MONASH University

Thymic recovery following chemotherapy damage – the impact of sex hormones and ageing

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Abstract

The thymus is responsible for development of self-tolerant T lymphocytes (thymopoiesis). Following establishment of the peripheral T cell pool, the thymus undergoes functional contraction, with a major reduction in thymopoiesis most evident from the onset of puberty, coincident with the increase in sex hormone production, and then continuing at a slower rate during ageing. Immune ageing is associated with an increased incidence of cancer, opportunistic infection and poor response to vaccination. Cytoablative treatments such as chemotherapy and irradiation to remove proliferating cancerous cells and as pre-conditioning prior to bone marrow transplantation, have severe damaging side effects to the immune system. Recovering immune competence therefore poses a problem for the aged and elderly due to the natural age-related involution of the thymus. There is a clinical need to develop strategies to enhance immune recovery following damage.

Previous work has demonstrated that blocking sex steroid production in middle-aged males through surgical castration or luteinising hormone releasing hormone (LHRH) receptor agonist treatment resulted in regeneration of thymopoiesis. In this study, we investigated a more clinically relevant LHRH analogue, the LHRH receptor antagonist Degarelix, that binds and blocks the LHRH receptor with fewer detrimental side effects as seen with LHRH receptor agonists. Preliminary investigations in healthy middle-aged male mice demonstrated a step-wise regeneration of thymocyte cellularity, demonstrating suitability as an alternative LHRH analogue.

While the precise mechanisms have not been fully resolved, age-related reduction in thymopoiesis occurs concomitant with loss of thymic epithelial cells (TECs) and we propose herein, may involve the changing capacity for postnatal bipotent TEC progenitors (TEPC) and lineage specific precursors to maintain mature TEC subsets required to support T lymphocyte production. Given the critical importance of TECs for thymopoiesis, and with the

majority of research into thymus ageing and impact of sex steroid blockade restricted to male mice, we performed an extensive comparative analysis of TEC subsets in females and males during ageing. We found better maintenance of mature TECs and thymopoiesis in females by middle age, suggesting a greater impact of male sex hormones on TEPC function.

We next investigated gender disparity in endogenous recovery from chemotherapy treatment and following Degarelix administration in middle-aged female and male mice. Cyclophosphamide treatment had a profound impact on thymocytes and TECs, with double positive thymocytes and mTEC^hi cellularity being the most affected. Endogenous thymic recovery in females involved immediate mobilisation of TEPC to replenish single lineage precursors to support recovery of mature cTEC^hi and mTEC^hi. Degarelix treatment enhanced the homeostatic differentiation process already evident in endogenous TEC recovery. However, in males, endogenous recovery was limited to immediate proliferation of residual mTEC^lo and mTEC^hi for their replenishment. Degarelix treatment had a greater impact in males, with sex steroid inhibition releasing the post-pubertal TEPC blockade, leading to replenishment of cTEC^hi and mTEC^lo through reactivation of TEPC differentiation. Enhanced thymopoiesis reached levels equivalent to that seen in females.

These findings stress the relevance of sexual dimorphism in adaptive immunity and highlight potential benefits of LHRH receptor antagonist treatment to enhance TEC recovery and T cell replenishment following cytoablative treatments in females and males.

Declaration

This thesis is an original work of my research and contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

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Publications during enrolment

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Conference Abstracts

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Alsharif A, Wong K, Hammett M, Boyd R, Chidgey A (2015) Damage and regeneration of thymic epithelial cells following chemotherapy treatment in an aged mouse model. Australasian Society of Immunology (ASI), Canberra, Australia (poster presentation).

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Dedication

To my mother Rahma, who always wanted the best for me:

You were an angel in the shape of my mum.... I miss seeing you and I know that you would be the happiest person in the world if you saw me finishing this degree.

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Finally, I deeply thank my father, Abdulmohsin and my brothers and sisters for their support.

Abbreviations

α6 ITG (ITGA6)	Alpha-6 integrin
β5t (ITGB5)	Beta-5 integrin
2D	Two dimensional
3D	Three dimensional
3PP	Third pharyngeal pouch
ACTH	Adrenocorticotropic hormone
ActR	Activin receptor
AIRE (Aire)	Autoimmune regulatory
ALK	Activin-like ligand
Ang-1	Angiotensin-1
APC	Antigen presenting cell
APS-1	Autoimmune polyendocrine syndrome type-1
AR	Androgen receptor
B220	Pan B-cell marker
BM	Bone marrow
BMP	Bone morphogenetic protein
BrdU	Bromodeoxyuridine / 5-bromo-2'-deoxyuridine
CAR	Chimeric antigen receptor
c-KIT	Proto-oncogene encoding the receptor tyrosine kinase
c-Myc	Transcriptional factor involved in cell-cycle transition events and proliferation
C57BL/6J	Black 6 wild type mice
CCL	Chemokine (C-C motif) ligand
CCR	Chemokine receptor
CCRL	Chemokine receptor ligand
CD	Cluster of differentiation
cDNA	Complementary DNA
CFE	Colony forming efficiency
CLP	Common lymphoid progenitor
CMJ	Cortico-medullary junction
CPA	Cyproterone acetate
cTEC	Cortical Thymic epithelial cell
CXCL	Chemokine (C-X-C motif) ligand
Cy	Cyclophosphamide
DAPI	4',6-diamidino-2-phenylindole
DC	Dendritic cell
DEC205	CD205
DES	Diethylstilbesterol
DLL	Delta-like ligand
DMSO	Dimethyl sulfoxide
DN	Double negative
DP	Double positive
E2A	Transcription factor 3, known as TCF3
E2F3	Family of transcription factors that regulate both cellular proliferation and differentiation
eGFP	*enhanced Green fluorescent protein*
EGFP	Epithelial green fluorescent protein
EpCAM	Epithelial cell adhesion molecule
ETP	Early thymic progenitor
FACS	Fluorescence-activated cell sorting
Fezf2	FEZ Family Zinc Finger 2
FGF	Fibroblast growth factor
FLT3	Cytokine receptor involved in normal hematopoiesis
FoxA2	Forkhead box protein A2
Foxn1	Forkhead Box N1
Foxp3	Forkhead box P3
FSH	Follicle Stimulating hormone
Fst	Follistatin
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GATA3	GATA (Erythroid transcription factor) Binding Protein 3
G-CSF	Granulocyte colony stimulating factor
GFP	*Green fluorescent protein*

GH	Growth hormone
GVHD	Graft versus host disease
H&E	Haemotoxylin and Eosin
hFT	Human fetal thymus
HLA	Human leukocyte antigen
HSC	Hematopoietic stem cell
HSCT	Hematopoietic stem cell transplantation
IFN-γ	Interferon Gamma
IGF-1	Insulin-like growth factor-1
IgG	Immunoglobulin G
IL	Interleukin
iPS	Induced pluripotent stem cell
JAG	Jagged ligand
KGF	Keratinocyte growth factor
Ki67	Marker Of Proliferation *Ki-67*
KLF	Krüppel-like factor
LGR5	Leucine-rich repeat-containing G protein coupled receptor 5
LH	Luteinizing hormone
LHRH-Ag	Luteinizing hormone releasing hormone agonist
LHRH-Ant	Luteinizing hormone releasing hormone antagonist
LiCl	Lithium Chloride
Lin28	Marker of undifferentiated human embryonic stem cells
LSK	$Lin^-Sca1^+c\text{-}Kit^+$
Ly364947	TGF-β inhibitor
Ly51	Mouse Anti-BP-1, Cortical thymic epithelial cell marker
MEFs	Mouse embryonic fibroblasts
mESC	Mouse embryonic stem cell
MFI	Mean fluorescent intensity
MHC	Major histocompatibility complex
MPP	Multi-Potent Progenitor
mRNA	Messenger RNA
MSC	Mesenchymal stem cell
mTEC	Medullary thymic epithelial cell
MTS	Mouse thymic stromal
NF-kB	*Nuclear factor kappa B*
NK	Natural killer
P53	Tumor protein 53, transformation-related protein/gene
P63	Tumor protein/gene 63, a member of P53 family of transcription factor
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PFA	Paraformaldehyde
PI	Propidium iodide
Plet1	Placenta Expressed Transcript 1
qRT-PCR	Real-Time Quantitative Reverse Transcription PCR
RANK	Receptor activator of nuclear factor kappa-B
RANKL	Receptor activator of nuclear factor kappa-B ligand
Rhox4	Reproductive homeobox 4
RNA	Ribonucleic acid
RPMI	Roswell Park Memorial Institute Medium
RT	Room temperature
RT-PCR	Real time Polymerase chain reaction/ Reverse transcription polymerase chain reaction
S1P	Sphingosine-1 phosphate
Sca-1	Stem cells antigen-1
SEM	Standard error mean
Shh	Sonic hedgehog
Six	Sineoculis homeobox
SM	Semi-mature
SP	Single positive
SSA	Sex steroid ablation
SYBR	Synergy Brands, Inc.
TBI	Total body irradiation
Tbx	T box transcription factor
TCF/LEF	Lymphoid enhancer binding factor/T-cell specific transcription factor

TCR	T cell receptor
TEC	Thymic epithelial cell
TEPC	Thymic epithelial progenitor cell
TESC	Thymic epithelial stem cell
Tfh	Follicular helper T cell
TGF	Transforming growth factor
Th	Helper T cell
Thy	Thymocyte antigen (CD90)
Tie	Angiopoietin receptor
TN	Triple negative
TNF	Tumour necrotic factor
TREC	T cell receptor excision circle
Treg	Regulatory T cell
TSH	Thyroid stimulating hormone
TSLP	Thymic stromal lymphopoietin
UEA-1	Ulex europaeus agglutinin I
Wnt	Wingless/integrated
XCL1	Chemokine ligand (lymphotactin)
YFP	Yellow fluorescent protein

CHAPTER 1:

LITERATURE REVIEW

1.1 A BRIEF INTRODUCTION TO THE THYMUS

The thymus is a bilobed gland located anterior to the heart in the superior anterior mediastinum. It is organised into phenotypically and functionally distinct outer cortical and inner medullary regions (**Figure 1.1**). These regions are comprised of thymic stromal cells (TSCs) and developing T cells (thymocytes), which account for approximately 1% and 99% of total thymic cellularity, respectively (reviewed in (Sakata, Ohigashi et al. 2018)). Interactions between thymocytes and the TSC compartment are essential for T cell development (thymopoiesis) (van Ewijk, Shores et al. 1994). Although TSCs include thymic epithelial cells (TECs), fibroblasts, endothelial cells, dendritic cells (DCs) and macrophages, it is the TECs that are most integral to thymopoiesis and central T lymphocyte tolerance (reviewed in (Takahama 2006)).
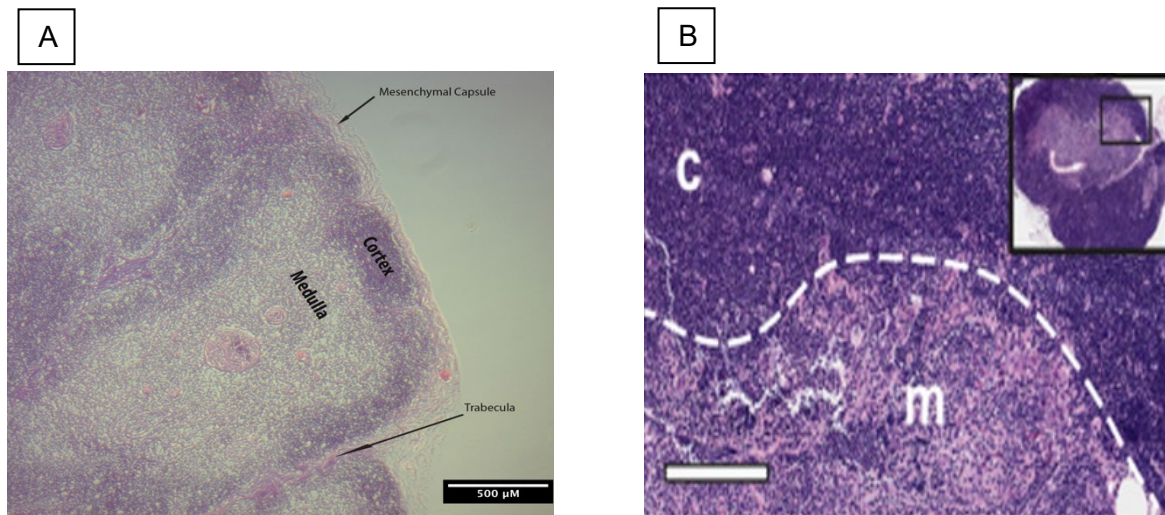
Figure 1.1 Thymic histology. A. H&E stained cross-section of paediatric thymus demonstrating a thymic lobule with an outer mesenchymal capsule, cortex and inner medulla. **B.** Histological section of a mouse thymic lobe showing an outer cortical and inner medullary region (Kim, Choi et al. 2017).

1.2 THYMUS ORGANOGENESIS

The thymus is a derivative of the third pharyngeal pouch endoderm which appears around day 9.5 of embryogenesis (E9.5) in mice, with spatial patterning of the thymus and parathyroid glands governed by the expression of forkhead box transcription factor n1 (Foxn1) and glial cells missing homologue 2 (Gcm2), respectively (Gordon, Bennett et al. 2001). Among other molecular queues, bone morphogenetic protein 4 (Bmp4) and sonic hedgehog (Shh) are also involved during these early stages of organogenesis, with the former thought to trigger Foxn1 expression (Patel, Gordon et al. 2006) and act against Shh signalling (Moore-Scott and Manley 2005). Precisely controlled Wnt signalling also has a role in initiating Foxn1 expression in the thymic rudiment (Balciunaite, Keller et al. 2002, Swann, Happe et al. 2017). By E11.5, while still attached to the pharynx, the thymus-parathyroid primordia have patterned into two separate organ domains. Pharyngeal detachment occurs at E11.5 – E11.75 and by E12.0 the thymus begins its caudal migration into the chest cavity (Gordon, Wilson et al. 2004).

While not essential for initial TEC specification, Foxn1 plays a key role in TEC regulation, including their differentiation, proliferation, and formation of the 3-dimensional (3D) TEC network (Blackburn, Augustine et al. 1996, Nowell, Bredenkamp et al. 2011). Null mutation in Foxn1 has been shown to produce the classical *nude* phenotype, with aberrant thymus development resulting in a primordial thymic rudiment that lacks the ability to attract hematopoietic progenitors (Nehls, Kyewski et al. 1996, Romano, Palamaro et al. 2013). This Foxn1-independent phase of early thymus organogenesis results in thymic epithelial progenitor cell (TEPC) formation, in which cells express the mouse thymic stromal (MTS) antigens reactive to the monoclonal antibodies MTS20 and

MS24 (Godfrey, Izon et al. 1990, Blackburn, Augustine et al. 1996, Gill, Malin et al. 2002). Foxn1 begins to be highly expressed from E11.25. As many as 450 genes are targeted by Foxn1 (Žuklys, Handel et al. 2016), including Delta-like ligand 4 (Dll4) the Notch ligand crucial for T lineage commitment and differentiation, and chemokines C-X-C motif chemokine 12 (Cxcl12) and Ccl25. Furthermore, Foxn1 directly enhances cTEC transcription of the thymoproteasome subunit β5t, which plays a pivotal role in positive selection for CD8$^+$ T cells (Murata, Sasaki et al. 2007, Ripen, Nitta et al. 2011, Xing, Jameson et al. 2013, Uddin, Ohigashi et al. 2017).

The ectodermal cell population comprising neural crest cells, surround the third pharyngeal pouch and eventually form the thymic mesenchymal capsule and become associated with the thymic vasculature. Interactions between thymic mesenchyme and TECs are essential for thymus organogenesis (Jenkinson, Jenkinson et al. 2003) with fibroblast growth factor-7 and -10 (Fgf7, Fgf10) from the mesenchyme signalling through the Fgf receptor 2 isoform IIIb (Fgfr2-IIIb) on TECs (expressed from E13), required for their growth but not initial formation (Revest, Suniara et al. 2001). Waves of hematopoietic precursors seed the thymic anlage by migrating through the perithymic mesenchyme to reach the thymic epithelium at E11.5, controlled by the expression of Ccl25 and Ccl21 (Liu, Ueno et al. 2005). By E13.5, reciprocal interactions between developing thymocytes and TECs/TSCs, commonly referred to as 'thymic crosstalk', influence further TEC differentiation and generation of the medullary compartment (van Ewijk, Shores et al. 1994).

1.3 THYMOPOIESIS

Postnatal thymopoiesis requires the migration of bone marrow (BM)-derived progenitors into the thymus, via blood vessels at the corticomedullary junction (CMJ). Controversy remains about the exact phenotype and lineage potential of the hematopoietic progenitors that colonise the thymus. In the BM, self-renewing hematopoietic stem cells (HSCs) generate non-renewing multipotent progenitors (MPPs) which then undergo lineage bifurcation to form either common lymphoid progenitors (CLPs) or common myeloid progenitors (CMPs). It is believed that CLPs efficiently colonise the thymus, however, single cell studies on the earliest thymic progenitors (ETPs) that are defined as lineage negative (Lin⁻; excludes B cells, T cells, myeloid cells, dendritic cells, erythroid cells and NK cells), CD25⁻, c-kit^{hi}, demonstrated multilineage potential, including T lymphocytes, dendritic and NK cells (Ardavin, Wu et al. 1993, Bell and Bhandoola 2008). Therefore, although not precluding CLPs, thymus colonising progenitors may also likely be derived from MPPs or lymphoid-primed multipotent progenitors (LMPPs) with potential to form T lymphocytes and myeloid lineage cells (Bhandoola, von Boehmer et al. 2007, Bell and Bhandoola 2008).

The multilineage potential of thymus colonising cells is maintained in the earliest thymocyte subsets that do not express markers of T lineage commitment (Porritt, Rumfelt et al. 2004), termed double negative 1 (DN1) cells (Lin⁻CD4⁻CD8⁻CD44⁺CD25⁻c-kit^{hi}) and DN2 cells (Lin⁻CD4⁻CD8⁻CD44⁺CD25⁺c-kit^{hi}). Non-T lineage potential is then lost at the DN3 stage (Lin⁻CD4⁻CD8⁻CD44⁻CD25⁺c-kit$^{-/lo}$) with T cell fate specification requiring Notch signalling (Radtke, Wilson et al. 1999). Thereafter, T cell receptor (TCR)-β chain rearrangements occur during the DN3 stage with successful assembly of the pre-TCRα

chain leading to differentiation towards a DN4 phenotype (Lin⁻CD4⁻CD8⁻CD44⁻CD25⁻).

DN4 to $CD4^+CD8^+$ double positive (DP) transition ensues, with the expression of mature

TCRαβ on DP thymocytes following successful TCRα chain rearrangements. Extensive

proliferation of DP cells, supported by the cytokines interleukin (IL)-7 and stem cell factor

(SCF) produced by cTECs, leads to a 10^5-fold expansion from the 10^2 precursors

estimated to enter the thymus each day (Shortman, Egerton et al. 1990). DP cells are

screened by cortical TECs (cTECs) for TCR functionality, through a process called

positive selection. This process ensures the survival of DP thymocytes with TCRs that

can bind self-peptide/MHC complexes. DP cells are then induced to differentiate into

$CD4^+$ or $CD8^+$ single positive (SP) T cells, which recognise major histocompatibility

complex (MHC) class II and class I, respectively (reviewed in (Kondo, Takada et al.

2017)). SP T cell lineage commitment at this step is dependent on whether the self-

peptide/MHC complex on cTECs is engaged by a CD4 or CD8 co-receptor molecule. DP

cells with TCRs incapable of binding such MHC complexes instead undergo 'death by

neglect'. Note that the degradation of intracellular self-peptides by thymoproteasomes

and proteases is essential for positive selection, with their absence resulting in a failure

of SP T cell lineage commitment (Honey, Nakagawa et al. 2002, Murata, Sasaki et al.

2007, Klein, Kyewski et al. 2014).

$CD4^+$ and $CD8^+$ SP T cells express C-C chemokine receptor type 7 (CCR7), which

attracts them towards the thymic medulla. Here, they interact with antigen presenting cells

(APCs) via a process known as negative selection. Medullary TECs (mTECs) and DCs

present self-peptides to test TCR affinity/avidity, with highly reactive T cells induced to

undergo apoptosis or differentiation into regulatory cells (Tregs), as preventative

measures against autoimmunity. Interestingly, some studies have suggested that negative selection also occurs within the thymic cortex, through interactions with hematopoietic APCs and cTECs (McCaughtry, Baldwin et al. 2008, Hu, Yap et al. 2016). Since few DCs reside in the cortex, it is hence unlikely that hematopoietic APCs contribute substantially to this process (Melichar, Ross et al. 2013). Moreover, CCR7 or CCR7 ligand (CCR7L) deficient mice present with autoimmunity (Kurobe, Liu et al. 2006), which implicates the importance of migration into the medulla for central tolerance. Promiscuous gene expression is a unique characteristic of mTECs and contributes to the establishment of T cell self-tolerance. About 75-90% of all genes are estimated to be expressed in mTECs, including tissue-restricted antigens (Sansom, Shikama-Dorn et al. 2014). The autoimmune regulatory transcription factor, Aire, is a crucial regulator of promiscuous gene expression in a subset of mTECs (Derbinski, Gäbler et al. 2005) with humans and mice expressing deficient or defective forms of Aire developing multiorgan autoimmune diseases (Anderson, Venanzi et al. 2002). The transcription factor Fez family zinc-finger 2 (FEZF2) also contributes to self-tolerance processes through AIRE-independent promiscuous gene expression in mTECs (Takaba, Morishita et al. 2015).

As T cells develop through these stages, they undergo sequential migration mediated by chemokines, from the CMJ entry point enriched in vasculature, through the cortex to the subcapsular zone, back through the cortex and CMJ and on to the medulla (Takahama 2006). Circulating precursors require expression of CCR7, CCR9 and the p-selectin glycoprotein ligand 1 (PSGL-1) for thymus seeding (Rossi, Corbel et al. 2005, Zlotoff, Sambandam et al. 2010). Migration from the CMJ through the cortex is regulated by chemokine signalling through the CXC-chemokine receptor 4 (CXCR4) and CCR7, with

further outward migration to the subcapsular region involving CCR9 signals. At the subcapsule, lining fibroblasts provide extracellular matrix components to facilitate DN2-DN3 differentiation (Anderson, Anderson et al. 1997). As the developing T cells become DP they return through the cortex where they interact with cTECs that mediate positive selection and subsequently develop into SP T cells. CXCL12 has been proposed to act as a cortex retention factor for DP cells (expressing CXCR4), enabling cells to remain in the cortex while undergoing maturational events (Kadakia, Tai et al. 2019). Increased surface expression of CCR7 and CCR4 facilitates their migration through the CMJ and into the medulla, with DCs and mTECs expressing the ligands CCL21 and CCL17/CCL22. The SP T cells interact with DCs and mTECs which mediate negative selection and the development of T regulatory cells (Cowan, McCarthy et al. 2014). This defined migratory sequence is illustrated in **Figure 1.2**. Naïve $CD4^+$ and $CD8^+$ SP T cells that survive the negative selection process transition from a $CD69^+CD62L^-$ to $CD69^-CD62L^+$ phenotype (Takahama 2006). The expression of sphingosine-1-phosphate (S1P) receptor 1 (S1P1) by $CD69^-CD62L^+$ naïve SP thymocytes subsequently orchestrates thymic egress, via a chemotactic gradient that attracts these cells into thymic capillary venules and the periphery (Weber, Krueger et al. 2009). Only about 3% of all DP thymocytes are selected to become SP $CD4^-CD8^+$ or $CD4^+CD8^-$ mature thymocytes, while the remaining unselected or rejected 97% die in the thymus (Shortman, Egerton et al. 1990). In the mouse thymus, this process of T cell development from entry of precursors to exiting of mature self-tolerant T cell has been estimated to take up to 3-4 weeks in mice (Berzins, Boyd et al. 1998) with thymus size regulated by competition among DN T cell precursors for stromal niches (Prockop and Petrie 2004).
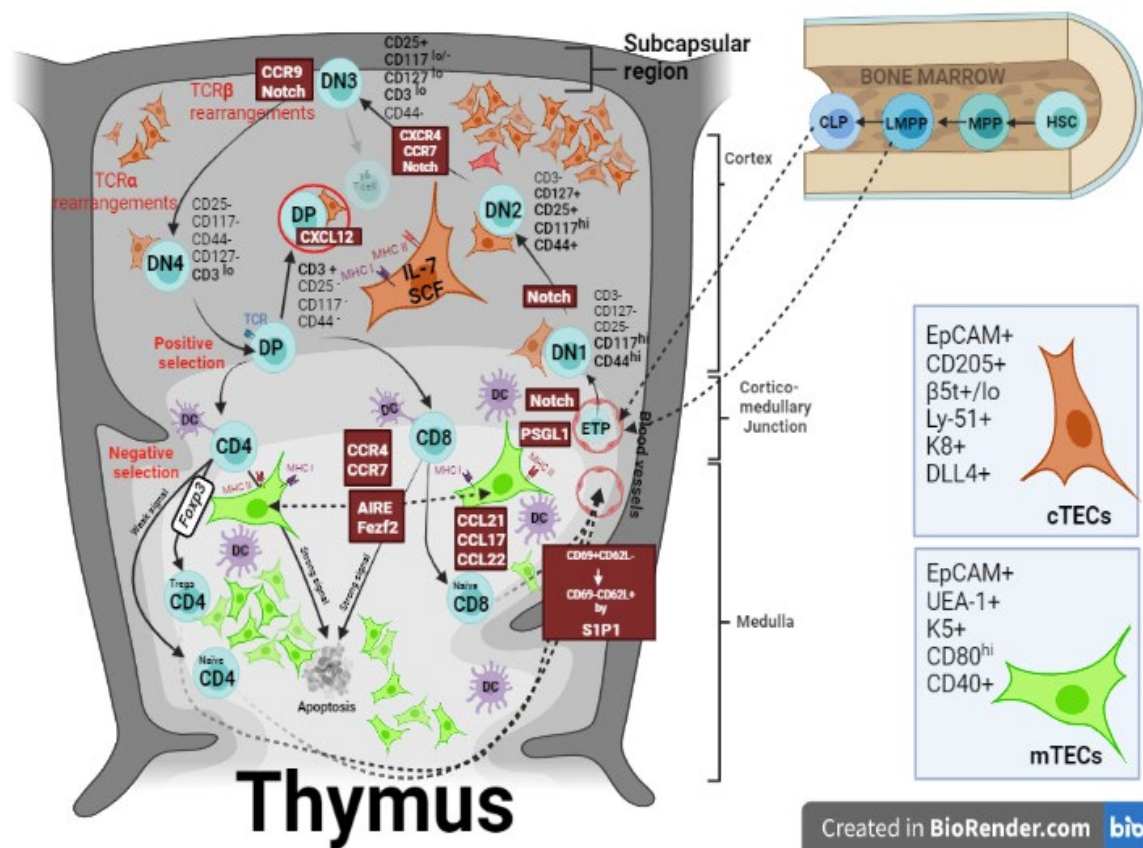
Figure 1.2 A schematic of thymopoiesis. Circulating PSGL1 expressing bone marrow derived lymphoid primed multipotent progenitors (LMPPs) and common lymphoid progenitors (CLPs) enter the thymus at the cortico-medullary junction (CMJ) and form the early thymic progenitors (ETPs). Continued Notch signalling from cortical epithelial cells (cTEC) is important for T lineage commitment as cells move from ETP to early DN3 stages, while migrating out to the subcapsular region, aided by chemokine signalling via receptors CXCR4, CCR7 and CCR9. T cell receptor (TCR)-β chain rearrangements occur during the DN3 stage with successful assembly of the pre-TCRα chain and Notch signalling leading to differentiation towards a DN4 phenotype. DN4 to CD4⁺CD8⁺ double positive (DP) transition follows with the expression of mature TCRαβ on DP thymocytes. DP retention in the cortex is aided by CXCL12. Proliferation of DP cells is supported by the cytokines interleukin (IL)-7 and stem cell factor (SCF) produced by cTECs. DP cells are screened by cTECs via their major histocompatibility complex (MHC) class II and class I, for TCR functionality (positive selection) and subsequently differentiate into CD4⁺ or CD8⁺ single positive (SP) T cells. DP cells expressing TCRs incapable of binding such MHC-peptide complexes instead 'die by neglect'. Newly generated CD4⁺ and CD8⁺ SP T cells expressing CCR7, migrate towards the thymic medulla. Here, medullary TECs (mTECs) and DCs present self-peptides to test TCR affinity/avidity, with highly reactive T cells induced to undergo apoptosis, known as negative selection, or differentiation into Foxp3⁺ T regulatory cells (Tregs), as preventative measures against autoimmunity. The autoimmune regulatory transcription factor, Aire, and *Fezf2* are crucial regulators of promiscuous gene expression in a subset of mTECs. Finally, self-tolerant naïve SP T cells migrate out of the thymus at the CMJ. Created with BioRender.com, adapted from (Takahama 2006).

1.4 THYMIC MICROENVIRONMENT

1.4.1 THYMIC EPITHELIAL PROGENITOR CELLS (TEPC)

1.4.1.1 Embryonic TEPC

Identifying markers of TEPC and the sequence of events regulating the emergence of cTEC and mTEC lineages, has importance in understanding the potential of TEPC in strategies to regenerate a functional thymus. Early studies investigated bipotent potential of cell populations present in the thymic primordium that express surface glycoproteins reactive to the antibodies MTS24 and MTS20 (Gill, Malin et al. 2002). These surface glycoproteins were expressed from E10, with the MTS24 binding glycoprotein subsequently identified as Plet1 (Depreter, Blair et al. 2008). Purified MTS20$^+$MTS24$^+$ TECs from E12 thymi (Bennett, Farley et al. 2002) and MS24$^+$ TECs from E15.5 thymi (Gill, Malin et al. 2002) were able to generate a functional thymic organoid containing both cortical and medullary TECs when transplanted under the kidney capsule as a cell aggregate with or without embryonic fibroblasts, termed reaggregate thymic organ cultures (RTOCs) (Anderson, Jenkinson et al. 1993, Anderson and Jenkinson 2007). Hence MTS24/Plet1 was proposed as a marker of a bipotent embryonic TEPC. However, these were not clonal studies, and later studies demonstrated that bulk populations of both MTS24$^+$ and MTS24$^-$ TECs from E14-E16 could give rise to functional thymic grafts (Rossi, Chidgey et al. 2007). By E18, MTS24$^+$ cells did not reaggregate well and did not form a graft, marking a significant developmental change, with postnatal MTS24/Plet1$^+$ expression becoming mostly restricted to mTECs and losing bipotent potential (Rossi, Chidgey et al. 2007, Wong, Lister et al. 2014). Plet1 expression has also been found on LGR5$^+$ intestinal stem cells that exhibited higher expression levels of late S-phase cyclins, and co-localised with Ki67$^+$ cells suggesting a highly proliferative cell population (Zepp,

Zhao et al. 2017). Thus, in the postnatal thymus, whether MTS24$^+$ mTECs represent a transit amplifying population, is speculative. Subsequent clonal studies argued the maintenance of postnatal bipotent potential remained only in the rare Plet1$^+$ cells found in cTECs expressing high levels of MHCII (Ulyanchenko, O'Neill et al. 2016). What distinguishes each of the MTS24$^+$ populations in cTECs and mTECs is yet to be investigated.

The existence of embryonic TEPCs with the potential to form cortical and medullary TECs, was more definitively illustrated using a clonal transplantation approach (Rossi, Jenkinson et al. 2006). Rossi *et al.,* microinjected single E12 EpCAM$^+$CD45$^-$ TECs marked with enhanced yellow fluorescent protein (eYFP), into a wildtype thymus which was then transplanted under the kidney capsule of wildtype mice and analysed after 4 weeks. The embryonic eYFP TECs were found to differentiate into both cTEC and mTEC lineages *in vivo* (Rossi, Jenkinson et al. 2006).

Around E11-E12 TECs begin to acquire the marker CD205, which is a marker for mature cTECs. By E13 some of these cells begin to express CD40, a marker associated with mTECs, with an emerging population of CD205$^-$CD40$^+$ evident from E14-E15 (Baik, Jenkinson et al. 2013). When E15 CD205$^+$CD40$^-$ TECs were reaggregated and transplanted under the kidney capsule, they gave rise to both cTECs expressing β5t and CD205 in the cortical regions, and ERTR5$^+$ mTECs including Aire$^+$ mTECs in the medullary regions. Collectively these results supported a serial progression model of mTEC emergence in the embryo, in which mTECs are derived from a bipotent TEPC that expresses markers of cTECs (Alves, Takahama et al. 2014), with an important role for

Notch signaling in the emergence of mTEC lineage progenitors that is required earlier than RelB and RANK mediated signalling (Liu, Kousa et al. 2020).

Unipotent mTEC progenitor/stem cells have been detected as early as E13.5 (Rodewald, Paul et al. 2001) and express SSEA-1 and high levels of Claudins 3 and 4 with self-renewing potential (Hamazaki, Fujita et al. 2007, Sekai, Hamazaki et al. 2014). They persist in the postnatal thymus to repopulate mTECs, but with substantially reduced capacity after 4 weeks of age in mice (Sekai, Hamazaki et al. 2014).

1.4.1.2 Postnatal TEPC

Mature TECs are a dynamic population with a limited lifespan and so need to be constantly replenished (Gray, Seach et al. 2006, Gray, Abramson et al. 2007, Rode and Boehm 2012). Given the postnatal thymus maintains some potential to regenerate after damage, the question of whether bipotent TEPCs exist in the postnatal/adult thymus or whether mature TECs are only maintained by immature unipotent precursors generated during thymic organogenesis, has attracted considerable interest. The precise identity of stem or progenitor epithelial cells in the adult thymus remains controversial.

Early studies identified cTEC progenitors that were enriched at the CMJ and expressed both keratin 5 and keratin 8 (K5+K8+) (Klug, Carter et al. 1998). Keratins are intermediate filament proteins expressed in all epithelial cells, with approximately 20 members identifying epithelial cell subsets in different tissues. Their intracellular expression is also regulated by stages of differentiation and proliferative activity. Klug *et al.* found two distinct subsets within the cortex: a predominant K8+K18+K5-K14- subset (K8+K5-) and a minor K8+K18+K5+K14- (K5+K8+) subset that was concentrated around the CMJ. Using various transgenic mice blocked at different stages of T cell development and thymic graft

experiments, they found that K5+K8+ precursors gave rise to K8+K5- progeny in the cortex, and this differentiation process depended on inductive interactions between TEC and early DN thymocyte subsets. While Klug *et al*. were unable to demonstrate direct evidence of precursor-progeny relationship in the medulla between K5+K8+ cells and K8-K18-K5+K14+ (K5+K14+) mTECs, it has since been proposed (Gill, Malin et al. 2002, Manley, Richie et al. 2011).

Using β5t-eGFP reporter mice Ohigashi *et al.* and Mayer *et al.* found that all mTEC, including Aire+ TEC in young adult mice expressed robust levels of eGFP and hence were originally derived from embryonic β5t+ cTEC (Ohigashi, Zuklys et al. 2013, Mayer, Žuklys et al. 2016). These results supported the developmental model in which TEPC of cortical phenotype in the embryo serially diverged to generate the mTEC lineage (Alves, Takahama et al. 2014). However, the existence of bipotent TEPC in the postnatal thymus, was first demonstrated, albeit indirectly, by Bleul *et al.* when neonatal reactivation of Foxn1 generated both cortical and medullary TECs (Bleul, Corbeaux et al. 2006).

Phenotypic identification based on surface markers would enable prospective isolation of live TEPC, important if they were to have clinical potential. Clonogenic studies identified post-natal TEPC, although the precise phenotype has not yet been resolved (Ucar, Ucar et al. 2014, Wong, Lister et al. 2014, Ulyanchenko, O'Neill et al. 2016). Wong *et al.* identified TEPC with hallmark features of bipotent progenitor cells within immature cTECs (cTEC^{lo}), which express the cTEC marker Ly51, low levels of MHCII and negative for the mTEC marker ulex europaeus agglutinin 1 (UEA-1^{neg}) (Wong, Lister et al. 2014). In this study, cTEC^{lo} were purified from ubiquitous-green fluorescent protein (GFP) mice and reaggregated with supporting embryonic stromal cells (non-GFP) before engrafting under

the kidney capsule of recipient mice. All the resulting reaggregate grafts contained pockets of GFP$^+$ cTEC and GFP$^+$ mTEC that were integrated throughout the cortical and medullary microenvironments, respectively. These GFP$^+$TECs expressed high and low levels of MHC II, including mature, Aire$^+$ mTEC. This demonstrated that the cTEC^{lo} subset contained a subset of TEPCs capable of differentiating into multiple TEC lineages *in vivo*. Other adult TEC subsets were also investigated for TEPC potential in this study. A small number of GFP+ cells were maintained in cTEC^{hi} RTOCs in half of the grafts, but virtually no GFP+ cells were recovered in mTECs grafts.

Further label retaining assays and colony forming assays using purified TEC populations from *Foxn1$^{eGFP/+}$* mice and an *in vitro* 3D culture system, localised the TEPC within the cTEC^{lo} subpopulation that expresses α6integrin (α6$^+$) and high levels of the stem cell antigen 1 (Sca1^{hi}) (Wong, Lister et al. 2014). Sca-1 and α6 integrin are markers of stem cells in other epithelial tissues (Welm, Tepera et al. 2002, Terunuma, Kapoor et al. 2007, Hayashi, Yamato et al. 2008, McQualter, Yuen et al. 2010). Progenitor potential was retained in the α6$^+$Sca1^{hi} cTEC^{lo} *Foxn1*-eGFP$^+$ colonies, evident in their capacity to self-renew and differentiate into downstream TEC lineages; reaggregate grafts from FACS purified colonies in RTOCs contained GFP$^+$ (Foxn1$^+$) thymic epithelial networks of cTECs and mTECs throughout the graft, including Aire$^+$ mTECs (Wong, Lister et al. 2014). Further studies by Lepletier *et al.*, identified an accumulation of postnatal α6$^+$Sca1^{hi}cTEC^{lo} TEPC during ageing, which was associated with increased loss of function (Lepletier, Hun et al. 2019). Alterations in the Bmp4 - activin A - follistatin axis as an underlying mechanism for age-related loss in TEPC function was proposed, and this was influenced in part by sex-hormones, given that sex steroid deprivation by physical

castration could reactivate TEPCs to generate downstream TEC lineages in aged male mice (Lepletier, Hun et al. 2019). A more recent study by Meireles *et al.,* also used a clonogenic assay to investigate progenitor capacity in postnatal TEC subsets (Meireles, Ribeiro et al. 2017) and found that colony-forming cells existed mainly within the cTEC population. These cells also expressed CD24 and Sca1 and lacked mTEC markers, somewhat consistent with the Wong *et al.,* phenotype. Isolating these cells from Actin-GFP[+] reporter mice and transplanting them as RTOCs, the GFP^{+} cells had integrated into the thymic microenvironment. They further suggested that clonogenic capacity is lost as mice transition into adulthood and this may relate to thymocyte-derived signals negatively regulating clonogenic activity (Meireles, Ribeiro et al. 2017).

In another study, Ucar and colleagues argued that thymosphere forming adult thymic epithelial stem cells (TESC) did not express Foxn1 or EpCAM (Ucar, Ucar et al. 2014). While it was possible that Ucar *et al.* identified stem cells more upstream and primitive than TEPC as all functional Foxn1^{+} TEC arise from Foxn1^{neg} precursors (Bleul, Corbeaux et al. 2006, Nowell, Bredenkamp et al. 2011), functional confirmation in RTOCs was not evaluated and a subsequent study demonstrated that these thymospheres were formed from contaminating mesenchymal cells, and did not generate TECs (Sheridan, Keown et al. 2017).

Ulyanchenko *et al.* also used a clonal approach with purified TEC subsets placed into RTOCs (Ulyanchenko, O'Neill et al. 2016). They proposed TEPC were exclusively in the Plet1^{+}cTEC[hi] population which had a greater bipotent capacity than cTEC[lo] which they argued were mostly single lineage cTEC precursors. Unfortunately, they did not investigate the $\alpha6^+$Sca1^{hi} cTEC[lo] TEPC enriched subpopulation identified by Wong *et al.*

Several other studies supported a post-natal progenitor subset that expresses markers typically associated with the cTEC lineage (Dumont-Lagacé, Gerbe et al. 2017, Tan and Nusse 2017). However, the precise phenotype of post-natal TEPC and to what extent the replacement of mature TECs in the adult relies on TEPC differentiation as opposed to single lineage precursors, are yet to be fully resolved. Ohigashi *et al.,* demonstrated that post-natal mTECs were maintained by lineage specific mTEC precursors, rather than bipotent TEPC (Ohigashi, Zuklys et al. 2015). This may be a consequence of the TEPC loss-of-function during ageing proposed by Lepletier *et al.,* with increased reliance on single-lineage cortical and medullary TEC precursors leading to gradual precursor senescence and the loss of TECs that distinguishes thymic involution (Lepletier, Hun et al. 2019). Further *in vivo* fate mapping at a single cell level and functional studies should be performed to resolve these issues.

1.4.2 TEC HETEROGENEITY

The TEC compartment undergoes a great deal of expansion and diversification in the early postnatal period correlating with escalating thymopoiesis, with lympho-epithelial interactions promoting molecular and functional changes, reviewed in Pinheiro and Alves (Pinheiro and Alves 2021). An overview of the different TEC subsets is illustrated in **Figure 1.3**.

1.4.2.1 Cortex

Cortical TECs are broadly defined by their expression of epithelial cell adhesion molecule (EpCAM) and CD205 (Ly75) or Ly51; while medullary TECs express EpCAM and UEA-1. Historical descriptions of TEC heterogeneity were limited to immature and mature

cTECs and mTECs with MHCII expression levels dividing TECs into MHCII^low immature TEC and MHCII^high mature TECs: cTEC^lo, cTEC^hi, mTEC^lo, mTEC^hi (Seach, Wong et al. 2012). Lineage-specific cTEC and mTEC progenitors within the cTEC^lo and mTEC^lo compartments are generated from bipotent progenitors in early ontogeny (reviewed in (Hamazaki 2015)) and are replenished in the adult by postnatal bipotent TEPC at least prior to the negative influences of thymus involution (Bleul, Corbeaux et al. 2006, Ulyanchenko, O'Neill et al. 2016, Lepletier, Hun et al. 2019). Thymic nurse cells are a β5t^+ cTEC subset found in the thymic cortex that seemingly engulf developing DP thymocytes with some phenotypically mature CD3^+CD4^+CD8^- cells, suggesting provision of a specialised microenvironment for optimising positive selection processes (Takahama, Ohigashi et al. 2017).

While broad functional properties of the thymic cortex are well understood, the functional heterogeneity of subsets within cTEC is less defined. The turnover rate of mature cTECs has not been well studied but recent research has more directly supported the postulation that cTEC^lo contain unipotent progenitors to replenish cTEC^hi (Shakib, Desanti et al. 2009, Wong, Lister et al. 2014, Ulyanchenko, O'Neill et al. 2016).

Expression of various markers implicates different functional roles, for example, expression of α6 integrin, Sca1, Plet1 are possible markers of TEPC as shown in **Figure 1.3** (Wong, Lister et al. 2014, Ulyanchenko, O'Neill et al. 2016, Meireles, Ribeiro et al. 2017, Lepletier, Hun et al. 2019). Dll4^+ specialised cTECs for T lineage commitment (Fiorini, Ferrero et al. 2008) expressed more highly in Plet1^- cTEC than in Plet1^+ cTEC and mTECs (Liu, Kousa et al. 2020); IL7 expressing TEC subsets that provide IL7 for the survival and differentiation of immature thymocytes (Alves, Richard-Le Goff et al. 2009,

Ribeiro, Rodrigues et al. 2013); TEC provision of chemokines and ligands for thymic seeding such as CCR7 and CCR9 ligands and CCL25 (Liu, Saito et al. 2006, Zlotoff, Sambandam et al. 2010); regulation and positioning of CXCR4+ immature thymocytes in the cortex by CXCL12 expressing cTEC (Plotkin, Prockop et al. 2003); β5t, the catalytic subunit of the thymoproteasomes in mature cTECS, important for CD8 positive selection (Murata, Sasaki et al. 2007); PRSS16, the thymic specific serine protease important for CD4 positive selection (Gommeaux, Grégoire et al. 2009) and reviewed in (Takahama, Ohigashi et al. 2017, Alawam, Anderson et al. 2020). However, these markers may not distinguish individual subsets, with cTECs possibly having overlapping roles, as might be suggested by only two major subsets identified in a single-cell RNA mapping approach (Bornstein, Nevo et al. 2018).

1.4.2.2 Medulla

mTECs produce chemokines important in controlling the migration of positively selected thymocytes from the cortex towards the medulla, such as CCL21-expressing mTEC[lo] (Lkhagvasuren, Sakata et al. 2013). The main role of the mTEC compartment is in final T cell maturation and central tolerance induction. Aire[+] mTEC[hi] and Aire[-] mTEC[hi] subsets present self-peptides to developing T cells to screen for autoreactivity and generation of regulatory T cells. The transcription factor Fez family zinc-finger 2 (FEZF2) has been reported to have a role in Aire-independent self-antigen expression for self-tolerance and is expressed in both mTEC[lo] and mTEC[hi] (Takaba, Morishita et al. 2015).

The rapid turnover of Aire[+] mature mTECs of up to two weeks (Gray, Seach et al. 2006, Gray, Abramson et al. 2007, Nishikawa, Nishijima et al. 2014) highlighted the critical requirement for ongoing mTEC renewal in the postnatal thymus. Lineage restricted,

unipotent mTEC precursors residing in the mTEC^{lo} subset that originated from β5t$^+$ embryonic TECs, were proposed to be the main cell population responsible for adult mTEC replenishment, with minimal input from postnatal bipotent TEPC (Ohigashi, Zuklys et al. 2015, Mayer, Žuklys et al. 2016). This reliance on unipotent progenitors is in keeping with the finding that postnatal TEPCs become increasingly quiescent following puberty in male mice (Lepletier, Hun et al. 2019).

