOC kidney capsule transplants		Offers different phenotype than the above for their stem-like bipotent TEPCs, but both place TEPCs at corticomedullary junction. Evidence a 3D culture system and an in vivo RTOC transplant system for their progenitor into cTEC and mTEC differentiation which has the pitfalls of being derived from a potentially heterogenous TEC population and does not show true *in situ* biopotency
Lepletier, A. et al., 2019 Interplay between Follistatin, Activin A, and Bmp4 signaling regulates postnatal thymic epithelial progenitor cell differentiation during aging[26]	Utilized thymus rejuvenation in aged mice via sex steroid ablation, they propose that MHCII^{lo} α6-integrin^{hi} Sca-1^{hi} corticomedullary junction located TEPCs described in *Wong, et al.*[25] become quiescent during puberty but can be give rise to cTECs and mTECs following androgen depletion	Describe mech. contributing to aged TEC dysfunction in which increased Follistatin A by TEPCs inhibits their own differentiation and maturation into functional cTECs and mTECs	
Dumont-Lagacé, M. et al., 2017 Detection of quiescent radioresistant epithelial progenitors in the adult thymus[27]	Use *ROSA26-rtTA;TetO-H2B-Gfp* mouse model allowing identification of slow-cycling cells through label retention in a pulse-chase assay to identify stem-like TEPC population at the corticomedullary junction that is UEA1$^-$ Krt5$^+$ Krt8$^+$ that becomes highly replicative during regeneration	Postnatal quiescent population that has features both mTECs and cTECs is more replicative than mature TEC subsets during regeneration	NO suggestion or experimentation of the bipotency of these TEPCs but does strengthen argument for postnatal stem-like population at the corticomedullary junction

Ucar, A. et al., 2014 Adult thymus contains FoxN1$^-$ epithelial stem cells that are bipotent for medullary and cortical thymic epithelial lineages[28]	*Ucar, et al.* describes an adult thymic epithelial progenitor population harvested ex vivo from adult mice and capable of long-lived spheroid formation that gives rise to mTECs and cTECs in vitro and *in vivo* when used for RTOC kidney capsule transplantation.	Identified bipotent adult thymus epithelial progenitor population that, surprisingly, is Foxn1$^-$.	These works do implicate a population of Foxn1$^-$ epithelium that can give rise to mTECs *in vitro* and under RTOC transplant conditioning, however their data suggest that TEPC progenitor differentiation into cTECs is *Foxn1* dependent. Moreover, neither demonstrate *in situ* adult bipotent progenitor capacity.
Nowell,C., et al., 2011 FoxN1 regulates lineage progression in cortical and medullary thymic cells but is dispensable for medullary sublineage divergence[11]	The above combined with *Nowell, et al.* which utilizes a mouse model with a conditionally revertible null *Foxn1* allele demonstrating cTEC and mTEC differentiation capability in absence of Foxn1 implicate the potential for Foxn1$^-$ TECP population.		
Nusser, A., et al., 2022 Developmental dynamics of two bipotent thymic epithelial progenitor types[29]	Mouse model utilizing CRISPR Cas-9 based scarring of exon 3 of the *Hrpt* gene driven by *Foxn1* expression to track specific cell progeny and identified barcode overlap between mTECs and cTECs in the embryonic and neonatal thymus but limited overlap in the adolescent thymus	Identify 2 progenitor populations: an early cTEC-biased progenitor type and.a postnatal mTEC-biased progenitor type	This elegant fate mapping study allows for specific progeny tracing via cell specific CRISR induced scars to measure actual in situ progeny without the need for any external manipulation. Though they identify progenitors with bispecific capacity they clearly show that most barcodes are shared within distinct TEC populations suggesting more contribution from cell type specific progenitors than from a true bipotent stem TEPC. Furthermore, as their model is not an inducible Cre, the self-renewal of these TEPCs cannot yet be answered.
Ragazzini, R., et al., 2023 *"Defining the identity and the niches of epithelial stem cells with highly pleiotropic multilineage potency in the human thymus"*[30]	Identified polykeratin BCAM$^+$ TEC populations with 'atypical stem cell signature' and show by spatial seq. and IHC their location in the subcapsular and perivascular niches in human thymus. Pseudotime analysis of scRNASeq support multilineage potential and BCAM$^+$ sorting and ex vivo culture system demonstrates mTEC and cTEC differentiation.	Subcapsular and perivascular niches containing adult TEC stem cells. They demonstrated *ex vivo* multilineage differential capacity of these isolated 'bona fide' stem cell TEPCs.	This study is unique for its use of postnatal human thymus however, and for that reason, they could not possibly show lineage tracing. Show differentiation of mTEC and cTEC in culture system. Pseudotime analysis, while supportive, cannot be evidence of functional bipotent differentiation that meaningfully contributes to adult mTEC and cTEC populations.
Propose that Bipotent Progenitors Have Minimal/No Postnatal Contribution			
Mayer, CE., et al., 2016 Dynamic spatio-temporal contribution of single β5t$^+$ cortical epithelial precursors to the thymus medulla[31]	Use of an inducible β5t-reporter fate mapping mice modified from *Ohigashi, et al.* show that embryonic cortical TEPC near the medulla gives rise to mTECs	Built on earlier study identifying embryonic bipotent progenitor population with cTEC footprint but shows minimal contribution to any adult thymus medulla	Together, argue existence of bipotent embryonic TEC population that does not contribute to maintenance of mTECs with age and rather suggests that mature cTECs and mTECs are maintained by downstream lineage-committed progenitors. Along with Nusser, et al., these fate mapping studies point to a more likely scenario than that painted by RTOC based studies – populations within the postnatal thymus may be *capable* of bipotency but *in situ* have very little actual contribution to TEC population maintenance.
Ohigashi, I., et al., 2015 Adult thymic medullary epithelium is maintained and regenerated by lineage-restricted cells rather than bipotent progenitors[32]	Similarly use β5t-reporter fate mapping and show that adult mTEC populations are not maintained by bipotent β5t expressing cTEC footprint TEPCs		

Lucas, B., et al., 2023 Embryonic keratin19^{+} progenitors generate multiple functionally distinct progeny to maintain epithelial diversity in the adult thymus medulla[33]	Fate mapped TEC progeny using an inducible *Krt19*^{CreERT}TdTom mouse model identified an embryonic Relb-independent K19^{+}MHCII^{-} mTEC biased progenitor population that gives rise to all mTEC subsets.	Identify an embryonic K19^{+}TEPC which serves as a nearly entirely medullary progenitor but capable of sustaining all mTEC populations for life	Notably, it does suggest that ~5% of these K19+ mTEC progenitors can differentiate into cTECs suggesting some biopotency. However, this study induces lineage tracing around E15.5 and does not refute earlier bipotent embryonic TEC studies but does suggest that adult mTEC populations are not maintained by a true bipotent stem-like progenitor
Farley, A., et al., 2023 Thymic epithelial cell fate and potency in early organogenesis assessed by single cell transcriptional and functional analysis[34]	*In vivo* lineage tracing using *Sox9-Cre* and *Psmb11-Cre* mice show E12.5 mice have dedicated Sox9^{+} mTEC progenitors and that previously believed bipotent TEPCs are heavily cTEC biased progenitors.	Changed prior interpretations and show lineage dedicated TCPC progenitors exist sooner than believed within the embryonic thymus	This recent study is notable as it comes from groups previously reporting postnatal bipotent progenitor capacity. It proposes that prior works showing early bipotency in *ex vivo* manipulated contexts (i.e., 3D culture, RTOCs) may be inducing a bipotency program that is minimal *in situ*.

FIGURE 1.1: Thymic Epithelial Progenitor Cells (TEPCs) at embryonic, postnatal and post-damage stages

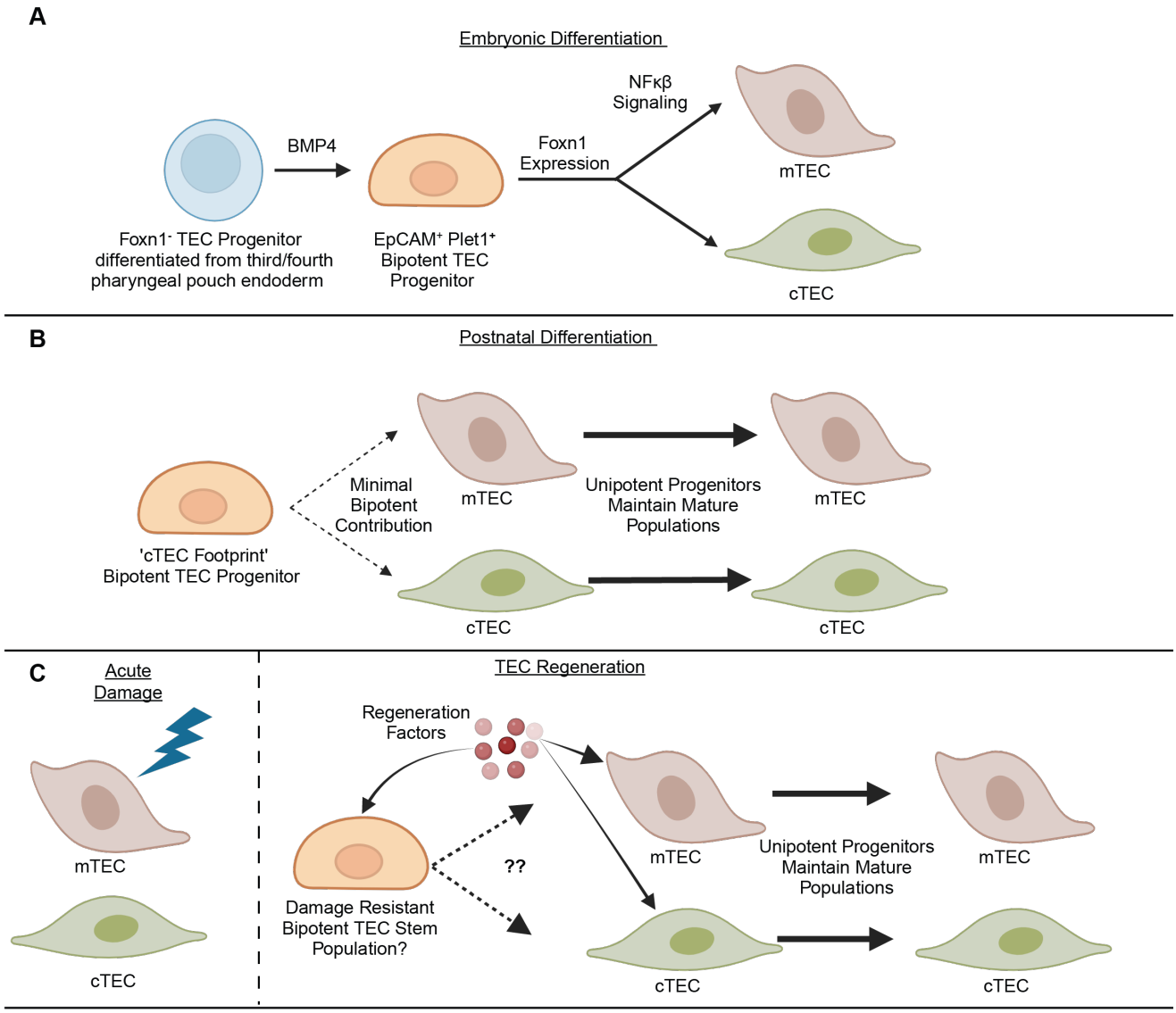


Figure 1.1: A, Thymic epithelial progenitor cells (TEPCs) at the embryonic stage express an EpCAM$^+$ PLET-1$^+$ phenotype. Progenitors do not require *Foxn1* for their initial differentiation toward the TEC lineage and are thus present within the thymic rudiment of *nu/nu* mice homozygous for non-functional *Foxn1*. Following *Foxn1* expression, TECs are directed toward the cTEC lineage in the absence of other signals. mTEC lineage direction requires RANKL:RANK and downstream NFκβ activaion. **B,** Studies disagree upon the phenotype and differentiation capacity of TEPCs in the postnatal thymus. Bipotentcy of adult murine TEPCs has been demonstrated by *ex vivo* TEPC culturing in reaggregared thymic organoid cultures (RTOCs) and their subsequent differentiatiation into mTECs and cTECs. While these studies show that TEC populations in the postnatal thymus *can* differentiate bipotently under manipulated conditions, they do not show that they *do* meaningully contribute to adult mTECs and cTECs *in situ*. *In situ* lineage tracing studies do show minor contributon (~5%) of a bipotent TEPC with a 'cTEC like footprint' (PLET-1$^+$ CD205$^+$ β5t$^+$) to adult mTECs and cTECs, however, adult cTECs and mTECs are mostly derived from distinct unipotent populations. **C,** Following damage to the thymus, regeneration factors such as BMP-4 and IL-22 act on TECs to initiate reconstitution. However, whether a regenration initiating TEC population exists and which population(s) contribute most to TEC reconstitution remain unanswered questions.

Foxn1 expression is required for TEC maturation, function and maintenance[13,35]. Induced *Foxn1* expression is even capable of reprogramming fibroblasts into functional TECs that can support thymopoieis *in vivo*[36]. Mice lacking functional *Foxn1* genes are known as 'nude' mice attributable to their hairless phenotype. Nude mice have a dysfunctional thymus rudiment and are incapable of endogenous T cell production. These athymic mice have proven essential in experimentation requiring allogeneic or xenogeneic tumor/tissue transplantation to avoid rejection of the engrafted tissue. Several genes downstream of *Foxn1* that control organ function and T cell differentiation have been identified. Among these, most recognized are *Ccl25* and *Cxcl12*, which are ligands required for hematopoietic progenitor recruitment and retainment within the thymus; *Dll4* a non-redundant Notch ligand required for T cell lineage commitment; and Stem Cell Factor (*Kitl*), a thymocyte survival signal [37-41]. However, the full downstream role of *Foxn1* is still being uncovered. *Zuklys, et al.,* utilized a transgenic mouse expressing a Flag-tagged FoxN1 and a doxycycline inducible *Foxn1* deletion mouse model to identify the most comprehensive list of *Foxn1* dependent genes yet[13]. They identify that, in addition to previously identified *Ccl25, Cxcl12, Dll4 and Kitl*, FoxN1 regulates other programs essential to TEC identity. These included antigen processing (i.e., *Tasp1, Psmb9, Psmb10, Psmb4, Psmb4, Psma4, Tap2, Prss16*), thymocyte selection (*Psmb11 and CD83*), transcription factors required for TEC proliferation and maturation (CREB1 and TP63), and even a complementary role to Aire in promoting promiscuous gene expression[13].

Foxn1 expression is dynamic during development; it is at its highest during embryonic development and until shortly after birth, and declines in adolescence and adulthood[42]. Mouse models in which postnatal *Foxn1* expression is reduced experience rapid thymus demise[12] and overexpression of *Foxn1* in aged mice can rescue thymus involution[43,44]; though more recent studies suggest that this effect requires extremely high *Foxn1* overexpression (>4-6 times endogenous levels)[45]. *Foxn1* is expressed in TECs but also in hair follicle cells (HFCs)[46,47]. As the upstream regulation of *Foxn1* remains incompletely understood, two groups recently probed

the differential *Foxn1* expression in these two cell types and identified a ~850 base pair cis-regulatory element located within the first intron of the gene necessary for TEC development but not HFC function[46,47]. Moreover, these works position this intronic region as a binding site for FoxN1 itself demonstrating a FoxN1 autoregulatory loop[13,46,47].

Human homozygous *Foxn1* mutation results in immunodeficiency known as lymphoid cystic thymic dysgenesis just as in Nude mice, though its incidence is <1/1,000,000[48]. However, heterozygous *Foxn1* mutations were only recently shown to result in severe T cell lymphopenia as well[49]. *Rota, et al.* demonstrated that *Foxn1* mutations can result in a dominant negative factor that displaces functional FoxN1 from nuclear condensates causing thymic hypoplasia potentially explaining the severe lymphopenia of heterozygotes and suggesting that inherited *Foxn1* mutations may be considered functionally autosomal recessive[50]. While *Foxn1* importance has been well-defined and its downstream regulation of TEC differentiation and homeostasis has been deeply probed, its regulation/function is still incompletely understood. Several thymus-regeneration promoting factors discussed below function by regulating TEC *Foxn1* expression[51,52].

Embryonic bipotent progenitors are believed to differentiate into cTECs by default[20]; mTEC differentiation requires NF-$\kappa\beta$ signaling within the bipotent progenitor leading to upregulation of RANK and a positive feedback NF-$\kappa\beta$ loop[53]. All TECs are $CD45^-$ Pan-$Cytokeratin^+$ $EpCAM^+$ $MHC\text{-}II^+$; cTECs can be identified by their expression of Keratin 8, CD205 and Ly-51, whereas mTECS are definable by their expression of UEA-1 and Keratins 5/14[19]. Once differentiated into cTECs and mTECs, there is further heterogeneity within each population[54,55]. cTECs are comparatively less heterogenous and can be broadly classified into $DLL4^{hi}$ or $DLL4^{lo}$ populations – it is likely that $DLL4^{hi}$ cTECs mature into $DLL4^{lo}$ cTECs[56]. There are also cTEC Nurse Cells that uptake large numbers of developing thymocytes aiding in their development or facilitating their apoptosis[19,57]. Though cTECs make up approximately 20% of all

TECs and far less than 1% of the total thymus makeup (when young and healthy), they have the weighty task of homing hematopoietic progenitors, directing T cell lineage commitment, facilitating their expansion/maturation and importantly, positively selecting T cells with receptors capable of recognizing antigen presentation machinery.

Medullary TECs, on the other hand, have considerably more diversity[58-60]. Recent works distinguish 4 main populations of mTECs **(Table 1.2)**. mTECs are responsible for promiscuous gene expression, transfer of tissue restricted peptides to antigen-presenting cells, negative selection of T cells and selection of regulatory T cells.

TABLE 1.2: mTEC Heterogeneity

mTEC Population	Proposed Function	Distinguishing Genes	Distinguishing Markers
mTEC I	Immature population that produces Ccl21 gradient by which developing thymocytes are drawn from the cortex to the medulla. Some exist at corticomedullary junction. If postnatal bipotent progenitor population exists, it is within this population	*Sox4, Ascl1, Itga6, Itgb4*	EpCAM^{+} UEA-1^{+} MHC-II^{lo} ITGB4^{+} L1CAM^{lo}
mTEC II	Differentiated from mTEC I population. Responsible for promiscuous gene expression	*Aire, Fezf2, H2-Aa, CD80, CD83*	EpCAM^{+} UEA-1^{+} MHC-II^{hi} Ly-6D^{-}
mTEC III	Corneocyte-like mTECs that form Hassals of Corpuscle (iselts within medulla consisting of fully mature cornified mTECs); their role in T cell selection is unknown	*Pigr, Ly6D, Krt10*	EpCAM^{+} UEA-1^{+} MHC-II$^{hi/lo}$ Ly-6D+
mTEC IV	IL-25 producing Tuft like TEC with an unknown homeostatic function	*L1cam, Sox9, Pouf23, Krt8, Dclk1*	EpCAM^{+} UEA-1^{+} MHC-II^{lo} ITGB4^{lo} L1CAMhi

Having established the organogenesis of the thymus and the populations of TECs that control its function, I turn now to reviewing the processes governing T cell commitment and selection.

1.2 THYMIC FUNCTION: THYMOPOIESIS

1.2a: Thymus Seeding Progenitors

We begin our journey of T cell production, termed thymopoiesis, from the perspective of a hematopoietic lymphoid progenitor cell released from the bone marrow into circulation. Cortical TEC production of CCL25 and CCL19/21 create a gradient that attracts CCR7/9

bearing hematopoietic progenitors to the thymus corticomedullary junction (CMJ)[61]. There, Thymic Seeding Progenitors (TSPs) enter the thymus via interaction of PSGL1$^+$ progenitors and P-selectin$^+$ endothelial cells at the CMJ[62]. Once within the thymus, cTECs provide non-redundant DLL4 signaling to Notch-1 Receptor to direct thymocyte lineage commitment of the newly imported TSPs[40]. Notch signaling is required to induce expression transcription factors *Tcf1, Gata3* (and subsequently *Bcl11b*), which in turn initiate T cell lineage gene expression including components of T cell receptor (TCR) formation (e.g., *Rag1/2*) [63]. At this point progenitors upregulate IL-7R and are termed Early Thymic Progenitors (ETPs) – however it should be noted here that phenotypic ETPs (CD4$^-$ CD8$^-$ cKit$^+$) within the thymus maintain differentiation capacity into non T cell lineages such as B cells and myeloid populations[64]. cTECs produce IL-7 and Stem Cell Factor (SCF, also known as KitL) needed for thymocyte survival and proliferation and CXCL12 to maintain CXCR4$^+$ thymocytes within the spongelike network of the cortex through which thymocytes mature and differentiate[61]. These earliest stages are defined by a lack of CD4 and CD8 expression and are thus termed “Double Negative” thymocytes. Following the DN1 stage (CD44$^+$ CD25$^-$), thymocytes upregulate IL-2Rα (CD25) at which point they become irreversibly committed to the T cell lineage[63]. TCRβ encoding genes begin their process of somatic VDJ segment recombination at the junction between DN2 (CD44$^+$ CD25$^+$) and DN3 (CD44$^-$ CD25$^+$) stages, and if successful will express the pre-TCR. The pre-TCR associates with CD3 to provide constitutive activation in the absence of stimulatory ligand; signaling involves the kinases LCK and ZAP70 and linker for activated T cells (LAT) and adaptor protein SLP76 which stimulate downstream activation cascades[65]. Note, this introduction to thymopoieis details prototypical $\alpha\beta$ T cell synthesis and not $\gamma\delta$ chain production. Pre-TCR signaling initiates massive proliferation and transition in to the (CD44$^-$ CD25$^-$) DN4 stage of development. DN4 thymocytes proliferate until they undergo successful

TCRα rearrangement and transition to TCR bearing $CD4^+$ $CD8^+$ Double Positive (DP) thymocytes **(Fig. 1.2).**

1.2b: Positive Selection

The cell mediated adaptive immune system solved the dilemma of creating a repertoire capable of diverse recognition through the massive degree of variety that somatic recombination yields – it has been estimated that the total paired $\alpha\beta$ chain with junctional diversity potential repertoire diversity is ~$2x10^{19}$ unique receptors[66,67]. Having established profound diversity, how does the thymus ensure that these TCRs are: 1. Functional – meaning they can recognize and react to antigen presentation and 2. Tolerant – what insurance mechanisms are in place such that these functional T cells are not self-reactive? Enter thymic selection.

cTECs are responsible for the positive selection stage of thymopoiesis. Highly polymorphic Major Histocompatibility Complex types I and II (MHC-I and MHC-II) present peptides derived from broken down proteins and T cells recognize the combination of this peptide:MHC complex. cTEC expression of the "self-peptide" loaded into MHC complexes for positive selection is owed to peptide processing and presentation machinery known as the "thymoproteosome". Expression of *Psmb11* encoding β5t – as opposed to other cells' expression of β5/β5i – processes peptides for presentation onto MHC-I while Thymus-specific Serine Protease (TSSP) and Cathespin L – as opposed to other cells' expression of Cathespin S – process endosomal peptides for MHC-II presentation[68]. Thus, cTECs express unique self-peptides in the service of TCR positive selection. During positive selection, newly formed TCRs are tested for their ability to recognize peptide:MHC complex (MHC-restriction). DP thymocytes with TCRs incapable of self-peptide:MHC recognition will have the opportunity to rearrange the TCRα chain and if the TCR is still incapable of self peptide:MHC recognition, the DP thymocyte will undergo apoptosis in a process known as death by neglect within 3-4 days. The vast majority of thymocytes are eliminated at this stage[69,70,71]. TCR recognition of MHC-I or MHC-II

directs T cell lineage direction; TCRs recognizing peptide:MHC-I complexes undergo *Runx3* dependent $CD8^+$ Single Positive differentiation and those recognizing MHC-II undergo *Thpok* $CD4^+$ Single Positive differentiation[68].

1.2c: Negative Selection and Thymus Egress

Next, the thymus has an elegant system for depleting potentially autoreactive T cells. mTECs positioned near the CMJ produce CCL21/CCL19, which attracts CCR7 bearing Single Positive (SP) thymocytes to enter the medullary space[69]. There, mTECs capable of what's known as 'promiscuous gene expression' (PGE) will express genes encoding tissue-restricted antigens (TRAs, i.e., Myelin Basic Protein and Insulin) that T cells may encounter once released into the periphery. Collectively, mature mTECs can express an impressive ~90% of the coding genome[72]. However, individual mTEC expression of all TRAs is an insurmountable metabolic task and it is estimated that a TRA may be expressed by only 0.4-3% of mTECs and thus tolerance is owed to a stochastic mTEC mosaic expression of TRAs[19,73]. This process is mediated largely by the gene *Aire*. AIRE acts to de-repress the expression of tissue specific sites within the genome[74]. It regulates ~4,000 genes – most of which encode TRAs – either directly or by enhancing their otherwise low expression[75]. Aire mutation results in a condition known as Autoimmune Polyendocrine Syndrome 1 characterized by autoimmunity targeting several organs (hypoparathyroidism, adrenocortical insufficiency). However, there remains a significant degree of TRA encoding genes that are AIRE independent. More recently another transcriptional regulator FEZF2 was implicated in the upregulation of ~400 of the Aire independent genes[75]. How the remaining TRAs are expressed remains a largely unanswered question. However, more recent evidence identifying the existence of so called "mimetic" mTECs which can co-opt lineage defining transcription factors of various cell types clearly play a major role in tolerance induction – though the processes regulating these mimetic mTECs require further investigation[76]. Once encoded, these TRA's are presented by $CD80/86^+$ $MHC\text{-}II^{hi}$

mTECs or transferred to hematopoietic antigen presenting cells, particularly XCR1^{+} cDC1s, for TCR recognition[70].

Single positive thymocytes with TCRs that are activated by self-antigen within the medulla undergo apoptosis (Negative Selection) or are shunted toward the regulatory T cell lineage[68]. Notably, negative selection is not isolated to the medulla and TCRs that bind self-peptide: MHC complexes presented by cTECs too tightly also undergo apoptosis[68]. The combination of positive and negative selection yields an affinity model or “goldilocks theory” of TCR affinity – TCRs must bind peptide:MHC complex tightly enough to dodge death by neglect, but not so tightly that they are negatively selected against. Overall, this leads to the cell death of ~98% of all developing thymocytes and the escape of only ~2% of developing thymocytes as naïve CD4/CD8 T cells at the end of the 3 weeks of thymopoiesis. Finally, after enduring these selection stages CD4/CD8 SP cells upregulate Sphingosine 1-phosphate Receptor (S1PR) and are induced by the high concentration of S1P in the peripheral blood stream to extravagate into circulation and begin their migration to secondary lymphoid organs[77].

FIGURE 1.2: Thymopoiesis Summarized

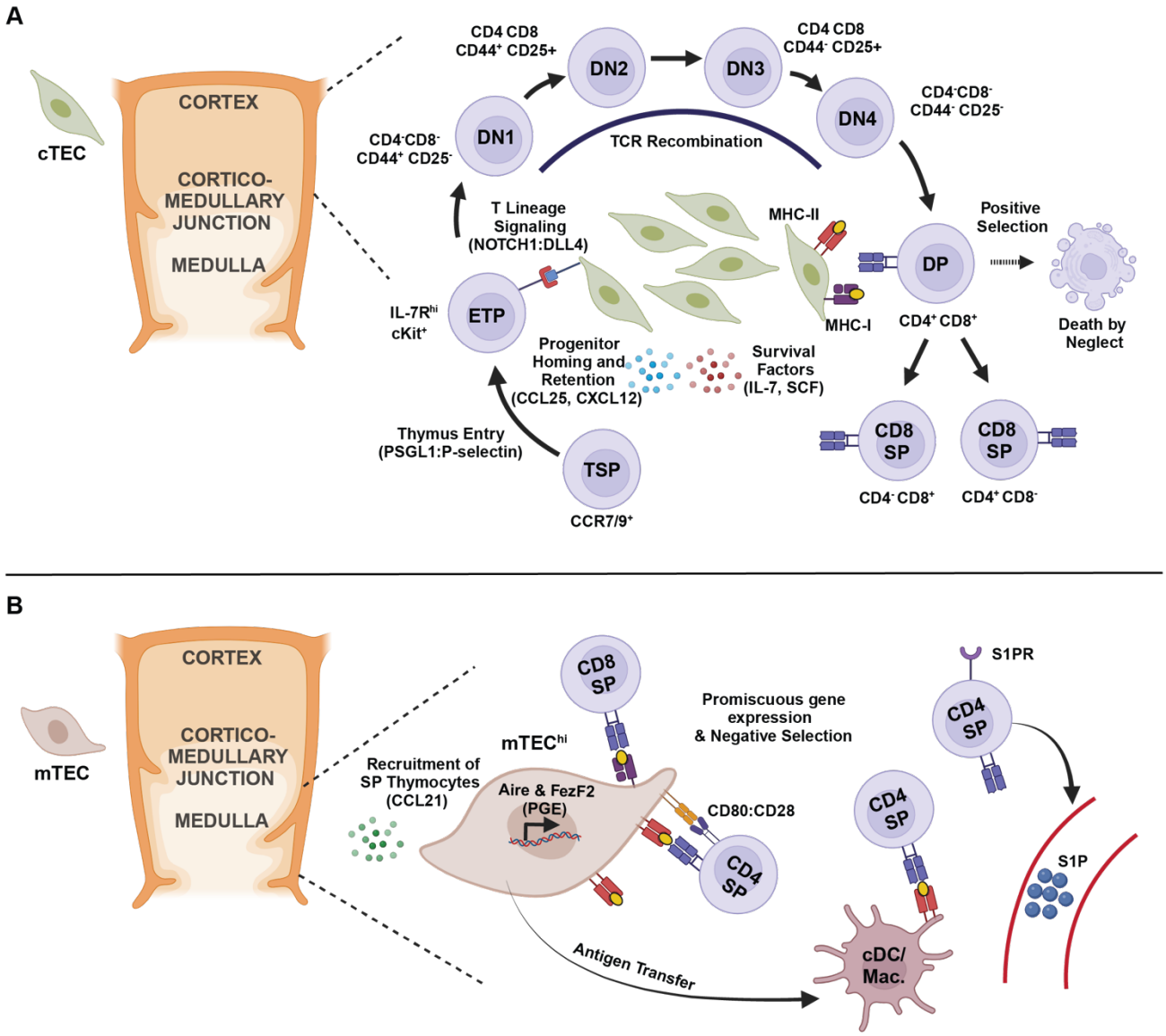


Figure 1.2: A, Thymic seeding progenitors (TSPs) are attracted to the corticomedullary junction by CCL25 and enter via P-Selectin Ligand binding to P-Selectin expresing endothelium. CXCL12 helps maintain thymocytes in the Cortex. cTECs present DLL-4 which binds NOTCH1 on TSPs to induce T cell lineage committment. Thymocyte populations differentiate into Early Thymic Progenitors (ETPs) and then into Double Negative 1 ($CD4^-CD8^-CD44^+CD25^-$), Double Negative 2 ($CD4^-CD8^-CD44^+CD25^+$), Double Negative 3 ($CD4^-CD8^-CD44^-CD25^+$), Double Negative 4 ($CD4^-CD8^-CD44^-CD25^-$) thymocyte developmental stages while undergoing TCR gene somatic recombination. Successful TCR expression results in $CD4^+CD8^+$ Double Positive (DP) thymocyte differentiation. Newly formed TCRs are tested against MHC:self-peptide complexes. Those incapable of MHC:self-peptide recognition will undergo apoptosis (death by neglect) and those which reocgnize MHC:self-peptide too tightly also undergo apoptosis (negative selection). DP thymocytes capable of MHC:self-peptide recognition with intermediate binding affinity will be positively selected and diferentiate into Single Positive $CD4^+$ (if recognizing MHC-II) or Single Positive $CD8^+$ (if recognizing MHC-I) thymocytes. **B,** mTEC secreted CCL21 draws SP thymocytes from the cortex into the medulla where, collectively, mTECs present antigens derived from proteins encoded by 90% of the genome. This promiscuous gene expression by mTECs is facilitated by the transcription regulators AIRE and FEZF2. If a SP thymocyte TCR binds too tightly to mTEC presented MHC:antigen complex, it will be negatively selected against and undergo apoptosis or be shunted toward the regulatory T cell lineage. After ~3 weeks of thymus development, CD4/CD8 thymocytes surviving slection stages upregulate Sphingosine-1 Phosphate Receptor (S1PR) and follow a gradient of Sphingosine-1 Phosphate (S1P) into the peripheral blood.

