IMPROVING SEX STEROID ABLATION-INDUCED THYMIC RECOVERY FOR CLINICAL APPLICATION

Melanie Natasha Hince

B.Sc. (Hons)

Monash Immunology and Stem Cell Laboratories

Faculty of Medicine, Nursing and Health Sciences

Monash University

Melbourne, Australia

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This thesis is dedicated to:

My loving husband

Daniel

“We can do anything now that science has invented magic”

-Marge Simpson

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THESIS SUMMARY

It is a widely accepted dogma that the immune system deteriorates with advancing age. This is best exemplified functionally by the increased incidence of opportunistic infections and cancer with older age, and structurally by the physical involution of the thymus. The latter directly leads to a reduced naïve T cell output and a homeostatic expansion of the peripheral memory T cell pool [1-3]. The impact is most prominent, however, following severe infections best exemplified by HIV and common immunodepletive therapies, such as chemotherapy and irradiation, where patients suffer extensive periods of immune insufficiency [4]. In addition, while pre-pubertal patients are able to recovery pre-treatment CD4+ T cell numbers within around 6 months, this time can be extended to at least 2 years in adults [5]. During this period, the risk of infections and disease relapse is significant and leads to high mortality and morbidity rates [6]. This indicates that there is a major clinical requirement to improve thymic function and T cell output in these patients.

While multiple factors can precipitate and exacerbate thymus atrophy, there is compelling evidence for a primary role of sex steroids: their removal through surgical gonadectomy reverses age-related involution and improves overall naïve T cell output following HSCT or chemotherapy [7-12]. Clinically, chemical sex steroid ablation using LHRH-agonists are preferential to surgical gonadectomy and are the standard care for many diseases including some forms of prostate and breast cancer. In both preclinical mouse models and human clinical trials we have demonstrated the LHRH-agonist to reverse age-related thymic atrophy and improve immune reconstitution following allogeneic and autologous HSCT [10, 13-15]. Although relatively successful, the number and effectiveness of responders during our clinical trials were limited when compared to pre-clinical animal studies. Thus we investigated the clinical parameters that may affect the transfer of LHRH-A therapy from animal models to humans.

While LHRH-A was successful in reversing age-related thymic atrophy in male mice, the degree of recovery was dependent on mouse genetic background and this demonstrates the need to customise the timing of LHRH-A therapy when combined with chemotherapy/irradiation in patients. Secondly, we compared LHRH-A induced thymic recovery in male and female mice. While LHRH-A has already been shown

to greatly increase thymic recovery following cyclophosphamide and to reverse age-related atrophy in males, within females, LHRH-A is not as successful. We hypothesised that this was due to extragonadal estrogen, which is produced by the adrenal gland following ovariectomy and in postmenopausal women. In addition, LHRH-A induces an initial sex steroid flare and could be interfering with thymic recovery. Thus, we combined LHRH-A treatment with an estrogen receptor antagonist (ER-A) in an attempt to block total estrogen interactions, particularly within the thymus. Interestingly, combination treatment with LHRH-A and Tamoxifen (a common clinical ER-A) enhanced early thymic atrophy indicating a combination of the LHRH-A induced surge plus a potential pro-estrogenic effect of the ER-A. This has been seen previously within the thymus [16, 17] and indicates the need to find an ER-A that is exclusively an antagonist for the immune system, or a global blocker of estrogen production, for future studies. The possibility that Tamoxifen may inhibit thymus function and hence immune capacity in these patients needs to be explored.

We also investigated whether a similar strategy of AR blockade would prevent the immunodepleting effects of the initial LHRH-A induced sex steroid flare and result in overall increased thymic hypertrophy in the male setting. We first formally demonstrated that an initial androgen flare induced thymic collapse then, with the combination of LHRH-A and an AR-antagonist (Cosudex), we showed that the immunosuppressive effect of an androgen flare is prevented and that thymic recovery is enhanced following chemotherapy treatment with cyclophosphamide. Together, the results in this thesis demonstrate that, while sex steroid ablation is a clinically viable therapy for the improvement of thymic regeneration, the combination of LHRH-agonist with AR-antagonist enhances the effectiveness, but usage of ER blockers in females needs substantial refinement in terms of inducing immune recovery in female patients.

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In accordance with Monash University Doctor of Philosophy regulations, the following declarations are made:

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university of 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.

This thesis comprises 1 first author review published in a peer reviewed journal, 1 primary manuscript ready for submission and 2 manuscripts being prepared for submission, presented as a total of 1 literature review chapter and 3 results chapters. The proportional contributions of the co-authors are described in the declarations of the authorship on the following pages.

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In the case of Chapter 3, the nature and extent of my contribution to the work was the following:

Nature of contribution	Extent of contribution (%)
Writing and drafting of manuscript, experimental design, execution and analysis of experiments and interpretation of results	70%

The following co-authors contributed to the work. Co-authors who are students at Monash University must also indicate the extent of their contribution in percentage terms:

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
Katerina Vlahos	Experimental design, execution and analysis of experiments and interpretation of results	
Richard Boyd	Supervisory role - intellectual input, experimental design and interpretation of results, drafting of manuscript	
Ann Chidgey	Supervisory role - intellectual input, experimental design and interpretation of results, drafting of manuscript	

Candidate's Signature	[redacted]	**Date** 2/7/10.

Declaration by co-authors

The undersigned hereby certify that:

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(2) they meet the criteria for authorship in that they have participated in the conception, execution, or interpretation, of at least that part of the publication in their field of expertise;
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Name	Nature of contribution	Extent of contribution (%) for student co-authors only
Katerina Vlahos	Experimental design, execution and analysis of experiments and interpretation of results	
Richard Boyd	Supervisory role - intellectual input, experimental design and interpretation of results, drafting of manuscript	
Ann Chidgey	Supervisory role - intellectual input, experimental design and interpretation of results, drafting of manuscript	

Candidate's Signature	[redacted]	**Date** 2/7/10

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The following co-authors contributed to the work. Co-authors who are students at Monash University must also indicate the extent of their contribution in percentage terms:

Name	Nature of contribution	Extent of contribution (%) for student co-authors only
Danika Khong	Intellectual input, execution and analysis of experiments and interpretation of results	10%
Richard Boyd	Supervisory role - intellectual input, experimental design and interpretation of results, drafting of manuscript	
Ann Chidgey	Supervisory role - intellectual input, experimental design and interpretation of results, drafting of manuscript	

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RESEARCH OUTPUT

Publications and manuscripts

1. **Hince M**, Sakkal S, Vlahos K, Dudakov J, Boyd, R and Chidgey A. (2008) The role of sex steroids and gonadectomy in the control of thymic involution. *Cellular Immunology*. 252:122-138

2. Barnard A, Layton D, **Hince M**, Sakkal S, Bernard C, Chidgey A and Boyd R. (2008) Impact of the neuroendocrine system on thymus and bone marrow function. *Neuroimmunomodulation*. 15: 7 – 18

3. Calder A, **Hince M**, Chidgey A and Boyd R. The impact of sex steroids on thymic function. *Molecular and Cellular Endocrinology*. (submitted manuscript)

4. Androgen receptor antagonist improves luteinising hormone-releasing hormone agonist mediated thymic recovery following chemotherapy (manuscript in preparation).

Presentations

Hince M, Vlahos K, Chidgey A and Boyd R. Sex steroid ablation enhances recovery of the female immune system following chemotherapy. Immunology Group of Victoria conference, Beechworth Victoria 2007 (Oral presentation)

Hince M, Vlahos K, Chidgey A and Boyd R. Sex steroid ablation enhances recovery of the female immune system following chemotherapy. Monash Immunology and Stem Cell Laboratories Student Symposium, Melbourne Victoria 2007 (Oral presentation)

Hince M, Chidgey A and Boyd R. Cosudex improves LHRH-agonist assisted thymic recovery following chemotherapy. Immunology Group of Victoria conference, Yarra Valley Victoria 2008 (Oral presentation)

Hince M, Chidgey A and Boyd R. Cosudex improves LHRH-agonist mediated thymic recovery following chemotherapy. Monash Immunology and Stem Cell Laboratories Student Symposium, Melbourne Victoria 2008 (Oral presentation)

Hince M, Khong D, Chidgey A and Boyd R. Cosudex improves LHRH-agonist mediated thymic recovery following chemotherapy. Infection and Immunity conference, Gold Coast Queensland 2009 (Poster presentation)

Hince M, Chidgey A and Boyd R. AR-antagonist improves LHRH-agonist mediated thymic recovery following chemotherapy. Immunology Group of Victoria conference, Yarra Valley Victoria 2009 (Oral presentation)

Hince M, Chidgey A and Boyd R. AR-antagonist improves LHRH-agonist mediated thymic recovery following chemotherapy. Australian Society for Immunology conference, Gold Coast Queensland 2009 (Poster presentation)

Hince M, Chidgey A and Boyd R. Strategies to enhance sex steroid ablation-induced thymic reconstitution following severe immunodepletion in male and female mice. Monash Immunology and Stem Cell Laboratories Student Symposium, Melbourne Victoria 2009 (Oral presentation)

Hince M, Chidgey A and Boyd R. Androgen receptor antagonist improves luteinizing hormone-releasing hormone agonist mediated thymic recovery following chemotherapy. ThymOz VI, Gladstone Queensland 2010 (Oral presentation)

Hince M, Chidgey A and Boyd R. Improving sex steroid ablation-induced thymic recovery for clinical application. Monash Immunology and Stem Cell Laboratories Seminar Series, Melbourne Victoria 2010 (Oral presentation)

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LIST OF ABBREVIATIONS

AR – Androgen receptor

CLP – Committed lymphoid progenitor

CRH – Corticotropic-releasing hormone

Cx – Castrated

Cy – Cyclophosphamide

DN – Double negative

DP – Double positive

EIA – Enzyme immunoassay

ER – Estrogen receptor

ER-A – Estrogen receptor analogue

ETP – Early thymic progenitor

FSH – Follicle stimulating hormone

G-CSF – Granulocyte-colony stimulating factor

GH – Growth hormone

GnRH – Gonadotrophin releasing hormone

GvHD – Graft versus host disease

GvL – Graft versus leukemia

HGF – Hepatocyte growth factor

HPG – Hypogonadal

HSC – Hematopoietic stem cell

HSCT – Hematopoietic stem cell transplantation

LH – Luteinising hormone

LHRH – Luteinising hormone-releasing hormone

LHRH-A – Luteinising hormone-releasing hormone-agonist

LHRH-R – Luteinising hormone-releasing hormone receptor

LSK – Lineage- Sca-1+ c-Kit+ cells

LT-HSC – Long term hematopoietic stem cell

MCP – Myeloid committed progenitor

MHC II – Major histocompatibility complex class II

Ovx – Ovariectomised

PBS – Phosphate buffered saline

PR – Progesterone receptor

PVS – Perivascular space

RIA - Radioimmunoassay

RTE – Recent thymic emigrants

SERM – Selective estrogen receptor mediator

SP – Single positive

SPF – Specific pathogen free

TCR – T cell receptor

Te – Testosterone

TEC – Thymic epithelial cell

TN – Triple negative

TREC – T cell receptor excision circle

Treg – T regulatory cells

CHAPTER 1 – Literature Review

THE ROLE OF SEX STEROIDS AND GONADECTOMY IN THE CONTROL OF THYMIC INVOLUTION

Manuscript information

Hince M, Sakkal S, Vlahos K, Dudakov J, Boyd, R and Chidgey A. (2008) The role of sex steroids and gonadectomy in the control of thymic involution. *Cellular Immunology*. 252:122-138.

This paper was an invited review, published in a 2008 edition of Cellular Immunology. The candidate, Melanie Hince, was primarily responsible for planning, writing, designing figures and collating the manuscript. Other co-authors contributed to the writing sections of the review and drafting. Detailed contributions are explained in the signed declaration on page

1.1 Abstract

A major underlying cause for aging of the immune system is the structural and functional atrophy of the thymus, and associated decline in T cell genesis. This loss of naïve T cells reduces adaptive immunity to new stimuli and precipitates a peripheral bias to memory cells against prior antigens. Whilst multiple mechanisms may contribute to this process, the temporal alliance of thymic decline with puberty has implicated a causative role for sex steroids. Accordingly ablation of sex steroids induces profound thymic rejuvenation. Although the thymus retains some, albeit highly limited, function in healthy adults, this is insufficient for resurrecting the T cell pool following cytoablative treatments such as chemo- and radiation-therapy and AIDS. Increased risk of opportunistic infections and cancer relapse or appearance, are a direct consequence. Temporary sex steroid ablation may thus provide a clinically effective means to regenerate the thymus and immune system in immunodeficiency states

1.2 Introduction

That the immune system deteriorates with age is the basic dogma used to explain the increased incidence of cancer, opportunistic infections and poor vaccine responsiveness in the elderly. At the cellular level this is best exemplified by a profound reduction in thymic size, T cell content and number of recent thymic emigrants (RTE) [1-3]. As this atrophy, in many mammalian species, is most evident from puberty onwards, it has been aetiologically linked to an increase in sex steroid production. In combination with peripheral T cell clonal expansion and gradual exhaustion of the naïve pool with progressive antigen contact, the decrease in thymic output leads to a gradual bias towards the memory phenotype [3-5]. Under normal circumstances there are sufficient pre-existing T cells to provide immune protection but when there is any severe insult to the immune system, such as chemotherapy/irradiation treatment or from chronic viral infections, best exemplified by HIV, the impact of the decline in thymic output becomes critical. The delay in T cell recovery in such conditions, including following hematopoietic stem cell transplant (HSCT), leads to an increased risk of opportunistic infections and disease relapse [6]. Consequently there is a higher rate of mortality in adults compared to pre-pubertal individuals [7]. In addition, as the number of virus-specific cells is reduced in the elderly, the ability to clear infections as well as respond to vaccines, is impaired [8, 9]. The need to replenish the peripheral immune repertoire and improve long-term immune reconstitution after HSCT has lead to a more recent focus on strategies to regenerate thymic tissue. Reversing the damage caused by sex steroids forms one of the major, logical strategies. This review will examine the endocrinology of thymic atrophy -the impact of sex steroids on the immune system and the use of gonadectomy to enhance immune reconstitution.

1.3 Age-associated Thymic Atrophy

The thymus undergoes significant structural involution with age. However, whilst there is a significant loss in cellularity, many studies have documented that the aged atrophic thymus remains functional, albeit limited [10]. A loss in thymic epithelial cells, an increase in the perivascular space (PVS) - which is infiltrated by adipocytes [11] and mature single positive (SP) T cells [12], and an increase in cystic cavities [13, 14] are all characteristic of the atrophic thymus. The remnant functional areas of

the cortical and medullary microenvironments are still capable of generating T cells albeit at greatly reduced levels [15, 16], and specific subsets of the medulla – such as major histocompatibility complex class II (MHCII) high expressing cells [17], are likely to still impart tolerance mechanisms [18, 19]. Qualitative changes in the microenvironment are also evident [20]; expression of pro-inflammatory cytokines by the thymic stroma such as TGF-β and IL-6 are increased with age, possibly contributing to an increase in apoptosis of thymocytes [3]. In terms of T cell precursors, the early T lineage progenitors (ETPs) (Lin^- $CD44^+$ $CD25^-$ $CD117^{hi}$ $CD127^{low/neg}$), currently the earliest known committed T cell progenitor in the thymus, are reduced in both frequency and number with age in mice [21, 22]. In addition, one study demonstrated ETPs from aged mice proliferate at a reduced rate when seeded into thymic lobes compared to those from young mice [22]. These alterations may be due to a reduction in both the number of progenitors entering the thymus, a loss in production of stromal derived cytokines and growth factors affecting proliferation, and/or intrinsic changes in the progenitor cells. The ultimate effect is that there is a decrease in the number of recent thymic emigrants, indicated by a reduction in expression of T cell receptor excision circles (TRECs), with age [3].

In the periphery, homeostatic mechanisms maintain total T cell numbers, which are kept relatively constant with age [23, 24], however, there is a major shift towards a memory phenotype [25, 26], their expansion facilitated by the decline in naïve T cell output [27, 28]. As a consequence there is a reduced variability within the TCR repertoire in the elderly, resulting in compromised adaptive immune responses to newly encountered antigen [29]. In addition, $CD4^+$ T cells from aged mice have a reduced response to antibody stimulation compared to those from young mice [30-32]. These effects of aging upon the activation of T cells have been partly attributed to defects in the Raf-1 and JNK-dependent protein kinase pathways [32]. Overall, the age-related reduction in naïve cells and in functional capacity of existing cells, leads to an increased rate of mortality due to infectious diseases such as pneumonia and urinary tract infections in the elderly [7, 9, 33, 34].

1.4 Understanding the Endocrinology of Thymic Atrophy

The link between the reproductive system, its role in the production of certain endocrine hormones such as sex steroids, and immunity was first reported in 1904, in a study on castrated cattle, which were found to have enlarged thymi [35]. Both castration and ovariectomy induced hypertrophy of the thymus, while testosterone re-administration suppressed this process. Subsequent investigations have revealed that imbalances in sex steroids may be detrimental too, with many immune abnormalities, such as an increase in autoimmune factors, occurring during periods of decreased fertility, spontaneous abortion, endometriosis and abnormal births as well as in hypogonadic patients [36-38].

1.4.1 Luteinising Hormone-Releasing Hormone and its Receptor

The hypothalamic decapeptide LHRH (luteinising hormone-releasing hormone) is an important focus of this review as it controls immune functions both directly and indirectly [36, 39]. Cyclic impulses of LHRH are able to activate the hypothalamus-pituitary-adrenal/gonadal axis, resulting in synthesis and release of luteinising hormone (LH) and follicle-stimulating hormone (FSH), which then control production of gonadal sex steroids [36] (Figure 1.1). However LHRH may also form part of an intracellular signalling system in the thymus. LHRH binding to its receptors on thymocytes and thymic stromal cells (but also splenocytes and possibly NK cells) activates downstream protein kinase C translocation resulting in an upregulation of IL-2 receptor expression [40], potentially improving the proliferative capacity of these cells. However, studies on endometriosis patients treated with LHRH-agonists suggested LHRH could also have a negative functional impact by reducing the cytotoxicity of NK cells [41, 42].

LHRH receptors have been observed on immune cells such as blood lymphocytes, mast cells, thymocytes and splenocytes [43-46] and are thought to play a role in the maturation of these cell types - suggesting a role for LHRH in early development as well as immune function and maintenance. A study involving treatment of male neonatal monkeys with a LHRH antagonist resulted in postnatal reduction in cortical cellularity and $CD8^+$ T cells within the thymus, comparative to vehicle-treated counterparts [47] while treatment of thymocytes with a LHRH-A reduced their

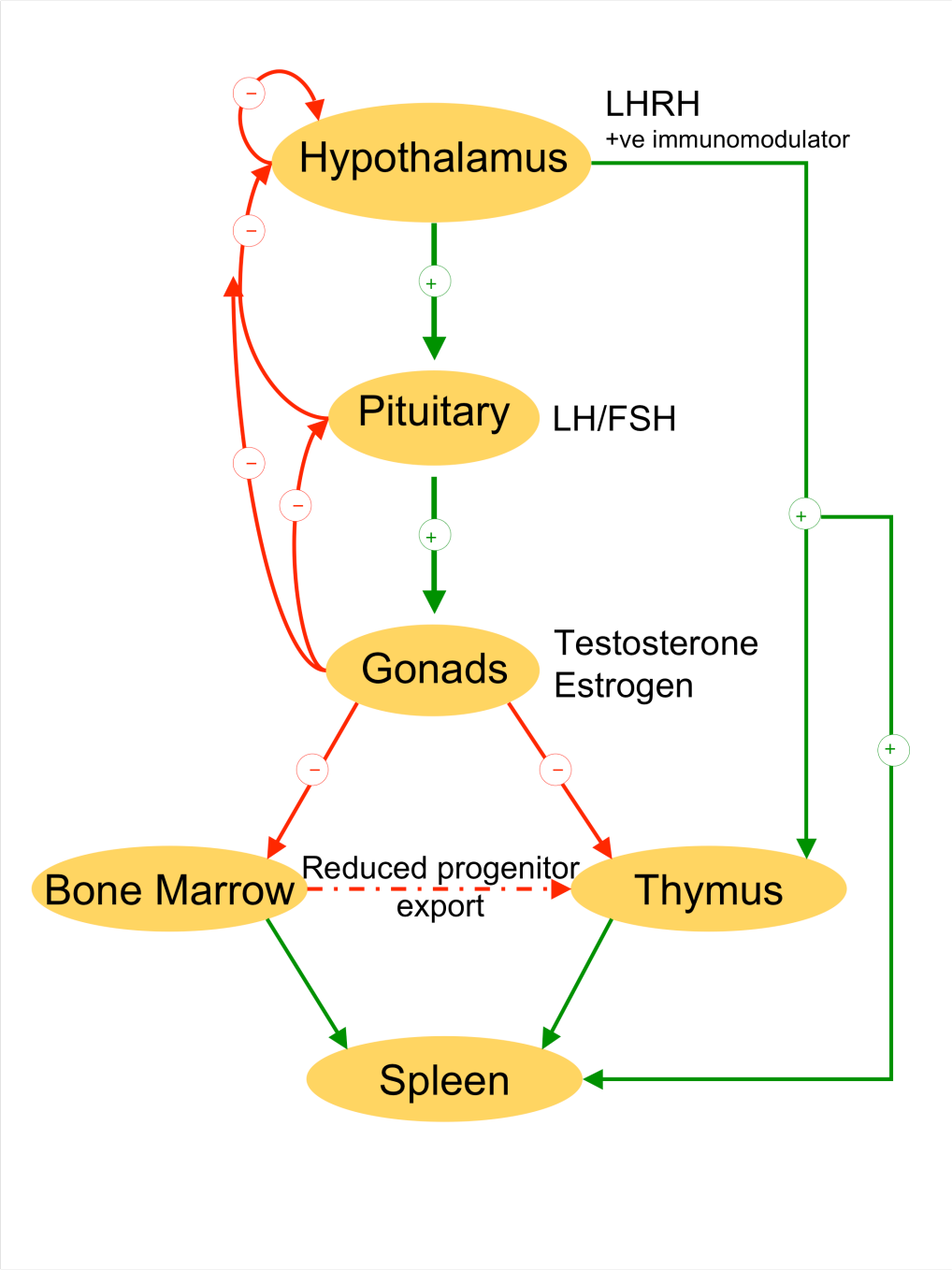


Figure 1.1 Simplified diagram of the HPG axis and the immunomodulatory effects of its components.

LHRH is produced by the hypothalamus and transported to the anterior pituitary where it controls the production and release of LH and FSH. These then govern production of gonadal steroids, which have negative effects on the thymus and bone marrow while LHRH itself is a positive immunomodulator. These effects are supported by experimental data obtained mainly from mice.

proliferation [48] thus indicating a probable direct interaction between LHRH and the thymus. Similarly, neonatal ERα knockout mice have a reduced thymic cellularity also implicating estrogen to positively regulate early thymic development [49]. To date, however, LHRH has thus been shown more commonly to act as a positive immunomodulator in regards to the thymus [36] but clearly this could be age dependent. While generally considered to be very safe clinically, side effects reflecting its role in bone development were evident in studies of prostate cancer patients treated with LHRH analogues, where bone density was greatly reduced within a few months of treatment [50, 51]. However, this is more likely to be due to the loss of androgen than a direct effect of LHRH.

1.4.2 Sex Steroids

The most compelling evidence for the role of sex steroids in thymic atrophy and diminished immune function with age, is that surgical or chemical gonadectomy (using LHRH analogues) reverses age-related thymic atrophy [52-54] and improves lymphocyte recovery following HSCT and chemotherapy [55-57] while the re-administration of androgen or estrogen prevents this reconstitution [58-60].

1.4.2.1 Androgens

The immunosuppressive role of testosterone is clearly evident in the rapid (within hours) induction of thymic atrophy after its administration and cessation of testosterone production by castration of males causing thymic hypertrophy [61-63]. Indeed, Olsen and colleagues have demonstrated the importance of both androgens and estrogens in the process of thymic involution [64]. Studies in transgenic mice that were defective in androgen expression on either the hematopoietic compartment or the stromal compartment, suggested that the direct interaction of sex steroids with their receptors on thymic stromal cells [64, 65] were responsible for thymic degeneration. However, testosterone administration also causes apoptosis of $CD4^+CD8^+$ double positive (DP) thymocytes both *in vitro* and *in vivo* at least in part by inducing the release of TNF-α [62, 66, 67]. Thus, TNF-α is a major mediator of sex-steroid induced apoptosis of DP thymocytes and possibly other thymocyte subsets to a lesser extent. The effects of enhanced androgen on the peripheral immune system have not been investigated as extensively as the thymus or bone

marrow. However, while testosterone administration decreases total splenic cellularity, there is no obvious preferential depletion of a specific lymphoid subset [62].

1.4.2.2 Estrogens

Estrogen-induced thymic degeneration post puberty in females occurs through a number of mechanisms [60] and is much more complex than that seen in males, not the least of which is the fact that estrogen is produced at multiple sites including the adrenals and adipose tissue. Increases in estrogens, such as during pregnancy, puberty or exogenous estrogen administration, enhance thymic atrophy [59, 68] characterised by reduction in DP thymocytes and increases in the proportion of $CD4^+$ and $CD8^+$ single positive T cell subsets (SP) [69, 70]. As with androgen administration in males, the SP T cells also decrease in number, however there is an opposing shift in the $CD4^+$ to $CD8^+$ ratio. With androgen replacement, $CD8^+$ T cells are increased relative to $CD4^+$ T cells, whereas this is the opposite with estrogen replacement [59, 71-73] thus favouring a T helper cell phenotype, although it is unknown why this dimorphism occurs. In the bone marrow, elevated estrogen levels directly inhibit the production of thymic precursors such as $Flt3^+$ LSK ($Lineage^-$ $Sca\text{-}1^+$ $c\text{-}Kit^+$) and in the thymus - ETPs and $CD4^-CD8^-$ double negative (DN) subsets [74]. This implies that the rise in female sex steroids impacts not only on the thymus, but also the proliferation of hematopoietic lineage cells - including potential thymocyte progenitors in the bone marrow. For the B lymphoid compartment, estrogen causes a major loss of pre B cells and their progeny [75, 76]. Re-administration of estrogen to ovariectomised females also causes a decline in B lymphopoiesis [77]. Estrogen administration, however, does not seem to as robustly affect the peripheral immune organs as it does the thymus and bone marrow. Following even high-dose estradiol treatment of male and female rats, total splenic cellularity was only slightly reduced but this does not preclude functional loss [68, 78]. There was, however, a significant decrease in total splenic T cells ($CD3^+$) in both sexes [78]. It is thus possible that T cells are the only major cell type negatively affected in the spleen by estrogen treatment, although destruction of B cell progenitors in the marrow ultimately leads to a loss of mature B cells [75, 76, 79].

1.4.3 Distribution of Sex Steroid Receptors

It is clear that increases in circulating androgen and estrogen levels have major consequences on both central and peripheral immune organs and their cellular subsets. Identifying sex steroid receptors on immune cells can help assess whether these effects are direct and/or indirect. Originally, the consensus was that androgen receptors (ARs) are only present on thymocyte and thymic stromal cell subsets [80, 81] but not bone marrow or splenic lymphocytes. In the thymus, direct binding of androgen to ARs on DP thymocytes results in the apoptosis of these cells. However, what role these receptors play when in the presence of elevated androgen is still unclear as the only specific subset yet identified to decrease following androgen administration are DP thymocytes. With the advent of more sophisticated analytical techniques such as RT-PCR, further ARs have been described in the spleen and bone marrow [82, 83]. In the bone marrow, androgens act via intracellular receptors in stromal cells to release TFG-β, which in turn suppresses B cell development [84]. In the spleen, both intracellular and "unconventional" surface ARs have been identified on both $CD4^+$ and $CD8^+$ T cells [82, 83, 85]. Thus, as testosterone administration promotes proliferation of these subsets, it can be assumed that there is a direct interaction with androgens.

Estrogens and estrogen receptors (ER) play seemingly conflicting, yet major roles in normal thymic development and maintenance in both males and females. ERs are present on many immune cell subsets including thymocytes, splenic T cells and splenic B cells [69, 85]. In a study of ERα deficient male mice, the thymus and spleen did not develop to the same size as their wild type counterparts, indicating the importance of ERα in promoting initial thymus development [86]. The second ER (ERβ) was only discovered in 1995 and has since been shown to also be necessary for normal immune function in studies on ERβ deficient mice [87, 88]. As with ovariectomised mice, ERβ deficient mice developed myelogenous hyperplasia and splenomegaly within the first 18 months post partum. Post puberty, however, activation of ERα and ERβ through exposure to estrogen induces significant atrophy of the thymus [59] and atrophy of myelogenous cells [89] implying both ERs (and estrogen) to be essential for homeostasis of the immune system. Estrogen thus seems to have both negative and positive influences on thymus development, differing

according to the age of the individual – estrogen appears to promote thymus growth early in life but converts to a suppressive effect presumably around puberty. This suggests shifts in the signalling pathways with development since the receptors seem the same; this is an important issue to resolve particularly with the current endeavours to manipulate sex steroids to influence immune system status.

1.5 Sexual Dimorphism in the Regulation of the Immune System

Autoimmune diseases are a major health issue as they are estimated to afflict 5% of the population [90]. Despite the fact that it is well known that females are more affected than males [91] to date there has been no consensus hypothesis as to why this is so. There are, however, two plausible explanations: that estrogen is less immunosuppressive than testosterone (which may be directly at the level of the effectors, or through lower levels of T regulatory cells being generated), or that estrogen positively influences autoreactive cells. Neither of these is conclusive yet. Furthermore, during pregnancy and post partum, disease severity is reported to fluctuate with hormone levels, with symptoms of pre-existing autoimmune diseases such as multiple sclerosis subsiding during pregnancy, only to return post partum [92] while in men with rheumatoid arthritis, elevated estrogen levels within the synovial fluid may be the cause of the inflammatory state [93, 94]. However this is not true for all kinds of autoimmune diseases [95]. Animal studies have shown that in the presence of estrogen, serum IgG levels are higher in females [96] compared to pre-pubertal individuals. Furthermore, estrogen has been reported to have stimulating effects on the immune system [97]; females produce more vigorous cellular and humoral immune reactions and are more resistant to certain infections compared to males [98]; again this would be consistent with fewer T regulatory cells in females but this is still unresolved (see below).

Alternatively in the absence of LHRH or sex steroids, such as female hypogonadal (HPG) mice, when estrogen was reintroduced, serum IgG levels were suppressed [99]. Thus the immunomodulatory effect of estrogen in potentially increasing autoantibodies may be linked to the presence of LHRH but it is more likely than in this instance of hypogonadism, the immunosuppressive function of (exogenous) estrogen is simply being more easily revealed. Alternatively gender differences in

autoimmune susceptibility maybe due to increased extrathymic T cell proliferation in response to elevated estrogen levels, to possibly counter the decline in intrathymic T cell proliferation. This has been shown to occur in the liver of young male mice administered estrogen [100]. Hence, this highlights a sexual dimorphism in the immune response and implies a role for sex hormones in immune regulation and autoimmunity and for estrogen at least in both a stimulatory and inhibitory capacity.

The increase in the frequency of immune disorders and autoimmune diseases with age has been linked closely with quantitative and/or qualitative defects of cells within the regulatory arm of the immune response. One major player is a subset of $CD4^+$ T cells; the $CD4^+CD25^+$ T regulatory cells (Tregs), which express the forkhead/winged helix transcription, factor Foxp3 [101-103]. Tregs follow the normal path of thymic development and are selected as part of the natural $CD4^+$ T cell repertoire. However, they appear late in ontogeny and are evident in the periphery around 3 days after birth in mice and humans [104]. With the thymus being integral to Treg generation, it is conceivable that thymic atrophy will lead to similar age-related changes as we see with conventional T cells.

There is little evidence on the effects of immunosenescence and sex steroids on Tregs. Contrary to expectations, human studies assessing Tregs have reported an increase in peripheral blood $CD4^+CD25^+$ and $CD4^+CD25^{hi}$ Tregs in the elderly compared to young adults [105]. This numerical increase does not explain why the elderly have an increased risk of autoimmune disease and therefore raises the issue of whether such cells are still functionally suppressive in older age. Similar to that observed with conventional T cells during age-related thymic involution, the Treg TCR repertoire could also be severely restricted due to homeostatic proliferation of pre-existing Tregs [106-109].

Sex steroids have been implicated to directly influence Treg number and function. As mentioned earlier, androgen and estrogen receptors are expressed in primary lymphoid organs and on mature peripheral B and T cells in mice and humans [110]. It was unknown whether these receptors are present on Tregs, however indirect evidence suggests they do possess estrogen receptors, with increase in circulating

estrogen either during pregnancy [111] or experimentally administered [112, 113], leading to the expansion and increased functional suppression of $CD4^+CD25^+$ Tregs. It cannot be excluded that this is an indirect effect, however a more recent study confirmed the presence of ERα by immunoblotting of resting human Treg cell lysates [114]. This was quite a significant finding as it explains at least in part the mechanisms by which some autoimmune diseases regress during times of increased sex hormone production.

1.6 Pregnancy and Thymic Atrophy

Atrophy of the maternal thymus during pregnancy has been explained as an evolutionary phenomenon designed to prevent rejection of the foetus [115]. Maximal involution occurs around gestational day 18.5 in mice, indicated by a five-fold reduction in thymocyte cellularity [116]. These studies show a block in T cell development evident at the early pre-T cell/DN2 ($CD3^-CD44^+CD25^+$) stage in mice, with all thymocyte subsets (ETP, DN2-4, DN, DP, $CD4^+$ and $CD8^+$) reduced in number. Since there are no major shifts in proportions of the subsets, the reduced thymopoiesis would be consistent with inhibition at the level of either reduced immigrant thymocyte progenitors of their intrathymic proliferation. We have also found a proportional loss in thymic stromal cells, including cortical and medullary epithelial cells, yet no proportional loss on the immature $CD4^+CD8^+$ thymocytes. (Sakkal et al., manuscript in preparation). This contrasts the well-known effect of corticosteroids to be primarily on DP thymocytes [117, 118], which we have confirmed (Sakkal et al., manuscript in preparation). Thus from this we can infer that glucocorticoids are perhaps not primarily responsible for pregnancy related thymic involution or are not produced at levels that affect the most glucocorticoid sensitive population of cells in the thymus [119]. We have also shown that corticosteroids reduce both the cortical and medullary thymic epithelium in a manner that is different to pregnancy (Sakkal et al., manuscript in preparation).

Whilst we would expect similar results during pregnancy in humans, this has not been established given the difficulties in obtaining maternal thymi. Indeed, there is a great paucity in our understanding of thymopoiesis in the human maternal thymus with the only reliable clinical data assessing leukocyte numbers in blood, which only

provide detail about thymic output, and not early thymocyte development or stromal cell interactions.