The discovery that Aire$^+$ mTEC^{hi} differentiate into post-Aire mTEC^{lo} cells with traits of terminally differentiated keratinocytes (Nishikawa, Hirota et al. 2010, Wang, Laan et al. 2012, Metzger, Khan et al. 2013), emphasized the possibility of greater heterogeneity in mTECs than first realised. Studies by Bornstein et al and Miller et al, identified a new subset of mTECs termed thymic tuft cells which are sensory epithelial cells and may play a role in central tolerance as Foxp3^{low} T regulatory cell precursors were reduced in tuft cell-deficient mice (Bornstein, Nevo et al. 2018, Miller, Proekt et al. 2018, Owen, Mahmud et al. 2019). The mTEC^{lo} compartment also contains Hassal's corpuscles, prominent in the human medulla, comprising flattened epithelial cells in concentric structures. These express keratin-10, involucrin, filaggrin and thymic stromal lymphopoietin (TSLP), that are also expressed by terminally differentiated epithelial cells such as keratinocytes (Nishikawa, Hirota et al. 2010). Single cell mapping approaches identified four major medullary TEC compartments with distinct functions and epigenetic landscapes (Bornstein, Nevo et al. 2018, Miller, Proekt et al. 2018, Park, Botting et al. 2020). A more recent study using single-cell transcriptomics identified even more TEC subsets (Baran-Gale, Morgan et al. 2020); nine TEC subsets were identified, including a potential bipotent

progenitor population which was termed intertypical TEC, that expanded during ageing as a direct consequence of their constrained differentiation.

Improved rare cell isolation techniques, fate mapping and single-cell genetic analytical approaches will help pave the way for deeper understanding of the phenotypic and functional heterogeneity of the thymic epithelial microenvironment.
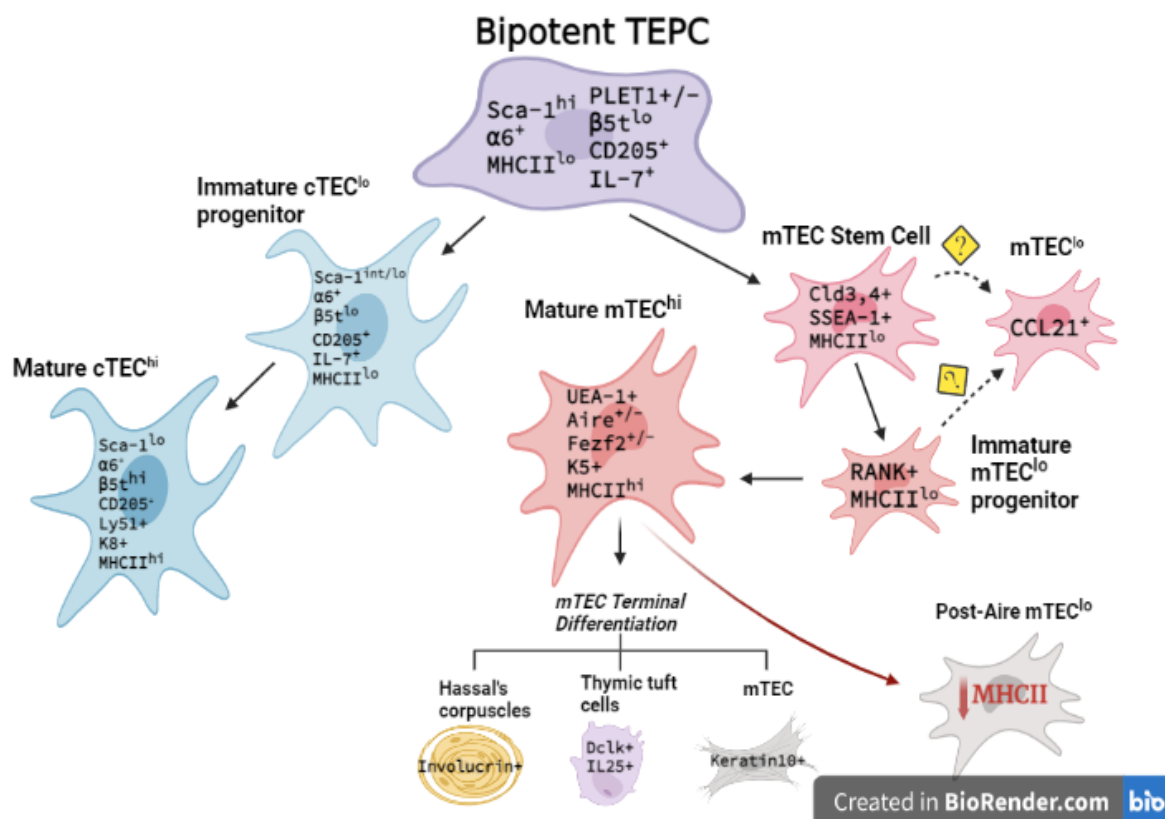
Figure 1.3. TEC heterogeneity. The precise phenotype of postnatal bipotent thymic epithelial progenitor cells (TEPC) has not yet been fully resolved, however, recent literature suggests possible expression of markers such as stem cell antigen-1 (Sca-1^{hi}), α6 integrin (α6$^+$), Placenta Expressed Transcript 1 (PLET1), MHCII^{lo}, CD205$^+$, and IL-7$^+$. TEPC have been proposed to differentiate into single lineage precursors that express low levels of MHCII, either as cortical TECs (CD205$^+$; cTEC^{lo}) or medullary TEC stem cells (Cld3$^+$ and SSEA-1$^+$) and/or RANK$^+$ mTEC progenitors (UEA-1$^+$; mTEC^{lo}). Further maturation of cTEC and mTEC single lineage precursors generate either mature cTECs (MHCII^{hi}β5t^{hi}Sca1^{lo}) and mature mTECs (MHCII^{hi}UEA1$^+$) respectively, with some mTECs further differentiating into subpopulations such as Hassal's corpuscles, thymic tuft cells and K10$^+$ cells. It should be noted that that some post-Aire+ mTEC^{hi} cells downregulate MHCII levels upon senescence (Post-Aire mTEC^{lo}). Adapted from (Alawam, Anderson et al. 2020).

1.5 AGE ASSOCIATED DETERIORATION OF THE IMMUNE SYSTEM

Many infectious diseases have been eradicated in the developed world and together with improved medical treatments and quality of life, humans are living much longer. However, age related decline in the effectiveness of the immune system, can lead to an increased vulnerability to infections and comorbidities, with higher morbidity and mortality rates in the ageing population, a tendency toward a pro-inflammatory state, increased prevalence of specific cancers and some autoimmune diseases, and reduced vaccine effectiveness due to diminished antigenic receptor diversity (reviewed in (Chidgey, Dudakov et al. 2007)). T cell senescence can be accelerated by repeated T cell stimulation and extensive replication, for example, chronic viral infections can lead to the acquisition of a senescent or exhausted phenotype that restricts T cell responsiveness (reviewed in (Mittelbrunn and Kroemer 2021). The elderly are more prone to developing complications from infections, and are susceptible to newly arising infectious organisms such as in West Nile fever, severe acute respiratory syndrome (SARS) (Peiris, Chu et al. 2003, Jean, Honarmand et al. 2007) and the current Corona Virus pandemic (COVID19). An accumulation of dysfunctional, terminally differentiated T cells occurs with ageing and as such, age-related immunosenescence has become a public health concern. One of the hallmarks of immune system ageing in all vertebrates with a thymus, is age-associated thymic involution.

1.5.1 AGE-ASSOCIATED THYMIC INVOLUTION

Chronic age-associated thymic involution begins early in life and is evolutionary conserved in all vertebrates (Torroba and Zapata 2003). In the early postnatal weeks and

months of life in mice and humans respectively, an expansion of thymopoiesis to populate the peripheral T cell pool is accompanied by expansion and diversification of thymic epithelial cells (reviewed in (Pinheiro and Alves 2021)). Thereafter contraction of this highly productive period occurs, and progressive thymus involution begins with global transcriptional profiling revealing the downregulation of cell-cycle regulators from as early as 4 weeks of age in mice (Ki, Park et al. 2014). Cortical thinning and the merging of medullary islands are evident by 4 weeks of age in mice (Park, Botting et al. 2020); **Figure 1.4A**). A further significant phase in the reduction of thymopoiesis is evident from the onset of puberty coincident with the surge in sex hormone production and is followed by a more gradual age-related decline. This decline in thymopoiesis is associated with alterations in thymic architecture. A loss of thymic epithelial tissue, an increase in non-epithelial perivascular spaces which in humans accumulate with lipid-laden adipocytes, disorganisation of the thymic microenvironment with cortical and medullary boundaries becoming less defined, are all hallmarks of the ageing thymus (**Figure 1.4B**). By mid-life, functional thymic tissue has reduced dramatically to only around 10% of its maximal capacity (Flores, Li et al. 1999) (**Figure 1.4**). Whether feed-back mechanisms from the peripheral T cell pool impact the thymus following establishment to enable diversion of energy resources, given greater than 90% of developing thymocytes undergo cell death, or whether thymic involution is due to an independent genetic or physiological program, is unclear and remains topical in ongoing evolutionary debates (reviewed in (Shanley, Aw et al. 2009)).

Minimal functional thymus tissue can persist into old age with cell tracking experiments demonstrating that recent thymic emigrants (RTEs) can still be detectable in 2-year-old

mice, relative to the size of remaining functional thymic tissue (Hale, Boursalian et al. 2006), RTEs maintain diversity of the TCR repertoire enabling efficient immune responses against newly encountered antigens and pathogens. However, with the reduction in naïve T cell production, clonal expansion through homeostatic proliferation plays a larger role in sustaining peripheral T cell numbers. This results in contraction of the antigen receptor repertoire diversity, leaving holes in the immune repertoire which compromises the detection of pathogens and leads to impaired immunity.

In addition to the reduced TCR repertoire and shifts in the proportion of naïve, effector/memory and regulatory T cells, the reduction in replenishment of newly generated naïve T cells due to thymus involution likely contributes to the accumulation of increasingly less efficient senescent or exhausted T cells, that collectively leads to progressive immunodeficiency (Sato, Kato et al. 2017, Thomas, Wang et al. 2020).
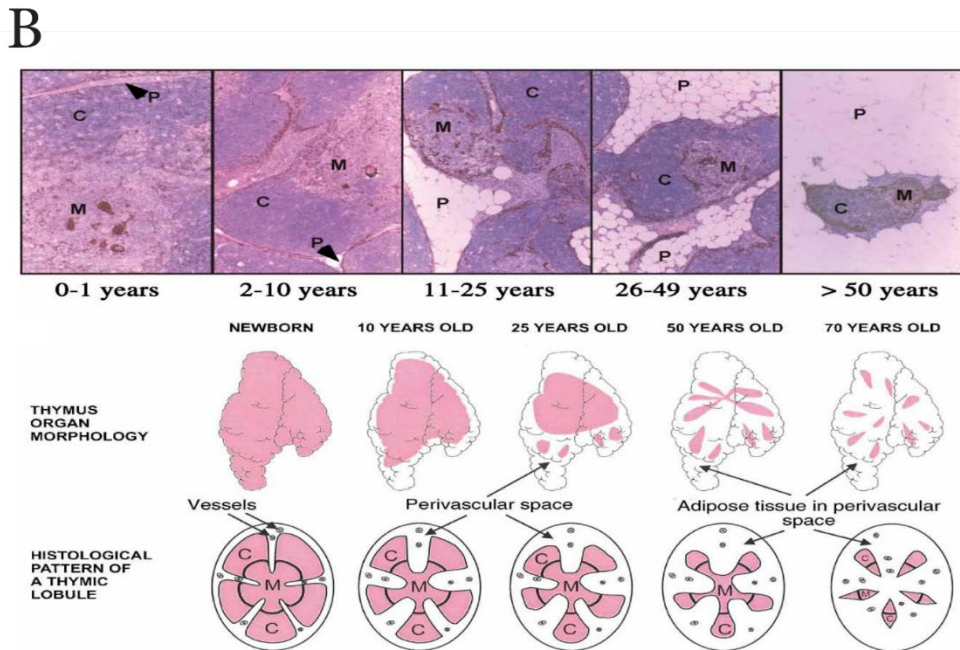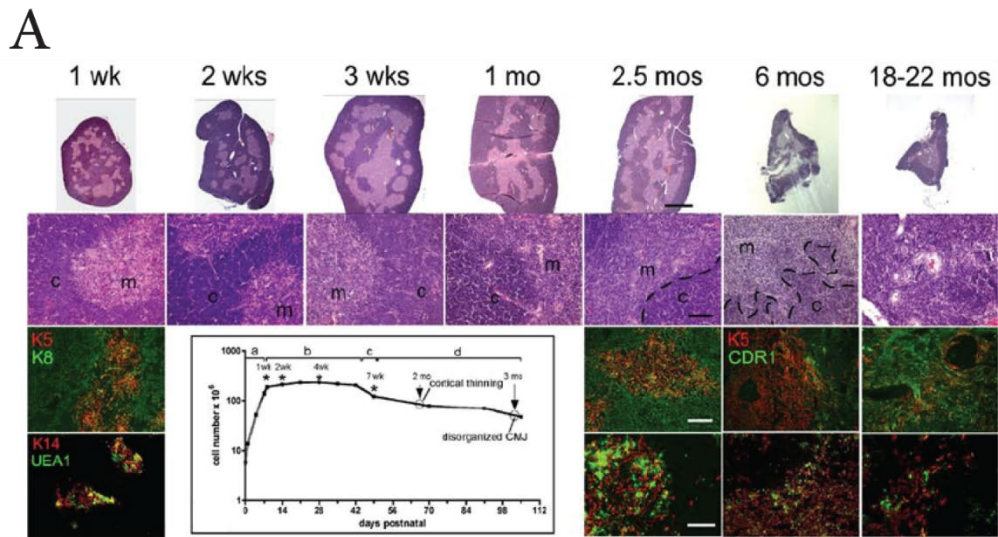
Figure 1.4 Age related thymus involution. (**A**)Thymus involution in mice is characterized by initial thinning of the cortical compartment, followed by loss of thymic tissue. (**B**) In humans, thymic involution is evident early in life with more significant loss following puberty and continuing into older age. It is characterized by loss of thymic functional tissue and an increase in adipose tissue infiltration in perivascular regions. Taken from (Manley, Richie et al. 2011) (mouse) and (Gruver, Hudson et al. 2007) (human).

1.5.2 TEC ALTERATIONS IN AGEING

In addition to the loss of TECs during ageing, thymopoiesis can be affected by alterations in the quality of aged TECs that contribute to the aged thymic microenvironment.

Age related loss of trophic cytokines impacts on the rate of both TEC and thymocyte proliferation. IL-7, produced by TECs, has a key role in thymocyte expansion during development and reduced levels of IL-7 in the ageing thymus (Andrew and Aspinall 2002) particularly within the cortex (Alves, Huntington et al. 2010, Ribeiro, Rodrigues et al. 2013). Global transcriptional profiling of TEC between 1-mo and 6-mo of age identified a decrease in E2F3 target genes associated with regulators of cell cycle, such as Aurkb, Cdc20, Cdc6, and Ccnb1, between 1 and 3 months in cTECs and between 1 and 6 months in mTEC^{lo} (Ki, Park et al. 2014). By 10 months, a three-fold reduced rate of TEC proliferation was observed compared to young adult mice (Gray, Seach et al. 2006). The canonical Wnt signalling pathway was also found to be significantly affected by ageing, with increases in Wnt5b which promotes adipogenesis, found in the cortical compartment, and diminished levels of Wnt7a and Wnt3a which promote TEC proliferation (Griffith, Fallahi et al. 2012). Increased oxidative DNA damage was also evident in TEC ageing (Barbouti, Evangelou et al. 2019).

An important role for cTECs is provision of Delta-mediated Notch 4 signals for T-cell lineage commitment (reviewed in (Takahama 2006, Anderson and Takahama 2012)). Notch receptors on lymphoid progenitors interact with Dll4 on cTECs, with thymic crosstalk regulating Dll4 expression during thymic organogenesis. Once reaching maximal thymocyte expansion in the neonatal period, the levels of Dll4 decrease dramatically (Fiorini, Ferrero et al. 2008). However, there was further loss of Dll4 in ageing

TECs in addition to other Foxn1 target genes such as Kit ligand (stem cell factor) and Cxcl12 (O'Neill, Bredenkamp et al. 2016).

Foxn1 expression is essential at multiple stages of TEC differentiation (Nowell, Bredenkamp et al. 2011) and has a role in thymic crosstalk between TECs and developing thymocytes, necessary for thymopoiesis (Su, Navarre et al. 2003). A loss of Foxn1 expression occurs in TECs during ageing with its downregulation implicated in thymus involution (Ortman, Dittmar et al. 2002, Chen, Xiao et al. 2009, Rode, Martins et al. 2015, Barsanti, Lim et al. 2017). Gene analysis studies identified a 2 fold decline in *Foxn1* in both cTEC and mTEC^{lo} subsets between 1 and 6 months (Ki, Park et al. 2014). Genetic approaches demonstrated that induced deletion of *Foxn1* or reduced expression leads to premature thymic involution (Chen, Xiao et al. 2009, Sun, Guo et al. 2010), and overexpression of *Foxn1* in the thymus attenuated age-related decline in thymopoiesis and naïve T cell output (Zook, Krishack et al. 2011). Furthermore, TEC-specific upregulation of Foxn1 in the fully involuted thymus resulted in robust thymus regeneration (Bredenkamp, Nowell et al. 2014). A proportional increase in Foxn1$^-$ TECs was also observed with ageing. Using transgenic mice for lineage tracing, O'Neill *et al.* demonstrated that the Foxn1$^-$ TECs increasing in the adult thymus, were not upstream progenitors, but were generated by the downregulation of *Foxn1* in previously *Foxn1*-positive TECs (O'Neill, Bredenkamp et al. 2016). This population, although decreased in number in the involuted thymus, showed a proportional expansion during ageing, particularly between 1-mo and 3-mo of age.

Alterations in proportions of TEC subsets with ageing also have implications in thymus involution. An accumulation of bipotent TEC progenitors at the expense of downstream

cTEC and mTEC unipotent lineage precursors and mature TECs, points to a loss in TEPC function during ageing (Lepletier, Hun et al. 2019). These increasingly quiescent Sca1^{hi} TEPC were influenced by an altered balance of follistatin, bone morphogenic protein 4 (Bmp4) and activin A signalling during ageing, with increased follistatin production by ageing TECs contributing to their own demise (Lepletier, Hun et al. 2019). Interestingly, *Fst* (encoding follistatin), *Bmp4* and *Inhba* (encoding Activin A) were specifically expressed in the Intertypical TEC sub-cluster described earlier that contains transcriptomic profiles suggestive of TEPC and which accumulates during ageing (Baran-Gale, Morgan et al. 2020).

Early T lineage progenitors have been shown to decline with ageing (Min, Montecino-Rodriguez et al. 2004), but were increased in the aged *Foxn1* overexpressing transgenic mice (Zook, Krishack et al. 2011). Given downregulation of *Foxn1* was more pronounced in cTEC between 1 and 3 months of age in wildtype mice (O'Neill, Bredenkamp et al. 2016), specific cortex downregulation of Foxn1 may be a key driver in early thymus involution, preceding CMJ disorganisation. What causes the downregulation of *Foxn1* is still under consideration.

1.5.3 INFLUENCES OF AN AGEING BONE MARROW ON THYMUS INVOLUTION

Given the bi-directional interactions between developing thymocytes and TECs, the role of ageing BM in provision of hematopoietic precursors for T cell development has also been considered (reviewed in (Dudakov, Khong et al. 2010)).

A numerical accumulation of long-term hematopoietic stem cells (HSCs) in the BM occurs with ageing, and these are functionally less effective as evidenced in their reduced capacity for self-renewal, engraftment and homing following transplantation and long-

term reconstitution potential (Morrison, Wandycz et al. 1996, Kamminga, van Os et al. 2005, Liang, Van Zant et al. 2005). Skewing away from lymphoid differentiation potential (Rossi, Bryder et al. 2005), alteration in p53 expression (Dumble, Moore et al. 2007), accumulation of mitochondrial and genomic DNA mutations (Geiger, Rennebeck et al. 2005, Yao, Ellison et al. 2006) and DNA repairing deficiency (Rossi, Bryder et al. 2007) also occur with ageing.

In addition to cell intrinsic alterations during ageing, changes in the BM niche/microenvironment to a more pro-inflammatory milieu are implicated in degeneration of HSC function (Wagner, Horn et al. 2008, Mayack, Shadrach et al. 2010, Lin, Elias et al. 2021). The BM is a complex multicellular network comprising spatially and functionally distinct niches for HSC subsets with different developmental potential, providing physical interactions and molecular cues essential for HSC localisation, maintenance and differentiation (Asada, Takeishi et al. 2017, Pinho, Marchand et al. 2018, Pinho and Frenette 2019). Ageing related alterations include reduced expression of osteopontin by BM stromal cells (Guidi, Sacma et al. 2017), skewing of mesenchymal stem cell function towards adipogenesis (Singh, Brennan et al. 2016), with the accumulation of adipocytes impacting on haematopoiesis (Naveiras, Nardi et al. 2009). Changes in BM vasculature during ageing, including leakiness and remodelling, can impact on HSC function and differentiation potential (Kusumbe, Ramasamy et al. 2016). With an increase in the incidence of haematological malignancies with ageing, understanding the mechanisms of HSC and niche ageing will be important in developing therapeutic approaches for its rejuvenation, particularly following cytoablative conditioning regimens for HSCT.

However, these age-related alterations in the provision and function of progenitors from an ageing BM niche is unlikely to trigger initial thymic involution, as the latter occurs earlier in life; functional thymic tissue begins to decrease in humans by the second year of life, reduces further with the onset of puberty, and continues to decline with ageing (Kumar, Connors et al. 2018). Moreover, transferring young HSCs or ETPs into aged animals, does not alone induce thymus regeneration (Zhu, Gui et al. 2007). Thus, it is likely that alterations in TEC function, particularly in the cortical compartment, is the primary cause for thymic involution, although, the decline in provision of functional BM derived progenitors exacerbates and prolongs thymic involution (Dudakov, Khong et al. 2010).

1.6 ENDOGENOUS THYMUS RECOVERY FOLLOWING CYTOREDUCTIVE THERAPIES

Cancer is a leading cause of death worldwide, accounting for around 10 million deaths in 2020 (Ferlay, Ervik et al. 2021). To eradicate malignant tumours, systemic chemotherapy and/or irradiation are frequently used, with these standard-of-care treatments considered to be the best approaches to rapidly eliminate proliferating tumorigenic cells. However, side effects of these agents cause profound damage to frequently cycling and proliferating cells, such as hematopoietic cells (including developing B cells, thymocytes, peripheral lymphocytes) and progenitor cells in the small intestine, epidermis, and hair follicles. Stromal cells are considered to be generally radiation resistant due to their lower cycling or post-mitotic states, however, the immune system can take years to fully recover, if at all, in the aged and elderly, indicating longer-term alterations to the BM and thymus niches (reviewed in (Chidgey, Seach et al. 2008, Velardi, Tsai et al. 2021).

1.6.1 BONE MARROW AND THYMUS DAMAGE CAUSED BY CHEMOTHERAPY AND IRRADIATION

The thymus has the capacity for repair in young patients, despite it taking up to 6 months to one year to recover T cells with a broad TCR repertoire (Krenger, Blazar et al. 2011). However, in older patients, where age-related thymic involution coincides with acute thymus damage, there is minimal to virtually no capacity for recovery of thymus function. Ongoing cytoreductive therapies or conditioning regimes associated with hematopoietic stem cell transplantation (HSCT), cause protracted immunodeficiencies, leading to increased morbidity and mortality and cancer relapse. The introduction of reduced-intensity conditioning for allogeneic HSCT with reduced associated risk, has enabled older patients or patients with co-morbidities to receive this treatment for haematological malignancies with better survival outcomes, however life-threatening infections are still a significant problem (Jenq and Van den Brink 2010).

Chemotherapy and irradiation treatment immediately deplete immune cells of both the innate and adaptive immune system. While the innate immune system, including neutrophils, natural killer cells and monocytes, recovers early, recovery of the adaptive immune system takes much longer. Both the BM and thymus microenvironment are significantly affected. A recent study demonstrated specific and prolonged failure in the post-transplant recovery of medullary thymic epithelial cells (mTECs) important to Foxp3$^+$ regulatory T cell development and formation of the intra-thymic dendritic cell pool with consequent implications for induction of autoimmunity (Alawam, Cosway et al. 2021).

Damage to the BM microenvironment has implications for thymus recovery and T cell reconstitution, given the reliance on hematopoietic precursors for T cell development. In fact, 2-month-old male and female mice receiving a single dose of sublethal irradiation,

showed reduced frequency of early thymic progenitors (ETP), 16 months later (Xiao, Shterev et al. 2017). They found damage to BM-derived HSCs was permanent, including Lin⁻Sca-1⁺cKit^hi (LSK) cells which contain lymphocyte progenitors, but this was partially compensated by the thymic microenvironment, with increased proliferation in immature DN thymocytes (Xiao, Shterev et al. 2017). Chemotherapy induced changes in the pro-haematopoietic factors in the bone marrow niche, such as downregulation of Notch ligands Dll4 and Dll1 by vascular epithelium (Tikhonova, Dolgalev et al. 2019), which can induce premature skewing of haematopoietic stem and progenitor cells (HSPCs) toward the myeloid lineage, contributing to the delay in lymphoid cell recovery (reviewed in (Velardi, Tsai et al. 2021)).

In the thymus, in addition to all thymocyte subsets being affected by chemotherapy, particularly DN and DP thymocytes, cTECs and mTECs are also depleted with recent evidence suggested unequal cTEC and mTEC damage by irradiation (Alawam, Cosway et al. 2021). The higher proliferating mature mTEC^hi subset including Aire⁺mTEC^hi, essential for self-tolerance induction and final maturation of developing T cells, are particularly sensitive (Fletcher, Lowen et al. 2009) and almost entirely rely on remaining mTEC lineage precursors (mTEC^lo) for replenishment (Ohigashi, Zuklys et al. 2015). Endothelial cells appear to be less affected by acute thymus injury although changes to vascular architecture were found after total body irradiation (Wertheimer, Velardi et al. 2018).

While chemotherapy/irradiation resistant peripheral T cells have reduced capacity for proliferation and cytokine production, and impaired TCR activation (Li, Wang et al. 2015), they do undergo lymphopenia induced homeostatic expansion but these have a restricted

TCR repertoire and limited antigen specificity, leaving the patient prone to opportunistic infections, cancer relapse and poor clinical outcomes. Moreover, to reduce the rate of malignant relapse, donor T cells are not depleted from HSCT grafts so they can mount an immune response against the tumour (graft-versus-tumour), however, graft-versus-host disease (GvHD) can also ensue with alloreactive donor T cells infiltrating and damaging host tissues (reviewed in (Jenq and Van den Brink 2010). Immunosuppression is used to balance the anti-tumour effects with GvHD, however, over 50% of patients with chronic GvHD still require immunosuppression five years post-transplant (Storb and Sandmaier 2016). Not only is the thymus damaged by GvHD (Clave, Busson et al. 2009) but immunosuppressive treatments can also damage the thymus, particularly mTEC^hi (Fletcher, Lowen et al. 2009), leaving patients susceptible to autoimmunity (Daikeler and Tyndall 2007).

There is a clinical need to enhance immune recovery to reduce adverse outcomes in cancer patients, particularly those receiving allogeneic HSCT and for optimal responses to cancer immunotherapies. Reconstitution of broad TCR diversity in the peripheral T cell pool requires thymus regeneration to produce and export new naïve T cells. Understanding the underlying mechanisms of endogenous thymic repair following damage may provide insight into developing clinical strategies to enhance T cell reconstitution in clinical settings of immune depletion.

1.6.2 MECHANISMS FOR ENDOGENOUS REPAIR

The mechanisms underlying endogenous thymic regeneration are poorly understood, however, recent studies have identified several factors produced by thymic endothelial cells that may play a vital role in TEC repair. Following sub-lethal irradiation, Wertheimer

et al. found a critical role for Bmp4 signalling in thymic regeneration with endothelial cell production of Bmp4 inducing the expression of *Foxn1* in TECs, and its downstream targets such as *Dll4*, important in thymocyte development (Wertheimer, Velardi et al. 2018). Bmp4 receptors (Bmpr2) are expressed by TEPC and lineage specific cTEC^lo and mTEC^lo progenitors (Barsanti, Lim et al. 2017) suggesting an important role for Bmp4 in promoting renewal of TEPC and immature cTEC and mTEC progenitors and/or their differentiation during the recovery process. Significant upregulation of IL-7, Kit-ligand (receptor for c-Kit expressed on ETPs), Cxcl12 and Fibroblast growth factor 7, all having important roles in thymus function, were also identified in gene analysis studies. This study also noted that endothelial cells were resistant to acute injury by sub-lethal irradiation and further confirmed this in scenarios of total body irradiation, cyclophosphamide and dexamethasone treatment, which reflects their significant role in regeneration following thymic insult (Wertheimer, Velardi et al. 2018). Previous work also suggested upregulation of IL-23 by radio-resistant CD103+ dendritic cells induced IL-22 production by lymphoid tissue inducer (LTi) cells, and this generated a cascade of events that support TEC regeneration (Dudakov, Hanash et al. 2012). IL-22 receptor is expressed in both cTECs and mTECs but not thymocytes, and induces *Foxn1* expression and Stat3 phosphorylation (Dudakov, Hanash et al. 2012, Pan, Liu et al. 2014) important for TEC maintenance (Sano, Takahama et al. 2001).

It is yet to be resolved whether thymic regenerative capacity is restricted only to activation of unipotent TEC precursors to replenish mature cTEC and mTECs or additionally involves reactivation of bipotent TEPC.

1.7 STRATEGIES TO ENHANCE THYMUS REGENERATION

Therapeutic restoration of immune competence in immunodeficiency is a major unmet clinical challenge. The therapeutic capacity to manipulate a dysfunctional ageing thymus or replace damaged thymus tissue, would be beneficial in a variety of clinical settings, such as restoring immune system function in the elderly or improving immune recovery following damage by irradiation and chemotherapy associated with cancer treatments and allogeneic HSCT. Promoting tolerance to allogeneic stem cell or organ transplants might also benefit by co-transplanting TECs derived from directed differentiation of donor pluripotent stem cells (Chidgey, Layton et al. 2008).

1.7.1 TRANSPLANTATION

1.7.1.1 Adoptive transfer of in vitro generated lymphoid progenitors or T cells

Adoptive transfer approaches may aid the recovery of thymopoeisis following severe thymus damage by total body irradiation or chemotherapy used in conditioning regimes for allogeneic HSCT (Arber, BitMansour et al. 2003). To overcome the limited availability of BM-derived lymphoid progenitors, *ex vivo* mouse and human culture systems have been developed that provide Notch receptor ligands for the directed differentiation of HSPC into immature T cells (reviewed in (Singh, Mohtashami et al. 2020)).

Immobilized Dll4 in a feeder free system (Reimann, Six et al. 2012) and mouse BM stromal cell line (OP9) monolayers expressing Notch receptor ligands, either Dll1 or Dll4, have been successful in generating large numbers of progenitor T cells from mouse HSPC (Schmitt and Zúñiga-Pflücker 2002), mouse and human pluripotent stem cells (Schmitt, de Pooter et al. 2004, Montel-Hagen, Seet et al. 2019) and human CD34+ cord blood cells (Reimann, Six et al. 2012). When transplanted together with HSCs into a

mouse model of allogeneic HSCT (Zakrzewski, Kochman et al. 2006, Awong, Singh et al. 2013) or into NOD/SCID/γc(-/-) mice (Reimann, Six et al. 2012), these T cell progenitors homed to the thymus and developed into mature, functional T cells that subsequently egressed and accelerated T cell reconstitution. The adoptive transfer of progenitors at the DN2 stage preferentially increased the lymphopoietic compartment (short-term recovery), while transfer of DN3s stimulated the recovery of mTECs (long-term recovery), suggesting transfer of both populations would be optimal (Smith, Reichenbach et al. 2017). However, Smith *et al.,* found that intrathymic migration of the *in vitro* generated progenitor T cells was hindered on irradiated stromal cells compared to healthy stromal cells, implicating functional damage to the thymic microenvironment (Smith, Reichenbach et al. 2017), such as reduced chemokine production (Zhang, Wang et al. 2014). Therefore, to increase the clinical effectiveness of this therapy, better recovery of T cell development may be achieved by combining other strategies that might enhance TEC repair/regeneration, and chemokine production. *In vitro* generated pro-T cells may also have therapeutic applications in generating tumor-infiltrating lymphocytes and chimeric antigen receptor (CAR)-T cells (reviewed in (Singh and Zúñiga-Pflücker 2018)). More recently, a serum-free, 3 dimensional (3D) artificial thymic organoid (M-ATO) system was developed using the MS5 murine bone marrow cell line transduced with human DLL1 (MS5-hDLL1) with greater effectiveness compared to the OP9-DLL1 monolayers, recapitulating human T cell development including positive selection, with seeded human HSPCs (Zhang, Wang et al. 2014) and human pluripotent stem cells (Montel-Hagen, Seet et al. 2019) generating early progenitor T cells which differentiated into functional CD4 and CD8 SP T cells and FoxP3+ regulatory T cells.

In recent advances, cell culture conditions at a single cell level have been developed, to enable more precise interrogation of the molecular programming that regulates long-term HSC properties in quiescence, self-renewal, differentiation and stress response (Oedekoven, Belmonte et al. 2021).

Developing *in vitro* culture conditions that ensure maintenance of HSC self-renewing capacity and function following transplantation, with a goal towards therapeutic applications is challenging. Issues with delayed engraftment, deficiencies in generating lymphoid progenitors, and maintaining capacity to generate daughter HSCs with full multi-lineage potential, need to be overcome. In addition, there is a need to overcome any usage of animal cells or products such as OP9 and MS5 cell lines before clinical translation.

1.7.1.2 Thymus transplantation

Neonatal thymus transplantation has had some success in DiGeorge Syndrome in which patients are congenitally athymic (Markert, Boeck et al. 1999). However, widespread use of thymus transplantation would be limited by donor tissue supply and histocompatibility. With the advent of induced pluripotent stem cell research, the challenge of generating therapeutically suitable TEC *in vitro*, that could form a functional thymus upon transplantation, has been explored in recent years. The enforced expression of Foxn1 in mouse embryonic fibroblasts was sufficient to reprogram these cells into functional TECs, which when transplanted, were able to form a complete functional thymus that supported CD4$^+$ and CD8$^+$ T cell development (Bredenkamp, Ulyanchenko et al. 2014). In an alternative approach, the directed differentiation of human embryonic stem cells and induced pluripotent stem cells into immature Foxn1$^+$ TECs was achieved using

combinations of growth factors, with further differentiation into functional thymic tissue requiring transplantation (Parent, Russ et al. 2013, Sun, Xu et al. 2013, Soh, Giudice et al. 2014). Bioengineered whole thymus organoids have also been investigated using decellularized endogenous thymic tissue (Tajima, Pradhan et al. 2016, Hun, Barsanti et al. 2017) and synthetic matrices (Fan, Tajima et al. 2015, Truong, Hun et al. 2016). However, bioengineered organs are still at the pre-clinical stages of development.

1.7.2 GROWTH FACTORS AND CYTOKINES

The prophylactic administration of growth factors and cytokines have been investigated to protect TECs during pre-transplant conditioning regimes, some of which have been based on knowledge gained from studying mechanisms of endogenous repair following acute thymus damage. These have included keratinocyte growth factor (KGF; also termed fibroblast growth factor 7) (Min, Taylor et al. 2002), KGF in combination with leuprolide acetate (Kelly, Highfill et al. 2008), and transient inhibition of p53 (Kelly, Goren et al. 2010). Exogenous administration of KGF has increased thymic cellularity in mouse models of ageing and following radiation/chemotherapy damage (Min, Panoskaltsis-Mortari et al. 2007) and in HSCT (Seggewiss, Loré et al. 2007) and GvHD (Rossi, Blazar et al. 2002) by enhancing TEC proliferation and function (Rossi, Jeker et al. 2007). However, KGF was recently found to have detrimental effects on recovery of thymic-dependent T cell development in a trial of relapsing-remitting multiple sclerosis (RRMS) patients receiving anti-CD52 antibody for lymphocyte depletion (Coles, Azzopardi et al. 2019). Trials investigating the effects of KGF on T cell reconstitution following HSCT are ongoing (reviewed in (Granadier, Iovino et al. 2021)).

The lymphopoietic cytokine IL-7 has been widely studied for immune regeneration as it can enhance the proliferation of lymphocytes and lymphocyte precursors (Mackall, Fry et al. 2011). Exogenous IL-7 treatment has led to the expansion of CD4+ and CD8+ T cells in patients with HIV infection and recipients of allogeneic HSCT, and increased virus-specific T cells (Levy, Sereti et al. 2012, Perales, Goldberg et al. 2012). A current clinical trial is evaluating the efficacy of recombinant IL-7 on T cell reconstitution in recipients of cord blood HSCT (NCT03941769).

Preclinical studies on the exogenous administration of IL-12, IL-15, IL-21, IL-22 and RANKL have individually, shown some potential in improving thymic function in age-related involution or after acute damage (Li, Hsu et al. 2004, Dudakov, Hanash et al. 2012, Dudakov, Hanash et al. 2015, Lopes, Vachon et al. 2017, Tormo, Khodayarian et al. 2017) and facilitating engraftment of hematopoietic progenitors after irradiation (Alpdogan, Eng et al. 2005, Chen, Burke et al. 2007, Pan, Wang et al. 2019).

1.7.3 HORMONES: EXOGENOUS ADMINISTRATION AND INHIBITION

1.7.3.1 Exogenous administration of hormones

Growth hormone has been extensively studied in the clinic and has shown some efficacy in regenerating the aged thymus (Taub, Murphy et al. 2010), enhancing BM repair following irradiation (Carlo-Stella, Di Nicola et al. 2004) and enhancing thymus function in HIV-1 patients evidenced by an increase in circulating CD4+ T cells and recent thymic emigrants (Napolitano, Schmidt et al. 2008, Fahy, Brooke et al. 2019) and improving their antiviral responses (Herasimtschuk, Westrop et al. 2008).

A study by Dixit *et al.,* showed that ghrelin declines with age and exogenous administration of ghrelin significantly improved thymopoiesis and the thymic architecture

in the aged thymus (Dixit, Yang et al. 2007). Thymosin-α1 is produced by TECs and regulates survival and proliferation of developing thymocytes. Phase I/II clinical trials in which thymosin-α1 was administered to recipients of allogeneic HSCT, showed enhanced T cells numbers and earlier appearance of pathogen-specific T cell responses against cytomegalovirus and *Aspergillus* species (Perruccio, Bonifazi et al. 2010). Interestingly a recent study demonstrated treatment with thymosin-α1 could reduce mortality in patients with severe coronavirus disease by restoring lymphocytopenia (Liu, Pan et al. 2020).

1.7.3.2 Inhibition of sex hormones

The immune-endocrine axis has been well described, with associations made between the rise in sex hormone production at puberty and decline in thymus and BM function, leading to deterioration in thymopoiesis, B-lymphopoiesis and availability of lymphoid precursors (Chidgey, Dudakov et al. 2007, Calder, Hince et al. 2011). Exogenous administration of androgen and estrogen induces thymic atrophy (Zoller and Kersh 2006, Velardi, Tsai et al. 2014) and conversely, gonadectomy induces enlargement of thymic tissue, an observation dating as far back as 1898 (Calzolari 1898).

Extensive studies on the removal of sex hormones by surgical gonadectomy in young male mice (Heng, Goldberg et al. 2005, Dudakov, Khong et al. 2010) and preclinical male mouse models of ageing and autologous- and allogeneic-HSCT, have demonstrated regenerative effects on both the thymus and BM, and enhanced immune reconstitution and hematopoietic recovery following HSCT (Goldberg, Sutherland et al. 2005, Sutherland, Goldberg et al. 2005, Dudakov, Goldberg et al. 2009, Dudakov, Goldberg et al. 2009, Khong, Dudakov et al. 2015). Surgical castration also induced increases in cellularity of the secondary lymphoid organs such as lymph nodes and spleen (Aboudkhil,

Bureau et al. 1991, Roden, Moser et al. 2004) and reduced the proportion of memory T cells consistent with an increased *de novo* production of thymic derived naïve T cells (Sutherland, Goldberg et al. 2005). However, it has also been demonstrated that thymus regeneration following surgical castration is only temporary and this may be due to persistent age-related qualitative changes in the medullary compartment (Griffith, Fallahi et al. 2012). This highlights the fact that sex hormone production is not the only mechanism underlying thymus involution during ageing. Nevertheless, a wave of newly produced naïve T cells may be adequate to rejuvenate the peripheral T cell pool.

Reversible sex hormone inhibition can also be achieved by administration of luteinising hormone-releasing hormone (LHRH) receptor analogues. LHRH receptor analogues are clinically approved pharmaceuticals used in the treatment of prostate cancer, endometriosis and breast cancer. LHRH is normally produced by the hypothalamus and acts on the pituitary to produce luteinising hormone (LH) and follicular stimulating hormone (FSH). These hormones stimulate the gonads to produce testosterone (in males) and estrogen (in females), which in turn acts on the production of LHRH and LH/FSH in a negative feedback system (**Figure 1.5**).
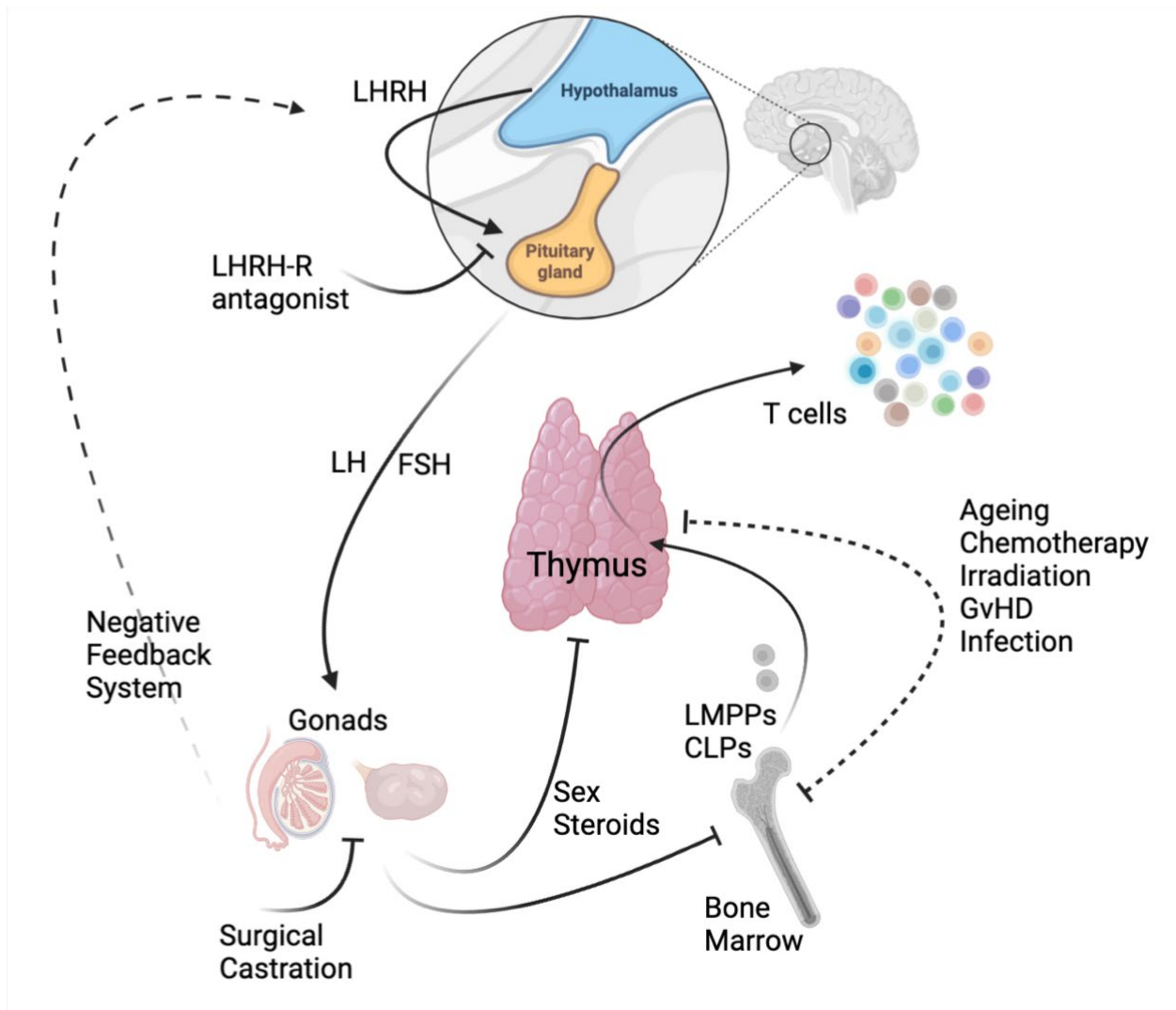
Figure 1.5 Overview of the hypothalamic-pituitary-gonadal-immune axis. T cells produced in the thymus originate from circulating bone marrow derived T lineage progenitors, such as lymphoid primed multipotent progenitors (LMPPs) and common lymphoid progenitors (CLPs). Luteinising hormone releasing hormone (LHRH) produced by the hypothalamus, binds to LHRH receptors on the pituitary, inducing the production of luteinising hormone (LH) and follicular stimulating hormone (FSH). These act on the gonads to produce sex steroids in a negative feedback system. Sex steroids have a negative impact on the thymus and bone marrow, in addition to ageing, chemotherapy and irradiation treatment, infections and graft versus host disease (GvHD). Surgical castration in males can regenerate the ageing thymus and bone marrow. LHRH analogues (agonist and antagonist), which can inhibit the production of sex steroids in a reversible manner, are being explored as a clinical strategy for thymus and bone marrow recovery following damage.