1.3 ACUTE THYMUS INJURY

Thymus function is not static and is rather highly variable in response to environmental stimuli. Here, I overview the several injurious stimuli that lead to acute thymus involution from every day minor injuries to profound thymus decimating ones.

1.3a: Steroids

Stressors leading to rises in systemic cortisol, a glucocorticoid hormone, are well known to cause thymic involution via apoptosis of thymocytes and clinical studies show a negative relationship between systemic corticosteroid levels and thymic function[78 79,80]. Glucocorticoids are central to many acute forms of thymic involution, directly inducing cell death of $CD4^+CD8^+$ DP thymocytes, which preferentially express the intracellular glucocorticoid receptor[79]; the same effect occurs following exposure to commonly used glucocorticoid immunosuppressives (i.e., dexamethasone, prednisone)[80]. The preferential glucocorticoid receptor expression within DP thymocytes may be attributable to a homeostatic role for TEC derived glucocorticoids in inducing DP apoptosis regulating thymopoiesis[81].

Sex steroids, or androgens, also contribute to thymus atrophy and in fact, the onset of puberty when sex steroid levels peak coincides with the period of most rapid age-related thymus involution[74,82,83]. Sex-steroid induced apoptosis of developing thymocytes themselves is mediated via glucocorticoid receptor as well as TNFα signaling[84,85]. However, androgen signaling is most impactful within the thymic epithelium in the context of involution. Androgen Receptor (AR) gene deletion within thymus stroma but not developing thymocytes increased thymus cellularity and improves thymus recovery post-HCT[86]. Androgen activity within TECs suppresses production of hematopoietic progenitor recruitment chemokines, most notably CCL25, thymocyte survival signal IL-7, and DLL4 production in cTECs[87-89]. It's long been appreciated (over a century!)[90] that sex steroid ablation, either surgically or, much more relevantly, with luteinizing hormone-releasing hormone (LHRH) receptor antagonism drugs

clinically approved in treatment of breast and prostate cancers, can rescue age-related thymus involution[91-94]. LHRH blockade can also improve T cell reconstitution following HCT and therefore represents an option for therapeutic intervention in multiple circumstances of thymus involution and T cell lymphopenia[95].

During pregnancy, rises in progesterone lead to thymus involution, a process required for normal fertility[96]. Progesterone mediates involution directly through cTEC downregulation of *Foxn1*, the master regulator gene of TEC function[97], as well as indirectly by suppressing the production of chemokines that attract hematopoietic progenitors[98]. Despite inducing thymus involution, progesterone leads to an increased development of regulatory T cells (Tregs), which is accomplished via progesterone mediated receptor activator of NF-κB ligand (RANK) signaling on mTECs[99]. RANK signaling promotes maturation of $CD80^{hi}$ mTECs and increased expression of transcriptional regulators of promiscuous gene expression (PGE) *Aire* and *Fezf2*[99].

1.3b: Infection

Infection can also lead to acute thymic involution through direct and indirect mechanisms[74,100,101]. Elevated intrathymic and systemic pro-inflammatory cytokines (i.e., IL-6, TNFα, IFNα, IFNγ) resulting from infections also can induce thymic atrophy[100-102]. Apart from depleting developing thymocytes, the overall thymic architecture, predominantly the loss of cortico-medullary distinction, is also linked to infection[101].

S.Typhimurium infection of thymus tissue causes apoptosis of double positive thymocytes due to, in part, IFNγ and endogenous glucocorticoids leading to organ atrophy persisting for weeks after infection resolution[102,103]. Other bacterial infections such as *E.coli,* streptococcus (*S.suis),* Mycobacterium (*M.avium and M.tubercolosis*), and the leakage of intestinal microbiota result in similar kinetics of thymic atrophy[104-108].

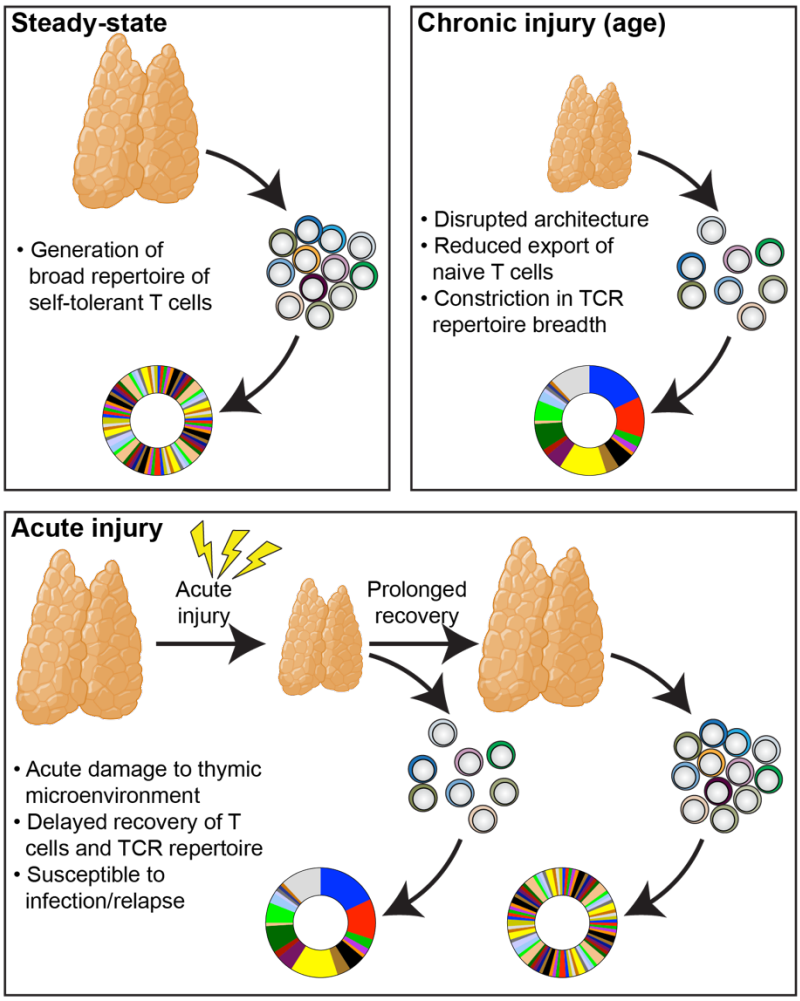
Viral infection can also damage the thymus. To group discussion of infection related injuries, chronic viral infection induced thymus injury is included here. Chronic Lymphocytic

Choriomeningitis virus (LCMV) infection leads to loss of a majority of their thymic cellularity in a mechanism thought to be driven by and explained by chronic LCMV activated $CD8^+$ T cell type I interferon response[109]. Human Immunodeficiency Virus (HIV) also targets the thymus, and is associated with decreased sjTREC (signal joint T cell receptor excision circles; a surrogate measurement for thymic T cell production) measurements[110]. However, anti-retroviral therapy (ART) can reverse this thymus damage[110,111].

1.3c: Chemotherapy and HCT Conditioning

While most stress and infection induced thymus damage is comparatively modest, the damage to the thymus caused by cytotoxic chemotherapy used to treat cancer and the conditioning received prior to Hematopoietic Cell Transplantation (HCT) is profound[112]. Alkylating chemotherapies such as cyclophosphamide are lymphotoxic to all thymocyte subsets[113,114]. Radiation damage especially has enduring effects on thymocyte development, as studies have shown both acute and chronic defects in thymopoiesis following a single dose of high intensity ionizing radiation[115-118] While thymocyte populations, particularly DP thymocytes, are highly sensitive to radiation and alkylating agents, the stromal compartment is not spared and its damage contributes to long-term deficiencies in thymic function[119]. Though peripheral expansion of T cell populations (thymus-independent T cell reconstitution) can partially compensate for reduced thymic function following cyto-ablation, this leads to a constricted, less diverse T cell repertoire **(Fig. 1.3)**. Patients are more vulnerable to opportunistic infection and relapse of primary malignancies during this period of deficient T cell numbers and diversity, thus underscoring the need to create thymus boosting therapies to aid immune reconstitution[120-122].

FIGURE 1.3: Acute and chronic thymus involution leads to decreased T cell receptor diversity



1.4 CELLULAR & MOLECULAR MECHANISMS OF ENDOGENOUS THYMUS REGENERATION

The pathways regulating endogenous thymus regeneration have begun to be uncovered. While TECs remain the functional units of thymopoiesis and are the master regulators of thymus regeneration, they do not act alone. Rather, organ recovery relies on cross talk between TECs and heterogenous mixture of hematopoietic and non-hematopoietic cells of the thymus stroma. The following section summarizes findings implicating these populations **(Fig. 1.4)**.

1.4a: Innate Lymphoid Cells

Innate lymphoid cells (ILCs) represent a rare population in the homeostatic thymus; however, they are highly damage resistant and are thus overrepresented following acute thymus injury[123]. Ours and other groups have identified multiple pathways by which ILCs support endogenous tissue recovery. After ionizing radiation induces loss of developing DP thymocytes, Roryt^{+} CCR6^{+} ILC3s are a potent source of IL-22[123]. IL-22 promotes proliferation of TECs in a Stat3/Mcl-1 signaling dependent fashion increasing their expression of *Foxn1*, *Aire*, and *Kgf*[124]. In Graft-vs-host disease (GvHD), ILC3 depletion leads to suppressed thymic regeneration, which is rescued upon exogenous IL-22 administration[125]. Notably, ILC derived IL-22 also has a protective effect on intestinal stem cells which represent an epithelial progenitor niche with several parallels to thymus epithelium[126,127]. These works became the basis of clinical trial NCT02406651 in which the effects of recombinant IL-22 in mitigating acute intestinal GVHD. Early results from clinical trial (NCT 02406651) suggest IL-22 (administered with corticosteroids) is well tolerated and alleviates dysbiosis in patients suffering from intestinal GVHD[128]. Though not yet available, the effect of IL-22 on thymic function and T cell reconstitution will be a secondary outcome in this cohort. IL-22 is also currently being explored in treatment of hepatitis (NCT02655510), and preliminary data results suggesting IL-22

treatment leads to increased hepatic regeneration and improved clinical scores of hepatic function (Lille and MELD), speaking to the potential shared regenerative pathways between epithelial progenitor cells of different organs[129]. Thymic ILC3s also express Receptor activator of nuclear factor κ-β ligand (RANKL), which induces NFκβ signaling by binding RANK expressing mTECs and promotes the maturation of medullary compartment[130,131]. During regeneration, ILC3s also produce Lymphotoxin-α that supports TEC proliferation and reconstitution, expression of *Ccl19* and *Ccl21*, and endothelial cell expression of *Icam-1*, *Vcam-1*, and *Selp*[61]. Finally, alarmins, IL-25 from thymus tuft cells and IL-33 from fibroblasts, are reported to stimulate ILC2 production of IL-4, IL-13, IL-5 and Amphiregulin (ligand for Epidermal Growth Factor) that contribute to organ repair[132-135]. Interestingly, tuft epithelial cells only contribute to regeneration after glucocorticoid and not radiation induced damage[132,134], suggesting that regenerative mechanisms may be specific to the modality of injury. These studies are examples of an emerging knowledge of a pro-regenerative Type 2 immunity axis[132,136,137]. Further supportive of this Type 2 coordinated response, though not ILCs, recirculating regulatory $CD4^+$ T cells also aid thymus regeneration via provision of Amphiregulin that promotes epithelialization[137].

1.4b: Endothelial Cells

Thymic endothelial cells (ECs) sense the loss of developing thymocytes post-acute damage and participate in production of regenerative factors[52,138]. Endothelial cells are a damage-resistant stromal cell type and are overrepresented within the thymus following acute damage. Following damage, ECs upregulate *BMP4*, a member of the TGFβ superfamily known to promote the development of thymus epithelium[139]. BMP4 primarily signals via cTECs increasing their *Foxn1* expression and downstream targets essential for T cell development including *Dll4* and *Ccl25*[52]. Given that TECPs share similar markers as cTECs, there is an exciting possibility that BMP4 may directly stimulate epithelial progenitor cells that rebuild the

stromal microenvironment. Interestingly, recent work implicates BMP4 antagonism in mediating TEC dysfunction and age-related thymus involution[26].

1.4c: Mesenchyme

Several studies of mesenchyme involvement in thymus repair point to their sentinel function as producers of alarmins (see above), however, fibroblast heterogeneity and their direct impact on thymic repair have begun to be elucidated[132,133]. Fibroblast growth factors (FGFs) produced by mesenchymal cells are known to be mitogenic, especially after injury, within epithelial cell lineages in skin, lung, bladder, gastrointestinal, and thymic tissue.[140,141] FGF molecules bind FGF receptors (FGFRs) that are encoded by a family of structurally similar tyrosine kinases.[140] The seventh FGF discovered, also known as Keratinocyte growth factor (KGF), has been shown to directly act on FGFR2IIIb expressing TECs and support the formation of the medullary epithelium[142,143]. Exogenous KGF treatment also protected thymic damage in models of GVHD, suggesting the important contribution of the molecule to restoring thymopoiesis[143]. Interestingly, another Fibroblastic growth Factor (FGF21) also aids in thymus rejuvenation in age-related chronic and post-HCT acute involution[144]. FGF21 full-body knockouts exhibited reduced numbers of major TEC subsets and defects in overall thymic function[144]. Recently, there has been increased interest in characterizing thymic fibroblast subsets to further elucidate their homeostatic and regenerative function. Through *In silico* analysis of mouse and human thymi, distinct populations of thymus fibroblasts have been characterized and their contributions to thymus function have begun to be appreciated[145,146]. A $Postn^{+}$ mesenchymal cell population residing along the corticomedullary region produces key lymphopoietic cytokines (CCL19, Flt3L, and IL-15) and upregulates these factors following acute injury to recruit of lymphoid progenitors[146].

1.4d: Myeloid Cells

In the context of acute irradiation damage, thymic dendritic cells (DC) sense the depletion of $CD4^+$ $CD8^+$ developing thymocytes and release IL-23 that stimulates ILC3 IL-22 prouduction[123]. Although IL-23 can be detected in different DC subsets, $CD103^+$ cDC1s are the crucial producers of functional IL-23 after damage. Thymus macrophages that make up ~0.1% of the organ cellularity are responsible for the clearance of the vast numbers apoptotic DP thymocytes, and are critical self-antigen presenters during negative selection highlighting this miniscule population's importance during homeostatic thymopoiesis[147]. However, remarkably little is known regarding their involvement in thymus regeneration. It has been posited that damage-induced *MafB* transcription factor expression within thymic macrophages and downstream production of IL-4 supports thymic regeneration[148] Eosinophils have also been implicated as contributors to thymus regeneration[134]. Eosinophils are recruited to the thymus post-radiation injury by ILC2 produced IL-5 and produce pro-reparative IL-4[134]. It is possible that both eosinophils and macrophages contribute to regenerative IL-4, however, it is likely that there is overlap especially considering shared phenotypic markers used in their identification, namely F4/80. Moreover, the exact mechanisms by which these myeloid populations and IL-4 support thymic regeneration remains incompletely understood.

Worth noting, however, is that factors outside of those implicated in endogenous processes also show promise in supporting thymic reconstitution. In contrast to blockade of sex-steroid hormones, provision of growth hormone (GH) and the "hunger hormone" Ghrelin support thymus function[149,150]. GH, of particular interest, is capable of improving HIV patient T cell production and antiviral response[151]. Thymosin-α1 is another exogenous potential thymus reparative factor as it may increase lymphocytes post HCT[152]. The cytokines IL-12, which acts by upregulating IL-7 production, and IL-21 also support T cell reconstitution in settings of age and acute damage[153-156].

Overall, organ repair and efficient re-epithelization is coordinated by a heterogenous mixture of hematopoietic and non-hematopoietic thymus stroma. While TECs remain at the

center, regeneration associated factors produced by other cellular participants aid in the regenerative response – or in other words, it takes a village to mediate endogenous thymus regeneration. Several of these factors are being investigated in clinical trials **(summarized in Table 1.3)**.

TABLE 1.3: Therapeutic strategies to boost thymic function: preclinical development and clinical translation

	Preclinical		Clinical		Trial Number	Refs
	Acute	Chronic				
IL-7	++	++	Increased T cells in HIV+ patients and improved reconstitution in allo-HCT recipients		NCT03941769	157-162
KGF	++	++	Used widely for mucositis, trials have not shown considerable benefit in T cell reconstitution in recipients of HCT.		NCT01233921, NCT03042585, NCT02356159, NCT00593554	163-170
IL-22	++	ND	Secondary readout in ongoing trial in steroid-refractory GVHD		NCT02406651	125,171-173
Thymosin-α1	++	++	Enhanced lymphocyte count and improved mortality in COVID-19 patients		NCT04320238 NCT04487444	152,174
SSA	++	++	Increased thymic function and output of RTEs in prostate cancer patients (aged) and in HCT recipients		NCT01746849 NCT01338987	175
GH	++	++	Increased thymic size and RTEs		NCT04375657	176

'++' = evidence of benefit to thymus repair/T cell reconstitution

FIGURE 1.4: Mechanisms of Thymus Regeneration

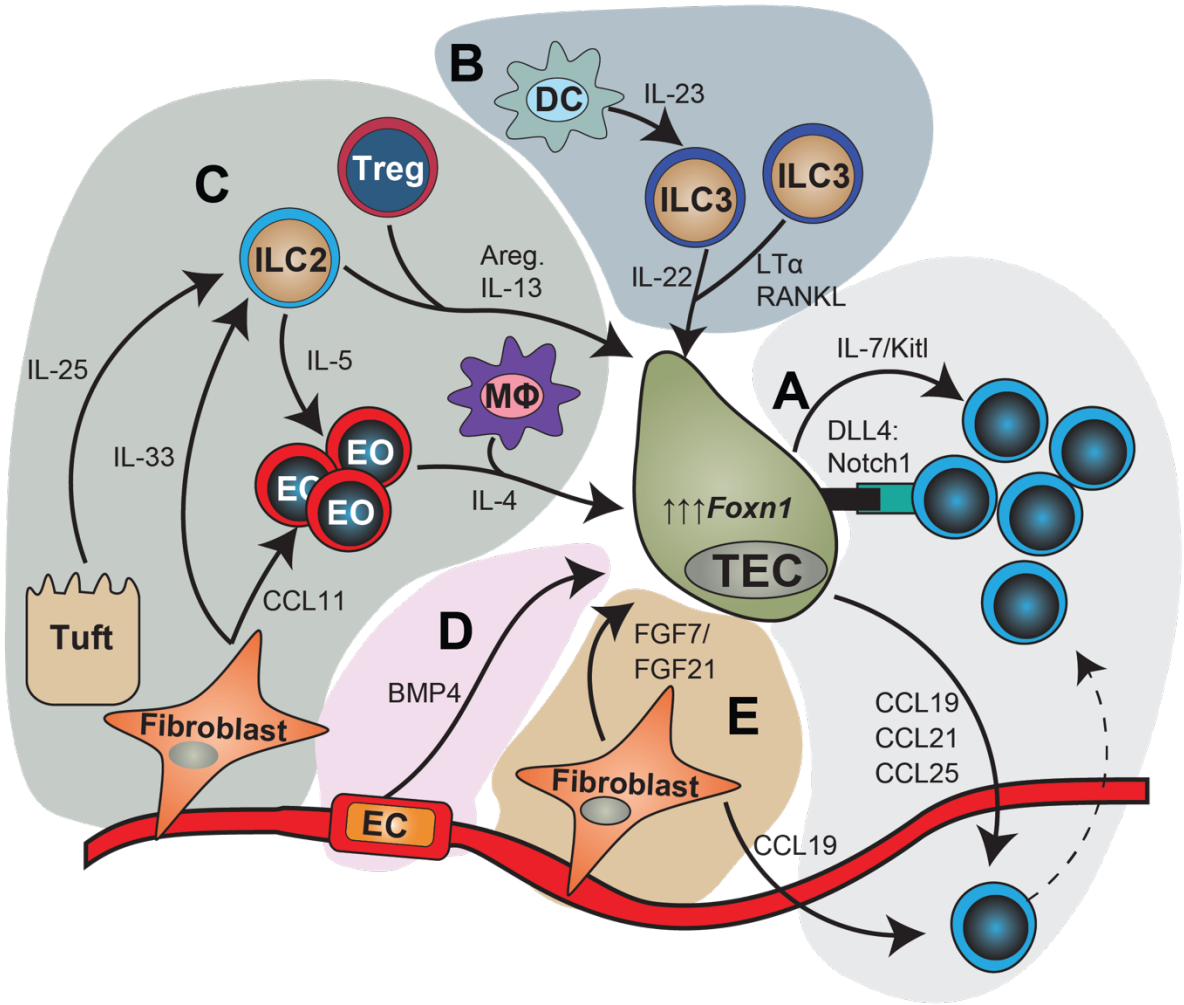


Figure 4: A, Thymic Epithelial Cells are at the center of thymus regeneration and the subsequently discussed factors act primarily on TECs. *Foxn1* expression within TECs leads to expression of 1) chemokines required for progenitor attraction to the thymus and thymocyte trafficking (CCL-19, CCL-21 and CCL-25), 2) DLL-4 which is required for T lineage directing of multipotent thymic seeding progenitors and 3) IL-7 and KitL (also known as Stem Cell Factor) thymocyte surivival and proliferation signals. **B,** Following acute damage, dendritic cells (DCs) sense the absence of developing thymocytes and release IL-23 that stimulates ILC3 production of IL-22 . Independent of DCs and IL-23, ILC3s also provide Lymphotoxin-α and RANK Ligand following damage. **C,** There is an emerging type 2 immune pro-repairative axis. Alarmins IL-33 and IL-25 are released from thymus fibrobalsts and tuft cells, respectively. IL-25 and IL-33 stimulate ILC2 production of type 2 immune factors IL-5, Amphiregulin and IL-13. IL-5 stimulates Eosinophil production of IL-4 (Macrophages have also been implicated as sources of repaiative IL-4 post-damage) while Amphi-reguling and IL-13 act directly on TECs. Fibrobalsts also support Eosinophil recruitment to the thymus via CCL-11. The mechanism by which IL-4 improves thymus funciton has not yet been reported but it is suspected to act on TECs. Recirculating Tregs accumulate in the thymus following injury and contribute to restroative Amphiregulin. **D,** Endothelial cells (EC), like DCs, sense the absence of developing thymocytes and secrete Bone Morphogenic Protein 4 (BMP4) which induces *Foxn1* upregulation in TECs. **E,** Fibrobalst release of Fibrobalstic Growth Factors FGF7 (also known as Keratinocyte Growth Factor, KGF) and FGF21 to stimulate TEC proliferation.

1.5 TRIGGERS OF ENDOGENOUS THYMIC REGENERATION

Developing thymocytes undergo tremendous proliferation, with an estimated 50 million thymocytes generated every day[177]. However, 95-98% of developing thymocytes will undergo apoptosis during selective stages[177]. Immature $CD4^-$ $CD8^-$ Double Negative (DN) thymocytes undergo intrinsic apoptosis in the absence of IL-7 signaling or if TCRβ gene rearrangement is unsuccessful and pre-TCR signaling cannot occur[71]. However, most cell death occurs at the $CD4^+CD8^+$ DP stage of development with positive selection in the thymus cortex during which only a small minority of DP thymocytes with TCRs of intermediate affinity for self-peptide: MHC complexes will continue to differentiate and an estimated ~95% of DPs will fail to recognize self-peptide: MHC and undergo death by neglect via intrinsic apoptosis[69,70,71]. In the thymus cortex and medulla, DP and $CD4^+$/$CD8^+$ SP thymocytes with TCRs of high affinity will undergo negative selection and die by apoptosis[178].

The common trigger to several of the mechanisms of endogenous thymus regeneration described above is the loss of developing thymocytes. Mice lacking DP thymocytes had increased levels of IL-23 and IL-22 suggesting that DP thymocytes provide a suppressive signal for the production these regenerative factors[138]. During thymic selection, dying cells bearing phosphatidylserine on their surface binds to TAM (Tyro, Axl, Mer) receptors on nearby dendritic cells and endothelial cells. This initiates activation of Rho1 GTPase, the pattern recognition receptor NOD2, and microRNAs to suppress the translation of regenerative factors BMP4 and IL-23 from ECs and DCs, respectively[138]. However, following acute-damage induced depletion of developing thymocytes such as that caused by gamma ionizing irradiation, the overall decreased phosphatidylserine interrupts this signaling cascade, lifting the brakes on damage resistant endothelium and DC production of BMP4 and IL-23[138].

Although there is a tremendous amount of thymocyte apoptosis during steady state T cell development, cytoreductive conditioning leads to orders of magnitude more cell death.

However, not only do such acute injuries lead to the rapid loss of developing thymocytes providing suppressive signals, but they also lead to a rapid rise in immunogenic cell death (ICD)[179,180]. Specifically, increased mitochondrial reactive oxygen species (ROS) stimulate the NLRP3 inflammasome in thymocytes following acute insult and subsequently lead to Caspase-1 cleavage and pyroptosis[138]. Forms of lytic cell death such as pyroptosis, necroptosis and necrosis lead to the release of intracellular contents known as damage associated molecular patterns (DAMPs) (i.e., HMGB1 and ATP) that signal tissue injury and promote an inflammatory response[181]. The pro-tissue regenerative effects of these DAMPs have recently come into focus in varied models of injury[182]. Among these, our group reports that the release of intracellular Zinc and ATP mediate endogenous thymus regeneration[179,180]. Though dietary zinc had long been appreciated as a stimulant for peripheral T cell replication, zinc that accumulates in thymocytes during their development is released during lytic cell death and stimulates EC production of BMP4 via the G-protein-coupled zinc receptor GPR39[180]. In addition to zinc, a recent preprint suggests that release of the prototypical DAMP ATP within the thymus, stimulates TECs and their expression of *Foxn1* by stimulating the purinergic receptor P2Y2[179]. Notably, *P2Y2*$^{-/-}$ mice show worsened wound closure supporting the regenerative capacity of ATP post-damage[183].

However, to suggest that ICD is only pro-regenerative within the thymus would be an oversimplification of what is likely a nuanced balance. Mice lacking NLRP3 – which is involved in mediating thymocyte pyroptosis and is activated by several DAMPs including ATP – have improved thymic recovery and T cell reconstitution post-HCT, as well as decreased age-related thymus involution[184]. Furthermore, activation of Caspase-1 leads to release of IL-1β and IL-18, which this thesis reports limits thymic regeneration after injury.

In summary, the thymus can maintain a robust regenerative response after acute injury that is triggered by a balance of the detection of various forms of cell death **(Fig. 1.5)**.