In addition to testosterone and estrogen, thymic involution has been associated with elevated levels of two other major hormones – progesterone [116, 120, 121], and corticotropic-releasing hormone (CRH) [122, 123]. Further, it has also been described that glucocorticoids regulate hormonal expression by suppressing the production of pituitary luteinising hormone and ovarian progesterone and estrogen, resulting in estradiol resistance by target tissues [122], presumably also limiting the extent of thymic atrophy. Since there was no proportional loss of DP cells, this again contrasts to the effects of glucocorticoids such as dexamethasone [124] Sakkal et al, manuscript in preparation). Thus the role of glucocorticoids in pregnancy induced thymic involution appears to be more of a regulatory one, rather than direct. Experiments using progesterone receptor deficient mice found that its expression on stromal cells was essential for thymic involution [121]. In this study progesterone receptor deficient thymi grafted under the kidney capsule of normal pregnant mice showed no involution. Whilst progesterone has clearly been implicated in maternal thymic involution, estrogen has also been suggested to contribute. Injection of 17-beta estradiol causes a reduction in ETP and DN2-4 populations [74] associated with reduced proliferation [116], suggesting the rising estrogen levels during pregnancy impact on the early thymic precursors.

Whilst many of the studies which assess thymocyte and stromal involution during pregnancy are primarily in rodent models [125, 126], they may not be directly applicable to humans although given the highly conserved nature of these hormonal interactions it is very likely to be equivalent. It is important to note that the pregnancy-induced thymic involution is reversible, with complete regeneration observed at four weeks post partum [127], thus making pregnancy a useful model of thymic regression and regeneration. The major changes in thymic function may also be linked to the need to prevent fetal immune rejection although this is still a poorly understood connection, which requires rigorous experimentation.

1.7 Other Reproductive Hormones

Other hormones associated with the reproductive system such as LH and FSH also play small but significant roles in immune maintenance. The presence of LH receptors on T cells has been documented [128] but in regards to direct interaction of LH and FHS with the thymus and/or other lymphoid components, no significant studies have been published.

Post-menopausal women suffer long-term withdrawal of progesterone and estrogens. Although there are other hormonal pathways affected during this stage of life, it has been suggested that the decline in total lymphocytes is due to the lack of progesterone/estrogen [129]. While female hypogonadal (HPG) mice do not show a significant decline in thymic cellularity with age [130, 131], the absence of LHRH as well as estrogen in these mice suggests that the inhibitory effect of estrogen post puberty relies on other members of the gonadal axis such as LHRH. There are conflicting reports on the role of sex steroids and LHRH in age-related thymic atrophy in males. In male HPG mice there is evidence of an increase in total thymic cellularity compared to their wild type counterparts [130]. Another group, however, observed no difference in thymic cellularity in both young and old HPG mice [132] although it was not stated what gender of mice was used in this case. Interestingly, it does appear that thymic atrophy still occurs in the HPG male mouse regardless of the absence of sex steroids [130, 132] supporting the involvement of other age related mechanisms, such as a decline in bone marrow derived progenitor cells or loss of LHRH which may have a direct stimulatory effect on the thymus and immune system. This is in fact a very important observation because it has great relevance to defining the approaches necessary to reverse thymic atrophy clinically.

1.8 Other Mechanisms of Thymic Involution with Age

Sex steroids are unlikely to be solely responsible for the global immune degradation with age. Changes in the production of other hormones and growth factors, as well as intrinsic changes to HSCs that impact on their proliferative and differentiation potential, are likely to be involved.

1.8.1 Alterations in the Bone Marrow Niche

Age-related deterioration of lymphopoiesis is not restricted to the thymus with significant effects also observed in the bone marrow. Deterioration in the ability of the bone marrow stroma to support lymphopoiesis has been reported in mice, particularly concerning the reduced production of IL-7 [133] and using in vitro B-cell culturing techniques, it was found that B-cell progenitors have a significantly reduced responsiveness to IL-7 with age [134]. Similarly within the committed lymphoid progenitors, there is a large reduction in the number and frequency of the common lymphoid progenitor-1 (CLP1) as well as a decline in their proliferative potential and their ability to respond to IL-7 [135, 136]. Further studies have shown enhanced mobilisation of stem cells in response to granulocyte-colony stimulating factor (G-CSF) in old mice compared to young mice, which may be due to a reduced adhesion of these stem cells to the bone marrow stroma with age [137]. Long term hematopoietic stem cells (LT-HSCs), defined as LSK $flk2^{-}CD34^{-}$, increase in frequency with age [138] and undergo progressive loss of differentiation and maturation potential [138-140]. With each self-renewal, HSCs undergo replicative stress and their reconstitution ability is reduced as shown in serial transplantation experiments of purified LSK cells, which in some ways mimic the natural aging process [139]. Changes in signalling with the stromal microenvironment may contribute to an apparent, but still controversial, bias away from the lymphoid lineage towards the myeloid lineage as well as age related intrinsic changes [138] and this could lead to the reduced frequency and number of ETPs evident with age [21].

Translating these findings into the human setting, it has been found that, in contrast to the mouse, there are fewer circulating $CD34^{+}$ HSCs [141] and following administration of G-CSF, aged individuals mobilise fewer $CD34^{+}$ cells into the periphery [142]. Functionally it has been found that $CD34^{+}$ cells from aged individuals have a reduced ability to develop into T-cells [143] as well as reduced self-renewal [144] and proliferative potential [145]. Therefore the impact of aging in the bone marrow can result in a reduction in progenitor proliferation, lymphoid lineage differentiation and export of hematopoietic progenitor cells, very likely contributing to the loss in total thymic cellularity.

1.8.2 Changing Profiles of Cytokines and Growth Factors

Changes in cytokine profiles in the thymic microenvironment have been associated with both a reduction in thymopoiesis and productive rearrangement of the TCR. This is most likely due to a reduction with age in the transcriptional regulator, E2A, which is involved in the rearrangement of the TCRβ genes thus leading to incorrect selection [146, 147]. Indeed there are major changes in expression of growth factors produced by thymocytes and thymic epithelial cells such growth hormone (GH), IGF-1 and Ghrelin, which promote thymopoiesis as well as the cytokines IL-13, IL-2, IL-9, IL-10, IL-14 and IL-7 [146-148, reviewed by 149]. In addition there is a reduction in the transcription factor Foxn1, an essential regulator of cortical thymic epithelial cell development [146, 150]. In contrast, there are increases in suppressive cytokines such as LIF, OSM and IL-6, all of which induce thymic atrophy following their exogenous administration, which admittedly may be supra physiological [147, 151]. Together, these results indicate that thymic atrophy with increasing age could also be due to both a reduction in the promotion of thymocyte maturation as well as an increase in T-cell apoptosis, which has been observed by many [152-154]. It is still unclear, however, whether these changes are completely independent of sex steroids or are a secondary effect from a functional impact of sex steroids on the stromal cells themselves.

1.9 Sex Steroid Ablation to Reverse Thymic Aging

Numerous studies over the years have demonstrated enhanced thymic growth and immune reconstitution following sex steroid ablation, be it surgical or chemical-induced androgen or estrogen deprivation. As mentioned earlier, surgical castration studies began with Henderson in 1904 where castrated cattle were shown to have significantly larger thymic size and weight compared to bulls and heifers [35]. Many studies have since confirmed this effect in several animal models along with an increase in thymopoiesis [155-161]. It is unclear what long–term health benefits this may translate to but eunuchs have been reported to live up to 12 years longer than non-castrated people [162].

1.9.1 Sex Steroid Ablation in Males - Surgical Castration

Castration by both surgical and chemical means has been shown to reverse age-related thymic atrophy (Table 1.1) [163]. In particular we have shown it enhances immune reconstitution in young (4-6 weeks), young-adult (3 months), middle-aged (9 months) and aged male mice (18-24 months) in several immunocompromised models including chemotherapy and allogeneic and autologous HSCT [22, 56, 164-167]. Following castration, we have shown an increase in proliferation and cellularity of early thymocyte subsets such that by 14 days post castration, the atrophic thymus resembles that of a young thymus [56, 164, 165, 168]. Reversal of age related alterations in the architectural organization between medullary and cortical regions follow. Importantly castration also induces an increase in immature cell types in the bone marrow (Figure 1.2), such as LSKs, HSCs and CLPs, which result in an increase in all immature B cell subsets [56, 164, 166] and may contribute to the increase along the thymocyte development pathway [69, 164-166].

Following castration, there is also an increase in the lymphoid cellularity of the peripheral organs, such as the spleen and lymph nodes [62, 169, 170]. Along with an increase in $CD4^+$ and $CD8^+$ T cells resulting from an increase in thymic output [165, 170], lymphocytes from castrated mice are more sensitive to antigen-mediated stimulation as demonstrated by ConA. Low ConA concentrations were able to induce proliferation of thymocytes from castrated male rats but not from T cells from their intact age-matched counterparts [159]. This was also evident in castrated mice vaccinated with OVA or TRAMP-C1 cells where peripheral T lymphocytes taken from the mice proliferated more readily when re-exposed to the nominated antigen than cells taken from sham-castrated mice [170]. Splenocytes from castrated male mice when co-stimulated with anti-CD3 and CD28, showed increased responsiveness to TCR stimulation above young intact counterparts [165].

Hence, surgical castration is able to enhance both the proliferative and functional capacities of thymocytes and their progenitors. It is extremely important that the effects of thymic regeneration following sex steroid ablation on the immune system encompasses all immune subsets in order to maintain immune balance as an

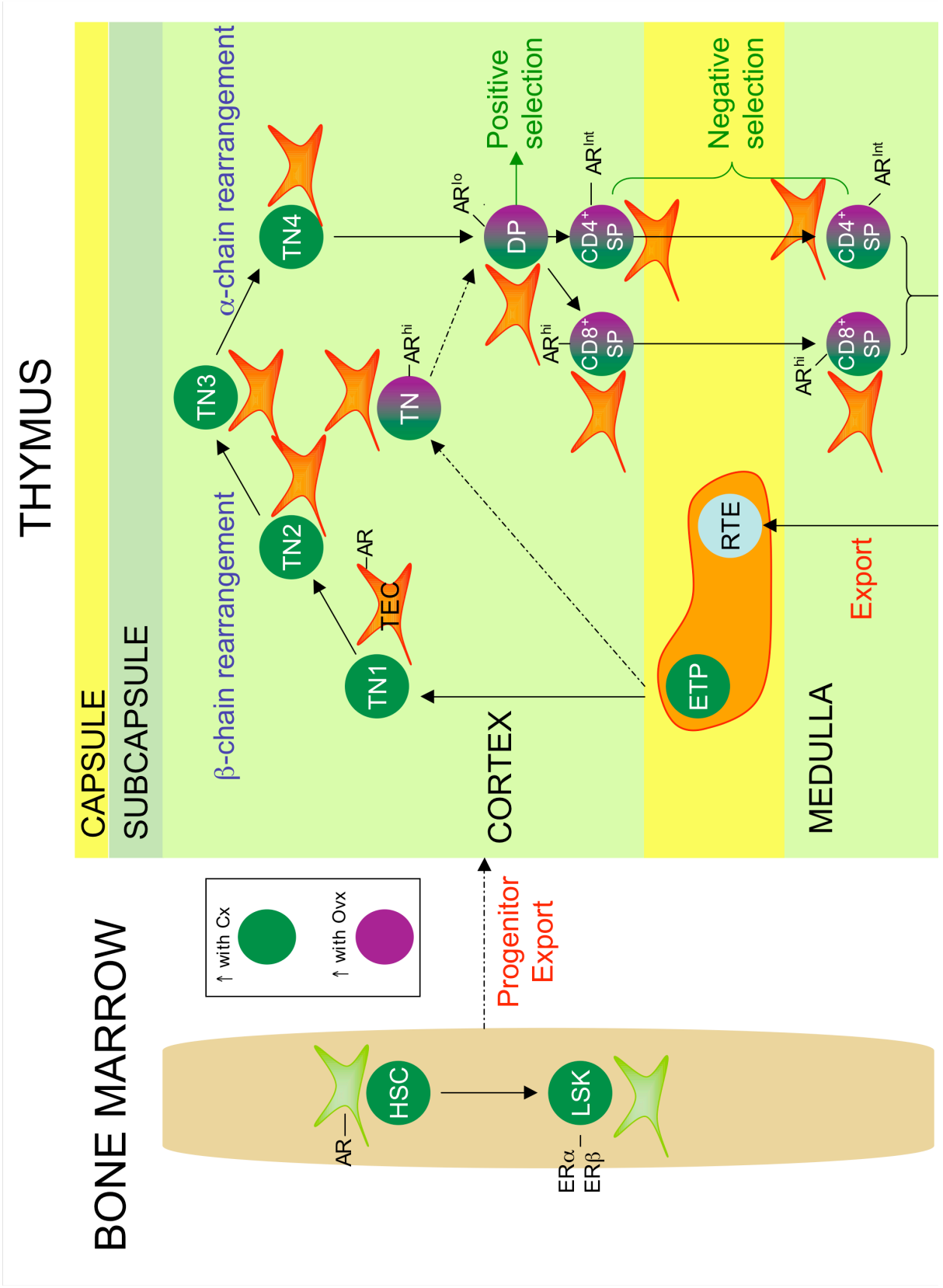
Table 1.1 Effects of aging upon the immune system and the aspects that can be corrected with surgical castration.

Aging on the immune system	**Effect of castration**
Increased memory:naïve T cell (Reduced peripheral $CD4^+$ and $CD8^+$ T cell numbers) [5, 24]	Increase in $CD4^+$ and $CD8^+$ peripheral T cells; reduced proportion and number of memory T cells [165]
Reduced TRECs in peripheral blood [3]	Increase in TREC levels with chemical castration [164]
Reduced proportion and proliferation of RTEs in the peripheral T cell pool [3]	Increase in RTE numbers [165]
Loss of functional tissue: cortical thymocytes [20]	Increase in absolute numbers of DN, SP $CD4^+$, SP $CD8^+$ and DP thymocytes [22, 163-165]*
Reduced thymic stroma [20]	Increase in thymic stromal cell numbers [64]
Increase in PVS: accumulation of adipocytes and connective tissue [11]	Reorganization of the thymic microenvironment/architecture [157]
Reduced ability to respond to sudden loss of peripheral T cells with an increase in T cell output [10]	Increased recovery rate of peripheral naïve T cell numbers following HSCT and chemotherapy [164]
Reduced proliferation of progenitors i.e. ETP and LSK [21, 31]	Increased number of ETP and LSK (reduced apoptosis of ETP) [22, 164, 165]

* Effect also seen following Ovariectomy [53]

Figure 1.2 Distribution of androgen and estrogen receptors in the thymus and bone marrow

Expression of the estrogen receptor (ER) has been shown on total thymocytes, but it is unclear on which particular subsets. Cessation of androgen or estrogen production by gonadectomy (Cx = male, Ovx = female) improves all thymocyte cell subsets, with those more susceptible depicted in the figure; ovariectomy results in the increased proportion of predominantly the DP thymocyte subset.


THYMUS
BONE MARROW
CAPSULE
SUBCAPSULE
CORTEX
MEDULLA
β-chain rearrangement
α-chain rearrangement
Positive selection
Negative selection
Progenitor Export
Export
TN1
TN2
TN3
TN4
TN
TEC
AR
AR^hi
AR^lo
AR^Int
DP
CD4+ SP
CD8+ SP
ETP
RTE
HSC
LSK
ERα
ERβ
↑ with Cx
↑ with Ovx

imbalance of one and not the other type of cell could lead to the increased risk of infections, cancer or autoimmune diseases.

In terms of immune regulation, reports on the effects of castration on Tregs are scarce. The first study reported an androgen dependent maintenance of Tregs in humans, where chemical castration (see next section), using the LHRH antagonist Acyline, reduced the percentage of peripheral blood $CD4^+CD25^+$ Tregs. This decline normalised during recovery with the return of normal hormone levels [171]. More recently, however, the effect of neonatal castration at post natal day 3 in rats was assessed [169]. In this case there was no significant difference in the percentage of thymic $CD4^+CD25^+Foxp3^+$ Tregs, although the absolute number was increased in castrated rats than in sham castrated rats due to thymic hypercellularity [169]. The differing reports here could obviously be due species differences but also to the timing of when sex steroids are removed, This issue of the effects of sex steroids and their removal on Tregs requires more detailed study as it could prove useful in the establishment of new treatments for autoimmune disease. In this regard, we have recently found that Tregs return to significantly higher levels in cancer chemotherapy patients undergoing HSC transplantation treated with LHRH, compared to the non-LHRH control group [57].

1.9.2 Sex Steroid Ablation in Females – Surgical Ovariectomy

Ovariectomy of female mice and rats has also been shown to cause enlargement of the thymus [60, 172] however to a lesser degree than that seen with castration in males [173]. The hormonal response to ovariectomy is, of course, more complex than castration of males as it causes a decrease in estrogens as well as prolactin, progesterone and dehydroepiandrosterone, which also have immunomodulatory effects [174]. It is further complicated by the production of estrogen by the adrenal glands by the conversion of local testosterone [175], which increases with age and can compromise the impact on thymus rejuvenation. Adrenalectomy of female (and male) mice results in thymic hypertrophy but this also involves a loss of glucocorticoids [176]. The impact of ovariectomy on thymocyte subsets also differs to that of castration in males. Although there is an increase in all thymocyte subsets (Figure 1.2) [53], thymic hypertrophy does not obviously include increased

proliferation of progenitors as is seen with castration, but rather an expansion of pre-existing subsets and mature subsets in the periphery [177]. Within the bone marrow compartment, ovariectomy of young-adult mice leads to an increase in total bone marrow cellularity reflected by an increase in total B cells ($CD19^+$) [77]. There is little change in total splenic cellularity or in either splenic T or B cell subsets however, similarly to castration, splenocytes taken from ovariectomised mice showed an increased proliferative capacity when introduced to LPS *in vitro* [172].

The long-term effects of sex steroid ablation on the immune system are of particular interest. While castration and ovariectomy appear to be long-term in respect to enhanced immune reconstitution (enhanced thymic size is evident for at least a year) it is unknown how this translates in the human setting. We have found that the thymus of castrated male mice is still enlarged (2-3 fold) twelve months post surgery (Sutherland, Dudakov unpublished observations). Interestingly we have also found that ovariectomy is very much age dependent: in young mice there is a profound increase in thymic size, as for males but there is little effect on female mice 6-12 months of age (Hince, Vlahos unpublished observations). This is possibly due to the contribution of adrenal and fat metabolism derived estrogen. Clinically, the treatment of females should therefore incorporate the use of ER blockers to eliminate the effect of peripheral estrogens. Long-term studies by others have shown that the thymus returns to age-matched controls by 20 months post ovariectomy in C57Bl/6 female mice [173] again presumably due to the gradual return of circulating estrogens [175] or as mentioned previously the potential reduction in supply of bone marrow progenitors.

1.9.3 Chemical Castration - LHRH-agonists

One very obvious major advantage of chemical castration is that it is completely reversible and has a very high safety record clinically. Most commonly it is affected by LHRH (otherwise referred to as gonadotrophin releasing hormone or GnRH) analogues. LHRH agonists are the most effective and are given in constant, supra-physiological levels to achieve sex steroid deprivation by first overstimulating the LHRH receptors in the pituitary. These then become desensitised to LHRH and stop production of LH and FSH, which results in the cessation of gonadal hormone

production and spermatogenesis [178]. This treatment has the advantage of being completely reversible with fertility returning to normal within weeks after cessation of treatment although closer to 1-2 months in man, [178]. It is obviously less invasive than the surgical alternative. LHRH has been used as the "standard of care" for over 25 years for millions of patients with sex steroid exacerbated clinical conditions. The most prominent group are prostate cancer patients who, having received LHRH-agonist, have an increased rate of remission as well as an improved quality of life [179-182]. The most likely effect on these patients is the simple reduction of testosterone to deprive the cancer cells of this growth factor but they also have improved T cells [183], which very likely contribute to the cancer control and may well be a "hidden jewel" in the treatment strategy. We have shown that these patients have increased levels of naïve T cells (both $CD4^+$ and $CD8^+$) [165, 183] and the Kwon group has found an increase in T cell infiltration of the prostate following androgen reduction [184].

The use of LHRH-agonist has shown a reversal as well as a delay of thymic atrophy in aged mice and rats [165, 185, 186]. Although the seminal vesicles do not reduce to the size seen with castration, thymus hypertrophy is greater with LHRH than that following surgical castration [185]. One likely explanation for this is the direct stimulatory effect of LHRH on the thymus itself since there are LHRH-Rs on thymic cells and indeed the thymus produces its own LHRH the level of which is upregulated by castration [58]. There is, however, an initial thymic atrophy caused by the agonist [186] most likely due to an indirect influence following the surge in sex steroids caused by the initial hyperstimulation of the pituitary prior to desensitising of the LHRH-R. Following this there is an increase in LHRH binding sites within the thymus [187] indicating a possible compensation mechanism with the lack of available LHRH in the thymus.

1.9.4 Chemical Castration - LHRH-antagonists

Whereas LHRH-agonists cause initial over-stimulation of LHRH receptors resulting in a surge of LH and FSH and subsequently a "flare" in gonadal steroid levels, LHRH-antagonists competitively bind the LHRH receptors in the pituitary causing an immediate suppression of LH and FSH release [188]. LHRH-antagonists require

constant binding with the LHRH receptors and if natural LHRH were to displace this event, sex steroids are produced once again. Such "leakage" together with the induction of some hypersensitivity, can occur clinically with some antagonists precluding them as the drug of choice for sex steroid suppression for prolonged periods. Clinically this can have very detrimental effects because it can allow promoted growth of AR^+ prostate cancer cells. LHRH-agonists, on the other hand, despite the initial "flare" are clinically safer because they maintain desensitised LHRH receptors for a period of time even after contact with the receptor is lost [179]. Another disadvantage of LHRH-antagonist treatment to enhance immune recovery is that even with the suppression of sex steroid production it actually suppresses thymocyte proliferation in response to ConA treatment as well as reduce the number of lymphocytes in the thymus and spleen [47, 189]. This may be in part due to blocking of the stimulatory effects of LHRH binding to LHRH-R on lymphoid cells. In this regard, LHRH-agonist treatment has been shown to improve T cell status in humans undergoing HSC transplantation following myeloablative chemotherapy [57]. Thus, further investigation into the comparative functional impact of LHRH antagonists on the thymus and immune responsiveness is required if it is to be considered an effective treatment to enhance immune recovery following chemotherapy or HSCT.

1.9.5 Androgen and Estrogen Receptor Blockers

As both ARs and ERs are present on thymocytes, thymic stromal cells and most other immune cell subsets, it is likely that the use of receptor blocking agents could prevent the ablative action of sex steroids on the thymus as well as the initial ablative action of sex steroid flaring with LHRH-agonists. This would not replace any beneficial effects of LHRH –agonist binding directly to its receptor on thymic and other lymphoid cells. Few studies have investigated the effect of these ER and AR blocking agents on the immune system, although they are routinely used in the treatment of breast, prostate and other sex steroid related cancers [180, 190, 191]. Use of Flutamide, an androgen receptor antagonist, has shown increases in spleen cellularity and thymic weight [68], although the thymic/splenic subsets affected by this treatment were not analysed. It could be assumed, however, that any subsets expressing the androgen receptor would benefit from the Flutamide treatment, as it

would restrict the negative impact of testosterone. Similarly, Tamoxifen, an anti-estrogen receptor agent, has been shown to increase relative total thymic weight [176, 192] as well as decrease circulating levels of testosterone and LH [193]. Thus, the action of this agent in males may be to simply decrease the inhibitory effects of testosterone and LH on the thymus whereas in females it prevents the suppressive action of total (both gonadal and adrenal) estrogens on the thymus.

1.10 Proposed Mechanisms of Immune Recovery

The mechanisms involved in the enlargement of the thymus following castration are not yet precisely known, but several likely theories have been proposed [169]. A direct impact of LHRH may be involved as intrathymic levels of LHRH are increased following surgical castration [58], which may be from the lack of negative feedback from the gonads to the hypothalamus, and lymphoid cells express receptors for LHRH (Figure 1.1). There is only scant evidence, however, for a functional impact of LHRH on T cells. When treated with LHRH, Jurkat cells, a human mature leukemic cell line that closely resembles mature T cells, had an enhanced proliferative activity [194]. Therefore, increased LHRH in the periphery may directly stimulate mature T cells and enhance proliferation, contributing to the increase in T cells in the spleen following castration but prior to the export from the thymus of newly derived naïve T cells. This ability of LHRH to directly impact on the immune system is of great functional relevance because it would provide reason to treat patients in whom sex steroids are already in part reduced such as menopausal women or following chemotherapy, which can damage gonadal function.

Following castration of male neonatal rats, a decrease in the number of apoptotic cells within the thymus is evident, aligning thymic recovery following castration with increased survival. Furthermore, increased thymocyte proliferation, shown by an increase in $BrdU^+$ cells, was seen in thymocyte cultures taken from castrated male rats [168, 169]. These both suggest an increased production of cytokines and growth factors, although we have shown that mRNA levels of IL-7, NGF, IGF-1, SCF or Fgf7 (KGF) in total thymic stromal cells did not change significantly after castration compared to sham-castrated controls [165]. Although KGF, for example, is required for thymic recovery following sublethal irradiation [195] we have shown that

castration of KGF-/- mice still results in thymic enlargement [56]. Subtle changes may not have been revealed in the heterogeneous population of cells in these early experiments and so this needs to be confirmed on purified stromal cell subsets. Indeed, Olsen and colleagues have demonstrated the importance of androgen receptors present on thymic stromal cells for the inhibitory effect of androgen on the thymus; chimeric mice deficient in AR expression on non-hematopoietic stromal cells did not undergo involution with androgen treatment [64]. It is also possible that the increase in thymic cellularity following castration is due to an increase in thymocyte progenitor entry.

1.11 Clinical Application - Sex Steroid Ablation to Enhance HSCT Engraftment and Thymic Recovery

It is well documented that the recovery period for T cells, particularly naïve cells, from treatments such as chemotherapy, radiotherapy and that associated with HIV infection is severely delayed with age, resulting in a long period of immunosuppression and subsequent high risk of opportunistic infections. In children (pre-pubertal) the recovery of $CD4^+$ T cells to protective (i.e. normal) levels takes ~ 6 months whereas in post-pubertal adults, with an atrophic thymus, these cells take years to recover and in some cases, never return to pre-treatment numbers [196]. Thus any method to enhance T cell-based immune reconstitution in these circumstances will be of major clinical benefit.

We have studied in detail the impact of either surgical or chemical (LHRH) castration in immunodepleted settings such as following chemotherapy and HSCT. When surgical castration was performed prior to autologous HSCT in mice, total LSK numbers were significantly increased by 2 weeks post transplant, and by 4 weeks the majority of LSKs were donor-derived [165]. In the thymus, there was a marked increased in all thymocyte subsets of both donor- and host-origin [164]. We have also shown castration-induced increased immune recovery in mice undergoing myeloablative radiation followed by allogeneic HSCT [56]. An important finding in that study was the graft versus host disease (GvHD) was not exacerbated by the loss of sex steroids but Graft versus Leukaemia (GvL) was retained. In both the autologous and allogeneic transplants there were increased levels of stem cells in the

bone marrow and more efficient engraftment as reflected by the total cellularity of the bone marrow, thymus and spleen [56, 164]. We further showed that prostate cancer patients treated for 4 months with an LHRH agonist, relative to pre-treatment controls, showed increased levels of total and naïve $CD4^+$ T cells; $CD8^+$ T cells were also increased [165]. This is also associated with increased T cell infiltration of the prostate [165, 184]. On the basis of extensive pre-clinical studies and these data on prostate cancer patients using an approved drug with a very high safety profile from over 25 years of clinical use, we undertook a pilot clinical trial on cancer patients that had undergone myeloablative chemotherapy and allogeneic or autologous HSCT with LHRH. The primary endpoint was increased levels of naïve $CD4^+$ T cells, given that this is normally "flat-line" for such adult patients even after ~2 or more years. Although the patient numbers are small, and not all responded, there was clearly a significant increase in $CD4^+$ T cells (naïve and total) from 9 months compared to ~6 months for children. In a similar setting children regain their T cells by ~6 months. These cells also had a broad TCR repertoire as determined by spectratyping [57]. These highly promising but preliminary findings are now being explored in greater detail in Phase II, double-blinded placebo controlled clinical trials on autologous HSCT patients. Sex steroid ablation, by chemical means, is thus a valuable tool to enhance both immune recovery and transplant engraftment following HSCT. LHRH potentially represents a major new tool in the clinical management of diseases of T cell origin, in particular immunodeficiency states. Such conditions are not restricted to cancer or HIV patients, however; it could also be used for corrective therapy of the immune system in autoimmune disease.

Another possible clinical application of sex steroid ablation therapy is during the lead up to major surgery and following major burn injury. In burn injuries, the cell-mediated response is suppressed with the stress involved in bodily damage. As males are more at risk of mortality from infection following such injury, androgen deprivation by Flutamide (androgen-receptor antagonist) treatment has been tested in mice. Mice castrated 2 weeks prior to trauma haemorrhage prevented the depression of MHCII expression on peritoneal macrophages as occurred in their intact counterparts [197]. Similarly, Flutamide treatment of male mice following burn injury enhanced the delayed-type hypersensitivity response [198]. Thus these

treatments would presumably lead to a decreased risk of developing sepsis in burn patients or those undergoing major surgery.

1.12 Other Models / Clinical Strategies to Reactivate the Thymus

While sex steroids are clearly linked to thymic atrophy with age, there are many other contributing factors. In the context of reversing thymic atrophy clinically, it is important to define these as alternatives to sex steroid disruption, or be incorporated as "adjuvants," enhancing the effectiveness of LHRH. Many growth factors effecting thymus growth are being investigated in pre-clinical studies. Hepatocyte growth factor (HGF) has been shown to both promote T cell reconstitution and inhibit chronic GVHD following HSCT in mice [199]. IL-7, which has the ability to enhance peripheral T cell proliferation in several species including primates [200] and increase TRECs in humans [201] is a promising therapeutic for immune deficiency and low-doses may reduce the occurrence of GVHD [202]. KGF has also been shown to protect epithelial cells from GVHD and to enhance thymic recovery after atrophy induced by aging [203] irradiation treatment and chemotherapy [195] while increasing naïve T cell proliferation as indicated by TREC levels [204]. GH also promotes immune reconstitution; in low doses it enhances immune recovery following HSCT as well as increasing total thymic size in mice and rats which is linked to a reduction in thymic adipocyte numbers which accumulate with aging [205-207]. However, in humans, there are very few reports on thymic reconstitution. Low doses of rhGH appear to enhance recovery of haematopoietic cell numbers following chemotherapy, and increase T cell recovery in HIV patients [208], but there were significant side effects in the latter study and the drug had the practical issue of having to be administered daily [209, 210]. Thus it seems likely that to effectively enhance thymus recovery in a raft of clinical conditions i.e. in the aged and, following chemotherapy or radiation, a combination of treatments may be required, with sex steroid ablation as the foundation platform, and the administration of one or more growth factors including IL-7.

1.13 Conclusions & Future Directions

It has been long recognised that there is a profound impact of sex steroids on the immune system in general and the thymus in particular. In this context, the effects of

sex steroids - androgens and estrogens, are complex and wide reaching. In the neonatal period, estrogen at least appears to be essential for normal immune development, while post-pubertal increases in sex steroid levels suppress stem and progenitor cells and downstream T and B cell differentiation and maturation, associated with a profound reduction in thymic output and an eventual increased risk of opportunistic infections. During periods of severe immunosuppression best typified by chemotherapy and radiotherapy, both surgical and chemical ablation of sex steroids improve immune recovery of the thymus and bone marrow and subsequently T and B lymphoid compartments. There is increased lymphocyte reactivity to antigen stimulation in both males and females post castration, although the improvement in females is not as impressive. While LHRH variants (particularly agonists) are shown to be very effective at enhancing immune recovery, their use in combination with either estrogen or androgen receptor blockers could prevent the initial surge in hormone/sex steroid levels and further increase thymic rebound. Thus, temporary sex steroid ablation has the potential to become an important tool for improving immune recovery following severe immunodepletion. In doing so, the ability to enhance thymus and bone marrow function provides a realistic platform for not only increasing T cell output, but also for manipulating the types and function of the T cells produced including the generation of T regulatory cells and thymic uptake of donor HSC to create tolerance in the recipient to transplants from that donor.

1.14 References

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CHAPTER 2

MATERIALS AND METHODS

2.1 Animals

Table 2.1 Animals used: strain, sex, age and place of purchase.

Strain	Sex	Age	Purchased
C57Bl/6	Male & Female	4-6 weeks (young)	Monash Animal Services (MAS; Melbourne)
		9 months (middle-aged)	Animal Resources Centre (ARC; Perth)
		12 months (aged)	Kindly donated by Glenda Bishop (Dept Psychology, Monash University, Melbourne)
		29 months (elderly)	
Balb/C	Male	3 months	MAS
		9 months	MAS

Mice were maintained in specific pathogen free (SPF) conditions at Mouseworks, Monash University. Techniques performed in this study received approval from the relevant Animal Ethics Committees.

2.2 Treatment of Animals

2.2.1 Ovariectomy

Mice were anaesthetised and two small dorsal incisions were made to expose the ovaries. These were removed and the wound closed using surgical staples. Sham-ovariectomy required the same surgical procedure, except for removing the ovaries. Ovariectomy was performed on the second day of chemotherapy treatment.

2.2.2 Luteinising Hormone-Releasing Hormone-Agonist administration

Mice received a single dose of 0.4mg Leuprorelin Acetate 3 month depot (Lucrin®, Abbott Australasia) via intra-muscular injection.

2.2.3 Androgen Receptor Antagonist administration

Androgen receptor antagonist (Cosudex/Bicalutamide) (AstraZeneca Ltd) was obtained in 50mg tablet form and crushed to form a powder. This was then dissolved in 50% ethanol to achieve three appropriate concentrations in a final volume of 50uL per dose; 16mg/Kg, 40mg/Kg and 75mg/Kg mouse body weight. Each dose administered orally 3 times per week.

2.2.4 Estrogen-receptor analogue administration

Standard estrogen-free feed was impregnated with 0.1g/Kg Tamoxifen (Sigma-Aldrich, Australia) by Specialty Feeds, Australia. Control mice were fed estrogen-free feed alone. All mice were observed daily for changes in weight and general health.

2.2.5 Testosterone administration (Hormone implants)

Testosterone (Te) silastic implants were prepared by packing Te powder (4-Androsten-17β-ol-3-one; Sigma Chemical Co., USA) into medical-grade polydimethylsiloxane tubing (Dow Corning, USA; ID 1.98mm, OD 3.18mm) and then sealing the ends with adhesive silicone (Dow Corning medical type A). The Te implants were 5mm in length. The hormone release rates have been described previously [1]. Following LHRH-A administration, a 1cm lateral incision was made and implants were places s.c. along the animal's dorsal surface.

2.2.6 Cyclophosphamide administration

Mice received two doses of cyclophosphamide (Endoxan®, Pharmacia Australia) suspended in filtered PBS at 200mg/kg over two days via intra-peritoneal injection.