Continued exposure to LHRH receptor agonists (eg. leuprolide acetate), hyperstimulates the LHRH receptor on the pituitary, leading to an initial surge in LH/FSH followed by increased sex hormone production in the first week, before continued LHRH receptor hyperstimulation blocks production of LH and FSH. Cessation of sex hormone production is generally achieved by day 21 of treatment (Thompson 2001). This initial flare in sex hormone production and associated clinical symptoms is considered a major drawback of this current treatment (Engel and Schally 2007). While the sex hormone surge negatively impacts the BM and thymus initially, sex steroid inhibition-induced regeneration becomes evident by day 28 after treatment (Sutherland, Goldberg et al. 2005, Sutherland, Spyroglou et al. 2008).

Alternatively, LHRH-receptor antagonists block signalling through the LHRH receptor (**Figure 1. 5**), with LH, FSH and sex hormones reduced within 24-48 hours (Velardi, Tsai et al. 2014). This has the clinical advantage of avoiding the sex hormone flare and initial thymic damage, and a shortened treatment period. As such, both Degarelix and Cetrorelix (and other LHRH receptor antagonists) have become the drug of choice in prostate and ovarian cancer treatments (Reissmann, Schally et al. 2000, Velardi, Tsai et al. 2014). The LHRH receptor antagonist may also block LHRH receptors on other cell populations, potentially providing additional direct effects.

The mechanisms underlying thymus regeneration following sex hormone inhibition, are not well understood. However, recent studies in surgically castrated male middle-aged mice have implicated reactivation of adult TEC progenitors, possibly through reversal of the age-related increase in follistatin production by cTECs and fibroblasts, allowing increased activin A signalling proposed to be important in TEPC and TEC differentiation

(Lepletier, Hun et al. 2019). Other studies have proposed upregulation of the Notch ligand Dll4 on cTECs, important for T lineage commitment (Velardi, Tsai et al. 2014), increased thymic CCL25 important for importation of hematopoietic progenitors from the circulation into the thymus (Williams, Lucas et al. 2008), and regenerated bone marrow with improved potential of HSCs and differentiation into lymphoid progenitors.

Conclusion

The thymus provides an inductive environment for the generation of a broad TCR receptor repertoire of naïve T cells. However, thymus involution begins early in life with further degeneration at the onset of puberty and during ageing. This deterioration in thymic function can also be exacerbated by acute and chronic damage from infections, multidose chemotherapy and other cytoablative cancer treatments such as total body irradiation used in conditioning regimes for allogeneic HSCT. Prolonged immune deficiency following acute injury associated with cytoablative treatments leads to increased morbidity and mortality. There are currently no approved clinical therapies to enhance T cell recovery in patients with lymphopenia. Based on pre-clinical and clinical research, proposed therapeutic strategies with real clinical potential include adoptive transfer of *in vitro* generated progenitor T cells, exogenous administration of cytokines and growth factors, and temporary sex steroid inhibition, each approach involving different signaling pathways and focus in the process of thymus regeneration. However, sex steroid inhibition appears to have a broader impact on immune recovery with rejuvenation evident in both the BM and thymus, compared to single targeted approaches.

This project follows on from previous research in our laboratory into the effects of androgen inhibition on thymus regeneration in middle aged male mice. It begins with

investigations into the effects of a third generation LHRH receptor antagonist on regeneration of thymopoiesis and TEC subsets in middle-aged male mice. Thymic involution has predominantly been studied in ageing male mice therefore a comparative analysis of pre-pubertal TEC subsets in females and males and their alterations at the onset of puberty and in middle age was performed, and the impact of LHRH receptor antagonist treatment in healthy middle-aged female and male mice. Finally, we investigated the TEC subsets most susceptible to chemotherapy treatment in 9-mo middle-aged female and male mice, and the rate of endogenous repair in comparison to mice treated with the LHRH receptor antagonist, Degarelix. The study focused on early recovery stages to elucidate the roles of TEPC and and/or unipotent lineage specific precursors (immature cortical and medullary epithelial cells) in endogenous repair vs LHRH receptor antagonist-induced repair.

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CHAPTER 2:

EFFECTS OF LHRH RECEPTOR ANTAGONIST TREATMENT ON THYMOPOIESIS IN THE AGEING MALE MOUSE

2.1 INTRODUCTION

Thymic stromal cells, comprised of thymic epithelial cells (TECs), thymic fibroblasts, endothelial cells and antigen presenting cells make up only 1-2% of the total cellularity of the young thymus (Gruver, Hudson et al. 2007). Yet they provide the thymic microenvironment necessary to support the other 98-99% of cells undergoing T cell development, with TECs being critical for T cell production. The thymus is anatomically structured into discrete cortical and medullary regions containing phenotypically and functionally distinct cortical TEC (cTEC) and medullary TEC (mTEC), and developing T cells (thymocytes) at defined stages of maturation (Prockop and Petrie 2000). The stepwise progression of thymocyte development through these regions, involving interactions with cTECs and mTECs (reviewed in (Takahama 2006)), is critical for generating a diverse pool of self-tolerant, naïve T cells. However, despite its importance, the thymus progressively declines in cellularity and function throughout life (Gruver, Hudson et al. 2007).

Thymic involution occurs in all vertebrates and is an evolutionary conserved event (Torroba and Zapata 2003). It begins early in life in humans and is most evident in mice from the onset of puberty, coincident with the increased production of sex steroids (Gruver, Hudson et al. 2007). It is characterised by a reduction in thymic size and cellularity and eventually, alterations in thymic architecture and microenvironment. The resulting gradual reduction in production of naïve T cells leads to an increasingly restricted T cell receptor (TCR) repertoire diversity in the periphery and reduced capacity to fight infections and cancer in the ageing population (Nikolich-Žugich, Slifka et al. 2004). Further thymic damage from immunosuppression and cytoablative treatments, such as

chemotherapy and radiotherapy used in cancer treatments and hematopoietic stem cell (HSC) transplantation (HSCT), can compound thymic involution (Fletcher, Lowen et al. 2009) and result in a greater delay in T cell recovery in the ageing population. Prolonged T cell deficiency can lead to increased morbidity and mortality due to opportunistic infections and malignant relapses. As such, various strategies are being explored with the aim to improve reconstitution of a broad T cell repertoire pool following damage in the aged population.

Given the significant role of sex steroids in post-pubertal thymic involution (Calder, Hince et al. 2011), many studies have investigated sex-steroid inhibition (SSI) as one approach to regenerate thymic cellularity and the naïve T cell pool. Pre-clinical studies utilised surgical gonadectomy to achieve androgen deprivation in young and ageing male mice, and in mouse models of chemotherapy, irradiation and bone marrow/HSCT (Goldberg, Sutherland et al. 2005, Heng, Goldberg et al. 2005, Sutherland, Goldberg et al. 2005, Goldberg, Alpdogan et al. 2007, Dudakov, Goldberg et al. 2009, Dumont-Lagacé, St-Pierre et al. 2015). These studies highlighted the plasticity of the ageing thymus in its capacity to rejuvenate thymopoiesis following androgen deprivation, albeit temporarily (Griffith, Fallahi et al. 2012). They also demonstrated an involvement of the bone marrow with respect to provision of hematopoietic T cell precursors following SSI, but not necessarily in the initiation of thymus regeneration (Dudakov, Goldberg et al. 2009, Khong, Dudakov et al. 2015). While the underlying mechanisms in thymus regeneration following SSI have not yet been fully resolved, Lepletier et al. proposed involvement of the activin A-follistatin-bone morphogenic protein 4 (Bmp4) axis in reactivation of bipotent

thymic epithelial progenitor cells in middle-aged castrated male mice (Lepletier, Hun et al. 2019).

A more clinically acceptable method of SSI utilises luteinizing hormone-releasing hormone (LHRH) receptor analogues to disrupt the hypothalamus-pituitary-gonadal (HPG) axis. LHRH receptor analogues block the production of sex steroids either through agonistic or antagonistic actions on the LHRH-receptor (Kovacs and Schally 2001). Administration of LHRH receptor agonists (LHRH-Ag) induce an initial surge in sex steroid production, before desensitisation of the pituitary LHRH receptors (Van Poppel and Nilsson 2008). With continuous exposure, sex hormone production drops to castrate levels after 21 days. In males, this initial flare in testosterone levels can further exacerbate thymus atrophy before androgen deprivation induced regeneration occurs. Despite initial thymic damage, thymic regeneration was evident one week after testosterone production had subsided to castrate levels (28 days following treatment). Our group and others have demonstrated LHRH-Ag induced thymus regeneration in mouse models of ageing and allogeneic HSCT (Marchetti, Guarcello et al. 1989, Goldberg, King et al. 2009). Thymic regeneration was demonstrated by increased thymic cellularity, increased lymphoid and myeloid bone marrow progenitors, and enhanced thymic reconstitution following bone marrow transplantation or HSCT, which led to enhanced peripheral T cell recovery without exacerbating graft-versus-host-disease. LHRH-Ag treatment was also investigated in clinical studies, showing increases in CD4 and CD8 T cell recovery and naïve T cells positive for T cell receptor excision circles (TREC[+]), in prostate cancer patients (Sutherland, Goldberg et al. 2005) and in recipients of allogeneic and autologous HSCT patients (Sutherland, Spyroglou et al. 2008).

An alternative approach to achieve SSI, is via LHRH receptor antagonists (LHRH-Ant). LHRH-Ant block the LHRH receptor, precluding the initial spike in sex steroids induced by LHRH-Ag (reviewed in (Reissmann, Schally et al. 2000)), and therefore offering a safer approach for immune regeneration. With more effective LHRH-Ant pharmaceuticals now available, Velardi *et al*, showed that treatment with a third generation LHRH-Ant, Degarelix (Firmagon®), reduced testosterone levels within 1-2 days of treatment in male mice. Increased total thymus cellularity was evident by day 7 post treatment, and this was associated with increased Delta-like 4 (DLL4) expression in cTECs (Velardi, Tsai et al. 2014). Moreover, Velardi and colleagues more recently found improved HSC recovery and survival when Degarelix was administered within one day following total body irradiation in a mouse model (Velardi, Tsai et al. 2018). They found an important role for luteinizing hormone (LH) levels in regulating HSC renewal. With LHRH-Ant induced LH suppression, HSCs were induced into quiescence, which protected them from exhaustion following TBI (Velardi, Tsai et al. 2018).

In this chapter, we investigated LHRH-Ant, Degarelix treatment on thymus regeneration in healthy middle-aged male mice, with a focus on thymopoiesis. Given the relatively rapid inhibition of testosterone production by LHRH-Ant compared to agonist analogues, we were interested in identifying earlier post-treatment changes in major thymocyte and TEC subsets, the sequence of regeneration, and the role of proliferation in the regenerative process.

2.2 MATERIALS AND METHODS

2.2.1 ANIMALS

C57BL6/J male mice aged between 7-12 months (middle aged) and 2-4 months (young adult) mice were used for our studies following the guidelines of the Australian National Health and Medical Research Council's Published Code of Practice for the Use of Animals in Research. Mice were obtained from the Monash Animal Research Platform and housed at the Animal Research Laboratory (Monash University, Australia) where they were maintained in a controlled environment and a standard diet and water *ad libitum*. All experiments were approved by the Monash University Animal Ethics Committee of Monash University (SOBSA/ADB/2015/039).

2.2.2 LHRH-ANTAGONIST TREATMENT

Chemical castration was achieved by a single subcutaneous injection of Degarelix (Firmagon®) at a concentration of 78µg/g per mouse (3-month depot), injected subcutaneously 48 hours prior (Day -2) to allow time for testosterone production to reach castrate levels by Day 0 (D0). Mice were analysed at various timepoints thereafter, following euthanisation by CO_2 asphyxiation.

2.2.3. INDIVIDUAL THYMUS DIGESTION FOR FLOW CYTOMETRY

Thymus digestion was based on our previously publication protocol (Seach, Wong et al. 2012). Mice were asphyxiated with CO_2 and thymuses extracted into RPMI before they were cleaned of fat and connective tissue. Thymic lobes were snipped with fine scissors and enzymatically digested individually in 0.0185% (w/v) Liberase Thermolysin Medium (Roche, Germany) and 0.02% (w/v) DNAse I in RPMI-1640 for 15 minutes (min) in a water

bath at 37°C. After incubation, fragments were agitated for 3-5 min using a wide-bore pipette tip and allowed to settle. The supernatants were collected in 2mL of cold fluorescence-activated cell sorting (FACS) buffer (0.1% BSA and 5 mM EDTA in PBS) to neutralize enzymatic activity. These steps were repeated, replacing fresh enzymes and using progressively smaller bore pipettes to gently disrupt remaining thymic fragments, until fully digested. After digestion, pooled thymic fractions were filtered through 100µM sterile nylon mesh followed by centrifugation at $500g_{max}$ for 3min at 4^0C. Cells were counted using a Z2 Coulter Counter (Beckman Coulter, U.S.A).

2.2.4 CELL SURFACE STAINING FOR FLOW CYTOMETRY

Cells were resuspended in primary antibody cocktail at a concentration of $1x10^6$ cells per 10µL and incubated for 15 min at 4°C in the dark. Cells were then washed with FACS buffer to remove any unbound antibody and centrifuged at $500g_{max}$ for 3 min. Cells were then resuspended in secondary antibody where relevant for 15 min at 4°C. Stained cells were then washed, resuspended in FACS buffer and filtered into round bottom polystyrene tubes. Lastly, propidium iodide (PI; Sigma Aldrich, U.S.A.) was added at a final concentration of 100ng/ml to exclude dead cells for live stain analysis.

Intracellular staining was used to investigate cell proliferation (Ki67) and Foxp3 expression (T regulatory cells; Tregs) in TEC and T cell populations. Cells stained with extracellular markers were fixed using Cytofix™ buffer (eBioscience U.S.A.) for 30 min at 4°C, according to manufacturer's instructions. Cells were then washed with Perm-wash buffer (BD Biosciences, U.S.A.), centrifuged at $500g_{max}$ for 3 min, and stained with intracellular antibodies or their isotype controls, for 30 min at 4°C. Stained cells were then

washed, resuspended in FACS buffer following centrifugation, and transferred into round bottom polystyrene tubes for FACS analysis.

Antibodies used to analyse thymocyte populations included: CD45, CD4, CD8, CD44, CD25, CD69, MHCI, Foxp3 (T regulatory cells), CD117 (Early T cell progenitor; ETP), Ki67 (proliferation). Antibodies used to analyse TEC populations included: CD45 (thymocytes), EpCAM (thymic epithelial cells), UEA-1 (mTEC), MHCII (TEC maturity), Ki67 (proliferation). Antibodies were obtained from BioLegend (USA) or BD Biosciences (USA).

2.2.5 FLOW CYTOMETRY- ACQUISITION AND ANALYSIS

Using the BD FACS Canto™ II flow cytometer (BD Biosciences, U.S.A.), stained cells were acquired using up to 8 channels with parameter, voltage and compensation settings established using BD FACSDiva v.6 software (BD Biosciences, U.S.A.). FCS file data were analysed using Flowlogic™ v7.2 (Inivai technologies, Australia).

2.2.6 STATISTICAL ANALYSIS

Statistical analysis was performed using Graph Pad Prism v7.0 software and IBM SPSS statistics (version 28). Following data normality assessments (*Shapiro-Wilk* normality test), independent *One-* or *Two-Way ANOVA* tests were run, with the appropriate post-hoc t-test performed for parametric data. Results are expressed as mean +SEM. A p value of less than 0.05 was considered statistically significant.

2.3 RESULTS

2.3.1 THYMOCYTE REJUVENATION FOLLOWING DEGARELIX ADMINISTRATION

To examine thymic effects of LHRH-Ant, middle-aged male mice were administered a single subcutaneous injection of Degarelix (Firmagon®) at a concentration of 78µg/g. Given testosterone levels subside within 24-48 hours following Degarelix treatment (Velardi, Tsai et al. 2014), we designated the day of treatment as day (D) -2, with castrate levels achieved by D0. Three cohorts with 4 mice per group were studied; untreated young adult (2-4 mo old) group, a middle-aged PBS treated control group, and the Degarelix treated group. Mice were sacrificed at D4, D7 and D14 **(Figure 2.1A)**. Thymuses were enzymatically digested and analysed by flow cytometry for changes in proportion and number of thymocyte subsets (FACS gating strategy shown in **Figure 2.1B**). Ageing results in a significant numerical loss in total thymocytes compared to young (young 132.5×10^6 ($\pm 2.96 \times 10^6$); aged D4 PBS control 92.3×10^6 ($\pm 8.53 \times 10^6$); p=0.002). A significant increase in total thymic cellularity was evident from D4 in the Degarelix treated cohort compared to PBS controls (**Figure 2.1C**).

A

LHRH-ant
S.C 78µg/g

Analyses

Day -2 -1 0   Day 4   7   14

B

non-debris

SSC-A
FSC-A

Viable cells

PI
FSC-A

TECs

Thymocytes

EpCAM
CD45

6.2 | 85
4.6 | 1.1

CD4
CD8

C

Young
PBS
Degarelix

Cell number/thymus
(x10⁸)

Young   D 4   D 7   D14

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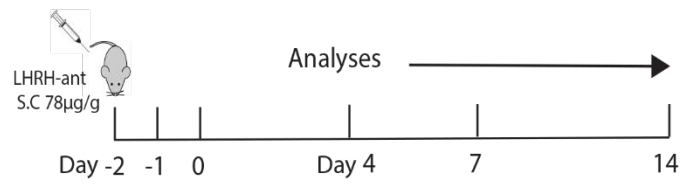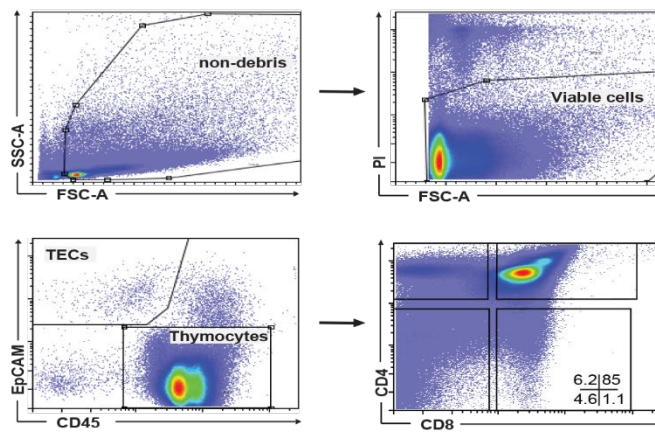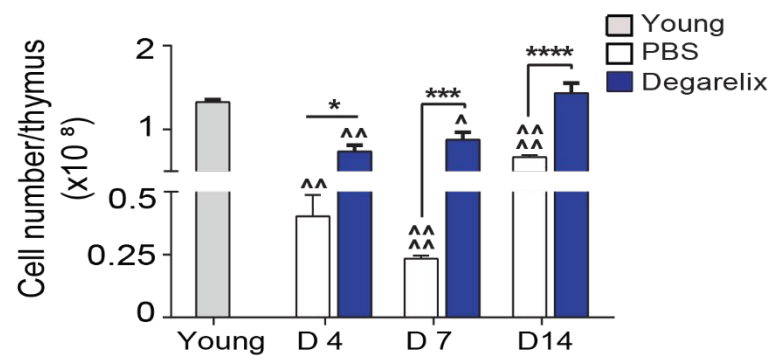
Figure 2.1 Analysis of thymocytes by flow cytometry following Degarelix treatment in male middle-aged mice shows significant numerical increase by day 7. (**A**) Timeline of Degarelix (Deg) treatment and analysis timepoints. (**B**) Flow cytometry gating strategy to analyse thymocytes. (**C**) Total thymus cellularity in untreated young male mice (grey bar), PBS treated middle-aged male controls (PBS; white bars) and Degarelix treated middle-aged male mice (Deg; blue bars) at D4, D7, D14. Data are expressed as mean +SEM (n=4 per group; >1 independent experiment). * denotes significant difference in Degarelix treated mice compared to PBS treated controls, where ***$p<0.001$ and ****$p<0.0001$, using Mixed-design Two-Way Repeated measures ANOVA with Bonferroni and R-E-G-W-Q post hoc multiple comparisons.

2.3.2 INCREASED PROLIFERATION IN THYMOCYTE SUBSETS CONTRIBUTE TO DEGARELIX INDUCED THYMUS REGENERATION

We investigated early phenotypic changes in broad thymocyte subsets, from D4, based on CD4 and CD8 expression. Flow cytometric analyses of CD4$^-$CD8$^-$ double negative (DN), CD4$^+$CD8$^+$ double positive (DP), CD4$^+$ single positive (SP) and CD8$^+$ SP T cells in young male mice and in middle-aged male mice after Degarelix or PBS treatment are shown in **Figure 2.2 A**.

Only the CD8 SP T cell subset showed a significant proportional change with ageing, demonstrating a more than two-fold drop between young (3.97%) and middle-aged mice (D4 PBS controls; 1.67%, p=0.0015) (**Figure 2.2 B**). Treatment with Degarelix did not result in any major proportional changes, however there was an initial significant increase in DP T cells and drop in proportion of CD4$^+$ T cells at D4 and D7, which was resolved by D14.

Ageing induced a numerical loss in all thymocyte subsets. The DN subset decreased from 4.16x10^6 ±0.17x10^6 in young to 1.74 x10^6 ±0.34 x10^6 in middle-aged mice (D4 PBS controls); DP cell counts decreased from 8.1x10^7 ±2.4x10^6 in young to 2.76 x10^7 ±6.4 x10^6 in middle-aged mice (D4 PBS controls); CD4$^+$ SP from 7.5 x10^6 ±0.4 x10^6 in young to 2.65x10^6 ±0.66x10^6 in middle-aged mice (D4 PBS controls); and CD8+ SP from 3.86x10^6 ±0.28x10^6 in young to 0.56x10^6 ±0.1x10^6 in middle-aged mice (D4 PBS controls) (**Figure 2.2 C).**

Following Degarelix treatment, an early statistically significant numerical change in the DN subset was evident from D4 (3.2 x10^6 ±0.15 x10^6 compared to PBS controls 1.7 x10^6 ± 0.34 x10^6), followed by a more than 4-fold increase in DPs from D7 (6.8x10^7 ±7.1 x10^6 compared to PBS controls 1.6x10^7 ±1.1 x10^6), and increase at D14 in CD4 SP T cells

($7.2 \times 10^6 \pm 1.1 \times 10^6$ compared to PBS controls $2 \times 10^6 \pm 0.12 \times 10^6$) and CD8 SP T cells ($2.5 \times 10^6 \pm 0.47 \times 10^6$ compared to PBS controls of $0.94 \times 10^6 \pm 0.05 \times 10^6$) (**Figure 2.2 C**). This suggests a normal sequence of maturation during Degarelix induced thymus regeneration. An early increase in proliferation at D4 across all subsets contributed to this numerical increase **(Figure 2.2 D)**. Degarelix treatment induced a statistically significant increase in the proportion of Ki67$^+$ cells across all subsets at D4, and this increase was maintained in DN cells across all timepoints analysed **(Figure 2.2 D),** with numerical increases in Ki67$^+$ cells evident in all subsets by D14 as the thymus expanded in cellularity **(Figure 2.2 E)**. These results suggest early increases in proliferation as one mechanism that contributes to the numerical increase in thymocyte cellularity.
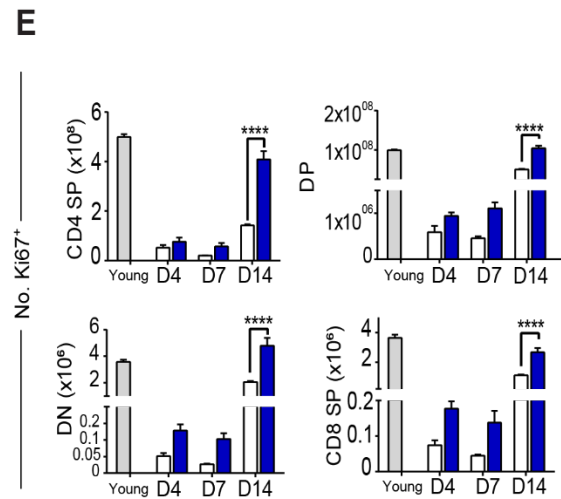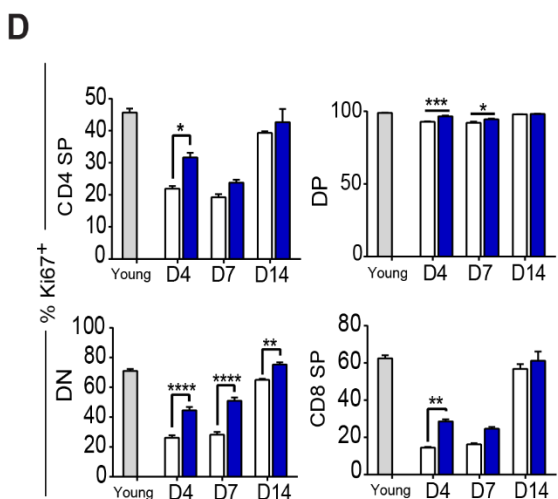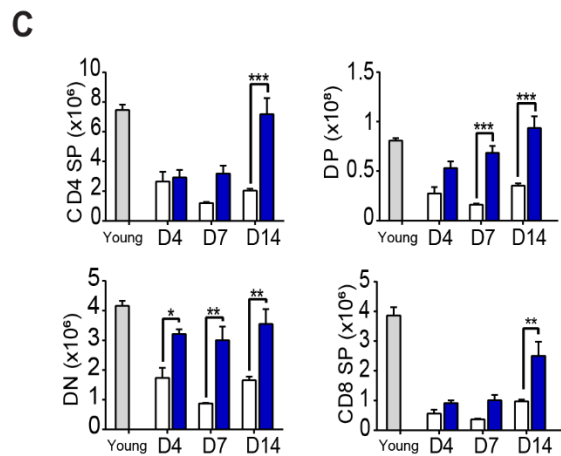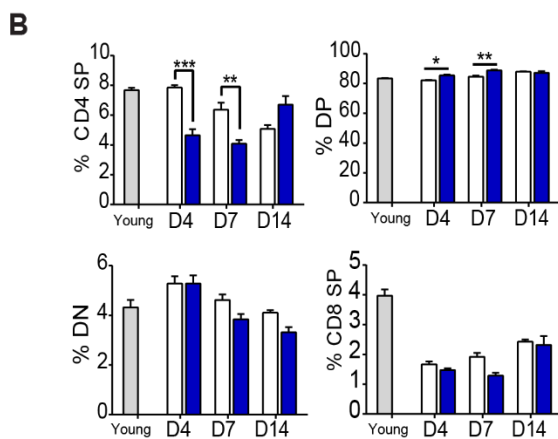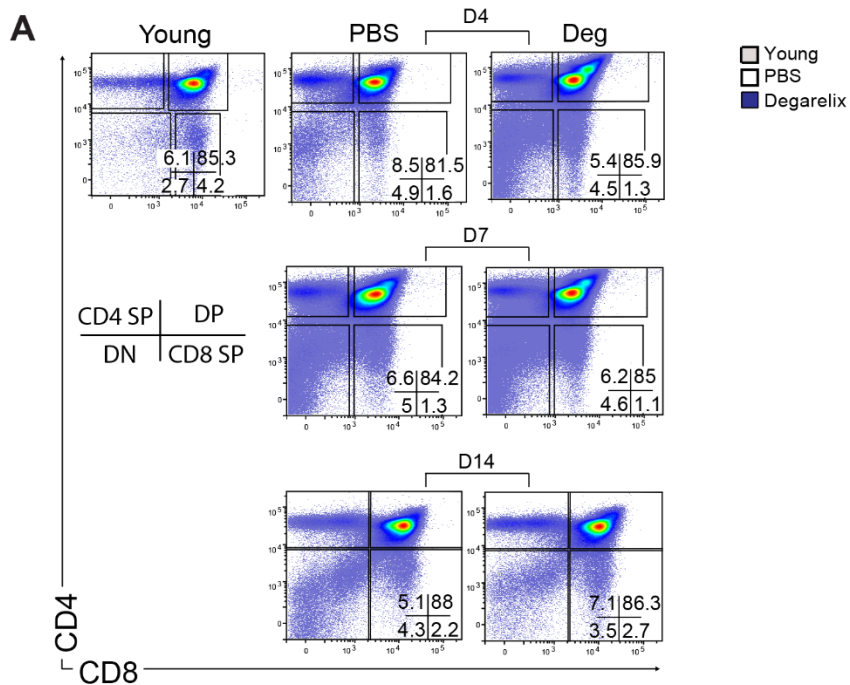
Figure 2.2 Increased thymocyte proliferation contributes to thymus regeneration after Degarelix treatment. (A) Representative dot plots show the proportions of T cells subsets; CD4 single positive (SP), CD8 SP, double positive (DP) and double negative (DN), in young and middle-aged mice treated with either PBS or Degarelix. Bar graphs show the proportions (**B**) and cell count **(C)** for each of these four subsets. Proportions **(D)** and number **(E)** of Ki67+ cells in young (grey bars), middle-aged mice treated with either PBS (PBS; white bars) or Degarelix (Deg; blue bars) for each of the T cell subsets are shown as bar graphs. Data are expressed as mean +SEM (n=4 per group; >1 independent experiment). * denotes a significant difference in Degarelix treated mice compared to PBS treated controls, where *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001. Data is representative of 1 experiment, using Mixed-design Two-Way Repeated measures ANOVA with Bonferroni and R-E-G-W-Q post hoc multiple comparisons.

2.3.3 EARLY REJUVENATION OF CD4-CD8- SUBPOPULATIONS WITH DEGARELIX TREATMENT

An increase in total thymus cellularity was evident from D4 with Degarelix treatment (**Figure 2.3 A**), and we found in the previous section that the DN subset was first to show significant regeneration in Degarelix treated mice at D4. We therefore further analysed the DN subset for changes in each of the major DN subpopulations. The phenotype of the four DN subpopulations are based on CD44 vs CD25 expression, DN1 ($CD44^+CD25^-$), DN2 ($CD44^+CD25^+$), DN3 ($CD44^-CD25^+$) and DN4 ($CD44^-CD25^-$). Representative dot plots from young, middle-aged PBS controls and middle-aged Degarelix treated DN subsets are represented in **Figure 2.3 B**. The proportions of each DN subset in Degarelix treated mice did not significantly alter during the early regenerative process (**Figure 2.3 C**), aside from an initial drop in the DN1 subset at D4 (3.68% ±0.4%) compared to PBS controls on the day (7.21% ±0.4%). Apart from no numerical change in the DN1 subset at D4, all other subsets demonstrated increased cellularity at all timepoints analysed (**Figure 2.3 D**). Early T lineage progenitors (ETPs) are found in the DN1 population (Porritt, Rumfelt et al. 2004) and defined by their positive c-Kit/CD117 expression (**Figure 2.3 E**). There there were no proportional changes in ETPs at each time point analysed. While increasing numerical trends were evident earlier, by D14 a significant three-fold numerical increase was evident with 1.5 $\times10^4$ ±0.4 $\times10^4$ ETPs in the Degarelix treated group compared to 0.55 $\times10^4$ ±0.015 $\times10^4$ in the PBS control group (**Figure 2.3 F**). Increases in cell number were not reflected in proportional alterations in the DN subsets or ETPs during the regenerative process to day 14, and as such no preference to a particular DN subset during thymus expansion. Therefore, collectively, these results

suggest Degarelix-induced expansion in DN subsets occurs through a normal process of

T cell development.
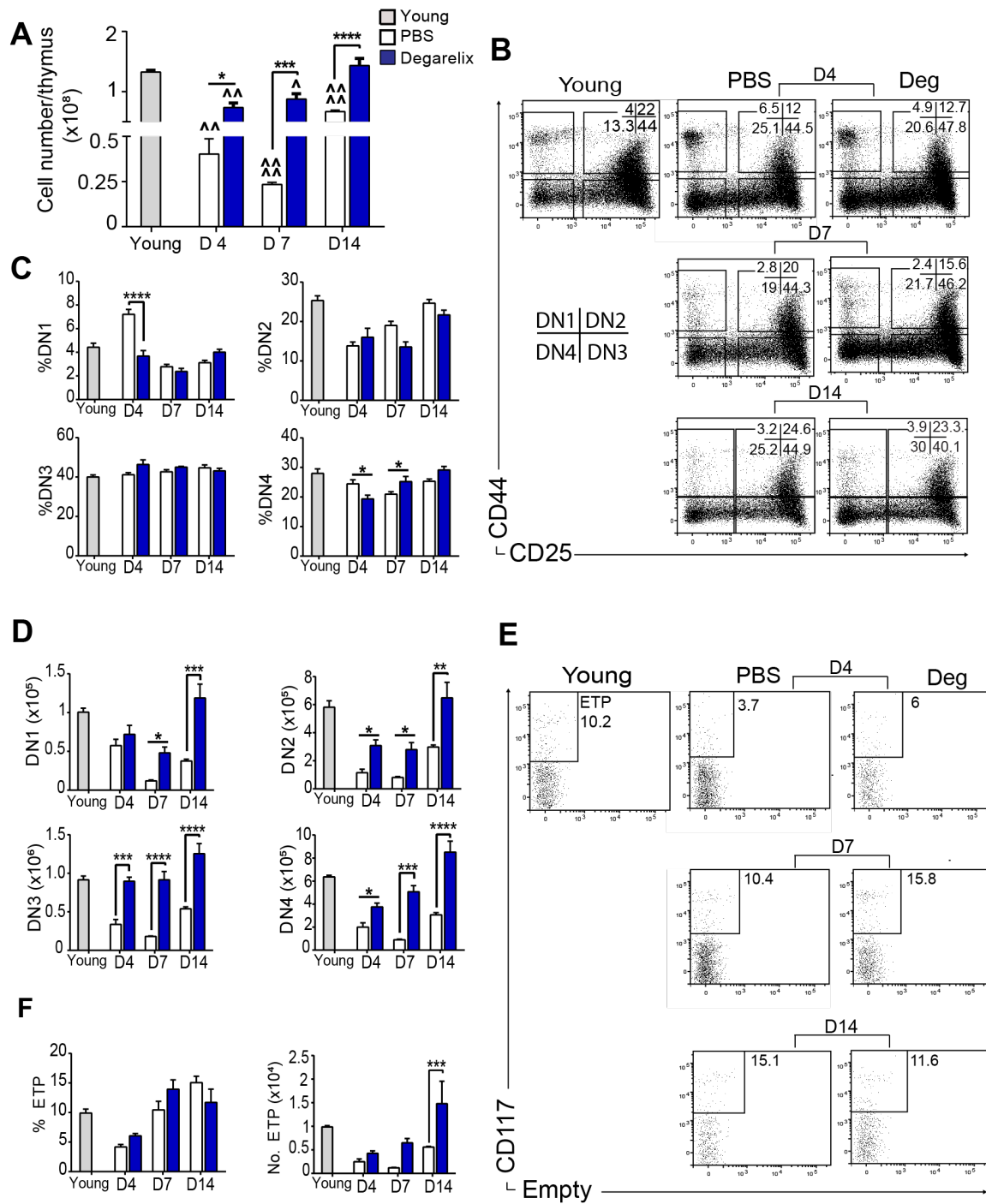
Figure 2.3 Numerical increase in CD4-CD8- subpopulations, including ETPs, with Degarelix treatment. **(A)** Total thymus cellularity of young (grey bars) and middle-aged male mice treated with either PBS (PBS; white bars) or Degarelix (Deg; blue bars). Proportions of the double negative subsets; DN1 (CD44$^+$CD25$^-$), DN2 (CD44$^+$CD25$^+$), DN3 (CD44$^-$CD25$^+$) and DN4 (CD44$^-$CD25$^-$) within the DN subset are shown as representative dot plots **(B)** or bar graphs (**C**) with cell counts shown in **(D)**. Representative dot plots **(E)** and bar graphs **(F)** display the proportion and number of ETPs. Data are expressed as mean +SEM (n=4 per group; >1 independent experiment). *denotes significant difference in Degarelix treated mice compared to PBS treated controls, where *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001, using Mixed-design Two-Way Repeated measures ANOVA with Bonferroni and R-E-G-W-Q post hoc multiple comparisons.

2.3.4 NORMAL PROCESS OF INTRATHYMIC CD4$^+$ AND CD8$^+$ T CELL MATURATION FOLLOWING DEGARELIX TREATMENT

To further assess the effect of LHRH-Ant on the numerical increase in SP T cells by D14, we analysed the three stages of development within CD4$^+$ and CD8$^+$ SP T cells; CD69$^+$MHC-I$^-$ Semi-mature (SM), CD69$^+$MHC-I$^+$ mature 1 (M1) and CD69$^-$MHC-I$^+$ mature 2 (M2) T cells (Kishimoto and Sprent 1997, Kurobe, Liu et al. 2006, Ebert, Jiang et al. 2009). Representative dot plots are shown in **Figure 2.4 A**.

There was no consistent alteration in the proportions of SM, M1 and M2 subsets in either the CD8$^+$ (**Figure 2.4 B**) or CD4$^+$ (**Figure 2.4 C**) subsets across all timepoints. The numerical increase in CD8$^+$ and CD4$^+$ SP cells at D14 was represented in all three sub-populations (**Figure 2.4 D&E**) and trending towards young levels.

These results support a normal process of SP T cell maturation following Degarelix-induced thymocyte regeneration.

2.3.5 NUMERICAL INCREASE IN T REGULATORY CELLS BY DAY 14 IN DEGARELIX TREATED MICE

We analysed CD3$^+$CD4$^+$CD25$^+$Foxp3$^+$ T regulatory cells (Tregs) in young and middle-aged mice treated with PBS or Degarelix (Deg). **Figure 2.5 A** shows representative dot plots. No alteration in the proportions of Tregs was evident at any of the timepoints analysed **(Figure 2.5 B)**. A significant numerical increase in Tregs was evident at D14 with 1.6 x10^5 ±0.4 x10^5 in Degarelix treated mice compared to 0.6 x10^5 ±0.04 x10^5 in PBS controls (**Figure 2.5 C**).

While there was no proportional increase in Ki67$^+$ Tregs, there was a numerical increase at D14 in Degarelix treated mice (**Figure 2.5 D-F**). These results suggest normal

expansion of Tregs by D14 with the increase in total thymus cellularity following Degarelix treatment, supporting normal T cell developmental processes.
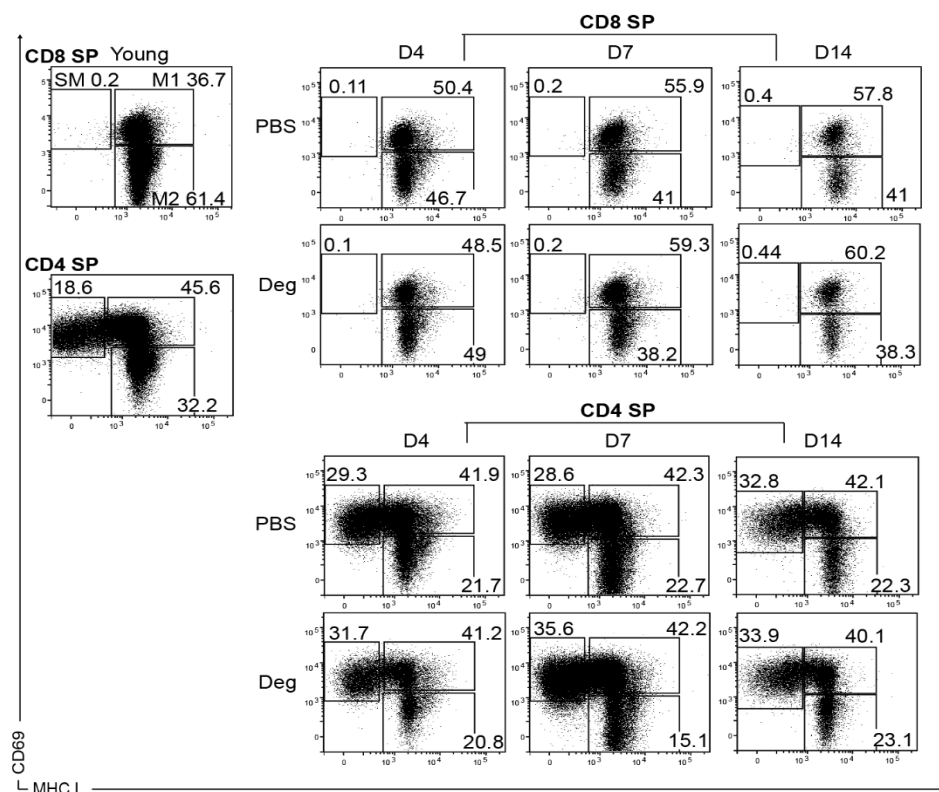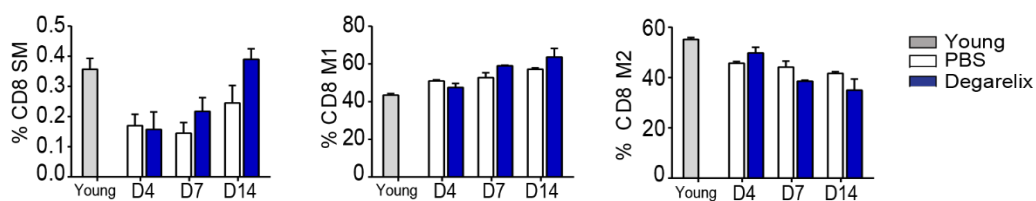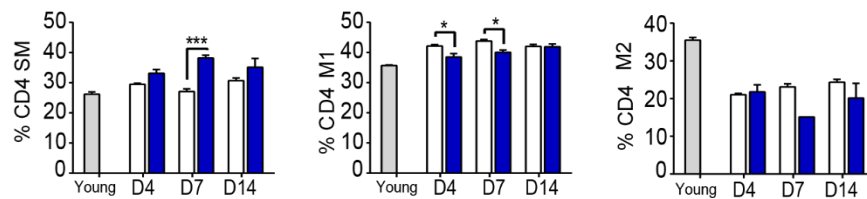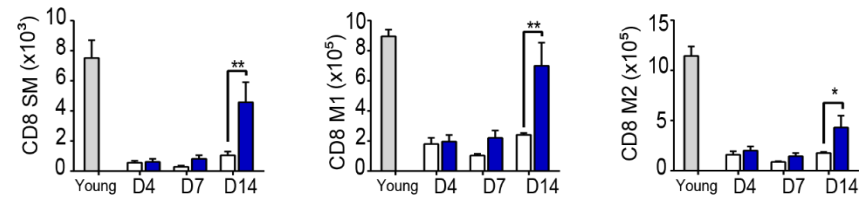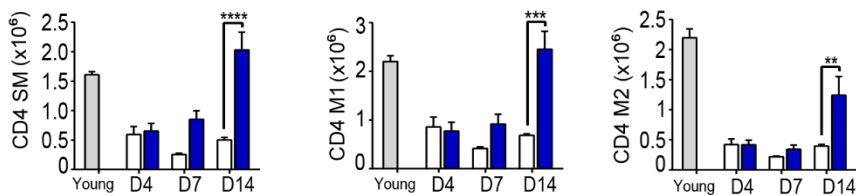
Figure 2.4 Degarelix-induced regeneration of CD4$^+$ and CD8$^+$ SP thymocytes follows a normal process of intrathymic T cell maturation. (A) Representative dot plots show the proportions of semimature CD69$^+$/MHC-II$^-$ (SM), mature CD69$^+$/MHC-II$^+$ (M1) and fully mature CD69$^-$/MHC-II$^+$ (M2) subpopulations within CD4$^+$ and CD8$^+$ T cells, in young (grey bars) and middle-aged mice treated with either PBS (PBS; white bars) or Degarelix (Deg; blue bars). Bar graphs show the prevalence of these subpopulations within CD8$^+$ **(B)** and CD4$^+$ **(C)** cells with their cell counts shown in (**D**) and (**E**). Data are expressed as mean +SEM (n=4 per group; >1 independent experiment). *denotes significant difference in Degarelix treated mice compared to PBS treated controls, where *$p<0.05$, **$p<0.01$, ***$p<0.001$ and ****$p<0.0001$. Data is representative of 1 experiment, using Mixed-design Two-Way Repeated measures ANOVA with Bonferroni and R-E-G-W-Q post hoc multiple comparisons.
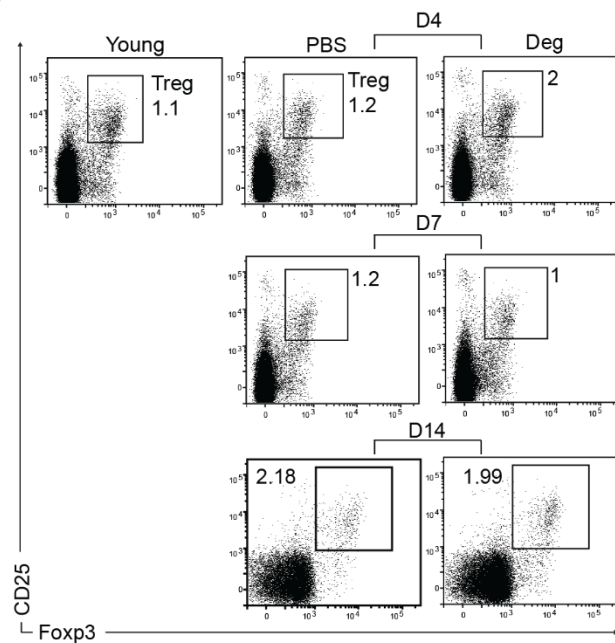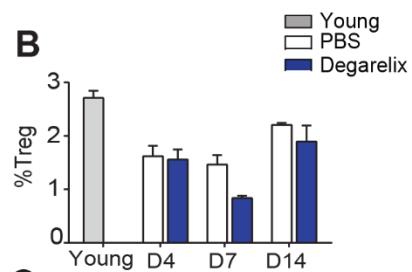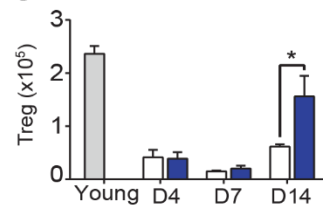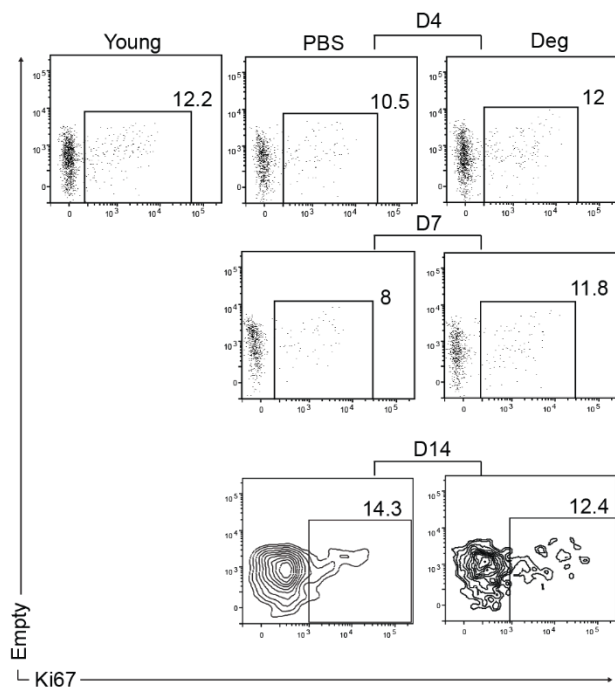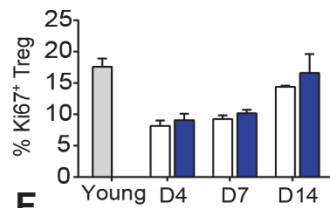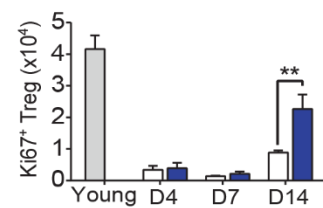
Figure 2.5 Increased number of CD25⁺ Foxp3⁺ Tregs is consistent with improved thymopoiesis following Degarelix administration. (A) Representative dot plots show the proportions of CD3⁺CD4⁺ Tregs in young, and middle-aged treated mice with either PBS or Degarelix. Bar graphs show proportions **(B)** and cell number **(C)** of Tregs in young (grey bars) and middle-aged mice treated with either PBS (PBS; white bars) or Degarelix (blue bars). **(D)** Representative dot plots of Ki67⁺ proliferating cells in young, and middle-aged mice treated with either PBS or Degarelix. Bar graphs show the proportions (**E**) and cell count (**F**) of the proliferating Ki67⁺ cells within the Tregs. Data are expressed as mean ±SEM (n=4 per group; >1 independent experiment). *denotes significant difference in Degarelix treated mice compared to PBS treated controls, where $*p<0.05$, $**p<0.01$. Data is representative of 1 experiment, using Mixed-design Two-Way Repeated measures ANOVA with Bonferroni and R-E-G-W-Q post hoc multiple comparisons.