FIGURE 1.5: Triggers of Thymus Regeneration

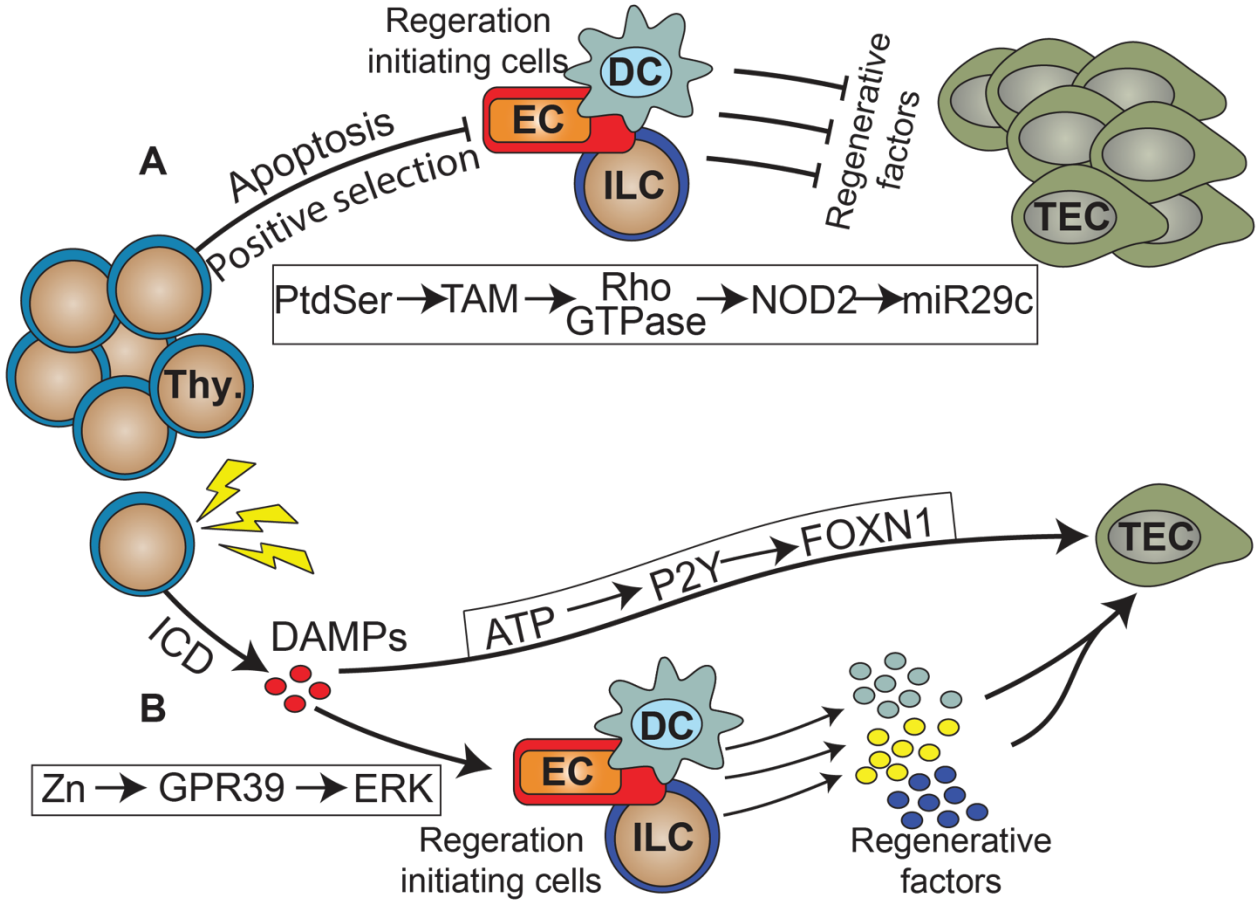


Figure 1.5: A, A homeostatic presence of apoptotic developing thymocytes presenting Phosphatidylserine to TAM receptor bearing sentinel Endothelial (ECs) and Dendritic Cells (DCs) initiates Rho GTPase --> NOD2 inflammasome activation and production of miR29c which suppresses the translation of regeneration factors *Il-23* and *Bmp4* transcripts. Therefore, the homeostatic presence of apoptotic cells suppresses regeneration factor production. **B,** However, upon acute damage the rapid depletion of developing thymocytes releases this brake and allows expression of regeneration factors IL-23 and BMP4. Moreover, increased immunogenic cell death leads to the release of damage associated molecular patterns (DAMPs) including ATP which can directly induce *Foxn1* upregulation in TEC cell lines and can mildly improve thymus repair, and Zinc which boosts thymic function via GPR39 signaling on ECs inducing their production of BMP4.

1.6 LIMITATIONS TO REPAIR

1.6a: Bone Marrow Contribution to Thymic Regeneration

As thymocytes are incapable of self-renewal, the thymus must recruit bone marrow derived multipotent progenitors, known as thymus seeding progenitors (TSPs), from the bloodstream[185]. TSPs migrate to and colonize the thymus where they undergo T cell lineage differentiation signals and become early T-cell progenitors. An integrated circuit regulates the production of multi-potent progenitors capable of T cell differentiation within the bone marrow, their exportation into the bloodstream, and subsequent importation into the thymus where they receive T cell differentiation signals[186,187]. HSCs reside in a specialized niche consisting of $LEPR^+$ mesenchymal stromal cells and vascular endothelial cells[188,189]. These stromal populations provide signals to hematopoietic precursors that lead to differentiation into $Flt3^+$ lymphoid primed multipotent progenitors (LMPPs) and subsequently $IL\text{-}7R^+$ common lymphoid progenitors (CLPs)[190-194]. In a poorly understood coordinated crosstalk between thymus and bone marrow, the availability of thymic microvascular and intrathymic developmental niches signal for bone marrow release of prothymocytes in waves into the bloodstream, which creates a "gated" system of bone marrow T cell progenitor release and thymus entry[186,187]. TSPs then migrate to and enter the thymus in a CCR7/9 and P-selectin Glycoprotein 1 (PSGL1) dependent fashion[62,195,196]. Early work defining thymus entry established that as few as 100-200 TSPs will colonize the thymus daily[197,198]. *Zietara, et al.* demonstrated that the adult thymus contains 160-200 niches for these rare TSPs to enter, however, most are occupied by dividing thymocytes and only ~10 of these niches remain readily available for colonization at steady state[199]. However, following bone marrow transplantation and other forms of acute damage, changes within the bone marrow niche contribute to the delay in T cell reconstitution[200]. Following damage from transplant or chemotherapy treatment, the architecture of the bone marrow microenvironmental niche are altered resulting in skewed differentiation away from lymphoid

and toward myeloid lineages[201-203] . Sinusoidal endothelial cells and mesenchymal cells are damaged and there is an expansion of bone marrow adipocytes[60,61]. Notch signaling is required for HSC differentiation into T-cell competent MPPs as well as for the regeneration of the endothelial cells of the microvascular niche critical for normal hematopoiesis[204-206]. Among other changes, vascular Notch delta-like ligands (*Dll1* and *Dll4*) are downregulated following irradiation contributing to premature bias of HSC differentiation toward myelopoiesis[203].

1.6b: Age Related Thymus Involution

Age related thymus involution is a natural phenomenon that begins in childhood and is most exacerbated during puberty[74]. While its occurrence was recognized even before the organ's immunological function was understood, the processes regulating involution remain incompletely elucidated[1,2]. Thymus involution is characterized by the gradual decline in organ cellularity, lost architecture of the cortex, medulla and corticomedullary junctions, and replacement of functional tissue by perivascular adipose tissue[207]. This results in a concomitant gradual loss in new naïve T cell production and, ultimately, decreased responsiveness to new antigens[208-210]. Importantly, while the young thymus is capable of endogenous repair following acute injury, this capacity diminishes considerably with age[150,211]. This contributes to the delayed T cell reconstitution in patients receiving thymus damage therapy, particularly the cytoreductive conditioning prior to HCT. This prolonged period of T cell immunodeficiency increases patient vulnerability to opportunistic infection and relapse of malignancy[212,213].

The initiation of involution can be tied to changes within the thymus stroma, particularly, to diminished TEC cellularity and turnover [214]. Among the most critical hallmarks of aged TEC dysfunction is decreased *Foxn1* expression and overexpression of *Foxn1* can rescue thymus function during aging.[12,43,44] Studies utilizing *Foxn1* reporter mice point to decreased *Foxn1* expression specifically in cTECs between 1 and 3 months as a key initiator of thymus involution[215]. These studies not only describe a reduction in TECs with age but also

demonstrate the persistence of *Foxn1*$^{-}$ TECs with age[215]. These findings are in line with others showing diminished expression of cell cycle, and particularly E2F3 target genes (one of which can be *Foxn1*) in TECs as early as 3 months of age[47,216-218].

In addition to these intrathymic stromal defects with age, one likely additional contributor to thymic involution is the decline in function in hematopoietic progenitors, even as early as in bone marrow. There is a wide body of evidence to support the notion that hematopoietic stem and progenitor cells in aged bone marrow are dysfunctional in reconstitution potential, self-renewal, and with a lineage bias toward myeloid rather than lymphoid differentiation[219] . Additionally, recent work suggests that reduced Notch signaling within bone marrow progenitors as early as 3 months of age in mice contributes to reduced thymic ETPs[220]. While BM progenitors exhibit reduced T lineage differentiation capacity, and this phenotype was maintained when aged HSCs were transplanted into young mice suggesting intrinsic HSC defects[221], competitive transplantation of aged and young marrow into irradiated recipients reflect similar capacities to reconstitute the thymus[222-224]. Therefore, while age-related alterations in hematopoietic progenitors contributes to reduced T cell production with age, it is the predominant viewpoint that the primary cause is the decreased functionality of the thymus. Studies exploring the “seed or soil” theory – colonizing TSPs are the seed and the thymus microenvironment the soil – in which young and old hematopoietic progenitors similarly colonized implanted fetal thymic tissue suggest that the destruction of the thymus microenvironment “soil” from age related thymus involution is the most influential factor contributing to reduced T cell output with age[225].

Consistent with this, a recent preprint suggests the emergence of aberrant age-associated TECs (aaTECs) that lose typical TEC hallmarks. Furthermore, in contrast with the lattice-work structure of TECs that are filled with developing thymocytes in the young organ, the aged, involuted thymus is instead characterized by high-density aaTEC clusters devoid of thymocytes[226]. These aaTECs resemble microenvironmental scars of former TECs and are not

only non-functional as inducers of thymopoiesis themselves, but also act as a sink for growth factors that could otherwise be used to expand functional TECs[226]. Critically, their presence severely limits thymic regeneration after injury.

1.7 Clinical Implications of Dysregulated Thymus Function

1.7a: Graft-Versus-Host Disease (GVHD)

Several factors determine the success of thymic dependent T cell reconstitution following HCT (i.e., diseases status, age, and the preconditioning received)[227]. Among the most significant hurdles post-transplantation for thymus-dependent T cell recovery is the incidence and severity of graft-versus-host disease (GVHD)[228]. During acute GVHD, T cells contained within the graft critical for targeting leukemic cells recognize and are activated by recipient allo-antigens. The skin, liver and intestinal tract are the primary organs targeted in GVHD; though not to be underappreciated, severe damage to the thymus is also characteristic (thymus atrophy, architecture disruption, and destruction of thymocytes and TECs) [228,229]. Clinical measurement of T cell production by peripheral signal joint T cell receptor excision circles (sjTRECs, quantifiable circular DNA excised furing TCR gene rearrangement) indicates that GVHD leads to a marked reduction in thymic function[230]. While it has long been appreciated that T cell immune reconstitution is important for patients following transplantation, a recent retrospective studies showed that CD4 T cell reconstitution within 100 days post transplantation is predictive of overall survival following HCT with and without acute GVHD (aGVHD)[231,232].

Damage to the thymus from pre-transplant cytoreductive conditioning, aGvHD and progression to chronic GVHD (cGVHD) are connected and multifactorial[227,233]. Highly replicative Aire$^+$ mTECs responsible for promiscuous gene expression (pGE) and tolerance induction are highly sensitive to pre-transplant conditioning[234]. *Alawarm, et al.* demonstrated how even in a syngeneic transplant model, failure of the thymus medulla to recover from pretransplant

conditioning injury leads to reduced Foxp3^{+} regulatory T cell production and subsequent autoimmunity[235]. Furthermore, in models allo-HCT, alloreactive donor T cells in the graft target thymic epithelial cells[236,237]. Damage to the medullary compartment from pre-transplant conditioning and allo-T cell targeting of these together lead to a long-lasting failure in central tolerance and the emergence of alloreactive, chronic GVHD (cGVHD) mediating T cells[237-240]. Alloreactivity is then exacerbated by autoreactive T cell targeting of secondary lymphoid organ stromal cells that induce peripheral immune tolerance[241] creating a “one-two punch” in the breakdown of central and peripheral tolerance contributing to cGVHD[233]. While GVHD itself leads to severe thymus atrophy, therapies aimed at reducing GVHD can decrease thymus function[150]. For example, rat Anti Thymocyte Globulin (rATG) is used as prophylaxis against aGvHD despite its direct cytotoxicity to thymic epithelium[242]. Comparison of more commonly used post-transplant GVHD prophylaxis of methotrexate/cyclophosphamide or calcineurin inhibitors tacrolimus/sirolimus show higher post-transplant TRECs than rATG treated recipients suggesting less thymus-toxicity[243]. In summary, an improved understanding of the balance between the type and intensity of immunosuppression to protect against allo-reactivity and endogenous T cell recovery is required – especially given the emerging use of haplo-identical donor transplantation that has the potential to greatly expand readily available donor options but also requires more intense GVHD prophylaxis[227].

1.7b: Clinical Relevance of Adult Thymus Function in Disease

The thymus is, of course, essential for the formation of a fully functional adaptive immune system. Children who undergo whole or partial thymectomy have persistent decreased naïve T cells, responsiveness to vaccine and overall numbers[244-246]. However, because the natural process of age-related thymus involution is evolutionarily conserved, the importance of thymic function into adulthood has long been questioned. This section summarizes findings

supporting the benefits of improving thymus function into adulthood and old age, specifically regarding increased adaptive immune function and prevention of autoimmunity and cancer.

The aging immune system is paradoxically simultaneously autoreactive ("inflammaging") and less effective ("immunosenescent"). Age-related thymus involution leads to decreased naïve lymphocyte output. Thymic production of T cells declines exponentially with an estimated half-life of ~16 years, meaning individuals 50 and over produce <10% of their original T cell capacity[207,247]. The combination of highly reduced new lymphocyte output and the peripheral expansion of antigen-experienced memory and senescent T cells – such as CMV-specific T cells – results in a constricted T cell receptor (TCR) repertoire, particularly among $CD8^+$ T cells **(Fig. 1.3)**. In fact, in aged mice, 70-80% of the T cell repertoire can be taken up by just 2-4 clones[248]. This reduced repertoire diversity combined with the outsized proportion of senescent memory cells contributes to increased susceptibility to infectious disease and reduced vaccine responsiveness in the elderly[247]. Illustrative of this, recent COVId-19 studies show that reduced TCR repertoire contributed to an age-related risk factor for COVID-19[249,250]. Furthermore, decreased CD4 TCR diversity with age could be linked to decreased COVID-19 vaccine responsiveness[251].

Not only does aging of the T cell immune system decrease pathogen responsiveness, but it also diminishes the capacity for cancer immune surveillance[252]. Increased TCR-diversity is reportedly a predictive prognostic biomarker for anti-tumor responsiveness in various cancers both with and without immune checkpoint blockade[253-256]. Mutational burden of the tumor is one prognostic indicator, as it is correlated with the immunogenicity of the tumor, but this immunogenicity is dependent on there being a sufficient diversity within the TCR repertoire such that a specific and sufficient T cell anti-tumor response can be mounted. Recently, in the largest yet study of its kind, 1420 patients having undergone thymectomy were compared to 6021 control patients and revealed that the risk of cancer incidence as well as all-cause mortality was

increased in the thymectomized group[257]. Experimentally, improving thymic output in aged mice following sex steroid ablation was capable of improving antitumor immunity[258].

The seemingly contradictory double-edged sword of immune aging is that despite T cells being less effective at combatting infection and tumorigenesis, they are simultaneously chronically active under sterile conditions leading to a chronic low-grade inflammatory state known as “inflammaging” that contributes to autoimmunity with age[150]. Loss of mTEC function in age-related involution can lead to failed central tolerance and output of autoreactive T cells[259,260]. Notably, a recent report demonstrated that mild caloric restriction could increase thymopoiesis in adult humans, and showed caloric restriction not only strengthened TCR repertoire diversity in aged mice but was also protective against inflammaging hallmarks[261]. In summary, recent interest in exploring the benefits of restored thymic function in aging have shed new light on its potential wide-ranging health benefits spanning improved immunity, cancer immunosurveillance, and decreased autoimmunity.

1.8 CONCLUSIONS

In summary, this introduction outlines the formation of the thymus organ and outlines the origin and central role that Thymic Epithelial Cells play in its function. The thymus is highly sensitive to acute injuries, most relevantly cytoreductive chemotherapy and pre-HCT conditioning, however it also has a remarkable capacity for regeneration following such injuries. However, this regenerative capacity diminishes with age such that it may take patients months-years and they sometimes may never fully recover their peripheral T cell numbers much less overall T cell diversity[211]. Study of endogenous mechanisms of thymus regeneration has identified several potential therapeutic targets **(Table 1.3)**, yet there remains no clinically approved therapy for improving thymus function to address T cell lymphopenia.

We identify that the initiation of regenerative processes is regulated by the balance between homeostatic cell death of developing thymocytes and their absence following injury as well as the products of immunogenic vs. non-immunogenic cell death. Clinical hurdles to improving thymus function include pre-thymic defects of hematopoietic progenitor T cell potential both following acute injury and during normal aging. Of even higher importance, the thymus itself deteriorates over the lifespan in a natural process known as age-related thymus involution driven by the dysfunction of TECs and downregulation of *Foxn1* that ultimately results in the accumulation on non-functional age associated TECs. And finally, we outline the potential health benefits for improving thymus function. Following HCT, improved thymic function may ameliorate leading causes of post-transplant mortality including aGVHD, opportunistic infection, and relapse of malignancy. And in the context of normal aging, thymic rejuvenation can aid in pathogen immunity, vaccine responsiveness as well as anti-tumor surveillance and immunotherapy.

Moving forward, we define new mechanisms regulating thymic regeneration tied to the sensitivity of thymic cells to undergoing immunogenic cell death following acute injury. We identify Interleukin-18 (IL-18) as a novel regulator of thymic function following acute injury. We identify thymic Natural Killer cells as actors of IL-18 induced suppression: IL-18 stimulated NK cells aberrantly target TECs to suppress regeneration. Moreover, we also implicate cytotoxicity in contributing to age-related thymus regeneration and identify CD8+ cytotoxic cells as likely effectors of Perforin-dependent age-related thymic demise.

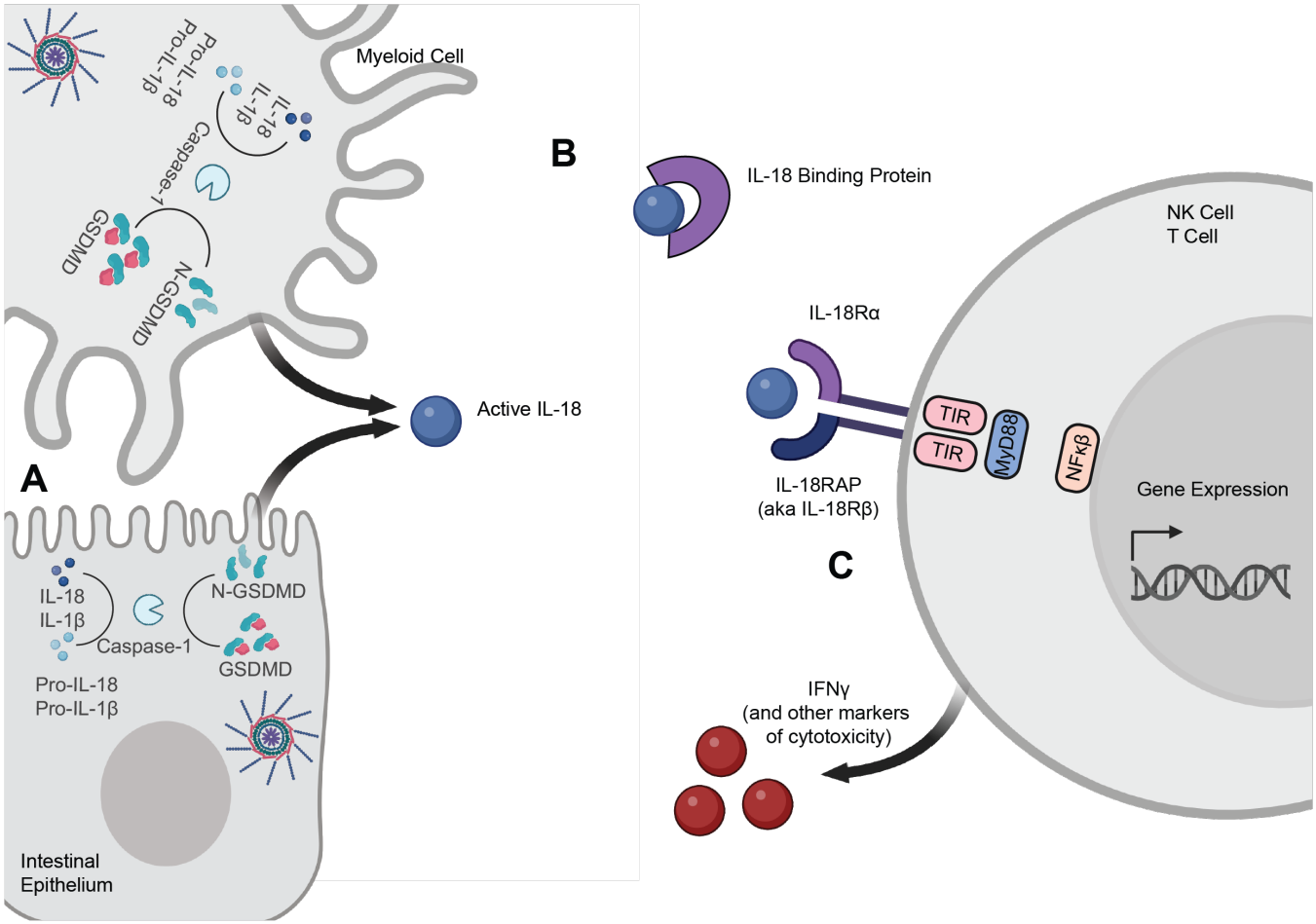


Figure 1.6: Simplified Diagram of IL-18 Synthesis and Signaling

A, Myeloid cells such as dendritic cells and macrophages are prominent sources of IL-18 as are epithelial cells lining the intestine. Inflammasomes NLRP3, NLRC4 and AIM2 once activated trigger the cleavage of Caspase-1. Caspase-1 mediates cleavage of the pro- inactive forms of IL-1β and IL-18 into their mature and active inflammatory states as well as the pro-form Gasdermin D into its active form. Gasdermin D creates pores in the membrane through which IL-18 and IL-1β exit the cell. Interestingly there is seemingly a degree of cl. Cas-1 that allows for IL-18 activation and release but that the cell can tolerate and not induce imunogenic cell death. Not shown above is that we posit that upon acute thymic damage, a redirection of pyruvate metabolism drives increased mitochondrial OXPHOS in cells that leads to increased reactive oxygen species activating the NLRP3 inflammasome and subsequently leading to the cleavage of Cas-1 *(Kinsella, S. et al. 2023)*.

B, Once in the periphery, active IL-18 can be sequestered by the endogenous antagonist of IL-18 known as IL-18 Binding Protein (IL-18 BP). It is estimated that under normal conditions ~95% of active IL-18 is bound by IL-18BP *(Landy, E., et al., 2024)*. However, during periods of rapid pyroptosis/acute inflammation, free IL-18 can exceed the neutralizing capacity of IL-18BP, allowing for increased free IL-18 and signaling onto target cells.

C, IL-18R expressed on $CD4^+$ Th1, $CD8^+$ T cells as well as NK and NKT cells. IL-18 can weakly signal through IL-18Rα alone however, strong signaling is only potentiated upon recruitment of its co-receptor IL-18RAP (aka IL-18Rβ). Reportedly, the presence of IL-12 or IL-15 is needed to induce type 1 immune gene expression in T cells and NK cells, however, recent evidence *(Cui, A., et al., 2024)* suggests that IL-18 alone can induce cytotoxicity gene expression in $CD8^+$ and particularly NK cells. Downstream IL-18R signaling is similar to IL-1R signaling: Toll-IL-1 Receptor dimerization recruits MyD88. Next, via IL-1-receptor Kinases (IRAKs) and TNF Receptor Associated Family 6 (TRAF6), NFκβ is stimulated for downstream immune signature gene expression *(Dinarello, C., et al., 2013)*. Most notably, IL-18 stimulates IFNγ production by Th1 $CD4^+$, $CD8^+$ and NK cells as well as other cytotoxicity associated genes including *Tnfα, Gzmb* and *Fasl (Cui, A., et al., 2024)*. Elements of this schematic figure were inspired by *Cui, A., et al.* and *Dinarello, C., et al.*

Chapter 2. DAMAGE-INDUCED IL-18 STIMULATES THYMIC NK CELLS LIMITING ENDOGENOUS TISSUE REGENRATION

This chapter will include the bulk of the experimental work for this thesis in which we show that IL-18 is a damage-induced inflammatory regulator of endogenous thymus regeneration, and that IL-18 acts via thymic Natural Killer (NK cells).

2.1 SUMMARY

Interleukin-18 is an acute phase pro-inflammatory molecule crucial for mediating viral clearance by activating Th1 $CD4^+$, cytotoxic $CD8^+$ T, and NK cells. Here, we show that mature IL-18 is generated in the thymus following numerous distinct forms of tissue damage, all of which cause caspase-1-mediated immunogenic cell death. We report that IL-18 stimulated cytotoxic NK cells limit endogenous thymic regeneration, a critical process that ensures restoration of immune competence after acute insults like stress, infection, chemotherapy, and radiation. NK cells suppressed thymus recovery by aberrantly targeting thymic epithelial cells (TECs), which act as the master regulators of organ function and regeneration. Together these studies reveal a novel pathway regulating tissue regeneration in the thymus and offer IL-18 as a potential therapeutic target to boost thymic function. Moreover, given the enthusiasm for IL-18 as a cancer immunotherapy for its capacity to elicit a type-1 immune response, these findings also offer insight into potential off-target effects.

2.2 INTRODUCTION

Despite its importance for the production of a diverse and tolerant T cell repertoire, the thymus is exquisitely sensitive to acute insult such as stress-induced rises in corticosteroids and infection, as well as more profound injuries such as chemotherapy and myeloablative

conditioning pre-hematopoietic cell transplantation (HCT) [112,150,211]. The thymus also harbors an endogenous capacity for regeneration following such acute injuries, however, tissue recovery is a prolonged process such that patients receiving intense thymus-damaging treatments may experience long periods of lymphopenia [51]. This is especially pertinent in HCT-recipients who are particularly vulnerable to opportunistic infection and malignant relapse – two leading causes of post-transplant mortality that are directly related to immune reconstitution – during a months to years-long duration of T cell immunodeficiency [212,213,232]. Therefore, understanding the mechanisms underlying thymus recovery could inform therapeutic targets to improve T cell reconstitution in such settings of organ damage [150]. We and others have identified several cells and molecules that mediate endogenous thymic regeneration including innate lymphoid cells, endothelial cells, eosinophils, and thymic epithelial cells (TECs) and the production of regeneration factors like IL-22, BMP4, and KGF [52,123,165,262]. However, despite these findings, there remains no clinically approved strategy for treating T cell lymphopenia.

We have previously reported that HCT-conditioning leads to an acute rise in not only apoptosis, but also pyroptosis – a form of immunogenic cell death [181,263,264]. During pyroptosis, intracellular contents are released causing local inflammation. We have recently reported that this immunogenic cell death leads to release of damage-associated molecular patterns (DAMPs) such as zinc and ATP that trigger regenerative responses via production of BMP4 by endothelial cells and induction of *FoxN1* expression within thymic epithelial cells (TECs), respectively [180,265]. However, pyroptosis also leads to the release of inflammatory cytokine IL-18 into the extracellular milieu [263]. IL-18 is a potent stimulator of type II interferon and cytotoxicity profiles in NK cells [266,267]. NK cells can regulate tissue regeneration and wound healing and recent works implicate NK cells in delaying epithelial recovery [268-270]. Here we interrogate the impact of this pro-inflammatory cascade and identified that acute thymus damage induced release of IL-18 suppressed endogenous mechanisms of organ recovery by stimulating resident cytotoxic NK cells that aberrantly target TECs.

Overall, this study demonstrates a novel pathway implicating injury induced local inflammation in the suppression of thymic function and isolates the cytokine IL-18 as a critical regulator of endogenous immune reconstitution.

Chapter 3. RESULTS

2.3a: Acute thymus injury leads to Caspase-1 cleavage and release of active IL-1β & IL-18

As part of normal T cell development, $CD4^+$ $CD8^+$ Double Positive (DP) Thymocytes and $CD4^+$/$CD8^+$ Single Positive Thymocytes undergo apoptosis as newly formed T cell receptors (TCRs) are tested against self-peptide:MHC complexes in positive and negative selection processes [70]. In fact, approximately 98% of all developing thymocytes fail to pass and survive thymic selection [177]. Importantly, apoptosis is an immunologically silent process and there is little inflammation within the homeostatic thymus [181]. Following acute damage such as that caused by the cytoreductive conditioning received prior to HCT, modeled with sublethal total body irradiation (SL-TBI, 550 cGy), thymus cellularity precipitously declines [123,138] **(Fig 2.1A)**. However, we have recently reported that ionizing radiation damage leads to cell death by both apoptosis and pyroptosis within the thymus [265]. In contrast to the immunologically silent apoptosis, pyroptosis is an immunogenic from of cell death mediated by cleaved Caspase 1 (cl-Cas-1) resulting in the release of damage-associated molecular patterns (DAMPs) [263]. Activation of Cas-1 occurred not only following ionizing radiation, but also following all other forms of acute thymus injuries tested: corticosteroid-induced stress, cytoreductive chemotherapy, and LPS **(Fig. 2.1A, S2.1A)**, all of which have been shown to induce acute thymic involution [51,211,271-273]. Although our studies have shown that release of DAMPs such as ATP and zinc can actually be pro-regenerative [180,265], cl-Cas-1 also mediates the proteolytic cleavage of the immature, inactive forms of IL-1β and IL-18 into their mature, active, inflammatory states **(Fig. 1.6)**. Accordingly, cleavage of Cas-1 led to the activation of IL-1β and

IL-18 into their mature states within the thymus following each of these acute damage models **(Fig 2.1B),** and we could detect no increase in activated IL-18 after TBI in mice lacking the catalytic domain of Cas-1 (**Fig. 2.1C**). IL-18 Binding Protein (IL-18BP), an endogenous antagonist of IL-18, was also upregulated following HCT-conditioning (**Fig. 2.1D)**; possibly in response to upregulation of activated IL-18 [274]. However, while early upregulation of IL-18BP reduced the ratio of IL-18 to IL-18 BP, this was rapidly reversed soon after allowing increased free IL-18 relative to baseline (**Fig. 2.1E)**.