2.3 Antibodies, Proteins and Immunoconjugates

Specificity	Clone	Form	Source
B220	RA3-6B2	PerCP, PE	BD Biosciences, USA
CD11c	HL3	FITC, Biotin	
CD3ε	145-2C11	Purified, FITC, PE	BD Biosciences, USA
CD4	RM4-5	FITC, PE	BD Biosciences, USA
CD8	53-6.7	PerCP, PE, FITC	BD Biosciences, USA
CD25	PC61.5	APC	BD Biosciences, USA
CD28	37.51	Purified	BD Biosciences, USA
CD43	1B11	PE	BD Biosciences, USA
CD44	IM7	FITC	BD Biosciences, USA
CD117 (c-kit)	2B8	APC	BD Biosciences, USA
CD127 (IL-Rα)	A7R34	PE	eBiosciences, USA

FcR	2.4G2	Hybridoma S/N	Boyd Lab, Australia
Human Ki67	B56	FITC	Becton-Dickinson,
IgM	R6-60.2	FITC	USA
Ly-6A/E (Sca-1)	D7	PE	BD Biosciences, USA
Ly-6G (Gr-1)	RB6-8C5	FITC, PE	BD Biosciences, USA
Mac-1 (CD11b)	M1/70	APC, FITC, PE	BD Biosciences, USA
MHCII (IA-IE)	M5/114.15.2	PE	BD Biosciences, USA
NK1.1	PK136	PE	BD Biosciences, USA
Streptavidin	-	PerCP	BD Biosciences, USA
TCRβ	H57-597	APC	BD Biosciences, USA
			BD Biosciences, USA

2.4 Flow Cytometry

2.4.1 Cell suspensions

Following killing by asphyxiation via CO2, organs (Femur, Tibia, Spleen and Thymus) were removed. Bone marrow was removed from the leg bones by flushing with cold PBS-BSA-Az through medullary canal using a syringe and 26-gauge needle. Single cell suspensions were made by repeated uptake and release from the syringe. Thymi and spleens were suspended by gentle teasing using frosted glass slides. Cell suspensions were filtered through 100μm filter mesh before washing with cold PBS-BSA-Az and recovered by centrifugation at 1500rpm for five minutes at 4°C.

Red blood cells in spleen and bone marrow suspensions were lysed via incubation with 1mL red blood cell lysis buffer for one minute at room temperature. Cell concentration and an approximate live count were determined via particle size and count analyser (Coulter, Germany).

2.4.2 Immunofluorescence staining of cells

$3x10^6$ cells/test were centrifuged at 1300rpm for three minutes in ninety-six well plates. The supernatant was then removed and cells were resuspended in 30μL primary antibody for fifteen minutes at 4°C. Cells were then washed in 150μL of cold PBS-BSA-Az and recovered by centrifugation at 1300rpm for three minutes at

4°C. Cells were suspended in 30µL secondary antibody where applicable and incubated for fifteen minutes at 4°C followed by a further wash step. Labelled cells were then suspended in a final volume of 200µL PBS-BSA-Az and transferred to round-bottom tubes for flow cytometry.

2.4.3 Intracellular staining for Ki67

Briefly, lymphocytes were fixed and permeabilised with eBioscience Fix/Perm working solution for 45 minutes at 4°C in the dark. Following two washes with Permeabilisation buffer, cells were stained with FITC conjugated anti-Ki67 diluted in Permeabilisation buffer for 30 minutes at 4°C in the dark, washed once more and prepared for flow cytometric acquisition.

2.4.4 Flow cytometric analysis

Labelled cells were analysed on a FACSCalibur (Becton Dickinson, USA). Viable cells were gated on their forward-side scatter profile. Electronic compensation was performed by adjusting for cells stained with a single fluorochrome. Data collection of triple negative (TN), LSK, common lymphoid progenitor (CLP) and myeloid committed progenitor (MCP) populations involved an initial gating on the lineage marker negative population then acquiring data only for cells within that gate. Flow cytometric data was analysed using Cell Quest software (Becton Dickinson, USA).

2.4.5 Antibody cocktails

Cell type	Lineage markers	Defined as
LSK	CD3, CD4, CD8, CD45R (B220), CD11b, Gr-1	$Lin^-CD117^+Sca\text{-}1^+$
TN1	CD3, CD4, CD8, CD11b, Gr-1	$Lin^-CD44^+CD25^-$
TN2	CD3, CD4, CD8, CD11b, Gr-1	$Lin^-CD44^+CD25^+$
TN3	CD3, CD4, CD8, CD11b, Gr-1	$Lin^-CD44^-CD25^+$
TN4	CD3, CD4, CD8, CD11b, Gr-1	$Lin^-CD44^-CD25^-$
CLP	CD3, CD4, CD8, CD45R, CD11b, Gr-1	$Lin^-CD117^{lo/int}CD127^+$
MCP	CD3, CD4, CD8, CD45R, CD11b, Gr-1	Lin-

Cell type	Defined as
B cell progenitors	CD45R, CD43, IgM
• Pro-B cells	$CD45R^+CD43^+IgM^-$
• Pre-B cells	$CD45R^+CD43^+IgM^+$
• Immature B cells	$CD45R^+CD43^-IgM^+$

Thymocyte subsets/T cells	TCRβ, CD3, CD4, CD8
• Triple negative (TN)	$TCR\beta^{lo}CD3^{-}CD4^{-}CD8^{-}$
• Double positive (DP)	$CD4^{+}CD8^{+}$
• CD4 single positive	$TCR\beta^{hi}CD3^{+}CD4^{+}CD8^{-}$
• CD8 single positive	$TCR\beta^{hi}CD3^{+}CD4^{-}CD8^{+}$
NKT	$TCR\beta^{+}NK1.1^{+}$
Splenic T cells:	CD3, CD4, CD8
• CD4	$CD3^{+}CD4^{+}CD8^{-}$
• CD8	$CD3^{+}CD4^{-}CD8^{+}$
Dendritic cells/granulocytes/macrophages	CD11b, CD11c, Gr-1, MHC-II I-A/I-E
	$MHC\text{-}II^{+}CD11c^{+}$
• Dendritic cells	$CD11c^{-}CD11b^{+}Gr\text{-}1^{lo/-}$
• Macrophages	$CD11c^{-}CD11b^{+}Gr\text{-}1^{+}$
• Granulocytes	

2.5 In vitro lymphocyte functional assays

2.5.1 Proliferation assay

Spleens were removed from mice and splenocytes were cultured in triplicate 96 well plates at a concentration of 2 x 10^5 cells/well in stimulation media (RPMI 1640 (Sigma St Louis, USA) supplemented with 10% heat-inactivated fetal calf serum (Sigma St Louis, USA), 100U/mL penicillin (Sigma, St Louis, USA), 100ug/mL streptomycin (Sigma, St Louis, USA), 2mM L-glutamine (Invitrogen, USA) and 50mM 2-ME (Invitrogen, USA)) and in the presence of 10ug/mL of either anti-CD3ε and anti-CD28 or media alone. Cells were stimulated for 66 hours at 37°C with 5% CO_2, with the addition of 1μCi/well [^{3}H] thymidine during the last 18 hours of culture. Cells were harvested onto filter mats and incorporated radioactive nucleic acid counted on a Top Count Harvester (Packard Biosciences, USA). Data are expressed as the mean $\pm$ 1 SD stimulation index values of triplicate measurements.

2.5.2 Proliferation assay with LHRH-A

Thymi were isolated from untreated young male mice and cultured in triplicate 96 well plates at a concentration of 1.25 x 10^5 cells/well, 2.5 x 10^5 cells/well or 5 x 10^5 cells/well in stimulation media (described above). 0.2ug, 1ug, 10ug or 50ug of LHRH-A was added to each cell concentration. Each concentration of cells were also cultured in the presence of anti-CD3ε and anti-CD28 but in the absence of LHRH-A as a positive control. Cells were incubated for 66 hours at 37°C with 5% CO_2, with the addition of 1uCi/well [^{3}H] thymidine during the last 18 hours of culture. Cells

were harvested onto filter mats and incorporated radioactive nucleic acid counted on a Top Count Harvester (Packard Biosciences, USA). Data are expressed as the mean ± 1 SD stimulation index values of triplicate measurements.

2.6 Serum sampling and Radioimmunoassay of Testosterone

2.6.1 Sera collection

Cardiac blood was collected from culled mice at days 0, 7, 14 and 28 following ovariectomy, LHRH-A and AR-A treatment combinations. Serum was isolated from each sample and stored at -70°C until assayed.

2.6.2 Serum Testosterone Radioimmunoassay

Serum testosterone concentration was assayed by radioimmunoassay with generous assistance by the Tilbrook laboratory, Department of Physiology, Monash University. Samples were extracted with Hexane and Toluene at 2:1 ratio. Samples were then vortexed and the aqueous layer was frozen using a dry ice/ethanol bath. The organic layer is decanted into fresh tubes and dried. This extraction method was repeated on the aqueous layer. Samples were reconstituted in an assay buffer. Antiserum (Krius Pty Ltd, C-6050) was added at a dilution of 1:17500. A tracer (GE Healthcare TRK921-250UCI) was added at a concentration to show 10,000cpm per 100uL. The samples were then centrifuged with dextran-coated charcoal and decanted samples were read using a gamma counter. Sample recovery ranged from 77.57 – 86.63% and sensitivity ranged from 0.27 – 0.34ng/mL.

2.7 Statistical analysis

For experiments where only two test groups were compared per time point, Student's T-Test was used. A *p* value of 0.05 or less was considered statistically significant.

For experiments that compared more than two test groups per time point, one-way ANOVA with Tukey's test was used to compare data. Statistical analysis was performed using SPSS version 15.0 software. A *p* value of 0.05 or less was considered statistically significant.

2.8 References

1. Robaire, B., et al., *Interactions of testosterone and estradiol-17 beta on the reproductive tract of the male rat*. Biol Reprod, 1979. **21**(2): p. 455-63.

CHAPTER 3 – Results section 1

EXPERIMENTAL PARAMETERS GOVERNING KINETICS AND MAGNITUDE OF THE THYMOPOIETIC RESPONSE TO LHRH-A TREATMENT

Manuscript information

This chapter is currently a manuscript in preparation with a planned submission date of August 2010. The candidate Melanie Hince was primarily responsible for performing and analyzing experiments as well as writing and editing. Other co-authors contributed technical assistance, interpretation of results and drafting of the manuscript. Detailed contributions are explained in the signed declaration on page VII.

3.1 Abstract

A major cause of immune aging is an increase in sex steroids. Investigations into the removal of sex steroids have demonstrated this to be a potential therapy for improving immune recovery following severe depletion such as chemotherapy and irradiation. Recent clinical trials into the use of Luteinising Hormone-Releasing Hormone agonist (LHRH-A) have indicated its potential to improve immune recovery following HSCT. We investigated the experimental parameters involved in the transfer from pre-clinical animal studies to human trials and potential methods to improve the number and quality of responders. While LHRH-A indeed increases thymic size in male and female mice, the degree of hypertrophy was age, gender and mouse strain dependent. We hypothesised that the low response in females was due to remaining estrogen, however the combination of LHRH-A and administration of an estrogen receptor analogue (ER-A) induced thymic atrophy. Together, these results highlight the complexities involved in translating a pre-clinical treatment to a clinical therapy.

3.2 Introduction

It is widely recognized that the immune system undergoes significant decline with advancing age, which is characterised by thymic atrophy and a reduction in naïve T cell output [1-3]. This decreased thymic output leads to a loss in naïve T cells balanced by a homeostatic expansion of pre-existing memory cells against prior antigens [2, 4]. While not a significant problem in healthy individuals, this presents an issue in regard to immune depletion therapies such as chemotherapy/irradiation or chronic viral infection such as HIV. Immune recovery following such treatments can be as little as 6 months in pre-pubertal patients, however this time is extended to at least 2 years in those of advanced age [5]. Consequently there is an increased occurrence of mortality in adults due to opportunistic infection and disease relapse [6, 7].

Investigations into the cause of immune depletion with age, which significantly increases around the time of puberty, have identified sex steroids as key instigators, specifically in the atrophy of the thymus. Accordingly, ablation of sex steroids either surgically or chemically has demonstrated reversal of age-related thymic atrophy as well as improved immune recovery following cytoablative depletion in animal models. We have previously shown surgical castration enhances thymic reconstitution in mice from 4 weeks- to 24 months-of-age following chemotherapy, irradiation and HSCT [8-11], which leads to improved immune function in response to TCR stimulation [11].

While surgical castration has demonstrated such positive results in animal studies, it is a clinically undesirable method of sex steroid ablation. Luteinising Hormone-Releasing Hormone agonists (LHRH-A) bind and over-stimulate the pituitary LHRH receptors and eventually desensitises them to LHRH stimulation, thus halting the downstream gonadal production of sex steroids [12]. This presents LHRH-A as a relatively non-invasive and reversible alternative to surgical SSA with a return to fertility within 1-2 months of cessation of treatment [12]. Administration of LHRH-A in pre-clinical animal models has demonstrated an enhanced immune recovery following allogeneic HSCT. Further to this, LHRH-A has been used in multiple

clinical human trials and has been observed to improve the recovery of the naïve CD4+ T cell population and thymopoiesis following localized irradiation therapy [11] as well as following allogeneic and autologous HSCT [13]. In the latter study, however, only around half of the patients responded in this manner. In addition, the time for this recovery to occur, while greatly reduced, is still considered delayed at 6-12 months.

Clearly, there are key differences between carrying out trials in pre-clinical animal models and humans. These include the differing age of subjects versus using age-matched animals, combining male and female patients within the same study, and the use of inbred mouse strains versus outbred humans. We present herein data that demonstrates each of these factors alters the thymic recovery kinetics. One drawback of LHRH-A treatment is the very nature of its mode of action. By initially overstimulating the LHRH-Rs within the hypothalamus, a surge in gonadal hormone production occurs termed the sex steroid flare before negligible levels of sex steroids are reached within approximately 3 weeks of treatment. Administration of estrogen or testosterone results in significant thymic atrophy. Clinically, this effect is prevented with the use of sex steroid receptor blockade: testosterone receptor analogues in males and estrogen receptor analogues in females. Tamoxifen is a selective estrogen receptor mediator (SERM) and was first approved by the FDA in 1977 for the treatment of advanced estrogen receptor (ER)+ and progesterone receptor (PR)+ breast cancer [14]. The combination of Tamoxifen and LHRH-A show an additive effect over either drug alone for the treatment of breast cancer [15]. Tamoxifen acts by binding nuclear ERs, to have an antagonistic effect within breast tissue, but has been shown to have agonistic action within bone and uterine tissues [14, 16]. Tamoxifen has also been shown to have differing effects in pre- and postmenstrual women in regards to bone maintenance due to the differences in competing 17β-estradiol levels [16]. In this study, we investigate the combination of LHRH-A with Tamoxifen (ER-A) in female mice.

3.3 Experimental design

3.3.1 LHRH-A in combination with Testosterone re-administration

0.4mg LHRH-A was administered intra-muscularly at day 0 and half of these were implanted with testosterone pumps at day 35 as described in Chapter 2, section 2.2.5. Another cohort of mice was implanted with testosterone pumps alone.

3.3.2 LHRH-A administration in comparison with ovariectomy

In middle-aged and aged female mice, LHRH-A was administered 21 days before two other cohorts were either ovariectomised or sham ovariectomised, as described in Chapter 2, section 2.2.1. This was to allow for recovery from a LHRH-A induced sex steroid flare.

3.3.3 LHRH-A administration in combination with ER-A

Estrogen receptor analogue (ER-A) was administered orally to young female mice daily for 28 days (Chapter 2, section 2.2.4), beginning 7 days before LHRH-A administration.

3.4 Results

3.4.1 LHRH-A increases thymic size and can be reversed with testosterone administration

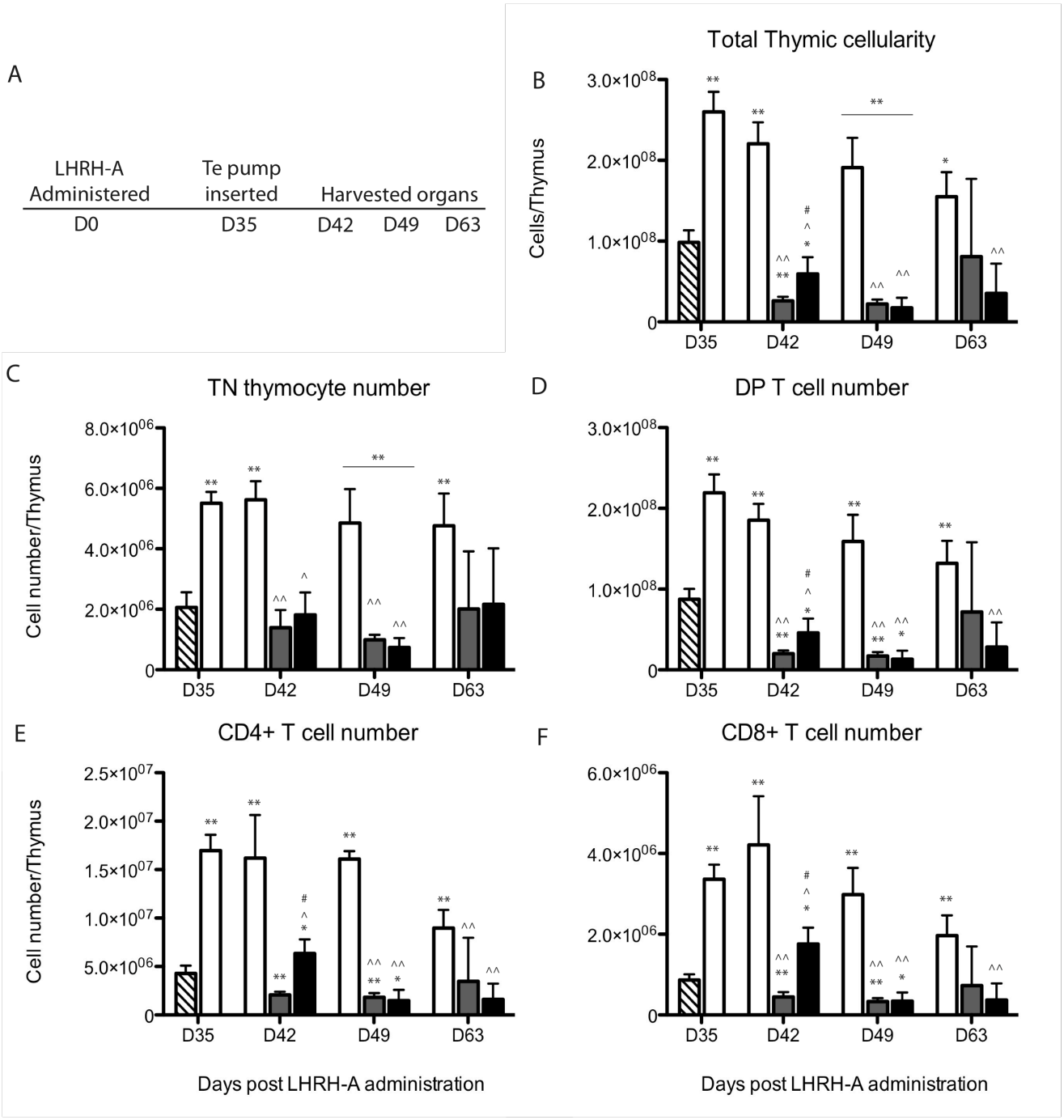
Numerous studies have demonstrated an increase in thymic size with the removal of sex steroids through surgical gonadectomy and that reintroduction of testosterone reverses this effect [17, 18]. Here, mice were administered a chemical equivalent to castration, an LHRH-A, according to the experimental time line in figure 1a. Castration levels of hormones were attained around 21 days (Chapter 5, section 5.4.2). By day 35 the thymi of young male C57/Bl6 mice were significantly increased in total viable cell number (Figure 3.1b). This was reflected in all maturing T cell subsets within the thymus (Figure 3.1c-f). Testosterone was reintroduced through the insertion of testosterone-filled implants at day 35 (Figure 3.1a). Within one week of testosterone release, total thymocyte number was significantly reduced in comparison with the untreated control and, with the combination of testosterone and LHRH-A treatment, compared to LHRH-A treated mice alone. This suggests that the removal of testosterone is the major inducer of thymic hypertrophy following LHRH-A treatment, which is consistent with previous studies [19, 20].

When compared to surgical castration, the magnitude of thymic hypertrophy was slightly increased following LHRH-A treatment. One possible explanation for this is a direct stimulatory effect of LHRH itself on the thymus, in addition to its indirect effect through reduced SS production. LHRH-Rs are expressed by thymic T cells and stromal cells, the thymus produces its own LHRH [21-23] and castration increases the intrathymic production of LHRH [23].

In an attempt to identify a potential direct effect of LHRH on thymocytes, whole thymocyte suspensions were cultured in the presence or absence of LHRH-A and cell proliferation was measured with the addition of [^{3}H]thymidine. As a positive control, thymocytes were also cultured with anti-CD3/CD28. As expected, thymocytes proliferated in response to anti-CD3/CD28 as shown by an increase in stimulation index (Figure 3.2a), however it did not appear that LHRH-A directly induced cell proliferation in comparison.

Figure 3.1 LHRH-A increases thymic size and can be reversed with testosterone administration

Young male mice were administered LHRH-A at day 0 and testosterone pumps were subsequently implanted at day 35 as per experimental time line (a). Total thymic cellularity following LHRH-A treatment (b); number of TN thymocytes (c); DP thymocytes (d); CD4+ T cells (e); CD8+ T cells (f). Each bar represents the mean $\pm$ 1 SD of five mice. * $p \leq 0.05$, ** $p \leq 0.01$ c.f. Untreated, ^ $p < 0.05$, ^^ $p < 0.01$ c.f. LHRH-A alone, # $p < 0.05$ c.f. Testosterone alone.


A
LHRH-A Administered
Te pump inserted
Harvested organs
D0
D35
D42
D49
D63
B
Total Thymic cellularity
Cells/Thymus
3.0×10^08
2.0×10^08
1.0×10^08
0
D35
D42
D49
D63
C
TN thymocyte number
Cell number/Thymus
8.0×10^06
6.0×10^06
4.0×10^06
2.0×10^06
D
DP T cell number
3.0×10^08
2.0×10^08
1.0×10^08
E
CD4+ T cell number
2.5×10^07
2.0×10^07
1.5×10^07
1.0×10^07
5.0×10^06
F
CD8+ T cell number
6.0×10^06
4.0×10^06
2.0×10^06
Days post LHRH-A administration
Key:
Untreated
LHRH-A
Testosterone
Combination

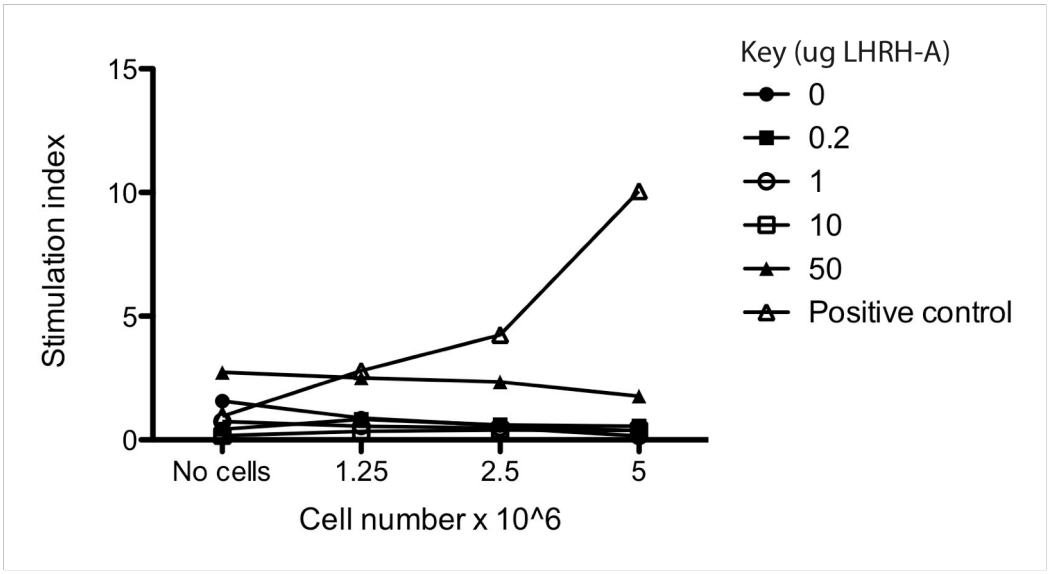


Figure 3.2 Total thymocytes do not show a proliferative response to LHRH-A addition

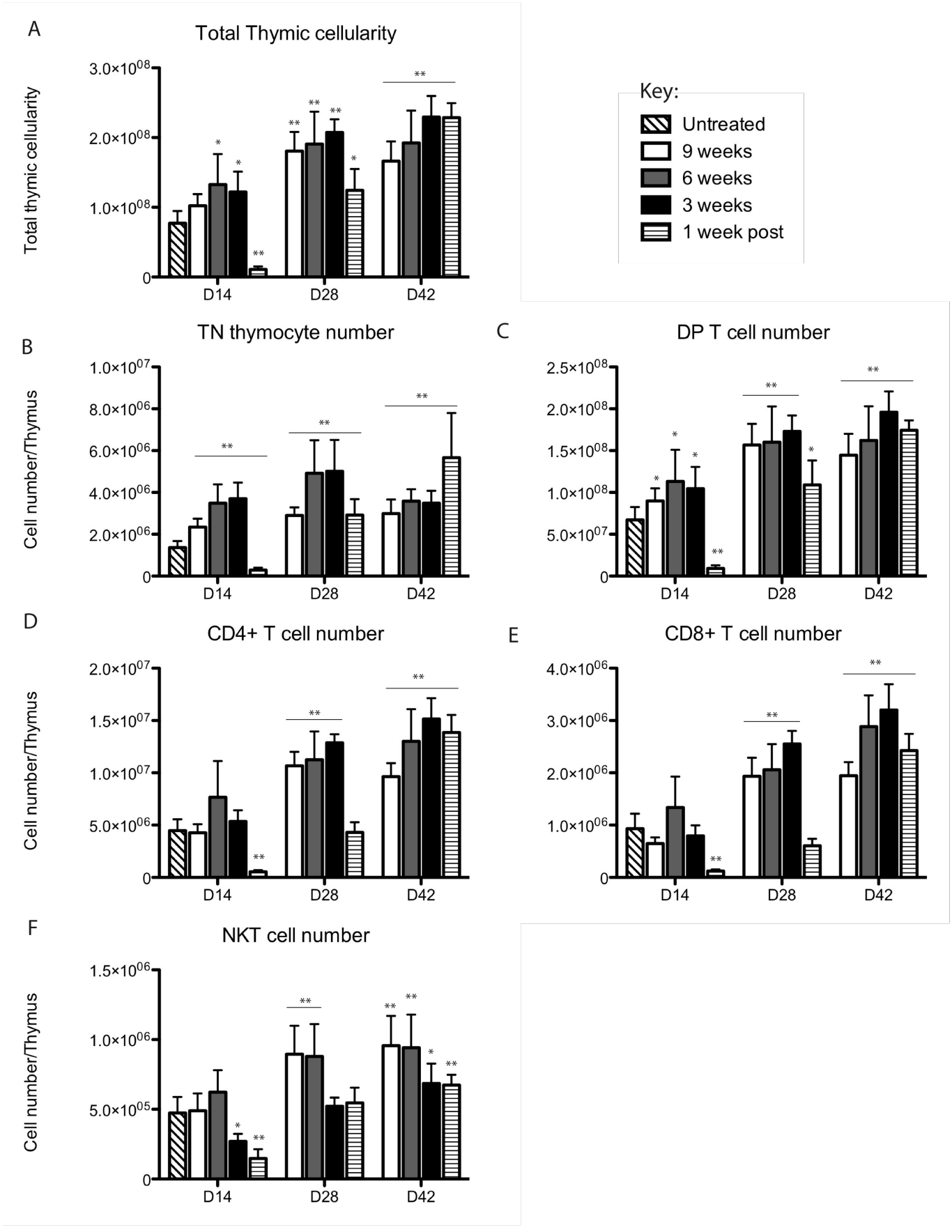
Total thymocytes were extracted from the thymi of young male mice and cultured with or without the addition of LHRH-A. Proliferation was measured following the addition of [^{3}H]thymidine and is expressed as the stimulation index.

3.4.2 Clinical timing of LHRH-A to improve thymic recovery following chemotherapy

Recent human clinical trials have demonstrated improved thymic recovery following short-term LHRH-A administration in patients undergoing HSC transplant rescue following high dose, myeloablative chemotherapy [13]. The protocol involved giving LHRH approximately 21 days prior to transplant. This was designed to allow the thymus and BM to recover from the initial sex-steroid flare induced by LHRH-A. During this trial, there were a proportion of patients who did not respond to LHRH-A treatment, with no increase in thymic output when compared with the control group. This could be because the thymus is too damaged to respond in these patients. We thus examined whether administration of LHRH-A earlier to initiate thymus recovery prior to chemotherapy, would minimize the damage and improve naïve T cell recovery. Young male C57Bl/6 mice were given 0.4mg LHRH-A (Chapter 2, section 2.2.2) at 3, 6 or 9 weeks before cyclophosphamide (Chapter 2, section 2.2.6), which was used as a model for chemotherapeutic immunodepletion. As expected, when LHRH-A was administered 3 weeks pre-cyclophosphamide treatment, the thymus showed full recovery and even hypertrophy by day 14 when compared to the untreated control (Figure 3.3a). In fact all groups, except for LHRH-A administered 1-week post cyclophosphamide, showed full recovery by day 14 in comparison to the untreated control (Figure 3.3a). When LHRH-A was administered 1 week post cyclophosphamide, recovery of the total thymus to untreated control levels was not seen until day 28 and this was reflected in all maturing thymocyte subsets (Figure 3.3b-e). By day 42 all treatment groups showed hypertrophy of the thymus when compared to untreated controls (Figure 3.3a-f). Interestingly, when LHRH-A was administered 3 weeks pre-cyclophosphamide, NKT cell numbers were decreased in comparison with untreated controls at day 14 indicating a delay in recovery (Figure 3.3f). NKT cell numbers in this treatment group returned to untreated controls by day 28. Together these results indicate that LHRH-A needs to be administered pre-, not post-chemotherapy, to better help patients respond. Also, the timing of LHRH-A administration does not alter the degree of thymic recovery following depletion.

Figure 3.3 Clinical timing of LHRH-A to improve thymic recovery following chemotherapy

LHRH-A was administered to young male mice followed by cyclophosphamide at 9, 6 or 3 weeks after LHRH-A or one week before LHRH-A. Total thymic cellularity following LHRH-A and cyclophosphamide treatment (a); number of TN thymocytes (b); DP thymocytes (c); CD4+ T cells (d) CD8+ T cells (e); NKT cells (f). Each bar represents the mean $\pm$ 1 SD of five mice. * $p<0.05$, ** $p<0.01$ c.f. NY.


A
Total Thymic cellularity
Total thymic cellularity
3.0×10^08
2.0×10^08
1.0×10^08
0
D14
D28
D42
Key:
Untreated
9 weeks
6 weeks
3 weeks
1 week post
B
TN thymocyte number
Cell number/Thymus
1.0×10^07
8.0×10^06
6.0×10^06
4.0×10^06
2.0×10^06
C
DP T cell number
2.5×10^08
2.0×10^08
1.5×10^08
1.0×10^08
5.0×10^07
D
CD4+ T cell number
2.0×10^07
1.5×10^07
1.0×10^07
5.0×10^06
E
CD8+ T cell number
4.0×10^06
3.0×10^06
2.0×10^06
1.0×10^06
F
NKT cell number
1.5×10^06
1.0×10^06
5.0×10^05
Time post Cyclophosphamide treatment

3.4.3 The effect of LHRH-A is strain-dependent in male mice

Given that the timing of LHRH-A administration before immune depletion did not cause marked differences in thymic hypertrophy, we further investigated why a proportion of LHRH-A trial patients did not respond. For genetic influences, we compared the effect of LHRH-A treatment in two strains of middle-aged (9mo) male mice. Our previous experiments into the ability of LHRH-A to enhance thymic recovery used C57Bl/6 mice, thus we began by ensuring the dose used was appropriate in the BALB/c strain. There was little difference between all three doses used within the total thymus (Figure 3.4a) and maturing thymocyte subsets (Figure 3.4b-e); 0.4mg was thus used per mouse for future experiments.

When directly comparing the effect of LHRH-A in BALB/c and C57Bl/6 male mice, there was a three-week delay in the recovery from a testosterone flare induced by LHRH-A treatment in the BALB/c strain. While C57Bl/6 mice showed a return to untreated control levels between days 14 and 28 (one week post androgen depletion), all thymocyte subset numbers were still depleted in BALB/c mice at this time. Thymic hypertrophy was not observed in Balb/C mice until day 56 (Figure 3.5b) and was reflected in all thymocyte subsets (Figure 3.5d, f, h, j) although serum testosterone was depleted by day 21 (Figure 3.6a). Thymic recovery of C57Bl/6 and Balb/C mice can be further expressed as a comparison of the fold increase of total thymic cellularity following LHRH-A treatment when compared to PBS controls (Figure 3.6b). C57Bl/6 mice demonstrated a 3-fold increase at day 28, while Balb/C mice only showed recovery and a following increase at day 42, which was further increased at day 56.

Within the bone marrow, increases in the total cellularity (Figure 3.7a) and all maturing B cell subsets (Figure 3.7c, e, g, i) were seen in C57Bl/6 male mice at day 28 and only Pre B cell numbers were reduced as a consequence of an androgen surge at day 14 (Figure 3.7g). In comparison, total bone marrow cellularity was unchanged in Balb/C mice (Figure 3.7b) and all maturing B cell subsets underwent decreases between days 7 and 28 (Figure 3.7d, f, h, j) indicating a key difference between these two strains in response to LHRH-A treatment.