2.3.6 ANALYSIS OF THYMIC EPITHELIAL CELLS FOLLOWING DEGARELIX TREATMENT

Given the apparent normal processes of thymocyte development evident from as early as D4 in Degarelix treated mice, we sought to investigate changes in thymic epithelial cells (TECs). Middle-aged male mice were administered a single subcutaneous injection of Degarelix (Firmagon®) at a concentration of 78µg/g. Three cohorts with 5 mice per group were studied in two independent replicate experiments; untreated young control group, a middle-aged PBS treated group, and the test Degarelix group. Mice were sacrificed at D4, D7, D10, D14 and D28 and thymi were enzymatically digested and analysed by flow cytometry **(Figure 2.6 A)**.

Total TECs were isolated according to their negative expression of CD45 and positive expression of EpCAM. TEC subsets can be further isolated according to their expression of either the cTEC marker Ly51, or the mTEC marker Ulex Europaeus Agglutinin-1 (UEA-1), in combination with MHCII. **Figure 2.6 B** shows UEA-1 based delineation of cortical (UEA1$^-$) and medullary (UEA1$^+$) TEC (cTEC and mTEC), while MHCII expression broadly divides these TEC subsets into mature cells expressing high levels of MHCII, and immature cells expressing low levels of MHCII.

In this series of experiments, total thymic cellularity was significantly increased by D7 post treatment (166 $\times10^6$ ± 29 $\times10^6$), compared to middle-aged PBS controls (54.5 $\times10^6$ ± 6.3 $\times10^6$), and remained at this level until day 28, which was the final timepoint analysed **(Figure 2.6 C)**. Surprisingly, given the antagonist nature of this LHRH-analogue, total TEC cellularity temporarily decreased at D4, and returned to middle-aged control levels by D7 **(Figure 2.6 D)**. These results indicate that total TEC cellularity does not increase alongside thymocyte expansion during Degarelix-induced regeneration.

A

LHRH-ant (Degarelix) S.C

Analyses

Day -2   -1   0   Day 4   7   10   14   28

B

Debris exclusion

Non-debris
88.9

SSC-A / FSC-A

Viable cells

Viable
98.7

PI / FSC-A

Hematopoietic cell exclusion

CD45^neg
0.3

SSC-A / CD45

Thymic stromal cells

TEC
33.5

Non-TEC
61.9

MHC-II / EpCAM

UEA-1 negative control

27.8   0

68.8   1

MHC-II / UEA-1

TEC subsets

cTEC^hi
5.8

mTEC^hi
13.4

cTEC^lo
44.9

mTEC^lo
35.9

MHC-II / UEA-1

C

Total thymic cellularity (x10^8)

D

TEC (x10^5)

Young (UT)   PBS   Degarelix

Figure 2.6 Analysis of thymocytes and total TECs by flow cytometry following Degarelix treatment in male mice. (**A**) Timeline of Degarelix treatment and analysis timepoints. (**B**) Gating strategy to analyse TECs by flow cytometry. Bar graphs show total thymic cellularity in untreated young (grey bars) and middle-aged mice treated with either PBS (white bars) or Degarelix (blue bars) (**C**) and total thymic epithelial cells (**D**). Data are expressed as mean +SEM (n=4 per group; >1 independent experiment). * denotes significant difference in Degarelix treated mice compared to PBS treated controls *$p<0.05$, **$p<0.01$, ***$p<0.001$ and ****$p<0.0001$. Data is representative of at least 2 independent experiments, using Mixed-design Two-Way Repeated measures ANOVA with Bonferroni and R-E-G-W-Q post hoc multiple comparisons.

### 2.3.7 AN INCREASE IN mTEC^{lo} CELLS PRECEDES AN INCREASE IN mTEC^{hi} FOLLOWING DEGARELIX ADMINISTRATION

We performed a brief analysis of TEC subsets based on their UEA-1 and MHCII expression. Representative dot plots demonstrate the proportion of TEC subsets in young untreated mice, and middle-aged mice across different time-points following either PBS or Degarelix treatment (**Figure 2.7 A**).

Notably, an increase in the proportion of mTEC^{lo} at D7 was followed by an increase in the proportion of mTEC^{hi} at D10 with Degarelix treatment, compared to middle-aged controls (**Figure 2.7 A and B**). This increase in mTEC^{lo} only showed significance at D7 post-treatment compared to controls, but the increase in mTEC^{hi} at D10 (34.7% ±2.2%) compared to controls (5% ±0.7%), was sustained through to D28 in both proportion and number (**Figure 2.7 B and C**).

We previously identified an accumulation of cTEC^{lo} in male mice during ageing (Lepletier, Hun et al. 2019). Degarelix treatment induced an early significant numerical and proportional reduction in cTEC^{lo} cells towards young levels, evident from D4 and D7, respectively, and maintained to Day 28. A significant proportional increase in cTEC^{hi} was evident at D10 in the Degarelix treated group (6.4% ±0.7%, compared to 3.7% ±0.5% PBS controls) and at D28. While a significant numerical increase was also noted at D28 in the cTEC^{hi} Degarelix treated group (7950 ±1763, compared to 2790 ±561 PBS controls), there was substantial numerical variability in cTEC^{hi} across all time-points in PBS control groups. This data demonstrates a proportional and numerical return of mTEC^{hi} cells to young levels following Degarelix treatment, with an initial expansion of mTEC^{lo} precursors preceding this event. It also demonstrates a reversal in the age-related accumulation of cTEC^{lo} cells from as early as day 4.

A

Young untreated | Aged untreated | Deg D4

cTEC^hi | mTEC^hi
cTEC^lo | mTEC^lo

Young untreated: 4 | 12 / 15 | 61
Aged untreated: 4 | 8 / 49 | 32
Deg D4: 4 | 9 / 47 | 33

Deg D7 | Deg D10 | Deg D14 | Deg D28

Deg D7: 2 | 12 / 26 | 55
Deg D10: 7 | 35 / 24 | 29
Deg D14: 5 | 43 / 10 | 39
Deg D28: 4 | 24 / 19 | 48

MHC II

UEA-1

B

mTEC^hi | mTEC^lo | cTEC^hi | cTEC^lo

C

mTEC^hi (x10^4) | mTEC^lo (x10^5) | cTEC^hi (x10^4) | cTEC^lo (x10^4)

Young (UT) | PBS | Degarelix
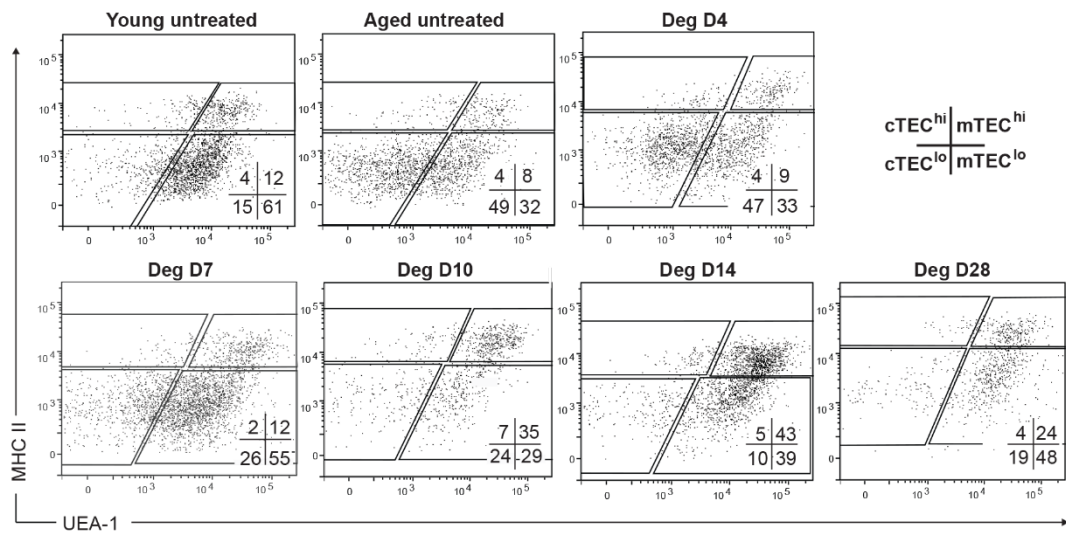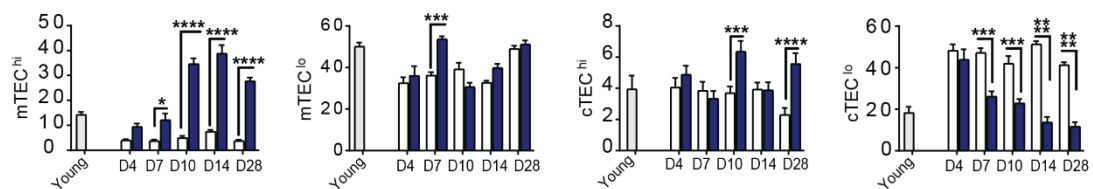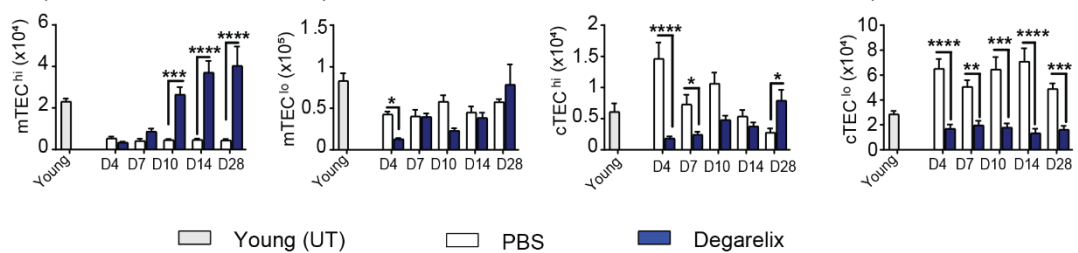
Figure 2.7 Reduced accumulation of cTEC^{lo} and increased mTEC^{hi} cells following Degarelix. (A) Representative dot plots of TEC subsets in untreated young (Young UT), middle-aged mice following PBS (PBS) or Degarelix (Deg) treatment. Bar graphs show proportional (**B**) and numerical (**C**) changes in medullary and cortical thymic epithelial cell subsets (mTEC^{hi}, mTEC^{lo}, cTEC^{hi} and cTEC^{lo}). Data are expressed as mean ±SEM (n=4 per group; >2 independent experiments). * denotes significant difference between Degarelix treated mice (blue bars) compared to middle-aged PBS treated mice (white bars), where n=5 per group, *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001, using Mixed-design Two-Way Repeated measures ANOVA with Bonferroni post hoc multiple comparisons.

2.4. DISCUSSION

Thymus involution is a hallmark of ageing. It is one of the first organs to diminish in activity early in life. In humans, thymus involution begins in the first years of life and further declines after the onset of puberty reducing to <10% of total thymic tissue by 70 years of age (Steinmann, Klaus et al. 1985). In mice, thymus atrophy is most evident from the onset of puberty (around 4-6 weeks of age) and characterised by a decline in developing thymocytes and supporting TECs (Sempowski, Gooding et al. 2002, Gray, Seach et al. 2006). The progressive loss of thymus tissue and thymopoiesis results in a decreased output of naïve T cells, leading to a restricted T-cell repertoire in the periphery and decline in immune function. This renders the aged/elderly more susceptible to infectious diseases, leading to increased morbidity and mortality, and reduced efficacy of adult vaccinations.

The association of thymic decline at puberty and the collapse of the thymus with exogenous testosterone administration led to investigations into inhibiting sex steroid production by surgical gonadectomy or interfering with the hypothalamic derived luteinising-hormone-releasing-hormone (LHRH) signalling to the pituitary. Most of the earlier mouse studies utilised agonist analogues of LHRH (LHRH-Ag) but the initial supersaturation of pituitary signalling, before downregulation of the LHRH receptor, led to a spike in testosterone production in the first week following treatment before inhibition of testosterone production was achieved 21 days later (Velardi, Tsai et al. 2014). This testosterone spike induced a loss of all thymocyte subsets, but thymus regeneration could still be achieved 28 days following administration of LHRH-Ag. Velardi et al compared a third generation LHRH receptor antagonist (Degarelix, Firmagon®) with the LHRH

receptor agonist used in previous studies (Lupron; leuprolide acetate) and found evidence of thymus regeneration by day 7 (Velardi, Tsai et al. 2014).

In this study, we were interested in investigating an earlier stage in the regenerative process, to gain some insight as to whether thymocytes progressed normally from early immature stages through to single positive T cells, or aberrant progression occurred possibly through stage specific proliferation. We were also interested in the timing and phenotypic integrity of regeneration in specific TEC subsets. We treated middle-aged healthy male mice with either PBS or Degarelix. Thymocyte recovery was evident before TEC recovery and appeared to progress in a stepwise manner with the most immature DN subset regenerating as early as day 4, followed by increases in DP by day 7 and SP CD4 and CD8 T cells increasing by day 14. This involved early proportional increases in proliferation of all subsets with DN cells continuing to increase to day 14. Within the DN subset, early increases were mostly evident in DN3 and DN4 subsets. Cells within the DN3 (CD44$^-$CD25$^+$) subset are exclusively committed to the T cell lineage and progress to DN4 following T cell receptor (TCR)-β selection. This early regeneration may be intrinsic to thymocytes rather than through cortical TEC regeneration, given the initial numerical loss in cTEC^{hi} at day 4. However, reversal of the age-induced accumulation of cTEC^{lo} cells evident at this early day 4 timepoint, may reflect an early qualitative improvement of the thymic microenvironment through production of thymic growth factors. There was no significant proportional increase in ETPs at this early stage.

Significant numerical changes in the CD4 and CD8 SP T cell subsets and their subpopulations (SM, M1, M2) was only evident by day 14. This timing reflects a normal progression of T cell development from the early increase in immature DN cells. It also

coincides with rejuvenation of the medullary TEC compartment, which was evident by the dramatic proportional and numerical increase in mTEC^{hi} cells from day 10. Compared to our earlier research on castration induced TEC recovery (Gray, Seach et al. 2006), more recent studies from our laboratory found that androgen deprivation via castration did not increase the total number of TECs (Lepletier, Hun et al. 2019). We believe this was due to improved TEC isolation techniques using more effective enzymes to break up the aged thymus and releasing more TECs (Seach, Wong et al. 2012). However, importantly, we found that androgen deprivation-induced thymus regeneration led to recovery of mature TEC subsets, particularly mTEC^{hi} cells critical to self-tolerance induction (Lepletier, Hun et al. 2019). With similar findings using a LHRH receptor antagonist found here, it may mitigate the concern that androgen deprivation produces autoreactive T cells in the absence of numerical total TEC recovery (Griffith, Fallahi et al. 2012). In further support, a numerical increase in Treg cells was also evident by day 14. Tregs are important for maintaining peripheral self-tolerance and the fact that the proportion of Tregs was maintained with increased thymocyte production, supports a normal process of T cell development and back-up peripheral support for any escaping autoreactive T cells.

CONCLUSION

In conclusion, we found the LHRH receptor antagonist, Degarelix was effective in early thymus regeneration in middle-aged male mice. The enhancement of normal thymopoiesis led to increases in CD4 and CD8 T cell production in a stepwise manner. This was possibly supported by an early qualitative recovery of the thymic microenvironment, evidenced by early reversal of the aged-induced accumulation of cTEC[lo], followed by regeneration of the mature mTEC compartment critical for self-

tolerance induction. Timing of the mTEC^{hi} recovery likely also involves crosstalk with newly derived SP CD4 and CD8T cells. In further studies, it will be important to determine whether Degarelix is equally as effective in females.

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CHAPTER 3:

GENDER DIFFERENCES IN THYMUS AGEING AND A COMPARATIVE ANALYSIS OF LHRH RECEPTOR ANTAGONIST TREATMENT ON THYMIC EPITHELIAL CELLS FROM MIDDLE-AGED MALE AND FEMALE MICE

3.1 INTRODUCTION

We and others have demonstrated decreased thymopoiesis and alterations in thymic epithelial cell (TEC) subsets during ageing. Links between the endocrine and immune axis and in particular, the impact of increased sex steroid production from puberty on thymus involution, has led to research into sex steroid inhibition (SSI) as one strategy to replenish a broad T cell repertoire (Calder, Hince et al. 2011). Investigations predominantly performed in male mice, demonstrated that blocking sex steroid production via surgical castration or use of luteinizing hormone releasing hormone (LHRH) analogues, commonly used in the treatment of breast and prostate cancer, resulted in transient thymus regeneration (Sutherland, Goldberg et al. 2005, Dudakov, Goldberg et al. 2009, Goldberg, King et al. 2009, Goldberg, Dudakov et al. 2010, Griffith, Fallahi et al. 2012, Dumont-Lagace, St-Pierre et al. 2015). LHRH receptor agonists (LHRH-Ag) induce an initial flare in testosterone levels that can further damage the ageing thymus, prior to LHRH-receptor downregulation and sex steroid inhibition (SSI) and as such, LHRH receptor antagonists (LHRH-Ant) have more recently been investigated for thymus regeneration (Velardi, Tsai et al. 2014, Velardi, Tsai et al. 2018). LHRH-Ant directly bind to and block the LHRH-receptor, and in males, leads to a reduction in testosterone to castrate levels within 24 to 48 hours (Velardi, Tsai et al. 2014). We demonstrated in the previous chapter 2, that thymus regeneration begins within 4 days following LHRH-Ant treatment in male mice. An early increase in the most immature DN ($CD4^-CD8^-$) T cell subset was evident by day 4, followed by the regeneration of downstream T cell subsets in an apparent stepwise manner, with increased production of mature CD4 and CD8 T cells by day 14. An early qualitative recovery of the thymic microenvironment was

proposed based on early reversal of the age-induced accumulation of cTEC[lo] followed by recovery of the mature medullary compartment, critical for final maturation of T cells and self-tolerance induction. Such phenotypic changes in TEC subsets during ageing and regeneration, led us to question the underlying mechanisms for TEC subset maintenance in the postnatal thymus and the potential role of thymic epithelial stem cells in TEC development, maintenance and malfunction (Wong, Lister et al. 2014, Lepletier, Hun et al. 2019). In the embryonic thymus, bipotent thymic epithelial progenitor cells (TEPC) generate both cortical and medullary TECs (Bennett, Farley et al. 2002, Gill, Malin et al. 2002, Rossi, Jenkinson et al. 2006, Baik, Jenkinson et al. 2013). This was proposed to occur via serial progression, with the initial generation of cTECs followed by the emergence of mTECs later during thymogenesis (Alves, Takahama et al. 2014). Bipotent progenitors also exist in the postnatal thymus (Bleul, Corbeaux et al. 2006), however, their precise phenotype remains controversial. In the postnatal thymus, it has been proposed that mature medullary TECs are mainly maintained by lineage restricted mTEC precursors rather than bipotent progenitors (Ohigashi, Zuklys et al. 2015). Single lineage medullary TEC precursors have been identified based on expression levels of the major histocompatibility complex II (MHCII). Mature medullary epithelial cells express high levels of MHCII (mTEC[hi]) and are derived from single lineage precursors expressing low levels of MHCII (mTEC[lo]) (Gray, Seach et al. 2006, Ohigashi, Zuklys et al. 2015). The mTEC[lo] subset also contains post-Aire expressing cells (Metzger, Khan et al. 2013) and recent single cell transcriptome studies have identified even more heterogeneity within these broad mTEC subsets (Takahama, Ohigashi et al. 2017, Bornstein, Nevo et al. 2018, Miller, Proekt et al. 2018). Investigations into the postnatal cortical compartment have not

traditionally segregated cTECs according to their MHCII expression levels and stages of maturation, as we have performed in our laboratory (Seach, Wong et al. 2012), although mature cTECs have been recognised by their high levels of CD205, MHCII and β5t expression (Ohigashi, Kozai et al. 2016). Therefore, only more recently were alterations in cTEC^{lo} and cTEC^{hi} subsets identified in the ageing and SSI-induced regenerating thymus (Lepletier, Hun et al. 2019).

Our laboratory phenotypically and functionally characterised a bipotent TEPC in a subset of the cTEC^{lo} compartment expressing high levels of stem cell antigen-1 (Sca-1^{hi}) and alpha-six integrin (α6^{hi}), that is predominantly localised at the cortico-medullary junction (Wong, Lister et al. 2014, Lepletier, Hun et al. 2019). It was demonstrated in male mice, that loss of mature TEC subsets in the ageing thymus is due to increasing quiescence of the Sca-1^{hi} α6^{hi} cTEC^{lo} post-natal TEPC (Lepletier, Hun et al. 2018), with an increased reliance on single lineage precursors (mTEC^{lo}) to maintain mature mTEC^{hi} populations (Ohigashi, Zuklys et al. 2015). Age-induced alterations in the thymic production of follistatin (Fst) and bone morphogenic protein 4 (Bmp4) that interferes with activin A signaling, have been proposed to play a role in the reduced differentiation of TEPC into downstream single lineage cortical and medullary TEC precursors (Lepletier, Hun et al. 2018). Majority of pre-clinical research into thymic involution and SSI induced thymic reactivation has been carried out in post-pubertal and aged male mice. Therefore, we have here investigated gender differences in the rate of thymic involution, with a particular focus on identifying changes in TEC subsets in pre-pubertal, post-pubertal and middle-aged groups, including TEPC. Finally, we performed a comparative analysis of TEC subsets following LHRH-Ant treatment in middle-aged females and males.

3.2   MATERIALS & METHODS

3.2.1 ANIMALS

C57BL/6J female and male mice (pre-pubertal, 4-week-old; post-pubertal, 7-week-old; and middle-aged, 7-12-month-old) were obtained from Monash Animal Research Platform and housed at Animal Research Laboratory (Monash University, Australia). Mice were maintained in a controlled environment with a standard diet and water *ad libitum*. All experiments were conducted according to the Australian National Health and Medical Research Council Guidelines of Animals Used for Scientific Purposes (2008) and were approved by Monash University Animal Ethics Committee (SOBSA/ADB/2015/039).

3.2.2 LHRH-Ant ADMINISTRATION

Middle-aged female and male mice were treated with Degarelix (Firmagon®; LHRH-antagonist) at a dose of $78\mu g$/gram/mouse, injected subcutaneously 48 hours prior (Day -2) to allow time for sex steroids to reach castrate levels by Day 0. Mice were subsequently analysed following euthanasia through CO_2 asphyxiation at indicated time points.

3.2.3 SERUM ANALYSIS FOR FSH AND LH

On day 4 following LHRH-Ant treatment, animals were sacrificed and whole blood samples were withdrawn via cardiac puncture using a 26G needle (BD Bioscience, U.S.A), and collected in a 1.5 ml microcentrifuge tube. Blood samples were allowed to clot for 30 minutes at RT, then centrifuged at 1000-2000g for 10 minutes at 4°C. The supernatant (blood sera) was transferred into a new microcentrifuge tube and stored at

minus 80°C. FSH and LH levels were examined via radioimmunoassay (collaboration with Prof Mark Hedger's laboratory, Hudson Institute of Medical Research, Australia).

3.2.4 THYMIC LOBE ENZYMATIC DIGESTION

Thoracotomy was performed to collect mouse thymi in RPMI medium 1640 (Gibco, U.S.A.), and each thymus cleaned of connective and adipose tissue. Thymi were snipped with fine scissors and transferred into 10ml conical tubes for enzymatic digestion using 0.03% (w/v) DNase I and 0.3% (w/v) Liberase Thermolysin Medium (Roche, Germany) in RPMI medium 1640, for 15 minutes at 37°C (Seach, Wong et al. 2012). Thymic fragments were gently agitated using a wide-bore tip pipette and allowed to settle. Supernatant was collected, fluorescence-activated cell sorting (FACS) buffer (0.1% BSA and 5mM EDTA in 1x phosphate-buffer saline (PBS) mouse tonicity), added to neutralise enzymatic activity, and placed on ice. Remaining fragments were then digested with fresh enzymes, and the cycle repeated until complete tissue digestion. Smaller-tip pipettes were used for agitation as the digestion process progressed. The pooled thymic fractions were filtered through a 100μm nylon mesh into a new collection tube and centrifuged at 300g for 5 minutes at 4°C. Cell pellets were resuspended in FACS buffer, and both total and viable cell counts were acquired using a Z2 Coulter Counter (Beckman Coulter, U.S.A.).

3.2.5 CELL STAINING FOR FLOW CYTOMETRY ANALYSIS

Cells were resuspended in primary antibody cocktail at a concentration of 1×10^6 cells per 10μL of conjugated/ unconjugated antibody and incubated for 15 minutes at 4°C in the dark. Cells were then washed twice with FACS buffer to remove unbound antibodies and centrifuged at 1300 rpm for 5 minutes. Where relevant, the cells were then stained with

secondary antibody for 15 minutes at 4°C.Stained samples were washed and resuspended in FACS buffer after centrifugation, then filtered into round-bottom polystyrene tubes. Propidium Iodide (PI) was added at a final concentration of 100ng/ml to exclude dead cells for live cell FACS analysis.

Intracellular staining was performed to identify proliferation (Ki-67) and Aire expression in TEC subpopulations. Cells previously stained with extracellular markers were fixed using Cytofix™ buffer (BD Biosciences, U.S.A.) for 30 minutes at 4°C. Samples were subsequently washed with Perm-wash buffer (BD Biosciences, U.S.A.), centrifuged at 300g for 5 minutes, and stained with intracellular markers or their isotype controls for 30 minutes at 4°C. Stained cells were then washed, resuspended in FACS buffer following centrifugation, and transferred into round-bottom tubes for flow cytometric analysis.

3.2.5.1 Antibodies utilised for immunofluorescent staining of TEC subsets

Primary antibodies: CD45, thymocyte; EpCAM, thymic epithelial cells; UEA-1, mTEC; MHCII, TEC maturity; α6 integrin and Sca-1, TEPC; Ki-67, proliferation; Aire, autoimmune regulator. Antibodies obtained from BioLegend (USA) or BD Biosciences (USA).

3.2.6 CD45 HEMATOPOIETIC CELL DEPLETION

To enrich for CD45- thymic stromal cells, anti-mouse CD45 Microbeads (Miltenyi Biotec, Germany) were added to pooled thymic digests (5µL beads + 95µl FACS buffer per 1×10^7 cells) and gently rotated for 20 minutes at 4°C. Samples were then washed with FACS buffer and centrifuged at 300g for 5 minutes to remove unbound magnetic beads, and resuspended in FACS buffer at a concentration of 0.5×10^8 cells/ml. Isolation of CD45- fractions was achieved using the 'Deplete' function of an AutoMACS Pro Separator (Miltenyi Biotec, Germany). The enriched CD45- thymic stromal cells were then

resuspended in 2mL of red blood cell lysis buffer that consists of 0.1M Tris-HCl in ammonium chloride 0.83% (w/v) and incubated in a water bath at 37°C for 2 minutes. Then, CD45- stromal cells were neutralized with FACS buffer and centrifuged at 1500 rpm for further 5 minutes, ready for immunofluorescent staining.

3.2.7. FLOW CYTOMETRY

3.2.7.1. Acquisition and Analysis

Using the BD FACS Canto™ II flow cytometer (BD Biosciences, U.S.A), 5×10^6 cells were stained and acquired using up to 8 channels. FCS files obtained from Canto™ II were analysed using Flowlogic™ v7.2 (Inivai Technologies, Australia). Parameter, voltage and compensation settings were established using BD FACSDiva v.6 software (BD Bioscience, U.S.A.).

3.2.7.2. Cell Sorting

Stained CD45- thymic stromal cell subsets were sorted with a BD Influx™ I cell sorter (BD Bioscience, U.S.A) at FlowCore (Monash University, Australia). Sorted cells were collected in RPMI medium 1640 containing 30% (v/v) foetal bovine serum (FBS).

3.2.8. 3D THYMIC EPITHELIAL PROGENITOR CELL (TEPC) CULTURE

1×10^4 purified TEC subsets acquired from cell sorting by FACS were co-cultured with 2×10^5 irradiated mouse embryonic fibroblasts (MEFs) in 50% growth-factor-reduced Matrigel® (BD Biosciences, U.S.A.), placed into 24-well 0.4μm transwell inserts (Millipore, Merck, U.S.A.) and incubated with TEC media (Table 3.1) as previously described (Wong, Lister et al. 2014) for seven days at 37°C in a hypoxic environment (5% O_2, 10% CO_2). Media was changed every 48 hours. On day 7, colony number was determined using an

optical (Zeiss Primo Vert, Germany) or multicolour confocal (Leica DMi8, Germany; Monash Micro Imaging) microscope. Colony forming efficiency was determined by dividing the number of colonies formed by the number of seeded cells x 100.

Reagents	Final concentration	Sources
RPMI Medium 1640	neat	Gibco by Life Technologies™, U.S.A.
Foetal Bovine Serum (Batch 086)	10% v/v	Gibco by Life Technologies™, U.S.A.
2-Mercaptoethanol	55uM	Gibco by Life Technologies™, U.S.A.
HEPES Buffer Solution	10mM	Gibco by Life Technologies™, U.S.A.
Penicillin +	100U/ml	Gibco by Life Technologies™, U.S.A.
Streptomycin	100ug/ml	
Sodium Pyruvate	1mM	Gibco by Life Technologies™, U.S.A.
Glutamax	2mM	Gibco by Life Technologies™, U.S.A.
MEM Non-Essential Amino Acids	0.1mM	Gibco by Life Technologies™, U.S.A.
Insulin-Transferrin-Selenium	5ug/mL	Gibco by Life Technologies™, U.S.A.
5-Triiodo-L-thyronine	2×10^{-9}M	Sigma-Aldrich, U.S.A.
Hydrocortisone	0.4ug/mL	Sigma-Aldrich, U.S.A.
Adenine	24ug/mL	Sigma-Aldrich, U.S.A.

Table 3.1. Composition of TEC media for *in vitro* TEPC cultures

3.2.9. IMMUNOCYTOCHEMISTRY OF IN VITRO CULTURED TEC COLONIES

Trans-well inserts were washed twice with PBS and fixed with 4% paraformaldehyde (PFA) in PBS for 20 minutes at room temperature (RT). Antigen retrieval was achieved by submersion in sodium citrate buffer (10mM sodium citrate, 0.05% Tween 20, pH 6.0 in PBS) for 30 minutes at 95°C. Samples were then washed with washing buffer (0.1% Triton-X in PBS) for 2 hours and blocked with 1% BSA in washing buffer for another 1 hour at RT. Colonies were stained with primary and secondary antibody cocktails (listed

below) for 2 hours each at RT in the dark. Samples were twice washed for 5 minutes in washing buffer between staining. Lastly, nuclear staining with DAPI was performed for 15 minutes, followed by two further washes. Trans-well insert membranes were cut with a scalpel and placed on a coverslip, the membrane gently removed from the stained colonies, and mounted on a glass slide with mounting media (Dako®, U.S.A.). Images were acquired using a confocal fluorescence microscope (Nikon Eclipse Ti, U.S.A.; Monash Micro Imaging) and analysed with Fiji-ImageJ software v.2.0.0.

3.2.9.1. Antibodies used for immunofluorescent staining

β5t, cTEC (MBL International USA) plus secondary antibody, rabbit IgG (Abcam, UK); Keratin-14, mTEC (BioLegend, USA) plus secondary antibody, chicken IgY (Invitrogen, USA); Aire, autoimmune regulator (eBioscience, USA) plus secondary antibody, rat IgG2c (Invitrogen, USA).

3.2.10. RT-qPCR

Total RNA was isolated using a RNAqueous™ Micro Kit (Invitrogen, U.S.A.) as per manufacturer's instructions. FACS-purified $CD45^-$ thymic stromal cell subsets (TEC subsets and $CD45^-$, $EpCAM^-$ non-TECs: fibroblasts + endothelial cells) were lysed immediately after sorting in RNAqueous™ Lysis Solution. The cell lysate was mixed with 100% ethanol and transferred into a silica-based filter which binds RNA. Total RNA was extracted following column purification, sample elution, and DNase treatment, with its concentration and purity measured using a NanoDrop (Thermofisher, U.S.A.).

First-strand cDNA synthesis was achieved using Superscript III reverse transcriptase kit (Invitrogen, U.S.A.). Total RNA was denatured in conjunction with oligo(dT)$_{20}$ (50μM)

primer and dNTP mix for 5 minutes at 65°C. Samples were immediately placed on ice for 2 minutes to allow for primer-RNA adherence. The Superscript III reverse transcriptase master mix was then added, and the reaction run for 50 minutes at 50°C. Following inactivation for 5 minutes at 85°C, *E. coli* RNase H was added for 20 minutes at 37°C to remove mRNA and acquire the cDNA template for RT-qPCR. RT-qPCR reactions were performed using SYBR Green Supermix-UDG (Invitrogen, Australia), pre-validated primer sequences (Fst: QT00105483) and Bmp4: QT00111174; Qiagen, Germany) and cDNA template, in a Corbett Rotor-Gene 3000 (Corbett Research, Australia). Expression of target genes was then analysed with Rotor-Gene software version 6.1 (Qiagen, U.S.A), relative to GAPDH using the standard $2^{-\Delta\Delta Ct}$ method (Lepletier, Hun et al. 2018).

3.2.11. STATISTICAL ANALYSIS

Statistical analysis was performed using Graph Pad Prism v7.0 software and IBM SPSS statistics (version 28). Following data normality assessments (*Shapiro-Wilk* normality test), independent *One-* or *Two-Way ANOVA* tests were run, with the appropriate post-hoc t-test performed for parametric data. Results are expressed as mean +SEM. A p value of less than 0.05 was considered statistically significant.

3.3. RESULTS

3.3.1. DELAYED NUMERICAL LOSS IN TOTAL TECS WITH AGEING

Thymic epithelial cells (TEC) represent a rare, heterogeneous population, making up less than 1% of the adult thymus cellularity (Gray, Chidgey et al. 2002). Therefore, flow cytometric analysis involves complicated processing of the thymus in the attempt to retrieve as many viable TECs as possible for accurate FACS analyses and sorting for *in vitro* cultures. Thymi from pre-pubertal (4-wk-old), post-pubertal (7-wk-old) and middle-aged (8-mo-old) C57BL/6J mice were individually enzyme digested using Liberase Thermolysin and DNAse and stained with a panel of antibodies (Seach, Wong et al. 2012). **Figure 3.1 A** illustrates the FACS gating strategy used for TEC subset analysis. Standard non-debris and viability gates (using propidium iodide (PI) staining) were applied to analyse intact and live cells. Hematopoietic cells were excluded using the leukocyte marker CD45. TEC were defined based on positive EpCAM and negative CD45 expression. TECs were further divided into medullary TEC (mTEC) and cortical TEC (cTEC) subpopulations by positive expression of Ulex Europaeus Agglutinin-I (UEA-1) and MHC-II. Differential levels of MHC-II expression within both subsets allowed further fractionation into four distinct sub-populations that reflect their maturity: cTEC^{hi}, cTEC^{lo}, mTEC^{hi} and mTEC^{lo} subsets.

A numerical analysis of pre-pubertal (4-wk), post-pubertal (7-wk) and middle aged (8-mo) female and male mice was conducted on thymocytes **(Figure 3.1 B)** and TECs per thymus **(Figure 3.1 C)** with their ratio illustrated in **Figure 3.1 D**. A dramatic decline in thymocyte number at the onset of puberty was evident in both male and female mice, to almost 50% of pre-pubescent levels by 7-wks. A further 2-fold decline in total thymocyte

cellularity by 8-mo was only evident in males; this later decline was not observed in females. While TEC numbers in 4-wk-old females were significantly higher than those found in 4-wk-old males, they were similar in TEC cellularity by 7-wks of age and both showed a dramatic numerical decline by 8-mo (**Figure 3.1 C**). The thymocyte/TEC ratio was significantly higher in female middle-aged mice compared to males and significantly higher than both 4-wk-old and 7-wk-old female mice (**Figure 3.1 D**).

Thus, in females, the greatest numerical decline in thymocytes occurs at the onset of puberty, with no further decline to middle-age. However, males show a two-step decline from the onset of puberty and continuing to middle age. TECs on the other hand, do not show an immediate numerical loss at the onset of puberty, but their greatest decline occurs between 7-wks and 8-mo of age in both genders.

A TEC gating strategy
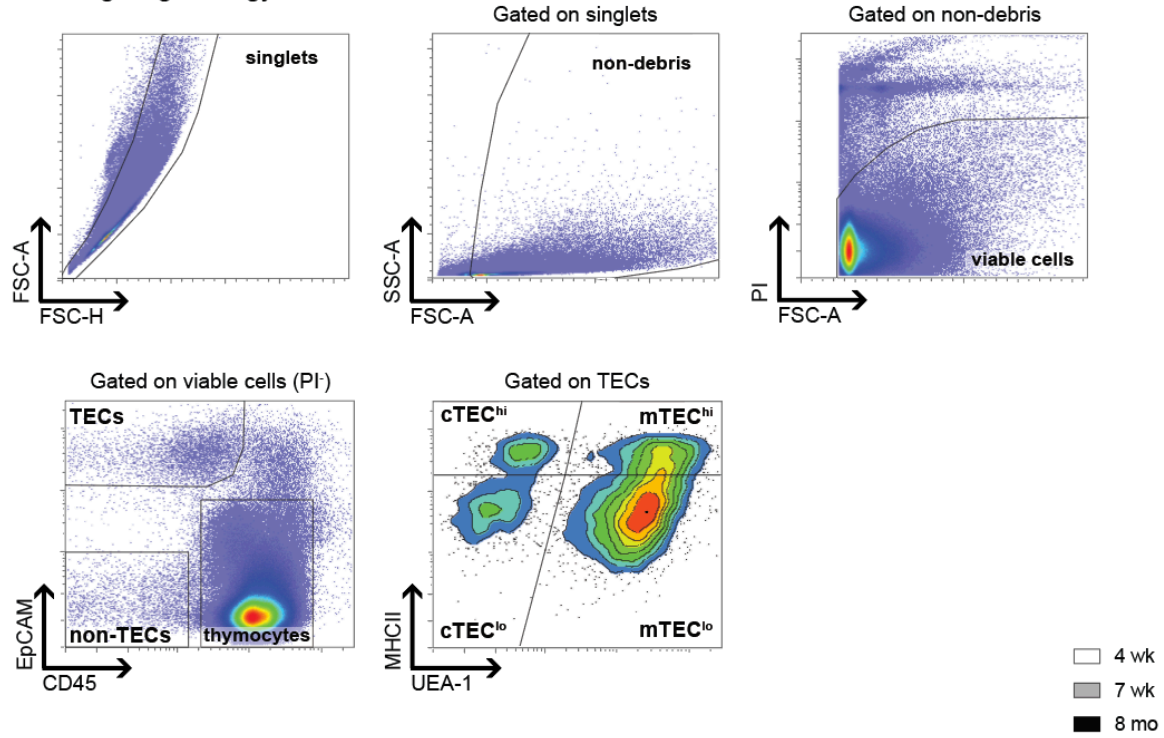
B Thymocytes
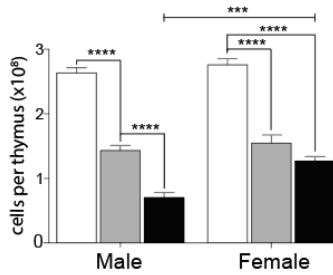
C TECs
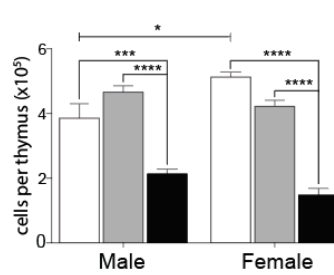
D Thymocyte/TEC ratio
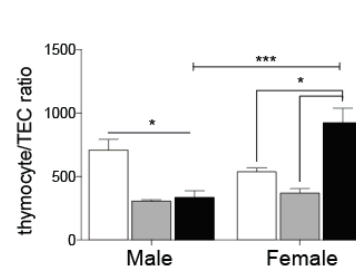
Figure 3.1. Flow cytometric analysis of thymocytes and total TECs in relation to age and gender differences. (**A**) Gating strategy to identify thymocytes, TECs and non-TECs. Number of thymocytes (**B**), TECs (**C**) and thymocyte/TEC ratio (**D**) in 4-wk-, 7-wk- and 8-mo-old male and female C57BL/6J mice. Data are expressed as mean ± SEM (n=4-6 per age group; 2 independent experiments). *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$, ordinary two-way ANOVA with Tukey's multiple comparisons. Data is representative of two independent experiments.

3.3.2. GENDER DISPARITY IN TEC SUBSETS WITH AGEING

Since a greater number of thymocytes can be supported by fewer TECs in middle-aged females, we investigated whether there were any gender differences in the maintenance of mature TEC subsets during ageing. As a recent study demonstrated numerical underestimation of TECs associated with enzyme digestion (Sakata, Ohigashi et al. 2018), we therefore assessed the changes in TEC subsets based on proportional alterations. TEC subsets were gated based on phenotypic expression of Ulex Europaeus Agglutinin-I (UEA-1) and MHCII expression, with cTECs identified as UEA-1$^-$ MHCII$^{lo/hi}$ and mTECs as UEA-1$^+$ MHCII$^{lo/hi}$ (**Figure 3.2 A**).

Results show an accumulation in proportion of cTEC^{lo} with ageing, following the onset of puberty (7-wk-old) and continuing to middle-age (8-mo-old) in both males and females (**Figure 3.2 B**). It should be noted that cTEC^{lo} showed a significantly greater accumulation in 8-mo-old males (28.6% ±1.5%) than their female counterparts (19.8% ±2.4%). The proportion of mature cTEC^{hi} subset was significantly higher in post-pubertal (12.6% ±0.3%) and middle-aged (17.9% ±1.5%) female cohorts compared to male counterparts (**Figure 3.2 C**). This might suggest a better post-pubertal maintenance of the cortical compartment in females and as such may have some bearing on the greater thymocyte:TEC ratio seen in females by middle age.

In the mTEC compartment, there was a proportional increase in mTEC^{lo} in 7-wk-old males following the onset of puberty with a concomitant loss in mTEC^{hi} (**Figure 3.2 D & E**). While there were no significant changes in mTEC^{lo} proportions during ageing in females, there was a significant reduction in mTEC^{hi} from the onset of puberty and this level was maintained in 8-mo-old groups. The proportions of mTEC^{lo} in 8-mo-old groups were significantly higher in males compared to female cohorts (**Figure 3.2 D**) and the

proportion of mTEC^{hi} in 8-mo-old groups were significantly higher in females, compared to male cohorts **(Figure 3.2 E)**.