To explore the functional involvement of these ICD-associated cytokines in regulating endogenous thymus regeneration, we assessed thymus regeneration after SL-TBI in mice with germline deletion of IL-1β signaling (*Il1r1*$^{-/-}$) or IL-18 signaling (*Il18*$^{-/-}$ and *Il18r1*$^{-/-}$). Although *Il1r1*$^{-/-}$ mice showed no significant changes in their capacity for thymic recovery, suggesting little role for IL-1 signaling, mice deficient for either IL-18 itself or its primary receptor IL-18R1 showed significantly improved thymus regeneration relative to WT controls **(Fig 2.1F)**. Moreover, mice lacking IL-18 had significantly larger thymuses at baseline but saw similar degrees of damage as WT controls suggesting greater regenerative capacity and not increased damage resistance **(Fig S2.1B-C)**. Mice lacking the catalytic domain of Cas-1 also showed significantly increased thymus regeneration **(Fig 1G)**, although to a lesser degree than IL-18-signaling deficient mice possibly reflective of the loss in pro-regenerative effects of DAMP release [180,265]. Consistent with these findings, administration of recombinant IL-18 (rIL-18) 3 days following SL-TBI – the point at which organ cellularity reaches a nadir and regenerative processes begin to take effect – significantly delays thymic reconstitution **(Fig. 2.1H)**. Taken together, these data demonstrate that damage-induced activation and release of IL-18 suppresses endogenous thymus repair after acute damage.

We also assessed the therapeutic potential of abrogating IL-18 signaling to improve thymus reconstitution by treating HCT-recipient mice with monoclonal αIL-18 antibody. αIL-18

mAb treatment began prior to transplantation and continued for 2-3 weeks following HCT. We hypothesized that capturing not only the acute spike in active IL-18 but also homeostatic IL-18 presence during the initial 2-3 weeks post-transplant would support organ recovery. We find that 50 days post transplantation, mice receiving αIL-18 mAb showed significantly higher thymus cellularity demonstrating that IL-18 can be pharmacologically targeted post transplantation **(Fig. 2.1I)**. Having established that IL-18 regulates endogenous thymus tissue recovery and that it can be pharmacologically targeted in the context of syngeneic HCT, we set out to identify its source(s) and mechanism of action within the organ.

FIGURE 2.1: Acute thymic damage triggers cleavage of caspase 1 and activation of IL-18 which suppresses thymus regeneration

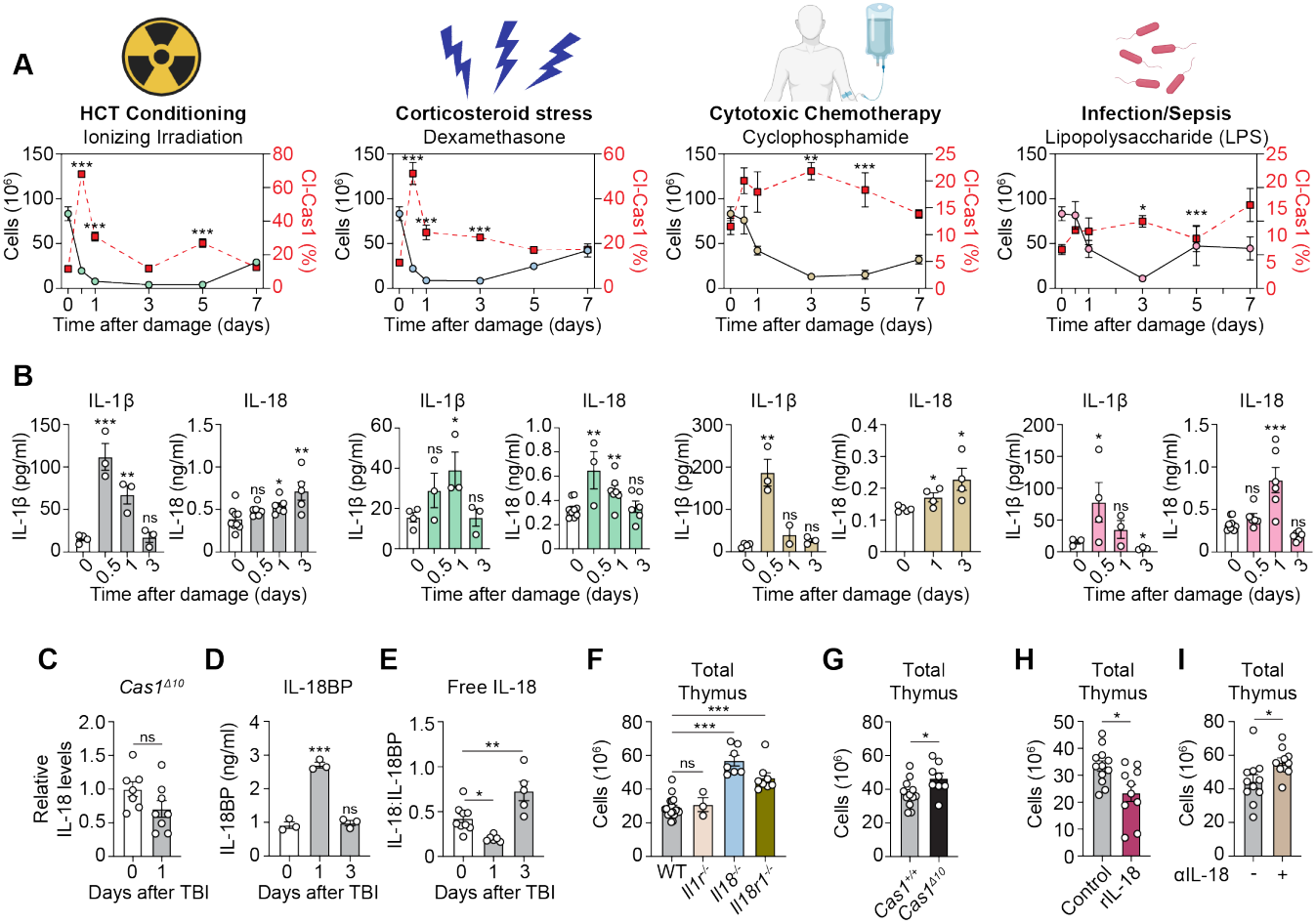


Figure 2.1: Acute thymic damage triggers cleavage of caspase 1 and activation of IL-18 which suppresses thymus regeneration

A-C, Female 1-2 mo C57/BL6 mice were given sublethal total body irradiation (SL-TBI, 550 cGy), Dexamethasone (i.p., 20 mg/kg), Cyclophosphamide (i.p., 200 mg/kg), or LPS (i.p., 1.5mg/kg). **A,** Thymus cellularity (Black) and Cleaved Cas-1 (Red) was measured by fluorescently conjugated FAM-YVAD-FMK at the given timepoints post-treatment (n=4-15/group/timepoint); all statistics compared to Day 0. **B,** Thymic amount of active IL-1β and active IL-18 (n=3-8/group) measured by ELISA at the indicated timepoints; all statistics compared to Day 0. **C,** Amount of active IL-18 measured by ELISA in female 1-2 mo C57/BL6 WT or *Cas1*$^{\Delta 10}$ mice at day 1 after SL-TBI (n=6-7/group). **D,** Amount of IL-18 binding protein (IL-18BP) at days 0, 1 and 3 after SL-TBI (n=3/group). **E,** Ratio of active IL-18 to IL-18BP average at days 0, 1 and 3 after SL-TBI representing free active IL-18 (n=5-8/group). **F,** Female 1-2 mo C57/BL6 WT, *Il1r1*$^{-/-}$, *Il18*$^{-/-}$ and *Il18r1*$^{-/-}$ mice were given SL-TBI and thymus cellularity was measured 7 days later (n=3-23/group/genotype). **G,** Female 1-2 mo C57/BL6 WT or *Cas1*$^{\Delta 10}$ mice were given SL-TBI and thymus cellularity measured at day 7 (n=5-10/group). **H,** Female 1-2 mo C57/BL6 WT mice were given SL-TBI and then administered recombinant IL-18 (rIL-18) on day 3 (s.c., 2.5 mg/kg). On day 7 thymus cellularity was measured (n=10-12/group). **I,** Female 1-2 mo C57BL/6 mice were lethally irradiated and transplanted (i.v.) with 5x10^6 CD45.1$^+$ WT bone marrow hematopoietic cells. Recipient mice were treated with 200μg αIL-18 monoclonal antibody or equal volume control PBS -1, 1, 3, 6, 9, 12, 15 and 18 days and thymus cellularity measured at day 50 post-transplant (n=10-11/group). Graphs represent mean $\pm$ SEM. *p<0.05, **p<0.01, ***p<0.001

FIGURE S2.1

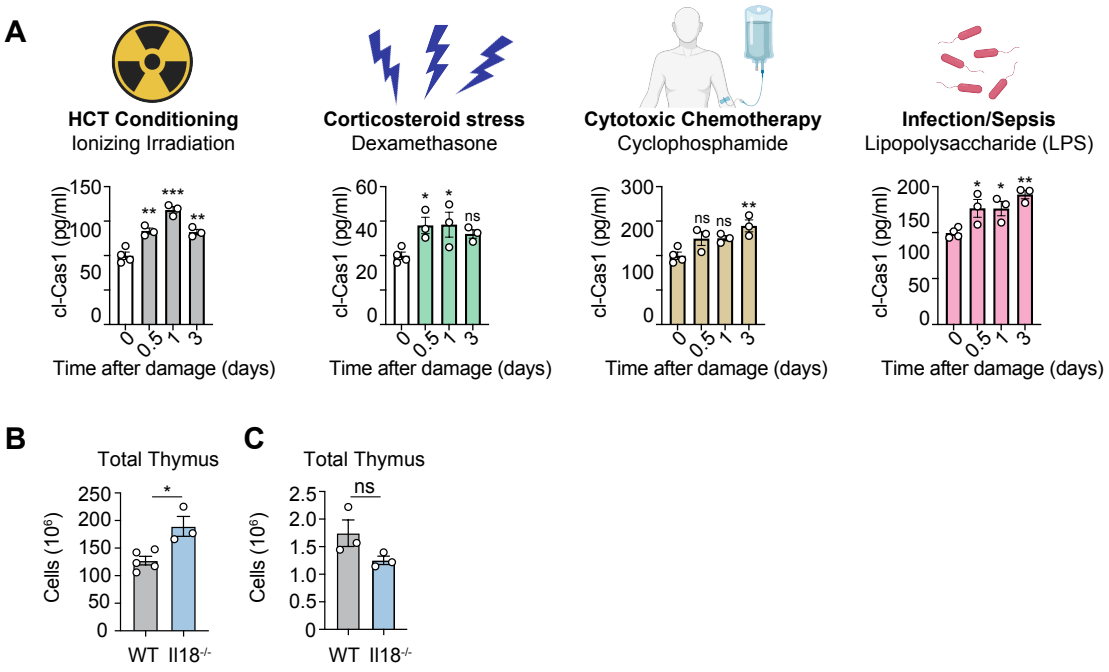

A
HCT Conditioning
Ionizing Irradiation
Corticosteroid stress
Dexamethasone
Cytotoxic Chemotherapy
Cyclophosphamide
Infection/Sepsis
Lipopolysaccharide (LPS)
cl-Cas1 (pg/ml)
Time after damage (days)
B
Total Thymus
Cells (10^6)
WT
Il18-/-
C
Total Thymus
Cells (10^6)
WT
Il18-/-

Figure S2.1: Cleaved Caspase 1 increases within the thymus following acute damages

A, Thymus supernatants generated from female 1-2 mo C57BL/6 mice given SL-TBI (550cGy), Dexamethasone (i.p., 20mg/kg), Cyclophosphamide (i.p., 200mg/kg) or LPS (i.p., 1.5mg/kg) and mature Caspase-1 assayed by ELISA on days 0, 0.5, 1 and 3 (n=3-4/group). **B,** Female 1-2 mo C57/BL6 WT or *Il18*$^{-/-}$ thymus cellularity was measured in the absence of any treatment/damage (n=3-5/group/genotype). **C,** Female 1-2 mo C57/BL6 WT or *Il18*$^{-/-}$ were given SL-TBI thymus cellularity was measured 3 days later (n=3-5/group/genotype). Graphs represent mean ± SEM. *p<0.05, **p<0.01, ***p<0.001

2.3b: IL-18 is produced by discrete populations of hematopoietic and non-hematopoietic stromal cells

Unlike *Il-1β* which is upregulated following inflammasome stimulation, *Il18* is constitutively expressed and is therefore present within the cytoplasm in its pro-form in several cell types awaiting activation by proteolytic cleavage [267,274]. To identify the source of IL-18 following acute damage, we investigated *Il18* gene expression from previously published gene expression datasets to narrow the possible source(s) **(Fig S2.2A)** [218]. At baseline, *Il18* was only expressed within the diverse $CD4^-CD8^-CD44^+CD25^-$ DN1 population that includes early T cell precursors, but also myeloid cells, B cells and Innate Lymphoid Cells (ILCs) [64]. With the knowledge that *Il18* expression was not found in mature thymocytes, we utilized scRNASeq performed on thymus stromal populations that interrogates all non-thymocyte populations by using *Rag2gfp* mice to exclude all Rag2-GFP^+ T cell lineage committed cells **(Fig. 2.2A, S2.2B)** [137,226]. We isolated *Il18* was only expressed by non-hematopoietic mesothelial cells and capsular fibroblasts, as well as a subset of dendritic cells (cDC1) and, to a lesser degree, macrophages **(Fig. 2.2B)**. Prior work using the high dimensional flow cytometric method 'infinity flow' to interrogate non-thymocyte organ heterogeneity helped us design panels to identify rare populations, particularly myeloid cells **(Fig S2.3)**[275]. We could also detect increased cleavage of Cas1 within cDC1 and non-epithelial $CD45^-$ cells early after TBI (**Fig. 2.2C**). This indicates that cDC1 and rare $CD45^-$ capsular mesothelial (MEC)/fibroblast (FB) populations meet the qualifications of 1) expressing *Il18* at baseline and therefore are likely to have cytoplasmic pools of inactive pro-IL-18 prior to damage, and 2) increase Cas-1 cleavage following injury necessary for proteolytic cleavage of immature pro IL-18. To functionally interrogate these sources, we generated mice with a specific deletion of *Il18* in conventional dendritic cells using the Zbtb-cre line ($Il18^{\Delta cDC}$) [276]. While WT mice increased their levels of IL-18 at day 1 after TBI, $Il18^{\Delta cDC}$ mice failed to significantly increase levels of activated IL-18 (**Fig. 2.2D**). To assess the potential

contribution of non-hematopoietic stromal cells like MECs and FBs, we performed bone marrow chimeras using WT or *Il18*$^{-/-}$ mice as recipients of WT bone marrow to preserve *Il18* expression in hematopoietic cells but remove its expression ins tromal populations such as capsular fibroblasts and mesothelial cells. Recipient mice were allowed to recover for 10 weeks following transplantation at which point they received a sublethal dose of TBI and IL-18 levels were measured at day 1. Similarly to *Il18*$^{\Delta cDC}$ mice, these chimera mice demonstrated an increase in IL-18 in WT recipient mice but failed to increase activated IL-18 in *Il18*$^{-/-}$ recipients (**Fig. 2.2E**). However, while not significant, we did observe a modest increase in activated IL-18 following injury in both models, consistent with active IL-18 being derived from multiple sources following acute damage.

FIGURE 2.2: Dendritic cells and non-hematopoietic stroma are sources of damage induced IL-18

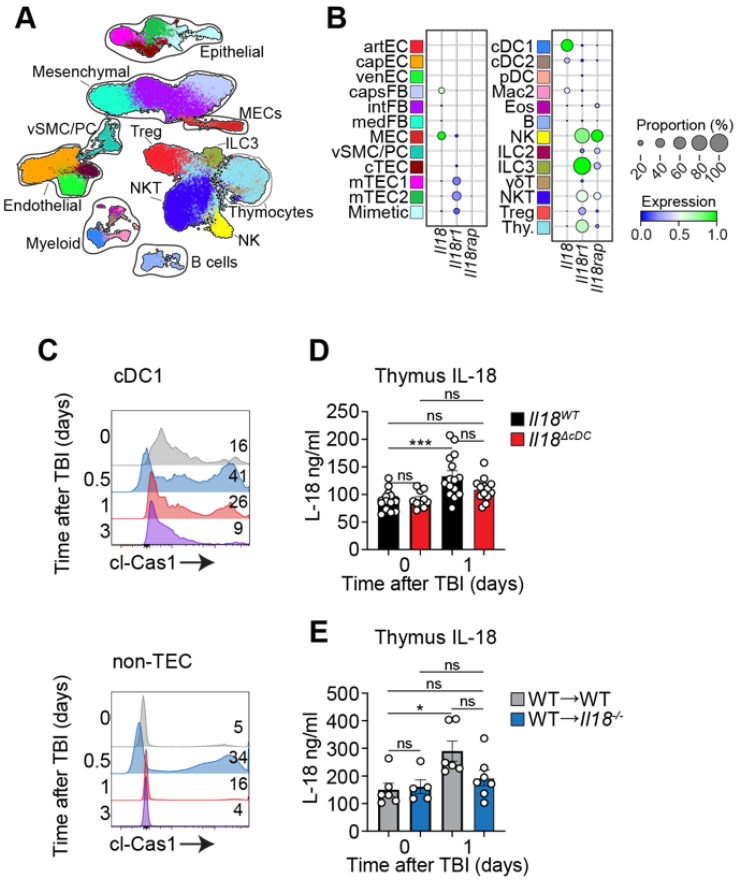


Figure 2.2: Dendritic cells and non-hematopoietic stroma are sources of damage induced IL-18

A-B, Single cell RNA sequencing was performed on (1) non-thymocyte $CD45^+$ stromal cells ($CD45^+$ *Rag2*-GFP^- isolated from female 1-2 mo *Rag2-gfp* mice) and (2) $CD45^-$ stromal cells isolated from female 1-2 mo C57BL/6 mice at baseline, 1, 4 and 7 days following SL-TBI. Data was previously integrated together and published in Lemarquis *et al.* (2024). **A**, Integrated UMAP of both datasets showing major clusters and annotation. **B,** Expression of *Il18, Il18r1* and *Il18rap* at baseline. Population abbreviations: arterial Endothelial Cell (artEC), capillary Endothelial Cell (capEC), veinous Endothelial Cells (venEC), capsular Fibroblasts (capsFBs), intermediate Fibroblasts (intFBs), medullary Fibroblasts (mFBs), Mesothelial Cells (MECs), vascular smooth muscle/Pericyte (VSM/PC), cortical Thymic Epithelial Cell (cTEC), medullary Thymic Epithelial Cells (mTEC), proliferating mTEC ($mTEC^{prol}$), cDC (classical Dendritic Cell), Macrophage (Mac), Eosinophils (Eos), Natural Killer (NK), Innate Lymphoid Cells (ILC), Natural Killer T Cell (NKT), Regulatory T cell (Treg), γδ T cell (γδ), Thymocytes (Thy). **C,** cl-Cas1 expression measured by fluorescently conjugated FAM-YVAD-FMK in $CD45^+CD11c^+MHCII^+XCR1^+$ cDC1 and $CD45^-EpCAM^-$ non-epithelial stromal cells at days 0, 0.5, 1 and 3 after SL-TBI (n=3-5/group). **D,** Amount of active IL-18 measured by ELISA in female 1-2 mo *Il18*$^{fl/fl}$ *Zbtb-Cre*$^-$ (*Il18*^{WT}) and *Il18*$^{fl/fl}$ *Zbtb-Cre*$^+$ (*Il18*$^{\Delta DC}$) mice at day 0 or 1 following SL-TBI (n=10-16/group). **E,** Female 1-2 mo C57/BL6 WT (WT→WT) or *Il18*$^{-/-}$ (WT→ *Il18*$^{-/-}$) mice were lethally irradiated (2 x 550cGy) and transplanted with $5x10^6$ $CD45.1^+$ WT bone marrow hematopoietic cells. 10 weeks after transplantation recipient mice were given a second dose of TBI (sublethal, 550cGy) and active IL-18 was measured on day 1 after this subsequent damage (n=5-7/group). Graphs represent mean ± SEM. *p<0.05, **p<0.01, ***p<0.001

FIGURE S2.2

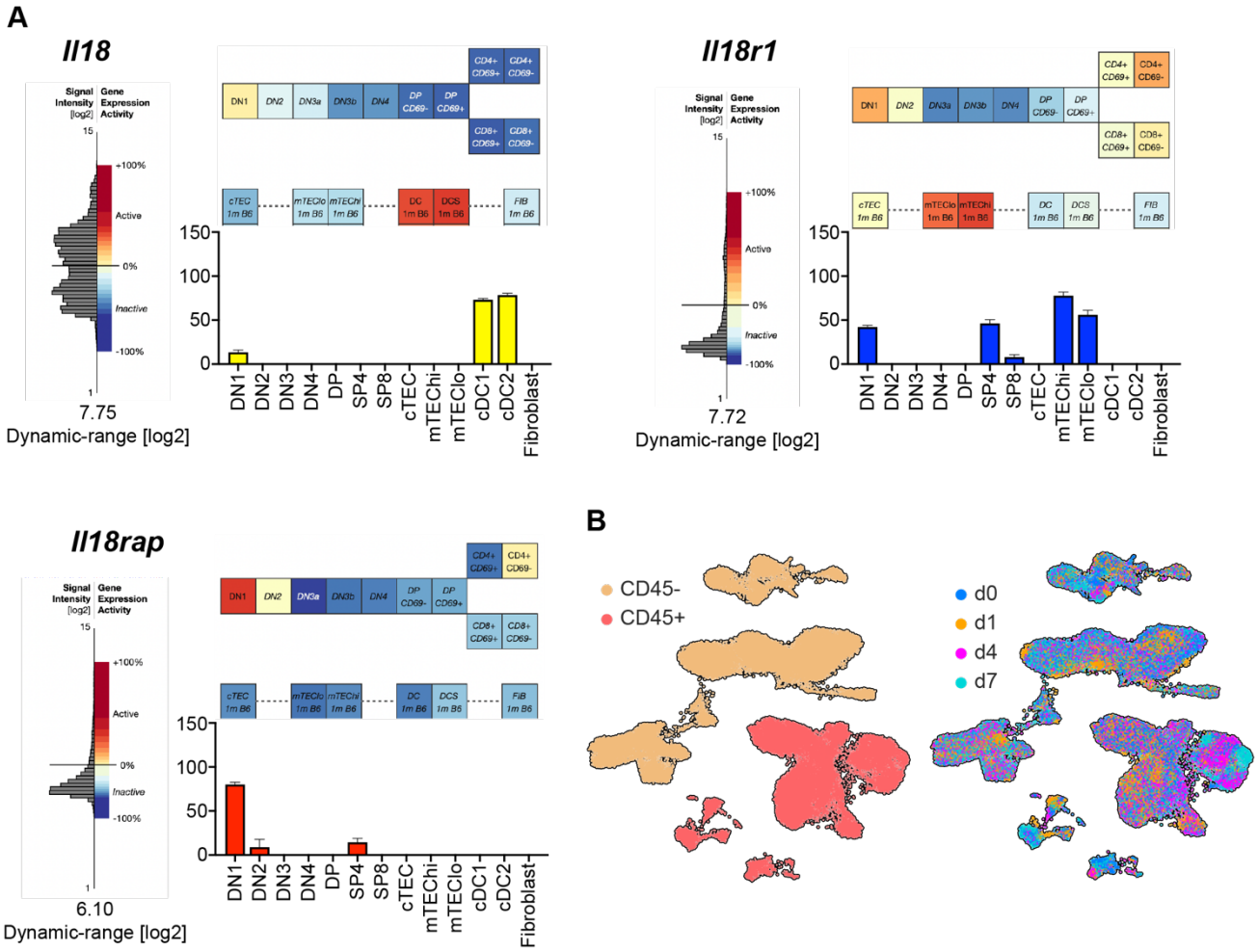

A
Il18
Signal Intensity [log2]
Gene Expression Activity
7.75
Dynamic-range [log2]
Il18r1
7.72
Dynamic-range [log2]
Il18rap
6.10
Dynamic-range [log2]
DN1
DN2
DN3
DN4
DP
SP4
SP8
cTEC
mTEChi
mTEClo
cDC1
cDC2
Fibroblast
B
CD45-
CD45+
d0
d1
d4
d7

Figure S2.2: Cleaved Caspase 1 increases within the thymus following acute damages

A, Relative expression of *Il18*, *Il18r1* and *Il18rap* on subsets of thymus populations at baseline in 1mo mice. Data extracted from Gene Expression Commons using the "Complete thymocyte:stromal interaction model dataset" (https://gexc.riken.jp/models/475/) which is based on GSE56928 [218]. **B**, UMAPs from Fig. 2A with color overlays indicating dataset (Left, RAG2-GFP-CD45+ or CD45-) or timepoint (right, day 0, 1, 4, 7 after SL-TBI).

FIGURE S2.3: High dimensional immunophenotyping of thymus stroma

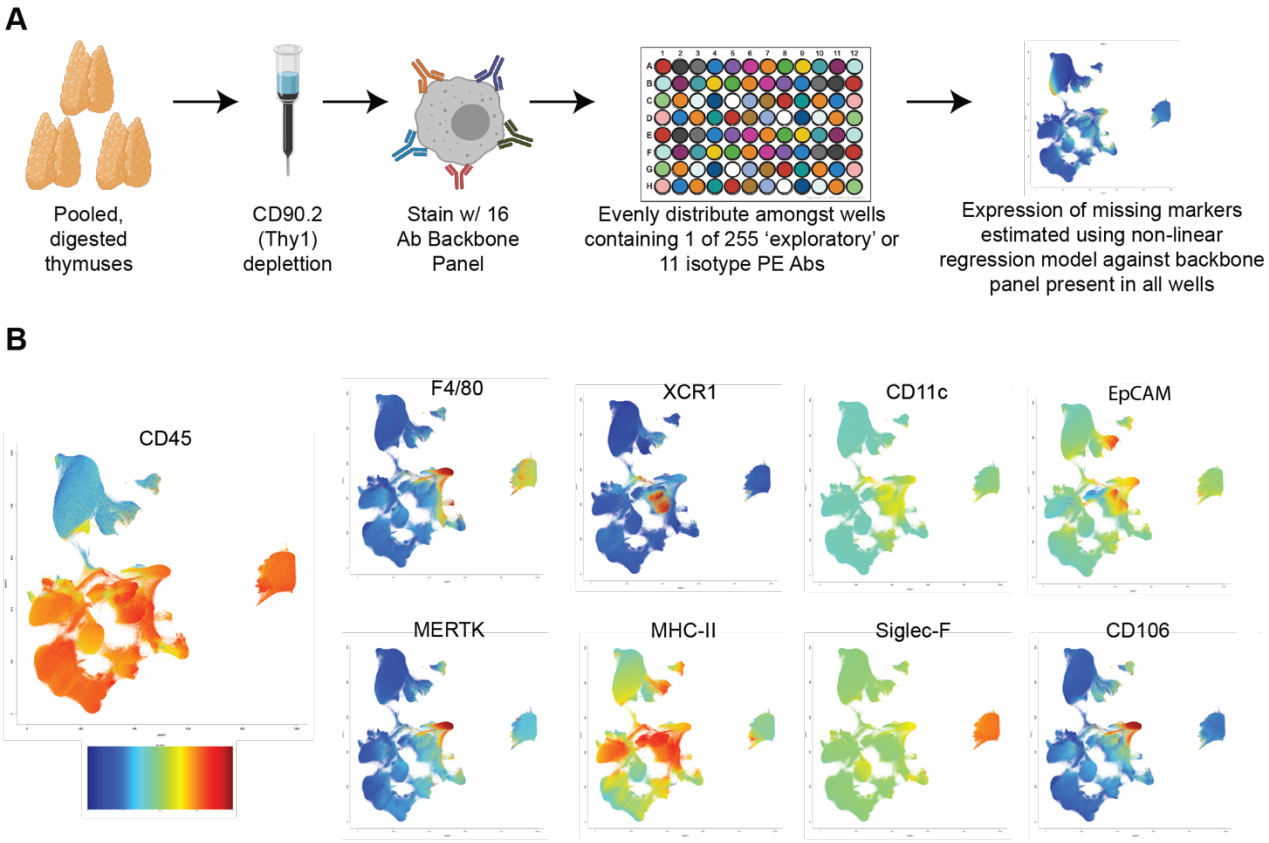


Figure S2.3: High dimensional immunophenotyping of thymus stroma

A, Thymuses were harvested from Female 1-2 mo C57BL6 mice and pooled for digestion and CD90.2 negative depletion performed to enrich for non-thymocyte hematopoietic and non-hematopoietic stromal cells. $CD90.2^-$ cells were thn stained with a 16 marker backbone of antibodies [CD45, Ly6G, CD24, CD11c, MHC-II, Siglec F, Ly6C, CD11b, PDGFRα, CD31, Ly-51, UEA-1, EpCAM, Ter119*, Thy-1*, B220*, NK1.1*, Viability] (*same fluorescent channel). Cells were then split equally for staining into 3 96-well plates containing with each well containing one of 255 exploratory antibodies and 11 respective isotype controls in the PE channel (Biolegend #700009). Acquired data was run through Infinity Flow pipeline that utilizes a non-linear regression model to estimate the expression of each 255 exploratory antibody on the populations identifiable with the backbone panel. **B,** Examples of resultant UMAPs from Infinity Flow pipeline characterizing myeloid populations.

2.3c: IL-18 suppression of thymus regeneration is not mediated via direct effect on TECs or hematopoiesis

To determine potential cellular targets for IL-18, we first interrogated previously described transcriptome datasets for expression of the two IL-18R subunit encoding genes [137,218,226]. We found at baseline that the *Il18r1* subunit was expressed by multiple populations including cortical and medullary thymic epithelial cells (TECs), Tregs, ILCs, NK, and NKT cells (**Fig. 2.2B, S2.2A**). TEC expression was especially notable given their role as master regulators of thymus function and regeneration [74], but we observed only minimal IL-18R protein expression on TEC subsets **(Fig. 2.3A-B, S2.3)** and deletion of *Il18r1* in TECs (using *Foxn1-cre*) demonstrated no change in their capacity for thymus regeneration compared to Cre^- littermate controls **(Fig. 2.3C)**.