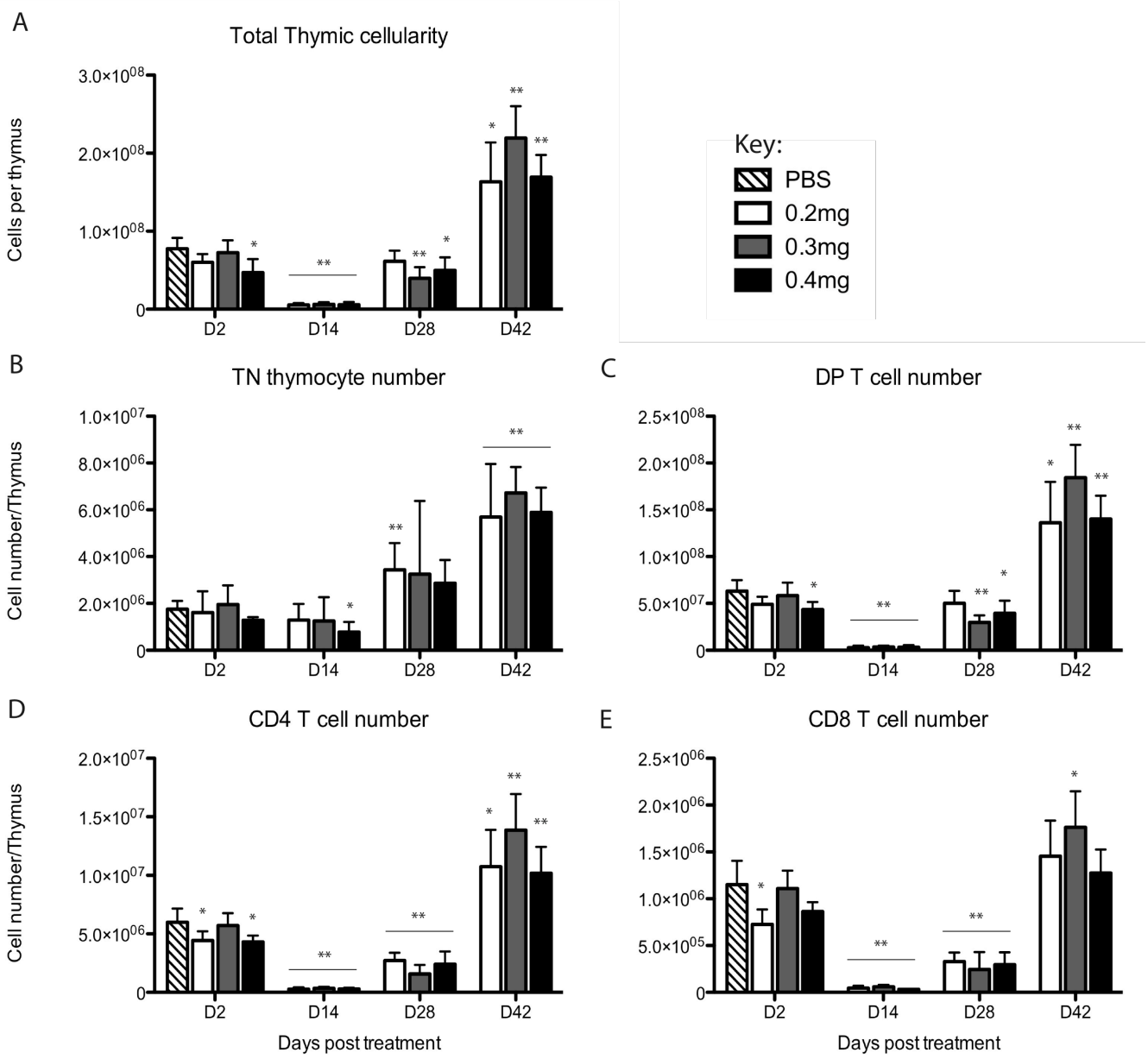
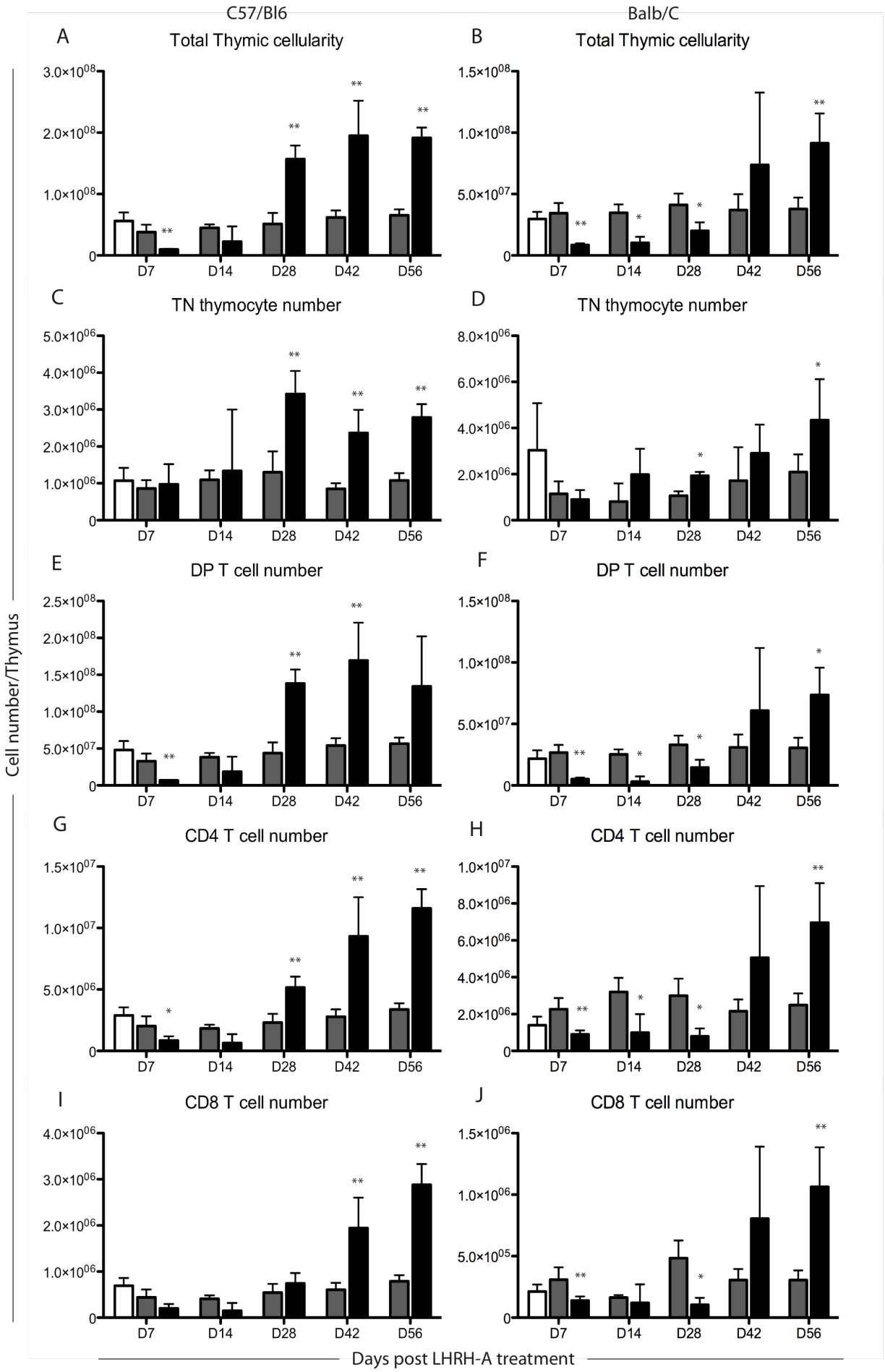


Figure 3.4 LHRH-A dose response in middle-aged Balb/C male mice

0.2, 0.3 and 0.4mg of LHRH-A were administered intra-muscularly to male Balb/C mice. Total thymic cellularity following LHRH-A administration (a); number of TN thymocytes (b); DP thymocytes (c); CD4+ T cells (d); CD8+ T cells (e). Each bar represents the mean $\pm$ 1 SD of five mice. * $p<0.05$, ** $p<0.01$ c.f. PBS treated control.

Figure 3.5 The effect of LHRH-A on the thymus of C57Bl/6 and Balb/C middle-aged mice

0.4mg of LHRH-A was injected intramuscularly into C57Bl/6 and Balb/C male mice. Total thymic cellularity of C57Bl/6 mice following LHRH-A administration (a); number of TN thymocytes (c); DP thymocytes (e); CD4+ T cells (g); CD8+ T cells (i). Total thymic cellularity of Balb/C mice following LHRH-A administration (b); number of TN thymocytes (d); DP thymocytes (f); CD4+ T cells (h); CD8+ T cells (j). Each bar represents the mean $\pm$ 1 SD of four to five mice. * $p<0.05$, ** $p<0.01$ c.f. Untreated. White bar = Untreated, Grey bar = PBS, Black bar = LHRH-A.


C57/Bl6
Balb/C
A
Total Thymic cellularity
B
Total Thymic cellularity
C
TN thymocyte number
D
TN thymocyte number
E
DP T cell number
F
DP T cell number
G
CD4 T cell number
H
CD4 T cell number
I
CD8 T cell number
J
CD8 T cell number
D7
D14
D28
D42
D56
Cell number/Thymus
Days post LHRH-A treatment

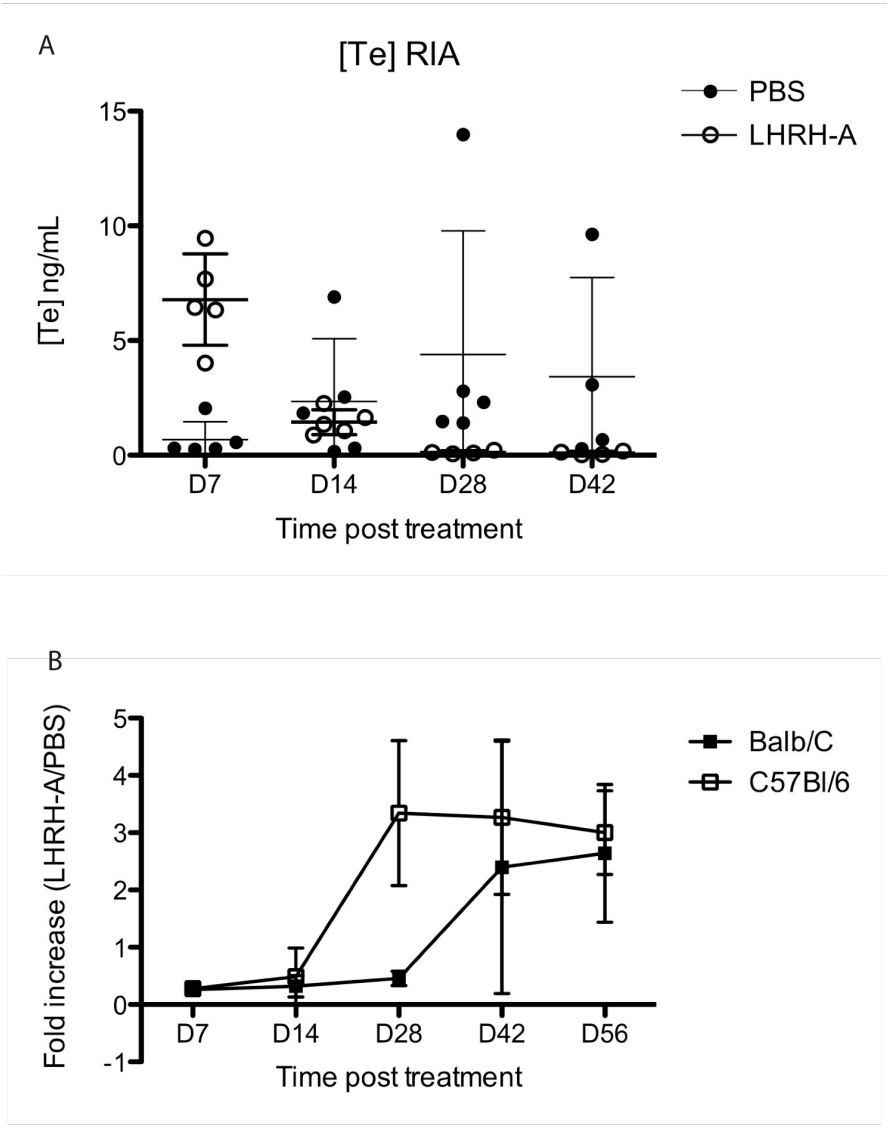
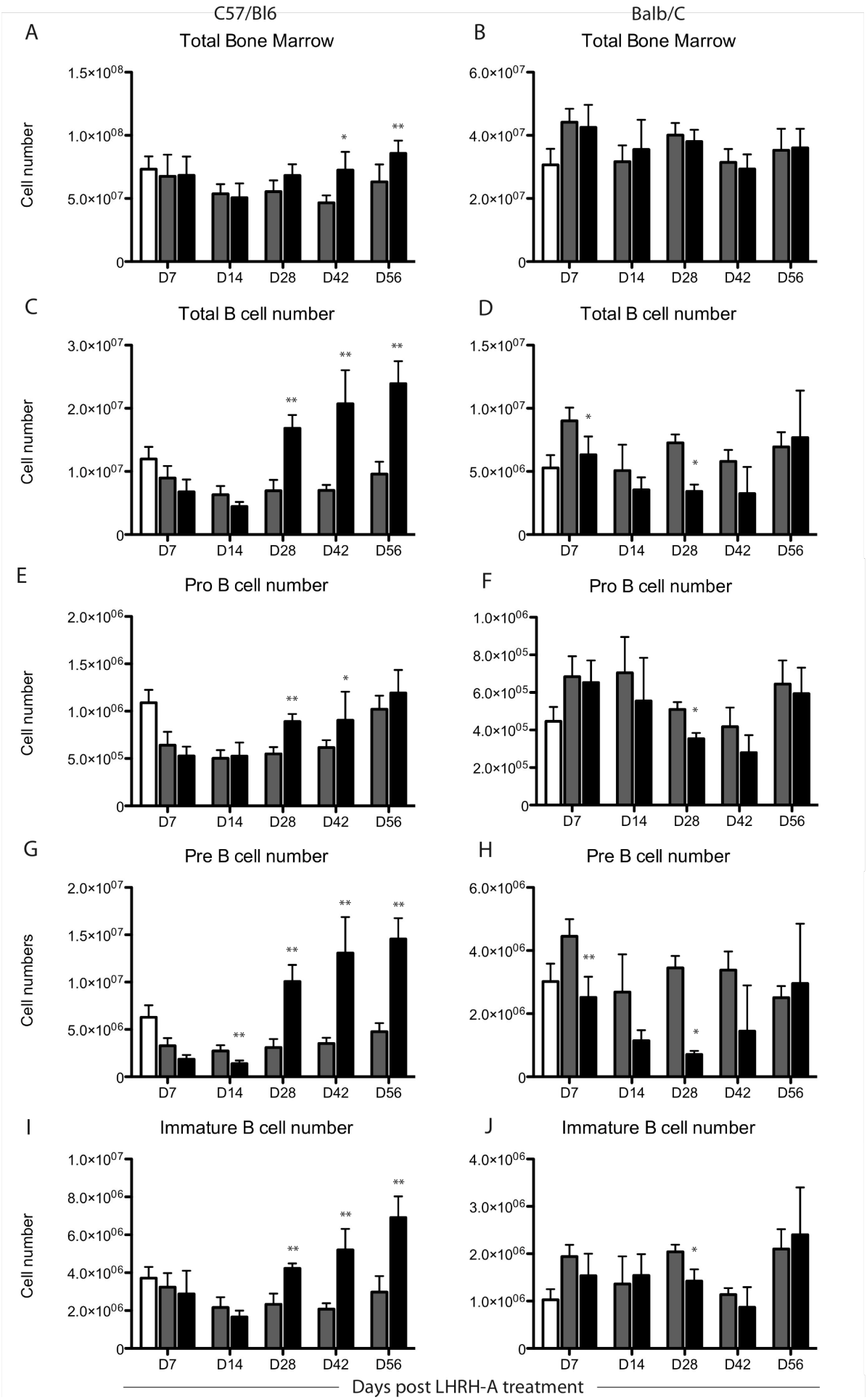


Figure 3.6 LHRH-A increases thymic cellularity more significantly in C57Bl/6 male mice than Balb/C male mice

0.4mg of LHRH-A was administered intra-muscularly to C57Bl/6 and Balb/C 9mo male mice. Concentration of serum testosterone measured by RIA (a). Fold increase of the total thymus cellularity (b). Each point represents the mean $\pm$ 1 SD of four to five mice.

Figure 3.7 LHRH-A increases bone marrow cellularity in C57Bl/6 but not Balb/C middle-aged male mice

0.4mg of LHRH-A was administered intra-muscularly to C57Bl/6 and Balb/C 9mo male mice. Total bone marrow cellularity in C57Bl/6 mice following LHRH-A administration (a); total number of B cells (c); Pro B cells (e); Pre B cells (g); immature B cells (i). Total bone marrow cellularity in Balb/C mice following LHRH-A administration (b); total number of B cells (d); Pro B cells (f); Pre B cells (h); Immature B cells (j). Each bar represents the mean $\pm$ 1 SD of four to five mice. * $p<0.05$, ** $p<0.01$ c.f. Untreated. White bar = Untreated, Grey bar = PBS, Black bar = LHRH-A.


C57/Bl6
Balb/C
A
Total Bone Marrow
B
Total Bone Marrow
C
Total B cell number
D
Total B cell number
E
Pro B cell number
F
Pro B cell number
G
Pre B cell number
H
Pre B cell number
I
Immature B cell number
J
Immature B cell number
Cell number
Cell numbers
D7
D14
D28
D42
D56
Days post LHRH-A treatment

3.4.4 The effect of LHRH-A is age- and gender-dependent in mice

The majority of studies using LHRH-A to improve thymic recovery have been undertaken in male subjects, be it mice or humans. Thus we investigated the effect of LHRH-A in comparison with surgical ovariectomy in female mice of varying ages. When administered to young female mice LHRH-A induced a slight reduction in total thymocyte number, which is likely due to an estrogen flare, at day 14 when compared to PBS treated controls (Figure 3.8a). This was observed in all thymocyte subset numbers (Figure 3.8b-e) though only significant in the TN thymocyte population (Figure 3.8b). While an increase in thymic cell number was observed in female mice at day 28 and 42, this was short-lived and total cell number returned to PBS controls at day 63. Within the bone marrow, a decline in Pre- and Immature B cell numbers was seen at day 7 with a return to PBS control levels at day 24 (Figure 3.9d, e). While an increase in total bone marrow cellularity was seen at day 42 (Figure 3.9a), which was attributed to total B cell numbers (Figure 3.9b), again this was short-lived with only Pre- and Immature B cell numbers still increased at day 63. While there was little change within the LSK and MCP cell population (Figure 3.10a-b), besides a short-lived increase in LSK cell numbers at day 42, CLP cell numbers were declined at days 7 and 14 followed by a significant increase from day 21 and numbers remained elevated at day 42 (Figure 3.10c).

There are clearly multiple factors, not just the loss of sex steroids, governing thymic atrophy, such as increasing age and changes in the production of other cytokines, hormones and growth factors [24-27]. Hence, we investigated the effect of LHRH-A on an age-depleted thymus in mice of 9 months (middle-aged), 18 months (aged) and 29 months (elderly) of age. However, while there was negligible decline in thymus size in both middle-aged and aged female mice (Figure 3.11a, b), there was again a short-lived increase in total thymic cellularity at day 14 in both age groups. The increase following LHRH-A treatment was slightly improved in comparison with surgical ovariectomy in both age groups. Interestingly, the effect of estrogen removal on thymocyte subsets was different between these two age groups. In middle-aged female mice, TN and DP thymocytes were increased with LHRH-A treatment (Figure 3.11c, e) while SP CD4+ and CD8+ thymocytes were increased with ovariectomy although no difference was seen between LHRH-A and ovariectomy

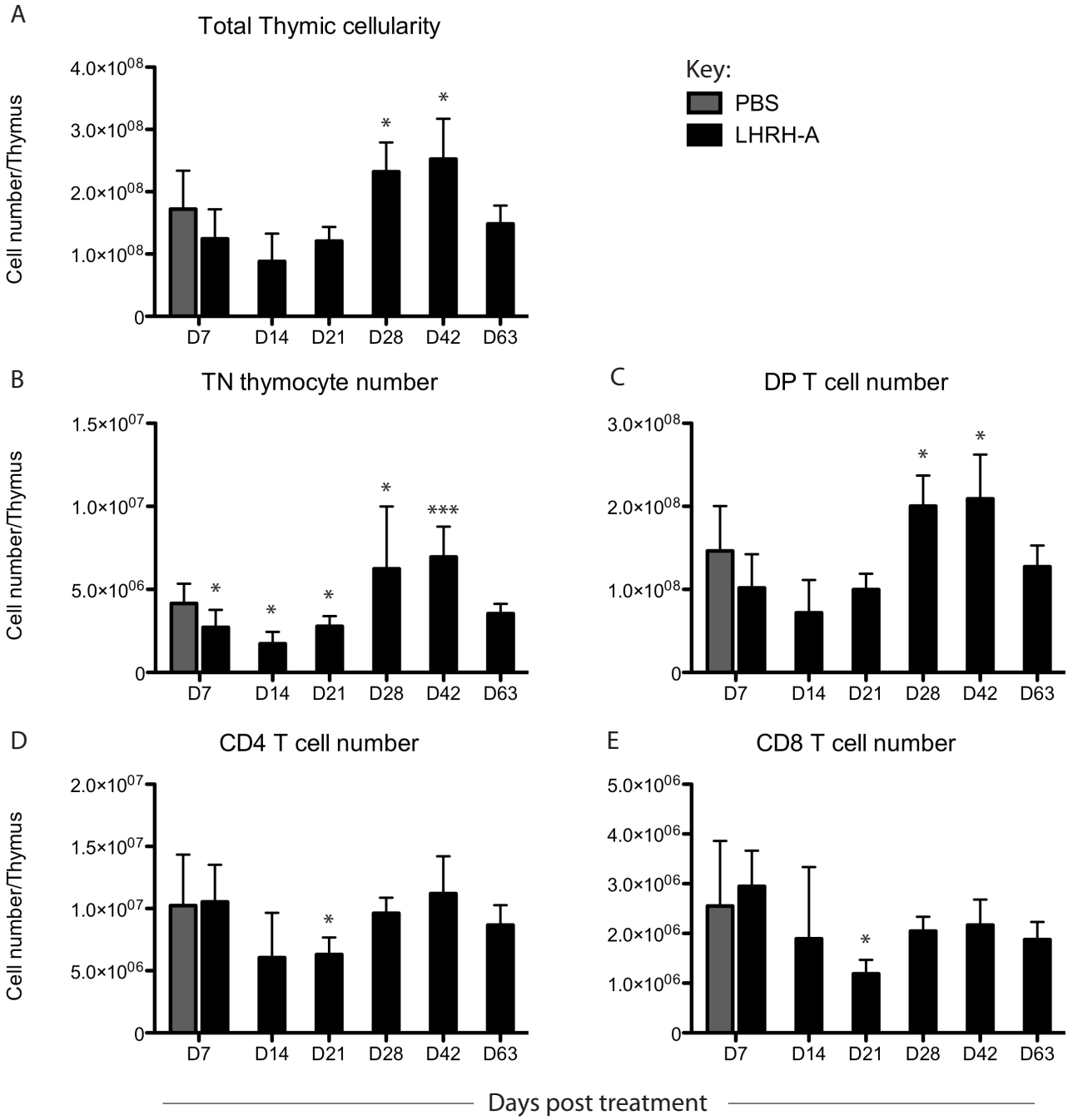


Figure 3.8 LHRH-A induces thymic hypertrophy in young female mice

0.4mg of LHRH-A was injected intramuscularly into young female C57Bl/6 mice. Total thymic cellularity following LHRH-A administration (a); number of TN thymocytes (b); DP thymocytes (c); CD4+ T cells (d); CD8+ T cells (e). Each bar within the PBS group represents the mean ± 1 SD of thirty mice. Each bar within the LHRH-A treated group represents the mean ± 1 SD of five mice. * $p<0.05$, *** $p<0.001$ c.f. PBS treated control.

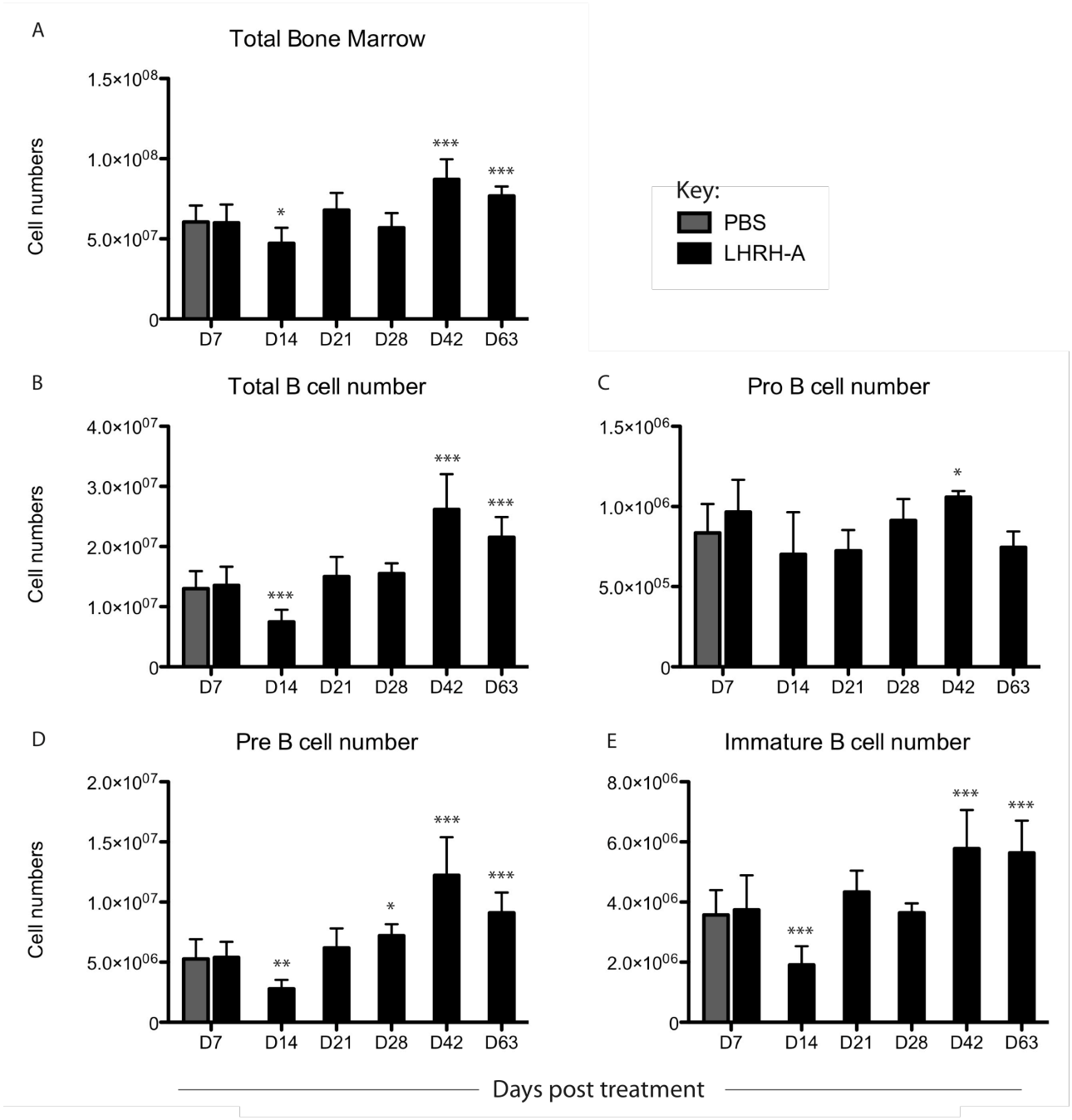
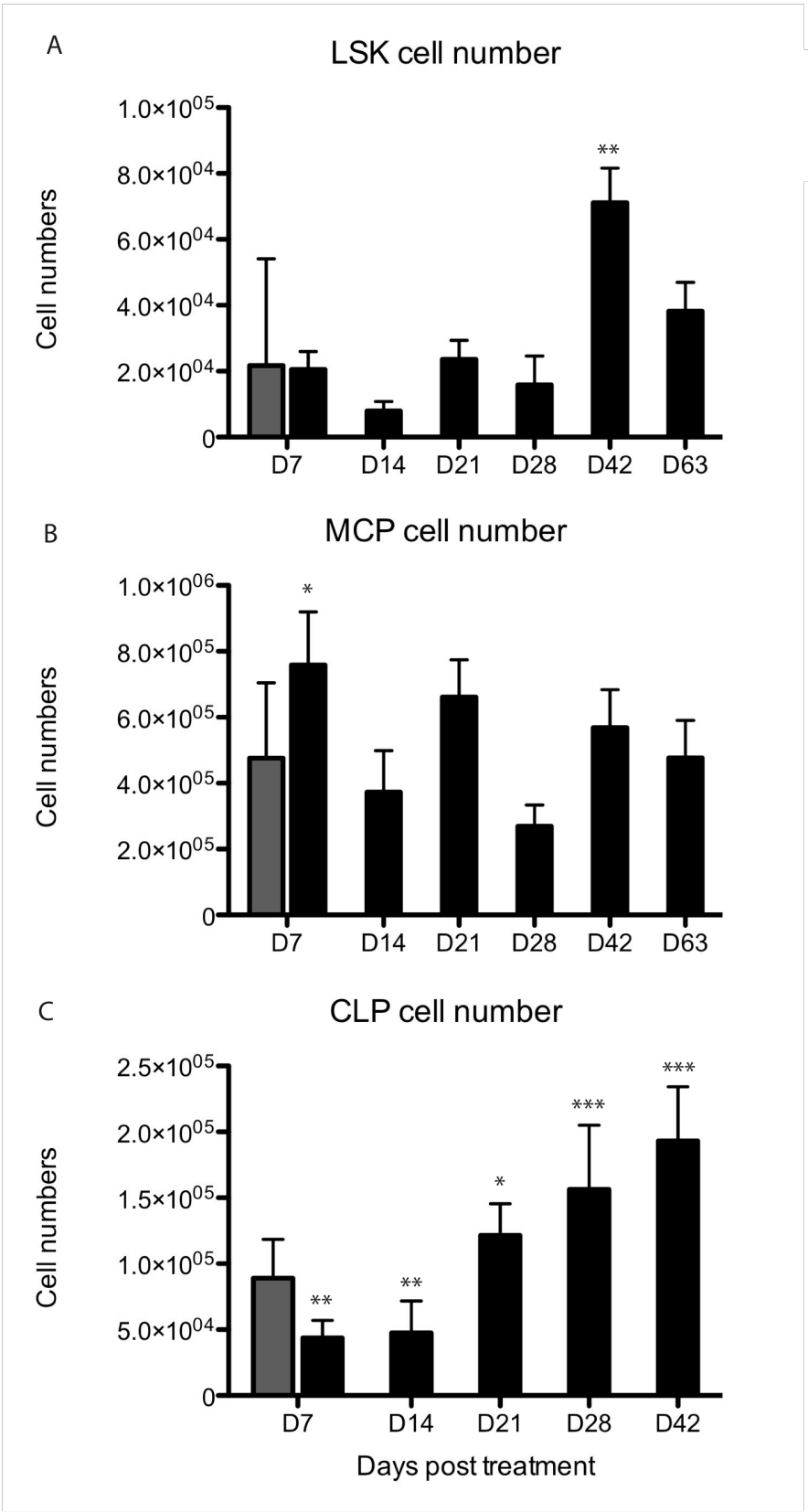


Figure 3.9 LHRH-A increases maturing B cells in young female mice

0.4mg of LHRH-A was injected intramuscularly into young female C57Bl/6 mice. Total bone marrow cellularity following LHRH-A administration (a); total number of B cells (b); Pro B cells (c); Pre B cells (d); Immature B cells (e). Each bar within the PBS group represents the mean $\pm$ 1 SD of thirty mice. Each bar within the LHRH-A treated group represents the mean $\pm$ 1 SD of five mice. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ c.f. PBS treated control.

Figure 3.10 LHRH-A increases lymphoid and myeloid progenitors in young female mice

0.4mg of LHRH-A was injected intramuscularly into young female C57Bl/6 mice. Number of LSK cells within the bone marrow following LHRH-A administration (a); MCP cells (b); CLP cells (c). Each bar within the PBS group represents the mean $\pm$ 1 SD of twenty-five to thirty mice. Each bar within the LHRH-A treated group represents the mean $\pm$ 1 SD of five mice. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ c.f. PBS treated control.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D14
D21
D28
D42
D63
B
MCP cell number
Cell numbers
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D7
D14
D21
D28
D42
D63
C
CLP cell number
Cell numbers
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**
**
*
***
***
D7
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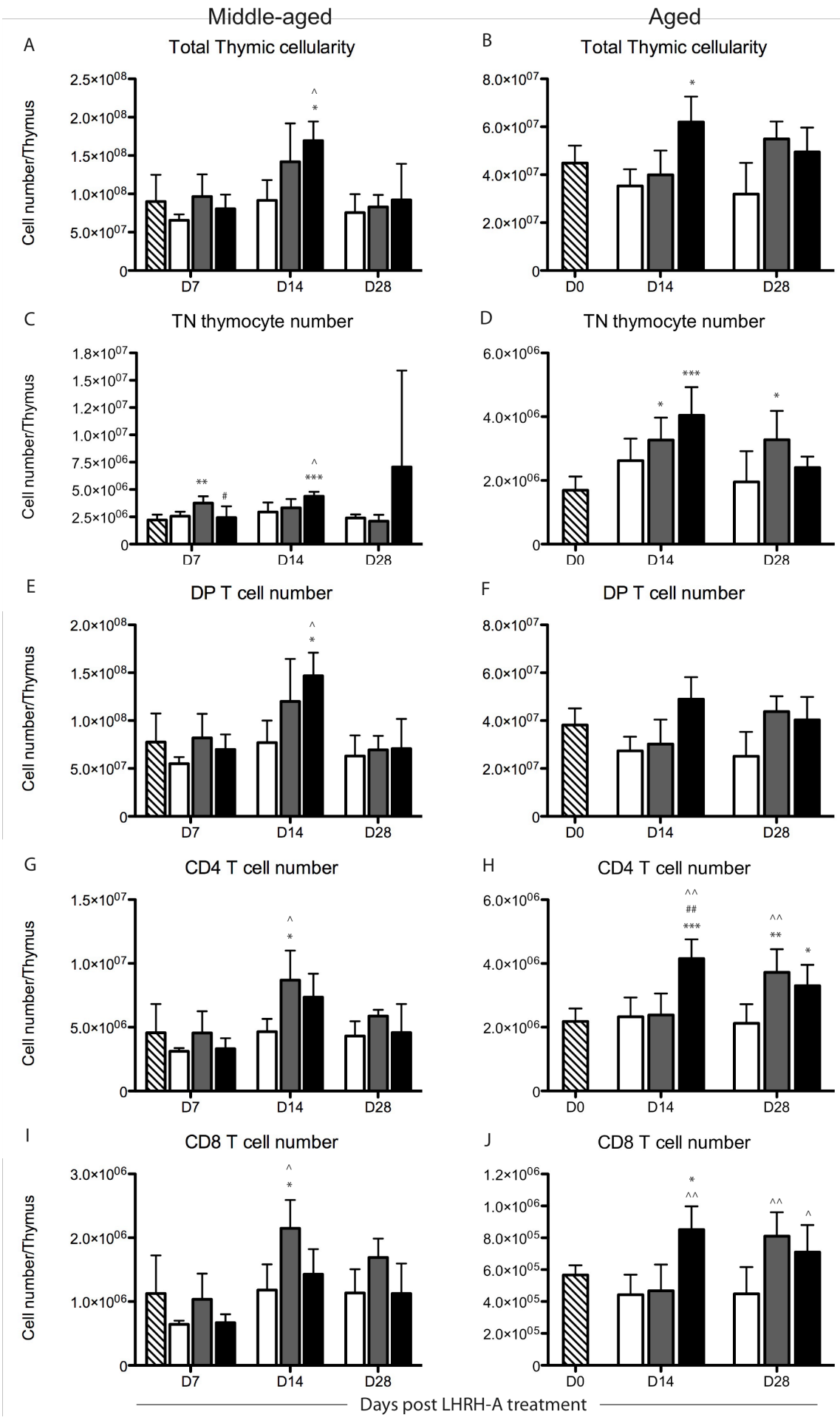
D42
Days post treatment


Middle-aged
Aged
A
Total Thymic cellularity
B
Total Thymic cellularity
C
TN thymocyte number
D
TN thymocyte number
E
DP T cell number
F
DP T cell number
G
CD4 T cell number
H
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Cell number/Thymus
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D7
D14
D28
Days post LHRH-A treatment

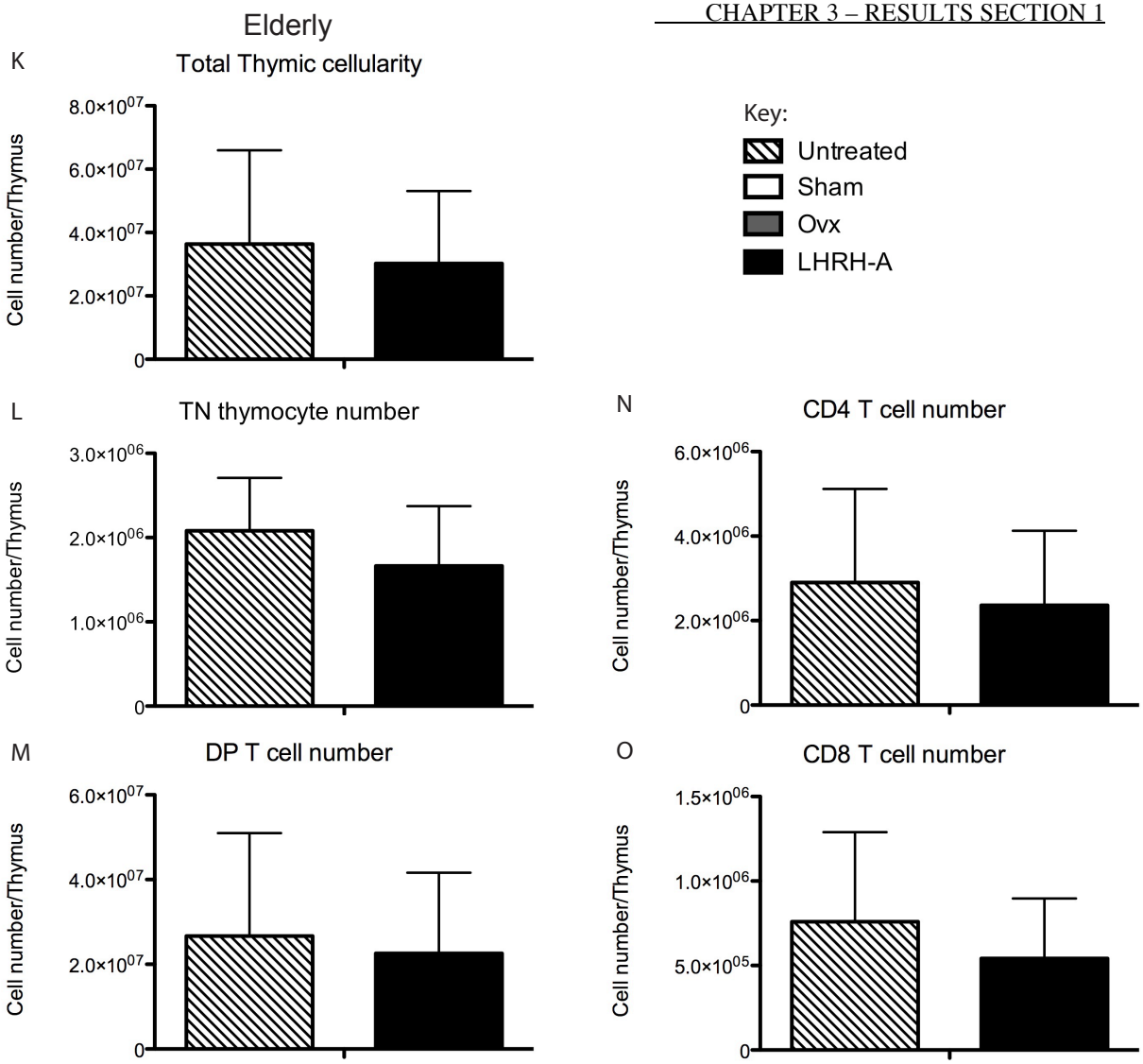


Figure 3.11 LHRH-A has limited effect on the thymus in middle-aged, aged and elderly female mice

Middle-aged and aged female C57Bl/6 mice were either ovariectomised, sham ovariectomised at day 0 or injected intramuscularly with 0.4mg of LHRH-A 21 days prior. Total thymic cellularity in middle-aged mice following treatment (a); number of TN thymocytes (c); DP thymocytes (e); CD4+ T cells (g); CD8+ thymocytes (i). Total thymic cellularity in aged mice following treatment (a); number of TN thymocytes (d); DP thymocytes (f); CD4+ T cells (h); CD8+ thymocytes (j). Elderly mice were either administered PBS as a control of LHRH-A. Total thymic cellularity 35 days following LHRH-A administration (k); number of TN thymocytes (l); DP thymocytes (m); CD4+ T cells (n); CD8+ T cells (o). Each bar represents the mean $\pm$ 1 SD of five mice. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ c.f. Untreated, ^ $p<0.05$, ^^ $p<0.01$ c.f. Sham control, # $p<0.05$, ## $p<0.01$ c.f. Ovx.

groups (Figure 3.11g, i). In aged mice, LHRH-A induced in increase in TN, SP CD4+ and SP CD8+, but not in DP thymocyte numbers, at day 14 (Figure 3.11d, f, h, j). Ovariectomy had little effect, except for a rise in TN and SP CD4+ thymocyte numbers at day 28 in comparison with untreated controls. When LHRH-A was administered to elderly female mice, there was no change seen in either the spleen, bone marrow (data not shown) or thymus in comparison to PBS treated controls at day 35 (Figure 3.11k – n). These mice were too frail to undergo ovariectomy.