Collectively, these data show that while there was no obvious difference in total TEC number at the onset of puberty, there were dramatic changes occurring in TEC subsets. The sharp proportional decline evident in both male and female mTEC^{hi} proportions following the onset of puberty coincided with an equally dramatic accumulation of cTEC^{lo} populations. Females maintained a higher proportion of mature cTEC^{hi} and mTEC^{hi} by middle age, with less accumulation of immature cTEC^{lo} and mTEC^{lo} compared to males, which together may underly better maintenance of thymopoiesis during ageing in females.

A TECs gated on PI⁻CD45⁻EpCAM⁺

8.8	38.3
6.2	46.7

4 wk

8.8	18.5
13.5	59.2

7 wk

8.0	11.7
27.9	52.7

8 mo

♂

cTEC^hi | mTEC^hi
───────────────
cTEC^lo | mTEC^lo

11.1	40.3
4.3	44.3

4 wk

13.5	24.1
13.2	49.2

7 wk

19.6	15.4
20.0	45.0

8 mo

♀

MHCII

UEA-1

B cTEC^lo

C cTEC^hi

□ 4 wk
■ 7 wk (grey)
■ 8 mo

D mTEC^lo

E mTEC^hi

% TECs per thymus
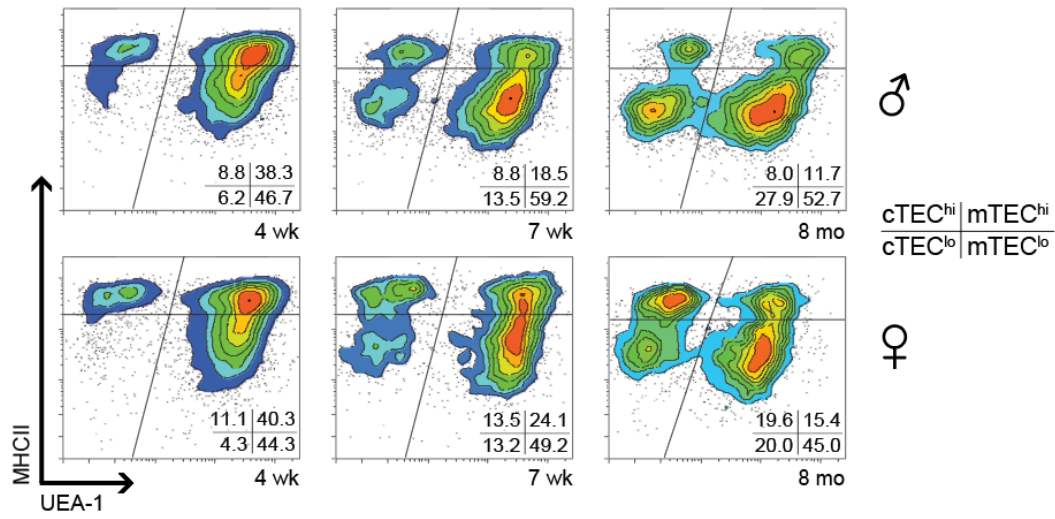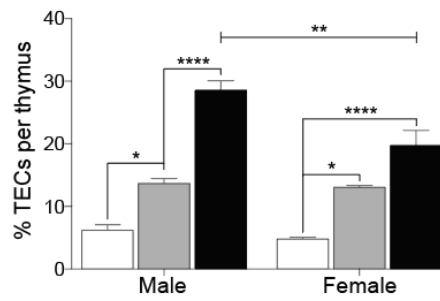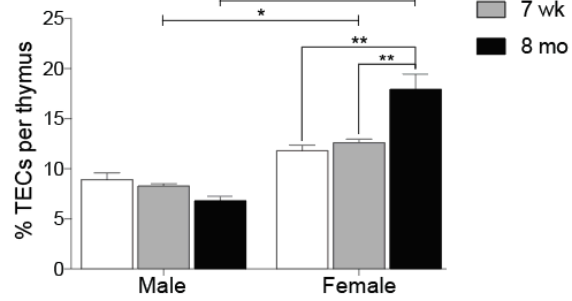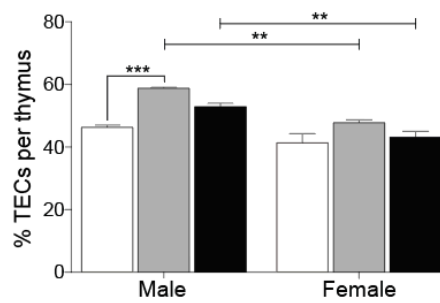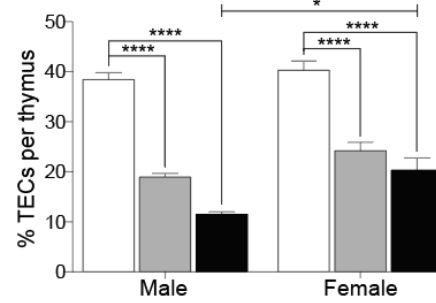
Male   Female

Figure 3.2 Reduced accumulation of cTEC^{lo} and better maintenance of mature TEC^{hi} subsets with ageing in middle-age females than males. (**A**) Representative flow cytometry contour-dot plots to portray alteration of TEC subsets with ageing. Proportion of cTEC^{lo} (**B**), cTEC^{hi} (**C**), mTEC^{lo} (**D**) and mTEC^{hi} (**E**) from TECs per thymus in 4-wk-, 7-wk- and 8- mo-old male and female mice. Data are represented as mean ± SEM (n=4-6 per age group; 2 independent experiments). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, using ordinary two-way ANOVA with Tukey's multiple comparisons.

3.3.3 THE DECLINE IN MATURE MEDULLARY TECs DURING AGEING DOES NOT INVOLVE REDUCED CAPACITY FOR PROLIFERATION

To gain some insight into the underlying mechanisms for alterations in TEC subsets following puberty, we next investigated the role of TEC proliferation by Ki-67 expression (**Figure 3.3 A**). In both male and female pre-pubertal groups, 12% of total TECs expressed Ki-67, whereas this declined significantly to approximately 7% post-puberty. A further decline was evident only in middle-aged males to approximately 5% (±0.2%), leaving a notable significant difference between middle-aged male and female cohorts (**Figure 3.3 B**).

Further investigation into individual TEC subsets showed significant changes in proliferation in the cortical compartment (**Figure 3.3 C & D**). In post-pubertal male mice, a transient decline in proportion of Ki-67 expressing cells was evident in the cTEC^{lo} subset, but this was followed by recovery in the middle-aged group. On the other hand, in females, there was a decline in proportion of Ki-67$^+$ cells in the cTEC^{lo} subset and this trend continued to middle-age (**Figure 3.3 C**). This trend is contrary to the observed accumulation of cTEC^{lo} during ageing and may suggest a block in transition of cTEC^{lo} to cTEC^{hi} cells. In the cTEC^{hi} compartment, female mice presented with a higher proportion of Ki-67$^+$ cells in both pre-pubertal (60.7%) and post-pubertal (46.8%) groups compared to males (33% and 29% respectively).

In the medullary compartment, while the decline in proportion of proliferating mTEC^{lo} was most evident following the onset of puberty in males, in females the decline was most evident between puberty and 8mo of age (**Figure 3.3 E**). By 8mo both genders demonstrated the same proportion of proliferating mTEC^{lo} (**Figure 3.3 E**). The proportion

of Ki-67$^+$ mTEC^{hi} demonstrated no significant difference between genders and across all age groups (**Figure 3.3 F**).

This data suggests the loss in mTEC^{hi} during ageing is not due to a reduced capacity to proliferate. However, there was a reduced proportion of proliferating cells in mTEC^{lo}, despite no change in their overall proportion with ageing. Whether this difference reflects an increase in the number of post-AIRE mature mTEC^{hi} moving to the mTEC^{lo} compartment (Metzger, Khan et al. 2013) would require further in-depth analysis, not within the scope of this current study. Within the cTEC compartment, maintenance (males) or reduction (females) in proliferating cells during ageing, is contrary to the accumulation of cTEC^{lo} evident by middle-age in both groups. This suggests an underlying mechanism for the accumulation cTEC^{lo} with ageing may be due to a block in differentiation of cTEC^{lo} to cTEC^{hi}.

A Gated on TEC/TEC subsets

Ki-67 iso

Ki-67$^+$ (%)

Ki-67

B Ki-67$^+$ TECs

% Ki-67$^+$ cells (from TEC)

□ 4 wk
▨ 7 wk
■ 8 mo

Male   Female

C Ki-67$^+$ cTEC^{lo}

% Ki-67$^+$ cells (from cTEC^{lo})

Male   Female

D Ki-67$^+$ cTEC^{hi}

% Ki-67$^+$ cells (from cTEC^{hi})

Male   Female

E Ki-67$^+$ mTEC^{lo}

% Ki-67$^+$ cells (from mTEC^{lo})

Male   Female

F Ki-67$^+$ mTEC^{hi}

% Ki-67$^+$ cells (from mTEC^{hi})

Male   Female
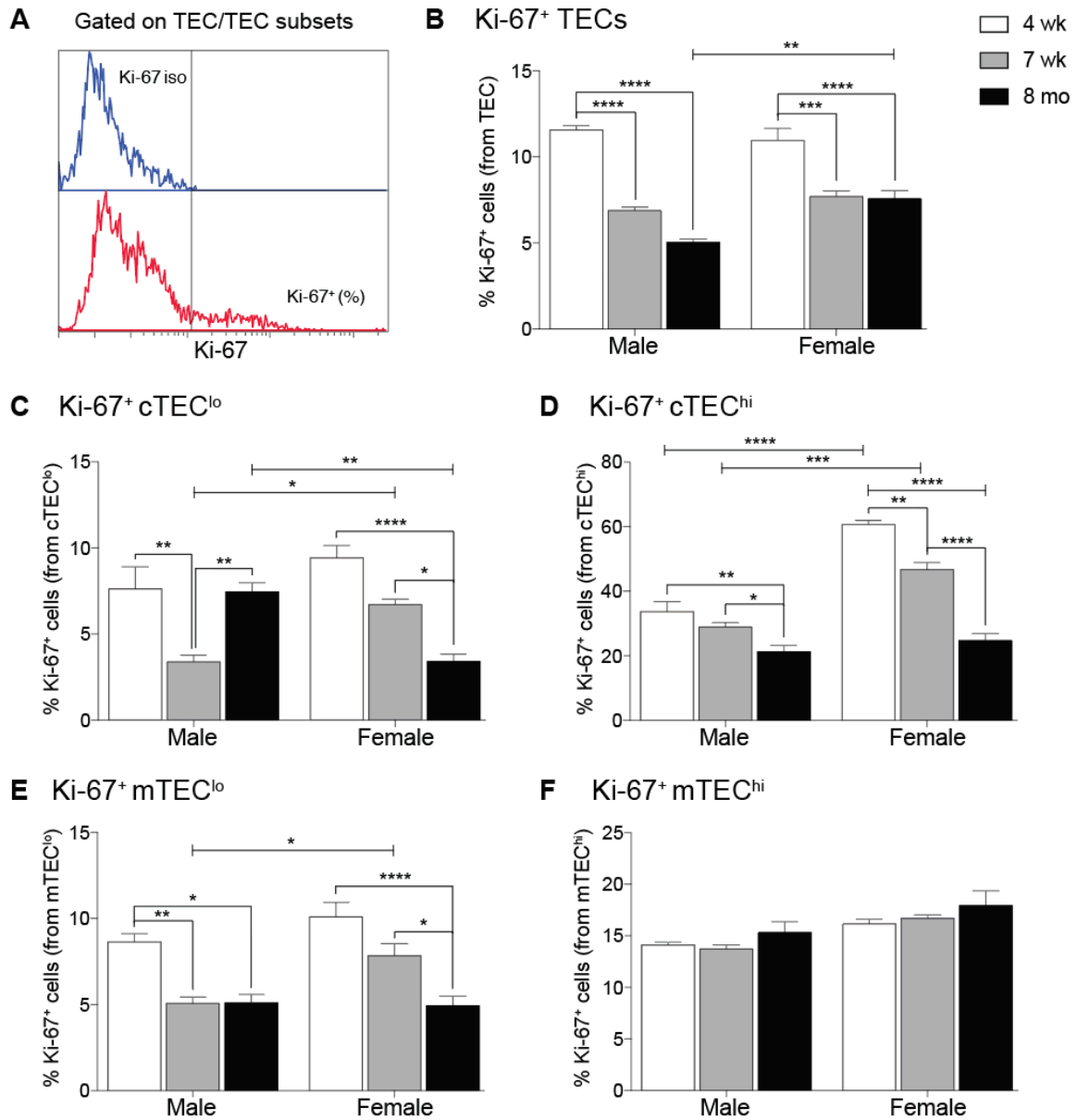
Figure 3.3 Analyses of Ki-67$^+$ TEC and TEC subsets in relation to age and gender differences. (**A**) Representative Ki-67 gating strategy with Ki-67 isotype (iso) as a negative control. Proportion of Ki-67$^+$ TECs (**B**), cTEC^{lo} (**C**), cTEC^{hi} (**D**), mTEC^{lo} (**E**) and mTEC^{hi} (**F**) in 4-wk-, 7-wk- and 8-mo-old male and female mice. Data are expressed as mean ± SEM (n=4-6 per age group; 2 independent experiments). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, using ordinary two-way ANOVA with Tukey's multiple comparisons.

3.3.4 GENDER DISPARITY IN DIFFERENTIATING AND RECOVERING TEPC WITH AGEING

Our group previously identified a post-natal bipotent TEPC in the cTEC^{lo} subset expressing high levels of Sca-1 and α6-integrin (Wong, Lister et al. 2014). The cTEC^{lo} compartment can be divided into three subsets: α6^{hi}Sca-1^{hi}, Sca-1^{int} and Sca-1^{lo}. As TEPCs differentiate they reduce their Sca-1 expression (Lepletier, Hun et al. 2019). Given the age and gender related alterations in the cTEC compartment, we investigated these transitional stages in the cTEC^{lo} subset across our three age groups. Overall, the dot plots showed a similar trend of α6^{hi}Sca-1^{hi} accumulation in both males and females (**Figure 3.4 A**). A significant increase in the proportion of α6^{hi} Sca-1^{hi} TEPC was evident post-puberty in both genders (**Figure 3.4 B**). No further accumulation was evident by middle-age. Interestingly, a dramatic increase in Ki-67 expressing TEPC and downstream Sca-1^{lo} cells was evident in 8-mo-old males, while females showed a reduction in Ki-67 expression in these subsets **(Figure 3.4 C)**. Together with the continued reduction in production of downstream Sca-1^{lo} cells to middle-age in males, despite increased proportion of Ki-67 expressing cells, this may hint at both a reduced capacity for TEPC differentiation and greater TEPC senescence in middle-aged males compared to females. It may additionally suggest males need to rely more heavily on proliferation for maintenance of unipotent Sca-1^{lo}cTEC^{lo} progenitors than on TEPC/Sca-1^{int} differentiation. Females maintained a slightly higher percentage of downstream Sca-1^{lo} cells at 8-mo of age (15.1% in female compared to 9.4% in male), which may contribute to maintenance of the higher proportion of cTEC^{hi} cells in females at 8-mo compared to males, shown earlier in **Figure 3.2 C**.

A cTEC^{lo} gated on UEA-1$^-$ MHCII^{lo} TECs

$\Diamond$

Sca-1^{lo} $\dfrac{\text{TEPC}}{\text{Sca-1}^{int}}$

$\female$

α6-integrin

Sca-1

4 wk 43.9 | $\dfrac{33.3}{22.1}$

7 wk 26.3 | $\dfrac{61.4}{14.9}$

8 mo 9.9 | $\dfrac{59.5}{29.5}$

4 wk 36.0 | $\dfrac{32.7}{31.3}$

7 wk 24.1 | $\dfrac{58.4}{17.6}$

8 mo 16.1 | $\dfrac{59.3}{22.9}$

☐ 4 wk
▨ 7 wk
■ 8 mo

B TEPC   Sca-1^{int} cTEC^{lo} Sca-1^{lo} cTEC^{lo}

% cTEC^{lo} per thymus

Male   Female

C Ki-67$^+$ TEPC   Ki-67$^+$ Sca-1^{int} cTEC^{lo} Ki-67$^+$ Sca-1^{lo} cTEC^{lo}

% Ki-67$^+$ cells (from TEPC)

% Ki-67$^+$ cells (from Sca-1^{int} cTEC^{lo})

% Ki-67$^+$ cells (from Sca-1^{lo} cTEC^{lo})

Male   Female

Figure 3.4. Accumulation of TEPC with ageing may involve increased proliferation in males, but not females. (**A**) Representative cTEC^{lo} contour plots based on α6-integrin and Sca-1 expression during ageing in male and female mice. (**B**) Proportion of TEPC, Sca-1^{int} and Sca-1^{lo} within cTEC^{lo} and (**C**) the proportion of Ki67^{+} cells within these cTEC^{lo} subpopulations, from 4-wk-, 7-wk- and 8-mo-old male and female mice. Results are presented as mean ± SEM (n=4-6 per age group; 2 independent experiments). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, using ordinary two-way ANOVA with Tukey's multiple comparisons.

3.3.5 ACCUMULATION OF SCA-1^{hi} PHENOTYPE IN mTEC^{lo} WITH AGEING

Interestingly, we found a similar trend in the mTEC compartment with regards to Sca-1 expression, with an accumulation of Sca-1^{hi} cells during ageing (**Figure 3.5 A**). However, it was previously found mTEC^{lo} cells do not harbour any TEPC progenitors (Wong, Lister et al. 2014) and recent studies have shown that a post-AIRE population also resides within the mTEC^{lo} subset (Michel, Miller et al. 2017).

Similar trends in these Sca-1$^{hi/int/lo}$ subpopulations within mTEC^{lo} were evident in both males and females across the age groups analysed (**Figure 3.5 A**). Unlike the cortical TEPC subset, which increased from the onset of puberty (7-wk-old), the proportional rise in α6^{hi}Sca-1^{hi} mTEC^{lo} was not evident until middle-age in both males (30.1% at 7-wks vs 45.3% at 8-mo) and females (22.1% at 7-wks vs 40.3% at 8-mo). However, in males, a gradual decline in Sca-1^{lo} mTEC^{lo} was first evident at 7-wks and continued to decline to middle-age (**Figure 3.5 B**). In females, this decline in Sca-1^{lo} mTEC^{lo} was delayed, with a reduction only significant at middle-age. Females also maintained a significantly higher proportion of Sca-1^{lo} mTEC^{lo} by middle-age, compared to males.

Unlike the Sca-1$^{hi/lo}$ populations in cTEC, there were no dramatic changes in the proportion of Ki-67 expressing cells within the equivalent mTEC^{lo} subsets during ageing **(Figure 3.5 C)**, despite a significant loss in Sca-1^{lo}mTEC^{lo} proportions. This data suggests a Sca-1^{hi} mTEC^{lo} differentiation blockade contributes to the decline in generation of Sca-1^{lo} mTEC^{lo}.

A mTEC^lo gated on UEA-1^+ MHCII^lo TECs
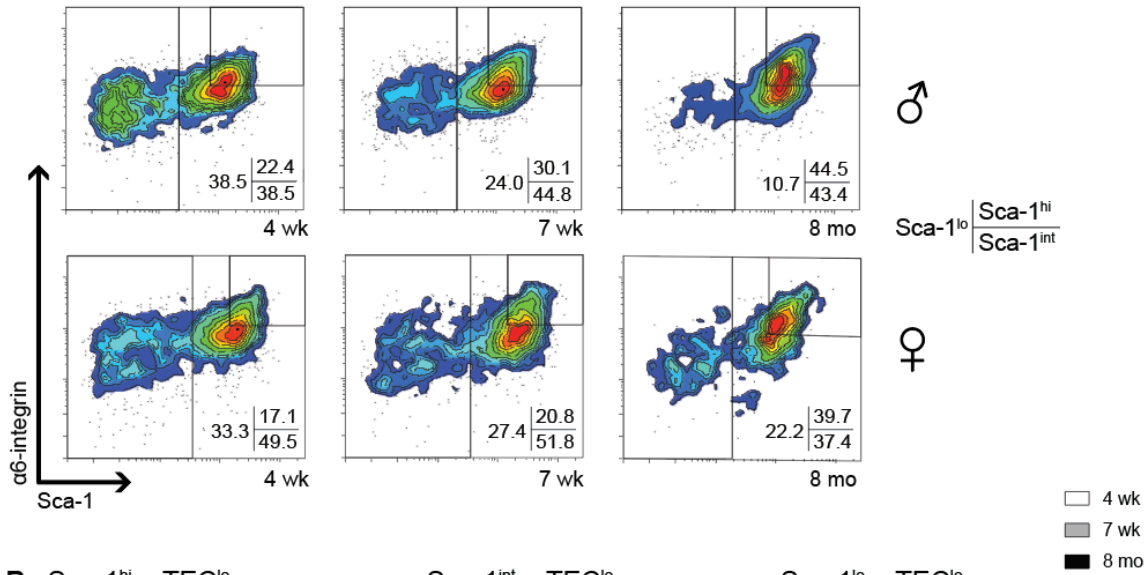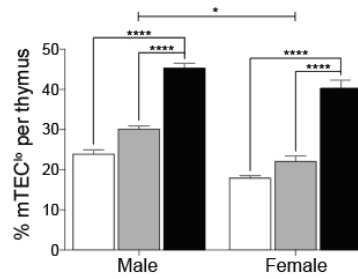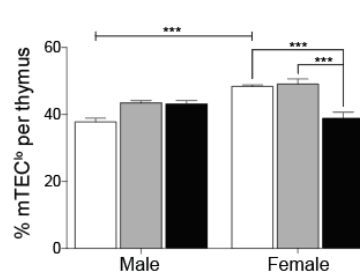
B Sca-1^hi mTEC^lo   Sca-1^int mTEC^lo   Sca-1^lo mTEC^lo
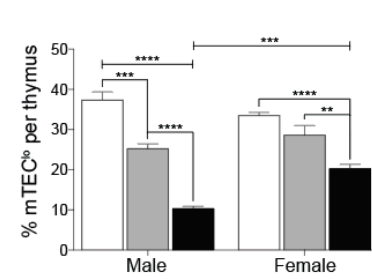
C Ki-67^+ Sca-1^hi mTEC^lo   Ki-67^+ Sca-1^int mTEC^lo   Ki-67^+ Sca-1^lo mTEC^lo
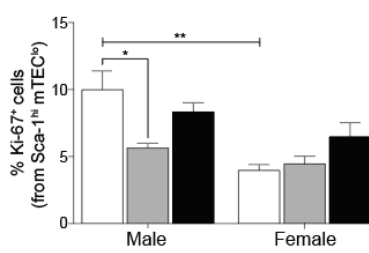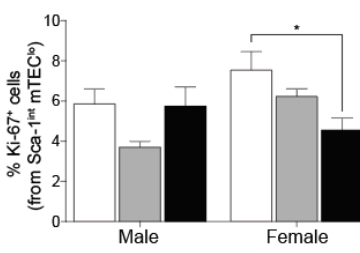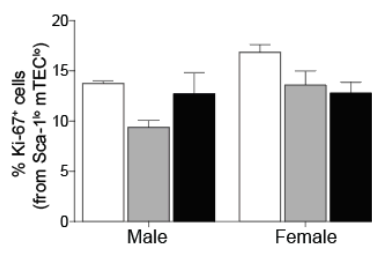
Figure 3.5. Accumulation of Sca-1^{hi} mTEC^{lo} with ageing. (**A**) Representative mTEC^{lo} contour plots based on α6-integrin and Sca-1 expression during ageing in male and female mice. Proportion of (**B**) mTEC^{lo} subsets: Sca-1^{hi} mTEC^{lo}, Sca-1^{int} mTEC^{lo}, Sca-1^{lo} mTEC^{lo} and (**C**) proliferating cells within, from 4-wk-, 7-wk- and 8-mo-old male and female mice. Results are presented as mean ± SEM (n=4-6 per age group; 2 independent experiments). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, using ordinary two-way ANOVA with Tukey's multiple comparisons.

3.3.6. MATURE cTEC^{hi} AND mTEC^{hi} SUBSETS EXPRESS LOW LEVELS OF SCA-1

Bipotent TEC progenitors in the cTEC^{lo} subset have been found to express high levels of α6 and Sca-1 (Lepletier, Hun et al. 2019), with Sca-1 expression levels proposed to reduce with differentiation into single lineage progenitors, such that Sca-1^{lo}cTEC^{lo} differentiate into cTEC^{hi} and Sca-1^{lo}mTEC^{lo} differentiate into mTEC^{hi}. We therefore analysed Sca-1 levels in mature cTEC^{hi} and mTEC^{hi}.

We found both cTEC^{hi} and mTEC^{hi} expressed low levels of Sca-1 in all age groups and in both males and females **(Figure 3.6 A & B)**. mTEC^{hi} also showed reduced expression of α6-integrin in all age groups, whereas cTEC^{hi} maintained high-intermediate levels of α6-integrin. Interestingly, the mTEC^{hi} subset does maintain a proportion of cells expressing intermediate levels of Sca-1. We might speculate mTEC^{hi}Sca-1^{int} represents a transit amplifying population, but further research would be required to support this concept. Together, these data support the reduction of Sca-1 expression levels as a model for TEC differentiation into mature cells, with mature mTEC^{hi} also showing reduced α6-integrin levels.

A cTEC^{hi} gated on UEA-1$^-$ MHCII^{hi} TECs

♂	95.0 $\frac{0.7}{4.2}$	94.6 $\frac{1.1}{4.3}$	90.4 $\frac{3.8}{5.8}$
	4 wk	7 wk	8 mo

Sca-1^{lo} $\frac{\text{Sca-1}^{hi}}{\text{Sca-1}^{int}}$

α6 / Sca-1

B mTEC^{hi} gated on UEA-1$^+$ MHCII^{hi} TECs

Sca-1^{lo} $\frac{\text{Sca-1}^{hi}}{\text{Sca-1}^{int}}$

α6 / Sca-1
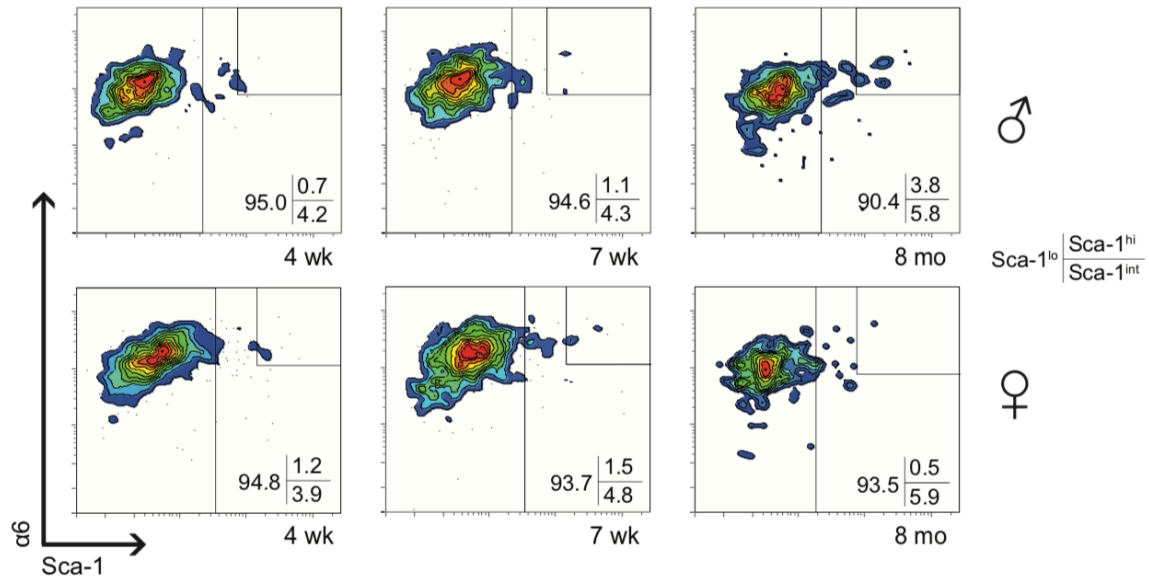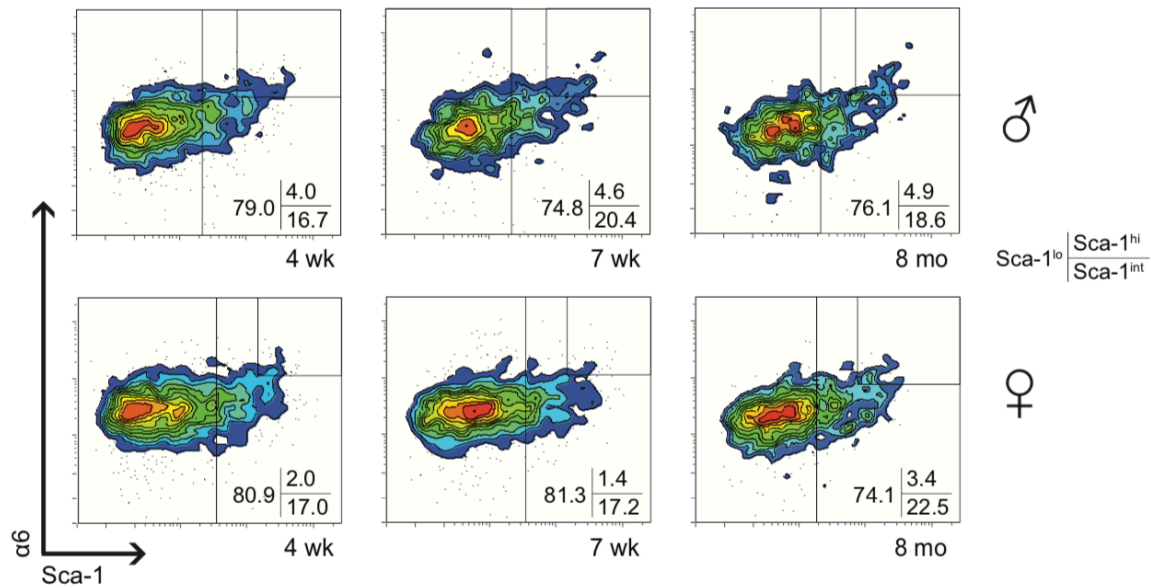
Figure 3.6. Mature cTEC^{hi} and mTEC^{hi} predominantly express low levels of Sca-1 in male and female mice. (**A**) Representative cTEC^{hi} contour plots based on α6-integrin and Sca-1 expression during ageing in 4-wk-, 7-wk- and 8-mo-old male and female mice. (**B**) Representative mTEC^{hi} contour plots based on α6-integrin and Sca-1 expression during ageing in male and female mice. n=4-6 per age group; 2 independent experiments.

3.3.7 PROPORTIONAL AND NUMERICAL LOSS OF AIRE$^+$ mTEC^{HI} AND AIRE$^+$ cTEC^{HI} WITH AGEING

The expression of the autoimmune regulator (Aire) in mature mTECs is crucial for central tolerance induction, as it controls promiscuous expression of a large set of tissue restricted antigens in mice (Anderson, Venanzi et al. 2002, Derbinski, Gäbler et al. 2005). Given the loss of mTEC^{hi} with ageing, we next investigated alterations in Aire$^+$ mTECs in pre-pubertal, post-pubertal and middle-aged male and female mice.

The gating strategy for Aire expression as a proportion of total mTECs is shown in **Figure 3.7 A**. As expected, the Aire$^+$ cells co-express high levels of MHCII. A significant proportional decline in Aire$^+$ mTECs occurred from the onset of puberty in both males and females and remained at this level to middle-age, with the level of decline significantly less in females (**Figure 3.7 B**). Numerically, both males and females showed a significant two-fold decline from the onset of puberty at 7-wks, and a further two-fold decline by middle-age (**Figure 3.7 C**), reflecting the proportional loss in mTEC^{hi} from puberty onwards previously seen in **Figure 3.2 E**. We found earlier, a reduced loss of thymocytes in females compared to males during the ageing process, but both genders showed an equal numerical loss in total TECs. While females maintained a slightly higher proportion of mTEC^{hi}, here we find similar numbers of Aire$^+$ mTECs to males. We can visualise this in the significantly reduced Aire$^+$ mTEC^{hi} / thymocyte ratio in middle-aged females (**Figure 3.7 D**).

Traditionally, cTECs are not usually analysed for expression of Aire. However, a recent publication investigating 3-wk-old C57BL/6 mice identified intermediate levels of Aire in cTEC^{hi}, which was reduced by two-fold in 3-wk-old mice born from germ free mothers (Hu, Eviston et al. 2019). We therefore investigated Aire expression in total cTECs in

males and females during ageing, with the gating strategy shown in **Figure 3.7 E**. Pre-pubertal females expressed almost three-fold more Aire$^+$ cTECs than their male counterparts, proportionally and numerically **(Figure 3.7 F & G)**. However, a two-fold drop occurred following the onset of puberty and remained at that level to middle-age. A gradual proportional drop was also evident in males. While Aire$^+$ cTEC^{hi} are a minor population, females did maintain a higher proportion than males by middle-age.

Thus, both genders demonstrate similar trends in the loss of Aire expression in mTEC and cTEC from the onset of puberty, with females maintaining a higher proportion by middle-age, reflecting their slightly better cTEC^{hi} maintenance overall. The Aire$^+$ cTEC^{hi} cells were previously found to reside at the cortico-medullary junction in 3-wk-old mice (Hu, Eviston et al. 2019), and co-expressed β5t, a proteosome subunit expressed in mature cTECs (Murata, Sasaki et al. 2007). This presents the intriguing question, whether a novel non-canonical pathway exists for the generation of Aire$^+$ mTEC^{hi}, specifically whether Aire$^+$ cTEC^{hi} cells are precursors to Aire$^+$ mTEC^{hi}. Alternatively, whether Aire$^+$ β5t$^+$ cTEC^{hi} found near the cortico-medullary junction may be involved in negative selection of early stage CD4+ and CD8+ SP cells as they migrate into the medullary region. This is beyond the scope of the current study but warrants further investigation.

A Aire⁺ mTECs gated on UEA-1⁺ TECs
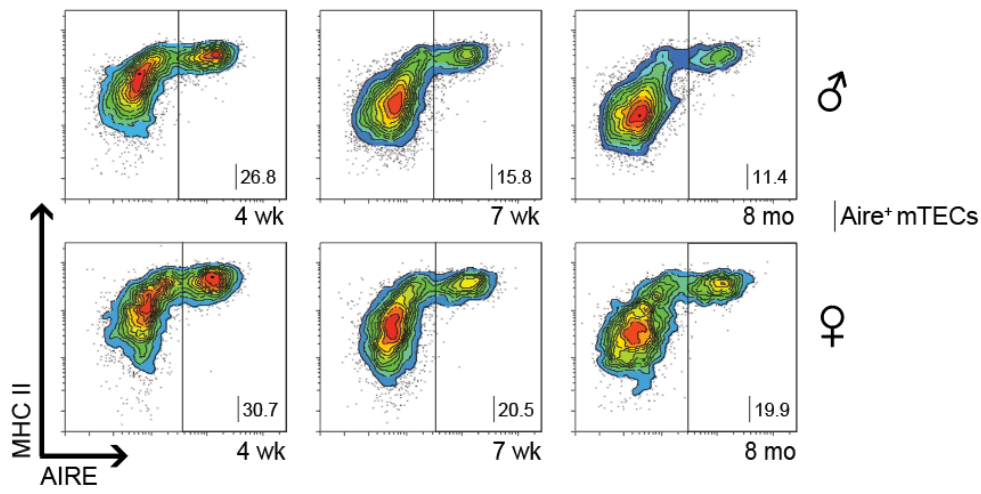
B
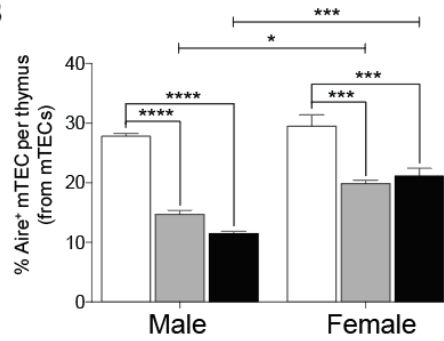
C
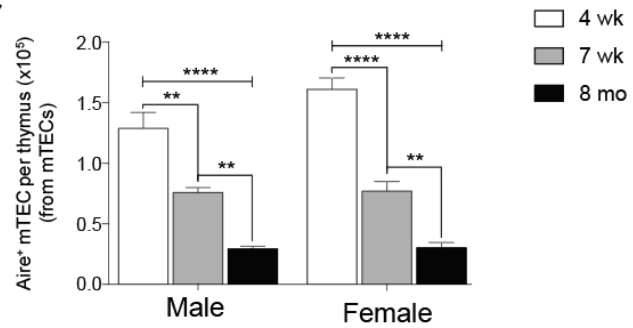
D
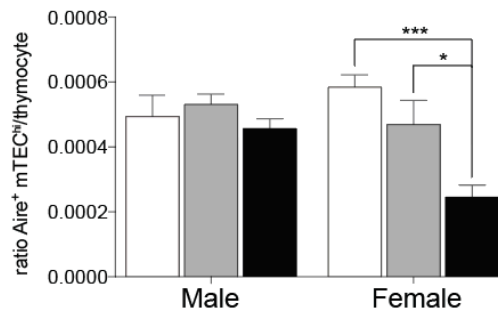
Figure 3.7 (I) Aire$^+$ mTEC^{hi} diminishes with ageing in male and female C57BL/6J mice. (**A**) Representative contour dot plots of Aire expression in mTECs from 4-wk-, 7-wk- and 8-mo-old male and female mice. Proportion (**B**) and number (**C**) of Aire$^+$ mTEC. (**D**) Ratio of Aire$^+$ mTEC to thymocyte. Data are expressed as mean ± SEM (n=4-6 per age group n=4-6 per age group; 2 independent experiments). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, using ordinary two-way ANOVA with Tukey's multiple comparisons.

E Aire⁺ cTECs gated on UEA-1⁻ TECs
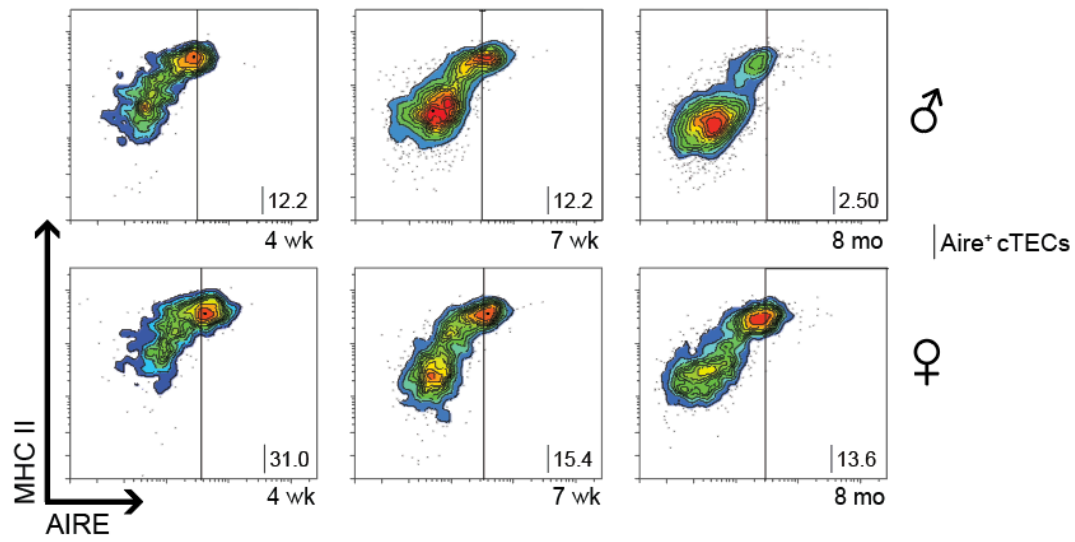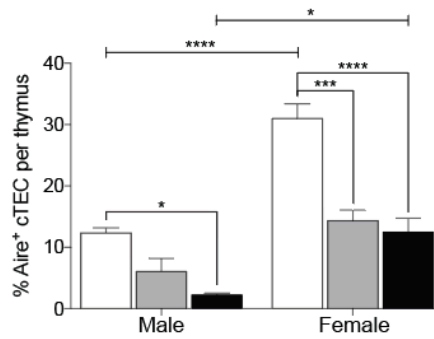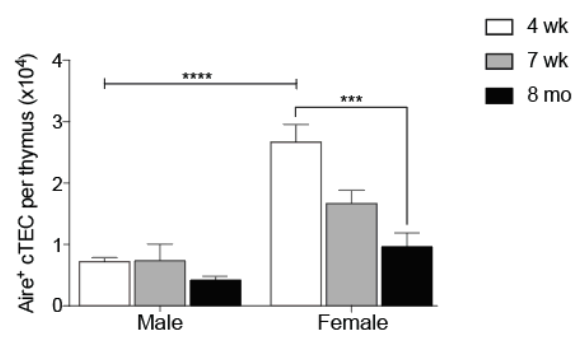
Figure 3.7 (II) Aire⁺ cTEC^hi diminishes with ageing in male and female C57BL/6J mice. (E) Representative contour dot plots of Aire expression in cTECs from 4-wk-, 7-wk- and 8-mo-old male and female mice. Proportion (**F**) and number (**G**) of Aire⁺ cTEC. Data are expressed as mean ± SEM (n=4-6 per age group n=4-6 per age group; 2 independent experiments). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, using ordinary two-way ANOVA with Tukey's multiple comparisons.

3.3.8 DIMINUTION OF TEPC FUNCTION FOLLOWING PUBERTY

Given the increased accumulation of TEPC ($\alpha6^{hi}$ Sca-1^{hi} cTEC^{lo}) from the onset of puberty, we next wanted to test for alterations in their capacity for differentiation using an *in-vitro* 3D clonogenic experimental assay previously developed in our laboratory (Wong, Lister et al. 2014, Lepletier, Hun et al. 2019). Purified TEPCs obtained from male and female 4-wk-old pre-pubertal, 7-wk-old post-pubertal and 8-mo-old middle-aged C57BL/6J mice were seeded for 7 days in 24-well plates to assess colony forming efficiency (CFE). No gender-based differences were apparent across all age groups, but a significant decline in CFE% was evident in post-pubertal and middle-aged groups (**Figure 3.8 A**). This suggests the phenotypic accumulation of TEPC from the onset of puberty coincides with a functional attenuation in both males and females.

To assess whether bipotent potential was maintained by the TEPC colonies that did form, we investigated individual colonies generated from *in vitro* TEPC cultures from 4-wk-old pre-pubertal mice and 8-mo-old middle-aged, male and female mice. TEPC colonies were isolated and analysed by immunohistochemistry for expression of β5t in cTECs, keratin-14 (K14) expressed in mTECs, and the nuclear marker DAPI to visualize cells **(Figure 3.8 B & C)**. Colonies from 4-wk-old female TEPC cultures demonstrated a higher proportion of K14$^-$ colonies than males, however, there was no notable difference with age in relation to the percentage of K14 expression in colonies (**Figure 3.8 B**). Three main types of colonies were found, which were classified into K14$^-$ cTEC (β5t$^+$ K14$^-$); differentiating colonies containing both cTEC (β5t$^+$ K14$^-$) and mTEC (β5t$^-$ K14$^+$); and fully differentiated mTEC colonies (β5t$^-$ K14$^+$) (**Figure 3.8 C**). Therefore, while functional attenuation of TEPC occurs from the onset of puberty as determined by their CFE, the

bipotent capacity of some TEPC is still evident. These observations also further support the proposal that bipotent TEPCs reside within the α6^{hi} Sca-1^{hi} cTEC^{lo} population.

It was previously proposed that activin A signalling is important in the activation of TEPC and the differentiation of TEC precursors into mature TECs, while Bmp4 maintains a pool of TEPC and immature TECs, as a counterbalance. An increase in *Fst*, which leads to reduced activin A availability, was found in cTEC, mTEC and fibroblast subsets and an increase in *Bmp4* in cTEC and mTEC was evident in 9-12 mo-old male mice, altering the Fst-activin A-Bmp4 axis and disrupting the normal balance of differentiation (Lepletier, Hun et al. 2019). We therefore investigated expression of *Fst* and *Bmp4* in female 4-wk-old pre-pubertal and 7-wk-old post-pubertal mice by RT-qPCR. Results showed a significant rise in *Fst* expression after the onset of puberty in cTEC and mTEC^{lo} subsets and a four-fold increase in *Bmp4* expression by non-TECs (**Figure 3.8 D**). These findings support a similar role in males and females for these TGF-β superfamily molecules in post-pubertal attenuation of TEPC and TEC differentiation.