Prior work has established that IL-18 can induce hematopoietic stem cell quiescence [277-279]. While we did not observe IL18R expression on bone marrow resident precursor populations at baseline **(Fig. 2.3D-E, 2S.4)**, we did find low levels of IL-18R expression on Early Thymic Progenitors (ETPs), the earliest stage of thymocyte development **(Fig. 2.3A-B, S2.4)**. Therefore, we explored whether *Il18r1*$^{-/-}$ thymocytes showed increased reconstitution capacity relative to WT thymocytes in a competitive transplantation model **(Fig 2.3F)**. 2 weeks post transplantation, an early timepoint at which initial thymus recovery is measured, $CD45.2^+$ *Il18r1*$^{-/-}$ cells had no competitive advantage over $CD45.2^+$ WT cells in seeding the thymus of $CD45.1^+$ WT recipient mice **(Fig 2.3G)**. Measuring longitudinal contribution of donor derived hematopoiesis by peripheral blood monitoring over 120 days following competitive transplantation showed similar reconstitution of both overall hemopoietic cells and T cells regardless of genotype **(Fig 2.3H)**. 120 days post-competitive transplantation, when all hematopoietic cells are of donor origin, we gave sublethally irradiated mice again to directly observe functional regenerative effects of *Il18r1*$^{-/-}$ vs. WT thymocytes and saw similar capacities to recolonize the thymus one week later **(Fig. 2.3I)**. Additionally, these chimeric recipient

thymuses showed similar degrees of regeneration by total organ cellularity one week post subsequent injury **(Fig 2.3J)**.

Consistent with prior literature, we observed that IL-18 did increase thymocyte differentiation and proliferation in studies coculturing *ex vivo* bone marrow derived hemopoietic precursors with the OP9-DLL1 co-culture system **(Fig. S2.5A-C)**[280]. From these data we conclude that IL-18 does not suppress thymus function by directly regulating TEC function, nor does it act directly via T-lineage progenitors. Therefore, we hypothesized that IL-18 activity was instead mediated by a thymus intrinsic mechanism.

FIGURE 2.3: IL-18 suppression of thymus function is not mediated directly through TECs or hematopoietic progenitors

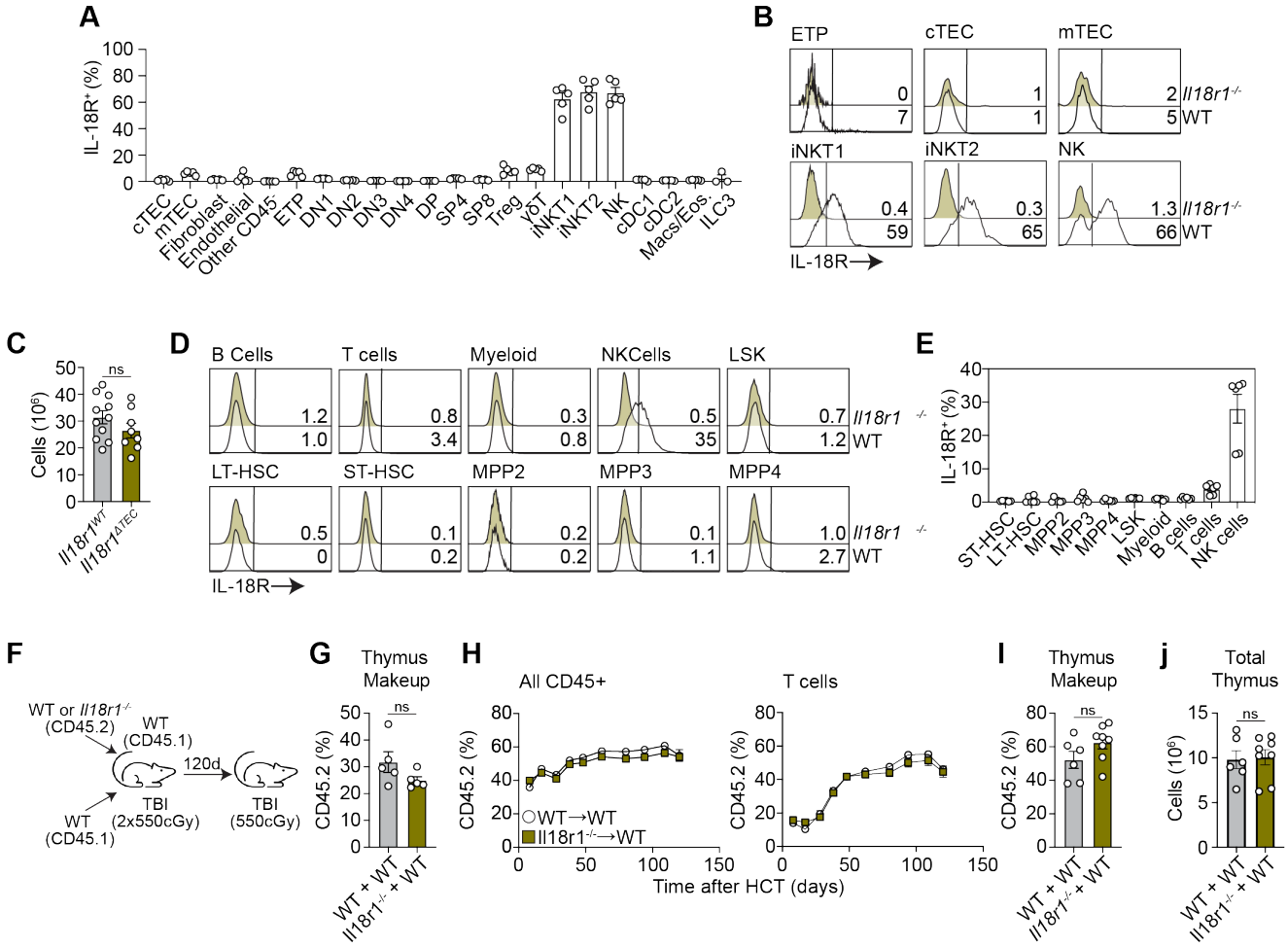
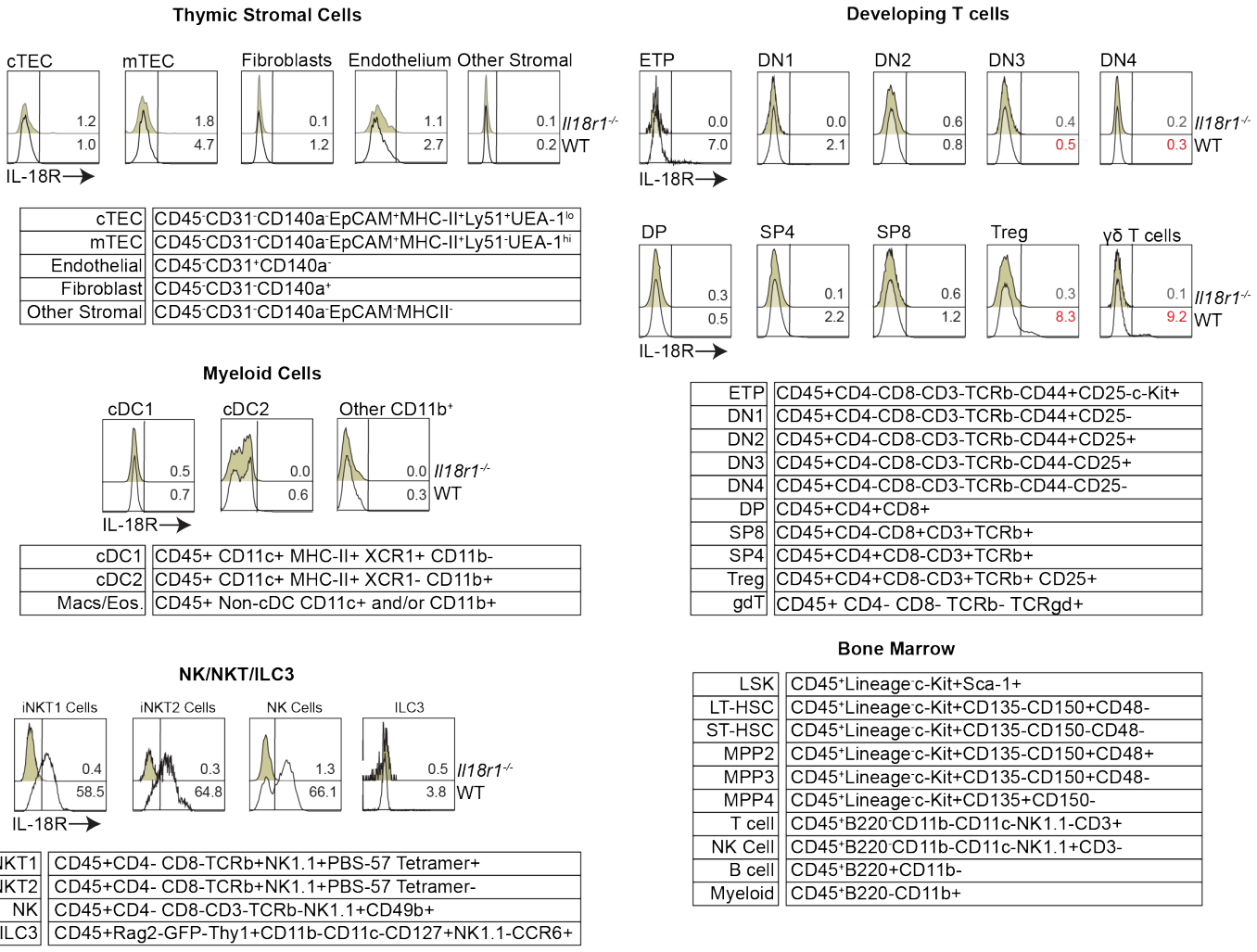


Figure 2.3: IL-18 suppression of thymus function is not mediated directly through TECs or hematopoietic progenitors

A-B, IL-18R expression on thymic cell populations assessed by flow cytometry at baseline (n=3-5/group). **B,** Concatenated flow plots showing expression of IL-18R on early T-lineage progenitors (ETPs), cortical TECs (cTECs), medullary TECs (mTECs), invariant natural killer cells 1 and 2 (iNKT1/2), and natural killer (NK) cells. Gates were based on expression in *Il18r1*$^{-/-}$ mice **C,** Female 1-2 mo *Il18r1*$^{fl/fl}$*:FoxN1-Cre*$^{-}$ (*Il18r1*^{WT}) and *Il18r1*$^{fl/fl}$*:FoxN1-Cre*$^{+}$ (*Il18r1*$^{\Delta TEC}$) were given SL-TBI and thymus cellularity assessed 7 days later (n=8-11/group). **D-E,** Bone marrow populations were measured for IL-18R expression by flow cytometry (n=5-6/group). **F-J, (F)** Female 1-2 mo WT CD45.1^{+} mice were lethally irradiated and transplanted (i.v.) with 2.5x10^{6} WT CD45.1^{+} bone marrow cells and 2.5x10^{6} bone marrow cells from either CD45.2^{+} WT or *Il18r1*$^{-/-}$ mice. **G,** Contribution of CD45.2^{+} cells in the thymus at 2 weeks following transplant (n=5/group). **H,** Contribution of CD45.2^{+} cells to peripheral blood total CD45 (left) or T cell (right) reconstitution over 17 weeks post transplantation (n=6-8/group). **I-J,** 17 weeks after transplantation, recipient mice were given a subsequent dose of SL-TBI (550cGy). 7 days later, thymuses were harvested and measured for **(I)** CD45.2^{+} percentage of all thymus CD45^{+} cellularity and **(J)** total thymus cellularity (n=6-8/group). Graphs represent mean ± SEM. *p<0.05, **p<0.01, ***p<0.001

FIGURE S2.4



cTEC	CD45-CD31-CD140a-EpCAM+MHC-II+Ly51+UEA-1lo
mTEC	CD45-CD31-CD140a-EpCAM+MHC-II+Ly51-UEA-1hi
Endothelial	CD45-CD31+CD140a-
Fibroblast	CD45-CD31-CD140a+
Other Stromal	CD45-CD31-CD140a-EpCAM-MHCII-

cDC1	CD45+ CD11c+ MHC-II+ XCR1+ CD11b-
cDC2	CD45+ CD11c+ MHC-II+ XCR1- CD11b+
Macs/Eos.	CD45+ Non-cDC CD11c+ and/or CD11b+

NKT1	CD45+CD4- CD8-TCRb+NK1.1+PBS-57 Tetramer+
NKT2	CD45+CD4- CD8-TCRb+NK1.1+PBS-57 Tetramer-
NK	CD45+CD4- CD8-CD3-TCRb-NK1.1+CD49b+
ILC3	CD45+Rag2-GFP-Thy1+CD11b-CD11c-CD127+NK1.1-CCR6+

ETP	CD45+CD4-CD8-CD3-TCRb-CD44+CD25-c-Kit+
DN1	CD45+CD4-CD8-CD3-TCRb-CD44+CD25-
DN2	CD45+CD4-CD8-CD3-TCRb-CD44+CD25+
DN3	CD45+CD4-CD8-CD3-TCRb-CD44-CD25+
DN4	CD45+CD4-CD8-CD3-TCRb-CD44-CD25-
DP	CD45+CD4+CD8+
SP8	CD45+CD4-CD8+CD3+TCRb+
SP4	CD45+CD4+CD8-CD3+TCRb+
Treg	CD45+CD4+CD8-CD3+TCRb+ CD25+
gdT	CD45+ CD4- CD8- TCRb- TCRgd+

Bone Marrow

LSK	CD45+Lineage-c-Kit+Sca-1+
LT-HSC	CD45+Lineage-c-Kit+CD135-CD150+CD48-
ST-HSC	CD45+Lineage-c-Kit+CD135-CD150-CD48-
MPP2	CD45+Lineage-c-Kit+CD135-CD150+CD48+
MPP3	CD45+Lineage-c-Kit+CD135-CD150+CD48-
MPP4	CD45+Lineage-c-Kit+CD135+CD150-
T cell	CD45+B220-CD11b-CD11c-NK1.1-CD3+
NK Cell	CD45+B220-CD11b-CD11c-NK1.1+CD3-
B cell	CD45+B220+CD11b-
Myeloid	CD45+B220-CD11b+

Figure S2.4: IL-18R expression on thymus populations

IL-18R expression on thymic cellular populations taken from female 1-2 mo C57BL/6 WT, Rag2gfp or Il18r1-/- mice (n=3-5/group). Tables show phenotypes used to gate individual populations.

2.3d: Damage Resistant NK Cells Suppress Thymus Regeneration after acute injury

IL-18 can signal through the Il-18R1 subunit but its signaling is potentiated by co-expression of the IL-18 receptor accessory protein Il18rap [274,281]. Consistent with their lack of functional signaling after damage, transcriptome datasets indicate that neither TECs nor thymocytes express *Il18rap* (**Fig. 2.2A, 2S.2A**). Expression of both *Il18r1* and *Il18rap* was largely restricted to ILCs, including Natural Killer (NK) cells, and invariant Natural Killer T (NKT) cells (**Fig. 2.2A**). Consistent with this, protein expression suggested NK1.1$^+$ populations including iNKT cells and NK cells strongly expressed IL-18R at baseline within the thymus **(Fig 2.3A-B)**. This was supported by CellChat analysis of scRNASeq which allows for simple and intuitive interactome visualization of sources and targets of IL-18. Targets of IL-18 included NK, NKT and ILC subsets, although NK cell transcriptomes exhibited the strongest aggregate interactome score **(Fig. 2.4A)**. Following HCT, although both recipient IL-18R$^+$ iNKT and NK cells are damage-resistant compared to more abundant thymocyte populations, such that their relative frequency within the organ is significantly higher in the days following transplantation, only NK cells maintained their absolute number with a considerable decrease in NKT cells **(Fig 2.4B-E)**.

To assess the role of NK1.1$^+$ cells in limiting thymic recovery, we dosed mice with αNK1.1 monoclonal antibody and induced thymus damage by SL-TBI, which showed near complete ablation of thymic IL-18R$^+$NK1.1$^+$ cells **(Fig 2.4F).** Mice depleted of NK1.1$^+$ cells had increased thymus cellularity compared to control mice **(Fig. 2.4G)** suggesting that either iNKT and NK cells are likely mediators of IL-18. Notably, improved regeneration observed upon αNK1.1 antibody treatment in WT thymuses was not recapitulated when performed in either *Il18*$^{-/-}$ and *Il18r1*$^{-/-}$ mice, suggesting that NK1.1$^+$ cell control of thymus recovery is IL-18 dependent **(Fig. 2.4H)**. To distinguish between the effects on IL-18R$^+$ NK vs iNKT cells in regulating thymus regeneration, *Cd1d*$^{-/-}$ mice lacking the antigen presenting machinery for iNKT

cell development were given SL-TBI to test the effects of thymus suppression in the absence of iNKT cells [282]. *Cd1d*$^{-/-}$ mice had no difference in their thymus cellularity one-week post injury compared to WT control mice **(Fig. S2.5D**) indicating that IL-18R^{+} iNKT cells, while more abundant than IL-18R^{+} NK cells, do not mediate the IL-18-dependent mechanism limiting thymus regeneration. Finally, to specifically identify NK cells as the target of IL-18 after acute injury, we generated mice with a specific deletion for *Il18r1* in NK cells by using the Ncr1-cre strain, which restricts deletion to cells expressing Nkp46 [283]. We found that these *Il18r1*$^{\Delta NK}$ mice exhibited a significant increase in thymus regeneration after SL-TBI and subsequent rIL-18 supplementation as in Fig. 1H (**Fig. 2.4I**), strongly indicating that NK cells are mediating the IL-18 response.

FIGURE 2.4: Damage resistant IL-18R⁺ NK cells suppress thymus repair

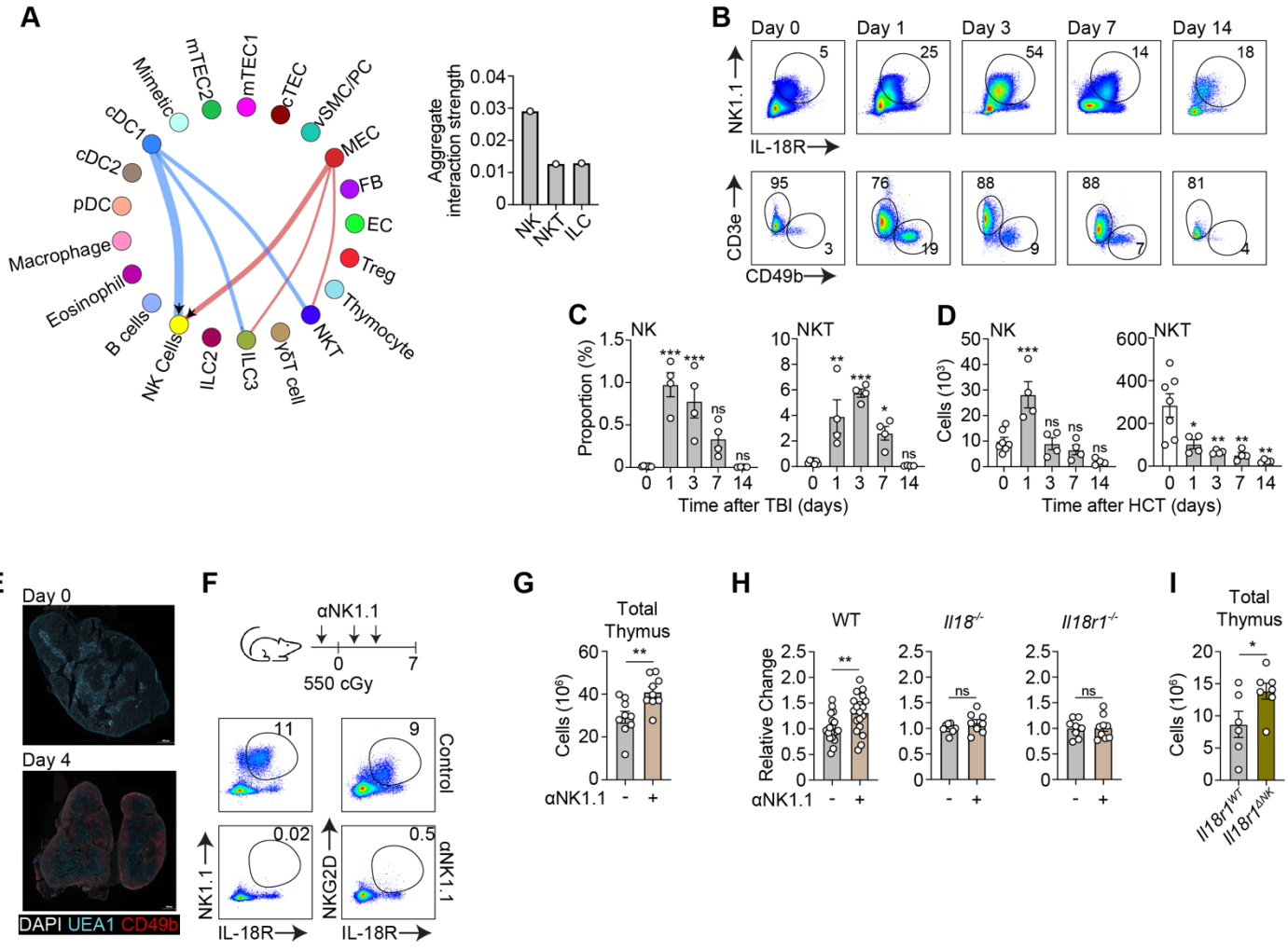


Figure 2.4: Damage resistant IL-18R$^+$ NK cells suppress thymus repair

A, CellChat interaction analysis for IL-18 from scRNASeq dataset described in 2A with quantification of the aggregate signal strength coming into any one IL-18 target cell. **B-D,** Female 1-2mo C57BL/6 CD45.2$^+$ mice were lethally irradiated and (i.v.) transplanted with 5x10^6 WT CD45.1$^+$ bone marrow cells. **B,** Concatenated flow cytometry plots showing recipient IL-18R-expressing cells gated on CD45$^+$CD45.1$^-$CD4$^-$CD8$^-$(upper) and expression of CD3 or CD49b gated on NK1.1$^+$IL-18R$^+$ cells at the indicated timepoints after HCT. **C**, Proportion of recipient NK or NKT cells at the indicated timepoints after HCT (n=4-7/group). **D**, Total number of NK or NKT cells at the indicated timepoints after HCT (n=4-7/group). **E,** Female 1-2mo C57BL/6 mice were given SL-TBI and thymus was visualized 4 days later by *in situ* by fluorescent imaging for CD49b$^+$ (NK) and UEA-1$^+$ (mTECs). **F-G,** Female 1-2 mo C57BL/6 mice were administered 200μg αNK1.1 monoclonal antibody or control PBS (i.p) at days -1, 1 and 3 days post SL-TBI and thymuses assessed at day 7. **F,** Thymus NK1.1$^+$IL-18R$^+$ (Left) and NKG2D$^+$IL-18R$^+$ (Right) cells (parent gated on viable CD45$^+$CD4$^-$CD8$^-$ cells) (n=9/group). **G,** Thymus cellularity measured 7 days post SL-TBI (n=9/group). **H,** Female 1-2 mo C57BL/6 WT, *Il18*$^{-/-}$ and *Il18r1*$^{-/-}$ mice were administered with 200 μg αNK1.1 monoclonal antibody or isotype/ PBS (i.p.) at -1, 1 and 3 days post SL-TBI and thymus assessed at day 7 days. Relative change in thymus cellularity comparing control treated to αNK1.1 treated within each strain (n=8-19/group). **I,** Female 1-2 mo *Il18r1*$^{fl/fl}$*:Ncr1-Cre*$^-$ (*Il18r1*^{WT}) and *Il18r1*$^{fl/fl}$*:Ncr1-Cre*$^+$ (*Il18r1*$^{\Delta NK}$) mice were given SL-TBI and administered with rIL-18 (s.c., 2.5 mg/kg) at day 3. Thymus cellularity measured on day 7 post SL-TBI (n=6-7/group). Graphs represent mean ± SEM. *p<0.05, **p<0.01, ***p<0.001

FIGURE S2.5

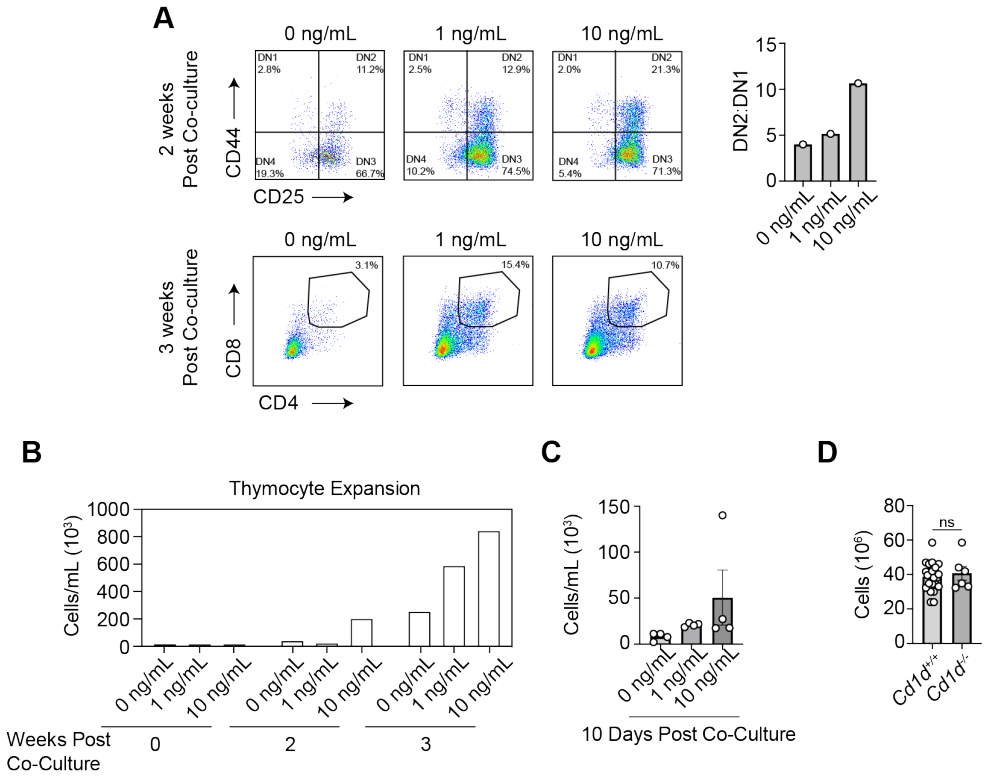

A
0 ng/mL
1 ng/mL
10 ng/mL
2 weeks Post Co-culture
CD44
CD25
DN1 2.8%
DN2 11.2%
DN4 19.3%
DN3 66.7%
DN1 2.5%
DN2 12.9%
DN4 10.2%
DN3 74.5%
DN1 2.0%
DN2 21.3%
DN4 5.4%
DN3 71.3%
DN2:DN1
3 weeks Post Co-culture
CD8
CD4
3.1%
15.4%
10.7%
B
Thymocyte Expansion
Cells/mL (10³)
Weeks Post Co-Culture
C
10 Days Post Co-Culture
D
Cells (10⁶)
ns
Cd1d+/+
Cd1d-/-

Figure S2.5: IL-18 promotes thymocyte expansion and differentiation *in vitro*

A-C, 50,000 lineage depleted bone marrow cells were co-cultured with OP9-DLL1^{GFP} fibroblasts confluent in a 6-well dish with 5ng/mL Flt3L plus 1ng/mL IL-7 and 0, 1 or 10 ng/mL rIL-18 (n=1/group). **A,** (Top) CD45^{+} Thy1^{+} CD4^{-} CD8^{-} DN1-4 thymocyte differentiation measured according to CD44 and CD25 expression 2 weeks post co-culture and (Bottom) CD45+Thy1+ thymocyte differentiation into CD4^{+} CD8^{+} Double Positive population 3 weeks post co-culture. (Right) Ratio of DN2:DN1 thymocyte differentiation 2 weeks post co-culture. **B,** Thymocyte expansion measured by non-adherent cell count quantified 2 and 3 weeks post co-culture with OP9-DLL1^{GFP} adherent cells. **C,** 50,000 bone marrow CD45+ Lineage- cKit+ Sca-1+ LSKs were FACS purified and co-cultured with OP9-DLL1^{GFP} fibroblasts confluent in a 6-well dish with 5ng/mL Ftl3L plus 1ng/mL IL-7 and 0, 1 or 10 ng/mL rIL-18 (n=4/group) and 10 days later, thymocyte expansion was quantified by measuring non-adherent cell expansion. **D,** Female 1-2 mo C57BL/6 WT (*Cd1d* $^{+/+}$) and *Cd1d* $^{-/-}$ mice were given SL-TBI and thymus cellularity was measured 7 days later (n=7-8/group). Graphs represent mean ± SEM. *p<0.05, **p<0.01, ***p<0.001

2.3e: Acute thymic damage activates NK Cells and induces a cytotoxic response

Given that our data demonstrated that IL-18R$^+$ NK cells are the main targets of IL-18 following acute thymic damage, we sought to further characterize the functional role of these innate lymphoid cells in regulating organ repair. Analysis of our scRNAseq dataset revealed upregulation following cytoreductive conditioning of a broad program of molecules involved in NK cell function, including the effectors *Ifng*, *Prf1, Gzma* and *Gzmb* **(Fig 2.5A)**. Comparison with other potential IL-18R$^+$ targets such as NKT cells revealed no such program (**Fig. 2.5A**). Protein analysis of thymus NK cells 3 days after HCT-conditioning supported the transcriptomics with increased NK expression of IFNγ, Granzyme B, Perforin, and surface marker NKG2D demonstrating broad induction of NK cell activation and cytotoxicity **(Figs 2.5B)**. Notably, thymus IL-18R$^+$ NK cells make up the largest population of Perforin$^+$ cells within the thymus following damage **(Fig 2.5C)**. Consistent with this, we also found a significant increase in the global thymic levels of IFNγ, Granzyme B, and Perforin early after HCT-conditioning (**Fig. 2.5D**).

Having demonstrated that NK cells are stimulated by cytoreductive conditioning induced thymic damage, we attempted to determine whether this increased NK activation profile directly resulted in increased NK cytotoxicity and killing of target cells. To address this aim, we cocultured IL-18R$^+$ NK cells FACS purified from either undamaged thymuses or from acutely involuted thymuses 2 days following HCT conditioning with RMA-S target cells. These *ex vivo* cytotoxicity assays revealed that NK cells from HCT-conditioning damaged thymuses were significantly more cytotoxic than those from the undamaged thymus **(Fig 2.5E)**. Taken together our data show that acute damage to the thymus such as that caused by HCT-conditioning activates resident NK cells, increasing their cytotoxicity and capacity for killing nearby target cells.