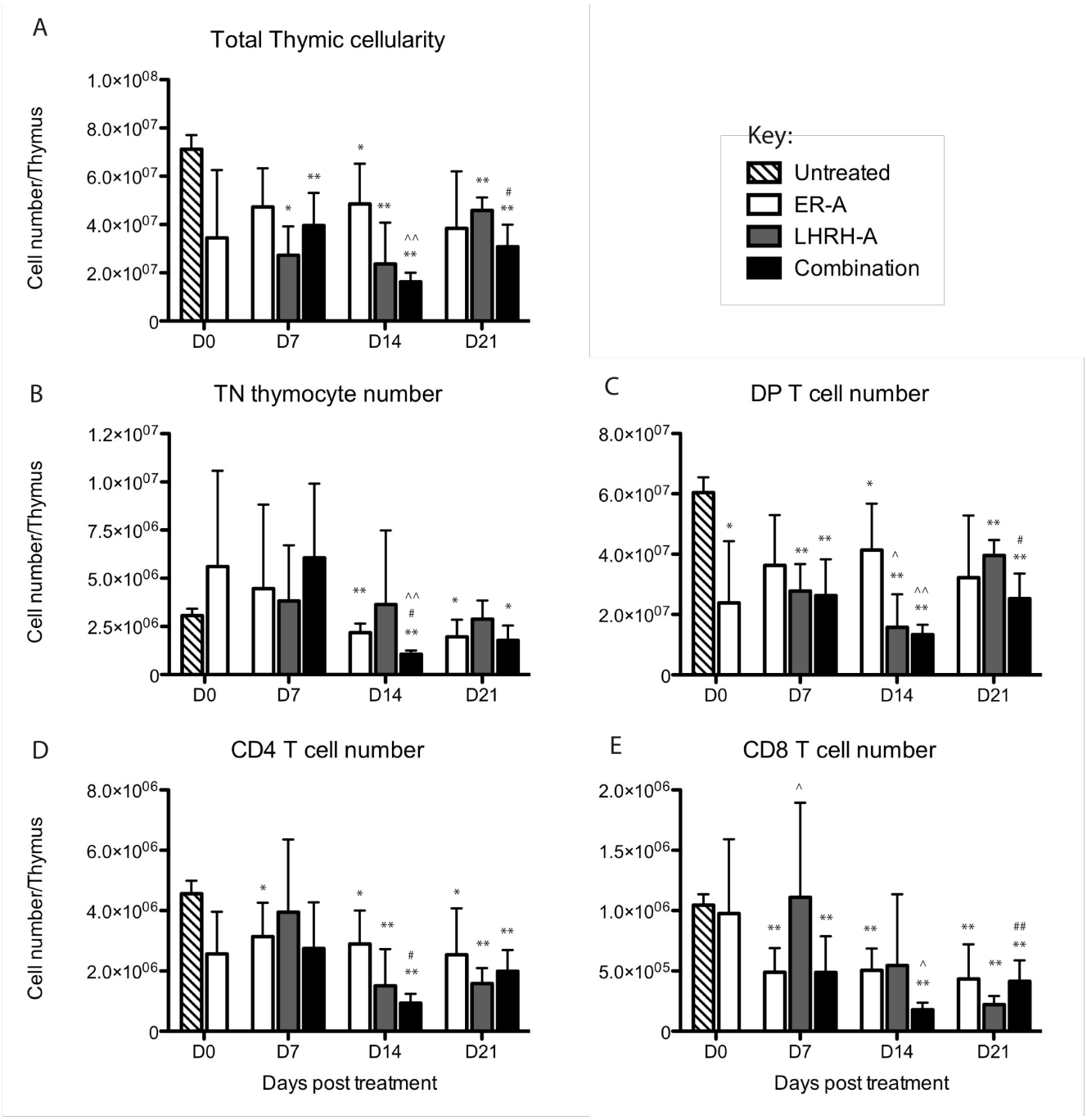
3.4.5 Combination of LHRH-A and ER-analogue in young female mice

Following ovariectomy, residual estrogen is still produced by the adrenal gland through testosterone aromatization, albeit at a relatively low level. However it may be sufficient to reduce the efficiency of LHRH-A induced thymic hypertrophy particularly in older female mice. A sex steroid flare is also a cause of significant adverse symptoms in patients, including enhanced growth of androgen receptor (AR)+ or ER+ tumor cells [28-30]. We hypothesised that blocking ERs would negate the effect of both extra-gonadal estrogen and a sex steroid flare. Estrogen receptor analogue (ER-A) was administered orally to young female mice daily for 28 days (Chapter 2, section 2.2.4), beginning 7 days before LHRH-A administration. Interestingly, the combination of ER-A and LHRH-A did not improve thymic size or prevent the initial thymic atrophy due to LHRH-A treatment (Figure 3.12a) and this was seen in all thymocyte subsets (Figure 3.12b-e). At day 0, ER-A had been administered for 7 days to ensure ERs were sufficiently bound. At this stage, total thymic number was reduced, and remained so throughout the experimental time line. Both lymphoid and myeloid progenitors were reduced in number following all treatment combinations (Figure 3.13), however LHRH-A when administered alone increased LSK cell number above untreated controls at day 21, when gonadal estrogen production has ceased (Figure 3.13a).

Within the bone marrow, all treatment combinations reduced total cell number as well as maturing B cell numbers (Figure 3.14). These did not recover to untreated control levels by day 21. Mice treated with LHRH-A alone showed faster B cell recovery compared to both ER-A treated groups. Thus it appears that ER-A accentuates the immune cell decline caused by an estrogen flare, hindering recovery

Figure 3.12 The combination of LHRH-A and ER-A worsens thymic recovery in young female mice

ER-A was given orally in combination with 0.4mg of LHRH-A, which was injected intramuscularly, into young female mice. Total thymic cellularity following treatment (a); number of TN thymocytes (b); DP thymocytes (c); CD4+ T cells (d); CD8+ T cells (e). Each bar represents the mean $\pm$ 1 of five mice. * $p<0.05$, ** $p<0.01$ c.f. Untreated, ^ $p<0.05$, ^^ $p<0.01$ c.f. ER-A alone, # $p<0.05$, ## $p<0.01$ c.f. LHRH-A alone.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D0
D7
D14
D21
Key:
Untreated
ER-A
LHRH-A
Combination
B
TN thymocyte number
Cell number/Thymus
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7.5×10^06
5.0×10^06
2.5×10^06
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C
DP T cell number
8.0×10^07
6.0×10^07
4.0×10^07
2.0×10^07
D
CD4 T cell number
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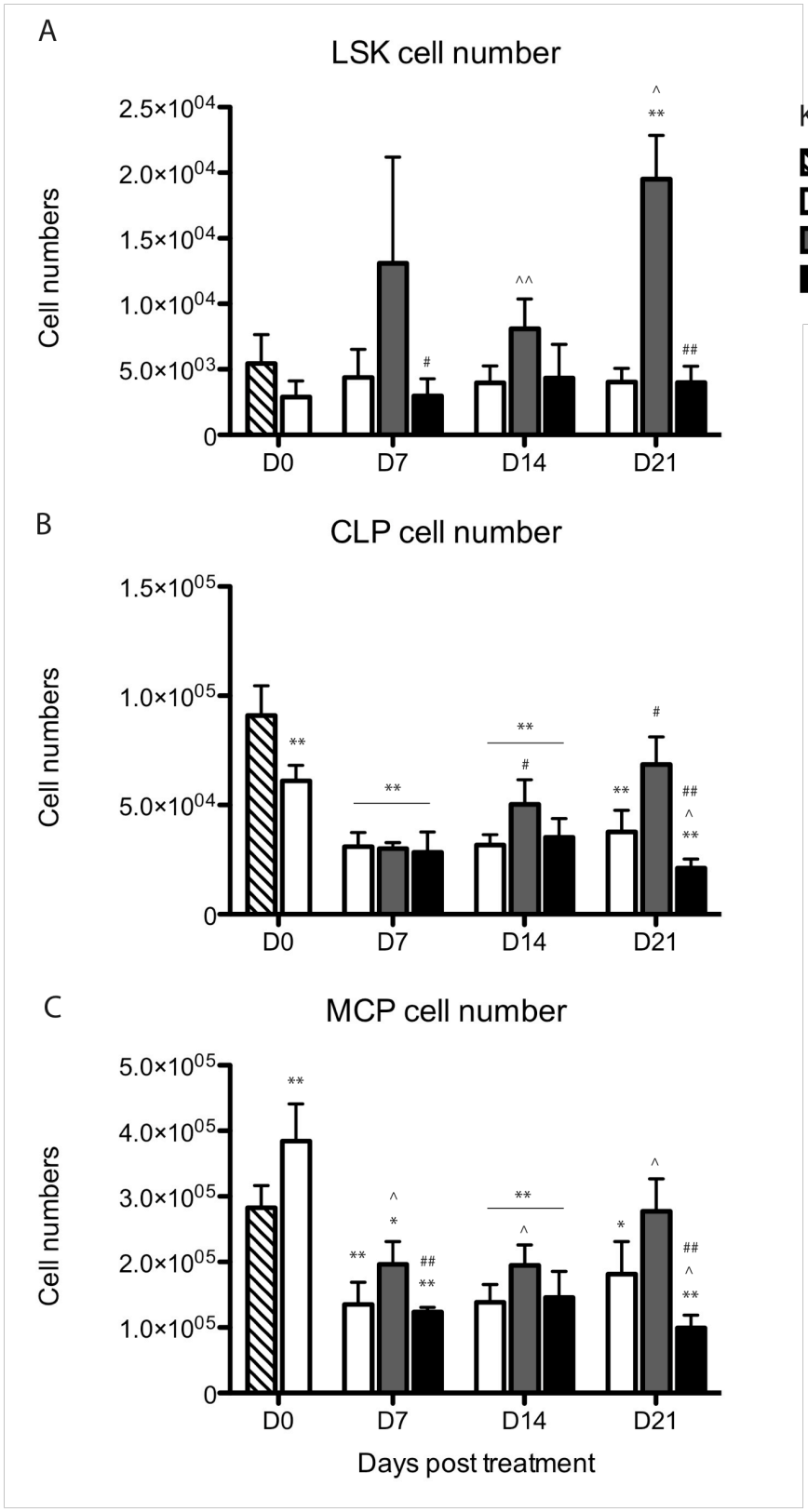
2.0×10^06
Days post treatment
E
CD8 T cell number
2.0×10^06
1.5×10^06
1.0×10^06
5.0×10^05
Days post treatment

Figure 3.13 The combination of LHRH-A and ER-A worsens lymphoid and myeloid progenitor cell recovery in young female mice

ER-A was given orally in combination with 0.4mg of LHRH-A, which was injected intramuscularly, into young female mice. Number of LSK cells within the bone marrow following treatment (a); MCP cells (b); CLP cells (c). Each bar represents the mean $\pm$ 1 of five mice. * $p<0.05$, ** $p<0.01$ c.f. Untreated, ^ $p<0.05$, ^^ $p<0.01$ c.f. ER-A alone, # $p<0.05$, ## $p<0.01$ c.f. LHRH-A alone.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D7
D14
D21
B
CLP cell number
Cell numbers
1.5×10^05
1.0×10^05
5.0×10^04
0
C
MCP cell number
Cell numbers
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4.0×10^05
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Days post treatment
Key:
Untreated
ER-A
LHRH-A
Combination

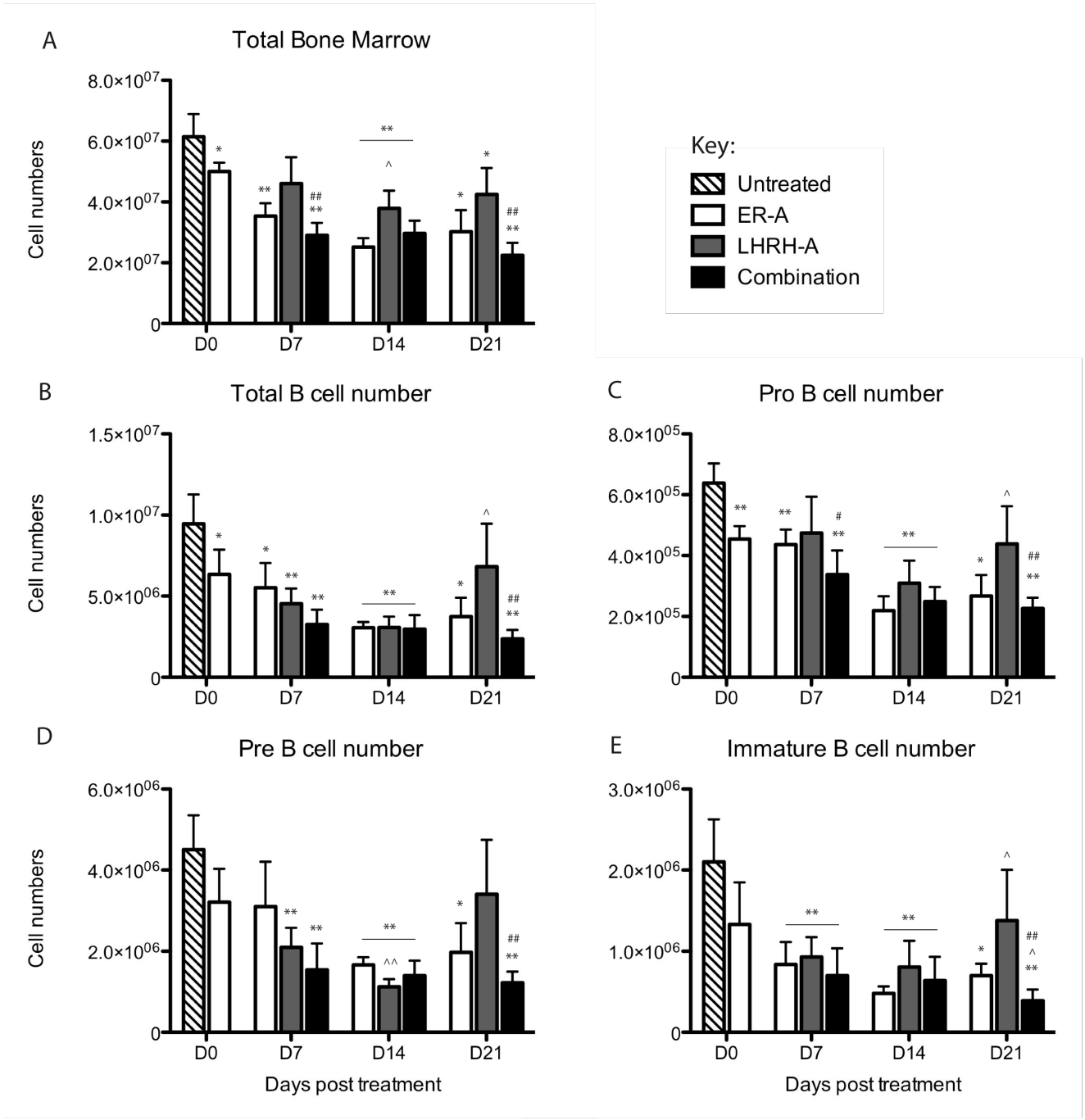

Figure 3.14 The combination of LHRH-A and ER-A worsens B cell recovery in young female mice

ER-A was given orally in combination with 0.4mg of LHRH-A, which was injected intramuscularly, into young female mice. Total bone marrow cellularity following treatment (a); total number of B cells (b); Pro B cells (c); Pre B cells (d); Immature B cells (e). Each bar represents the mean ± 1 SD. * $p<0.05$, ** $p<0.01$ c.f. Untreated, ^ <0.05, ^^ $p<0.01$ c.f. ER-A alone, # $p<0.05$, ## $p<0.01$ c.f. LHRH-A alone.

to untreated control levels. B cells within the spleen were reduced temporarily at day 7 with both ER-A treatment combinations (Figure 3.15b), but both CD4+ and CD8+ T cell subsets were significantly reduced from day 7 to 21 (Figure 3.15c-d).

We then sought to establish whether effects of ER-A were different from, or synergistic with the established LHRH-A protocol. In young female mice that had been administered cyclophosphamide at days -1 and 0, total thymic cellularity and all thymocyte subsets were reduced, as expected, but ER-A treatment resulted in a further decline in comparison with LHRH-A treatment alone (Figure 3.16). Thus the combination of ER-A and LHRH-A does not improve thymic recovery following chemotherapy-induced depletion. Together, these results highlight a potential detrimental effect with the use of ER-A in the clinic in regards to a functional immune system.

3.5 Discussion

It is well documented that sex steroid ablation dramatically improves immune recovery from immunodeficient states in mice and rats [11, 19, 31]. Since surgical gonadectomy is invasive and irreversible, cessation of sex steroid production through chemical means, such as through the use of an LHRH-A, is a clinically relevant alternative. In humans, LHRH-A administration in patients receiving allogeneic HSCT resulted in a recovery of naïve CD4+ T cells to pre-HSC levels within 9 months [13] compared with at least 2 years [5]. However, these studies identified a number of unresponsive patients. We thus investigated methods that may improve the degree of response, the proportion of responders as well as relevant factors governing a patient's lack of response.

One substantial unknown is the significance of any direct effect of LHRH-A on thymocytes themselves. This is particularly relevant since chemotherapy accompanying LHRH-A treatment in many patients destroys the thymus as a byproduct of destroying malignancy, or creating marrow space prior to transplant. Our observations indicate that LHRH-A improves thymic size more significantly than surgical castration (unpublished observations) in addition LHRH-Rs are expressed within the total thymus [32, 33]. However, in our hands, LHRH-A itself

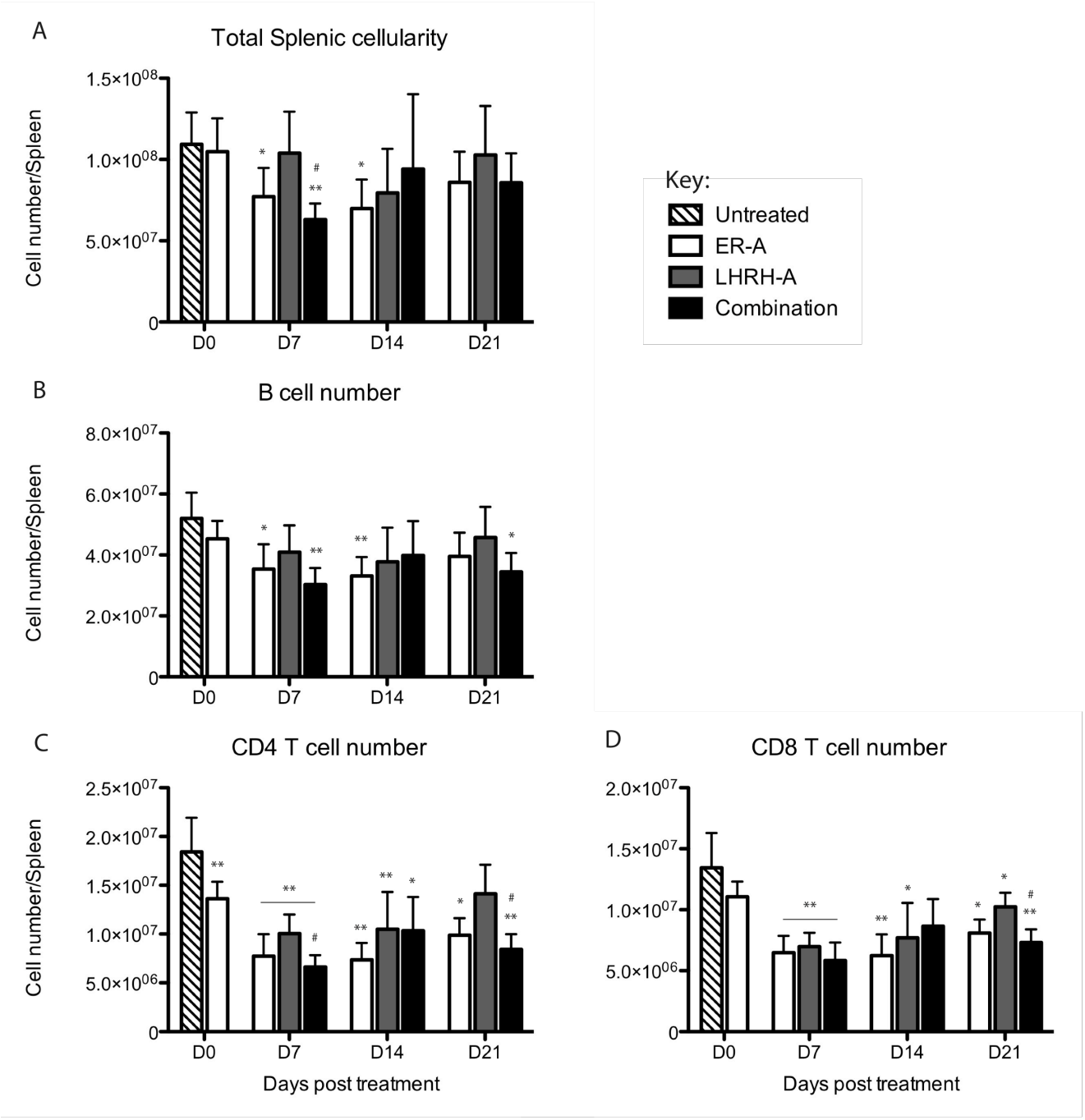

Figure 3.15 The addition of ER-A to LHRH-A treated female mice has little additive effect in the spleen

ER-A was given orally in combination with 0.4mg of LHRH-A, which was injected intramuscularly, into young female mice. Total splenic cellularity following treatment (a); number of B cells (b); CD4+ T cells (c); CD8+ T cells (d). Each bar represents the mean $\pm$ 1 SD. * $p<0.05$, ** $p<0.01$ c.f. Untreated, # $p<0.05$ c.f. LHRH-A alone.

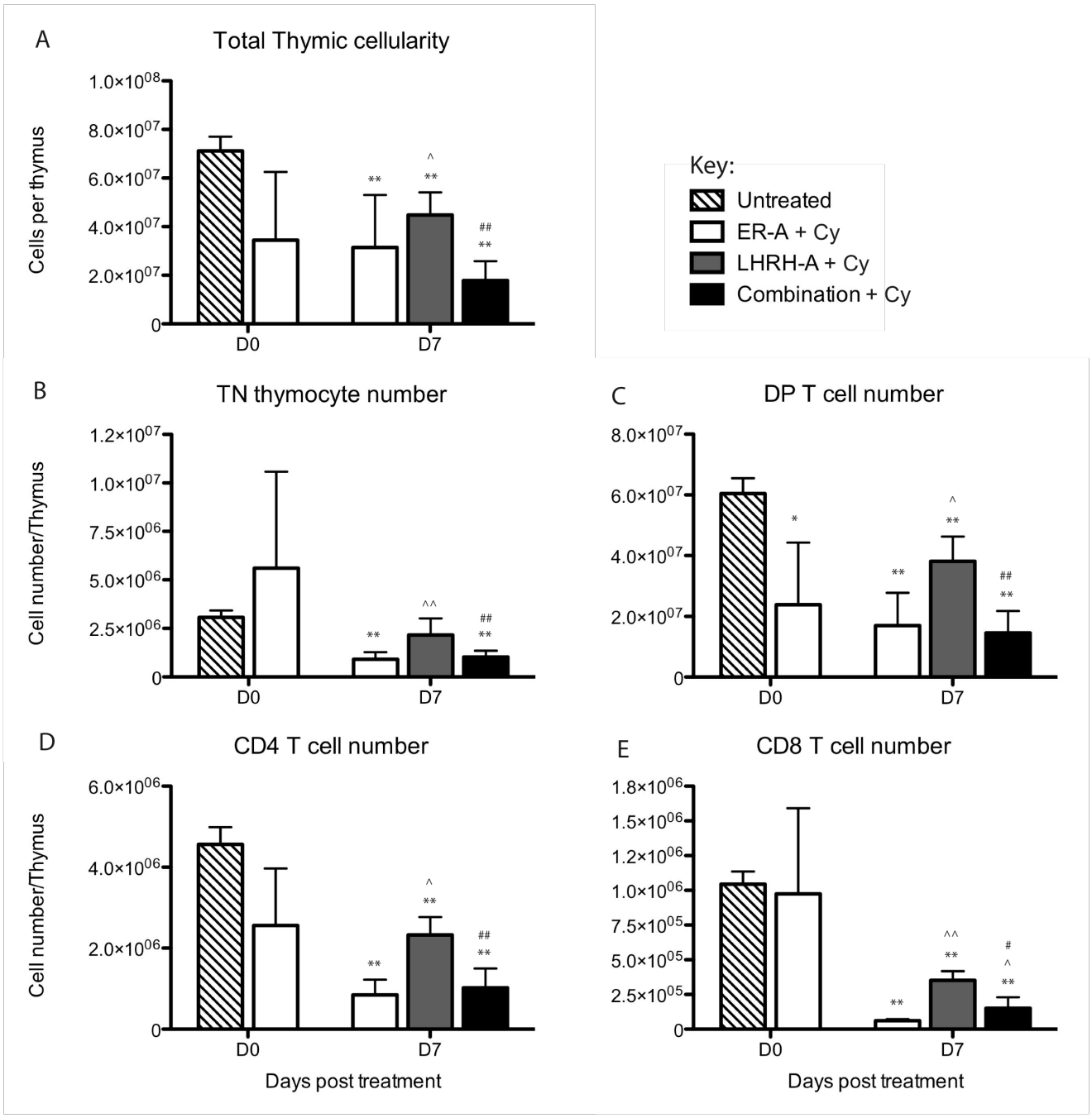

Figure 3.16 The combination of LHRH-A and ER-A worsens total thymic recovery following chemotherapy in young female mice

Young female mice were administered a split dose of cyclophosphamide following LHRH-A and/or ER-A. Total thymic cellularity following treatment (a); number of TN thymocytes (b); DP thymocytes (c); CD4+ T cells (d); CD8+ T cells (e). Each bar represents the mean ± 1 SD. * $p<0.05$, ** $p<0.01$ c.f. Untreated, ^ $p<0.05$, ^^ $p<0.01$ c.f. ER-A + Cy, # $p<0.05$, ## $p<0.01$ c.f. LHRH-A + Cy.

did not appear to directly influence thymocyte number when testosterone was reintroduced or when LHRH-A was injected intrathymically (data not shown). LHRH-A also did not increase proliferation of a whole thymocyte preparation.

Our *in vitro* experimental design did not include the supportive thymic stroma or allow for secondary effects, such as androgen-independent changes. It is also possible that our *in vivo* experimental timing was not sufficient to observe an effect. An additional problem may be the formulation of the LHRH-A. Better than the slow release formulation of Lucrin (used herein) could be purified LHRH peptide to further delineate any direct intrathymic effects of this hormone. More precise mapping of the expression of LHRH-R within cell populations of the thymus, including the thymic stroma, would also be instructive. Given that LHRH-R mRNA within thymic and splenic tissues mirrors that in the brain, it is likely that any direct effect of LHRH is short-lived and LHRH-Rs may be downregulated as they are in the brain after prolonged LHRH-A administration.

Recent clinical trials using LHRH-A in humans, while successful, only showed improved naïve CD4+ T cell recovery in around half the patients studied [13]. We investigated the importance of timing of LHRH-A administration, since it can be observed in many human studies that the rate of immune recovery differs between patients of the same age following induced depletion. When LHRH-A was administered at 3, 6 or 9 weeks before chemotherapy, rate of thymic regrowth was unchanged, indicating that scheduling for LHRH-A administration may not explain delayed immune recovery. To investigate the impact of genetic background on immune recovery and subsequent thymic hypertrophy, we administered LHRH-A to Balb/C male 9mo mice and compared to C57Bl/6 male mice. We observed both a delay in the recovery in Balb/c mice when compared to C57Bl/6 mice as well as a reduced hypertrophy. It is likely that the initial testosterone surge reduced the thymus recovery capacity. In situations of sex steroid exacerbated clinical disease, such as prostate cancer and breast cancer, a surge in androgens or estrogens induced by LHRH-A leads to significant negative side effect such as promoted growth of the AR+ or ER+/PR+ cancer cells [28, 30]. Combine this with a delay in immune recovery and patients could be left with an increase in tumor size and a decline in the

immune response to clear these cells. Thus, future studies will need to investigate the clinical utility of combining LHRH-A with AR blockade.

While we have observed that the kinetics of thymic regeneration is strain dependent, we also demonstrated it to be age- and gender- dependent. It is well documented that the recovery capacity of the immune system is decreased with age [34] partially owing to the reduced reconstitution potential of HSCs and a homeostatic expansion of memory cells [35-37]. Interestingly, thymic recovery was significantly reduced in young and almost non-existent in aged to elderly female mice studied while LHRH-A and surgical castration induces significant hypertrophy in male mice from 4 weeks to 24 months of age [8-11]. This could also have been due to residual estrogen produced by the adrenal gland. However, when LHRH-A was combined with ER blockade, thymic atrophy was increased. Tamoxifen has been shown to act as both an estrogen agonist and antagonist depending on the tissue of action [14, 29]. Our data suggests that Tamoxifen acts as a pro-estrogen within the thymus to induce apoptosis of thymocytes. This is supported by two studies whereby Tamoxifen, when administered to castrated rats, decreased thymic weight by thirty percent and increased serum estrogen [38, 39]. Notably, TGF-β expression was significantly increased in the more recent of these studies indicating a possible mechanism in regards to Tamoxifen-induced thymic atrophy [38]. Thus, further investigation is required into the precise action of Tamoxifen within the thymus to determine whether it is a viable treatment with the combination of LHRH-A in females.

While SERMs have been overtaken by third-generation aromatase inhibitors (AI) as the most active drug for ER+ and PR+ positive breast cancers [40, 41], Tamoxifen is still the preferential treatment over chemotherapy/irradiation for cancer reduction [14]. In animal studies, Tamoxifen administration to intact male rats was shown to increase thymic and splenic weight. However, given that the combination of LHRH-A and Tamoxifen is a standard treatment for postmenstrual women, it is possible that a subsequent thymic atrophy would prolong the period of immunosuppression. This would indicate the clinical need for true ER blockade throughout all tissues.