A

Female ○   Male ●

B

K14⁻ colony ■   K14⁺ colony ■

Female   Male

4 wk   8 mo

C

β5t   K14   DAPI   Merge

K14⁻ colony

K14⁺ colony

10 μm

D Female

□ 4 wk   ■ 7 wk

relative *FST* expression

cTEC   mTEC^lo   mTEC^hi   non-TEC

relative *BMP4* expression

cTEC   mTEC^lo   mTEC^hi   non-TEC
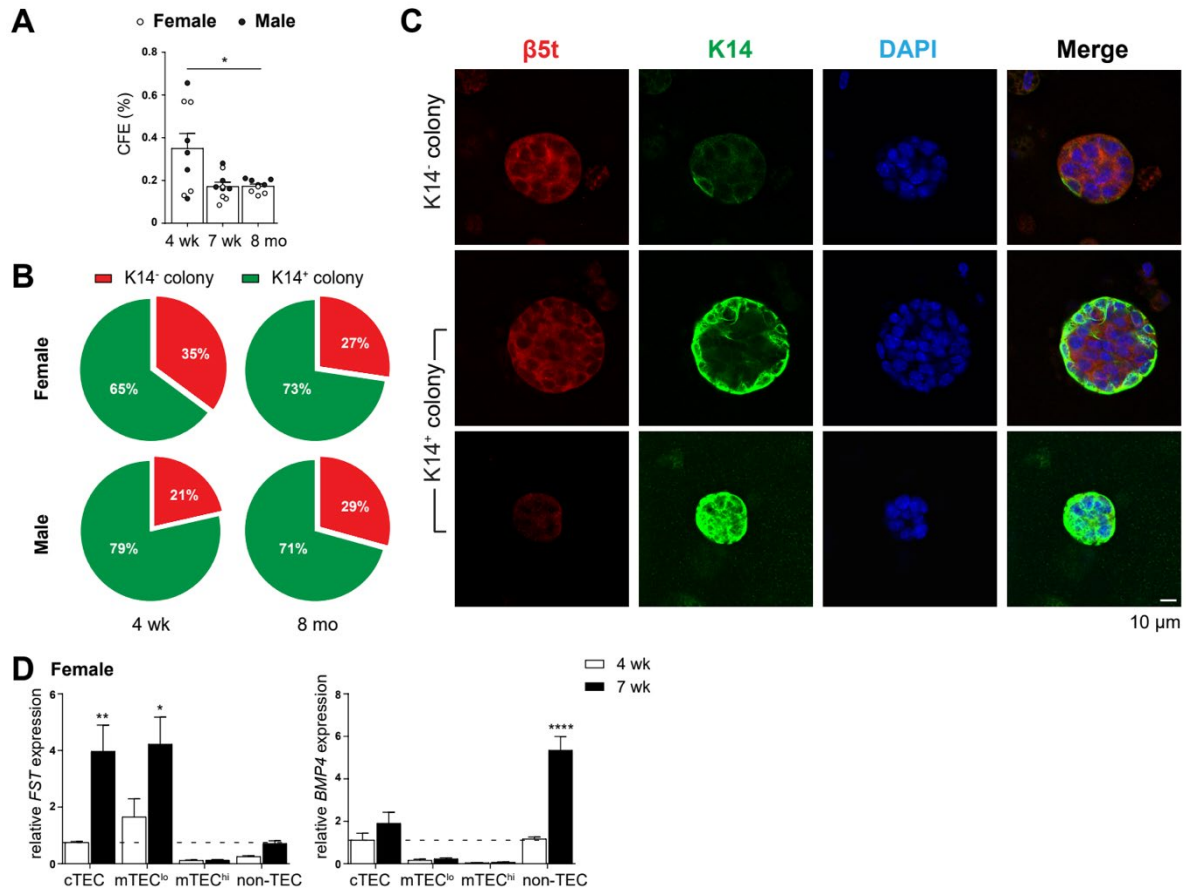
Figure 3.8. Attenuation of TEPC function following puberty in male and female mice. (**A**) Colony forming efficiency (%) of 4-wk and 7-wk, and 8-mo-old C57BL/6J male and female mice. Results are presented as mean ± SEM (n=4-10 per age group). ****p<0.0001, ordinary one-way ANOVA with Tukey's multiple comparisons. (**B**) Proportions of colony phenotypes from total count of colonies. (**C**) Representative immunofluorescent images of day 7 colonies generated from purified TEPC isolated from 4-wk-old male and female C57BL/6J mice. Colonies are stained with β5t (cortical marker; red colour), K14 (medullary marker; green) and DAPI (nuclear marker; blue). Scale bar = 10 μm. (**D**) Relative Fst and Bmp4 expression in cTEC, mTEC^{lo}, mTEC^{hi} and non-TEC subsets from 4-wk- and 8-wk-old C57BL/6J female mice; normalised to 4-wk-old cTEC. Results are presented as mean ± SEM. Five biological replicates, three independent experiments. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, using ordinary two-way ANOVA with Tukey's multiple comparisons.

3.3.9. DEGARELIX TREATMENT INDUCES DOWNREGULATION OF LUTEINISING HORMONE AND FOLLICLE STIMULATING HORMONE, IN FEMALES

Given the evidence of gender disparity in TEC ageing, we next performed a comparative analysis of the effects of sex steroid inhibition in middle-aged female and male mice using the LHRH receptor antagonist investigated in Chapter 2. The LHRH receptor antagonist, Degarelix, was injected subcutaneously (78µg/g/mouse) at day -2, to achieve complete castration by day 0 and mice were analysed at various timepoints thereafter, up to day 28 (**Figure 3.9 A**).

Previous studies showed that testosterone was reduced to castrate levels within two days following Degarelix treatment in male mice (Velardi, Tsai et al. 2014). We therefore investigated levels of luteinizing hormone (LH) and follicle stimulating hormone (FSH) in females in untreated (UT) mice and following Degarelix treatment. Our results show a significant drop in LH and FSH following Degarelix administration with LH reaching to almost zero levels, and this decline persisted until day 28 which was the final timepoint analysed (**Figure 3.9 B**).
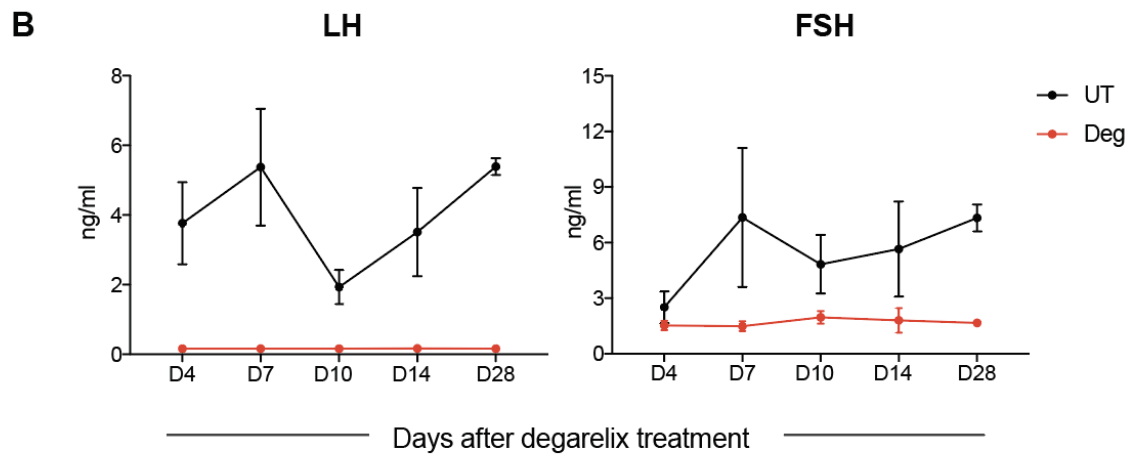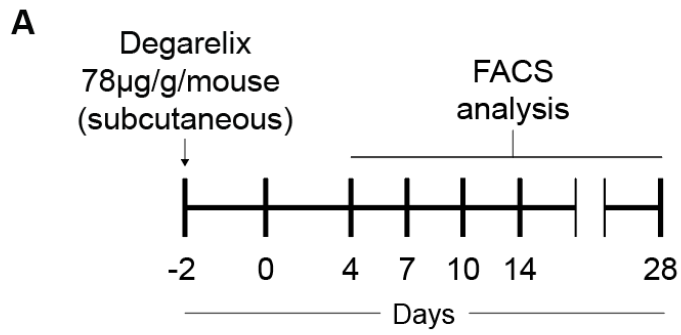
Figure 3.9. Reduced serum LH and FSH with Degarelix treatment. (**A**) Timeline of Degarelix injection and thymus analyses. (**B**) Serum LH and FSH concentrations (ng/ml) in 8-month-old female mice following Degarelix treatment from day 4 to day 28. Data are presented as mean ± SEM. Three biological replicates per time point.

3.3.10 SUBSTANTIAL RISE IN MIDDLE-AGED THYMOCYTES FOLLOWING LHRH-ANTAGONIST TREATMENT WITH GENDER DISPARITY

We next performed flow cytometric analysis on thymic subsets from female and male mice at different timepoints following Degarelix administration. Females showed an earlier numerical increase in thymocytes evident at day 7 and reaching maximal levels by day 10. In this set of experiments, the increase in thymocytes following Degarelix treatment in males was more delayed, reaching significance at day 10 and continuing to increase until day 28, the last timepoint analysed (**Figure 3.10 A**). Interestingly, females did not show the initial loss in TECs at day 4 evident in males and instead showed a gradual increasing trend that reached significant levels at day 10, before returning to untreated levels by day 14 (**Figure 3.10 B**). TEC numerical recovery to UT levels in males was not evident until day 14. Given there was no obvious loss in thymocyte cellularity at the day 4 timepoint, it would suggest a TEC specific phenomenon as well as gender specific.

Degarelix treated females showed no significant changes in proportion of TEC subsets (**Figure 3.10 C**). In contrast, Degarelix treated males showed a significant decline in cTEC^{lo} proportions and increase in mTEC^{hi} populations surpassing UT levels by day 10.
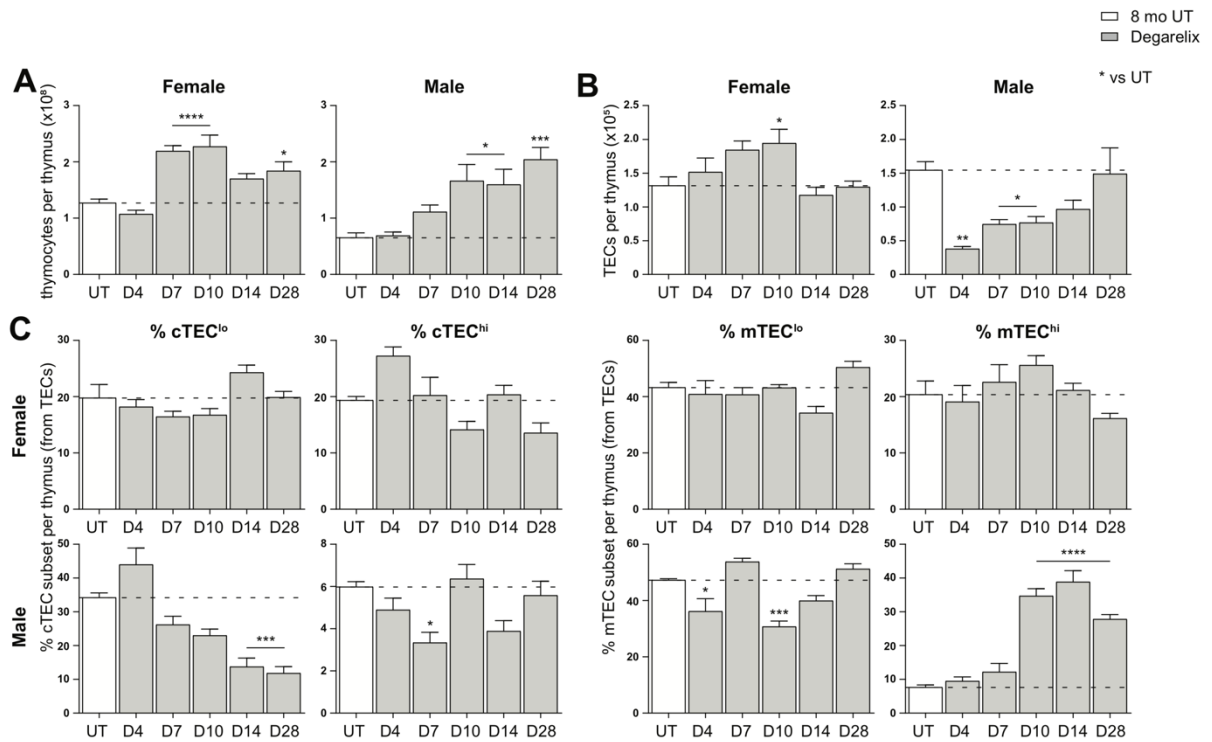
Figure 3.10 Phenotypic analysis of TEC subsets in middle-aged female and male mice following Degarelix treatment. (**A**) Numerical analysis of total thymocyte cellularity and (**B**) TEC cellularity, following Degarelix treatment in middle-aged female and male C57BL/6 mice from day 4-28 compared to untreated (UT) control mice. (**C**) Proportional analysis of TEC subsets following Degarelix treatment in female and male C57BL/6 mice from day 4-28 compared to untreated (UT) mice. Data are shown as mean ± SEM (n=5 per time point). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, using ordinary one-way ANOVA with Dunnett's multiple comparisons.

3.3.11 TEPC REACTIVATION FOLLOWING DEGARELIX

To investigate whether Degarelix induced TEPC reactivation, we further analysed the Sca-1 subpopulations within the cTEC^{lo} and mTEC^{lo} subsets (**Figure 3.11**). In males, there was an immediate decline in proportion of TEPC at day 4, concomitant with an increase in Sca-1^{int} cTEC^{lo}, suggesting early TEPC reactivation (**Figure 3.11 A**). A gradual return to untreated levels by day 28 occurred thereafter. Only transient reactivation was evident in females, with a loss in TEPC accompanied by an increase in Sca-1^{int} cTEC^{lo} at day 7. The Sca-1^{lo} cTEC^{lo} did not show any significant change other than a transient drop in females at day 10.

Figure 3.11 B shows the percentage of Sca-1 subpopulations within the mTEC^{lo} subset. Males showed a similar but more persistent trend in Sca-1 mTEC^{lo} subpopulations seen in cTEC^{lo}. An early reduction in Sca-1^{hi} mTEC^{lo} from day 4 persisting to day 28, was accompanied by an increase in Sca-1^{int} mTEC^{lo}. Females only showed a transient loss of Sca-1^{lo} mTEC^{lo} at day 7.

Together, results suggest the slight increase in TEC number associated with an increase in thymopoiesis in females occurred in a homeostatic manner with transient reactivation of TEPC. While in males, the increase in thymopoiesis and mTEC^{hi} generation following Degarelix treatment may have been via reactivation and mobilization of both TEPC and mTEC^{lo} progenitor populations.

A % cTEC^lo subset per thymus (from cTEC^lo)

B % mTEC^lo subset per thymus (from mTEC^lo)

□ 8 mo UT
■ Degarelix

* vs UT
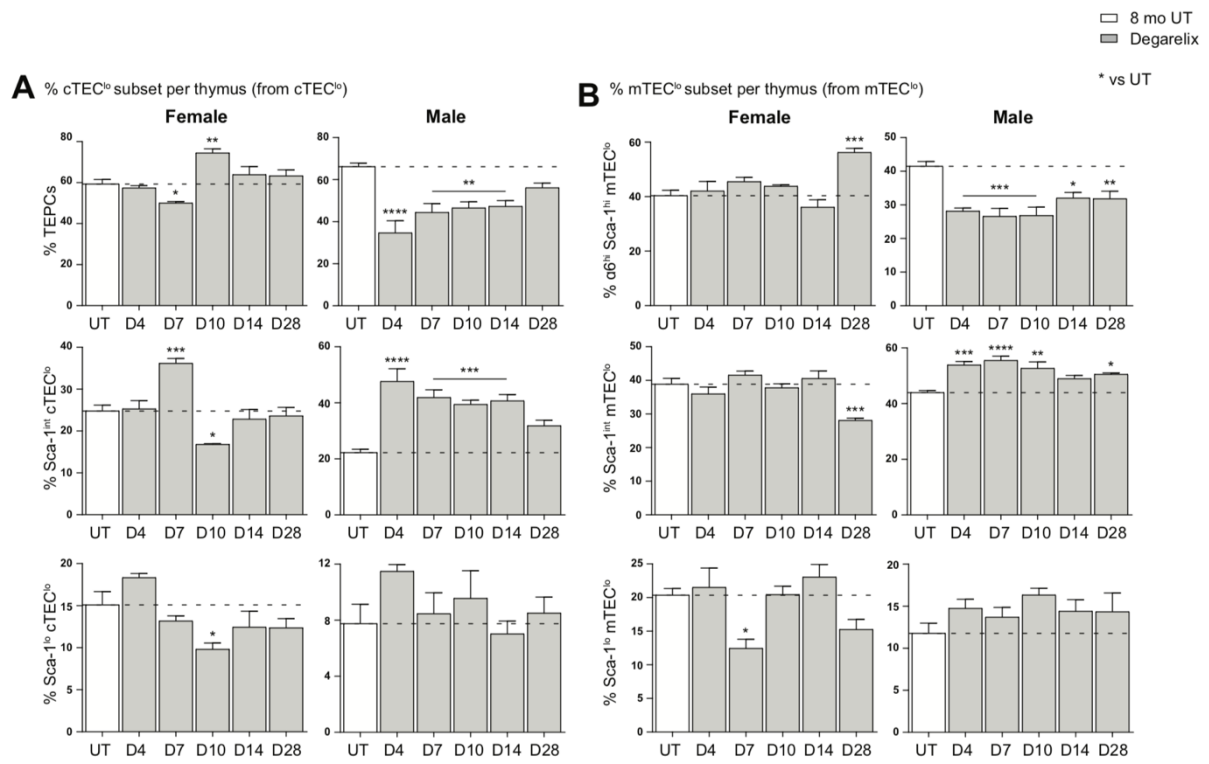
Figure 3.11. Phenotypic analysis of progenitor TEC populations in Degarelix treated middle-aged females and males. (**A**) Proportional analysis of cTEC^{lo} subsets based on α6-integrin and Sca-1 expression: TEPC, Sca-1^{int} cTEC^{lo} and Sca-1^{lo} cTEC^{lo}, following Degarelix treatment in middle-aged males and females, compared to untreated (UT) controls. (**B**) Proportional analysis of mTEC^{lo} subsets based on α6-integrin and Sca-1 expression: Sca-1^{lo} cTEC^{lo}, Sca-1^{int} cTEC^{lo} and Sca-1^{lo} cTEC^{lo}, following Degarelix treatment compared to untreated (UT) controls. Data are shown as mean ± SEM (n=5 per time point). *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, using ordinary one-way ANOVA with Dunnett's multiple comparisons.

3.3.12. DISPARATE CHANGES IN FOLLISTATIN EXPRESSION FOLLOWING DEGARELIX TREATMENT IN FEMALES AND MALES

We identified increases in *Fst* expression in cTEC and mTEC^{lo} and increases in *Bmp4* expression by non-TECs (endothelial cells and fibroblasts) associated with the phenotypic changes in TEPC and TEC subsets after the onset of puberty in both males and females (**Figure 3.8 D**). We therefore wanted to investigate whether changes in *Fst* and *Bmp4* expression preceded TEC subset modifications following Degarelix treatment and whether there was any gender disparity.

We investigated the relative expression of *Fst* and *Bmp4* in purified TEC subsets from untreated and day 5 Degarelix treated 8-mo-old middle-aged male and female mice (**Figure 3.12**). A decline in *Fst* production in cTEC^{lo} and mTEC^{lo} subsets was evident in Degarelix treated male mice, although only significant in mTEC^{lo} (**Figure 3.12 A**), which was consistent with our group's previous findings in male mice following surgical castration (Lepletier, Hun et al. 2019). Conversely in females, *Fst* expression was significantly increased in cTEC^{lo} (**Figure 3.12 C**). Both male and female groups demonstrated a significant rise in *Bmp4* expression by cTEC^{lo} in day 5 Degarelix treated mice (**Figure 3.12 B&D**). These disparate alterations in *Fst* expression between males and females with Degarelix treatment, reflect the gender differences in TEC subset regeneration seen earlier, with greater rejuvenation of cTEC^{lo} and mTEC^{hi} subsets evident in males.
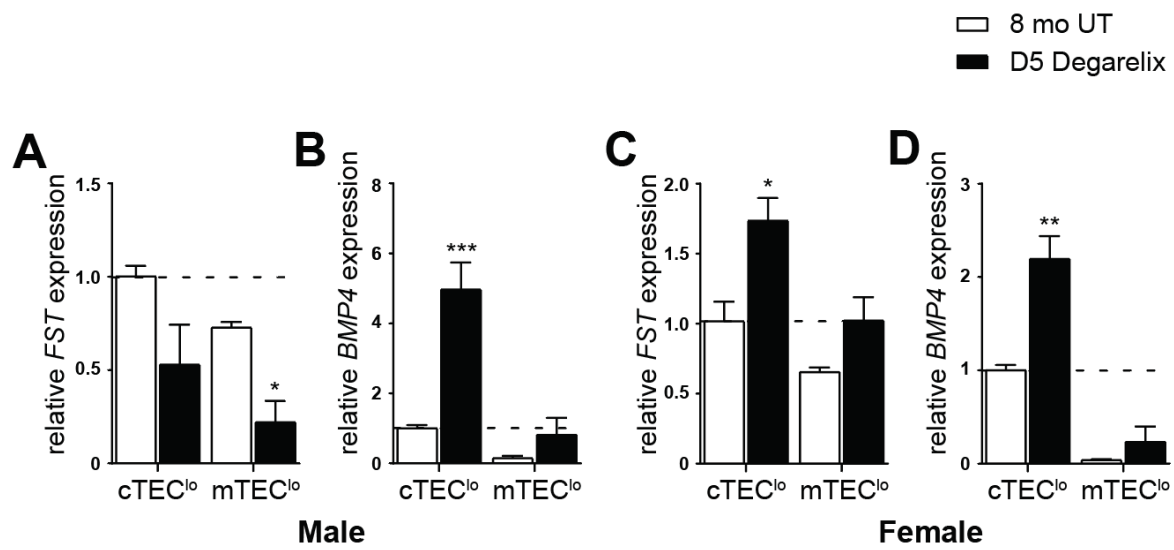
Figure 3.12. Gender differences in expression of *Fst* and *Bmp4* at day 5 following Degarelix treatment in 8-mo-old male and female mice. Relative *Fst* (**A&C**) and *Bmp4* (**B&D**) expression in cTEC^{lo} and mTEC^{lo} subsets at D5 post-Degarelix treatment in males (left) and females (right) compared to untreated (UT) control mice. Results are presented as mean ± SEM. Five biological replicates, three independent experiments. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, using ordinary two-way ANOVA with Tukey's multiple comparisons.

3.4. DISCUSSION

Investigations into thymus involution and sex steroid inhibition (SSI)-induced thymus regeneration have predominantly been carried out in male mice, with relatively minimal research into sexual dimorphism in thymus ageing and regeneration. Nevertheless, it has been recognized that while androgens and estrogens exert direct effects on the thymus, thymic involution progresses at different rates in males and females (Olsen and Kovacs 1996, Zoller and Kersh 2006). As thymic epithelial cells are critical for T cell development, we performed detailed investigations into phenotypic alterations in TEC subsets during ageing with a focus on sexual dimorphism. We extended this research to explore gender related differences in SSI-induced regeneration using a second generation LHRH-receptor antagonist.

We investigated the impact of the surge in sex steroids at puberty and longer-term ageing to middle age. Using flow cytometric analyses, we examined the phenotypic differences in thymic subsets in pre-pubertal, post-pubertal and middle-aged mice. Sexual maturity in mice occurs around 35 days of age, therefore we defined 4-wk-old mice as pre-pubertal, 7-wk-old mice as post-pubertal, and 8-mo-old mice nearing middle-age (Flurkey, Currer et al. 2007).

We initially identified two phases of thymic involution evident in both males and females; numerical loss of thymocytes at the onset of puberty, followed by a later numerical loss of total TECs by middle-age. However, while total TEC numbers had not changed at the onset of puberty, there were dramatic phenotypic changes within the major TEC subsets with evidence of sexual dimorphism.

Males continued to lose thymocyte cellularity through to middle-age, maintaining their post-pubertal thymocyte:TEC ratio, however females maintained thymocyte levels post-puberty which resulted in a greater thymocyte:TEC ratio by middle-age. This may be due to the overall better maintenance of cTEC^{hi} and mTEC^{hi} by 8mo of age able to support T cell development in females compared to males. Nevertheless, a two-fold loss in mTEC^{hi} occurred at the onset of puberty in both genders, concomitant with an accumulation of cTEC^{lo} which continued to middle-age. This imbalance was significantly greater in males.

A subset of mature mTECs express Aire, which is a transcription factor controlling the expression of tissue-restricted antigens (TRAs), important for self-tolerance induction during T cell development. Defects in central tolerance to TRAs, can lead to risks in developing certain autoimmune diseases such as multiple sclerosis, autoimmune polyglandular syndrome, type 1 diabetes mellitus (Anderson, Venanzi et al. 2002). Loss of Aire expressing mTEC^{hi} was evident at the onset of puberty in both males and females. Therefore, even though females maintained a higher level of mTEC^{hi} than males, the ratio of Aire$^+$ mTEC to thymocytes was reduced in females two-fold by middle age, while the Aire$^+$ mTEC to thymocyte ratio in males did not change from pre-pubertal levels. Single mTECs can express a wide range of TRAs, and TRAs appear to be spatially distributed in a random manner within the thymic medulla (Dhalla, Baran-Gale et al. 2020). The loss of Aire$^+$ mTECs during ageing may therefore reduce the likelihood of interaction with single thymocytes migrating through the thymic medulla. This potentially incomplete central tolerance induction of developing T cells in females may contribute to their higher predisposition to some autoimmune diseases (Olsen and Kovacs 1996, Fairweather, Frisancho-Kiss et al. 2008) alongside other risk factors (Ngo, Steyn et al. 2014).

Our group previously proposed that bipotent progenitors reside within the cTEC^{lo} compartment, and further identified the TEPC subpopulation within, that expresses high levels of α6-integrin and Sca-1, in males (Wong, Lister et al. 2014, Lepletier, Hun et al. 2019). Here we performed a comparative analysis in males and females to investigate TEPC and other Sca1 expressing subpopulations in immature and mature TEC compartments for gender disparity during ageing. Our results demonstrate TEPC (Sca-1^{hi} cTEC^{lo}) accumulation also occurs in females from the onset of puberty, with concomitant loss in downstream Sca-1^{lo} cTEC^{lo}, but to a slightly lesser extent compared to males. cTEC^{hi} express low levels of Sca-1, supporting the following cTEC differentiation progression:

TEPC (Sca-1^{hi} cTEC^{lo})➡ Sca-1^{int} cTEC^{lo} ➡ Sca-1^{lo} cTEC^{lo}➡Sca-1^{lo}c TEC^{hi}.

Overall, females did maintain over two-fold more cTEC^{hi} than males. However, cTEC^{hi} in females exhibited a greater reduction in proportion of proliferating cells from the onset of puberty, as did TEPC and Sca-1^{lo} cTEC^{lo}. Thus, the loss in cTEC^{hi} in females appeared to be due to both a block in TEPC differentiation and significantly reduced cTEC^{hi} proliferation. However, in males the more apparent block in TEPC differentiation into Sca-1^{lo} cTEC^{lo} was compensated by increased levels of proliferating Sca-1^{lo} cTEC^{lo}, possibly underlying the lack of any change in proportion of cTEC^{hi} by middle-age. This apparent functional block in TEPC differentiation was supported by their reduced colony forming efficiency, in both males and females, but with some TEPC maintaining their capacity to differentiate into both β5t$^+$ cTECs and K14$^+$ mTECs. Further *in vivo* studies on TEPC function could include single cell analysis using reaggregate organ cultures (Rossi et al.

2006).

With respect to the mTEC compartment, we also identified a similar Sca-1 expression gradient in mTEC^{lo}. The mTEC^{lo} compartment contained Sca-1^{hi} cells, suggesting direct differentiation from TEPC. Thereafter, Sca-1 expression reduces during differentiation, with mTEC^{hi} mostly expressing low levels of Sca-1. We propose a similar mTEC differentiation progression as cTECs with respect to Sca-1 expression:

TEPC➡ Sca-1^{hi} mTEC^{lo} ➡Sca-1^{int} mTEC^{lo} ➡ Sca-1^{lo} mTEC^{lo} ➡ Sca-1^{lo} mTEC^{hi}

A minor mTEC^{hi} population maintained intermediate levels of Sca-1. Whether this population represents transit amplifying cells given 15% of mTEC^{hi} are proliferating, and the Sca-1^{int} sub-population makes up around 20% of the mTEC^{hi} subset. Or they may represent post-Aire expressing cells which are in the process of reverting to Sca-1$^{int/hi}$ mTEC^{lo} as recently proposed (Ferreirinha, Ribeiro et al. 2020) and add to the age-related accumulation of this subpopulation. Future research using more sophisticated cell tracing techniques may resolve this. Thus, the major loss in mTEC^{hi} evident in both male and females appears to be due to reduced TEPC differentiation, and a block in differentiation of Sca-1^{hi}mTEC^{lo} to Sca-1^{lo} mTEC^{lo} lineage progenitors, rather than loss of proliferation in mTEC^{lo} from puberty. Mature mTECs may also be affected by an increased rate of senescence.

Alterations in the Fst-activin A-Bmp4 axis previously identified in males (Lepletier, Hun et al. 2019) also seem to be present in females with cTEC and mTEC^{lo} populations showing a dramatic increase in Fst, which leads to reduced availability of activin A proposed to be important in TEPC differentiation. The increase in Bmp4 production is also evident in

females and has previously been proposed to hold cells in a more undifferentiated progenitor state (Barsanti, Lim et al. 2017). Bmpr2, which is the receptor for Bmp4, are primarily expressed by TEPC, cTEC^{lo} and mTEC^{lo} progenitors and found to have a role in progenitor maintenance (Barsanti, Lim et al. 2017). The recent finding that Bmp4 is important in regeneration of TECs following irradiation-induced damage (Wertheimer, Velardi et al. 2018), is consistent with our findings, in that an initial expansion in the number of progenitors could occur prior to differentiation into- and replenishment of- the mTEC^{hi} population.

Overall middle-aged female mice illustrated better maintenance of cTEC^{hi} and mTEC^{hi} subsets compared to males, but sexual disparity appears to exist in the level of TEPC and lineage specific progenitor blockade, and capacity for maintaining mature TEC proliferation.

Finally, we found sexual dimorphism in the impact of SSI on TEC replenishment. Thymopoiesis did increase earlier in females which may be due to females maintaining a higher starting population of mature cTECs and mTECs at middle age, than males. But increased thymopoiesis continued in males and was accompanied by reactivation of TEPC evidenced by the reversal of cTEC^{lo} accumulation and early rapid increase in differentiation of TEPC (Sca-1^{hi} cTEC^{lo}) to Sca-1^{hi-int} mTEC^{lo}, leading to increases in mTEC^{hi}. Thus, in males, after an initial loss in TEC cellularity, SSI mainly impacted on mTEC regeneration through TEPC reactivation. Some reactivations of TEPC were apparent in females but to a much lesser extent than males, was more delayed and did not result in as obvious an expansion of mature TEC populations as seen in males. SSI did not appear to have an impact on reducing Fst levels in females, as was evident in

males following castration (Lepletier, Hun et al. 2019), and this may explain the reduced impact of SSI in reactivating TEPC in females.

CONCLUSIONS

Our results demonstrate gender disparity in the underlying mechanisms and rates of thymic involution. The onset of puberty impacted on thymopoiesis and TEPC differentiation in both males and females, with the mTEC^{hi} compartment showing the greatest loss in cellularity. However, TEPC blockade and mTEC^{hi} loss was attenuated in females, resulting in better maintenance of mature TECs and thymopoiesis by middle age. Nevertheless, females did lose Aire^{+} mTEC^{hi} to a similar degree as males resulting in a greater thymocyte:Aire^{+} mTEC^{hi} ratio during ageing. This imbalance may explain observations that females display a stronger immune response to vaccination and infections but have a higher predisposition to many autoimmune diseases. SSI via administration of LHRH-Antagonist, Degarelix, was less effective in females possibly due to minimal impact on the TEPC differentiation blockade and *Fst* expression.

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CHAPTER 4:

SHORT-TERM LHRH RECEPTOR ANTAGONIST TREATMENT AS A STRATEGY FOR ENHANCED THYMIC RECONSTITUTION FOLLOWING CHEMOTHERAPEUTIC DAMAGE

4.1 INTRODUCTION

Thymic involution involves the progressive decline of immunocompetence with ageing. The reduced output of naïve T cells and subsequent accumulation of memory T cells diminishes T-cell receptor repertoire diversity, which impairs the ability of the host to fight new infections and impacts negatively on immune surveillance against malignant cells (Fulop, Larbi et al. 2011). This process also leads to prolonged immune cell recovery following cytoablative therapies such as chemotherapy or irradiation associated with cancer treatments, particularly in the elderly (Mackall, Fleisher et al. 1994, Mackall 2000). Moreover, T cell deficiency in older allogeneic hematopoietic stem cell transplantation (HSCT) recipients can result in opportunistic infections and increased morbidity and mortality (Small, Papadopoulos et al. 1999, Maury, Mary et al. 2001). Given that recovery of a broad T cell receptor repertoire is dependent on thymus function, it is clear that clinical strategies to expedite thymus restoration and immune reconstitution following damage are needed.

Cytoablative agents used in chemotherapy can be characterised into approximately five different categories: alkylating agents, antimetabolites, topoisomerase inhibitors, microtubular poisons and cytotoxic antibiotics (Galluzzi, Buque et al. 2015). Cyclophosphamide (Cy), which is an alkylating agent, eliminates proliferating cells through interfering with DNA duplication and is commonly used in chemotherapy to treat cancers such as lymphoma, leukemia, multiple myeloma, breast cancer, ovarian cancer and small cell lung cancer. It is also used as an immune-suppressor in some autoimmune diseases and following organ transplants. Like many other chemotherapeutic agents, it causes severe immunological side effects, such as immunodeficiency.

In young mice, both Cy and dexamethasone treatment resulted in thymus damage, with a severe loss of thymocytes and TECs, particularly mature medullary thymic epithelial cells (mTEC^{hi}) important for late-stage T cell development and self-tolerance induction (Fletcher, Lowen et al. 2009). However, chemotherapy induced damage to TEC subsets, including recently identified adult bipotent thymic epithelial progenitor cells (TEPC), and the process of endogenous regeneration in ageing males and females has not been systematically investigated.

We previously found that androgen deprivation by surgical gonadectomy can enhance T cell recovery (Heng, Goldberg et al. 2005) and bone marrow lymphopoiesis (Dudakov, Goldberg et al. 2009) in young adult male mice following chemotherapy. Furthermore, luteinizing hormone releasing hormone (LHRH) receptor agonist treatment in middle-aged male mice undergoing allogeneic HSCT accelerated engraftment and enhanced T cell reconstitution (Goldberg, King et al. 2009). Clinical trials using LHRH receptor agonist treatment in allogeneic and autologous HSCT recipients, showed improved immune recovery with increased CD4+ T cell regeneration and enhanced T cell receptor repertoire and T cell function (Sutherland, Spyroglou et al. 2008). However, LHRH receptor agonists hyper-stimulate the LHRH receptor which induces an initial surge in sex steroid production, and this causes further thymus damage before castration levels are reached 3 weeks later (Hirakata, Okumi et al. 2010, Velardi, Tsai et al. 2014). Therefore, LHRH receptor antagonists which directly block the LHRH receptor, have been investigated as an alternative to the agonist analogues for a more immediate inhibition in sex steroid production that does not induce an initial spike in sex steroids.

Using a third generation LHRH receptor antagonist to treat young adult male mice, Velardi *et al.* demonstrated an increase in total thymic cellularity beyond vehicle controls by D7, whereas animals treated with the LHRH receptor agonist remained thymocyte depleted at D7 and D14, showing recovery only by D28, consistent with the 3 week lag to reach castrate levels of sex steroids (Velardi, Tsai et al. 2014). Interestingly, in a brief analysis, females did not respond as effectively to LHRH receptor antagonist treatment compared to males, for total thymocyte recovery. Velardi *et al.* further demonstrated improved HSC recovery when LHRH receptor antagonist treatment was administered prior to sub-lethal total body irradiation in male mice (Velardi, Tsai et al. 2018).

In this final chapter, we investigated gender disparity in middle-aged female and male mice undergoing one round of Cy treatment, with a focus on the phenotypic recovery of TEC subsets, both endogenously and following LHRH receptor antagonist treatment. We found that Degarelix treatment had a more profound impact on TEC subset recovery in males, with release of the age-related TEPC blockade contributing to enhanced kinetics of mature TEC replenishment. As such, we additionally analysed thymocyte recovery in males, to determine whether enhanced sequential recovery of T cell subsets occurred with Degarelix treatment, following Cy damage.

4.2 MATERIALS AND METHODS

4.2.1 ANIMALS

C57BL6/J male and female mice aged between 2-4 months (young adult) and 8-months (middle-aged) were used in our studies following the guidelines of the Australian National Health and Medical Research Council's Published Code of Practice for the Use of Animals in Research. Mice were maintained in a controlled environment at the Animal Research Laboratory (Monash University, Australia), with a standard diet and water *ad libitum*. All experiments were approved by Monash University Animal Ethics Committee (SOBSA/ADB/2015/039).

4.2.2 DEGARELIX AND CYCLOPHOSPHAMIDE TREATMENT

Middle-aged male and female mice were injected subcutaneously with Degarelix (Firmagon®; LHRH receptor antagonist) at a dose of 78μg/gram/mouse, 48 hours prior (Day -2) to allow time for sex steroids to reach castrate levels by Day 0. The following day mice were injected intraperitoneally (IP) with cyclophosphamide (Baxter, U.S.A) at a dose of 100mg/kg/day over two days (Day -1, Day 0). Control mice included gender matched, untreated middle-aged mice and middle-aged mice treated with cyclophosphamide and PBS. Mice were euthanised by CO_2 asphyxiation and analysed at days 4, 7, 10, 14 and 28, with *n=4-5* per time-point.

4.2.3 INDIVIDUAL THYMUS LOBE DIGESTION FOR FLOW CYTOMETRY

Mice were asphyxiated with CO_2 and thymuses extracted, placed into RPMI medium 1640 (Gibco, U.S.A.) then cleaned of adipose and connective tissue. Thymic lobes were snipped with fine scissors and transferred into 10ml conical tubes for enzymatic digestion using 0.03% (w/v) DNase I and 0.3% (w/v) Liberase Thermolysin Medium (Roche, Germany) in RPMI medium 1640, for 15 minutes at 37°C (Seach, Wong et al. 2012) Thymic fragments were then incubated for 10 minutes in a water bath at 37°C before gentle agitation using a wide-bore pipette for one minute and allowed to settle. The supernatant was collected in 2mL of cold fluorescence-activated cell sorting (FACS) buffer (0.1% BSA and 5mM EDTA in 1x phosphate-buffer saline (PBS) mouse tonicity) to neutralize enzymatic activity and samples placed on ice. Remaining thymic fragments were digested in fresh enzymes and the process repeated, using progressively smaller bore pipettes until tissue was fully digested. The thymic fractions were pooled and filtered through sterile 100µM nylon mesh into a new collection tube and centrifuged at 300g for 5 minutes at 4°C. Cell pellets were resuspended in FACS buffer, and cells counted using a Z2 Coulter Counter (Beckman Coulter, U.S.A.).

4.2.4 CELL STAINING FOR FLOW CYTOMETRY ANALYSIS

Cells were resuspended in primary antibody cocktail at a concentration of $1x10^6$ cells per 10µL of conjugated/ unconjugated antibody and incubated for 15 minutes at 4°C in the dark. Unbound antibodies were removed by washing twice with FACS buffer and centrifuged at 1300 rpm for 5 minutes. Where required, cells were then stained with secondary antibody for 15 minutes at 4°C. Stained samples were washed and

resuspended in FACS buffer after centrifugation then filtered into round-bottom polystyrene tubes. Propidium Iodide (PI) was added at a final concentration of 100ng/ml to exclude dead cells for live cell FACS analysis.

4.2.4.1 Antibodies used for immunofluorescent staining of TEC subsets

Primary antibodies: CD45, thymocyte; EpCAM, thymic epithelial cells; Ly51, cTEC; UEA-1, mTEC; MHCII, TEC maturity; α6 integrin and Sca-1, TEPC; Ki-67, proliferation; Aire, autoimmune regulator. Antibodies obtained from BioLegend (USA) or BD Biosciences (USA).

4.2.4.2 Intracellular staining

Intracellular staining was performed to identify proliferation (Ki67), Aire and Foxp3 expression in TEC and/or T cell populations. Cells previously stained with extracellular markers were fixed using Cytofix™ buffer (BD Biosciences, U.S.A.) for 30 minutes at 4°C. Samples were subsequently washed with Perm-wash buffer (BD Biosciences, U.S.A.), centrifuged at 300g for 5 minutes, and stained with intracellular markers or their isotype controls for 30 minutes at 4°C. Stained cells were then washed, resuspended in FACS buffer following centrifugation, and transferred into round-bottom tubes for flow cytometric analysis.

4.2.4.3 Antibodies for thymocyte and lymph node immunofluorescent staining

Primary antibodies: CD45, thymocyte; CD4 and CD8, thymocyte/T cell subsets; CD44 and CD25, subpopulations within CD4⁻CD8⁻ double negative/DN subset (DN1-DN4); CD117, ETPs within DN1 subset; CD69 and major histocompatibility complex I (MHCI), stages of immaturity in single positive/SP CD4⁺ and CD8⁺ subsets; CD3, CD4, CD25 and

Foxp3, T regulatory cells; Ki67, proliferation. Antibodies obtained from BioLegend (USA) or BD Biosciences (USA).

4.2.5 FLOW CYTOMETRY-ACQUISITION AND ANALYSIS

Using the BD FACS Canto™ II flow cytometer (BD Biosciences, U.S.A), $5×10^6$ cells were stained and acquired using up to 8 channels. FCS files obtained from Canto™ II were then analysed using Flowlogic™ v7.2 (inivai technologies, Australia). Parameter, voltage and compensation settings were established using BD FACSDiva v.6 software (BD Bioscience, U.S.A.).

4.2.6 STATISTICAL ANALYSIS

Statistical analysis was performed using GraphPad Prism 7 software and IBM SPSS statistics version 28. Independent two-way ANOVA, Mixed-designed Two-Way Repeated Measures ANOVA, Mann-Whitney U test (two tailed) and unpaired t-tests were used. Post hoc analyses were performed using Bonferroni, Tukey, Sidak and R-E-G-W-Q for multiple comparisons. Results are expressed as mean +SEM unless otherwise specified. A p-value of < 0.05 was considered statistically significant.

4.3 RESULTS

4.3.1 DEGARELIX TREATMENT ENHANCED THYMOCYTE RECOVERY FOLLOWING CHEMOTHERAPY IN BOTH FEMALES AND MALES

We performed a comparative analysis on the impact of chemotherapy alone or in conjunction with the LHRH receptor antagonist, Degarelix (Deg), on thymus cellularity in female and male mice. A subcutaneous injection of Degarelix (78µg/g/mouse) was administered to middle-aged (8-mo-old) female and male C57BL/6J mice at day -2, to achieve castration levels of sex steroids by day 0. Two intraperitoneal injections of cyclophosphamide (Cy; 100mg/kg/day) were then administered over two days (day -1, day 0), and mice analysed at days 4, 7, 10, 14 and 28 (Figure 4.1 A). Control mice received two intraperitoneal injections of cyclophosphamide only (100mg/kg/day; day -1, day 0), or no treatment at all (untreated; UT).

Cy treatment induced a dramatic reduction in total thymocyte cellularity in females and males, as evident in both the Cy and Cy+Deg groups at D4 compared to UT controls (Figure 4.1 B). Endogenous thymocyte recovery from Cy treatment reached UT levels by D28 in both females and males. Degarelix treatment enhanced the kinetics of thymocyte recovery from Cy damage, reaching UT levels by D10 in females, with males surpassing UT levels at this timepoint. Males showed a substantially greater response to Degarelix treatment, achieving thymocyte cellularity levels beyond 3-fold UT levels by D28, equivalent to pre-pubertal levels (Ch 3; Figure 3.1 A), while females achieved about 1.6-fold UT levels at this timepoint, noting that males had an almost two-fold lower UT base level. These findings suggest androgens have a more suppressive effect than estrogens on thymopoiesis.

Total TEC number remained relatively unchanged in females following Cy treatment compared to UT controls (Figure 4.1 C); whereas in males, Cy treatment induced a substantial reduction in TECs evident at D4, which suggests a greater reliance on proliferation for TEC maintenance in males. Endogenous recovery to UT levels was not yet achieved by D28 in males. Degarelix treatment did not substantially alter total TEC number in females, and it did not enhance numerical recovery of TECs in males by D28 following Cy, which was the final analysis timepoint in this study (Figure 4.1B).
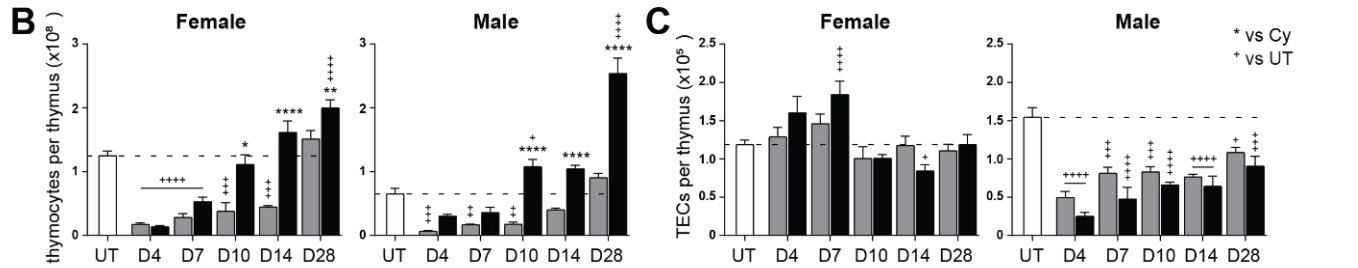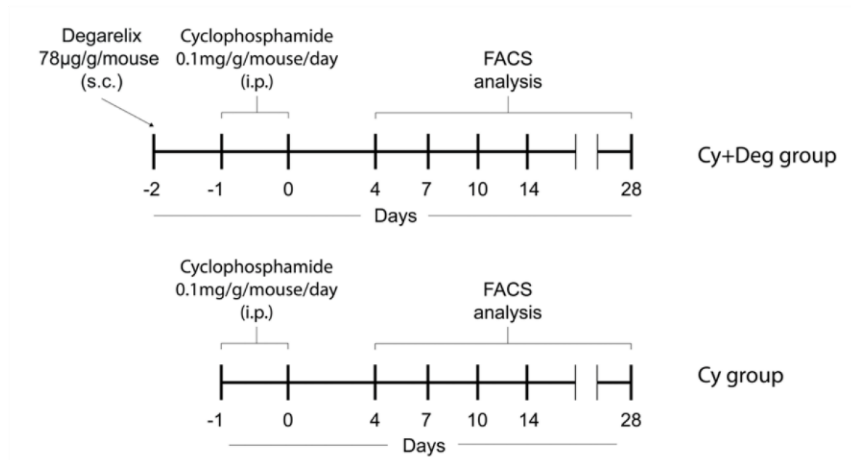
Figure 4.1 Degarelix treatment enhances thymocyte recovery following chemotherapy. (**A**) Timeline of Degarelix and Cy treatment and analysis timepoints. (**B**) Total thymus cellularity and total TEC cellularity (**C**) in 8-mo-old female and male mice treated with Cy+Deg, compared to UT and Cy alone treated controls that were age and sex matched. Data presented as mean +SEM (n>3; two independent experiments). * vs. Cy, + vs. UT. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, using ordinary two-way ANOVA with Sidak's multiple comparisons.