FIGURE 2.5: HCT conditioning activates thymic NK cells

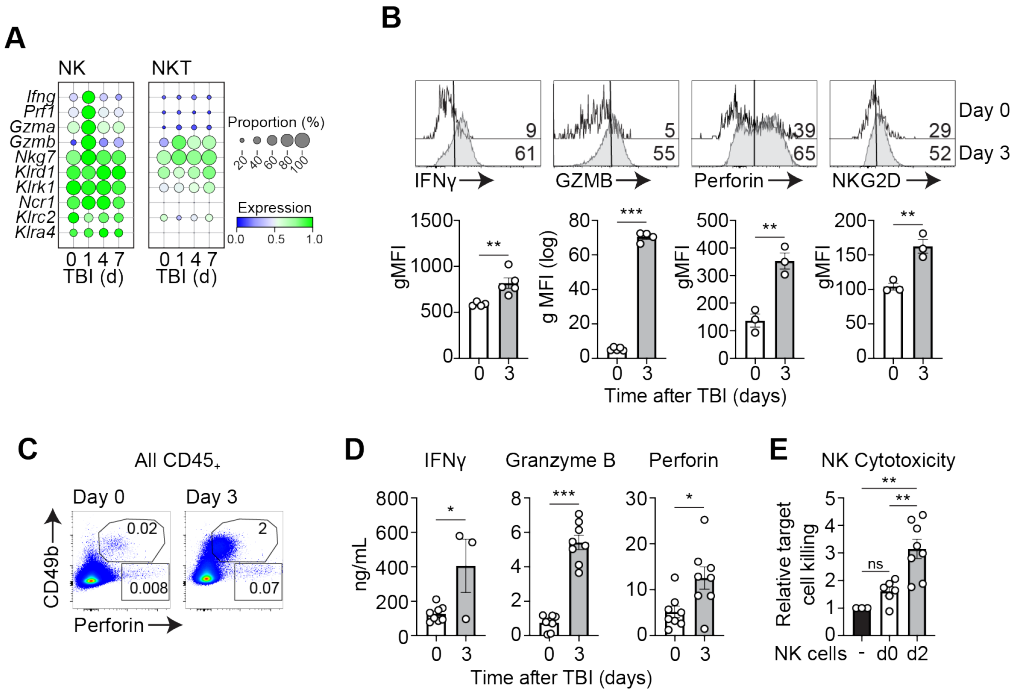


Figure 2.5: HCT conditioning activates thymic NK cells

A, NK and iNKT cell gene expression of cytotoxicity factors *Ifng, Prf1, Gzma, Gzmb* and activation markers *Nkg7, Klrg1, Klrk1* at days 0, 1, 4 and 7 post SL-TBI taken from scRNASeq dataset described in 2A. **B,** IL-18R^{+}NK1.1^{+}CD49b^{+} NK cell expression of *Ifng*-GFP, Granzyme B (GzmB), Perforin and surface NKG2D at days 0 and 3 following SL-TBI in female 1-2 mo C57BL/6 WT or *Ifng*-reporter (GREAT) mice (n=3-5/group). **C,** Concatenated plots showing perforin and IL-18R expression in CD45^{+} cells at baseline or 3 days post SL-TBI. **D,** Amount of thymic IFNγ, Granzyme B, and Perforin measured by ELISA from female 1-2 mo C57BL/6 mice at day 0 and 3 post SL-TBI (n=3-8/group). **E,** NK1.1^{+}IL-18R^{+}TCRβ^{-}CD49b^{+} NK cells were FACS purified from female 1-2 mo C57BL/6 mice at baseline (day 0) or 2 days post SL-TBI and cocultured with cell-trace labeled RMA-S target cells at a 2:1 effector to target ratio. RMA-S Target cell Annexin V expression was measured at 5 hours post co-culture and cell death assessed relative to RMA-S cells cultured without NK cells present (n=3-8/group). Graphs represent mean ± SEM. *p<0.05, **p<0.01, ***p<0.001

2.3f: IL-18 mediates the NK cell effector program after acute damage

IL-18 was first known as IFNγ inducing factor [284], but recent evidence suggests a broader role in activating cells such as NK cells [266,274]. Consistent with this broader role we observed that following cytoreductive conditioning, expression of the activation and effector genes *Klrk1, Nkg7, Gzma, Gzmb, Pfr1* and *Ifng* in NK cells correlated with the expression of the restricted coreceptor *Il18rap* shortly following acute damage*,* which was not the case in NKT cells **(Fig 2.6A)**. These data strongly suggests that IL-18 signaling mediates the activation of this program. However, to functionally determine whether IL-18 directly induced activation of thymic NKs, we administered rIL-18 unmanipulated mice and assessed NK cell derived cytotoxic factors 2 days later. Although in this model thymic size was unaffected 2 days following administration, rIL-18 alone did increase the number of NKs within the thymus and their expression of the effector molecules Perforin and IFNγ **(Figs. 2.6B-D)**. Furthermore, NK cells isolated from thymuses of mice receiving rIL-18 3 days post cytoreductive conditioning and harvested 4 days thereafter (as in Fig 1H) showed significantly higher cytotoxicity than thymic NK cells from mice receiving control PBS **(Fig. 2.6E)**. Together, these findings suggest that IL-18 release following HCT-conditioning increases thymus NK cell cytotoxicity.

The most widely studied function of IL-18 is its capacity to induce IFNγ production from T cells and NK cells [285] Our scRNAseq dataset suggested widespread expression of IFNγ receptor genes, including on crucial stromal subsets of thymic epithelial cells (**Fig. 2.6F**); a pathway that has previously been implicated in mediating TEC cell death in acute graft versus host disease after HCT [236] This led us to hypothesize that IL-18 activated NK cell released IFNγ contributes to suppression of thymus regeneration via direct targeting of TECs. To address this, we generated mice with a TEC-specific deletion for *Ifngr1;* however, absence of IFNγR on TECs identified no difference in thymic regenerative capacity, suggesting that TECs are not a direct target of IFNγ after injury (**Fig. 2.6G**). To assess if IFNγ could be affecting other cells, we assessed

regeneration after SL-TBI in mice with a germline deletion for *Ifngr*, which similarly did not show significantly higher thymus cellularity one week post conditioning compared to WT controls **(Fig 2.6H)**. NK cell mediated killing involves degranulation releasing preformed cytotoxic proteins, mainly granzymes and perforin [286] Mice lacking Perforin (*Prf*$^{-/-}$) did in fact show significantly improved thymus regeneration compared to wildtype control mice **(Fig. 2.6I)**. Therefore, we demonstrate that Perforin-dependent direct cytotoxicity of NK cells is capable of suppressing thymus repair post-injury. Moreover, bulk RNASeq comparison of WT vs. *Il18*$^{-/-}$ thymic NK1.1^{+} IL-18R^{+} cells at baseline or 3 days post SL-TBI suggested that *Prf1* upregulation is in part IL-18 dependent **(Fig. 2.6J)**. As Perforin expression suppresses regeneration, we attempted to utilize newly discovered small molecule (benzene sulfonamide) Perforin inhibitor[287,288] during thymus repair. However, there were no differences in thymic cellularity between Perforin inhibitor and control treated mice 7 days post SL-TBI (**Fig. 2.6K)**, however we were not able to assess the amounts of released Perforin in these treated mice and therefore could not validate inhibition.

FIGURE 2.6: IL-18 stimulation of cytotoxic NK cells suppresses thymus regeneration

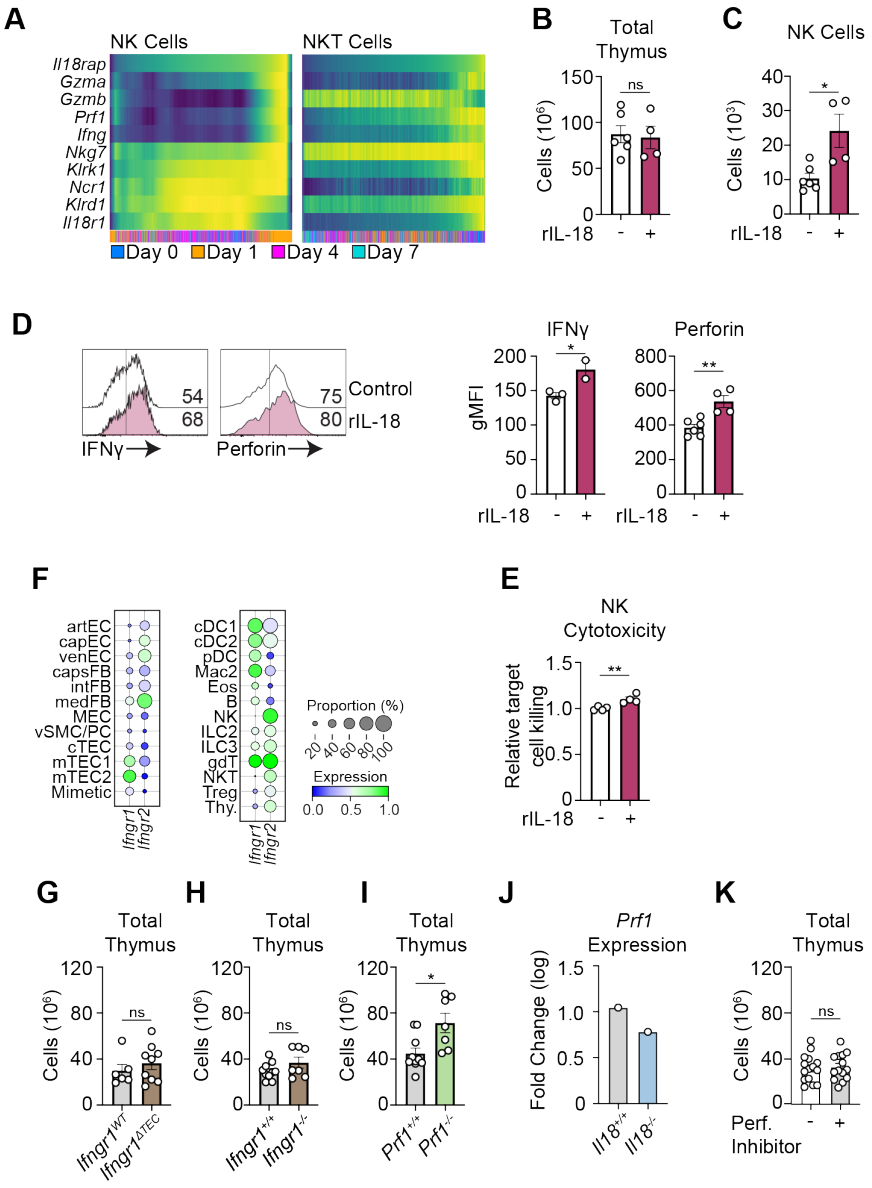


Figure 2.6: IL-18 stimulation of cytotoxic NK cells suppresses thymus regeneration

A, Heat map of thymus NK and iNKT cell gene expression of *Gzma, Gzmb, Prf1, Ifng, Nkg7, Klrk1, Ncr1, Klrd1 and Il18r1* 1, 4 and 7 days following SL-TBI relative to expression of *Il18rap* (taken from scRNASeq dataset described in 2A). **B-D,** Female 1-2 mo C57BL/6 WT or *Ifng*-reporter mice were given rIL-18 (s.c., 2.5mg/kg) or PBS and total thymus cellularity (**B**) or IL-18R$^+$ NK cell number (**C**) assessed on day 3 (n=4-6/group). **D**, Expression of *Ifng*-GFP or Perforin at day 3 (n=2-6/group). **E,** Female 1-2mo C57BL/6 mice were given SL-TBI and 3 days later administered with rIL-18 (s.c., 2.5mg/kg) or PBS. ON day 7 NK1.1$^+$IL-18R$^+$TCRβ^-CD49b$^+$ NK cells were then FACS purified and cocultured with cell-dye labeled RMA-S target cells at a 5:1 Effector to Target ratio. RMA-S target cell Annexin V expression was measured 5 hours post co-culture (n=4/group). **F,** *Ifngr1* and *Ifngr2* expression at baseline taken from the scRNASeq dataset described in 2A. **G,** Female 1-2 mo C57BL/6 *Ifngr$^{fl/fl}$:Foxn1-Cre$^-$* (*Ifngr^{WT}*) and *Ifngr$^{fl/fl}$:Foxn1-Cre$^+$* (*Ifngr$^{\Delta TEC}$*) mice were given SL-TBI and thymus cellularity measured on day 7 (n=6-9/group). **H,** Female 1-2 mo C57BL/6 WT (*Ifngr $^{+/+}$*) or *Ifngr1$^{-/-}$* mice were given SL-TBI and thymus cellularity was measured at day 7 (n=7-8/group). **I,** Female 1-2 mo C57BL/6 WT (*Prf $^{+/+}$*) and *Prf $^{-/-}$* mice were given SL-TBI and thymus cellularity was measured at day 7. **I,** Bulk RNASeq was performed on equal numbers of NK1.1$^+$ IL-18R$^+$ cells were FACS purified from thymuses of female 1-2 mo C57BL/6 WT (*Il18$^{+/+}$*) and *Il18$^{-/-}$* mice at baseline and 2 days post SL-TBI and the log(2) fold upregulation of *Prf1* between day 0 and 3 measured (n=3/group). **J,** Female 1-2 mo C57BL/6 WT mice were treated with 2.5mg benzenesulfonamide Perforin Inhibitor or equal volume vehicle (20% Cyclodextrin) (i.p) every 12 hours beginning 12 hours before SL-TBI through 5 days post SL-TBI and thymus cellularity assessed at day 7 post SL-TBI (n=14-15/group). Graphs represent mean ± SEM. *p<0.05, **p<0.01, ***p<0.001

2.3g: Cytotoxic NK Cells Aberrantly Target Thymic Epithelial Cells

Ly49 family receptors on NK cells recognize constitutively expressed MHC-I on cells to inhibit NK cytotoxicity [289]. Several viruses evolved to express proteins interfering with MHC-I antigen presentation, and NK cells are equipped to recognize virally infected cells' downregulation of MHC-I to mediate their killing. Tumor cells, which similarly downregulate MHC-I to evade $CD8^+$ T cell mediated immune surveillance, are also targeted by NK cells[289].

Having established that NK cell cytotoxicity suppresses thymus regeneration, we sought to identify populations that may be targeted by NK cells following cytoreductive conditioning. We observed significant downregulation of MHC-I component H2-kb in both cTECs and mTECs following acute thymus damage **(Fig 2.7A)**. TECs not only downregulate NK cytotoxicity inhibitory factor MHC-I, but they also upregulate NK stimulating NKG2D ligand RAE-1 following cytoreductive conditioning **(Figs 2.7B)**. Together, downregulation of H2-kb and upregulation of NKG2D ligands makes TECs vulnerable to NK cell cytotoxicity. Given the crucial function of TECs during normal T cell development as well as thymic regeneration [74], we hypothesized that deletion of TECs by IL-18 triggered NKs following cytoreductive conditioning suppresses thymus function. Consistent with the differential degree of MHC-I downregulation and Rae1 upregulation, we found that mTEC cellularity was significantly greater in *Il18*$^{-/-}$ mice while cTEC cellularity was not significantly affected 7 days post SL-TBI **(Fig 2.7C)**. We also found a similar differential effect on TEC subsets in mice whose thymus recovery was improved by treating with αNK1.1 mAb (as in Fig 3I) **(Fig 2.7D)**. These data suggested that IL-18 triggered cytotoxic NK cells preferentially mediate mTEC cell destruction.

Taken together, we have identified that following cytoreductive conditioning, increased cl-Cas-1 leads to the release of activated IL-18 which triggers the cytotoxicity of organ-resident NK cells, and that these NK cells target TECs, particularly mTECs, to suppress thymus recovery and T cell reconstitution. Furthermore, these data also demonstrate that IL-18 abrogation holds promise as a therapeutically feasible strategy that can improve thymus recovery post-HCT.

FIGURE 2.7: Cytotoxic NK cells aberrantly target Thymic Epithelial Cells

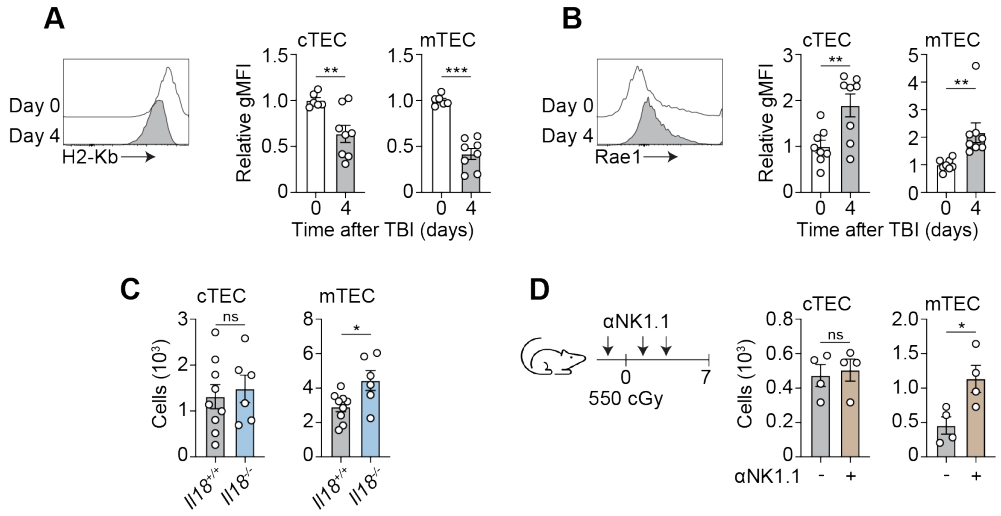


Figure 2.7: Cytotoxic NK cells aberrantly target Thymic Epithelial Cells

A-B, Expression of MHC-I (**A**) and pan-Rae1 (**B**) at day 0 and 4 following SL-TBI of 2mo C57BL/6 mice. Concatentaed flow plots gated on all TECs ($CD45^{-}EpCAM^{+}MHC\text{-}II^{+}$) and graphs showing MFI within specific cTEC and mTEC populations (n=6-8/group). **C,** Female 1-2 mo C57BL/6 WT and *Il18*$^{-/-}$ mice were given SL-TBI and thymus assessed at day 7 for number of $CD45^{-}EpCAM^{+}MHC\text{-}II^{+}Ly51^{+}UEA\text{-}1^{-}$ cTEC and $CD45^{-}EpCAM^{+}MHC\text{-}II^{+}Ly51^{-}UEA\text{-}1^{+}$ mTEC were quantified (n=6-9/group). **D,** Female 1-2 mo C57BL/6 mice were administered 200μg αNK1.1 monoclonal antibody or PBS at -1, 1 and 3 days post SL-TBI. Thymus was analyzed on day 7 days for number of cTEC and mTEC (n=4/group). Graphs represent mean $\pm$ SEM. *p<0.05, **p<0.01, ***p<0.001

2.4 CONCLUSION

In this study, we have identified a crucial role for IL-18 in limiting thymic regeneration following acute damage via stimulation of tissue-resident NK cells, which aberrantly targeted key stromal populations such as mTECs. Despite its importance for generating and maintaining a pool of naïve T cells, the thymus is extremely sensitive to multiple forms of acute injury including everyday insults like stress and infection, but also more profound damage like cytoreductive chemotherapy and ionizing radiation. We recently reported that thymocyte depletion following ionizing radiation is mediated by sharp rises in both caspase-1-mediated immunogenic cell death (ICD) as well as caspase-3 mediated apoptosis in thymocytes [265]. Products of this lytic cell death including Zinc and ATP support thymic regeneration post-injury by directly or indirectly acting on TECs, the primary stromal cell supporting T cell development [180,265,290]. Here, we demonstrate that increased ICD not only occurs following radiation injury, but also during the depletion phase of other acute thymus insults including common chemotherapy regimens, corticosteroid treatment and sepsis.

During infection, myeloid cells such as DCs and macrophages release caspase-1 activated IL-18 that stimulates potent IFNγ production by $CD4^+$ Th1, $CD8^+$ and NK cells [291]. As an acute-phase effector response, constitutive expression in cells leads to cytoplasmic stores of the inactive immature form of IL-18 that await proteolytic cleavage [274,292]. Our data confirms that in the thymus, hematopoietic constitutive expression of *Il18* was restricted largely to cDC1s. Indeed, abrogation of *Il18* expression specifically within DCs did mitigated the rise in IL-18 following conditioning. We could also detect a relatively high baseline amount of cl-Cas-1 in cDC1 and low levels of active IL-18 in the organ, which could be consistent with a previously reported homeostatic role for IL-18 in maintaining a population of thymus-resident regulatory T cells [293]. However, protein still rose in cDC conditional *Il18* knockout mice consistent with there being alternate sources of activated IL-18 post-injury. Supporting the possibility of an alternate

source of IL-18, we also found constitutive expression of IL-18 in non-hematopoietic cells such as capsular fibroblasts and mesothelial cells lining the organ parenchyma. This expression was supported by increased cl-Cas1 in the non-hematopoietic compartment after TBI and functionally confirmed by bone marrow chimeras that showed decreased levels of protein following damage in *Il18*$^{-/-}$ recipients. This alternate source of IL-18 is consistent with reports suggesting nonhematopoietic sources are functional contributors of intestinal integrity regulating IL-18 [294,295]. This study demonstrating that capase-1 dependent IL-18 is a novel suppressant of thymus regeneration is also consistent with previous work in which mice deficient for NLRP3 inflammasome upstream of caspase-1 showed improved thymus function [184].

IL-18 has been shown to regulate intestinal barrier function via epithelial cell maturation and function [294-296]. Given existing parallels between intestinal epithelium and TECs, we first explored the possibility that IL-18 signaling via TECs may directly inhibit their function. However, although there was detectable *Il18r1* expression within various mTEC subsets, neither the *Il18rap* coreceptor, nor significant levels of IL-18R protein expression was found on any TEC subset. Crucially, conditional genetic deletion of *Il18r1* within *Foxn1* expressing TECs did not alter thymus recovery following TBI. One additional mechanism that has been demonstrated for IL-18 is in the regulation of bone marrow hematopoiesis [278,279] which, given the fundamental requirement for bone marrow progenitor seeding of T cell development, was a distinct possibility for a mechanism underlying the role of IL-18 in thymic regeneration. However, we could detect no difference in the capacity for *Il18r1* deficient hematopoietic stem and progenitor cells to 1) reseed the recovering thymus shortly following transplantation and 2) reconstitute overall hematopoietic and T cell populations longitudinally 120 days post-transplant. Furthermore, recipients receiving WT and *Il18r1*$^{-/-}$ donor cells showed similar capacity for recovery after a subsequent insult of SL-TBI 4 months following transplantation. Thus, despite prior evidence for an effect of IL-18 on epithelial cells in the gut and on hematopoietic progenitors in the BM, these mechanisms did not explain the IL-18 suppression of tissue repair in the thymus.

IL-18 canonically signals on T cells, NKT and NK cells to mediate a TH1 response, primarily by inducing expression of IFNγ [267,274]. Indeed, although there was some expression of *Il18r1* on TECs, ETPs, Tregs, and ILCs, by far the greatest expression was restricted to NKT cell progenitors and NK cells. While we found that depletion of both NK and NKT populations with αNK1.1 mAb improved thymus function after damage. In contrast with studies implicating NKT cells in regeneration [262], in our hands mice deficient for NKT cells did not show any alteration in thymic repair. Furthermore, expression of the coreceptor *Il18rap*, which significantly potentiates IL-18 signaling [274,281], was largely absent on NKT cells and was restricted in the thymus to NK cells. Importantly, specific deletion of *Il18r1* in NK cells revealed improved thymus regeneration. However, despite previous studies linking IFNγ with TEC cell death during graft versus host disease [236], surprisingly IFNγ did not seem to be the effector molecule used by NK cells to limit thymic regeneration. Instead, IL-18 induced a broad activation program including upregulation of effectors of direct cytotoxicity such as granzymes and perforin, and mice deficient for perforin demonstrated improved thymus reconstitution. IL-18 requires co-stimulation with either IL-12 or IL-15 to activate of $CD4^+$ Th1 and $CD8^+$ T cytotoxic cells, however, recent reports suggest that IL-18 alone is capable of stimulating a broad activation program in NK cells [266], and accordingly we found that sole rIL-18 administration increased NK cell Perforin, GzmB and IFNγ production.

This study represents the first recognition of tissue-resident NK cells negatively regulating thymus function. In fact, donor NK cells are reportedly beneficial in the setting of HCT, promoting engraftment, reducing GVHD by targeting HLA-mismatched antigen presenting cells, and increasing TEC proliferation [297,298]. However, we suggest a different mechanism whereby radio-resistant *recipient* NK cells are activated as a consequence of damage and counteract thymus regeneration. While ionizing radiation has been shown to induce expression of MHC class I in tissues such as gut [299,300], this was not the case in TECs which showed both a

decrease in MHC I expression but also upregulation of the NK-activating ligand Rae1, making them vulnerable to cytotoxicity in an HLA-mismatch independent fashion. However, the skewing toward mTEC targeting could be explained by their higher rate of cell turnover and proliferation, contributing to their sensitivity to radiation damage, both of which can be drivers for NKG2D ligand expression [214,301-303]. Therefore, there is a distinction between the pro and anti-reparative functions of donor and recipient NK cells, respectively. Furthermore, our work is consistent with reports showing that IL-18 can stimulate NK cells that aberrantly target epithelium during HSV-2 infection and delay re-epithelization in wound healing, as well as with NK cell directed cytotoxicity targeting HSPCs that upregulate NKG2D ligands in Fanconi Anemia due to genotoxic stress [268-270,301]. Our study therefore contributes to a growing body of literature revealing a role for NK cells in regulation of tissue injury and recuperation.

This work positions IL-18 as a potential therapeutic target for improving thymus function after acute injury such as HCT conditioning. However, given the reported context-dependent effects of IL-18 in GVHD, along with its emerging promise in immunotherapy, careful examination of its abrogation will be required to balance its pro-reparative and graft-versus-tumor effects [267,304-309]. In summary, this study identifies a novel pathway regulating T cell immune reconstitution following acute thymus damage and presents multiple opportunities for potential therapeutic targeting to improve T cell reconstitution not only in HCT patients, but also those exposed to other acute thymus damages due to chemotherapy, stress, and infection.

CHAPTER 3. IL-18 INDEPENDENT CYTOTOXICITY CONTRIBUTES TO AGE-RELATED THYMUS INVOUTION

3.1 SUMMARY

Despite the remarkable capacity of the young and healthy thymus to produce a diverse and tolerant T cell repertoire and regenerate following acute injury, both the T cell productivity and ability to recover are severely diminished with age. As we had previously implicated inflammation within the thymus microenvironment – and specifically inflammatory cytokine IL-18 – in suppressing thymus function, we explored the effects of IL-18 in age-related thymus involution. Though we identify increased IL-18 bioavailability and representation of IL-18R+ cells within the organ, IL-18 deficiency did not alter organ involution in aged thymuses. However, we do identify a novel role of Perforin signaling in contributing to chronic thymic damage. Unlike in our study of acute damage in which NK cells were the predominant sources of thymus regeneration suppressing Perforin, we identify Perforin expressing mature T cell populations that represent more likely candidates of chronic damage mediators. Overall, these data begin to uncover a novel pathway of cytotoxicity regulating age-related thymus involution that may be therapeutically targetable to improve T cell production and overall immunity with age.

3.2 INTRODUCTION

The thymus is at its largest and most productive during infancy and will chronically decline as a natural process of aging in a process known as age-related thymus involution. Though this phenomenon has long been recognized, the processes underlying this process are incompletely understood[112]. Forces including rises in sex steroids and decreased hematopoietic T cell potential contribute to this involution[95,150]. Central to age-related thymus decline though is

the diminished functionality of TECs – reduced turnover and overall cellularity – and chiefly, reduced *Foxn1* expression[12,42-44]. While 'inflammaging' – state of chronic activation under sterile conditions leading to a chronic low-grade inflammatory state – is in part attributable to reduced tolerance induction of the aged thymus, little is known regarding the effects of age-related inflammation on regulating thymus involution itself[310]. The studies described here utilize 12-month-old and 18-month-old mice to capture ongoing damage and the near completely involuted organ.

Given our reported involvement of the inflammatory cytokine IL-18 in the overall suppression of thymic regeneration and, importantly, that we observe increased thymus cellularity in the absence of damage in *Il18*$^{-/-}$ adolescent mice **(Fig. S2.1B)**, we explored the role of IL-18 in the context of age-related thymus involution. We hypothesized that abrogating IL-18 can improve thymic function in age and ameliorate involution. This hypothesis was supported by literature demonstrating that in the absence of the NLRP3 inflammasome upstream of Cas-1 cleavage, thymus size was increased in age[184]. Furthermore, abrogation of IL-33 – which like IL-18 is an IL-1 family cytokine that acts as an alarmin – also improves aged thymus size and T cell function at least in the context of Schistosome infection[311]. In our models of acute thymus injury, thymic cytotoxic NK cells target TECs, thus we also sought to explore the function of NK cells in the chronically, involuted thymus. Finally, to test a broader role of cytotoxicity and because of our identified role of Perforin in regulating thymus recovery from acute damage, we investigate the role of Perforin signaling in regulating age-induced chronic damage.

3.3 RESULTS

3.3a: Increased IL-18 signaling in aged, involuted thymus

To assess the potential involvement of IL-18 in regulating organ function during aging, we first assessed the changes in 'free' thymic IL-18 across the murine lifespan. We found that

active IL-18 increased within the thymus during aging **(Fig. 3.1A)** and that IL-18BP decreased to undetectable levels in the aged thymus **(Fig. 3.1B)**. Therefore, we concluded that there was increased 'free' IL-18 in the aging thymus strengthening our hypothesis that increased organ inflammation with age could contribute to IL-18 mediated thymic dysfunction.