LHRH-A is a promising treatment to improve immune recovery following chemotherapy/irradiation-induced depletion. Of course, implications of total sex steroid ablation must be considered. As has been discussed, estrogen plays a key role in the maintenance of bone density in both males and females. In pre-menstrual women, Tamoxifen has been shown to have little effect on changes in bone density, as it is less potent than 17β-estradiol. LHRH-A treatment in males and post-menopausal women, however, induces bone loss within a few months of treatment, which is due to increased bone remodeling and resorption [42]. In addition, LHRH-A directly stimulates residual peripheral T cells and increases cytokine production in both male and female mice [11, 43] inferring a possible inflammatory and/or autoimmune response. LHRH-A treatment in female mice does not change the incidence of GVHD [43]. If LHRH-A were to be transferred to the clinic, its intended use is short-term, minimizing sex steroid depletion-associated side effects.

Together, these results highlight the complexities involved in translating a successful pre-clinical treatment to a successful clinical therapy. The immune system is highly responsive to hormones in multiple overlapping pathways and cell types. LHRH-A treatment, however, does show great promise for reducing immune recovery post-chemo. Here we show that the degree of patient response, however, is likely to depend on age, gender, residual sex steroids or concomitant hormone-altering treatment regimens such as Tamoxifen, and unknown genetic factors shown through use of different mouse strains. Further elucidation of the underlying mechanisms could permit effective pre-screening of patients to identify non-responders, who may require more careful monitoring or additional supportive therapy.

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CHAPTER 4 – Results section 2

SEX STEROID ABLATION ENHANCES RECOVERY OF THE FEMALE IMMUNE SYSTEM FOLLOWING CHEMOTHERAPY

Manuscript information

This chapter is currently a manuscript in preparation with a planned submission date of August 2010. The candidate Melanie Hince was primarily responsible for performing and analyzing experiments as well as writing and editing. Other co-authors contributed technical assistance, interpretation of results and drafting of the manuscript. Detailed contributions are explained in the signed declaration on page VIII.

4.1 Abstract

There is an increased incidence of malignancies with age and prolonged periods of immunodeficiency with high risk of opportunistic infections associated with chemotherapy and radiation therapy. This highlights the overwhelming need to improve immune competence in adults, primarily by overcoming age-induced thymus atrophy. We have previously demonstrated significant thymus rejuvenation and hence immune recovery by removing sex steroids in young and aged males, through both surgical and reversible chemical means. The latter is based on a luteinising hormone-releasing hormone agonist (LHRH-A). Here, we have investigated the effectiveness of sex steroid ablation on immune recovery in young and middle-aged females. Both ovariectomy and treatment with LHRH-A consistently induced increases in total thymic cellularity, including early after chemotherapy in young females, with the effectiveness becoming less pronounced in middle-aged female mice.

4.2 Introduction

Chemotherapy agents such as Cyclophosphamide (Cy) act by targeting and destroying highly proliferative cells such as cancerous cells, but they also impact severely on cells of the gut epithelium, hair follicle and immune system. As a consequence, patients remain immunocompromised for extended periods of time following treatment, leaving them with an increased risk of mortality from opportunistic infections and cancer relapse. In addition, as patients advance in age, the delay in lymphocyte recovery in number and function is exacerbated; young, pre-pubertal patients recover to pre-treatment CD4+ T cell levels within 6 months following Cy treatment, whereas this time can be extended beyond 2 years or not at all in middle-aged adults [1]. In such cases, peripheral expansion of pre-existing T cells occurs, resulting in a greatly diminished lymphocyte pool diversity [2].

One of the major causes of immune deterioration with age is the suppressive influence of sex steroids [3, 4]. It has been clearly demonstrated that the addition of sex steroids, specifically testosterone and estrogen, induces DP thymocyte apoptosis in part through the release of TNF-α [5, 6]. In contrast, the removal of sex steroids by both surgical and chemical means (through the use of an LHRH-A) has been shown to increase thymic and splenic cellularity, essentially reversing thymic atrophy [7]. Previous studies by our group have also demonstrated an improved immune recovery in male mice following HSCT and chemotherapy, with the removal of sex steroids by surgical castration [8-10]. In addition, the clinical use of LHRH-A in patients undergoing either autologous or allogeneic hematopoietic stem cell transplantation (HSCT), resulted in an increase in thymic production of naïve thymocytes - as measured by T cell receptor excision circles (TRECs), and improved T cell function, leading to an enhanced CD4+ and CD8+ T cell recovery [7, 11].

While studies in females have been limited in comparison, past publications have demonstrated an increase in total thymic size following ovariectomy in both young and middle-aged females [12]. In Chapter 3 we presented data indicating that sex steroid ablation, through the use of an LHRH-A, induces thymic hypertrophy and reverses age-related atrophy in female mice. We therefore sought to determine whether LHRH-A could also enhance immune recovery following chemotherapy in

females. We show that both Ovx and LHRH-A lead to a temporary increase in lymphocyte number in the bone marrow, thymus and spleen in young and middle-aged females following chemotherapy. This rejuvenation of naïve B and T cells in the periphery further supports the clinical use of LHRH-A to improve immune recovery.

4.3 Experimental Design

4.3.1 LHRH-A administration in combination with cyclophosphamide therapy in young and middle-aged female mice

0.4mg of LHRH-A was administered intramuscularly twenty-one days before cyclophosphamide injection, which was administered at 200mg/Kg split over two days as detailed in Chapter 2, section 2.2.6. Separate cohorts of mice were either ovariectomised or sham ovariectomised with the second cyclophosphamide dose. Mice were culled and organs harvested at days 7, 14 and 28 following cyclophosphamide treatment.

4.4 Results

4.4.1 Effects of LHRH-A on total immune organ cellularity is more pronounced in males compared to females

Following a single dose of 0.4mg of LHRH-A, sex steroid levels diminish to zero within 21 days (see Chapter 5, section 5.4.2). As we have previously demonstrated a significant increase in thymic cellularity by 7 days following surgical castration [10], we therefore selected analysis at day 28 following LHRH-A treatment as this equates to 7 days following castrate levels of sex steroids being achieved by the drug. In male mice, total cellularity within the bone marrow compartment, thymus and spleen had increased significantly by 28 days when compared to PBS administered controls (Table 4.1), consistent with previous observations [3, 7, 10]. However, whilst there was an increase in thymic cellularity in age-matched young females, it was not as pronounced compared to males and there were no changes in bone marrow or splenic cellularity at this timepoint (Table 4.1). In the middle-aged cohort (Table 4.2), thymic cellularity was increased in both males and females, albeit to a lesser extent compared to the young cohort, and the effect of LHRH-A was again not as pronounced in females compared to males. The middle aged females showed no increase in bone marrow cellularity and both males and females showed no increase in splenic cellularity at this timepoint.

4.4.2 LHRH-A improves T cell recovery following chemotherapy in both young and middle-aged females

Given that LHRH-A treatment enhances thymic size in male and, to a lesser degree, female mice, we investigated the impact of sex steroid ablation on immune recovery following chemotherapy in female mice. To ensure full ablation of sex steroids close to commencement of chemotherapy, LHRH-A was administered 20 days prior to the initial dose of Cyclophosphamide (Cy). In both young and middle-aged mice, sex steroid ablation by either treatment showed no significant change in bone marrow cellularity when compared to sham controls (Figure 4.1a, b). In young mice LHRH-A treatment increased thymic cellularity as early as 7 days following chemotherapy compared to sham controls (Figure 4.1c) while ovariectomy increased cellularity at 14 days to sham control levels. Whilst there was no significant difference in the middle aged females between sex steroid ablated groups and sham controls at day 7

	Male control	Male + LHRH-A	Female control	Female + LHRH-A
Bone Marrow	$6.74x10^7$ $\pm$ $5.96x10^6$	$9.24x10^7$ $\pm$ $9.35x10^6$ **	$5.11x10^7$ $\pm$ $1.08x10^7$	$5.70x10^7$ $\pm$ $9.10x10^6$
Thymus	$1.26x10^8$ $\pm$ $1.85x10^7$	$2.81x10^8$ $\pm$ $1.51x10^7$ ***	$1.63x10^8$ $\pm$ $1.94x10^7$	$2.32x10^8$ $\pm$ $2.11x10^7$ *
Spleen	$9.47x10^7$ $\pm$ $2.08x10^7$	$1.53x10^8$ $\pm$ $2.27x10^7$ **	$1.51x10^8$ $\pm$ $3.02x10^7$	$1.21x10^8$ $\pm$ $1.55x10^7$

Table 4.1 LHRH-A increases organ size in young male mice and in young female mice 28 days after administration

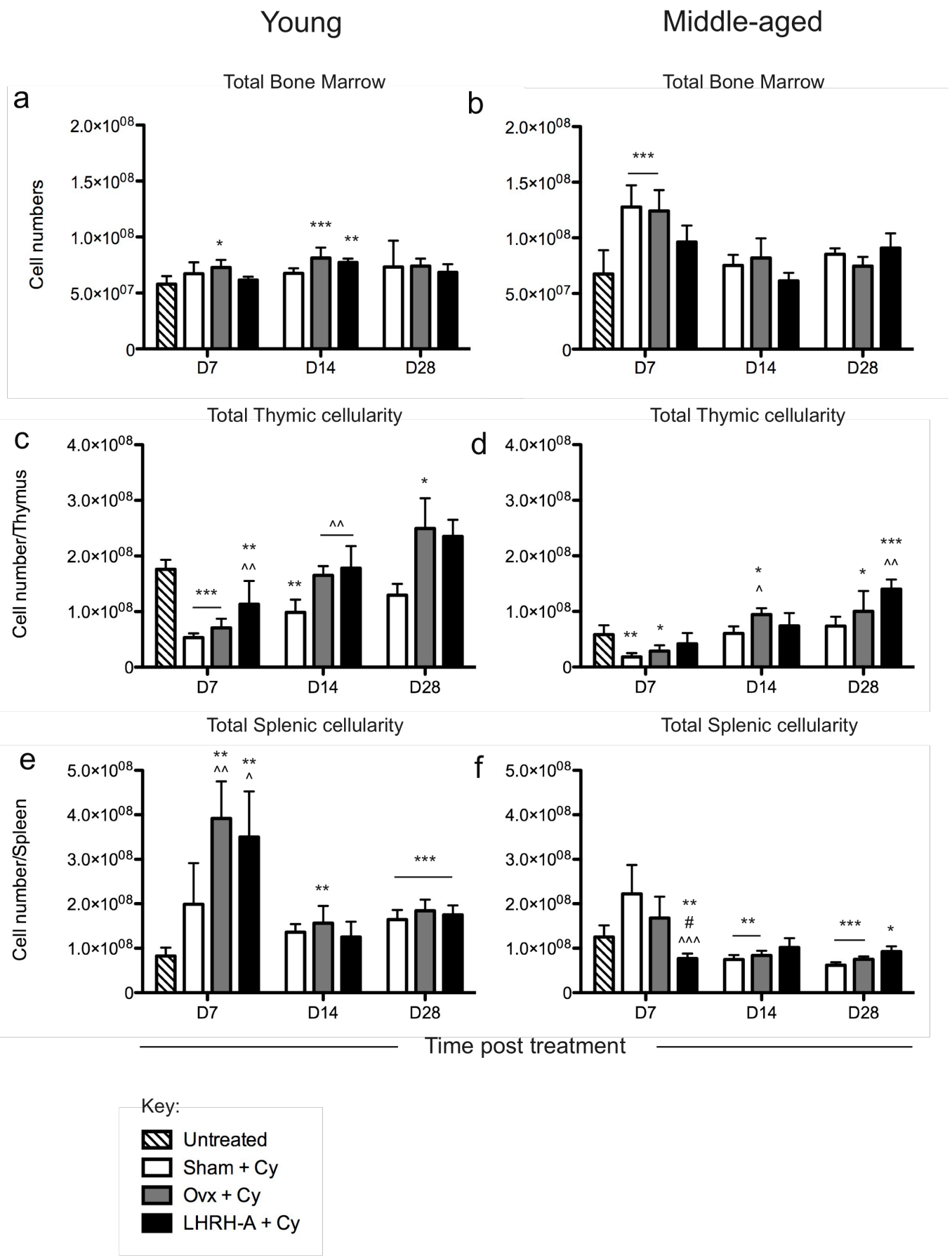
Numbers are represented as the mean $\pm$1 SD of four to six mice. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ c.f. respective control. Female data is summarised from Chapter 3, section 3.4.4.

	Male control	Male + LHRH-A	Female control	Female + LHRH-A
Bone Marrow	$4.66x10^7$ $\pm$ $5.83x10^6$	$7.25x10^7$ $\pm$ $1.45x10^7$ *	$1.23x10^8$ $\pm$ $1.64x10^7$	$1.51x10^8$ $\pm$ $1.32x10^7$
Thymus	$6.20x10^7$ $\pm$ $1.12x10^7$	$1.95x10^8$ $\pm$ $5.70x10^7$ **	$9.00x10^7$ $\pm$ $3.49x10^7$	$1.69x10^8$ $\pm$ $2.52x10^7$ *
Spleen	$1.26x10^8$ $\pm$ $3.45x10^7$	$1.74x10^8$ $\pm$ $3.31x10^7$	$8.38x10^7$ $\pm$ $1.41x10^7$	$7.62x10^7$ $\pm$ $5.55x10^6$

Table 4.2 LHRH-A increases overall organ cellularity in aged male mice and in aged female mice following administration. 35 days in females, 42 days in males
Numbers are represented as the mean $\pm$1 SD of four to six mice. * $p<0.05$, ** $p<0.01$ c.f. respective control. Female thymus data is summarised from Chapter 3, section 3.4.4.

Figure 4.1 LHRH-A improves total organ recovery following chemotherapy in both young and middle-aged females

Young (4-6wks) and middle-aged (9 months) female mice were administered LHRH-A 21 days before chemotherapy. Total bone marrow cellularity in young mice (a); total thymic cellularity (c); total splenic cellularity (e). Total bone marrow cellularity in middle-aged mice (b); total thymic cellularity (d); total splenic cellularity (f). Each bar represents the mean $\pm$1 SD of four to six mice. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ c.f. untreated, ^ $p<0.05$, ^^ $p<0.01$, ^^^ $p<0.001$ c.f. Sham, # $p<0.05$ c.f. Ovx.


Young
Middle-aged
a
Total Bone Marrow
Cell numbers
2.0×10^08
1.5×10^08
1.0×10^08
5.0×10^07
0
D7
D14
D28
b
Total Bone Marrow
c
Total Thymic cellularity
Cell number/Thymus
4.0×10^08
3.0×10^08
2.0×10^08
1.0×10^08
d
Total Thymic cellularity
e
Total Splenic cellularity
Cell number/Spleen
5.0×10^08
4.0×10^08
3.0×10^08
2.0×10^08
1.0×10^08
f
Total Splenic cellularity
Time post treatment
Key:
Untreated
Sham + Cy
Ovx + Cy
LHRH-A + Cy

following chemotherapy, both treatment groups did later increase thymic cellularity: ovariectomy at day 14 and LHRH-A at day 28 (Figure 4.1d). It is interesting to note that with LHRH-A treatment, total thymic cellularity was not decreased with chemotherapy when compared to untreated controls, but it was seen with sham-ovariectomised and ovariectomised mice. Both ovariectomy and LHRH-A temporarily increased splenic cellularity when compared to sham and untreated controls at Day 7 and 28 following chemotherapy in young mice (Figure 4.1e). However, no increase was evident in the spleen with sex steroid ablation by either treatment in middle-aged females (Figure 4.1f).

4.4.3 The effect of sex steroid ablation on bone marrow-derived lymphoid progenitors in female mice following chemotherapy

The progenitors studied in the BM were $Lin^{-}Sca1^{+}c\text{-}kit^{+}$ (LSK) and $Lin^{-}IL7R^{+}c\text{-}kit^{+}$ (CLP) subsets. In young female mice, while the day 7 proportional data were variable, at day 7 and 14 there was a numerical decrease in LSK in all treatment groups relative to the untreated controls (Figure 4.2a, c) – no doubt reflecting the impact of Cy. By day 28 these had fully recovered in the young but there was no impact of Ovx or LHRH-A relative to the sham controls. In the middle-aged mice, there were no major changes proportionally between the treated groups (Figure 4.2b) but LHRH-A treated group had significantly more total number of LSK at day 7, 14 and 28 (Figure 4.2d) compared to untreated controls.

For CLPs, the proportion in young mice was temporarily increased with all treatments at day 7 following chemotherapy in comparison to untreated controls (Figure 2e), however this did not translate into an increase in cell number. By day 28 there was a trend towards an increase in number and proportion of CLP in Ovx and LHRH mice. In middle-aged mice, a temporary increase in both CLP proportion (Figure 2f) and cell number (Figure 2h) was seen following LHRH-A treatment at day 7.

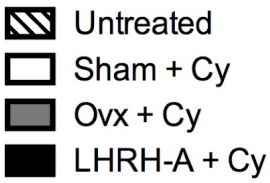
4.4.4 Thymocyte recovery is improved by sex steroid ablation following chemotherapy

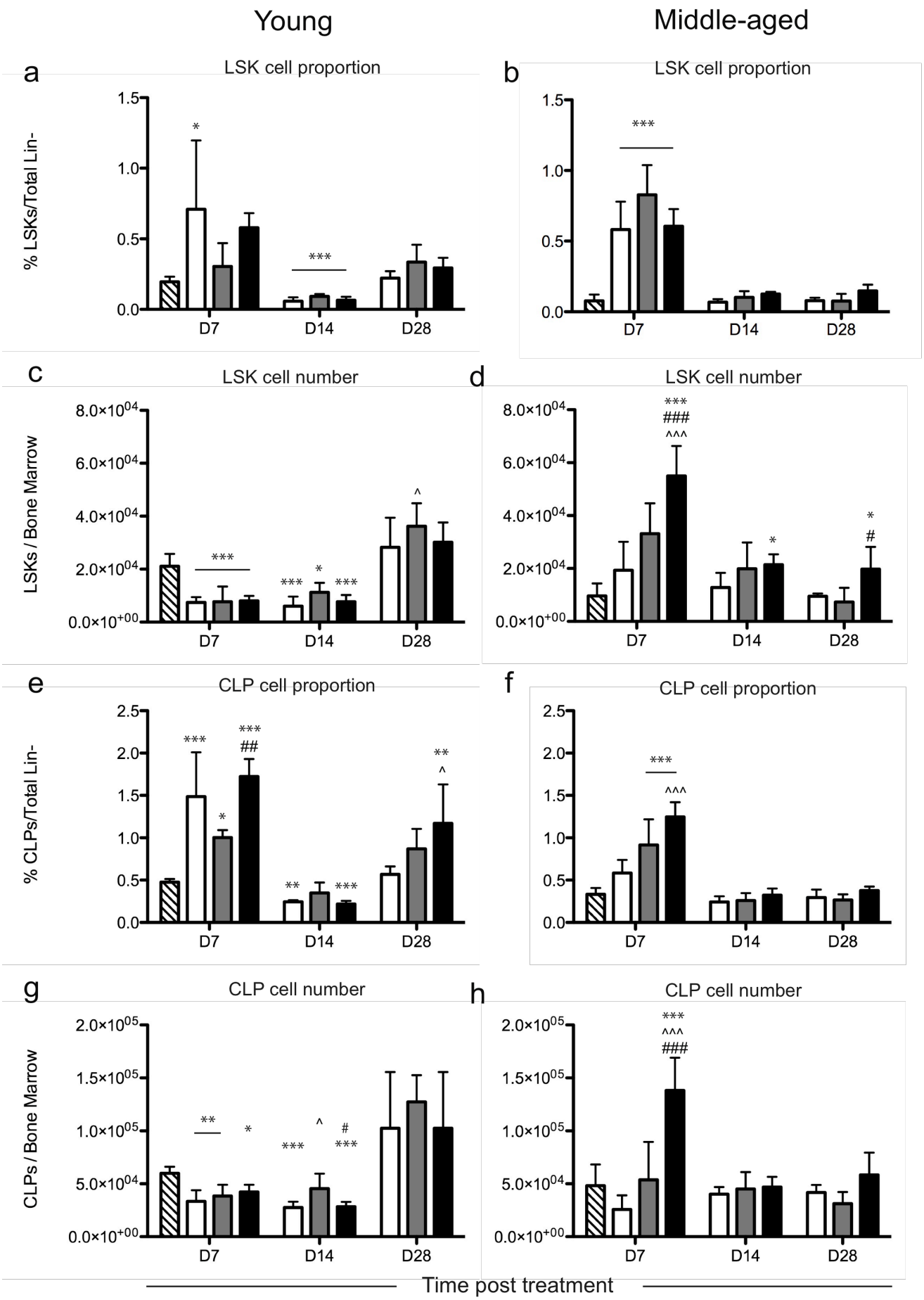
Since both LHRH-A and ovariectomy increased total thymic cellularity following chemotherapy, we investigated whether their effects were global or preferential to

Figure 4.2 Bone marrow derived lymphoid progenitors are increased with sex steroid ablation in female mice following chemotherapy

Young (4-6wks) and middle-aged (9 months) female mice were either administered LHRH-A 21 days before chemotherapy or ovariectomised/sham ovariectomised at day 0. LSK cell proportion in young mice (a); LSK cell number (c); CLP cell proportion (e); CLP cell number (g). LSK cell proportion in middle-aged mice (b); LSK cell number (d); CLP cell proportion (f); CLP cell number (h). Each bar represents the mean $\pm$1 SD of four to six mice. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ c.f. Untreated, ^ $p<0.05$, ^^^ $p<0.001$ c.f. Sham, ## $p<0.01$, ### $p<0.001$ c.f. Ovx.

Key:




Young
Middle-aged
a
LSK cell proportion
% LSKs/Total Lin-
b
LSK cell proportion
c
LSK cell number
LSKs / Bone Marrow
d
LSK cell number
e
CLP cell proportion
% CLPs/Total Lin-
f
CLP cell proportion
g
CLP cell number
CLPs / Bone Marrow
h
CLP cell number
D7
D14
D28
Time post treatment

specific thymocyte subsets. Cy caused a major loss by day 7 in all subsets as expected. For the CD3-CD4-CD8- (TN) precursor subset (Figure 4.3a, b), LHRH-A had recovered by day 7 and Ovx by day 14, in both the young and middle-aged with LHRH-A still elevated at day 28 in the latter.

For CD4+CD8+ (DP) immature thymocytes (Figure 4.3c, d) in the young, again LHRH-A had recovered their numbers by day 7 and together with Ovx continued to increase them above the sham control by day 14 and through to day 28. In the middle-aged, LHRH-A had a protective effect at day 7, while Ovx increased cell number by day 14 and LHRH-A again by day 28.

The CD3hi CD4+CD8- (Figure 4.3e, f) and CD3hiCD4-CD8+ (Figure 4.3g, h) mature single positive (SP) cells were significantly reduced in all groups at day 7, reflecting the longer time for their generation from TN through to DP cells. However Ovx and LHRH-A increased their levels to variable degrees relative to sham-controls, particularly by day 28 in the young and middle-aged.

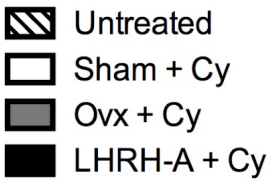
4.4.5 Sex steroid ablation has little effect on Splenic T cell recovery following chemotherapy

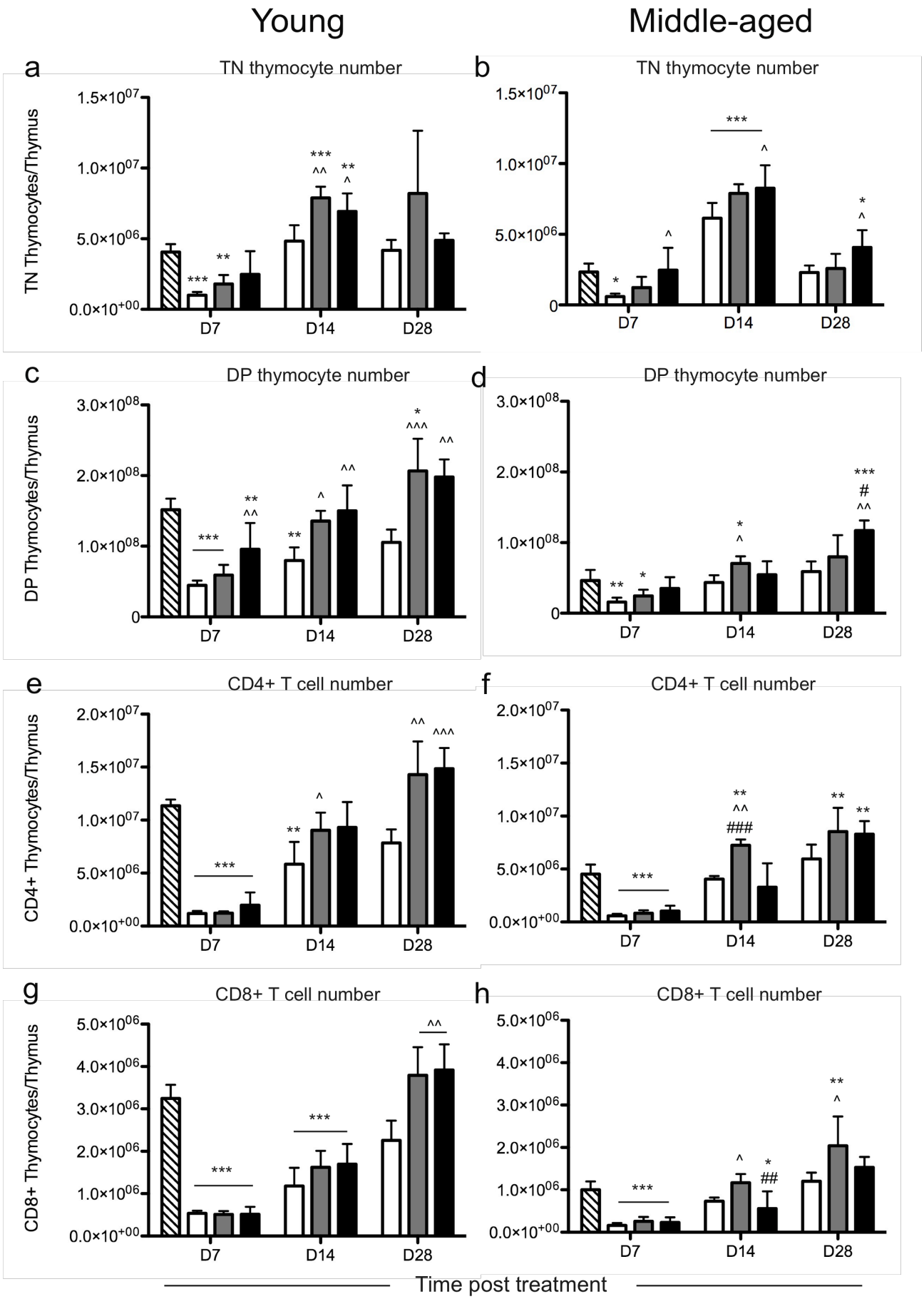
While sex steroid ablation improves the recovery of peripheral CD4+ and CD8+ mature T cells following chemotherapy and HSCT in both pre-clinical animal models and humans, sex steroid ablation by ovariectomy or LHRH-A treatment had little effect in either young or middle-aged female mice. As expected, Cy induced a decline in CD4+ T cell numbers in both young and middle-aged female mice (Figure 4.4a, b). However, there were no significant differences in CD4+ and CD8+ T cell number between sex steroid ablative treatments and sham controls in young mice (Figure 4.4a, c). Both ovariectomy and LHRH-A treatment improved the recovery of CD4+ T cell numbers in middle-aged mice with sham control still depleted at day 28 (Figure 4.4b).

Figure 4.3 Thymocyte subset cell numbers are increased with LHRH-A treatment following chemotherapy

Young (4-6wks) and middle-aged (9 months) female mice were either administered LHRH-A 21 days before chemotherapy or ovariectomised/sham ovariectomised at day 0. TN thymocyte number in young mice (a); DP thymocytes (c); CD4+ T cells (e); CD8+ T cells (g). Total TN thymocyte number in middle-aged mice (b); DP thymocytes (d); CD4+ T cells (f); CD8+ T cells (h). Each bar represents the mean $\pm$1 SD of four to six mice. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ c.f. Untreated, ^ $p<0.05$, ^^ $p<0.01$, ^^^ $p<0.001$ c.f. Sham, # $p<0.05$, ## $p<0.01$, ### $p<0.001$ c.f. Ovx.

Key:




Young
Middle-aged
a
TN thymocyte number
TN Thymocytes/Thymus
b
TN thymocyte number
c
DP thymocyte number
DP Thymocytes/Thymus
d
DP thymocyte number
e
CD4+ T cell number
CD4+ Thymocytes/Thymus
f
CD4+ T cell number
g
CD8+ T cell number
CD8+ Thymocytes/Thymus
h
CD8+ T cell number
D7
D14
D28
Time post treatment

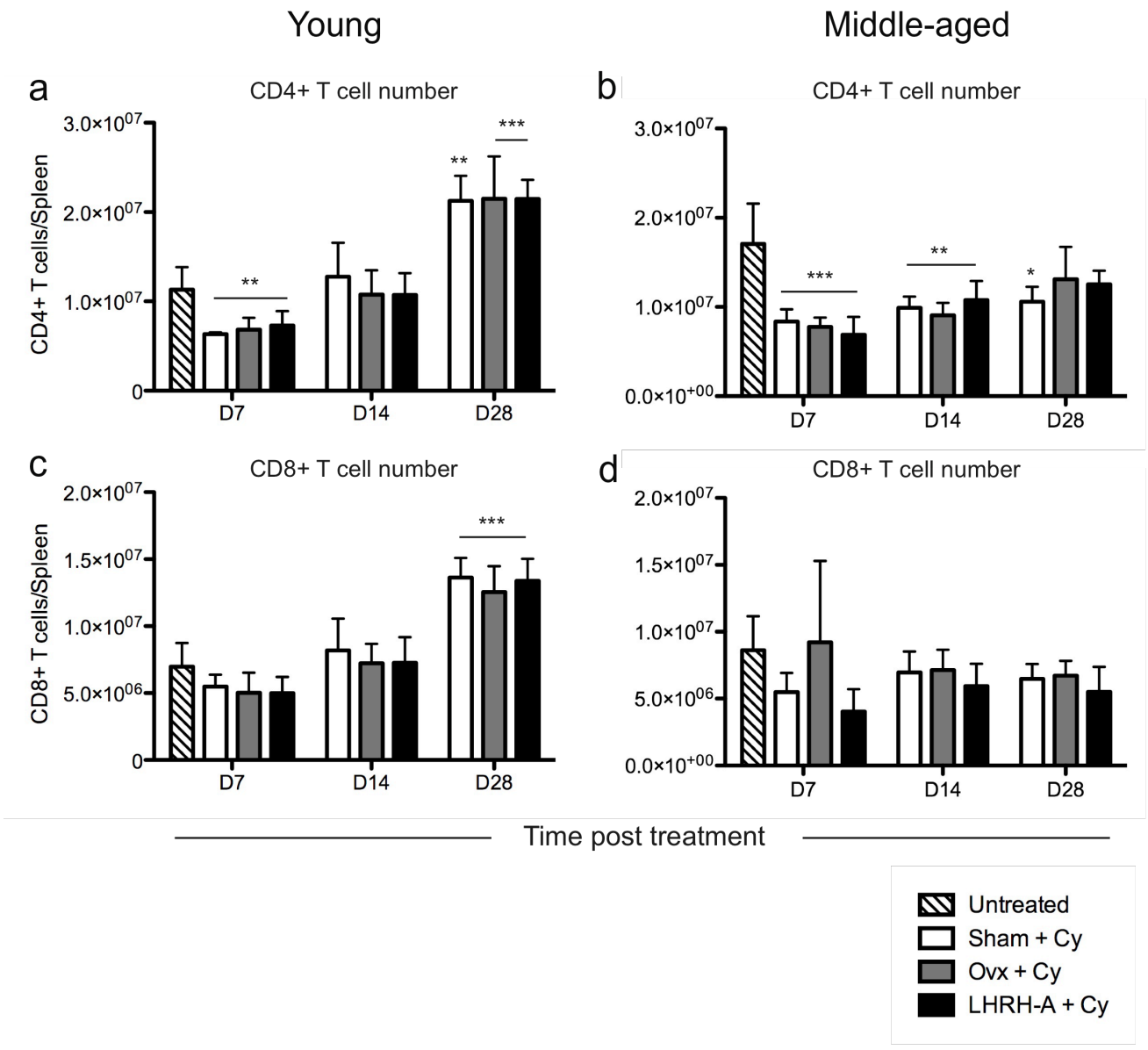


Figure 4.4 Sex steroid ablation has little effect on Splenic T cell recovery following chemotherapy

Young (4-6wks) and middle-aged (9 months) female mice were either administered LHRH-A 21 days before chemotherapy or ovariectomised/sham ovariectomised at day 0. CD4+ T cell number in young mice (a); CD8+ T cell number (c). CD4+ T cell number in middle-aged mice (b); CD8+ T cell number (d). Each bar represents the mean $\pm$1 SD of four to six mice. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ c.f. Untreated.

4.4.6 LHRH-A increases B cell recovery in middle-aged but not young females following chemotherapy

Given that we observed an early increase in CLP cell numbers within the bone marrow following LHRH-A in middle-aged mice and that the preferred lineage for these cells is into the B cells [13], we analysed changes induced by sex steroid ablation on maturing B cell subsets within the bone marrow and spleen following chemotherapy. Total B cells were defined as being B220 (CD45R)+ve and were subdivided into the following subsets based on CD43 and IgM expression; Pro B cells (CD43+ve, IgM-ve), Pre B cells (CD43+ve, IgM+ve) and Immature B cells (CD43-ve, IgM+ve). As for T cells, Cy severely depleted all B cell subsets in both young and middle-aged animals. In young mice, there was significant spontaneous rebound with little difference observed between treatment and sham control groups in regards to total B cell numbers besides a temporary increase with ovariectomy at day 14 following chemotherapy (Figure 4.5a). This increase was reflected in the Pre B cell subset (Figure 4.5e). There appeared to be no advantage in the rate of recovery of maturing B cells in young mice with sex steroid ablation. In middle-aged mice, however, while all subsets were reduced in number in the sham control group, LHRH-A treatment appeared to increase total B cell numbers (Figure 4.5b), which was reflected in Pre B cell subsets (Figure 4.5f). At day 28, LHRH-A treatment induced an increase, relative to shams, in total B cell numbers (Figure 4.5b), which was reflected in Pre B cell numbers (Figure 4.5f), with a similar trend for immature B cell number with LHRH-A treatment (Figure 4.5h), but this was not significant.

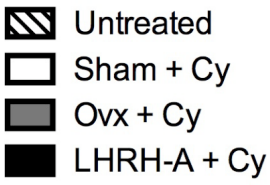
4.4.7 LHRH-A decreases splenic myeloid cell number in middle-aged but not young females following chemotherapy

In contrast to lymphoid cells, Cy did not deplete myeloid cells (DC, granulocytes, macrophages; Figure 4.6). In young mice both Ovx and LHRH-A caused an increase in these three classes of cells at day 7 (Figure 4.6a, c, e), this was also generally maintained at days 14 and 28 except for granulocytes at D28. For the older middle-aged mice there were few consistent changes for all cell types.