4.3.2 DEGARELIX INDUCES EARLIER AND ENHANCED RECOVERY OF MATURE TEC POPULATIONS FOLLOWING CYCLOPHOSPHAMIDE DAMAGE

We further examined the impact of Cy and Cy+Deg treatment on proportional changes in major TEC subsets which revealed greater sensitivity of Cy treatment in mature mTECs (Figure 4.2). Flow cytometric analysis of major TEC subsets can be assessed using either the cTEC marker Ly-51 or mTEC marker UEA-1 in combination with MHCII. **Figure 4.2 A** shows representative contour plots using Ly51 vs MHCII to divide TECs into immature (MHCII^{lo}) and mature (MHCII^{hi}) cTEC (Ly51$^+$; cTEC^{lo} and cTEC^{hi}) and mTEC (Ly51-; mTEC^{lo} and mTEC^{hi}) subsets, in UT, Cy treated and Cy + Deg treated 8-mo-old females. Of note are the different TEC subset proportions in untreated females compared to males, with males demonstrating a greater accumulation of cTEC^{lo} and at least 2-fold less cTEC^{hi} and mTEC^{hi} (**Figure 4.2 B**).

The mTEC^{hi} subset was the most affected by Cy treatment, evident by the dramatic proportional loss at D4 in both females and males (**Figure 4.2 B**). Complete endogenous recovery of mTEC^{hi} to UT levels was achieved between D14 and D28 in both females and males.

In females, no obvious reduction in mTEC^{lo} preceded the almost two-fold recovery of mTEC^{hi} by D10, despite a two-fold loss of Ki67+mTEClo at D4 and D7. However, there was a reduction at D10 in the cTEC^{lo} subset in which TEPC reside (Wong, Lister et al. 2014, Lepletier, Hun et al. 2019). This suggests endogenous replenishment of the mTEC^{lo} subset for mTEC^{hi} recovery is likely via activation of TEPC in females, in addition to the immediate proliferation of mTEC^{hi}.

In males, the recovery of mTEC^{hi} by D14 was preceded by mobilization of mTEC^{lo} from D10 which persisted until D28. Interestingly, there was no reduction in Ki67$^+$mTEC^{lo} at D4 in males as seen in females (**Figure 4.2 C**), which could suggest an immediate proliferative response to maintain mTEC^{lo} following mobilization. The increase in Ki67$^+$ mTEC^{hi} at D4 in males also suggests an immediate endogenous recovery response from Cy-damage by proliferation of either the remaining chemo-resistant cells or newly differentiated cells.

There was no significant proportional loss of cTEC^{hi} from Cy damage at D4. However, endogenous mobilization of cTEC^{lo} was apparent at D10 in females, coincident with an increase in cTEC^{hi} at D10 and D14, before returning to UT levels by D28. In contrast, there was no apparent mobilization of cTEC^{lo} in males, rather the proportion of cTEC^{lo} in males increased immediately following Cy damage, compared to UT levels, and persisted until D28. Both Ki67$^+$cTEC^{lo} and Ki67$^+$cTEC^{hi} were depleted following Cy treatment, with recovery of cycling cTEC^{lo} to UT levels by D7 and cTEC^{hi} by D14.

Thus, endogenous recovery of mTEC^{hi} involves both differentiation of mTEC^{lo} and immediate proliferation of mTEC^{hi}. Males appear to rely more heavily on proliferation for replenishment of mTEC^{lo} for continued mTEC^{hi} recovery following Cy damage, while TEPC activation may play a role in endogenous TEC recovery in females.

Degarelix treatment induced earlier mTEC^{hi} recovery following Cy damage compared to endogenous recovery, reaching significance by D7 in females and D14 in males (Figure 4.2B). Significant cTEC^{lo} mobilization was evident from D7 to D14 in females, returning to UT levels by D28, and from D10 to D28 in males. This was accompanied by a wave of

cTEC^{hi} expansion at D4 in females and a dramatic expansion of cTEC^{hi} at D7-D10 in males. Degarelix may enhance both differentiation of single lineage cTEC^{lo} progenitors and reactivation of TEPC, given both have been proposed to reside in the cTEC^{lo} compartment.

This data suggests Degarelix treatment enhanced gender-specific endogenous repair through progenitor differentiation in the early stages, with enhanced proliferation at later stages. Sequential recovery of cTEC^{hi} and mTEC^{hi} possibly occurred in parallel with recovery of thymocyte populations.

A TECs gated on PI⁻CD45⁻EpCAM⁺

Female / UT panel labels: MHCII (y-axis), Ly-51 (x-axis)

mTEC^hi | cTEC^hi
mTEC^lo | cTEC^lo

Legend:
- □ 8 mo UT
- ▨ Cy
- ■ Cy + Deg

* vs Cy
⁺ vs UT

B

% cTEC^lo   % cTEC^hi   % mTEC^lo   % mTEC^hi

Female / Male rows

% cTEC subset per thymus (from TECs)
% mTEC subset per thymus (from TECs)

C

% Ki67+ cTEC^lo (from cTEC^lo)   % Ki67+ cTEC^hi (from cTEC^hi)   % Ki67+ mTEC^lo (from mTEC^lo)   % Ki67+ mTEC^hi (from mTEC^hi)

Female / Male rows

% Ki67⁺ cTEC subset per thymus
% Ki67⁺ mTEC subset per thymus

Figure 4.2 Degarelix treatment induces an earlier and more enhanced recovery of mature TEC populations following chemotherapy. (**A**) Representative flow cytometry contour plots depicting proportional changes in 8 mo old female TEC subsets with or without Degarelix (Deg) post-Cy treatment. Ly51 and MHCII were used to divide TECs into cTEC^{lo}, cTEC^{hi}, mTEC^{lo} and mTEC^{hi} subpopulations. (**B**) Proportions of cTEC^{lo}, cTEC^{hi}, mTEC^{lo} and mTEC^{hi} subpopulations in females and males (**C**) Proportions of Ki67+ cells in cTEC^{lo}, cTEC^{hi}, mTEC^{lo} and mTEC^{hi} subpopulations in females and males. Data presented as mean +SEM (n>3; two independent experiments). * vs. Cy, $^+$ vs. UT. *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$, using ordinary two-way ANOVA with Sidak's multiple comparisons.

4.3.3 DEGARELIX ENHANCES RECOVERY OF AIRE$^+$ mTEC FOLLOWING CYCLOPHOSPHAMIDE DAMAGE

Due to the enhanced recovery of thymocytes with Degarelix treatment following Cy damage, we evaluated Aire expression within mTECs to determine whether self-tolerance mechanisms were in place. **Figures 4.3 A & C** show representative flow cytometry contour plots of Aire expression in CD45$^-$EpCAM$^+$UEA-1$^+$ mTECs and CD45$^-$EpCAM$^+$Ly51$^+$ cTECs, in UT, Cy and Cy+Deg treated 8-mo-old females and males and at D4, D7 and D14.

Cy treatment severely impacted Aire+ mTECs, shown by the substantial proportional and numerical loss at D4 and D7 in both females and males, with proportional recovery to UT levels achieved by D14 (**Figure 4.3 B**).   Degarelix treatment enhanced proportional and numerical recovery of Aire$^+$mTECs in both females and males at D14, surpassing UT levels and reaching at least a two-fold increase in cellularity compared to Cy alone groups. These data suggest maintenance of central tolerance, although further investigations into Aire-dependent tissue-restricted antigen (TRA) expression would be required for confirmation.

No significant numerical changes were observed in Aire$^+$cTECs following Cy and Cy+Deg treatments, although a proportional increase was evident at D14 in both genders (**Figure 4.3 D**).

A mTECs gated on CD45⁻EpCAM⁺UEA-1⁺

C cTECs gated on CD45⁻EpCAM⁺Ly-51⁺

Figure 4.3 Phenotypic analysis of Aire⁺ mTECs and cTECs in middle-aged female and male mice with Degarelix treatment following Cy damage. (**A**) Representative flow cytometry contour plots depicting proportional changes in the Aire⁺mTEC subpopulation with or without Degarelix following Cy treatment. (**B**) Proportion and number of Aire⁺mTECs per thymus. (**C**) Representative flow cytometry contour plots depicting proportional changes in the Aire⁺cTEC subpopulation with or without Degarelix following Cy treatment. (**D**) Proportion and number of Aire⁺cTECs per thymus. Data are presented as mean +SEM (n>3, two independent experiments). * vs. Cy, ⁺ vs. UT. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, using ordinary two-way ANOVA with Sidak's multiple comparisons.

4.3.4 DEGARELIX INCREASED TEPC RECOVERY FOLLOWING CYCLOPHOSPHAMIDE TREATMENT

We proposed mobilization and differentiation of progenitor populations as an underlying mechanism for Degarelix-enhanced recovery of mTEC^{hi} following Cy damage. To analyse the involvement of bipotent TEPC in the recovery process, we investigated proportional changes in the cTEC^{lo} subset where bipotent TEPC reside (Wong, Lister et al. 2014, Lepletier, Hun et al. 2019). As previously described, cTEC^{lo} subpopulations were analysed according to expression of α6 integrin and Sca-1; α6 integrin$^+$ Sca-1^{hi} bipotent TEPCs, Sca-1^{int} and Sca-1^{lo} single lineage progenitors. **Figure 4.4 A** shows representative flow cytometry contour plots of UT, Cy and Cy+Deg treated groups at D4, D7, D10, D14 and D28 in females and males.

In females, a substantial reduction of TEPC was evident at D4 in Cy and Cy+Deg groups (**Figure 4.4 B**), alongside significant increases in Sca-1^{int} cTEC^{lo} and Sca-1^{lo} cTEC^{lo} proportions. This suggests Cy damage to TECs induced a prompt endogenous response in females through TEPC mobilization but does not rule out some direct damage to TEPC given the significant loss of Ki67$^+$ TEPC at D4 (**Figure 4.4 B & C**). TEPC homeostasis gradually recovered in females, reaching UT levels by D28, with Degarelix treatment enhancing an earlier recovery at D14; this appears to be associated with proliferation, with a significant enhancement of Ki67$^+$ TEPC at this timepoint (**Figure 4.4 C**). However, both treatment groups in males had not reached UT levels by D28.

In males, mobilization of TEPC to Sca-1^{int} cTEC^{lo} was evident, but no further differentiation into Sca-1^{lo} cTEC^{lo} was apparent. Degarelix induced transient mobilisation of TEPC at D7, coincident with a spike in Sca-1^{int} and Sca-1^{lo} subpopulations without

enhanced proliferation (**Figure 4.4 B**); a dramatic surge in cTEC^{hi} was also observed at this timepoint (**Figure 4.2 B**). Thus, Degarelix treatment enhanced TEPC activation in females and released the TEPC differentiation blockade in males.

We therefore propose that gender disparity is apparent in endogenous recovery following Cy damage, with immediate TEPC activation and proliferation events in females, while males relied heavily on immediate proliferation events due their TEPC differentiation blockade. Degarelix treatment augmented TEPC activation in females and induced TEPC reactivation in males, which resulted in faster recovery kinetics of cTEC^{hi} and mTEC^{hi}. Enhanced proliferation of cTECs and mTEC^{lo} at D14 may relate to the wave of increased thymopoiesis with Degarelix treatment.

A cTEC^lo gated on UEA-1^−MHCII^lo TECs
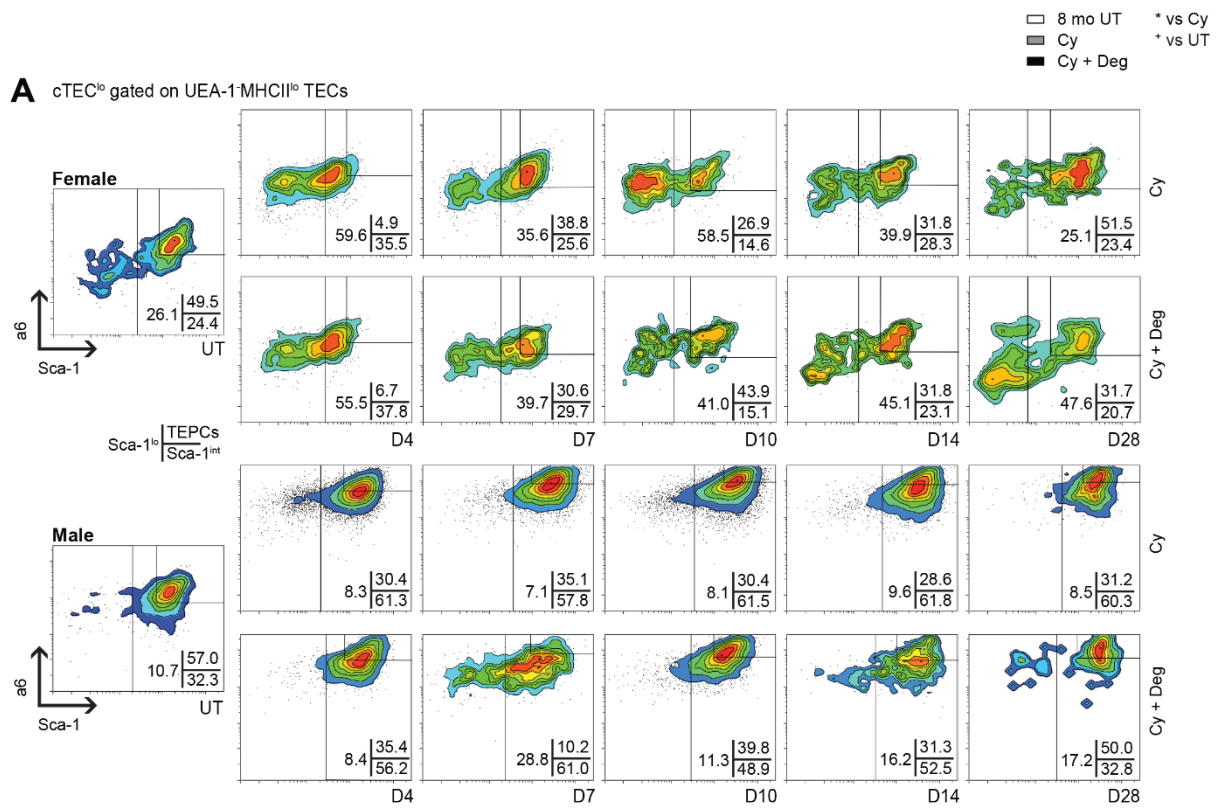
B % cTEC^lo subset per thymus (from cTEC^lo)
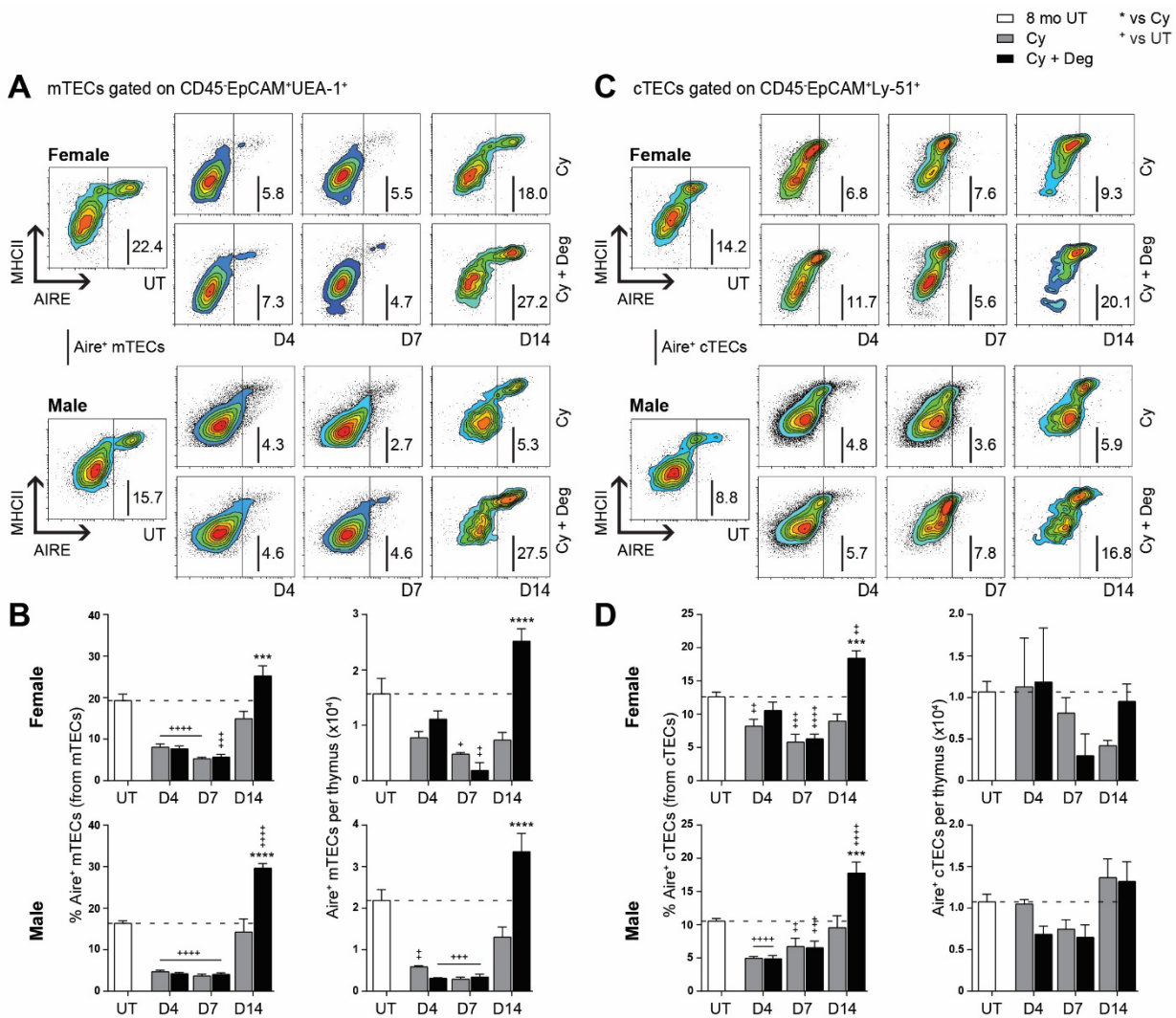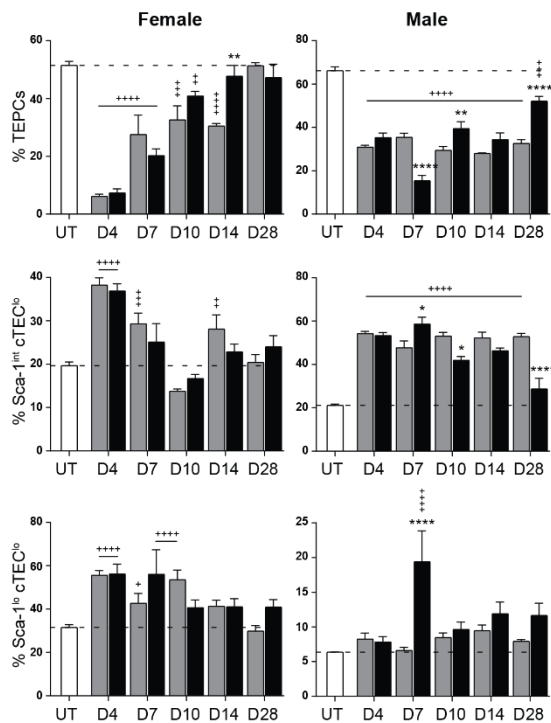
C % Ki67^+ cTEC^lo subset per thymus
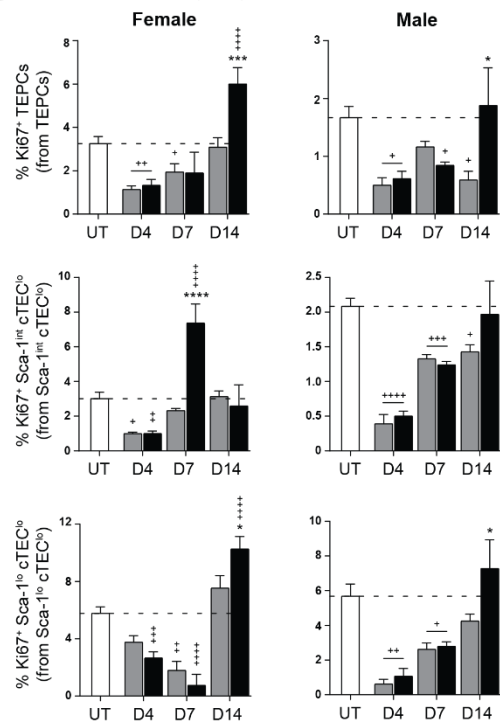
Figure 4.4 Analysis of TEPC reactivation in middle-aged female and male mice with Degarelix treatment following Cy damage. (**A**) Representative flow cytometry contour plots depicting proportional changes in cTEC^{lo} subpopulations with or without Degarelix following Cy treatment. Expression of α6-integrin and Sca-1 were used to divide cTEC^{lo} into TEPC (α6^{hi}Sca-1^{hi}), Sca-1^{int} cTEC^{lo}, and Sca-1^{lo} cTEC^{lo} subpopulations. (**B**) Proportion of TEPC (α6^{hi}Sca-1^{hi}), Sca-1^{int} cTEC^{lo}, and Sca-1^{lo} cTEC^{lo} subpopulations. (**C**) Proportion of Ki-67+ cells within cTEC^{lo} subpopulations. Data are presented as mean +SEM (n>3, two independent experiments). * vs. Cy, $^+$ vs. UT. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, ordinary two-way ANOVA with Sidak's multiple comparisons.

4.3.5 DEGARELIX TREATMENT ENHANCED DIFFERENTIATION AND PROLIFERATION WITHIN mTEC^{lo} SUBSETS FOLLOWING Cy DAMAGE

In Ch3 we proposed the following TEPC-mTEC^{hi} differentiation sequence:

TEPC➡Sca-1^{hi} mTEC^{lo}➡Sca-1^{int} mTEC^{lo}➡Sca-1^{lo} mTEC^{lo}➡Sca-1^{lo} mTEC^{hi}

Since we previously proposed single lineage mTEC^{lo} precursors originate from TEPC and give rise to mTEC^{hi}, we investigated changes in the Sca-1$^+$mTEC^{lo} subpopulations following Cy and over the course of Degarelix treatment. Subpopulations of mTEC^{lo} were divided according to expression of α6-integrin and Sca-1; α6-integrin$^+$ Sca-1^{hi}, Sca-1^{int} and Sca-1^{lo}. Representative flow cytometry contour plots of UT, Cy and Cy+Deg treated groups at D4, D7, D10, D14 and D28 in females and males are shown in **Figure 4.5 A**.

Similar immediate mobilization trends evident in the cTEC^{lo} subpopulations, were apparent in female mTEC^{lo} subpopulations in both treatment groups, transitioning from Sca-1^{hi} to Sca-1^{int} and Sca-1^{lo} at D4 (**Figure 4.5 B**), which preceded the significant increase in mTEC^{hi} at D7 in females (**Figure 4.2 B**). This trend was not evident in endogenous recovery in males, and delayed in the Degarelix treated group, with a transient reduction in proportion of α6hi Sca-1^{hi} mTEC^{lo} at D7. This gender disparate mobilization of Sca-1^{hi} mTEC^{lo} in Cy+Deg groups may explain the accelerated recovery of mTEC^{hi} reaching 20% by D7-10 in females, but males only achieving this level by D14.

Cy treatment induced a loss of Ki67$^+$Sca-1^{lo} mTEC^{lo} at D4 in females, however this population had increased in males, supporting immediate proliferation as a mechanism for immediate endogenous recovery of existing Sca-1^{lo} mTEC^{lo} precursors for mTEC^{hi}

regeneration in males (**Figure 4.5 C**). This was enhanced with Degarelix treatment at D14 which may relate to replenishment of mTEC^{lo} at this timepoint.

Within the mTEC compartment, these data implicate immediate differentiation of Sca1^{hi} mTEC^{lo} rather than proliferation as the driving force of mTEC^{hi} restoration in females, enhanced with Degarelix treatment. While endogenous recovery in males engaged immediate proliferation for mTEC^{lo} replenishment, with Degarelix treatment activating Sca1^{hi} mTEC^{lo} mobilization.

A mTEC^lo gated on UEA-1⁺MHCII^lo TECs

Legend (top right):
□ 8 mo UT   * vs Cy
■ Cy   + vs UT
■ Cy + Deg

Female

a6 / Sca-1

Cy row values:
- D4: 27.5 | 10.1 / 62.4
- D7: 16.2 | 52.5 / 31.3
- D10: 12.5 | 70.0 / 17.5
- D14: 9.9 | 62.8 / 27.3
- D28: 12.0 | 68.9 / 19.1

UT (Female): 17.2 | 59.1 / 23.7

Sca-1^lo | a6^hi Sca-1^hi / Sca-1^int

Cy + Deg row values:
- D4: 28.8 | 10.2 / 61.0
- D7: 35.1 | 36.5 / 28.4
- D10: 13.7 | 77.0 / 9.3
- D14: 13.7 | 64.1 / 22.2
- D28: 9.0 | 66.5 / 24.5

Male

a6 / Sca-1

Cy row values:
- D4: 10.6 | 38.1 / 51.3
- D7: 12.2 | 34.9 / 52.9
- D10: 12.4 | 29.6 / 58.0
- D14: 13.6 | 28.7 / 57.7
- D28: 9.4 | 38.2 / 52.4

UT (Male): 10.3 | 40.1 / 49.6

Cy + Deg row values:
- D4: 17.8 | 35.2 / 47.0
- D7: 28.2 | 19.5 / 52.3
- D10: 15.7 | 37.6 / 46.7
- D14: 19.6 | 29.2 / 51.2
- D28: 14.7 | 51.5 / 33.8

B % mTEC^lo subset per thymus (from mTEC^lo)

Female / Male

Top: % a6^hi Sca-1^hi mTEC^lo
Middle: % Sca-1^int mTEC^lo
Bottom: % Sca-1^lo mTEC^lo

x-axis: UT, D4, D7, D10, D14, D28

C % Ki67⁺ mTEC^lo subset per thymus

Female / Male

Top: % Ki67⁺ a6^hi Sca-1^hi mTEC^lo (from Sca-1^hi mTEC^lo)
Middle: % Ki67⁺ Sca-1^int mTEC^lo (from Sca-1^int mTEC^lo)
Bottom: % Ki67⁺ Sca-1^lo mTEC^lo (from Sca-1^lo mTEC^lo)

x-axis: UT, D4, D7, D14

Figure 4.5 Analysis of mTEC precursor subpopulations in middle-aged female and male mice with Degarelix treatment following Cy damage. (**A**) Representative flow cytometry contour plots depicting proportional changes in mTEC^{lo} subpopulations with or without Degarelix following Cy treatment in females and males. Expression of α6-integrin and Sca-1 were used to divide mTEC^{lo} into α6^{hi}Sca-1^{hi} mTEC^{lo}, Sca-1^{int} mTEC^{lo}, and Sca-1^{lo} mTEC^{lo} subpopulations. (**B**) Proportion of α6^{hi}Sca-1^{hi} mTEC^{lo}, Sca-1^{int} mTEC^{lo}, and Sca-1^{lo} mTEC^{lo} subpopulations. (**C**) Proportion of Ki-67+ cells within mTEC^{lo} subpopulations. Data are presented as mean +SEM (n>3, two independent experiments). * vs. Cy, $^{+}$ vs. UT. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001, using ordinary two-way ANOVA with Sidak's multiple comparisons.

4.3.6 DEGARELIX ENHANCES NUMERICAL RECOVERY OF MAJOR T CELL SUBSETS FOLLOWING CYCLOPHOSPHAMIDE DAMAGE

Females demonstrated enhanced endogenous TEC recovery from Cy damage with Degarelix treatment via TEPC differentiation. However, with the greater negative impact of androgens on TEPC function and progenitor TEC mobilisation in males, the recovery from Cy damage was even more enhanced by Degarelix treatment compared to females. As such, we additionally investigated early stages of thymocyte recovery with Degarelix treatment in males.

Representative flow cytometry contour plots illustrate the four major thymocyte subsets based on CD4 and CD8 expression; double negative (DN; $CD4^-CD8^-$), double positive (DP; $CD4^+CD8^+$), CD4 single positive (CD4 SP; $CD4^+ CD8^-$) and CD8 single positive (CD8 SP; $CD4^- CD8^+$) cells in young untreated (UT), 8-mo-old PBS treated (PBS), Cy and Cy+Deg treated males at D4, D7 and D14 (**Figure 4.6 A**).

DP thymocytes showed the greatest proportional loss at D4 following Cy treatment, recovering by D7 (**Figure 4.6 B**), however, all subsets suffered a significant numerical loss with recovery to PBS aged levels by D14 (**Figure 4.6 C**). Degarelix treatment did not alter proportional recovery in any subset, however, it did significantly enhance the numerical recovery by D14 (**Figures 4.6 C**). Degarelix induced a greater proportional increase in proliferating $CD4^+$ and $CD8^+$ cells at D7 (**Figure 4.6 D**). A numerical increase in $Ki67^+$ proliferating cells was apparent across all subsets by D14 and enhanced with Degarelix treatment (**Figure 4.6 E**). This data suggests Degarelix-induced recovery of thymocytes involves enhanced proliferation, particularly in DN, $CD4^+$ and $CD8^+$ T cells.
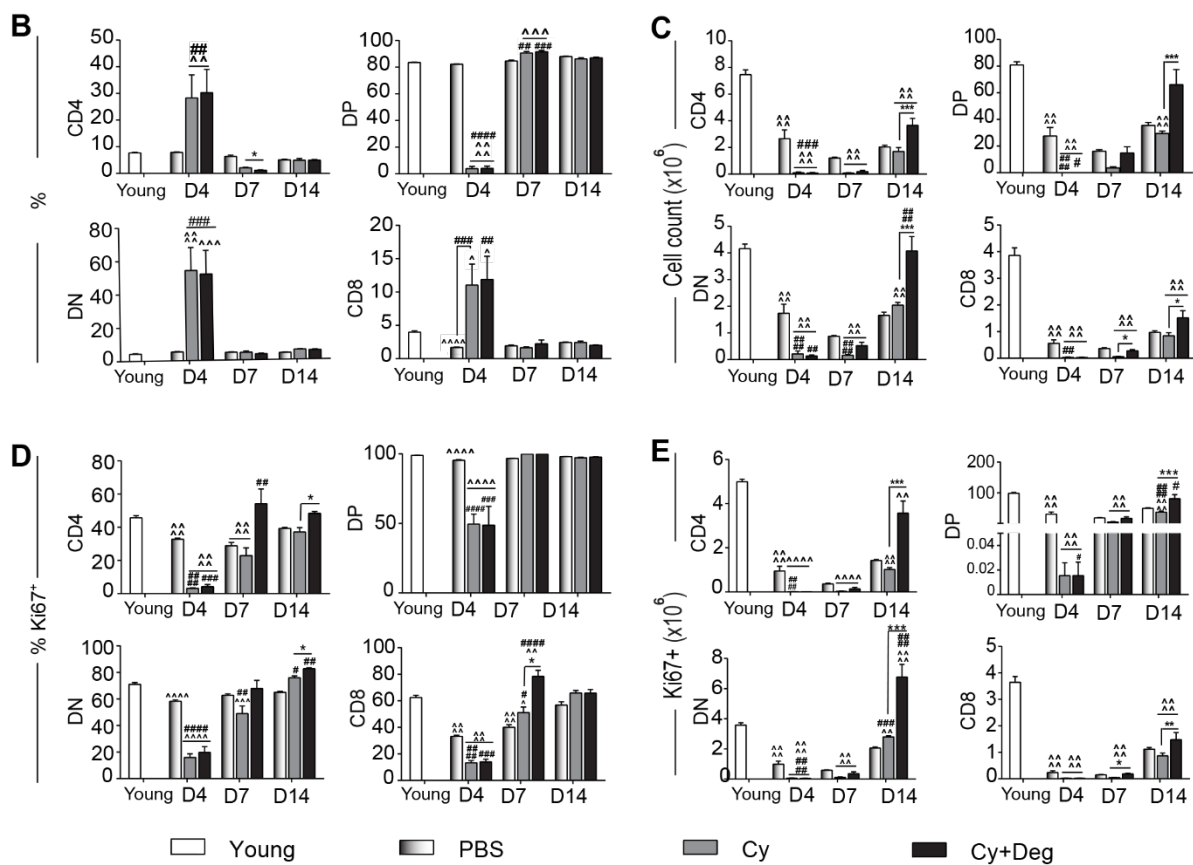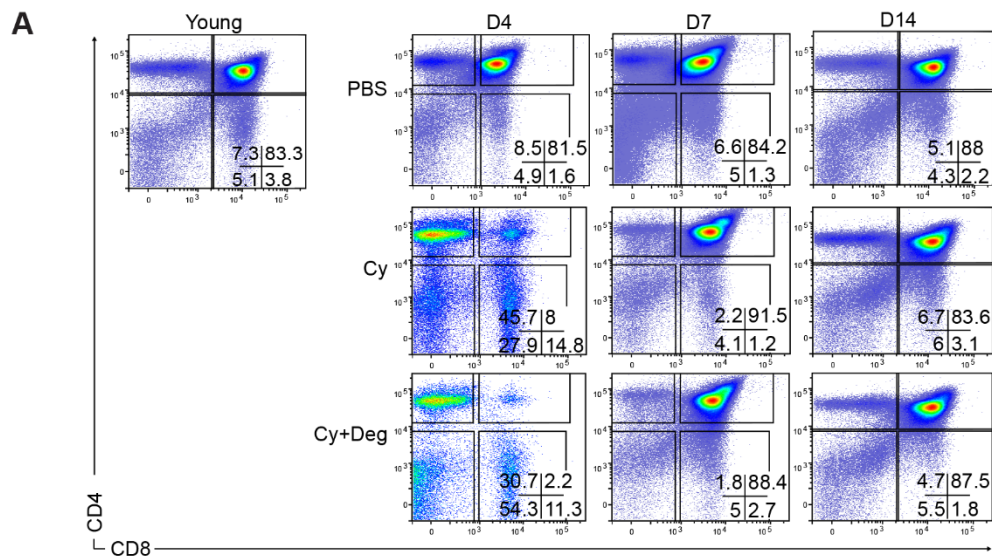
Figure 4.6 Analysis of Cy damage to major T cell subsets and recovery with Degarelix treatment in males. (**A**) Representative flow cytometry contour plots illustrating proportional changes in T cell subsets: CD4⁻CD8⁻ (double negative, DN), CD4⁺CD8⁺ (double positive, DP), CD4⁺CD8⁻ single positive (SP, CD4) and CD4⁻CD8⁺ (SP, CD8) cells in young UT controls and 8 mo old Aged UT controls and Cy and Cy+Deg treated groups. Proportions (**B**) and cell numbers (**C**) of the four T cell subsets at D4, D7 and D14 timepoints. Proportions (**D**) and cell numbers (**E**) of Ki67⁺ cells within T cells subsets. Data are presented as mean +SEM (n=5 per group, two independent experiments). ^ compared to Young UT, [#] compared to PBS and * Cy vs. Cy+Deg, where * [#] ^ $p < 0.05$, ** ^{##} ^^ $p < 0.01$, *** ^{###} ^^^ $p < 0.001$ and **** ^{####} ^^^^ $p < 0.0001$, using Mixed-design Two-Way Repeated measures ANOVA with Bonferroni and R-E-G-W-Q post hoc multiple comparisons.

4.3.7 DEGARELIX INDUCED EARLY RECOVERY OF CD4⁻CD8⁻ SUB_POPULATIONS FOLLOWING Cy-INDUCED DEPLETION

While numerical recovery of total thymocytes did not reach significance until D10 with Cy+Deg treatment (**Figure 4.7 A**), we sought to determine whether early transitional changes were evident in DN subpopulations, including ETPs.

The DN immature thymocyte subset can be further divided based on CD44 vs CD25 expression profiling into DN1 (CD44$^+$CD25$^-$), DN2 (CD44$^+$CD25$^+$), DN3 (CD44$^-$CD25$^+$) and DN4 (CD44$^-$CD25$^-$) subpopulations, with early T lineage progenitors (ETPs; CD117$^+$) residing in the DN1 group. **Figure 4.7 B** shows representative flow cytometry dot plots of DN subpopulations in UT young, PBS treated middle-aged controls and D4, D7 and D14 in Cy and Cy+Deg treated males. DN2 was most affected by Cy damage at D4, with proportions still not quite recovering to UT levels at D14 (**Figure 4.7 B&C**). However, numerical analysis revealed severely depleted DN1-4 at D4 and D7 (**Figure 4.7 D**). Early recovery was not significantly enhanced by Degarelix treatment until D14 when it surpassed PBS controls. DN1, DN2, DN3 and DN4 subpopulations showed a significant increase in cellularity with Degarelix treatment, with DN1 showing a trend of increase.

Numerically, ETPs were also severely affected by Cy treatment, with Cy+Deg groups surpassing endogenous recovery and young levels by D14, from 7010 ±571 (Cy) to 20497 ±3395 (Cy+Deg) (**Figure 4.7 E&F**). Therefore, early thymocyte recovery kinetics with Degarelix treatment, does not appear to involve an immediate increase of incoming precursors, possibly due to substantial Cy-induced damage. The first wave of thymopoiesis may be generated from remaining chemotherapy-resistant DN cells, with a second wave generated from incoming ETPs between D7 and D14.

A

thymocytes per thymus (×10⁸)

UT D4 D7 D10 D14 D28

B

D4

Young   PBS   Cy   Cy + Deg

DN1 | 4.7 | 24.4
29.7 | 39
DN4 | DN3

PBS: 6.5 | 12 / 25.1 | 44.5
Cy: 11.4 | 4.3 / 7.4 | 70.7
Cy + Deg: 11.8 | 5.8 / 20.1 | 53.3

D7

PBS: 2.8 | 20 / 19 | 44.3
Cy: 10.5 | 7.6 / 57.6 | 19.2
Cy + Deg: 3.4 | 3 / 39.3 | 46.4

D14

PBS: 3.2 | 24.6 / 25.2 | 44.9
Cy: 2.2 | 17.5 / 24.5 | 53.8
Cy + Deg: 7.3 | 13.3 / 39.9 | 37.6

CD44 / CD25

C

%DN1, %DN2, %DN3, %DN4
Young D4 D7 D14

D

DN1 (×10⁵), DN2 (×10⁶), DN3 (×10⁶), DN4 (×10⁶)
Young D4 D7 D14

E

D4

Young   PBS   Cy   Cy + Deg

ETP 10 | 3.7 | 2.1 | 3.1

D7

9.8 | 7.6 | 8.9

D14

13 | 10 | 5.4

CD117 / Empty

F

%ETP, ETP (×10⁴)
Young D4 D7 D14

☐ Young   ▨ PBS   ▨ Cy   ■ Cy+Deg

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Figure 4.7 Degarelix treatment enhances recovery of DN subpopulations following Cy damage. (A) Total number of thymocytes per thymus. **(B)** Proportions of DN subpopulations in representative dot plots; DN1 (CD44$^+$/CD25$^-$), DN2 (CD44$^+$/CD25$^+$), DN3 (CD44$^-$/CD25$^+$) and DN4 (CD44$^-$/CD25$^-$). Proportions **(C)** and cell numbers **(D)** of DN subpopulations in UT young (grey) PBS middle-aged (white), Cy (black) and Cy+Deg (red) treated groups at D4, D7 and D14. **(E)** Representative dot plots display the proportion of CD4$^-$CD8$^-$ CD117$^+$ DN1 early T lineage progenitors (ETP). **(F)** Proportion and cell number of ETPs. Data are presented as mean +SEM (n=5, 2 independent experiments). * Cy+Deg vs. Cy, $^\#$ compared to PBS, ^compared to Young UT, where *$^\#$^p<0.05, **$^{\#\#}$^^ p<0.01, ***$^{\#\#\#}$^^^ p<0.001, ****$^{\#\#\#\#}$^^^^ p<0.0001, using Mixed-design Two-Way Repeated measures ANOVA with Bonferroni and R-E-G-W-Q post hoc multiple comparisons.

4.3.8 ENHANCED NUMERICAL RECOVERY OF SEMI MATURE CD4 SUBPOPULATION IN DEGARELIX-TREATED MICE

Semi-mature (SM), mature 1 (M1) and mature 2 (M2) CD4 and CD8 T cell subsets were evaluated in young UT and middle-aged PBS controls, and D4, D7 and D14 in Cy and Cy+Deg treated males, with representative flow cytometry dot plots shown in **Figure 4.8 A**. Cy treatment induced an immediate proportional loss of semi-mature CD8 and CD4 subsets, and subsequent proportional loss of the $CD8^+$ and $CD4^+$ M1 populations at D7, with no apparent impact from Degarelix treatment (**Figure 4.8 B&C**).

Cy treatment induced a profound numerical loss of SM, M1 and M2 $CD8^+$ and $CD4^+$ T cells at D4 - D7, recovering to PBS control levels by D14 and enhanced with Degarelix treatment (**Figure 4.8 D&E**). Overall, improved recovery from Cy damage with Degarelix treatment was only evident by D14.
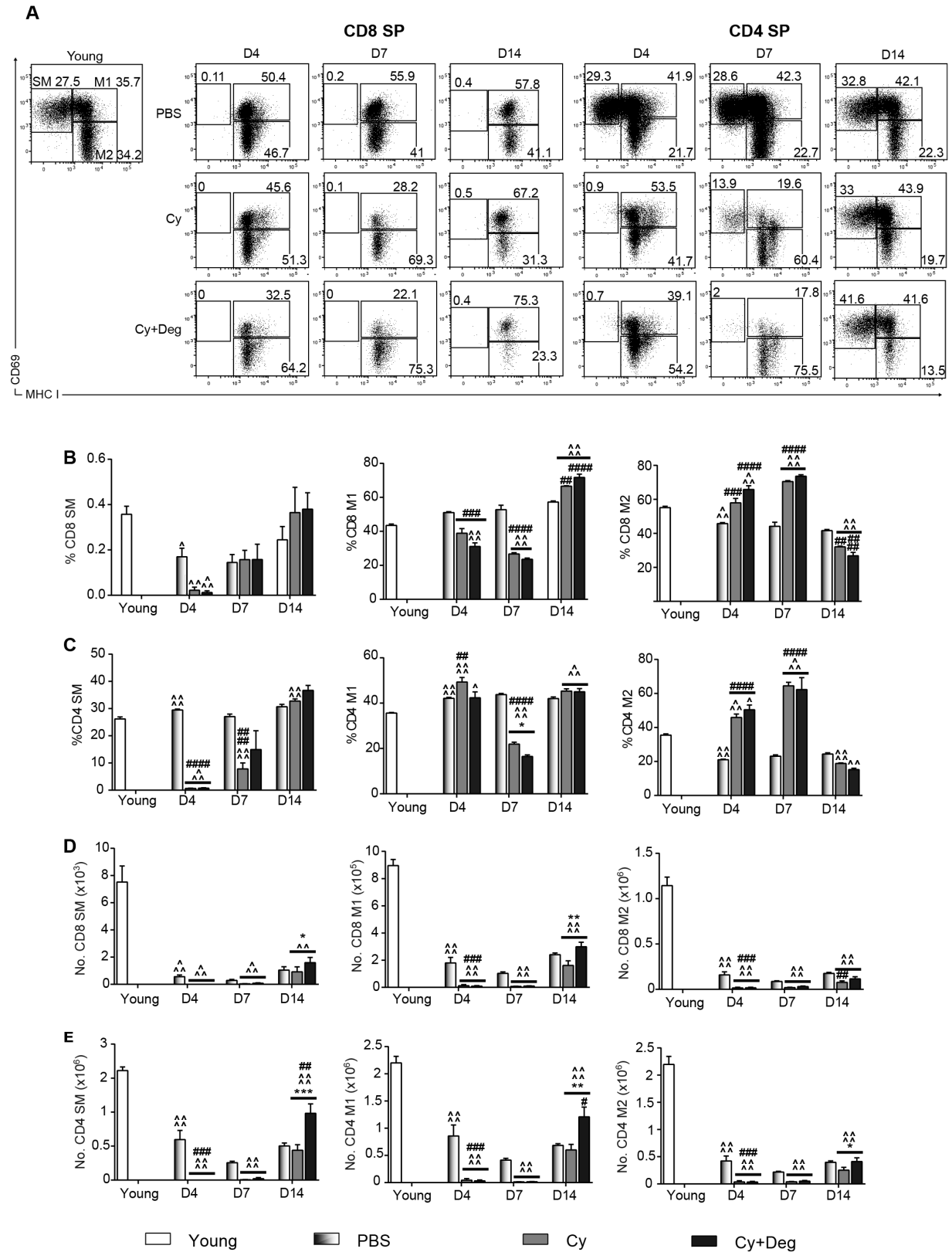
Figure 4.8 Analysis of semi-mature and mature CD4 and CD8 subpopulations. (**A**) Representative dot plots show proportions of semi-mature CD69$^+$/MHC-II$^-$ (SM), mature CD69$^+$/MHC-II$^+$ (M1) and fully mature CD69$^-$/MHC-II$^+$ (M2) thymocytes in young untreated (UT) and middle-aged PBS (PBS) controls, Cy and Cy+ Deg treated cohorts at D4, D7 and D14. Proportions (**B&C**) and cell numbers (**D&E**) of these subpopulations within CD8$^+$ and CD4$^+$ T cells, respectively. Data are presented as mean +SEM (n=5, 2 independent experiments). * Cy+Deg vs. Cy, # compared to PBS, ^compared to Young UT, where *#^p<0.05, **##^^ p<0.01, ***###^^^ p<0.001, ****####^^^^ p<0.0001, using Mixed-design Two-Way Repeated measures ANOVA with Bonferroni and R-E-G-W-Q post hoc multiple comparisons.