The increase in active IL-18 and decreased IL-18BP we observed by ELISA was supported by our scRNASeq data set on non-hematopoietic and hematopoietic Rag2-GFP^{-} stroma **(Fig. 3.1BC-D)**. *Il18* gene expression was mildly increased in thymic 18-month-old Mesothelial cells and DCs/Macrophages compared to baseline 2-month-old thymic control cells **(Fig. 3.1C)**. Interestingly, there was also the emergence of *Il18* expression in B cells in the 18-month-old thymus **(Fig. 3.1C)**. *Il18bp* baseline gene expression is not noticeably decreased in stromal populations during aging, however, *Il18bp* response to SL-TBI is blunted during aging **(Fig. 3.1D)**. We also performed spatial RNASeq on 2-month-old and 18-month-old thymuses using the 10X Visium platform to capture changes in gene expression tied to the architectural disruption of the chronically involuted thymus. Overall, this platform proved challenging to interpret within the thymus. Cellular density was simply too high, particularly within the young thymus, to be able to reliably quantify gene expression or to assign cell identity to gene expression, even when combined with serial sectioning and immunofluorescent staining for epithelial and myeloid markers (images not shown). Our overall conclusion was that the thymus is a suboptimal setting for this spatial sequencing platform which was more suited for organ sections characterized by less cell density and more delineated architecture. However, we could take away the relative concentration of gene expression per organ (i.e., in how much of the organ area was a gene of interest expressed). Consistent with our analyses, we observed increased *Il18* expression in the aged thymus compared to control 2-month-old organ **(Fig. 3.1E)**. Both the young and old thymic sections showed similarly high levels of *Il18bp* gene expression, however **(Fig. 3.3E)**. Addressing the IL-18 signal receiving end, we also observed increased *Il18r1* gene expression concentration within the 18-month-old thymus. Overall, protein

expression and available single cell and spatial RNA sequencing data supported increased IL-18 signaling within the aged, involuted thymus.

FIGURE 3.1: Exploring IL-18 signaling in age-related thymus involution

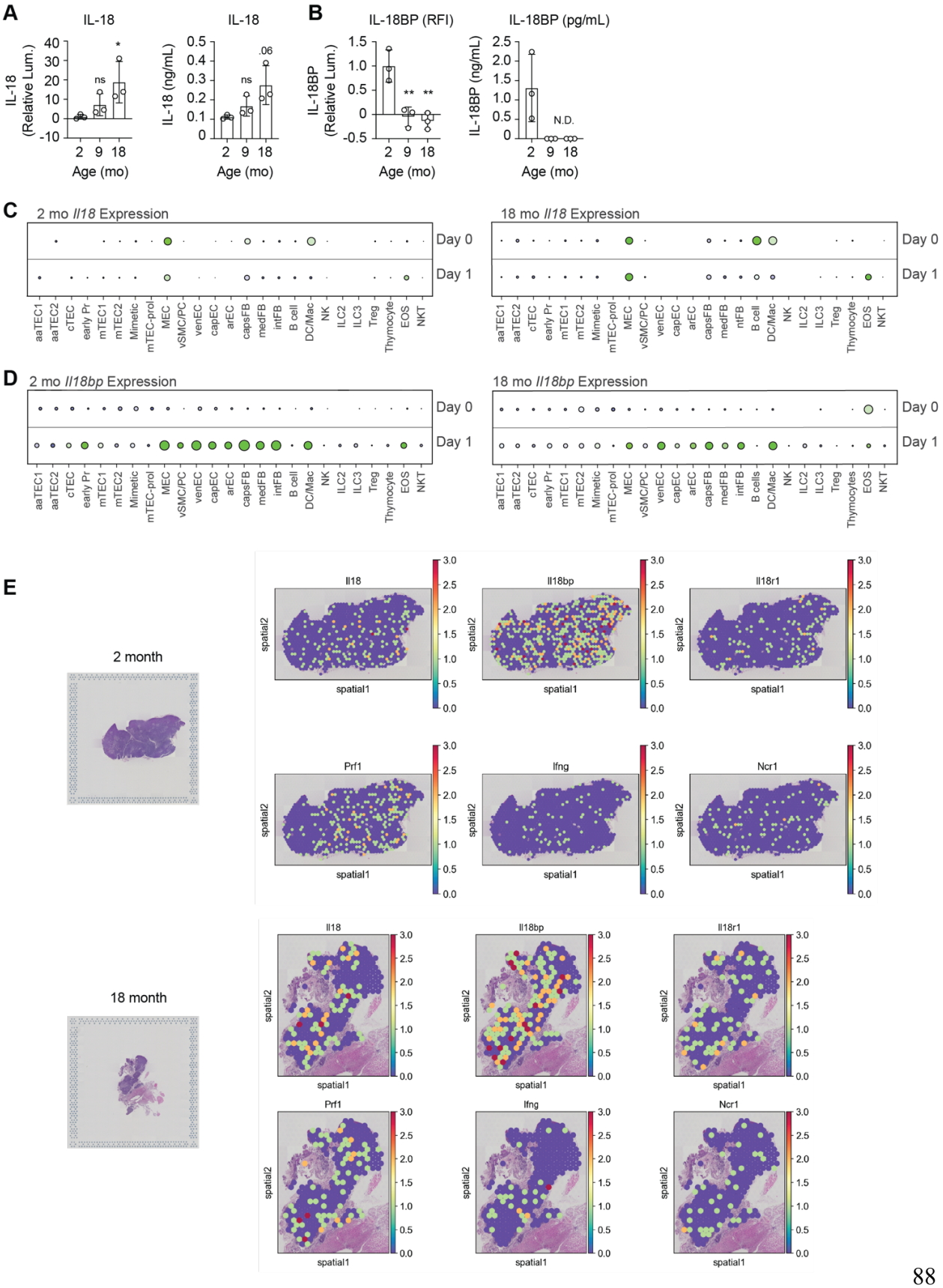


Figure 3.1: Exploring IL-18 signaling in age-related thymus involution

A-B, Thymuses from male 2 mo, 9 mo, and 18 mo C57/BL6 WT mice were harvested absent any additional treatments and measured for **(A)** mature IL-18 and **(B)** IL-18BP. As standard curves were unable to interpolate all values relative luminescence (Left) and protein concentrations where available (Right) are provided (n = 3/group). **C-D,** Single cell RNA sequencing was performed on (1) non-thymocyte $CD45^+$ stromal cells ($CD45^+$ *Rag2*-GFP^- isolated from female 1-2 mo *Rag2-gfp* mice) and (2) $CD45^-$ stromal cells isolated from female 1-2 mo and 18 mo C57BL/6 mice at baseline, 1, 4 and 7 days following SL-TBI as described in 2.2. Data (from 2 mo mice) was previously integrated together and published in Lemarquis *et al.* (2024). **(C)** *Il18* gene expression and **(D)** *Il18bp* shown at days 0 and 1 post SL-TBI in 2mo (Left) and 18 mo thymuses (Right). **D,** Thymuses from male 2 mo, 9 mo, and 18 mo C57/BL6 WT mice were harvested and preserved in FFPE blocks then used for 10x Visium Spatial Sequencing. Spatial expression for 2 mo old (Top) and 18 mo (Bottom) of the genes *Il18, Il18bp, Il18r1, Prf1, Ifng* and *Ncr1* shown. Graphs represent mean ± SEM. *p<0.05, **p<0.01, ***p<0.001

3.3b: IL-18 abrogation cannot rescue thymus involution

To better understand IL-18 target cells in age, we sought to characterize the IL-18R$^+$ populations within the old, involuting thymus and compare it to that of young, healthy one. 12-month-old thymuses had overall similar makeups of their IL-18R$^+$ population as young ones with both being dominated by NKT cells, however, there were increased contribution from IL-18R$^+$ CD8$^+$ cells and CD8$^-$ CD4$^-$ cells **(Fig. 3.2A)**. When measuring the makeup of the total thymus, there was an increase in the relative frequency of IL-18R$^+$ NK cells and IL-18R$^+$ CD8$^+$ T cells in the 12-mo-old thymus compared to the 2-mo old thymus **(Fig 3.2B)**. These data show that aging is associated with increases in the representation of IL-18R$^+$ populations of interest, particularly NK cells, to go along with the increase in free IL-18 **(Fig 3.1A-B)**. The potential for increased IL-18 driven inflammation was supported by spatial sequencing showing that there was an increase in the relative concentration of *Prf1* and *Ifng* expressing populations, though NK specific Nkp46 encoding *Ncr1* expression showed similar frequency in the aged thymus compared to young one **(Fig 3.1E)**. Therefore, we explored whether IL-18R$^+$ NK cells contributed to the chronic organ damage of aging similar to the mechanism idenitifed for IL-18R$^+$ NK cell suppression of organ recovery following acute injury.

However, both 12-month-old and 18-month-old *Il18*$^{-/-}$ thymuses showed similar overall cellularity to age-matched WT controls **(Fig. 3.2C, F)** allowing us to conclude that – despite the increased free IL-18, higher frequency of IL-18R$^+$ NK cells, and role for IL-18 in regulating acute thymus injury recovery – IL-18 does not regulate age-related thymus involution **(Fig. 3.2C, F)**. At 12-months of age, *Il18*$^{-/-}$ mice showed similar numbers of cTECs, mTECs, and Ly51$^-$ UEA-1$^-$ age-associated TECs (aaTECs) **(Fig. 3.2D-E)**. Note, we along with collaborators recently reported the existence of these aaTECs as a nonfunctional TEC population characterized by an epithelial to mesenchymal transition gene signature and that act as a sink for TEC growth factors[226]. Interestingly though, there are significantly increased cTECs, mTECs and aaTECs in *Il18*$^{-/-}$ 18-month-old thymuses compared to WT age-matched controls **(Fig. 3.2G-H)**. This raises

several questions. Given that TEC numbers were no different at 12-months, does IL-18 affect TEC cellularity and turnover between 12-months and 18-months of age? If so, what are the mechanisms by which IL-18 may be rescuing TEC cellularity in the involuted 18-month-old thymus? Though there is a trend of increased cellularity in *Il18*$^{-/-}$ 18-month-old thymuses, it does not rise to the level of significance, which leads us to wonder why the observed dramatically improved TEC cellularity in 18-month-old *Il18*$^{-/-}$ thymuses does not translate to overall organ cellularity as we would expect? Presently aging *Il18*$^{-/-}$ mice will help us explore these unanswered questions.

We were also interested in exploring the effects on peripheral T cell differentiation in these aged mice lacking IL-18. *Il18*$^{-/-}$ mice showed decreased CD4+ central memory and increased CD4^{+} effector cells and decreased CD8^{+} naïve cells and increased CD8^{+} effector cells **(Fig. 3.2I)**. These effects may be more attributable to IL-18 signaling on peripheral T cell populations than a secondary effect from altered thymic function[285,305,312]. Finally, as thymic output is presumably higher in the absence of IL-18 at least until the onset of age-related thymus involution, we sought to answer whether the absence of IL-18 was associated with any resultant autoimmunity. To address this concern, we performed histological analysis on kidney, lung, pancreas, liver and small intestines of 12-month-old and 18-month-old *Il18*$^{-/-}$ mice. There was broadly increased inflammatory pathological scoring characterized by more lymphoplasmacytic infiltrate in *Il18*$^{-/-}$ mice; inflammation was particularly increased in the pancreas and intestines of Il18-/- mice **(Fig. 3.2I, Table 3.1, Fig S3.1)**. Note, however, that the increased inflammation within the organs of *Il18*$^{-/-}$ mice cannot be solely attributed to the impact of IL-18 on the thymus rather than any of its many effects on peripheral T cells or other immune cells. Overall, these data suggest that despite a potential protective role in preserving TEC cellularity, abrogating IL-18 is not capable of rescuing overall thymus size during age-related chronic involution and that systemic IL-18 deficiency may lead to increased autoimmunity.

FIGURE 3.2: IL-18 abrogation does not ameliorate age-related thymus involution

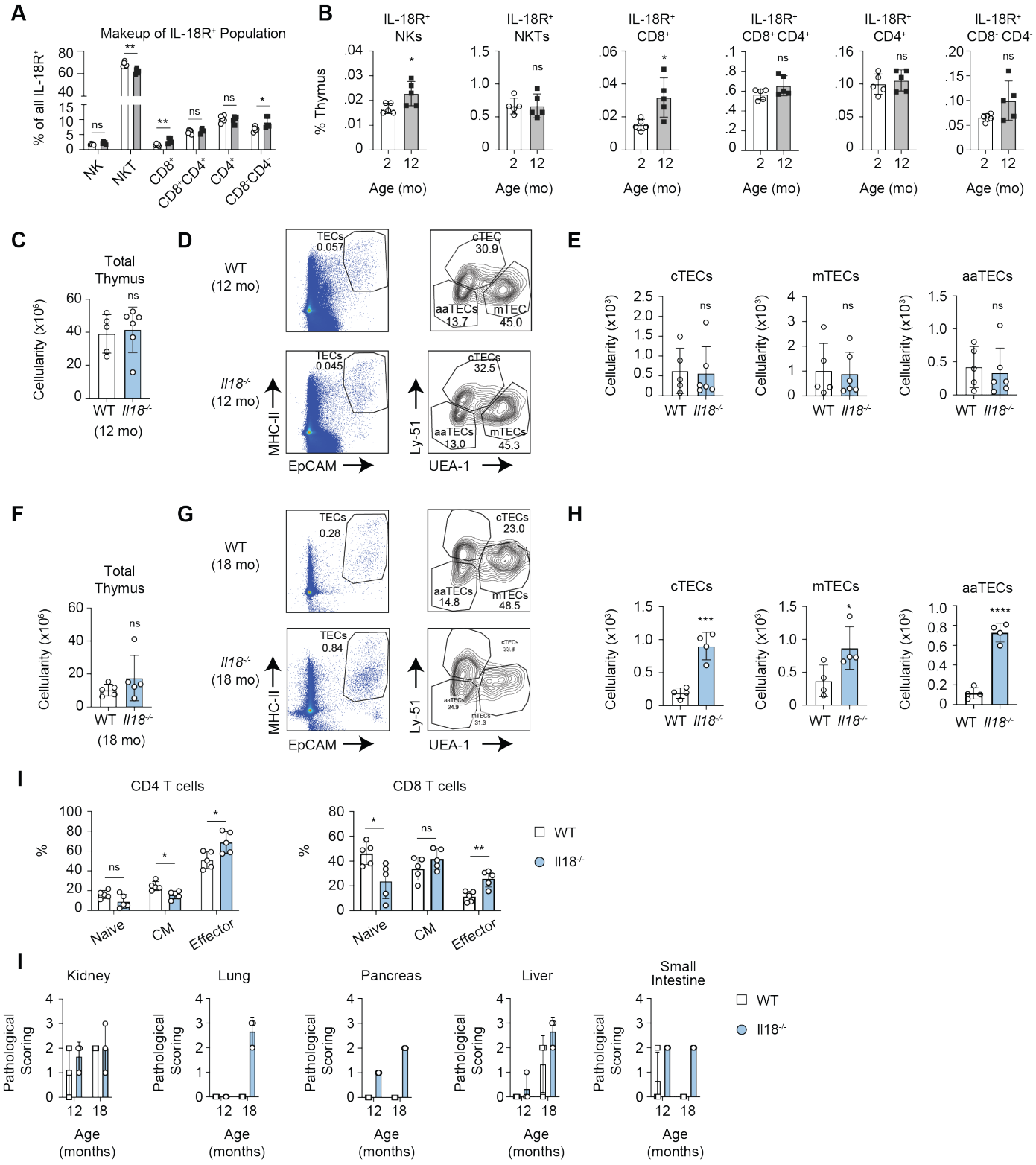
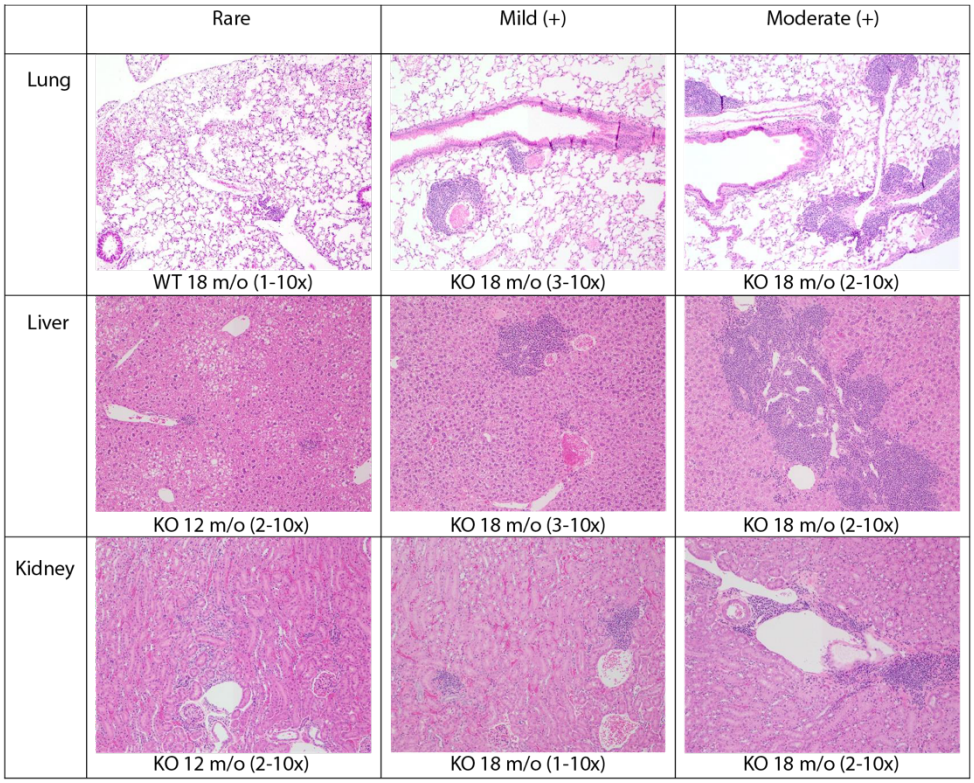


Figure 3.2: Abrogating IL-18 does not ameliorate age-related thymus involution

A, Cell identity [$CD4^-CD8^-CD49b^+TCR\beta^-$ NK cells, $CD4^-CD8^-CD49b^-TCR\beta^+$ NKT, $CD8^+CD4^-$, $CD4^+CD8^+$, $CD4^+CD8^-$ and $CD4^-CD8^-$ cells] and their frequencies within the $CD45^+$ IL-$18R^+$ population of thymuses from male 2 mo and 12 mo C57/BL6 WT mice were absent any additional treatments (n=5/group). **B,** Relative frequency of overall thymus cellularity of IL-$18R^+$ NK, NKT, $CD8^+CD4^-$, $CD4^+CD8^+$, $CD4^+CD8^-$ and CD4- CD8- cells of male 2 mo and 12 mo C57/BL6 WT thymuses (n=5/group). **C,** Thymus cellularity of 12 mo C57/BL6 WT or *Il18*$^{-/-}$ mice (n = 5-6/group). **D-E,** Cortical, Medullary, and Age-associated TEC populations **(D)** gating shown (all $CD45^-$ parent gate) and **(E)** quantified of thymuses of 12 mo C57/BL6 WT or *Il18*$^{-/-}$ (n = 5/group). **F,** Thymus cellularity of 18 mo C57/BL6 WT or *Il18*$^{-/-}$ mice (n = 5-6/group). **G-H,** Cortical, Medullary, and Age-associated TEC populations **(G)** gating shown (all $CD45^-$ parent gate) and **(H)** quantified of thymuses of 12 mo C57/BL6 WT or *Il18*$^{-/-}$ (n = 5/group). **I,** Frequencies of $CD62L^+$ $CD44^-$ naïve, $CD62L^+$ $CD44^+$ Central Memory and $CD62L^-$ $CD44^+$ Effector T cell populations within all $CD3^+$ $CD4^+$ T cells (Left) and $CD3^+$ $CD8^+$ T cells (Right) of 12-month-old C57/BL6 WT and *Il18*$^{-/-}$ spleens. Kidneys, lungs, pancreas, liver, and small intestine were harvested from 12- and 18-mo old male C57/BL6 WT or *Il18*$^{-/-}$ mice and preserved in formalin for subsequent H&E and pathological scoring of tissue inflammation (n=3/group). Graphs represent mean ± SEM. *p<0.05, **p<0.01, ***p<0.001.

FIGURE S3.1: 12mo and 18mo WT vs *Il18*$^{-/-}$ Pathology Report



Pathology Summary Report written by Dr. Raisa Glabman:

Overall, there were numerous tissue changes evidence predominantly by lymphoplasmacytic cell infiltrates in the KO groups. Additioanlly, there were age-related lesions that occurred in both KO and WT mouse groups, as noted. Lungs were clearly affected throughout the lung fields. Occasionally, the mesothelium was segmentally or focally lined by plump cells, however the significance of this is unclear. There was a benign lung tumor (papillary adenoma) present in a WT 18 mo mouse, considered to be incidental and frequently observed in mice. Pancreatic tissue in the WT group appeared normal. There were rare to mild perivascular lymphoplasmacytic infiltrates particuarly in the mesenteric fat of both KO age groups. In two 18 mo KO mice, there were also focal pancreatic tissue loss. Hepatic tissue appeared more affected in the 18 mo groups and both WT and KO liver tissue contained lymphoplasmacytic cell infiltrates although severity in the KO group was higher. The kidneys of aged KO was similarly more affected than WT. There was rare to mild inflammation in WT groups, presumed to be age-related, paticularly in the medulla. Within the intestines, no major lesions were observed, however there were regions of lamina propria which were segmentally expanded by lymphoplasmacytic and neutrophilic infiltrates in the KO groups. This trend supports autoimmune effects of IL-18 knockout in aged mice.

Table 3.1: 12 and 18-month-old tissue pathology				
Phenotype	Age	Tissue	Diagnosis	Inflammation Score
WT	12 mo (1)	Lung	WNL	0
WT	12 mo (2)	Lung	WNL	0
WT	12 mo (3)	Lung	WNL	0
Il18$^{-/-}$	12 mo (1)	Lung	WNL	0
Il18$^{-/-}$	12 mo (2)	Lung	Segmentally plump mesothelial cells Segmentally plump mesothelial cells WNL	0
Il18$^{-/-}$	12 mo (3)	Lung	Segmentally plump mesothelial cells Segmentally plump mesothelial cells WNL	0
WT	12 mo (1)	Pancreas	WNL	0
WT	12 mo (2)	Pancreas	WNL	0
WT	12 mo (3)	Pancreas	WNL	0
Il18$^{-/-}$	12 mo (1)	Pancreas	rare perivascular lymphoplasmacytic infiltrates	1
Il18$^{-/-}$	12 mo (2)	Pancreas	rare perivascular lymphoplasmacytic infiltrates	1
Il18$^{-/-}$	12 mo (3)	Pancreas	+ perivascular lymphoplasmacytic infiltrates	1
WT	12 mo (1)	Liver	WNL	0
WT	12 mo (2)	Liver	WNL	0
WT	12 mo (3)	Liver	WNL	0
Il18$^{-/-}$	12 mo (1)	Liver	WNL	0
Il18$^{-/-}$	12 mo (2)	Liver	rare perivascular lymphoplasmacytic infiltrates with single cell necrosis	1
Il18$^{-/-}$	12 mo (3)	Liver	WNL	0
WT	12 mo (1)	Kidney	WNL	0
WT	12 mo (2)	Kidney	rare interstitial lymphoplasmacytic infiltrates surrounding renal pelvis	1
WT	12 mo (3)	Kidney	+ medullary lymphoplasmacytic infiltrates; radiating inflammation with karyorrhectic debris	2
Il18$^{-/-}$	12 mo (1)	Kidney	+ interstitial lymphoplasmacytic infiltrates	2
Il18$^{-/-}$	12 mo (2)	Kidney	rare interstitial lymphoplasmacytic infiltrates	1
Il18$^{-/-}$	12 mo (3)	Kidney	+ perivascular lymphoplasmacytic infiltrates	2
WT	12 mo (1)	Intestine	+ lymphoplasmacytic infiltrates in LP, proliferative and dilated microvasculature	2
WT	12 mo (2)	Intestine	WNL	0
WT	12 mo (3)	Intestine	WNL	0

Il18$^{-/-}$	12 mo (1)	Intestine	+ lymphoplasmacytic infiltrates in LP	2
Il18$^{-/-}$	12 mo (2)	Intestine	+ lymphoplasmacytic infiltrates in LP	2
Il18$^{-/-}$	12 mo (3)	Intestine	+ lymphoplasmacytic infiltrates in LP	2
WT	18 mo (1)	Lung	WNL; focal papillary adenoma	0
WT	18 mo (2)	Lung	Foci of plump mesothelial cells	0
WT	18 mo (3)	Lung	WNL	0
Il18$^{-/-}$	18 mo (1)	Lung	++ perivascular and peribranchial infiltrates	3
Il18$^{-/-}$	18 mo (2)	Lung	++ perivascular and peribranchial infiltrates	3
Il18$^{-/-}$	18 mo (3)	Lung	+ perivascular and peribranchial infiltrates	2
WT	18 mo (1)	Pancreas	WNL	0
WT	18 mo (2)	Pancreas	WNL	0
WT	18 mo (3)	Pancreas	WNL	0
Il18$^{-/-}$	18 mo (1)	Pancreas	+ perivascular lymphoplasmacytic infiltrates	2
Il18$^{-/-}$	18 mo (2)	Pancreas	+ perivascular lymphoplasmacytic infiltrates	2
Il18$^{-/-}$	18 mo (3)	Pancreas	+ perivascular lymphoplasmacytic infiltrates WNL	2
WT	18 mo (1)	Liver	WNL	0
WT	18 mo (2)	Liver	+ perivascular lymphoplasmacytic to neutrophilic infiltrates with single cell necrosis	2
WT	18 mo (3)	Liver	+ perivascular lymphoplasmacytic to neutrophilic infiltrates with single cell necrosis	2
Il18$^{-/-}$	18 mo (1)	Liver	++ perivascular lymphoplasmacytic to neutrophilic infiltrates with vacuolation, lacy cytoplasm, regneration	3
Il18$^{-/-}$	18 mo (2)	Liver	++ perivascular lymphoplasmacytic infiltrates	3
Il18$^{-/-}$	18 mo (3)	Liver	+ perivascular lymphoplasmacytic infiltrates	2
WT	18 mo (1)	Kidney	+ interstitial lymphoplasmacytic infiltrates	2
WT	18 mo (2)	Kidney	+ interstitial lymphoplasmacytic infiltrates, multifocal tubular mineralization	2
WT	18 mo (3)	Kidney	+ interstitial lymphoplasmacytic infiltrates	1

Il18$^{-/-}$	18 mo (1)	Kidney	rare interstitial lymphoplasmacytic infiltrates	1
Il18$^{-/-}$	18 mo (2)	Kidney	++ interstitial lymphoplasmacytic infiltrates, loss of parenchyma associated with focal infarct	3
Il18$^{-/-}$	18 mo (3)	Kidney	+ interstitial lymphoplasmacytic infiltrates, rare glomerular edema	2
WT	18 mo (1)	Intestine	WNL	0
WT	18 mo (2)	Intestine	WNL	0
WT	18 mo (3)	Intestine	WNL	0
Il18$^{-/-}$	18 mo (1)	Intestine	+ lymphoplasmacytic, neutrophilic infiltrates in LP	2
Il18$^{-/-}$	18 mo (2)	Intestine	+ lymphoplasmacytic, neutrophilic infiltrates in LP	2
Il18$^{-/-}$	18 mo (3)	Intestine	+ lymphoplasmacytic, neutrophilic infiltrates in LP	2

Pathology amd scoring performed by Dr. Raisa Glabman

Key:

Inflammation was scored according to severity:

0: no inflammation

1: minimal inflammation

2: mild inflammation

3: moderate inflammation

4: marked inflammation

3.3c: Perforin abrogation ameliorates age-related thymus involution

Having established that increased IL-18 in aging does not regulate chronic involution, we explored whether other factors of cytotoxicity were involved in chronic damage. Though *Ifng* signaling did not appear to be altered in Rag2-GFP^{-} 18-month-old thymic populations **(Fig. 3.3A)**, there was increased *Pfr1* gene expression in thymic NK cells with age **(Fig. 3.3B)**. This was intriguing as TECs of 12-month-old mice downregulated MHC-I component H2Kb expression compared to control young mice **(Fig 3.3C)**. As our study of acute damage recovery implicated cytotoxic NK cell targeting of TECs with downregulated NKG2D inhibitory MHC-I expression, we hypothesized that a similar mechanism may be taking place in old age; in other words, we wondered whether NKG2D ligand expression changes on aging TECs may allow for cytotoxic NK cell targeting that contributed to age-related involution. However, in contrast to the scRNASeq data shown in 3.3B, the intensity of Perforin expression was decreased in thymic NKs of 12-month-old mice **(Fig 3.3D)**. Moreover, 12-month-old thymic NKs showed similar expression of activation marker NKG2D as control young counterparts **(Fig. 3.3D)**. Altogether, and surprisingly given the increased free IL-18 with age, it appears that thymic NK cells do not increase in their cytotoxicity with age.

Nevertheless, because we had implicated Perforin signaling in regulating thymus function following acute injury **(Fig. 2.6I)**, we also wanted to test its abrogation in the context of age-related involution. 12-month-old *Perforin*$^{-/-}$ mice showed significantly increased thymus cellularity compared to age matched WT controls allowing us to conclude that the Perforin mediated cytotoxicity does in fact contribute to chronic thymus involution **(Fig. 3.3E)**. Surprisingly, despite the increased cellularity of 12-month-old *Prf1*$^{-/-}$ thymuses, they did not have increases in any of the cTEC, mTEC and aaTEC populations **(Fig. 3.3F-G)**. However, it is possible that the increased thymus function is attributable rather to the ratio of TEC as12-month-old *Prf*$^{-/-}$ thymuses had an mTEC to cTEC ratio closer to that of a young mouse and decreased frequency of aaTECs compared to 12-month-old WT mice **(Fig. 3.3F)**. Nonetheless,

from these data we could conclude that Perforin signaling does indeed contribute to age-related thymus involution.