Figure 4.5 Sex steroid ablation improves maturing B cell reconstitution following chemotherapy in female mice

Young (4-6wks) and middle-aged (9 months) female mice were either administered LHRH-A 21 days before chemotherapy or ovariectomised/sham ovariectomised at day 0. Total B cell number in young mice (a); Pro B cells (c); Pre B cells (e); Immature B cells (g). Total B cell number in middle-aged mice (b); Pro B cells (d); Pre B cells (f); Immature B cells (h). Each bar represents the mean $\pm$1 SD of four to six mice. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ c.f. Untreated, ^ $p<0.05$, ^^ $p<0.01$ c.f. Sham, ## $p<0.05$ c.f. Ovx.

Key:



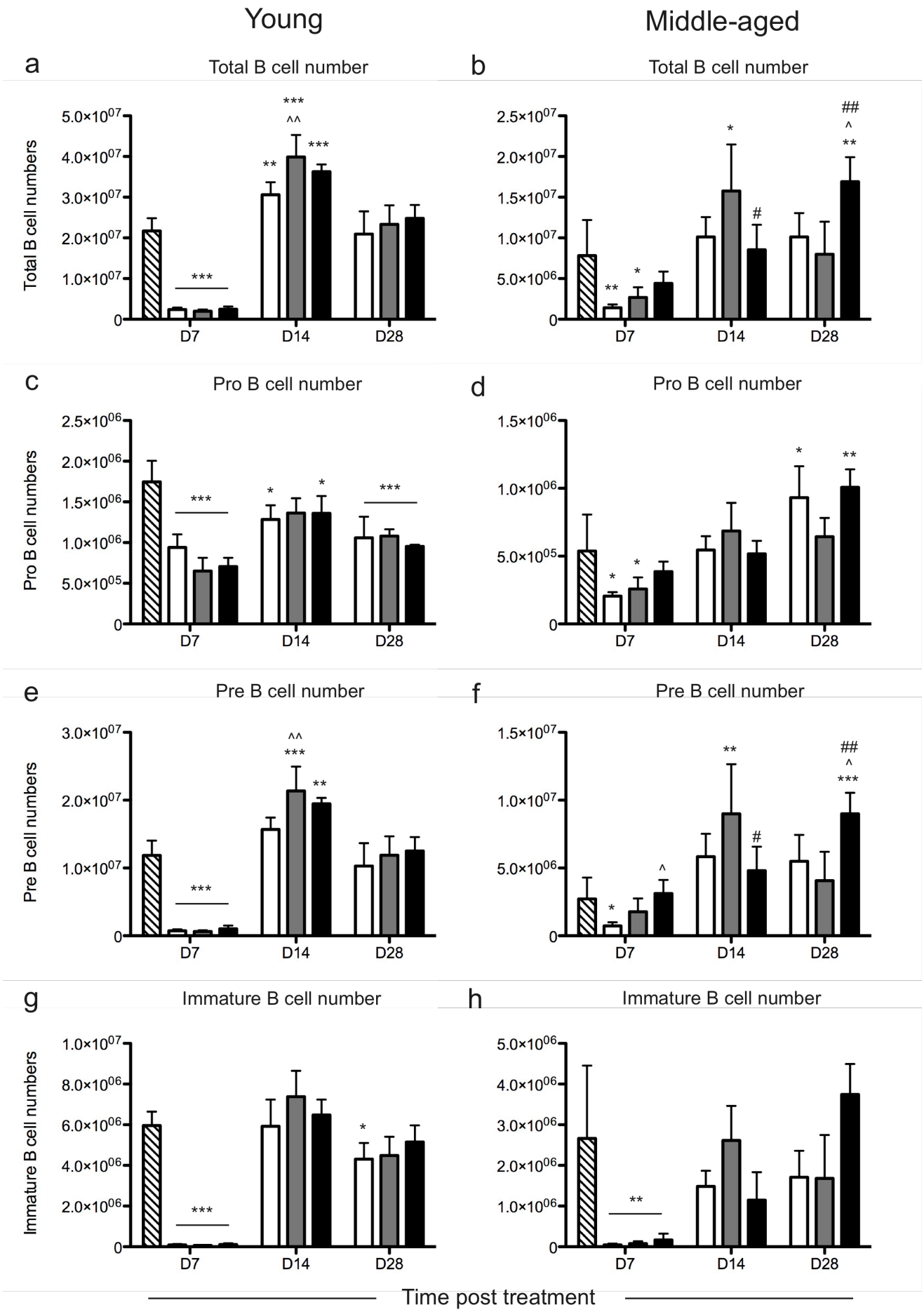
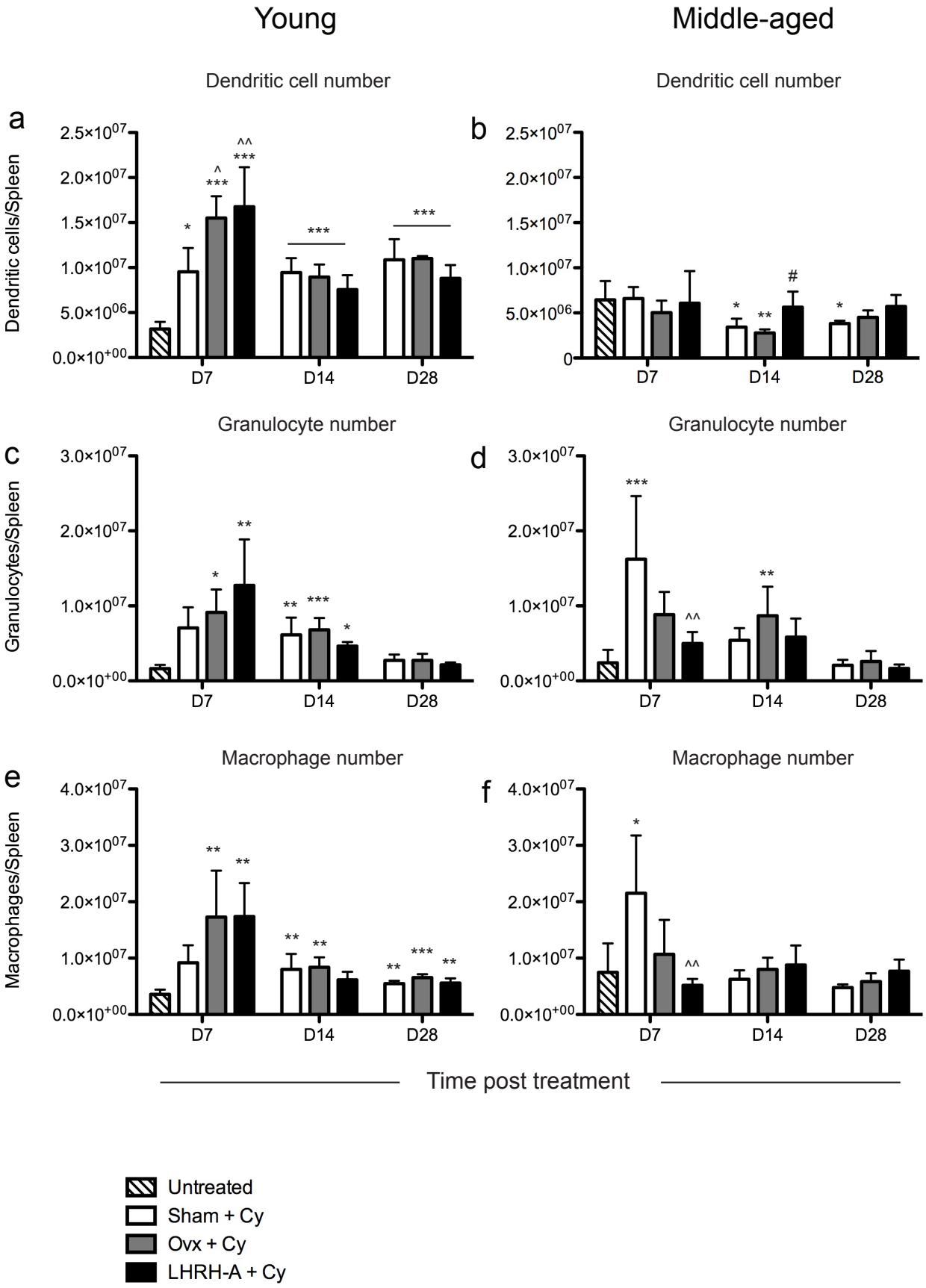

Young
Middle-aged
a
Total B cell number
Total B cell numbers
b
Total B cell number
c
Pro B cell number
Pro B cell numbers
d
Pro B cell number
e
Pre B cell number
Pre B cell numbers
f
Pre B cell number
g
Immature B cell number
Immature B cell numbers
h
Immature B cell number
D7
D14
D28
Time post treatment

Figure 4.6 LHRH-A treatment decreases splenic myeloid cell number in middle-aged but not young female mice following chemotherapy

Young (4-6wks) and middle-aged (9 months) female mice were either administered LHRH-A 21 days before chemotherapy or ovariectomised/sham ovariectomised at day 0. Dendritic cell number in young mice (a); granulocyte number (c); macrophage number (e). Dendritic cell number in middle-aged mice (b); granulocyte number (d); macrophage number (f). Each bar represents the mean $\pm$1 SD of four to six mice. * $p<0.05$, ** $p<0.01$, c.f. Untreated, ^ $p<0.05$, ^^ $p<0.01$ c.f. Sham, # $p<0.05$ c.f. Ovx.


Young
Middle-aged
Dendritic cell number
Dendritic cell number
a
b
Dendritic cells/Spleen
2.5×10^07
2.0×10^07
1.5×10^07
1.0×10^07
5.0×10^06
0.0×10^+00
0
D7
D14
D28
Granulocyte number
Granulocyte number
c
d
Granulocytes/Spleen
3.0×10^07
Macrophage number
Macrophage number
e
f
Macrophages/Spleen
4.0×10^07
Time post treatment
Untreated
Sham + Cy
Ovx + Cy
LHRH-A + Cy

4.4.8 Effect of Sex steroid ablation on splenocyte stimulation following chemotherapy in middle-aged female mice

To determine how the removal of estrogen impacts on the functional capacity of splenocytes following chemotherapy, whole splenocytes were stimulated with either 10ug/mL of anti-CD28 and anti-CD3 or media alone and proliferation measured by the integration of [^{3}H]thymidine (as described in Chapter 2, section 2.5.1). At day 7 after Cy both Ovx and LHRH-A increased proliferation with higher doses on anti-CD3 (Figure 4.7a); LHRH-A also gave higher counts at day 14 (Figure 4.7b). By Day 28 after Cy there were no differences between Ovx and LHRH-A and untreated mice (data not shown).

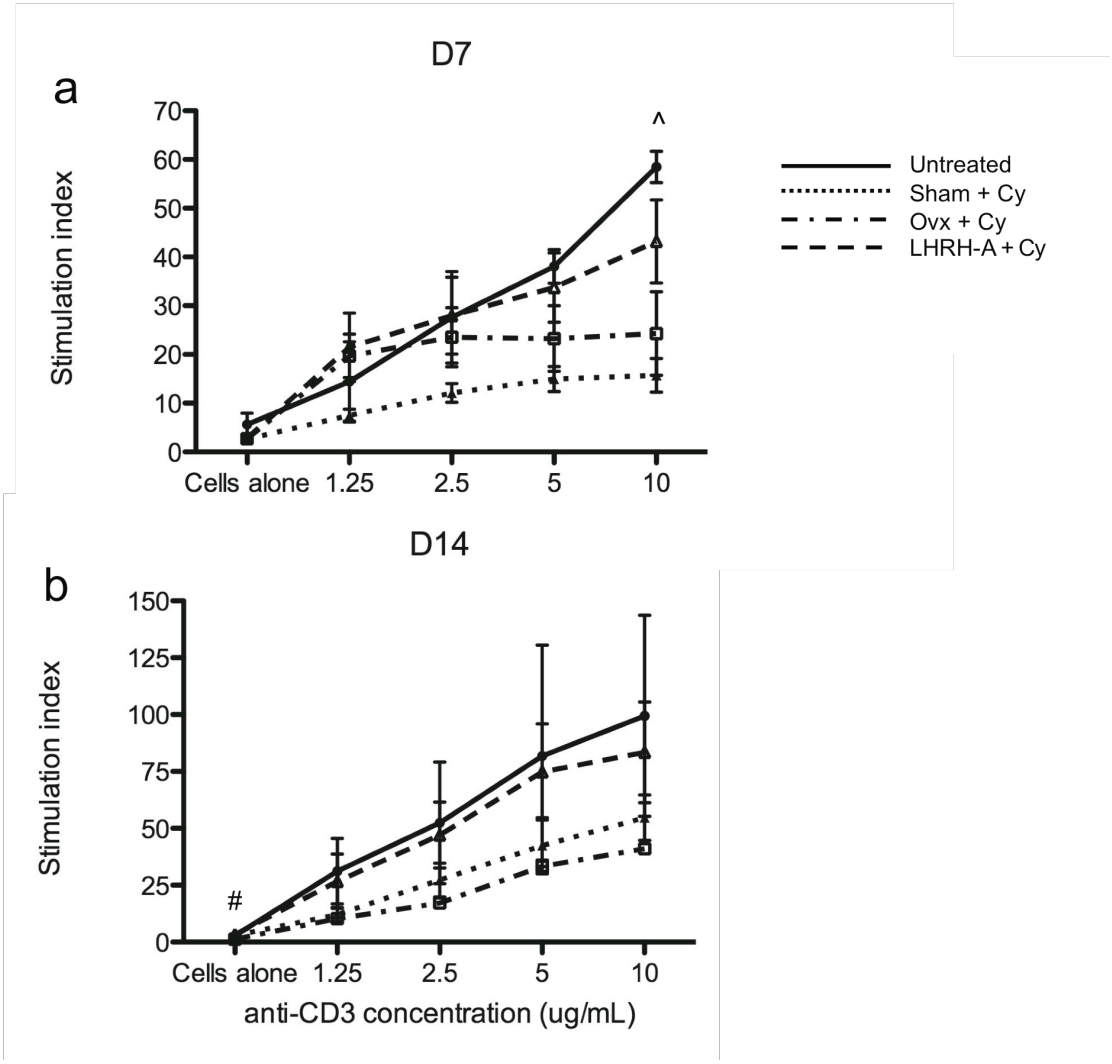


Figure 4.7 Sex steroid ablation decreases splenocyte proliferation when stimulated with anti-CD3/CD28 following recovery from chemotherapy in middle-aged mice

Total splenocytes were extracted from the spleens of middle-aged female mice at days 7 and 4 days following treatment and cultured with anti-CD3/CD28. Proliferation was measured following the addition of [^{3}H]thymidine and is expressed as the stimulation index. Each point represents the mean $\pm$1 SD of two to five mice. * p<0.05, ** p<0.01, *** p<0.001 Sham c.f. Ovx, ^^ p<0.01, ^^^ p<0.001 Sham c.f. LHRH-A, # p<0.05, ## p<0.01 Ovx c.f. LHRH-A.

4.5 Discussion

Following chemotherapy, patients undergo extended periods of immune deficiency. As a consequence they are commonly exposed to a high risk of opportunistic infection and cancer relapse, which are associated with an insufficiency in CD4+ T cells and immune surveillance. In young pre-pubertal patients, CD4+ T cell numbers generally recover to pre-treatment levels within 6 months, while in those of advanced age this time can extend to at least 2 years [14, 15]. As such, approaches to improve the rate of reconstitution have been based on investigations into the major cause of immune aging. As the rate of thymic atrophy accelerates from the time of puberty [16], the suppressive influences of sex steroids have been proposed to play a major role in this process. Further to this, extensive studies from our laboratory and others have shown that cessation of sex steroid production in aged mice, through both surgical and chemical castration, reverts thymic size and function to that of the young, pre-pubertal state [10]. We have also shown that castration of male mice enhances immune reconstitution through an increase in lymphoid precursors following HSCT [7-9]. There have, however, been limited investigations into the removal of sex steroids in females. We demonstrate here that the use of an LHRH-A in young and middle aged females enhances the recovery of thymic cellularity and peripheral CD4+ T cells following cytoablation.

The present study initially confirmed that chemical sex steroid ablation of males increases thymic size [10] and while also true for females, the effect was not as pronounced in both young and middle aged animals [12]. This is not surprising given the sexual dimorphism that exists within the immune system with autoimmunity being more prevalent in females. Investigations into the roles of the major sex steroids, testosterone and estrogen, in immune development and maintenance have revealed key differences between the two. Testosterone is generally regarded as a potent immunosuppressant [17, 18], and is likely to be the key instigator of thymic atrophy with age; exogenous testosterone reverses the thymic enhancing effect of surgical castration [19]. Estrogen on the other hand has been shown to play multiple roles depending on the developmental stage of the immune system, which is further complicated by the multiple sites of estrogen production. During neonatal development estrogen withdrawal in mice is shown to result in retarded thymic

growth while in post-pubertal mice this same treatment increases thymic size and absolute thymocyte numbers [20] thus revealing a bi-phasic, pivotal role for estrogen in both immune development and maintenance.

Within the thymus, LHRH-A treatment showed an enhanced recovery of DP T cells following chemotherapy, followed by a later wave in progenitor regeneration in comparison to ovariectomy. LHRH-A treatment could therefore have an effect additional to estrogen ablation. Firstly, LHRH-A may be acting to protect certain cell subsets from the damaging effects of chemotherapy. Whether this is through direct protection through an unknown mechanism or by increasing the proliferation of these cells to compensate for the loss following chemotherapy is yet to be determined. In a study by Kelly et al, LHRH-A administration in irradiated mice showed an accelerated recovery of thymic epithelial cells rather than a protection from irradiation [21]. This suggests that LHRH-A is improving the thymic microenvironment very early after immune ablation to support or even stimulate an increased rate of thymopoiesis. It is possible that the LHRH-A itself is interacting with LHRH receptors, which are expressed in the thymus, to improve thymic regrowth.

In adult female patients undergoing chemotherapy for breast cancer, it has been previously reported that reconstitution of peripheral CD4+ T cells do not arise from an increase in thymic output (i.e. TREC positive) as is seen in males; a peripheral expansion of CD45RO+ CD4+ T cells makes up the majority of the restored population [14]. Following both ovariectomy and LHRH-A, however, there was an increase in single positive CD4+ T cells in the thymus, indicating a potential increase in naïve T cell output, but this was only reflected in total CD4+ cells in the spleen of middle-aged mice by day 28. Again this 28-day time-point may be too early to detect a significant impact on the periphery but does indicate that peripheral expansion of peripheral T cells is not increased with sex steroid ablation in females. Alternatively, newly expanded CD4+ T cells following chemotherapy in aged females have been shown to have an increased susceptibility to apoptosis [14]. Thus it is possible that any newly generated CD4+ T cells in this study are quickly depleted. Studies of LHRH-A treatment in human patients, however, have

demonstrated an increased thymopoietic capacity with higher TREC and naïve CD4+ T cells in the peripheral blood in a combined study of both male and female patients, again this was over a much longer time frame (first increases were observed from ~6 months [11]

Splenocytes taken from middle-aged females following chemotherapy were more responsive to anti-CD3 and anti-CD28 with LHRH-A treatment in comparison to sham controls and were similar to untreated controls at day 7 (Figure 4.6a) while ovariectomy had little effect. This is contradictory to previous reports that estrogen deficiency increases T cell activation *in vivo,* via IFN-γ [22]. While it could be argued that these previous studies were carried out in young female mice at 12 weeks of age and not aged mice as were used in the current study, further publications including an investigation into post-menopausal women undergoing hormone replacement therapies have shown a possible improvement in immune function with an increase in mitogen-induced T cell proliferation [23, 24]. In addition, sham control showed an increased response at day 28 in comparison with both ovariectomy and LHRH-A treated groups.

Within the bone marrow, we demonstrated a positive impact of sex steroid ablation on lymphoid progenitors, both LSK and CLPs, though temporary, which later translated to increases in maturing B cell numbers. In addition, while sex steroid ablation temporarily increased myeloid cells in young mice, this was not observed in middle-aged mice. Again, while sex steroid ablation has been demonstrated to improve all aspects of immune recovery following depletion [7-10, 25, 26] in males, our results highlight that this is not seen to the same degree in females and that consistent changes are not seen as often with advancing age. However, as these are preliminary findings, repeated in depth investigation is required to make any definitive conclusions.

The removal of estrogen may introduce an increased susceptibility to chronic degenerative autoimmune diseases such as rheumatoid arthritis and osteoporosis such as that seen in post-menopausal women [27, 28]. However as females are more susceptible to autoimmune disease in comparison to males and women with

premature menopause show a reduced risk in comparison to age-matched fertile women [29], it is probable that the removal of estrogen will reduce the risk of onset of other, non-rheumatic autoimmune diseases. Also, LHRH-A can be used to only temporarily ablate estrogen levels and normal immune function can be restored thereafter.

While we have demonstrated estrogen ablation to improve immune recovery in young female mice, the effects are not as obvious in middle-aged mice. In males, castration removes 99% of total testosterone production [30], while ovariectomy only removes one source of estrogen leaving the adrenal glands and fat deposits intact. These peripheral tissues contribute around 10% of total estrogen produced [31]. Although this is a seemingly small proportion, adrenalectomy has been shown to increase thymic weight within 3 days of surgery [32]. The adrenal gland also produces 50% of total testosterone in females [33], thus it is probable that significant levels of immunosuppressive sex steroids remain in ovariectomised mice to hamper thymic recovery. This could be overcome with the combination of ovariectomy and adrenalectomy, but as the adrenal gland also produces glucocorticoids these mice may not recover from the surgical stress. A more clinically applicable option would be the combination of androgen and estrogen receptor blockade with LHRH-A to prevent the association of peripherally produced sex steroids with their respective receptors in immune organs.

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CHAPTER 5 – Results section 3

ANDROGEN RECEPTOR ANTAGONIST IMPROVES LUTEINISING HORMONE-RELEASING HORMONE AGONIST MEDIATED THYMIC RECOVERY FOLLOWING CHEMOTHERAPY

Manuscript information

This chapter is currently a manuscript in preparation with a planned submission date of August 2010. The candidate Melanie Hince was primarily responsible for performing and analyzing experiments as well as writing and editing. Other co-authors contributed technical assistance, interpretation of results and drafting of the manuscript. Detailed contributions are explained in the signed declaration on page IX.

5.1 Abstract

The kinetics of immune recovery following cytotoxic treatments such as chemotherapy and irradiation or chronic viral infections best exemplified by HIV are severely delayed in the elderly. This is a direct consequence of the gradual involution of the thymus with age, mostly attributed to an increase in sex steroid production from the onset of puberty. We have previously published that androgen ablation therapy by surgical gonadectomy or Luteinising Hormone-Releasing Hormone agonist (LHRH-agonist) treatment reverses age-related thymic involution and significantly improves thymic recovery following cytotoxic regimes in both young and aged preclinical animal models. However, the agonistic nature of the more clinically relevant LHRH-agonist treatment induces an initial sex steroid "flare", which causes further atrophy of the thymus about one week post administration, further delaying recovery kinetics. To counteract this, we used an androgen receptor (AR) antagonist in combination with the LHRH-agonist. The AR-antagonist prevented the immunosuppressive effects of the early sex steroid "flare" and following chemotherapy the combination treatment resulted in improved thymic recovery and overall increased thymic size. These studies demonstrate that the combined use of a sex steroid receptor antagonist together with an LHRH-agonist would be a more optimal clinical approach to enhancing immune recovery following immune depletion.

5.2 Introduction

It is widely accepted that the immune system becomes increasingly dysfunctional with advancing age [1, 2]. There is a decline in naïve T cell output [3] leading to a reduced capability to combat newly encountered antigens [2, 4, 5]. Immune aging becomes a particularly significant problem in the recovery from cytoablative therapies such as chemotherapy and irradiation, and following HIV infection, leading to extended periods of severe immunosuppression. Immune recovery of pre-pubertal patients can be achieved within 4-6 months, however in the elderly CD4+ T cells may not return to pre-treatment levels within 2 years [6] or not at all. Patients are thus exposed to a high risk of opportunistic infection and cancer relapse. Enhancement of immune recovery hence represents an important unmet clinical need.

Although sex steroids play are important for immune development and maintenance throughout life [7] they are generally considered to be immunosuppressive [8, 9]. As such, the rise in sex steroids that occurs during puberty has been shown to contribute to, if not precipitate, thymic atrophy, which leads to a decrease in T cell production [3, 10] and progressive disorganisation of the thymic architecture. Supporting this are numerous studies demonstrating that removal of sex steroids leads to reversal of thymic involution and an increase in naïve T cell output - as indicated by an increase in T cell receptor excision circles (TRECs) in peripheral T cells [11-17]. Further studies have shown that administering testosterone increases thymocyte apoptosis [18, 19].

While surgical castration in pre-clinical animal models has dramatic recuperative effects on the thymus and hence immune regeneration – with recovery within one week [12-14, 20], clinically it is obviously inappropriate. A more acceptable clinical treatment can be achieved through the use of luteinising hormone-releasing hormone (LHRH) or LHRH analogues. LHRH-agonists are most common: at sufficiently high doses they override the normally cyclic pulses of LHRH released from the hypothalamus and constantly bind with high affinity to their pituitary receptors. After an initial surge the pituitary becomes “desensitised” leading to loss of gonadotrophin production and ultimately failure to produce gonadal sex steroids.

LHRH thus offers a relatively non-invasive and reversible option for sex steroid ablation, with a return to fertility within 1-2 months of cessation of treatment [21]; indeed this drug is the "standard of care" for many diseases including some forms of prostate and breast cancer. In pre-clinical mouse models we have shown LHRH-agonist enhances immune recovery following allogeneic HSC transplantation (HSCT) [22, 23]. When used to treat human prostate cancer, we found that LHRH-agonist resulted in enhanced recovery of the naïve thymus-derived CD4+ T cell population [14]. Importantly, we have recently shown that LHRH-agonist given to patients undergoing high-dose chemotherapy requiring either allogeneic or autologous HSC transplant (HSCT) induces a reversal of age-related thymic atrophy with increased levels of naïve thymic-derived (TREC+) CD4+ T cells [24]

As successful as it is, the agonistic nature of the drug, however, initially causes an overstimulation of the LHRH receptors, leading to a surge in production of sex steroids. This "flare" occurs before negligible, castrate levels of testosterone are achieved which is usually within approximately 3 weeks of treatment [24]. While following ablation of sex steroids the thymus increases in size both in preclinical animal models and humans, the early flare induces a sudden decline in thymic size, exacerbating the age-induced atrophy and further delaying thymic recovery, particularly in the elderly. This also compounds any damage from chemotherapy or irradiation treatments. Indeed, this may explain why some patients show delayed immune recovery kinetics or at worst, do not appear to respond to LHRH-agonist treatment following bone marrow transplantation [24].

Clearly there needs to be a way in which the impact of the "testosterone flare" can be minimised. We have approached this problem by combining androgen receptor (AR) antagonists with LHRH-agonist to prevent any detrimental effects on the thymus by the initial surge in sex steroids. In this study, using an LHRH-agonist, we first demonstrated that there was an androgen flare, which caused thymus collapse. We then showed that the combination of an LHRH-agonist with an AR antagonist both prevents the immunosuppressive effect of an androgen flare and enhances thymic recovery following chemotherapy treatment with cyclophosphamide. This approach

offers an improvement in thymic regeneration that is clinically applicable and highly relevant to those undergoing depletion regimes for malignancies.

5.3 Experimental design

5.3.1 Androgen Receptor Antagonist dose response

Androgen receptor antagonist (AR-antagonist) was fed orally for seven days at 16mg/Kg, 40mg/Kg or 75mg/Kg mouse body weight as described in Chapter 2, section 2.2.3. Thymi were then harvested at day 7 and analysed by flow cytometry. Total thymocyte proliferation was measured by Ki67 expression.

5.3.2 Androgen Receptor Antagonist administration in combination with LHRH-agonist

AR-antagonist was fed orally for seven days pre LHRH-agonist intramuscular injection. AR-antagonist administration was continued throughout the experimental timeline. Mice were culled and thymi harvested at days 0, 7, 14, 21 and 28 and analysed by flow cytometry. Serum testosterone concentration was analysed by RIA (described in Chapter 2, section 2.2.3) at each of these time points.

5.3.3 Androgen Receptor Antagonist administration in combination with LHRH-agonist and Cyclophosphamide

AR-antagonist was fed orally for seven days pre LHRH-agonist intramuscular injection. A split dose of 200mg/Kg Cyclophosphamide (Cy) was administered at days -1 and 0. AR-antagonist administration was continued throughout the experimental timeline. Mice were culled and organs harvested at days 0, 1, 3, 5, 7, 14, 21 and 28 and analysed by flow cytometry.

5.4 Results

5.4.1 Androgen Receptor Antagonist increases thymocyte proliferation and total cellularity

Currently, AR blockade is used in combination with LHRH-agonist and either irradiation or chemotherapy as a standard therapy for advanced prostate cancer [reviewed by 25]. Using previously published doses that achieved androgen blockade and reduction in seminal vesicle size [26, 27], we assessed the impact of AR-antagonists on thymic regeneration. While relatively side effect free, AR-antagonists in high doses can cause liver abnormalities [28, 29]. We therefore selected three minimal doses of AR-antagonist (16, 40 or 75mg/kg; the clinical dosage is generally 50mg daily) to be administered into young male mice three times per week. Thymi were harvested and analysed at day 7 and thymocytes were labelled with anti-Ki67 to determine any increase in proliferation; Ki67 indicates exit from the G0 phase of the cell cycle. Whilst total thymic cellularity increased only with the highest dose administered (Figure 5.1a), all doses increased the number of cycling cells as determined by the increase in Ki67 expression compared to untreated controls (Figure 5.1b), indicating successful binding to the AR. As the lowest dose used (16mg/Kg) still showed an increase in Ki67 expression, it was used for all future experiments to prevent the sex steroid flare.

5.4.2 AR-antagonist prevents the immunodepleting effects of an LHRH-agonist induced androgen flare

To assess whether the combination treatment of AR-antagonist and LHRH-agonist is able to prevent the immunodepleting effects of an early androgen flare, we administered an AR-antagonist three times per week orally with a 3-month depot intra-muscular injection of an LHRH-agonist and assessed changes within the thymus.

As expected, the LHRH-agonist treatment resulted in a substantial increase in serum testosterone levels at day 7 (as measured by RIA) when compared to untreated controls at day 0 (Figure 5.2a). As expected the addition of an AR-antagonist did not alter serum testosterone levels since they are influenced by the LHRH-agonist, however it did impact significantly on the total thymic cellularity. Following LHRH-

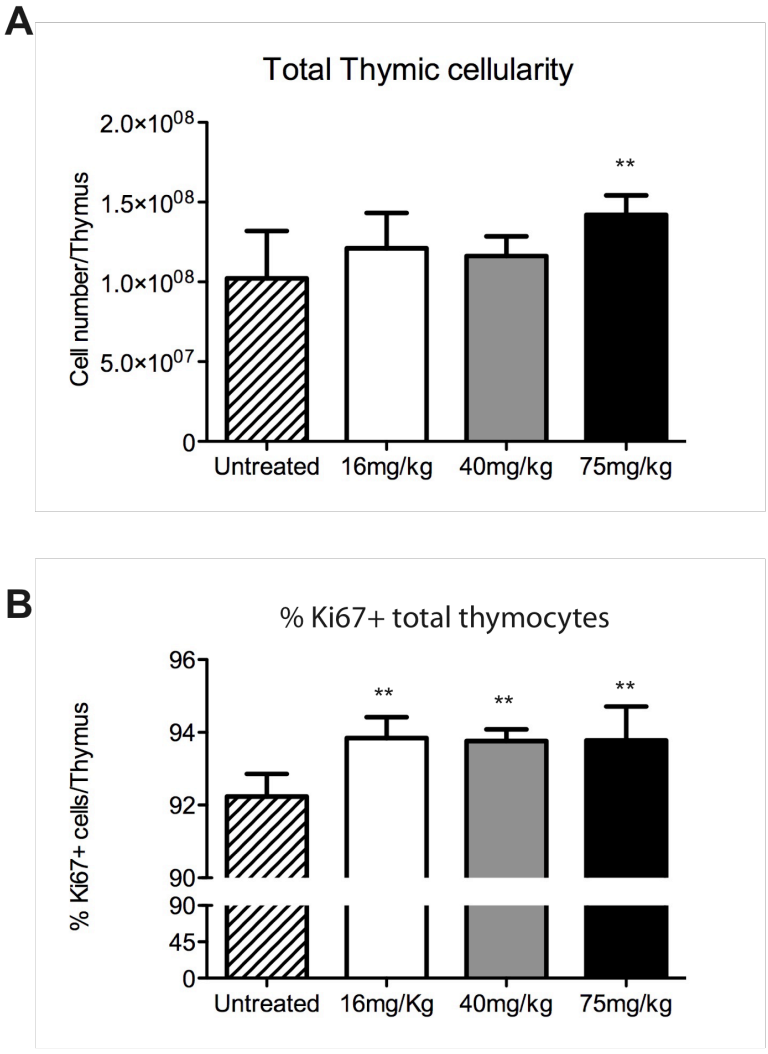
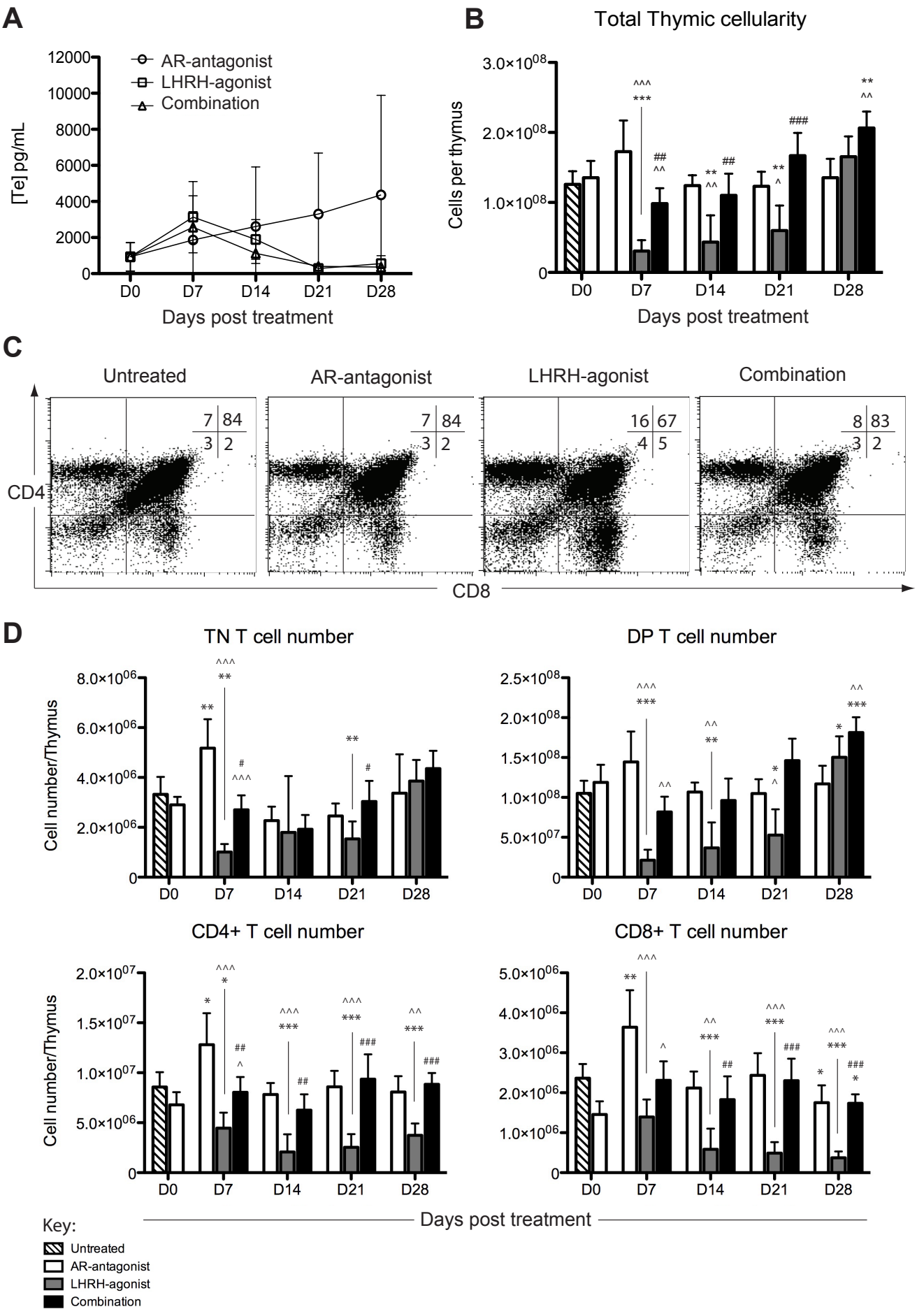


Figure 5.1 Thymic cellularity and proliferation was increased following AR-antagonist administration

Mice were fed an AR-antagonist ever second day at three different doses. Total thymic cellularity 7 days post treatment (a). Proportion of Ki67 expressing thymocytes per total thymus at 7 days post treatment (b). Each bar represents the mean $\pm$ 1 SD of five mice. ** $p \leq 0.01$ c.f. Untreated.