4.3.9. REGULATORY T CELLS DEMONSTRATED SLOWER RECOVERY THAN OTHER T CELL SUBSETS

We next performed flow cytometry analysis of thymic $CD25^+Foxp3^+$ T regulatory cells (Tregs) residing within the $CD4^+$ T cell subset (**Figure 4.9 A**).

A proportional increase in Tregs was evident immediately after Cy treatment, suggesting greater loss of $CD4^+$ non-regulatory T cells (**Figure 4.9 B**) However, Cy treatment induced a substantial numerical loss in Tregs evident at D4 and D7. Recovery to middle-aged PBS control levels was only achieved by D14 with Degarelix treatment (**Figure 4.9 C**) which was associated with enhanced proliferation (**Figure 4.9 D - F**).

Overall, Cy treatment depleted Treg numbers, with proliferation contributing to their endogenous recovery, and enhanced by Degarelix treatment.
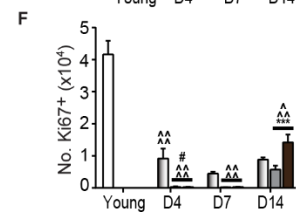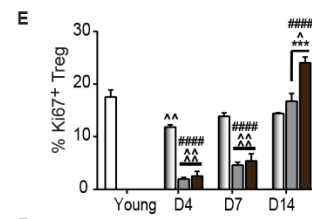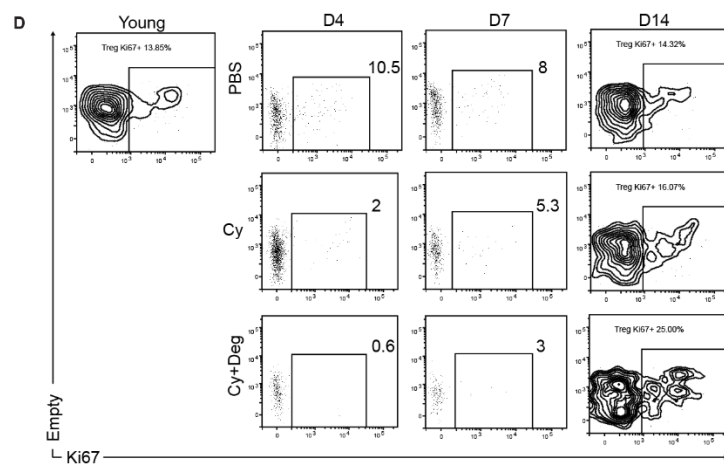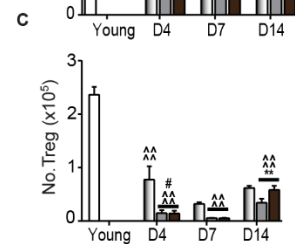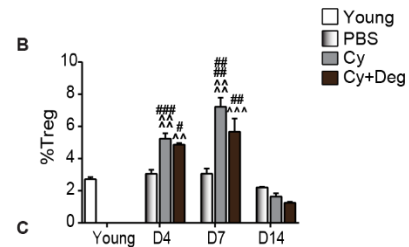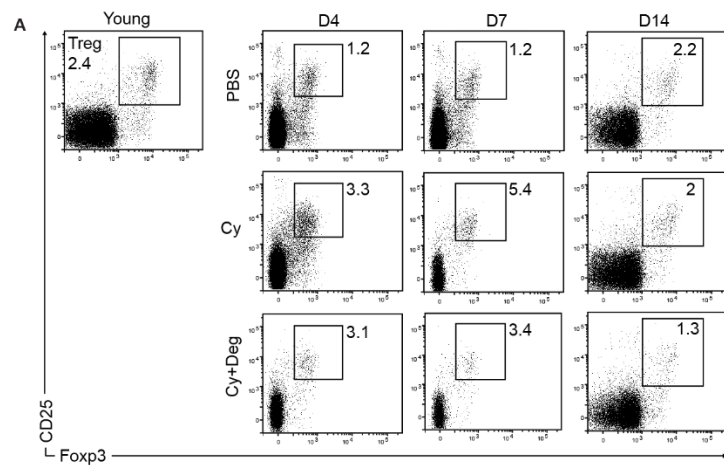
Figure 4.9. Cyclophosphamide damage depletes Tregs. (**A**) Representative flow cytometry dot plots of CD25$^+$Foxp3$^+$ Tregs (isolated from CD3$^+$CD4$^+$ thymocytes). Proportions (**B**) and cell numbers (**C**) of Tregs. (**D**) Representative flow cytometry dot plots of Ki67 expression in Tregs. Proportions (**E**) and cell number (**F**) of proliferating Ki67$^+$ Tregs. Data are presented as mean +SEM (n=5 per group, 2 independent experiments). * Cy+Deg vs. Cy, # compared to PBS, ^compared to Young UT, where *#^p<0.05, **##^^ p<0.01, ***###^^^ p<0.001, ****####^^^^ p<0.0001, using Mixed-design Two-Way Repeated measures ANOVA with Bonferroni and R-E-G-W-Q post hoc multiple comparisons.

4.3.10 NUMERICAL INCREASE IN naïve CD4$^+$ AND CD8$^+$ T CELLS IN LYMPH NODES WITH DEGARELIX TREATMENT

Due to pronounced enhancement of thymopoietic recovery with Degarelix treatment following Cy damage, we examined for enhancements in the peripheral T cell pool. Total splenocyte numbers were enhanced with Degarelix treatment by D28 (data not shown), although no notable differences within splenic subsets were evident. We detected higher peripheral T cell numbers within brachial and inguinal lymph nodes (**Figure 4.10 A**). This increase was evident in naïve (CD62L$^+$CD44^{lo}) CD4$^+$ and CD8$^+$ T cell subpopulations from D14, with enhancements also seen in central memory (CM, CD62L$^+$CD44^{hi}) and effector memory (EM, CD62L$^-$ CD44^{hi}) cell numbers. Ki67 analyses indicated involvement of homeostatic expansion of these populations following Cy depletion of Ki67$^+$ proliferating cells, evident from D10 (**Figure 4.10 B**).

A Bilateral brachial and inguinal lymph nodes

Legend: □ 8 mo UT, ▨ Cy, ■ Cy + Deg; * vs Cy, + vs UT
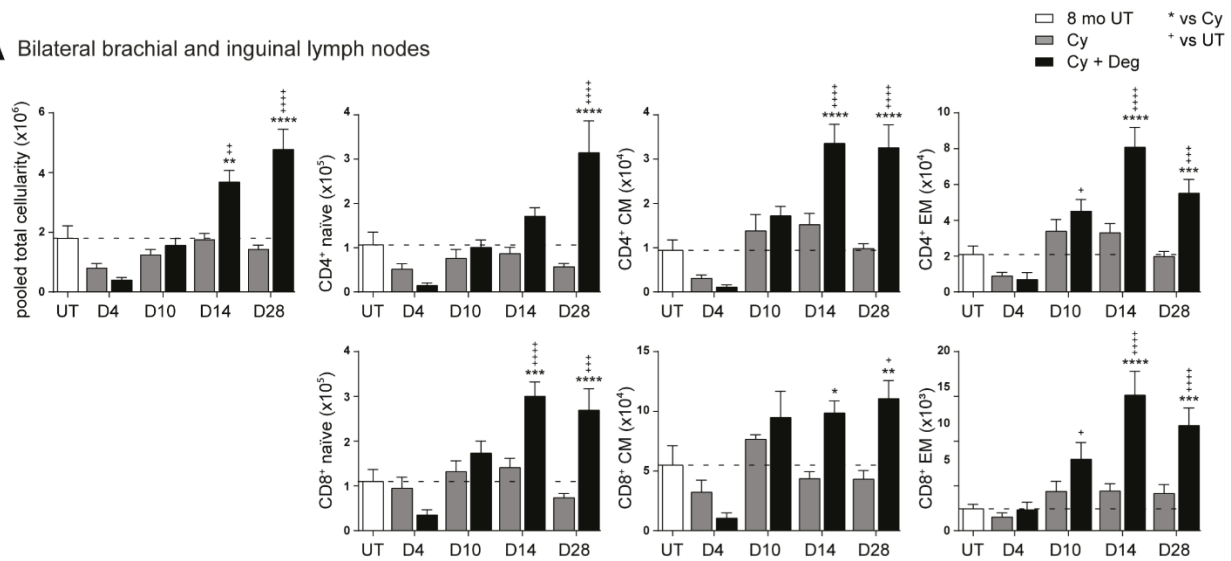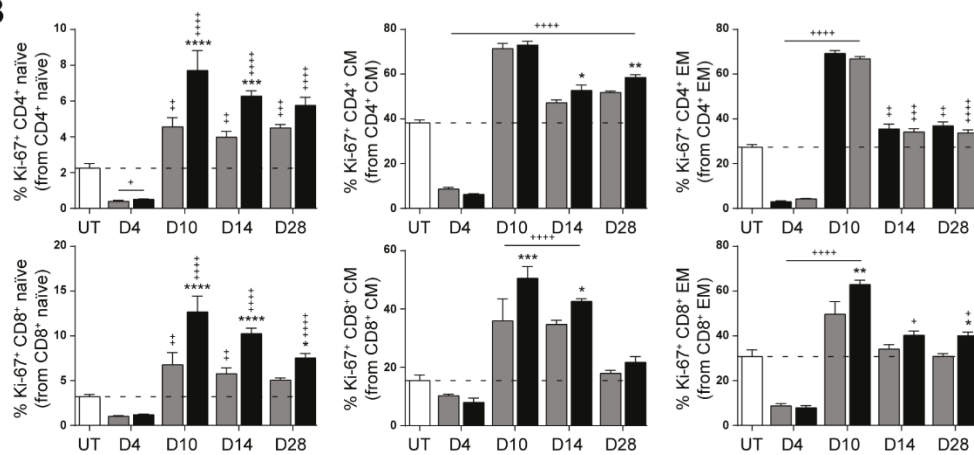
Figure 4.10 Degarelix treatment in middle-aged male mice following Cy damage enhances expansion of CD4+ and CD8+ T cells in lymph nodes. (**A**) Phenotypic T cell profiles from pooled bilateral brachial and linguinal lymph nodes of middle-aged male mice. Total cellularity, and naïve, central memory (CM), and effector memory (EM) T cell numbers are shown. (**B**) Proportion of Ki-67+ T cell subsets. Data are presented as mean + SEM (n>3). * vs. Cy, + vs. UT. *$p<0.05$, **$p<0.01$, ***$p<0.001$, ****$p<0.0001$, ordinary two-way ANOVA with Sidak's multiple comparisons.

4.4. DISCUSSION

Age-related progressive reduction in naïve T cell generation can lead to severe clinical consequences in the aged and elderly following damaging cytoablative treatments such as chemotherapy and irradiation, commonly used during cancer therapy and pre-conditioning for HSCT. These treatments have severe immunological side effects, causing profound damage to thymocytes and peripheral lymphocytes. Lymphopenia-induced peripheral expansion of residual chemotherapy or irradiation-resistant T cells results in a restricted TCR repertoire, leaving the patient prone to opportunistic infections and cancer relapse, with increased patient morbidity and mortality (Velardi, Tsai et al. 2021). In addition, disparate CD8^{+} and CD4^{+} T cell expansion can result in a prolonged imbalance in T cell subsets (Mackall, Fleisher et al. 1997). A clear need for immune reconstitution strategies is required.

Here we examined a promising approach to restore thymic function following Cy treatment, through the administration of LHRH receptor antagonist, Degarelix. We investigated endogenous recovery following Cy treatment and the effectiveness of Degarelix in enhancing recovery in middle-aged female and male mice, to determine whether gender disparity was apparent.

We previously identified murine sexual dimorphism in age-related thymic involution, with post-pubertal TEPC blockade evident in males, but attenuated in females, resulting in better maintenance of mature TECs and thymopoiesis in middle aged females. This impacted on the recovery process following Cy damage. In both females and males, the endogenous response to mTEC[hi] loss, the subset most sensitive to Cy damage, was an

immediate but temporary rise in proliferation of mTEC^{hi} at D4. However, gender disparate processes were involved to achieve full mTEC^{hi} recovery by D14-28.

In females, endogenous recovery from Cy damage involved immediate TEPC mobilization that led to expansion of cTEC^{hi} and contributed to recovery of mTEC^{hi} through continued provision of mTEC^{lo} progenitors. As such, no major loss in mTEC^{lo} was evident at any time-point despite the Cy-induced loss of proliferating mTEC^{lo} at D4 and D7. Differentiation of Sca1^{hi} mTEC^{lo} into Sca1$^{int/lo}$ mTEC^{lo} was already evident at D4 followed by increases in mTEC^{hi} by D10. Degarelix treatment enhanced the rate of TEPC self-renewal and differentiation into cTEC and mTEC progenitors, and further differentiation of mTEC^{lo} subpopulations, to achieve an earlier significant increase in mTEC^{hi} by D7.

In males, endogenous recovery from Cy damage involved immediate proliferation of mTEC^{lo} progenitors and mTEC^{hi} (two-fold greater than females). Some mobilization of TEPC to the Sca1^{int}cTEC^{lo} stage was evident at D4, but no further differentiation was evident. Whether this is due to a differentiation block, or partial TEPC differentiation towards the mTEC lineage requires further investigation. Thus, endogenous recovery following Cy damage in males relied heavily on mTEC progenitor differentiation and proliferation to replenish mTEC^{lo} and mTEC^{hi}. This is consistent with recent literature which proposed maintenance of adult mTECs relied on mTEC^{lo} single lineage progenitors and not bipotent TEPC following thymus damage from irradiation treatment; while the gender of mice was not specified in this publication, we would predict males were used (Ohigashi, Zuklys et al. 2015).

Degarelix treatment induced reactivation of TEPC by D7 in males, and early mobilisation of Sca1^{hi}mTEC^{lo} to Sca1^{lo} mTEC^{lo} progenitors from D4, leading to earlier recovery of mTEC^{hi} compared to endogenous recovery. Thus, in males, Degarelix treatment released the age-related TEPC blockade. However, self-renewal of TEPC was still not achieved by D28 even with Degarelix treatment, which may suggest continued mobilization beyond D28; overall numerical recovery of TECs had not been achieved by D28 in males, even though proportional recovery of mTEC^{hi} levels had returned to UT levels. Degarelix also enhanced later stage proliferation of cTEC^{lo} and cTEC^{hi} in both females and males at D14, which may relate to the increased number of thymocytes transitioning through the cortex following sex steroid inhibition.

Given the greater negative impact of sex steroids on TECs in males and more pronounced recovery, particularly in the cortical compartment following Degarelix treatment, we further analysed thymopoietic recovery in males. Of the main T cell subsets, DPs were the most affected by Cy damage, however, proportional recovery of DPs was already evident by D7 with no obvious enhancement of endogenous recovery with Degarelix treatment. These DPs likely developed from remaining chemotherapy-resistant DN cells, given the numerical loss of ETPs from Cy damage. Between D7 and D14, Degarelix treatment had enhanced DN and DP expansion, reaching young levels by D14, coincident with enhanced cTEC^{hi}. Increases in CD4$^+$ and CD8$^+$ T cell numbers beyond endogenous recovery were also becoming evident by D14, coincident with expansion of mTEC^{hi} and importantly, Aire$^+$mTEC^{hi}, essential to ensure continued central tolerance processes. Tregs had also shown numerical recovery to UT levels by D14.

Sequential recovery of thymocyte subsets from DN subpopulations through DP and SP subsets was not as obvious compared to Degarelix treatment in healthy mice (Chapter 2), likely due to the profound Cy damage affecting all thymocyte subsets in the first week following treatment. However, by D14 Degarelix treatment had enhanced the numerical recovery of all thymocyte subsets including ETPs, achieving equivalent to young levels in the immature DN and DP subsets. We would expect numerical recovery of SP subsets to young levels to be achieved 7-10 days later, given the findings from Ch2 demonstrating a 14-day regeneration of SP subsets to young levels following Degarelix treatment in healthy middle-aged males.

Enhanced total thymic cellularity was evident with Degarelix treatment from D10 in males, and while this timepoint was not included in the thymocyte subset analyses, there was evidence of a substantially greater number of CD4$^+$ and CD8$^+$ SP thymocytes by D14. Therefore, the D14 enhanced numerical increase in lymph node naïve T cells with Degarelix treatment, likely contains newly derived and egressed naïve CD4$^+$ and CD8$^+$ T cells in addition to homeostatic expansion of residual T cells.

CONCLUSION

Cy treatment has a profound impact on thymocytes and TECs, with DPs and mTEC^{hi} being the most affected. Endogenous recovery of mature TECs in middle-aged females involves immediate mobilisation of TEPC to replenish single lineage progenitors as they differentiate into cTEC^{hi} and mTEC^{hi}. Degarelix treatment enhanced this differentiation process as well as TEPC self-renewal. It also enhanced later stage proliferation of the cortical TEC compartment coincident with the increase in transitioning cortical thymocytes

following sex steroid inhibition. However, TEPC blockade is evident in middle-aged males with endogenous recovery involving immediate proliferation of mTEC^{hi} and mTEC^{lo} and differentiation of mTEC^{lo} single lineage progenitors. Degarelix treatment released TEPC blockade, with reactivation of TEPC differentiation contributing to cTEC^{hi}, mTEC^{lo} and mTEC^{hi} replenishment. More detailed investigations on the impact of T cell development, including thymopoiesis in females, may clarify whether regeneration of the cortical compartment is the underlying mechanism for improved thymopoiesis, such as greater Dll4 expression and IL7 production, or whether the removal of sex steroids additionally impacts directly on thymocyte proliferation capacity.

This study details for the first time, the relationship between sexual dimorphism and TEC recovery following chemotherapy damage. This data supports our earlier proposal of continued TEPC function in middle-aged females, which enables faster regeneration of damaged TECs following Cy treatment, while in middle-aged males the TEPC blockade is only released with Degarelix treatment-induced sex steroid inhibition. Otherwise, males rely heavily on proliferation of existing progenitors as a means to recovering mature TECs. This study indicates a greater suppressive effect of androgens than estrogens on thymopoiesis and TEPC function, and effectiveness of Degarelix as a therapeutic in both females and males for enhanced thymus regeneration. Collectively, this data suggests a plausible benefit into analyzing naïve T cell output in prostate and breast cancer patients who are treated with LHRH analogues.

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CHAPTER 5:

GENERAL DISCUSSION

5.1 GENERAL DISCUSSION

Sexual dimorphism in immunity and cancer susceptibility and survival are well recognised (Klein and Flanagan 2016, Özdemir and Dotto 2019). Females have reduced incidence and more potent immune defensive reactions against bacterial, fungal, and viral infections, better antibody formation and stronger immune-responses to vaccinations, relative to males (Klein 2000). Moreover, epidemiological studies have shown that females have a lesser risk and better prognosis than males in a wide range of cancer types that are unrelated to reproductive function (Clocchiatti, Cora et al. 2016, Haupt, Caramia et al. 2021). Nevertheless, females have a higher tendency than males in rejecting allografts and in developing autoimmune diseases, such as multiple sclerosis and rheumatoid arthritis (Pido-Lopez, Imami et al. 2001, Whitacre 2001, Gui, Mustachio et al. 2012). Generally, both the innate and adaptive immune responses are stronger in females than males, however, the underlying bases for these differences are complex and not completely understood.

Sex differences in peripheral immunity include a higher number of CD4^{+} T helper cells and B cells and a higher percentage of interleukin-2 producing lymphocytes in females, while males have a higher frequency of natural killer cells (Klein and Flanagan 2016), and there is a burgeoning interest in sex specific differences in gut microbiota shaping the peripheral immune response, such as via T regulatory cells (Gomez, Luckey et al. 2015, Elderman, De Vos et al. 2018, Taneja 2018). We herein demonstrate that sexual dimorphism is also evident in age-related progressive degeneration of the thymus, a

consequence of which is reduced production of naïve T cells, contributing to immune deterioration.

The progressive reduction in naïve T cell generation during ageing can lead to severe clinical complications following cytoablative treatments associated with cancer therapy and preconditioning regimes for hematopoietic stem cell transplantation (HSCT), such as chemotherapy and total body irradiation. The delay in immune recovery, particularly in older patients, increases the incidence of opportunistic infections and cancer relapse, and increases patient morbidity and mortality. There is a clear need to develop clinical strategies to improve immune reconstitution in older patients. We addressed this problem herein, by investigating a promising approach to increase the kinetics of T cell recovery following chemotherapy damage, using a reversible sex steroid inhibitor; specifically, a third generation luteinizing hormone releasing hormone (LHRH) receptor antagonist, Degarelix, which is kinetically more effective than LHRH receptor agonists for thymus regeneration. Additionally, since preclinical research in this field has been lacking in females, we conducted a comparative analysis of thymus ageing and underlying mechanisms, in relation to murine sexual dimorphism. Finally, we performed a comparative analysis of the impact of Degarelix on thymus recovery following cyclophosphamide (Cy) treatment, in middle-aged female and male mice.

Thymus involution in humans occurs in multiple phases, with contraction of thymopoiesis first apparent in the early years of life once the peripheral T cell pool has been fully established (Hale 2004). The second prominent phase follows the onset of puberty, and this is followed by a more gradual age-related decline (Flores, Li et al. 1999, Ventevogel and Sempowski 2013). Coincident with contraction of thymopoiesis during ageing, is a

loss of thymic epithelial cells (TECs), such that by mid-life only around 10% of maximal functional tissue remains in the thymus (Gruver, Hudson et al. 2007).

The influences of sex steroids on thymus involution have been known for many decades, predominantly based on observations of thymus regrowth following castration (Greenstein, Fitzpatrick et al. 1987, Windmill, Meade et al. 1993). There has been a resurgence of interest in sex steroid inhibition (SSI)-related immune regeneration approaches over the last two decades, however, it is only in recent years that underlying mechanisms for SSI-induced thymus regeneration are beginning to emerge (Velardi, Tsai et al. 2014, Lepletier, Hun et al. 2019). Both thymocytes and TECs express hormone receptors, but seminal work by Olsen et al. using bone marrow chimeric mice, demonstrated a greater role for androgen binding to TEC androgen receptors (AR) in thymus involution, as opposed to lymphocyte AR binding (Olsen, Olson et al. 2001). Further studies demonstrated sexual dimorphism in transcriptome analyses of TECs, with cortical TECs showing the greatest differences (Dumont-Lagacé, St-Pierre et al. 2015), including transcription of the Notch ligand Dll4 (Velardi, Tsai et al. 2014) which is indispensable for T cell specification, commitment and development (Shah and Zúñiga-Pflücker 2014). Age-related accumulation of cTECs was more evident in males (Dumont-Lagacé, St-Pierre et al. 2015); this cTEC accumulation was later shown to be entirely localised to the immature cTEC compartment (cTEC^{lo}) and influenced by sex hormones, as demonstrated by its reversal following castration in male mice (Lepletier, Hun et al. 2019). Moreover, the accumulation of cTEC^{lo} was proposed to be due to a block in differentiation potential of bipotent TEPC and single lineage TEC progenitors (Lepletier, Hun et al. 2019).

Given the critical requirement of TECs in enabling thymopoiesis, we performed an extensive comparative phenotypic and functional analysis of TEC subsets from healthy female and male C57BL/6J mice based on the main stages of thymus involution: pre-pubertal (4-wks-old), post-pubertal (7-wks-old), and middle-aged (7-12-mo-old) mice were investigated. Enzymatic digestion methods were used to isolate TECs from thymic tissue. We have extensive experience in TEC isolation and utilised improved enzyme cocktails and methods that do not cleave TEC subset markers and reduce TEC damage (Seach, Wong et al. 2012). However, cells containing large spreading processes, such as mature cTECs, may be more susceptible to physical and enzymatic disruption, with numbers potentially being underestimated. As such, with the assumption that such cells may be lost across all age groups and gender, we limited our study to a comparative proportional analysis of specific TEC subsets between age groups and gender.

We found attenuated accumulation of cTEC^{lo} in females compared to males and this was associated with better maintenance of mature cTEC^{hi} and mTEC^{hi} by middle-age. We therefore further analysed the cTEC^{lo} subpopulations which include bipotent TEC progenitors (TEPC).

We previously described a bipotent TEPC residing in the cTEC^{lo} compartment that expressed high levels of $\alpha6$ integrin and Sca-1 ($\alpha6^+$Sca1^{hi}cTEC^{lo}; (Wong, Lister et al. 2014)), with downregulation of Sca-1 linked to differentiation into unipotent cTEC^{lo} progenitors (Lepletier, Hun et al. 2019). We further demonstrated herein, that mature cTEC^{hi} express low levels of Sca-1, consistent with this differentiation progression. Interestingly, this hierarchy has also been recently described in hematopoietic stem and progenitor cells (HSPCs), with high expression of Sca-1 identifying quiescent cells with

superior self-renewal potential and elevated repopulation activity compared to the Sca1^{lo} HSPCs, and lower Sca-1 expression identifying downstream cycling cells (Morcos, Schoedel et al. 2017). In males, attenuation of TEPC function with ageing was linked to a significant increase in TEC and fibroblast production of Follistatin (FST) which impaired TEPC and mTEC differentiation through Activin A signaling antagonism (Lepletier, Hun et al. 2019). We also found an increase in TEC production of FST at the onset of puberty in females, coincident with attenuation of TEPC function and loss of mTEC^{hi}, albeit less than demonstrated in males (Figure 3.8D). Moreover, an increase in TEC production of Bmp4 in ageing males was proposed to support TEC progenitors at the expense of differentiation into their mature counterparts (Lepletier, Hun et al. 2019). This is consistent with findings from Wertheimer, who found increased Bmp4 following irradiation damage (Wertheimer and Velardi 2018). Given the Bmp4 receptor (Bmpr2) is primarily expressed by cTEC^{lo} and mTEC^{lo} (Barsanti, Lim et al. 2017), we propose this immediate injury-induced response of increased Bmp4 production may contribute to expanding TEC progenitors to enable ongoing support of mature TECs.

Males demonstrated a greater accumulation of cTEC^{lo} from the onset of puberty to middle-age compared to females. This was reflected by increased proportions of TEPC and Sca-1^{int}cTEC^{lo} subpopulations, with a profound loss of downstream Sca-1^{lo}cTEC^{lo} progenitors, despite a dramatic increase in proliferation of these cells in males. Together with diminished TEPC function from the onset of puberty, based on their colony forming efficiency, this may suggest enhanced senescence of TEPC in males, compensated by a greater reliance on proliferation to maintain progenitors for mature TEC maintenance. Future experiments that explore TEC senescence during ageing are required. Loss of

TEPC differentiation with ageing was attenuated in females compared to males, with no change in proliferation-based progenitor maintenance and continued cTEC^{hi} production. This suggests continued maintenance of mature TEC populations through progressive differentiation of TEPC and downstream progenitors in females, albeit attenuated post-puberty.

We previously identified a rare population of Aire$^+$cTEC^{hi} that expressed β5t$^+$ and was located at the cortico-medullary junction (Hu, Eviston et al. 2019). Pre-pubertal females demonstrated a three-fold higher proportion and quantity of this cell population compared to males. However, a two-fold proportional loss at the onset of puberty brought levels down to that of pre-pubertal males. This level was maintained to middle age, while a further proportional loss was evident in males. The loss of Aire$^+$cTEC^{hi} from the onset of puberty and during ageing is coincident with the loss of Aire$^+$mTEC^{hi} and warrants further investigation into whether it represents a precursor to the mature Aire expressing subset of mTEC^{hi}. Interestingly, Aire$^+$cTEC^{hi} were not numerically affected by cyclophosphamide treatment, suggesting a state of quiescence or low cycling progenitor population. However, as a proportion of cTECs, recovery to beyond untreated levels was evident by D14 following sex steroid inhibition. Their response to the receptor activator of nuclear factor B (RANK) signaling pathway, which has been shown to control Aire expression in mTECs, warrants investigation.

It is well accepted that unipotent progenitors of mTEC^{hi} exist in the mTEC^{lo} compartment (Gray, Seach et al. 2006) and do not have bipotent capacity (Wong, Lister et al. 2014, Ulyanchenko, O'Neill et al. 2016). Interestingly, since mTEC^{lo} demonstrated a similar α6 vs Sca-1 profile as cTEC^{lo}, we proposed that Sca1^{hi} TEPC generate Sca-1^{hi} mTEC^{lo}

unipotent progenitors, with downregulation of Sca-1 linked to differentiation into mTEC^{hi}, which express low levels of Sca-1 (Sca-1^{lo}mTEC^{hi}). There is an age-related accumulation of Sca1^{hi}mTEC^{lo} evident by middle-age, and profound loss of Sca-1^{lo} mTEC^{lo} and Sca1^{lo}mTEC^{hi} from the onset of puberty, more-so in males compared to females. This would suggest multiple levels of dysfunction in mTEC^{lo} progenitor differentiation. Further investigations should include determining the relationship between α6^{hi} Sca-1^{hi} mTEC^{lo} and the previously described mTEC 'stem cells' such as stage-specific embryonic antigen-1 (SSEA-1)$^{+}$ Claudin (Cld)3,4^{hi} TECs, which showed reduced functional capacity in the adult thymus (Sekai, Hamazaki et al. 2014, Hamazaki, Sekai et al. 2016). Aire^{+} mTEC^{hi} were profoundly reduced in both males and females from the onset of puberty, consistent with the overall loss of mTEC^{hi}, however, this left females with a reduced ratio of Aire^{+}mTEC^{hi} to thymocytes. This may be a contributing factor to the greater incidence of autoimmunity in females (Fairweather, Frisancho-Kiss et al. 2008).

Thus, diminution of TEPC function and progenitor differentiation blockade is more evident in males than females during ageing, and we propose this as a mechanism underlying the post-pubertal progressive loss of mature TECs. Given the sexual dimorphism associated with attenuation of TEPC and progenitor function from the onset of puberty, we performed a comparative analysis of SSI as an approach to thymus regeneration.

Based on extensive preclinical phenotypic analyses of thymocyte and bone marrow regeneration following surgical gonadectomy, the use of reversible SSI by LHRH receptor agonists was first investigated as a feasible clinical approach for thymus regeneration and immune recovery following damaging cytoablative conditioning regimes prior to HSCT (Sutherland, Goldberg et al. 2005, Sutherland, Spyroglou et al. 2008). However,

as described earlier, the process of SSI by LHRH receptor agonists involves an initial surge in sex steroid production in the first week of treatment, before desensitization and downregulation of LHRH receptors leads to reduced sex steroid production, with castrate levels achieved 21 days after treatment. In males, the testosterone surge causes further damage to an already involuting thymus and while thymus regeneration was evident 7 days after reaching castrate levels of testosterone, 28 days after LHRH receptor agonist administration, it is unclear whether this initial damage has any longer-term impact on thymus function. More recent investigations using a LHRH receptor antagonist demonstrated a faster and less damaging approach to thymus regeneration, with no initial surge in sex steroid production, and castrate levels of sex steroids achieved within 24-48 hours after administration (Velardi, Tsai et al. 2014).

Herein we examined the extent of TEC damage by a commonly used chemotherapeutic drug, cyclophosphamide (Cy), and the beneficial effects of a third generation LHRH receptor antagonist, Degarelix (Firmagon), over endogenous TEC recovery in middle-aged females and males. Cy treatment induced a profound loss of mTEC^{hi} in both females and males, with sexual dimorphism evident in both endogenous TEC recovery and impact of Degarelix treatment.

In females, endogenous recovery from Cy damage involved an immediate and profound mobilisation of TEPC, and differentiation of downstream Sca1^{lo}cTEC^{lo} and Sca1^{hi}mTEC^{lo} progenitors by day 4, to replenish the loss of proliferating mature TECs following Cy-induced apoptosis. Recovery of mTEC^{hi} additionally included an early increase in proliferation of mTEC^{hi}, evident at D4. While Degarelix treatment did not significantly enhance this process, it did induce earlier kinetics of TEPC self-renewal back to UT levels,

an early transient expansion of cTEC^{hi} at D4 and enhanced recovery kinetics of mTEC^{hi}, significant by D7. A summary schematic is shown in Figure 5.1 top panel.

Endogenous recovery from Cy damage in males, appeared to initiate partial TEPC mobilization but this was blocked at the Sca-1^{int}cTEC^{lo} stage. Focus was instead on the medullary compartment, with mobilization and proliferation-based replenishment of Sca1^{hi-lo}mTEC^{lo} subpopulations and early proliferation of mTEC^{hi}. This is consistent with the findings by Ohigashi et al., demonstrating that adult mTEC^{hi} maintenance and regeneration is restricted to mTEC lineage progenitors (Ohigashi, Zuklys et al. 2015). However, Degarelix treatment reversed the TEPC blockade in males, with TEPC mobilisation evident by D7, contributing to a profound increase in cTEC^{hi} at D7-D10. In addition, early mobilization of Sca1^{hi}mTEC^{lo} led to a significant increase in Sca1^{lo}mTEC^{lo} progenitors by D4 and enhanced recovery of mTEC^{hi}. Mobilisation of TEPC was sustained to D28; longer term analysis will reveal whether this continues with Degarelix treatment, or eventually subsides. A summary schematic is shown in Figure 5.1 bottom panel.

Thus, females maintain TEPC function by middle-age, albeit somewhat attenuated by estrogen from the onset of puberty. Cy damage induces immediate TEPC and Sca1^{hi}mTEC^{lo} mobilization for mature TEC recovery. Degarelix treatment enhances progenitor self-renewal and the kinetics of cTEC^{hi} (D4) and mTEC^{hi} (D7) recovery, which is reflected in increased thymopoiesis by D10. In contrast, androgens induce both a severe block in TEPC function in males, and attenuation of mTEC progenitor proliferation and function, leading to the profound loss of mTEC^{hi} from the onset of puberty. This impacts on endogenous TEC recovery following Cy damage, with males relying on early

proliferation events for recovery of mTEC^{hi}. Degarelix treatment releases mTEC progenitor mobilization (D4) and reverses TEPC blockade by D7, leading to an increase in cTEC^{hi} (D7) and mTEC^{hi} (D14).

While recovery is delayed in males compared to females, by D28 the proportion of mTEC^{hi} levels had reached levels equivalent to females (approximately 30-35% of TECs), which is three-fold greater than UT middle-aged male levels and almost equivalent to pre-pubertal levels. This further highlights the greater negative impact of androgens on mTEC^{hi} maintenance in males and the effectiveness of Degarelix treatment in enhancing mTEC^{hi} recovery.
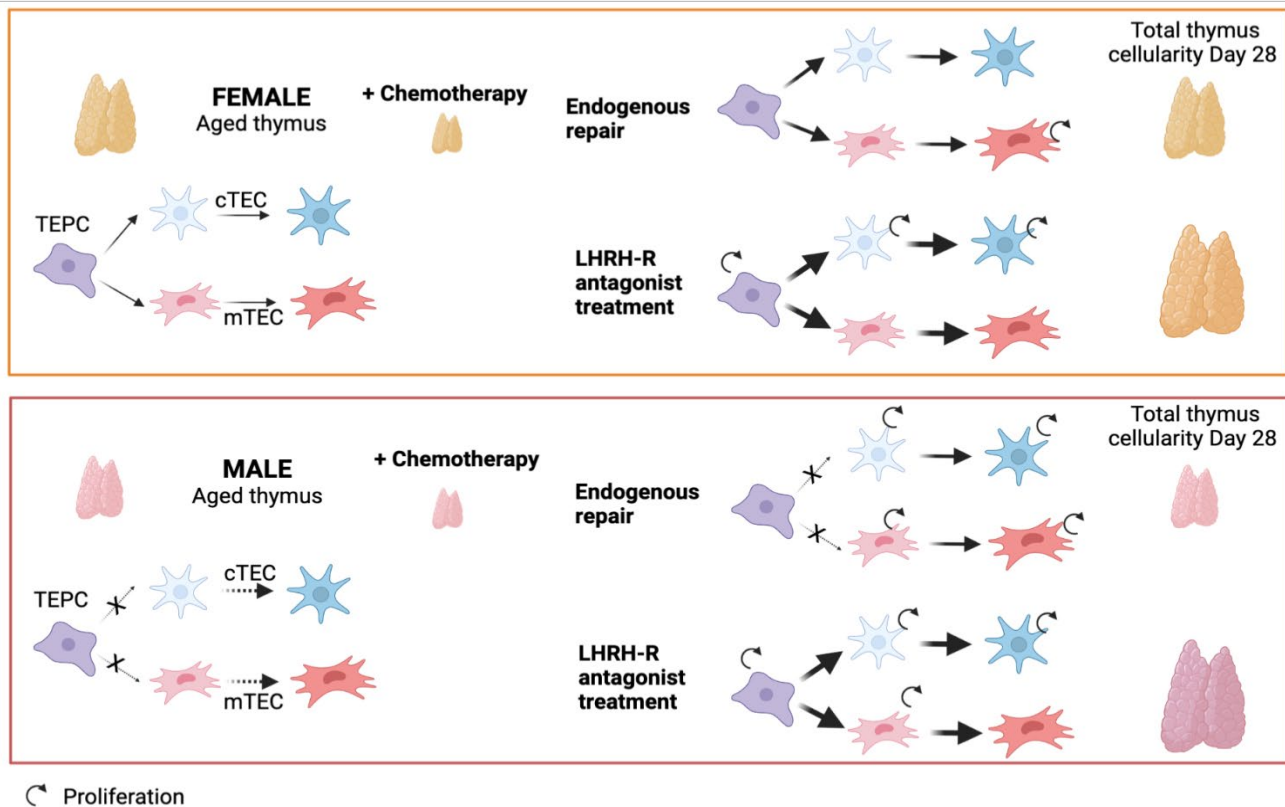
Figure 5.1 Thymus recovery following chemotherapy damage: endogenous recovery and with LHRH receptor antagonist treatment.

Top Panel. In females, while TEPC function is somewhat attenuated from the onset of puberty, endogenous recovery following chemotherapy damage activates TEPC for replenishment of immature cTEC^{lo} and mTEC^{lo}, which in turn generates mature cTECs and mTECs. LHRH receptor antagonist treatment enhances this homeostatic endogenous recovery, and increases TEPC self-renewal and proliferation of cTECs.

Bottom Panel. In males, due to a post-pubertal block in TEPC function, endogenous recovery following chemotherapy damage relies on proliferation of residual immature cTEC^{lo} and mTEC^{lo} and mature cTEC^{hi} and mTEC^{hi}. In addition to increased proliferation, LHRH receptor antagonist treatment releases the TEPC functional blockade, leading to replenishment of immature cTEC^{lo} and mTEC^{lo}, which in turn generates mature cTECs and mTECs.

Following endogenous repair, total thymus cellularity has returned to untreated aged levels by day 28. However, following LHRH receptor endogenous treatment, thymus recovery is enhanced to almost pre-pubertal levels by day 28 in both males and females.

Velardi *et al*. proposed sex steroid modulation of Notch signaling in cTECs as an underlying mechanism for reduced thymopoiesis during ageing and demonstrated an increase in delta like 4 (Dll4) and interleukin 7 (IL7) expression in total cTECs 7 days after Degarelix treatment in 8-12 wk-old males (Velardi, Tsai et al. 2014). We propose the increase in Dll4 and IL7 expression may be additionally due to an increase in production

of cTEC^{hi} following Degarelix treatment, with release of the post-pubertal TEPC blockade and functional attenuation of progenitors. This was profoundly evident in Degarelix treated middle-aged (8-mo-old) males by D7 following Cy damage (Figure 4.2B) and this strong influence on the cortical compartment is reflected in the profound numerical expansion of thymocytes, surpassing untreated control levels by D10.

In healthy middle-aged males, a numerical increase in DN cells was evident at D4 following Degarelix treatment, leading to a sequential increase in DP (D7) and SPs (D14). However, this sequence of recovery was less clear following Cy damage, with a more delayed numerical recovery of thymocyte subsets. However, proportional recovery of DPs, the most affected thymocyte subset, was achieved by D7 possibly through early differentiation of remaining Cy-resistant DNs, including ETPs in the DN1 subset (Figure 4.7F). Enhanced proliferation of all T cell subsets was evident between D7 and D14 with Degarelix treatment, which possibly reflects the improved cortical compartment.

The bone marrow is also damaged from Cy treatment, with surgical castration shown to enhance numerical recovery of CLPs by D4 and B cell recovery by D14 in young male mice (Dudakov, Goldberg et al. 2009). This recovery is likely to be more delayed in middle-aged mice. Therefore, longer-term investigations would confirm whether the first wave of thymopoiesis following Cy damage identified herein, originates from Cy-resistant DN cells, before ongoing thymopoiesis can be achieved from new entry of hematopoietic precursors following recovery of the bone marrow. Recovery of bone marrow function following damage will be important for sustained thymic regeneration following damage, despite intrathymic compensation such as increased proliferation. The impact of a single dose of sub-lethal radiation was found to be sufficient to cause permanent damage to

HSCs that ultimately resulted in long-term decline of thymocyte production, similar to accelerated ageing-associated thymic involution (Xiao, Shterev et al. 2017). Whether a similar level of damage occurs with single or multiple rounds of chemotherapy in females and males, warrants future investigation.

While numerical recovery of total TECs to UT levels was not yet reached by D28 in males despite enhanced thymopoiesis by D10 following Degarelix treatment, proportional recovery of Aire⁺mTEC^hi to beyond UT levels by D14 was coincident with the increase in SPs at this timepoint. This suggests self-tolerance mechanisms were sustained, but long-term analysis of peripheral T cells for evidence of autoimmunity would confirm this. To our knowledge, there has been no clinical evidence of enhanced autoimmunity in prostate cancer patients undergoing LHRH analogue treatment. Thymic production of Tregs returned to UT levels by D14 in Degarelix treated groups to support peripheral tolerance. We briefly addressed the question of whether newly derived naive T cells from Degarelix-enhanced thymopoiesis would be supported by ageing peripheral lymph nodes that are subjected to increased fibrosis (Thompson, Smithey et al. 2019). We found a two-fold increase in naïve CD8⁺ T cells in the brachial and inguinal lymph nodes, with naïve CD4⁺ T cells reaching an almost three-fold increase above UT middle-aged levels by D28; this significant increase likely occurred earlier given the increasing trend evident at D14. The respective contributions from newly derived T cells egressing from the thymus and proliferation of Cy-resistant naïve cells in the lymph node, requires further investigation. However, in older animals (18-23 mo-old), the extent of age-induced fibrosis appeared to limit the recruitment of newly generated naïve T cells from the thymus following Degarelix treatment in a recent study (Thompson, Smithey et al. 2019). This would suggest limited

immune regeneration outcomes in the very old without concurrent strategies for lymph node rejuvenation.

5.2 CONCLUSION

This study details for the first time, the relationship between sexual dimorphism and TEC aging from the pre-pubertal stages of life to middle-age. We identified better maintenance of TEPC function and differentiation of unipotent cTEC and mTEC progenitors by middle-age in females compared to males. This highlighted the greater negative impact of androgens on adult TEC maintenance, compared to estrogens, and was evident in the disparate kinetics of endogenous recovery. As such, and not surprisingly, sex steroid inhibition had a greater impact on TEC recovery in males following Cy-induced loss of mature TECs. In females, Degarelix enhanced the homeostatic differentiation process already evident in endogenous TEC recovery, including bipotent TEPC mobilization and downstream unipotent progenitor differentiation. In males, endogenous recovery relied primarily on proliferation of remaining Cy-resistant mTEC^{lo} and mTEC^{hi}. Degarelix treatment reversed the age-related TEPC blockade and attenuation of unipotent progenitor differentiation, to enhance homeostatic TEC recovery. The proportion of cTEC^{hi} reached similar levels to that in females, albeit a temporary increase, and the proportion of mTEC^{hi} recovered to female levels by D14. The proportions of mTEC^{hi} continued to increase to pre-pubertal levels in both females and males. Self-tolerance mechanisms were maintained following Degarelix treatment, with recovery of Aire$^+$mTEC^{hi} in place to support enhanced production of SP T cells. Taken together, these findings stress the relevance of sexual dimorphism in adaptive immunity and suggest a

substantial benefit of LHRH receptor antagonist treatment to enhance TEC recovery and the kinetics of T cell replenishment following cytoablative treatments, in both males and females, although possibly less effective in post-menopausal women.

Improved technological methodologies have resulted in the identification of new thymic epithelial cell subpopulations through more detailed phenotypic and gene expression profiling and single cell spatial mapping approaches (Ki, Park et al. 2014, Bornstein, Nevo et al. 2018, Miller, Proekt et al. 2018, Lepletier, Hun et al. 2019, Baran-Gale, Morgan et al. 2020). Moreover, there is a growing interest in identifying bipotent thymic epithelial progenitor cells in the adult thymus (Wong, Lister et al. 2014, Ulyanchenko, O'Neill et al. 2016) and which intrinsic and extrinsic factors affect their function (Lepletier, Hun et al. 2019). Further investigations will undeniably contribute towards a better understanding of age-related thymic dysfunction. Given the sexual dimorphism identified herein, it will be essential to include both females and males in these studies. Together with investigating the mechanisms of TEC recovery following severe damage these studies will further advance the development of appropriate clinical strategies for thymus and T cell recovery in states of immunodeficiency. The inclusion of sex/gender as a variable in preclinical and clinical studies will ensure that discoveries of new biological mechanisms can be translated effectively in both females and males, for personalized medicine and improved human health (Danska 2014).

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