Next, we sought to determine what the actors may be in the Perforin-dependent mechanism. Characterization of the Perforin$^+$ population within the baseline thymus of 2-month-old versus 12-month-old WT thymuses revealed that while most cells were still IL-18R+ NK1.1+, there were declines in the representation of IL-18R$^+$ NK and NKT cells and increases in the CD8$^+$ and CD4$^-$ CD8$^-$ populations within the aged Perforin$^+$ cells **(Fig. 3.3H)**. Moreover, the overall contribution to total organ cellularity was unchanged in the context of Perforin$^+$ IL-18R$^+$ NK cells, consistent with an IL-18 and NK cell independent mechanism **(Fig 3.3H)**. There were significant increases however in the representation of IL-18R$^-$ NK1.1$^-$ populations **(Fig. 3.3I)**. Rises in the frequency of CD8$^+$ Perforin$^+$ T cells **(Fig. 3.3I, J)** and of CD4$^-$ CD8$^-$ NK1.1$^-$ TCRβ^+ Perforin$^+$ T cells **(Fig. 3.3I, K)** are of particular interest. A rise in age-associated CD8$^+$ T cells that have an exhausted-like phenotype and are characterized by increased *GzmK*, *Tox*, and *Eomes* and decreased *Tbx21* and *Tcf7* expression have been described as a hallmark of inflammaging[313]. For this reason, the Perforin$^+$ CD8$^+$ T cells that rise in frequency in the aged thymus are of particular interest in potentially mediating a Perforin-dependent role in age-related thymus involution. Note, that the exact T cell identity of the increased Perforin$^+$ CD4$^-$ CD8$^-$ NK1.1$^-$ TCRβ^+ population **(Fig. 3.3K)** is currently unknown and requires further study. Finally, consistent with increased thymic output, aged *Prf1*$^{-/-}$ splenic T cell populations have higher proportions of naïve cells **(Fig. 3.3L)**.

In summary, we rejected our hypothesis that IL-18 contributed to age-related thymus involution, but we did identify a role for Perforin signaling in contributing to chronic organ damage and identified candidate populations, particularly CD8$^+$ T cells, in being the actors of the Perforin mechanism.

FIGURE 3.3: Perforin abrogation ameliorates age-related thymus involution

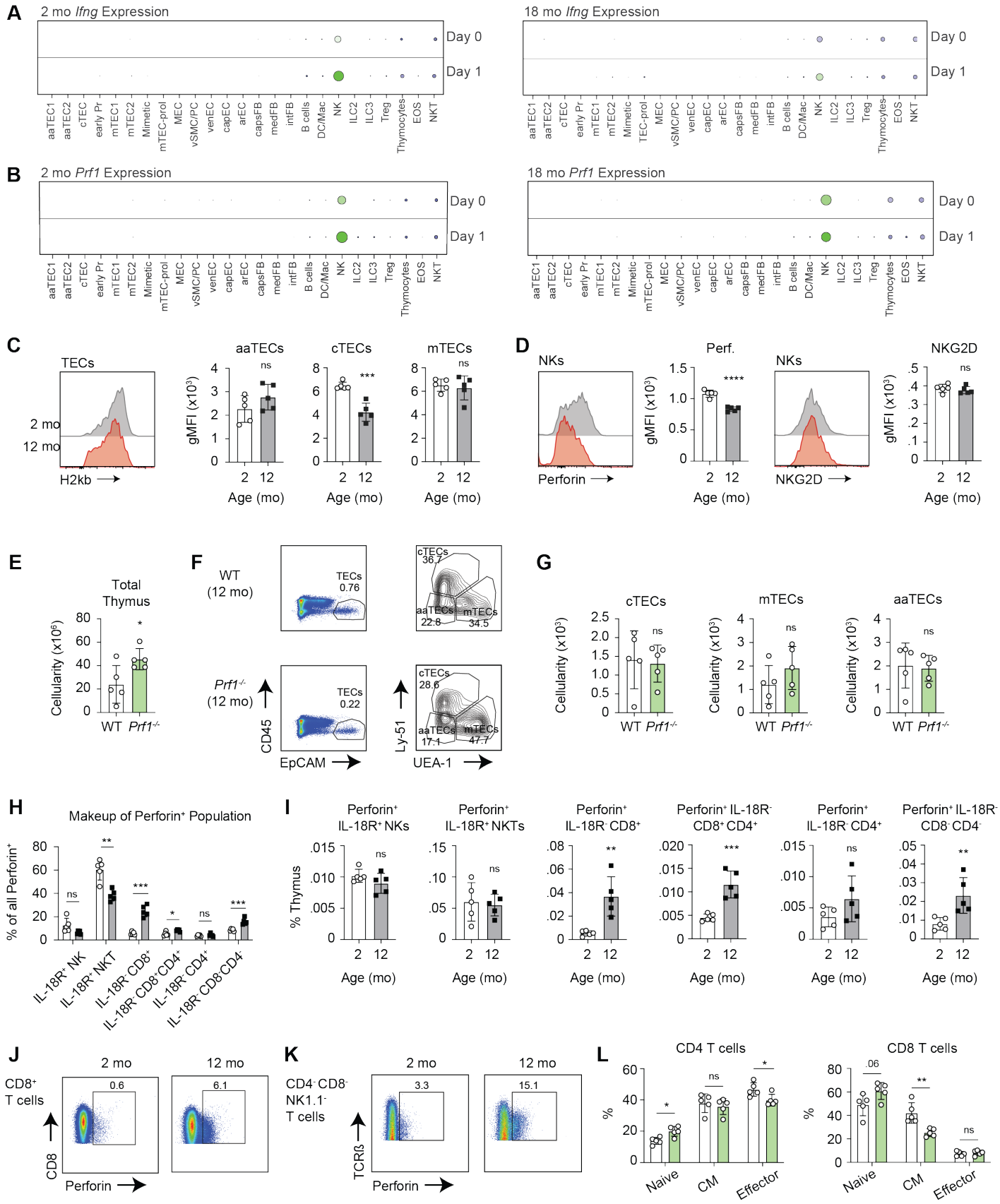


Figure 3.3: Perforin abrogation ameliorates age-related thymus involution

A-B, Single cell RNA sequencing was performed on (1) non-thymocyte CD45$^+$ stromal cells (CD45$^+$ *Rag2*-GFP$^-$ isolated from female 1-2 mo *Rag2-gfp* mice) and (2) CD45$^-$ stromal cells isolated from female 1-2 mo and 18 mo C57BL/6 mice at baseline, 1, 4 and 7 days following SL-TBI as described in 2.2. Data (from 2 mo mice) was previously integrated together and published in Lemarquis *et al.* (2024). **(A)** *Ifng* gene expression and **(B)** *Prf1* shown at days 0 and 1 post SL-TBI in 2mo (Left) and 18 mo thymuses (Right). **C,** H2kb expression on all CD45$^-$ EpCAM$^+$ MHC-II$^+$ TECs (Histogram) and H2kb gMFI on Ly51$^+$ cTECs, UEA-1$^+$ mTECs and Ly51$^-$ UEA-1$^-$ age-associated TECs of C57/BL6 male 2- and 12-month-old thymuses (n=5/group). **D,** Perforin (Left) and NKG2D (Right) expression of NK1.1$^+$ CD49b$^+$ NK cells of male 2- and 12-month-old thymuses (n=5/group). **E,** Thymus cellularity of 12 mo C57/BL6 WT or *Prf1*$^{-/-}$ mice (n = 5/group). **F-G,** Cortical, Medullary, and Age-associated TEC populations **(F)** gating shown (all CD45$^-$ parent gate) and **(G)** quantified of thymuses of 12 mo C57/BL6 WT or *Prf1*$^{-/-}$ (n = 5/group). **H,** Cell identity [IL-18R$^+$CD4$^-$CD8$^-$ CD49b$^+$ TCRβ^- NK cells, IL-18R$^+$CD4$^-$CD8$^-$ CD49b$^-$ TCRβ^+ NKT, IL-18R$^-$CD8$^+$CD4$^-$, IL-18R$^-$ CD4$^+$CD8$^+$, IL-18R$^-$ CD4$^+$CD8$^-$ and IL-18R$^-$ CD4$^-$ CD8$^-$ cells] and their frequencies within the CD45$^+$ Perforin$^+$ population of thymuses from male 2 mo and 12 mo C57/BL6 WT (n=5/group). **I,** Relative frequency of overall thymus cellularity of Perforin$^+$ IL-18R$^+$ NK, IL-18R$^+$ NKT, IL-18R$^-$ CD8$^+$CD4$^-$, IL-18R$^-$ CD4$^+$CD8$^+$, IL-18R$^-$ CD4$^+$CD8$^-$ and IL-18R$^-$ CD4- CD8- cells of male 2 mo and 12 mo C57/BL6 WT thymuses (n=5/group). **C, F,** Thymus cellularity of 18 mo C57/BL6 WT or *Il18*$^{-/-}$ mice (n = 5/group). **J-K,** Perforin$^+$ frequency of **(J)** TCRβ^+ CD8$^+$ T cells and **(K)** NK1.1- CD4- CD8- TCRβ^+ T cells within thymuses of 2- and 12-month-old male C57/BL6 mice (n=5/group). **L,** Frequencies of CD62L$^+$ CD44$^-$ naïve, CD62L$^+$ CD44$^+$ Central Memory and CD62L$^-$ CD44$^+$ Effector T cell populations within all CD3$^+$ CD4$^+$ T cells (Left) and CD3$^+$ CD8$^+$ T cells (Right) of 12-month-old C57/BL6 WT and *Prf1*$^{-/-}$ spleens. Graphs represent mean ± SEM. *p<0.05, **p<0.01, ***p<0.001.

3.4 CONCLUSION

In this follow up chapter, we begin to investigate the role of inflammatory mediators in regulating age-related thymus involution. Given previous reports of the NLRP3 inflammasome upstream of Capsae-1 cleavage involvement in contributing to declines in thymus cellularity with age, and our reports of active IL-18 suppressing thymus regeneration, we expected that that IL-18 may be similarly involved chronic organ demise. We detected increased free IL-18 within involuted thymus as well as higher frequencies of IL-18R$^+$ NK cells including IL-18R$^+$ NK cells and CD8$^+$ T cells. However, upon aging of *Il18*$^{-/-}$ mice there were no alterations in thymus cellularity of 12- and 18-month-old mice suggesting no involvement of IL-18 in mediating age-related organ involution. Worth mentioning, however, is that we did not perform any experiments addressing IL-18 involvement in thymus repair in the aged thymus. To do so, we would have given 12- and 18-month-old WT and Il18-/- mice our modalities of acute thymus damage (i.e. SL-TBI or Dexamethasone) and subsequently assessed thymus cellularity at least one week later.

Interestingly though, we did observe significant increases in TEC cellularity within 18-month-old but not 12-month-old *Il18*$^{-/-}$ mice. This requires further explanation as it is possible that increased IL-18 involved inflammation within the involuting organ can contribute to aberrant TEC destruction between 12- and 18-months of age. Initially, we focused on the roles of NK cells in chronic thymus involution given our close study of them in regulating acute thymic damage regeneration. Although it appears that particularly cTECs may become more vulnerable to cytotoxic NK cell targeting during aging as evidence by decreased NKG2D inhibitory MHC-I expression, NK cells of 12-month-old thymuses did not appear to be any more cytotoxic/activated than 2-month-old NK cells. Overall, the current evidence provided in this chapter suggests that NK cells do not contribute to age-related organ involution though further study is required to fully characterize the role of NK cells within the aged thymus.

While abrogating IL-18 itself was not capable of rejuvenating the thymus, we still sought to identify the broader role of cytotoxicity in regulating thymic function during aging. We observed an increased representation of Perforin expressing T cells within the 12-month-old thymus, and of particularly high interest, Perforin expressing $CD8^+$ T cells which may represent age-associated $CD8^+$ T cells implicated in inflammaging outside of the thymus. *Prf1*$^{-/-}$ 12-month-old thymuses were significantly larger than their age-matched WT controls, from which we concluded that Perforin$^+$ cell cytotoxicity are involved in suppressing thymic function during normal aging. Despite the increased overall thymic cellularity of these Perforin deficient elder mice, there was no significant difference in overall TEC cellularity. This does not necessarily reflect a TEC-independent mechanism behind the increased thymic function of the aged *Pfr1*$^{-/-}$ model and further study of TEC derived thymopoietic factors is required. One potential mechanism may be evidenced by 12-month-old *Pfr1*$^{-/-}$ mTEC to cTEC ratio (~5:3) being closer to the expected ratio of the young, healthy thymus than aged WT thymic cTEC to mTEC ratio (~1:1) and aaTECs made up less of the TEC compartment in aged *Prf1*$^{-/-}$ organs. Overall, we have only begun to uncover a potential mechanism for cytotoxicity regulation of thymus function in aging. Unfortunately, there is no readily available Prf1fl/fl mouse line that may help to address the underlying mechanism of Perforin involvement in involution. Moreover, as perforin binds directly to target cell membrane inducing pore formation and cell destruction, it is not as though we can identify specific receptor bearing populations onto which perforin signals. More likely, as in the case of IL-18 during thymus recovery from acute damage, is that perforin is the secondary mediator of other inflammatory mediators involved in regulating chronic involution. Careful examination of inflammatory molecules and receptor bearing immune cells in the aging thymus is necessary to better elucidate the upstream factors involved in the observed phenotype of 12-month-old Perforin deficient thymuses.

One of the consequences of reduced T cell output over time is decreased naïve T cells, expansion of memory populations and an overall decrease in T cell Receptor diversity. This

contributes to reduced pathogen immunity and vaccine responsiveness in the elderly. Highlighting the potential translational benefit to improving thymus function in age, 12-month-old *Prf1*$^{-/-}$ T cells were more enriched for naïve T cells and had less central memory T cells than age-matched WT controls. The recent advances in Perforin inhibitors may offer an opportunity for therapeutic intervention for thymic rejuvenation in old age. However, unlike during the initial weeks following acute thymic damage such as the period post-transplantation, there is not an identifiable temporal window for intervention in inhibiting Perforin signaling and long-term inhibition would come at the expense of reducing the cytotoxicity of peripheral immune cells required for pathogen clearance. This further highlights the need to further clarify the mechanisms by which cytotoxicity contributes to age-related involution so that other more thymus-specific therapeutic targets may be identified.

Materials & Methods

Key Resources

Antibodies				
CD45	BUV395	BD Bioscences	565967	AB_2739420
CD31	APC Fire-750	Biolegend	102434	AB_2629683
CD140a	APC	Biolegend	135907	AB_2043969
MHC-II	Pacific Blue	Biolegend	107620	AB_493527
EpCAM	PerCP-eFluor710	BD Biosceinces	46-5791-82	AB_10598205
Ly-51	BV786	BD Biosciences	740882	AB_2740531
UEA-1	FITC	Vector Labs	ZC0426	
CD8a	APC/Cy7	Biolegend	100714	AB_312753
CD4	BUV563	BD Biosciences	565709	AB_2739335
TCR-β	PE-Dazzle/594	Biolegend	109239	AB_2565654
CD3e	BV785	Biolegend	100232	AB_2562554
CD25	PerCP/Cy5.5	Biolegend	102030	AB_893288
CD44	BUV737	BD Biosciences	612799	AB_2870126
NK1.1	BV605	Biolegend	108753	AB_2686977
CD49b	FITC	BD Biosciences	561067	AB_2034010
c-Kit	APC	Biolegend	105811	AB_313220
TCR-γδ	PE	Biolegend	118107	AB_313831
CD1d PBS-57 Tetramer	BV421	NIH Tetramer Core		
CD11c	FITC	Tonbo	35-0114	AB_2621677
CD11c	BUV737	BD Biosciences	612796	AB_2870123
CD11b	BUV737	BD Biosciences	741722	AB_2871093
XCR1	BV785	Biolegend	148225	AB_2783119
B220	Alexa Fluor 700	Biolegend	103232	AB_493717
Thy-1 (CD90.2)	BV785	Biolegend	105331	AB_2562900
CD127	PE	Tonbo Biosciences	50-1271	AB_2621780
Sca-1	PE-Dazzle/594	Biolegend	122527	AB_2687354
CD135	PE	Biolegend	135305	AB_1877218
CD150	PerCP-efluor710	eBioscience	46-1502-82	AB_2016699
CD48	BV421	Biolegend	103427	AB_10895922
NKG2D	BV421	BD Biosceinces	562800	AB_2737805
H2kb	BV421	Biolegend	116525	AB_2876430
Pan-Rae1	PE	R&D	FAB17582P	AB_357086
Perforin	PE-Dazzle/594	Biolegend	154315	AB_2922482

Granzyme B	PE	Thermo Fisher	MHGB04	AB_10372671
IL-18R	PE/Cy7	Thermo Fisher	25-5183-82	AB_2762705
IL-18R	PE	Thermo Fisher	12-5183-80	AB_2572616
Annexin V	APC	Biolegend	640920	
NK1.1	Unconjugated	BioXCell	BE0036	AB_2562139
IL-18	Unconjugated	BioXCell	BE0237	
CD49b	Unconjugated	Biolegend	103501	AB_313024

Peptides & Recombinant Proteins		
Cyclophophamide	UW Medicine Pharmacy	NDC 16714-859-01
LPS	Invivogen	tlrl-eblps
Dexamethasone	Sigma-Aldrich	D2915-100MG
Recombinant murine IL-18	Biolegend	767008
Recombinant murine IL-7	Peprotech	217-17
Recombinant murine Flt3L	Peptrotech	250-31L
Recombinant murine IL-15	Biolegend	566302
CellTrace Yellow Cell Proliferation Kit	Thermofisher	C34573

Critical Commercial Assays		
Caspase-1 (active) Staining Kit - Green Fluorescence	Abcam	ab219935
IL-1β Mouse ELISA Kit	Invitrogen	88-7013-22
IL-18 Mouse ELISA Kit	Thermo Fisher	BMS618-3
Mouse Granzyme B ELISA	R&D	DY1865
Mouse Perforin ELISA Kit	Novus Biologicals	NBP3-00452
Mouse IFNγ ELISA Kit	ThermoFisher	KMC4021
Mouse Caspase-1 ELISA Kit	Adipogen	AG-45B-0002-KI01
Mouse IL-18 Binding Protein	Abcam	ab254509

Experimental Models: Cell Lines		
RMA-S	Courtesy of Dr. Martin Prlic FHCC	N/A
OP9 DLL1	Courtesy of Dr. Juan Carlos Zuniga-Pflucker, University of Toronto	N/A

Experimental Models: Organisms/Strains		
Il1r1-/-	Jackson Laboratories	3245
Il18-/-	Jackson Laboratories	4130
Il18r1-/- (aka IL-1Rrp KO)	Jackson Laboratories	4131
Casp1Δ10	Jackson Laboratories	32662

Il18fl/fl * Zbtb46 Cre (aka zDC Cre)		Courtesy of Dr. Roni Nowarski, HMS and Dr. Richard Flavell, Yale
	Jackson Laboratories	32662
Il18r1fl/fl * Foxn1 Cre		Courtesy of Dr. Giorgio Trinchieri, NCI
	Jackson Laboratories	18448
CD1d-/-	Jackson Laboratories	8881
Ifnγr-/-	Jackson Laboratories	3288
Ifnγrfl/fl * Foxn1 Cre	Jackson Laboratories	25394
	Jackson Laboratories	18448
Prf1-/-	Jackson Laboratories	2407
Il18r1fl/fl * Ncr1 Cre		Courtesy of Dr. Kevin Barry, FHCC
Rag2-eGFP	Jackson Laboratories	5688
B6 CD45.1 (aka Pep Boy)	Jackson Laboratories	2014
Great (Ifng reporter)	Jackson Laboratories	17580

Software Algorithms		
Prism v10.0	GraphPad Software	https://www.gsea-msigdb.org/gsea
FlowJo v10.9.0	BD	https://www.flowjo.com/
Biorender	Biorender	https://www.app.biorender.com/

Other		
TECAN Spark 10M	Tecan, Switzerland	Spark 10M
Z2 Coulter Particle and Size Analyzer	Beckman Coulter, USA	N/A
Bio-Rad CFX96 Real Time System	Bio-Rad	N/A
Luminometer	Turner Biosystems, USA	Veritas microplate Luminometer
Irradiator	Fred Hutchinson Cancer Research Center	Mark I series 30JL Shepherd irradiator

Deposited Data		
Bulk Microarray raw data	Ki, et al.[218]	GEO: GSE56928
Single cell RNA Sequencing raw data	Kousa, et al.[226]	GEO: GSE240016
Single cell RNA Sequencing raw data	Lemarquis, et al.	GEO: GSE244673

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mice

Inbred male and female C57Bl/6J (000664) and B6 CD45.1 (002014) mice were obtained from Jackson Laboratories (Bar Harbor, USA). *Il1r1*$^{-/-}$ (003245), *Il18*$^{-/-}$ (004130), *Il18r1*$^{-/-}$ (004131), *Casp1Δ10* (032662), *CD1d*$^{-/-}$ (008881), *Ifngr*$^{-/-}$ (003288), *Prf1*$^{-/-}$ (002407), *Rag2-eGFP* (005688) and *GREAT* ("interferon-gamma reporter with endogenous polyA transcript") mice were obtained from Jackson Laboratories and bred in house. Il18 flox mice (*Il18*$^{fl/fl}$) were obtained from R. Nowarski (Harvard Medical School) and R. Flavell (Yale School of Medicine) and crossed in house to *Zbtb46 Cre*$^{+}$ obtained from Jackson Laboratories (032662) to generate *Il18*$^{fl/fl}$ *Zbtb Cre*$^{+}$ (*Il18*$^{\Delta DC}$) mice. *Il18r1*$^{fl/fl}$ mice were obtained from G. Trinchieri (NCI) and crossed to *Foxn1 Cre*$^{+}$ mice obtained from Jackson Laboratories (018448) and *Ncr1 Cre*$^{+}$ obtained from K. Barry (Fred Hutch Cancer Center) to generate *Il18r1*$^{fl/fl}$ *Foxn1 Cre*$^{+}$ (*Il18r1*$^{\Delta TEC}$) and *Il18r1*$^{fl/fl}$ *Ncr1 Cre*$^{+}$ (*Il18r1*$^{\Delta NK}$), respectively. *Ifngr*$^{fl/fl}$ (025394) and *Foxn1 Cre*$^{+}$ (018448) mice were obtained from Jackson Laboratories and crossed to generate *Ifngr*$^{fl/fl}$ *Foxn1 Cre*$^{+}$ (*Ifngr*$^{\Delta TEC}$) mice. All experimental mice were between 6-10 weeks old. Mice were maintained at the Fred Hutchinson Cancer Research Center (Seattle, WA), and acclimatized for at least 2 days before experimentation, which was performed per Institutional Animal Care and Use Committee guidelines.

METHOD DETAILS:

Cell Isolation

Single cell suspensions of freshly dissected thymuses were obtained and enzymatically digested using 0.15% Collagenase D (Sigma 11088882001) and 0.1% DNase 1 (Sigma 10104159001) in DMEM, as previously described.[123] Cellularity was calculated using the Z2

Coulter Particle and Size Analyzer (Beckman Coulter, USA). For studies sorting rare populations of cells in the thymus, multiple identically-treated thymuses were pooled so that sufficient numbers of cells could be isolated; however, in these instances, separate pools of cells were established to maintain individual samples as biological replicates. BM was flushed from femors and tibias and then filtered through a 70 μm filter. Peripheral blood was collected into EDTA capillary pipettes (Fisher Scientific). Red Blood Cell Lysis was performed with ACK Lysis Buffer (A1049201, Fisher Scientific).

Flow Cytometry

Cells were stained with the following antibodies for analysis: CD45 (565967, BD Biosciences), CD31 (102434, Biolegend), CD140a (135907, Biolegend), MHC-II (107620, Biolegend), EpCAM (46-5791-82, BD Biosciences), Ly-51 (740882, BD Biosciences), UEA-1 (ZC0426, Vector Labs), CD8a (100714, Biolegend), CD4 (565709, BD Biosciences), TCR-β (109239), CD3e (100232, Biolegend), CD25 (102030, Biolegend), CD44 (612799, BD Biosciences), NK1.1 (108753, Biolegend), CD49b (561067, BD Biosciences), c-Kit (105811, Biolegend), TCR-γδ (118107, Biolegend), CD1d PBS-57 Tetramer (NIH tetramer Core), CD11c (35-0114, Tonbo), CD11c (612796, BD Biosciences), CD11b (741722, BD Biosciences), XCR1 (148225, Biolegend), B220 (103232, Biolegend), CD90.2 (105331, Biolegend), CD127 (50-1271, Tonbo), Sca-1 (122527, Biolegend), CD135 (135305, Biolegend), CD150 (46-1502-82, eBioscience), CD48 (103427, Biolegend), NKG2D (562800, BD Biosciences), H2-kb (116525, Biolegend), Pan Rae-1 (FAB17582P, R&D), IL-18R (25-5183-82, Thermo Fisher), IL-18R (25-5183-82, Thermofisher). Following fixation and permeabilization (554714, BD Biosciences), cells were stained with the following antibodies: Perforin (154315, Biolegend), Granzyme B (MHGB04, Thermofisher). Annexin V staining (640920, BioLegend) was performed in Annexin V binding buffer (422201, BioLegend). Flow cytometric analysis was performed on a Symphony S6 (BD Biosciences) and

cells were sorted on an Aria II (BD Biosciences) using FACSDiva (BD Biosciences) or FlowJo (Treestar Software).

In vivo Acute Damage Models

To induce thymus damage, mice were given sublethal total body irradiation at a dose of 550 cGy from a cesium source mouse irradiator (model here) with no hematopoietic rescue. Other models of thymus damage included i.p. injection of 20mg/kg Dexamethasone (Sigma-Aldrich D2915), 200mg/kg Cyclophosphamide (University of Washington Medical Pharmacy) or 1.5mg/kg LPS (Invivogen tlrl-eblps). For *in vivo* studies of rIL-18 administration, C57Bl/6J or GREAT mice were either given (s.c.) 2.5 mg/kg rIL-18 in the absence of other thymus damaging treatment (Day 0) or 3 days post SL-TBI.

In vivo Depletion and Transplantation Studies

To perform NK1.1$^+$ cell depletion studies, mice were injected (i.p.) with 200ug (10mg/kg) αNK1.1 mAb (BioXCell BE0036) at days -1, 1 and 3 post-SL-TBI. B6 HCT recipients received 1100 cGy TBI (2 × 550 cGy) prior to transplantation and within 24hrs received i.v. injection of 5 to 10 × 10^6 bone marrow (BM) cells. To perform IL-18 abrogation experiments, mice were dosed (i.p.) with 200ug (10mg/kg) αIL-18 mAb (BioXCell BE0237) at days -1, 1, 3, 6, 9, 12, 15 and 18 post-transplantation.

Protein quantification

For detection of supernatant active IL-1β, active IL-18, Granzyme B, Perforin, IFNγ, and mature Cas-1 (Figs 1B-C, 5C, and S1) were obtained by mechanically digesting thymic in defined volumes of buffer. The resulting supernatant was quantified using cytokine specific ELISA kits (IL-1β Invitrogen #88-7013-22; IL-18 Thermo Fisher #BMS618-3; GzmB R&D #DY1865;

Perforin Novus Biologicals # NBP3-00452; IFNγ Thermo Fisher #KMC4021; mature Cas-1 Adipogen #AG-45B-0002-KI01) and absorbance was measured on Tecan Spark 10M (Tecan, Switzerland).

For detection of whole organ active IL-18 and IL-18 Binding Protein (1B only post-cyclophosphamide IL-18, 1D, 2D-E) thymuses were homogenized in RIPA buffer (25mM Tris pH 7.6, 150nM NaCl, 1% NaCl, 1% NP-40, 0.1% SDS, 0.05% sodium deoxycholate, 0.5mM EDTA) with protease inhibitors (Thermo Fisher A32955), using a homogenizer 150 (Fisher Scientific) and normalizing by mass at a concentration of 20 mg thymus tissue/mL RIPA buffer. The resulting lysates was quantified using cytokine specific ELISA kits (IL-18 Thermo Fisher #BMS618-3 and IL-18 BP Abcam # ab254509), and absorbance was measured on the Tecan Spark 10M (Tecan, Switzerland).

In vitro cell culture

Co-culture experiments were performed by plating 50,000 *ex vivo* FACS purified bone marrow Lineage$^-$ selected or Lineage$^-$ Sca-1$^+$ c-Kit$^+$ FACS purified cells onto 6 well-plates confluent with OP9-DLL1^{GFP} cells in OP9 media previously described.[180,314] Co-cultures were performed in the presence of 5 ng/mL Flt-3L (Peprotech, 250-31L) and 1 ng/mL IL-7 (Peprotech 217-17) and either 0 ng/mL, 1ng/mL or 10 ng/mL rIL-18 (Biolegend 767008). Equal volumes of non-adherent cells were assessed by flow cytometry for differentiation at 10, 14 or 21 days post co-culture.

Cytotoxicity Assays

Cytotoxicity assays were performed by co-culturing NK1.1$^+$ IL-18R$^+$ CD49b$^+$ TCRβ$^-$ NK cells FACS isolated from either undamaged (Day 0) or 48 hours post SL-TBI with Cell-trace Yellow (Thermofisher C34573) labeled RMA-S cells at a 2:1 Effector to Target ratio in RPMI supplemented with 10ng/mL rIL-15 (Biolegend 566302). Co-cultures were incubated at 37°

degrees for 5 hours, after which cell death of Cell Trace Yellow (CTY) labeled RMA-S cells was assessed by flow cytometry according to Annexin V (Biolegend 640920) expression.

Imaging

Thymuses were mounted in optimal cutting temperature coumpound (OCT, TissueTek) and snap-frozen, and 10um sections were fixed in a 1:1 methanol/acetone solution. Sections were stained with anti-CD49b (Biolegend 108901), UEA-1 (Vector Laboratories), and detected with Alexa 568 (Thermo Fisher Scientific).

4′,6-diamidino-2-phenylindole (DAPI) was stained at a concentration of 300 nM, and sections were mounted with Vectashield Plus (Vecto Laboratories, Burlingame, CA) and imaged using a TissueFAX Plus (Tissuegnostic, Tarzana, CA). Fluorescent images of whole thymic tissue were analyzed using ImageJ 1.5i.

Single Cell RNA Sequencing

Previously generated and published single cell RNA-seq datasets of thymic CD45- non-hematopoietic cells (GSE240016; 50,890 cells) and RAG2-CD45+ hematopoietic cells (GSE244673; 37,879 cells) from 2-month-old mice at steady state and days 1, 4 and 7 after SL-TBI were used for this study[137,226]. The CD45- dataset can be viewed at https://thymosight.org/ along with all previously published thymus single cell sequencing datasets.

Statistics

Statistical analysis between two groups was performed with unpaired two-tailed t test. Statistical comparison between 3 or more groups in Figures was performed using a one-way ANOVA with Dunnett's multiple comparison test (2D-E, 4C-D). All statistics were calculated using Graphpad Prism and display graphs were generated in Graphpad Prism or R. Information on replicates, error bars and statistical significance can be found in the figures and their corresponding legends.

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VITA

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