Figure 5.2 AR-antagonist prevented the immunodepleting effect of an androgen flare

Mice were administered an AR-antagonist, a LHRH-agonist or a combination. Serum testosterone concentration was measure by radioimmunoassay (a). Each point represents the mean $\pm$ 1 SD of three to five mice. Total thymic cellularity following treatment (b). Representative dot plots of TN, DP, SP CD4+ and SP CD8+ thymocytes seven days following treatment (c). TN, DP, SP CD4+ and SP CD8+ thymocyte numbers following treatment (d). Each bar represents the mean $\pm$ 1 SD of five mice. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ c.f. Untreated, ^ $p<0.05$, ^^ $p<0.01$, ^^^ $p<0.001$ c.f. AR-antagonist, # $p<0.05$, ## $p<0.01$, ### $p<0.001$ c.f. LHRH-agonist.


A
[Te] pg/mL
AR-antagonist
LHRH-agonist
Combination
Days post treatment
B
Total Thymic cellularity
Cells per thymus
Days post treatment
C
Untreated
AR-antagonist
LHRH-agonist
Combination
CD4
CD8
D
TN T cell number
DP T cell number
CD4+ T cell number
CD8+ T cell number
Cell number/Thymus
Days post treatment
Key:
Untreated
AR-antagonist
LHRH-agonist
Combination

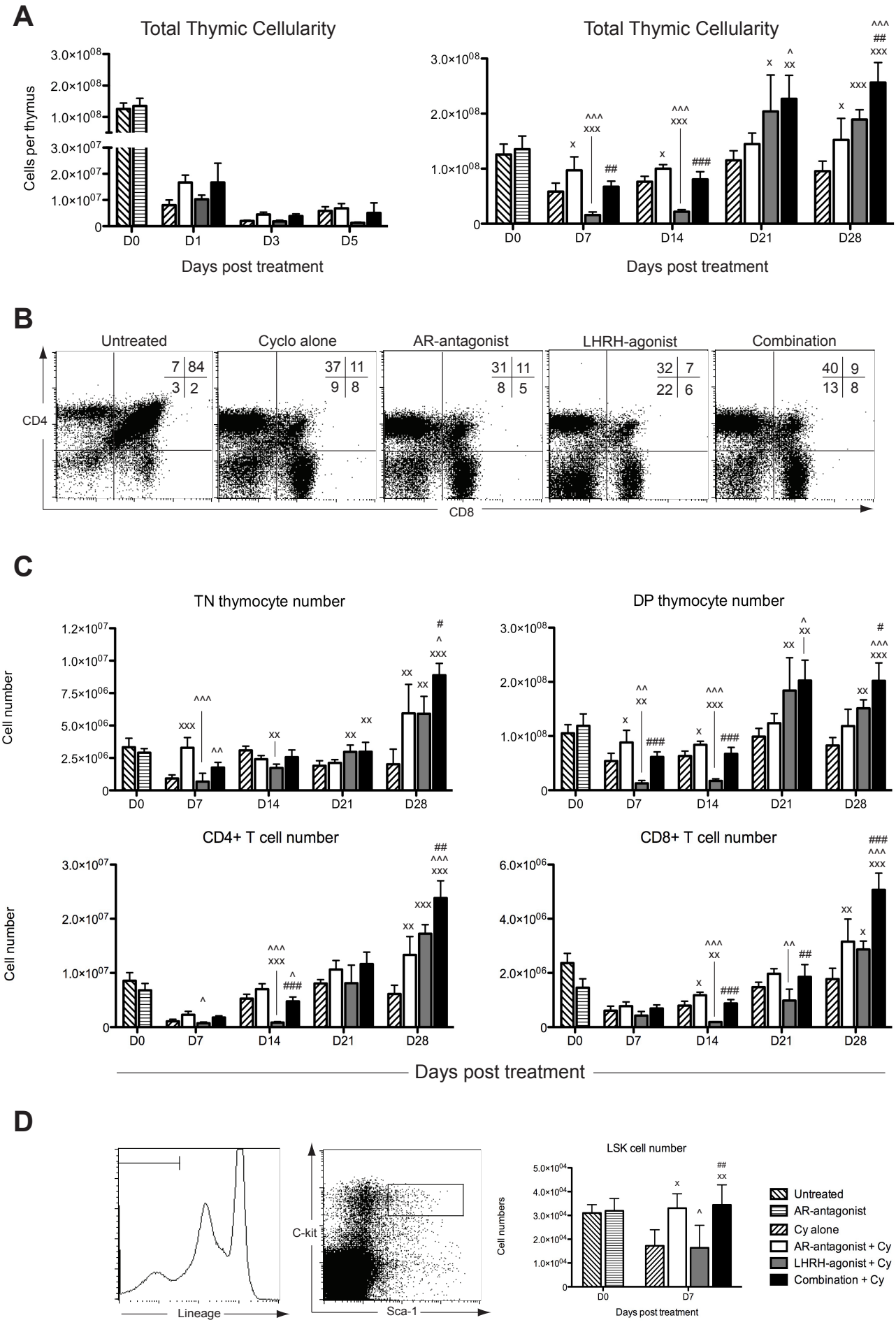
agonist treatment alone, the testosterone flare resulted in a significant decline in total thymic cellularity at day 7 (Figure 5.2b) and this flare was absent with treatment of AR-antagonist alone as indicated by the unaffected total thymic cellularity. Importantly, the combination treatment was able to prevent the initial reduction in cellularity induced by the LHRH-agonist. The subset most affected by LHRH-agonist treatment was the DP T cell subset, with a significant reduction in proportion and number at day 7 following treatment (Figure 5.2c). This is consistent with DP thymocytes being immature and well known for their sensitivities to corticosteroids [30]. A reduction in cell number can also be seen in triple negative (TN), CD4+ single positive (SP) and CD8+ SP T cell subsets, but not to such a significant degree as in the DP T cells (Figure 5.2d). Proportions were unchanged with AR-antagonist treatment alone, although there appeared to be a brief increase in TN, CD4+ SP and CD8+ SP T cell numbers at day 7 following treatment. The combination treatment of LHRH-agonist and AR-antagonist prevented the androgen flare-induced depletion of all T cell subsets, with cell number remaining unchanged in comparison to untreated samples at days 7, 14 and 21 (Figure 5.2d). By day 28, the combination of the LHRH-agonist and AR-antagonist resulted in an overall increase in total thymic cellularity when compared to untreated and AR-antagonist treated controls (Figure 5.2b). This was reflected in DP T cell numbers (Figure 5.2d), which were also increased with LHRH-agonist treatment in comparison to untreated controls.

5.4.3 AR-antagonist improves LHRH-agonist mediated thymic recovery following chemotherapy

We hypothesised that the initial thymic depletion induced by LHRH-agonist reduces the recovery capacity of the thymus following secondary depletion such as chemotherapy and that the use of an AR-antagonist would counter this. To investigate this, we administered cyclophosphamide as the chemotherapeutic agent, to groups treated with an AR-antagonist alone, LHRH-agonist alone or a combination of the two. Cyclophosphamide alone resulted in maximal thymocyte depletion between days 2 and 4 [13]. We found herein that total thymic cellularity was relatively unchanged with all treatments when compared to cyclophosphamide treatment alone (Figure 5.3a) during early time points (days 1-5). Chemotherapy-induced depletion by proportion was evident predominantly in the DP T cell subset

Figure 5.3 Combination of AR-antagonist and LHRH-agonist improved thymic and lymphoid progenitor recovery following chemotherapy-induced depletion

Mice were administered an AR-antagonist, a LHRH-agonist or a combination of the two followed by a split dose over two days of cyclophosphamide. Total thymic cellularity following treatment at early and later time points (a). Representative dot plots of TN, DP, CD4+ and CD8+ thymocytes three days post treatment (b). TN, DP, SP CD4+ and SP CD8+ thymocyte numbers following treatment (c). Representative flow cytometry plots of lymphoid progenitors (LSK), defined as lineage-, Sca-1+, c-kit+ by flow cytometry, and LSK cell numbers following treatment (d). Each bar represents the mean $\pm$ 1 SD of five mice. ^{x} $p<0.05$, ^{xx} $p<0.01$, ^{xxx} $p<0.001$ c.f. Cy alone, ^ $p<0.05$, ^^ $p<0.01$, ^^^ $p<0.001$ c.f. AR-antagonist + Cy, # $p<0.05$, ## $p<0.01$, ### $p<0.001$ c.f. LHRH-agonist + Cy.


A
Total Thymic Cellularity
Cells per thymus
Days post treatment
Total Thymic Cellularity
Days post treatment
B
Untreated
Cyclo alone
AR-antagonist
LHRH-agonist
Combination
CD4
CD8
C
TN thymocyte number
DP thymocyte number
CD4+ T cell number
CD8+ T cell number
Cell number
Days post treatment
D
Lineage
C-kit
Sca-1
LSK cell number
Cell numbers
Days post treatment
Untreated
AR-antagonist
Cy alone
AR-antagonist + Cy
LHRH-agonist + Cy
Combination + Cy

in all treated groups (Figure 5.3b, $p<0.001$) at day 3 and was further reduced with LHRH-agonist treatment in comparison with chemotherapy alone ($p<0.01$), indicating that thymocyte depletion induced by LHRH-agonist is additional to that induced by chemotherapy.

Numerically, chemotherapy-induced depletion of all maturing subsets (Figure 5.3c) at day 7 following treatment with further depletion evident following LHRH-agonist treatment. The rate of recovery of total thymocytes improved at day 7 with the use of the AR-antagonist in comparison to chemotherapy treatment alone (Figure 5.3a). This was reflected in the TN and DP T cell subsets, such that numbers were similar to untreated controls. Total thymocyte numbers recovered to untreated levels by day 21 with chemotherapy treatment alone. With the combination treatment of AR antagonist and LHRH-agonist, all thymocyte subsets were increased in number in comparison to LHRH-agonist treatment alone and by day 28 numbers had increased in comparison to all treatment and control groups, indicating an additional effect with the combination treatment.

To investigate the effect of androgen blockade on the recovery of lymphoid progenitors, we analysed the lineage-ve, Sca-1+ve, c-kit+ve (LSK) population of cells at day 7 following treatment. As expected, chemotherapy induced depletion of this subset in comparison to untreated controls (Figure 5.3d). The LHRH-agonist did not further reduce this population, however, those mice being treated with the AR-antagonist, showed LSK numbers similar to untreated levels at day 7 (Figure 5.3d). These results indicate an AR antagonist dependent improvement in the recovery of LSK numbers or alternatively, protection against the damaging effects of the chemotherapeutic agent.

5.4.4 Combination of AR-antagonist and LHRH-agonist improves peripheral T cell recovery following chemotherapy

Sex steroid ablation has already been shown by our lab to improve peripheral CD4+ T cell recovery [23, 24] however it may still be affected by the testosterone flare. We thus determined whether the AR-antagonist could further improve peripheral T cell

recovery. Following chemotherapy alone, splenic CD4+ T cells were decreased in comparison to untreated controls at days 7 ($p<0.01$) (Figure 5.4).

Treatment with LHRH-agonist and Cy significantly reduced both CD4+ and CD8+ T cell numbers at day 14 in comparison to untreated controls, which had recovered by day 21. The combination of AR-antagonist and LHRH-agonist again prevented the decline in both T cell subsets caused by the androgen flare and at day 28 CD4+ T cell number was increased in comparison to both the chemotherapy alone treatment group and untreated controls ($p<0.01$).

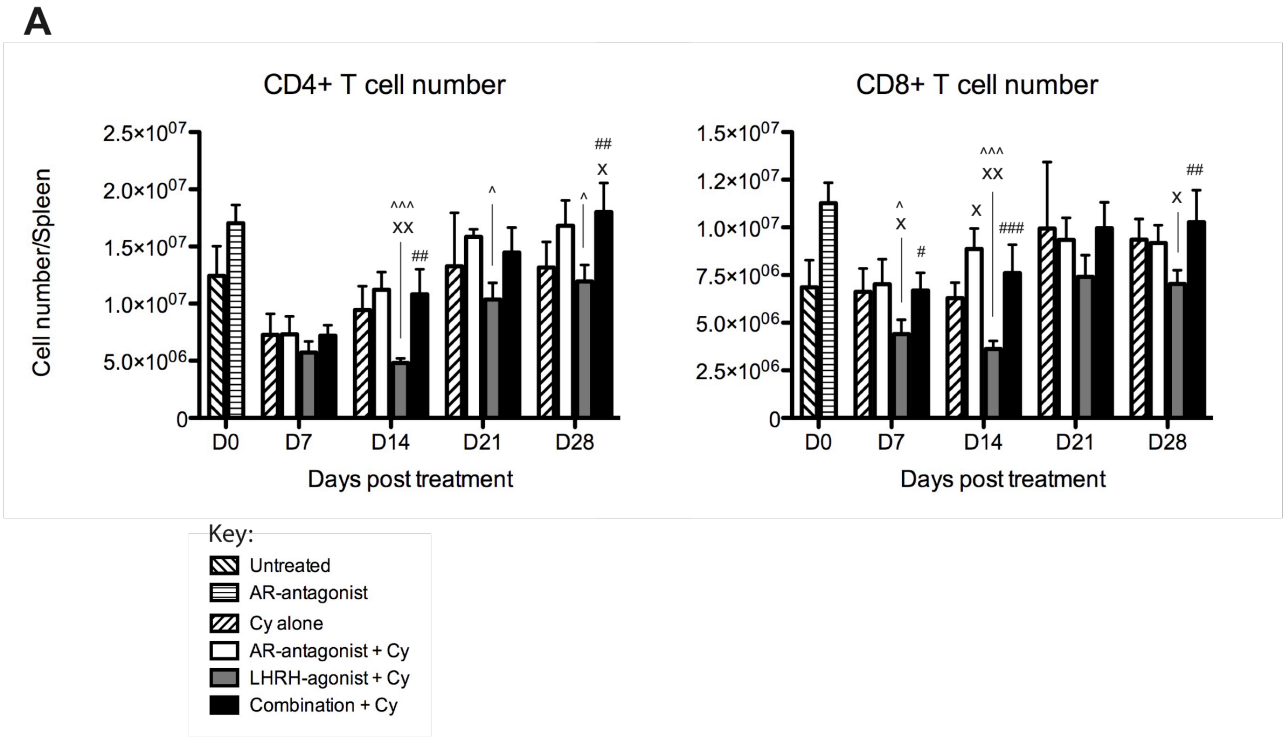


Figure 5.4 Peripheral CD4+ T cells are increased with combination AR-antagonist and LHRH-agonist treatment following chemotherapy

Mice were administered an AR-antagonist, a LHRH-agonist or a combination of the two followed by a split dose over two days of cyclophosphamide. CD4+ and CD8+ SP splenic T cell numbers following treatment (a). Each bar represents the mean ± 1 SD of five mice. ^{X} p<0.05, ^{XX} p<0.01 c.f. Cy alone, ^ p<0.05, ^^^ p<0.001 c.f. AR-antagonist + Cy, # p<0.05, ## p<0.01, ### p<0.001 c.f. LHRH-agonist + Cy.

5.5 Discussion

Following cytoreductive therapies such as irradiation and high dose chemotherapy, patients, are exposed to extended periods of immunosuppression primarily due to a lack of CD4+ T cells. Consequently they are at risk of opportunistic infection and to cancer relapse [31]. Recovery takes up to 6 months in children but is greatly delayed in adults, taking at least 2 years or more. During this time, the involuted thymus must recover both structure and function to support thymopoiesis and T cell output. We have previously shown that ablation of sex steroid production by surgical castration has a profound rejuvenating effect on thymus structure and function following age- or therapy-induced atrophy [12, 13, 20, 24]. An obvious caveat to these studies is their impractical clinical utility. However, LHRH-agonist treatment can achieve the same level of sex steroid reduction but is completely reversible and consequently has been used for nearly 30 years for the treatment of sex steroid exacerbated diseases such as prostate cancer. Not only has it been very successful, but is also has a long safety profile. Recently, we undertook clinical trials using an LHRH-agonist to reduce the time to haematopoietic and immune recovery in cancer patients undergoing high dose chemotherapy with haematopoietic rescue afforded by autologous and allogeneic HSCT The trial showed marked improvement of thymopoiesis, importantly manifest as a significant increase in naïve (TREC+) CD4 T cells [24]. While this treatment was successful for ~60% of the patients, not all responded and the period of immunosuppression was still extensive (~6 months).

Whilst more clinically acceptable, LHRH as an agonist (the most common therapeutic form) leads to an initial but temporary overproduction of gonadal hormones, termed a ‘sex steroid flare’. These sex steroids are strongly immunosuppressive and in males testosterone administration induces apoptosis through the interaction with androgen receptors expressed on thymocytes and peripheral T cells [32] as well as on thymic epithelial cells (TECs) [33]. We thus hypothesised that the androgen flare induced by LHRH-agonist treatment, would exacerbate the natural aging-induced thymus atrophy and cause irreversible damage, retarding the thymic recovery.

In this study we have indeed demonstrated that the surge in testosterone following LHRH-agonist administration results in a significant decline in thymic size at day 7, most notably within the CD4+ CD8+ DP T cell subset in line with previous studies [18]. Once castration equivalent (negligible) serum testosterone levels are achieved at day 21, thymic cellularity begins to recover to pre-treatment numbers. A logical clinical approach therefore, would be to overcome this initial thymic involution before chemotherapy; the more damaged the thymus, the longer it will take for recovery to immune competence. Thus, we aimed to prevent the immunodepletive effects of the sex steroid flare following LHRH-agonist treatment by co-administering an androgen receptor (AR) antagonist.

Interestingly, both low and high doses of AR-antagonist administration slightly increased serum testosterone concentrations, although not significantly. This increase has been previously reported in several experimental and clinical models [34, 35]. Testosterone production is controlled through a negative feedback mechanism involving the neuroendocrine LHRH pathway. When androgen receptors are blocked, testosterone production is increased due to the lack of feedback. While this would be a significant issue in androgen-dependent conditions such as testosterone sensitive prostate cancer, when used in combination with the LHRH-agonist, serum testosterone levels were still reduced to negligible levels by day 21 and were not increased in comparison to LHRH-agonist treatment alone between days 7 and 21.

Studies into the use of AR-antagonists for both early and advanced prostate cancer have reported instances of liver abnormalities with the continued use of high doses. A minimal dose was therefore selected to reduce the risk of additional side effects. Whilst this dose induced thymocyte cycling, as determined by an increase in cells exiting G0 phase of the cell cycle (Ki67 expression), when used alone, no increase in total thymic cellularity was evident throughout the experimental time course. Given the high expression of ARs on TECs [33] it is possible that the AR-antagonist activated the AR+ TECs to increase their number, but not total thymocyte numbers. Until further analysis of the thymic epithelial subsets is completed, this remains speculative. Importantly, when combined with the LHRH-agonist, there was no

apparent loss in thymic cellularity due to the sex steroid flare evident in the LHRH-agonist alone group at day 7. The combination treated groups maintained thymic cellularity at untreated levels until day 28 after which it increased further. Thus the co-administration of AR-antagonist with the LHRH-agonist in the current study appears to protect the thymus from the thymocyte depleting effects of a sex steroid flare induced by the LHRH-agonist.

Chemotherapy impacted dramatically on total thymic cellularity, affecting all T cell subsets. Treatment with LHRH-agonist did improve the extent of thymic recovery to levels greater than the age-matched untreated controls. However, the initial damage by the testosterone surge delayed recovery. By co–administering AR-antagonist with the LHRH-agonist, thymic atrophy induced by the sex steroid flare was negated and total thymic cellularity at day 28 was greater than LHRH-agonist alone.

Interestingly, while the AR antagonist did not increase total thymic cell number in otherwise untreated mice, when used in chemotherapy-induced immune depleted mice, cellularity was significantly greater at day 7, suggesting a direct effect on thymic recovery by androgen signalling blockade. Greater saturation of the AR antagonist may enhance the recovery kinetics even further to counteract the chemotherapy-induced damage.

Following surgical castration of young male mice in combination with allogeneic HSCT or chemotherapy, LSK (Lineage-ve, Sca1+ve, c-kit+ve) cell numbers were significantly increased within 6 weeks [11, 20]. LSKs have previously been shown to be sensitive to sex steroids, with a selective depletion of these cells within the bone marrow following estrogen administration [36]. In the current study we demonstrated that within one week of chemotherapy-induced depletion of LSK cells, numbers were increased with both AR-antagonist treatment alone and in combination with LHRH-agonist. This improved recovery of LSK cell numbers in the presence of the AR antagonist, may indeed contribute to an increase in T cell progenitor entry into the thymus, supporting the increase in thymopoiesis and eventual T cell output. Finally we show an increased peripheral CD4+ T cell number following the combination of AR antagonist and LHRH-agonist within 4 weeks of

treatment in comparison to either treatment alone. While LHRH-agonist alone did not induce an initial depletion of CD4+ T cells, the combination of AR-antagonist and LHRH-agonist improved cell number when compared to LHRH-agonist alone by day 21 ($p<0.05$) (data not shown).

In conclusion, the current study demonstrates the use of an AR antagonist to both reduce the immunosuppressive effects of a sex steroid flare following LHRH-agonist treatment and improve thymic recovery following chemotherapy-induced depletion. This could simply occur through the direct prevention of testosterone-receptor interactions, thus removing the atrophic effects that sex steroids have on most lymphocyte subsets and increasing thymocyte proliferation as has been previously shown following surgical castration [13]. TECs, the essential supporting cells for thymopoiesis, also express ARs and are therefore likely to have a role in thymic involution [33]. It is therefore likely that the sex steroid ablation has a direct impact on TEC recovery, both in cellularity and in their production of cytokines and growth factors crucial to T cell survival, differentiation and proliferation. The data presented here suggests a clinically relevant use for combination androgen blockade therapy for the improvement of thymic recovery following depletion.

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CHAPTER 6

GENERAL DISCUSSION AND THESIS CONCLUSIONS

That the immune system degrades with advancing age is a significant clinical issue. This is characterised by a decline in thymic structure and function with a subsequent reduction in naïve T cell output [1]. Consequently, in adults and the aged there is an increased incidence of opportunistic infections, new and relapsing cancers a reduced capacity to respond to newly encountered antigen, such as vaccines, yet paradoxically there is an increase in autoimmune disease [2-4]. In addition to this, there is a markedly reduced capacity with age to recover immune competence following HIV infection and common cytoablative therapies such as chemotherapy, radiotherapy. Patients suffer extensive periods of immune insufficiency and this manifests as high morbidity and mortality from opportunistic infection and cancer relapse [reviewed by 5]. Hence it is of paramount importance to improve both lymphopoiesis and immune function in not only such severe clinical conditions but also prophylactically in the elderly.

Sex steroids have long been known to be immunosuppressive. Given the temporal association of the acceleration of thymic atrophy with puberty, several studies have thus investigated the impact of sex steroid intervention on thymus function and hence immunity Withdrawal of sex steroids through both surgical gonadectomy and chemical means (using LHRH and its analogues) reverses age-related thymic atrophy [6, 7]. Importantly, we have shown it accelerates immune recovery in male mice following chemotherapy and both allogeneic and autologous HSCT [8-12]. In the present study, we have demonstrated the use of an LHRH-A to both reverse immune aging and improve immune recovery following chemotherapy, in female mice. In both males and females, the impact of sex steroid ablation is clearly age-dependent with middle-aged mice not responding as well as young mice. A surprising finding was that chemical castration did not appear to have any impact on thymic recovery in aged or elderly females. Given the fact that LHRH-As have been used very successfully in human females, one logical explanation is that the physiology of estrogen production and even function (in terms of the immune system) may be different in mice. In this regard, in the clinical trial of LHRH in HSCT cancer patients, some of the best responders in terms of thymus rejuvenation (generation of naïve, TREC+ CD4+ cells) were older females [13]. The kinetics and degree of response to LHRH-A therapy was also dependent on the strain of mice,

suggesting an impact of genetic background. Hence, the results of this thesis suggest the need to combine LHRH-A treatment with other therapies to improve both estrogen blockade and hopefully immune recovery.

While LHRH-As are used as a standard care in both pre- and post-menstrual females with some breast and endometrial cancers, they are generally combined with estrogen receptor (ER) analogues [14]. This is done for two main reasons; firstly, estrogen is produced not only by the gonads, but also by aromatisation of circulating testosterone by the adrenal glands and adipose tissue, which would not be depleted with LHRH-A treatment. Secondly, the agonistic action of this drug induces an initial overstimulation of the LHRH receptors within the pituitary. Subsequently, there is an overproduction of LH and FSH followed by a surge in gonadal sex steroid production. This sex steroid "flare" has major clinical consequences in cases of sex steroid sensitive conditions such as AR+ prostate cancers and ER+/PR+ breast cancers. The combination of ER-analogues and LHRH-As have shown an additive effect for the treatment of breast cancer [15].

The ER analogue used throughout the present studies, Tamoxifen, is classified as a selective estrogen receptor mediator (SERM). As such, Tamoxifen is reported to act as antagonist within breast tissue and an agonist within bone and uterine tissue [14, 16]. LHRH-A induces an initial decline in total thymic size in young female mice; therefore we aimed to demonstrate that the combination of LHRH-A and Tamoxifen would prevent this effect. Interestingly, we observed the opposite in young, otherwise untreated, female mice as well as following cyclophosphamide-induced thymic depletion. While Tamoxifen has previously been reported to induce thymic atrophy in castrated male rats [17, 18] indicating it is acting as an agonist in this organ, similar to bone, our findings suggest that this treatment regime would prolong the period of immune insufficiency and indicates the clinical need for true ER blockade. Aromatase Inhibitors (AIs) have overtaken Tamoxifen as the most active agent for estrogen receptor blockade however side effects, such as the occurrence of endometrial and uterine cancer [14], are increased. Alternatively, a study comparing Tamoxifen with a pure anti-estrogen, EM-800, has shown the latter to be more potent and without the undesirable estrogenic activation of endometrial cells [16]. These are thus logical candidates for applying to immune system regeneration.

Similarly in males, LHRH-A therapy is combined with androgen receptor (AR) blockade to achieve maximal sex steroid ablation and is a standard therapy for AR+ prostate cancer. The early sex steroid flare induced by LHRH-As can promote the growth of AR+ prostate cancer cells, and in regards to the immune system, induces apoptosis of androgen sensitive cells, most notably DP thymocytes [19]. Once circulating testosterone decreases to negligible levels at day 21, the thymus (in young animals) is able to recover to pre-treatment numbers. The combination of AR-antagonist and LHRH-A both prevented thymic depletion induced by an androgen surge and thus improved thymic recovery following chemotherapy in young male mice. Also, since early lymphocyte progenitors, LSKs, were increased in number with this combination therapy, this could indicate an increased progenitor cell entry into the thymus, or intrathymic proliferation, to promote thymopoiesis. Given that this combination therapy is the standard care for prostate cancer, it should be easily transferrable to a new patient group requiring immune system rejuvenation

While it might seem obvious to administer LHRH-antagonists in the place of LHRH-A, these drugs require constant contact with LHRH receptors to prevent sex steroid production. If natural LHRH were to displace the antagonist, sex steroid production by the gonads would return. This can occur clinically, thus LHRH-As remain the drug of choice for sex steroid sensitive conditions for prolonged therapy. LHRH-As, although they induce the initial sex steroid flare, safely maintain desensitised LHRH receptors even after the drug has lost contact [20]. Also, given that LHRH-As are even more successful at improving thymic size than surgical castration (unpublished observations), it is possible that it also interacts with LHRH receptors expressed intrathymically to have an additive, thymus endogenous, effect. While we were not able to demonstrate a direct impact of LHRH on thymocyte proliferation, LHRH administration has previously been shown to induce thymic hypertrophy [21] as well as prevent pregnancy-induced atrophy [22]. Hence, if LHRH-antagonists are to be considered for the purpose of immune recovery, further investigation is needed.

Sex steroid ablation therapy to improve immune recovery following significant depletion is not without clinical consequences. Sex steroids are obviously important hormones in regards to correct bodily function. Estrogen is essential for the preservation of bone density, which thus declines in post-menopausal women, and

are vasoprotective in pre-menopausal and early post-menopausal women. In men, loss of testosterone (and estrogen) is linked with a loss of libido, increased fat and reduced muscle mass, depression, anaemia, insulin resistance and heart disease [reviewed by 23]. While sex steroid ablation therapy would increase the risk of these conditions, it would preferentially only be administered until immune function has risen to a sufficient level. LHRH-A studies in humans indicate this period to be 6-9 months [13, 24], rather than 2 years, if at all, in untreated adults, but if combined with sex steroid receptor blockade, it is possible that this time would be reduced further. Following cessation of treatment, fertility and sex steroid production returns within a few months [25].

Intuitively this intervention at the level of sex steroids could also be combined with molecules known to have a positive impact on the immune system, in particular the thymus. These include growth hormone [26, 27], KGF/FGF7 [24, 28, 29], IL7 [30-34] and FLT3 ligand [35, 36]. Such combinations have already been shown to be additive [24], reducing the required treatment time for immune rejuvenation, and no doubt degree of enhancement will be of major clinical significance.

While the precise mechanisms of sex steroid ablation-induced thymic recovery are unclear, much data exists to identify both the supportive thymic and bone marrow microenvironments as important targets. Removal of sex steroids results in a definite switch between stromal cell production of immunosuppressive cytokines, such as TGF-β, and pro-thymopoietic cytokines and growth factors, such as CCL25, KGF and IL-7 [8, 9, 37-39]. These changes within the bone marrow allow for increased production of lymphoid progenitors followed by release and entry into the thymus [12, 40].

In conclusion, sex steroid ablation using common, standard of care, clinically approved drugs such as LHRH-A, with more than two decades of safety profiling, is a clinically viable method of regenerating immune function in a wide variety of patients suffering profound immunologically based diseases, many of whom not only suffer from a major loss in quality of life, but also risk mortality from prolonged periods of immunosuppression. In addition, we have identified maximal androgen blockade to enhance this process. Given the complex interaction between the immune system and hypothalamic-gonadal axis, these studies open the door to

investigation into hormone combination therapies for great improvement of immune recovery in the future.

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CHAPTER 7

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Review

The role of sex steroids and gonadectomy in the control of thymic involution

Melanie Hince, Samy Sakkal, Katerina Vlahos, Jarrod Dudakov, Richard Boyd [*,1], Ann Chidgey [1]

Monash Immunology and Stem Cell Laboratories (MISCL), Level 3, Building-75, Monash University, Wellington Road, Clayton, Melbourne 3800, Australia

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Abstract

A major underlying cause for aging of the immune system is the structural and functional atrophy of the thymus, and associated decline in T cell genesis. This loss of naïve T cells reduces adaptive immunity to new stimuli and precipitates a peripheral bias to memory cells against prior antigens. Whilst multiple mechanisms may contribute to this process, the temporal alliance of thymic decline with puberty has implicated a causative role for sex steroids. Accordingly ablation of sex steroids induces profound thymic rejuvenation. Although the thymus retains some, albeit highly limited, function in healthy adults, this is insufficient for resurrecting the T cell pool following cytoablative treatments such as chemo- and radiation-therapy and AIDS. Increased risk of opportunistic infections and cancer relapse or appearance, are a direct consequence. Temporary sex steroid ablation may thus provide a clinically effective means to regenerate the thymus and immune system in immunodeficiency states.

Keywords: Thymus; Aging; Thymic involution; Sex steroids; Gonadectomy; LHRH

1. Introduction

That the immune system deteriorates with age is the basic dogma used to explain the increased incidence of cancer, opportunistic infections and poor vaccine responsiveness in the elderly. At the cellular level this is best exemplified by a profound reduction in thymic size, T cell content and number of recent thymic emigrants (RTE) [1–3]. As this atrophy, in many mammalian species, is most evident from puberty onwards, it has been aetiologically linked to an increase in sex steroid production. In combination with peripheral T cell clonal expansion and gradual exhaustion of the naïve pool with progressive antigen contact, the decrease in thymic output leads to a gradual bias towards the memory phenotype [3–5]. Under normal circumstances there are sufficient pre-existing T cells to provide immune protection but when there is any severe insult to the immune system, such as chemotherapy/irradiation treatment or from chronic viral infections, best exemplified by HIV, the impact of the decline in thymic output becomes critical. The delay in T cell recovery in such conditions, including following hematopoietic stem cell transplant (HSCT), leads to an increased risk of opportunistic infections and disease relapse [6]. Consequently there is a higher rate of mortality in adults compared to pre-pubertal individuals [7]. In addition, as the number of virus-specific cells is reduced in the elderly, the ability to clear infections as well as respond to vaccines, is impaired [8,9]. The need to replenish the peripheral immune repertoire and improve long-term immune reconstitution after HSCT has lead to a more recent focus on strategies to regenerate thymic tissue. Reversing the damage caused by sex steroids forms one of the major, logical strategies. This review will examine the endocrinology of thymic atrophy—the impact of sex steroids on the immune system and the use of gonadectomy to enhance immune reconstitution.

* Corresponding author. Fax: +61 3 9905 0780.
E-mail address: richard.boyd@med.monash.edu.au (R. Boyd).
[1] These authors contributed equally to this work.

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2. Age-associated thymic atrophy

The thymus undergoes significant structural involution with age. However, whilst there is a significant loss in cellularity, many studies have documented that the aged atrophic thymus remains functional, albeit limited [10]. A loss in thymic epithelial cells, an increase in the perivascular space (PVS)—which is infiltrated by adipocytes [11] and mature single positive (SP) T cells [12], and an increase in cystic cavities [13,14] are all characteristic of the atrophic thymus. The remnant functional areas of the cortical and medullary microenvironments are still capable of generating T cells albeit at greatly reduced levels [15,16], and specific subsets of the medulla—such as major histocompatibility complex class II (MHCII) high expressing cells [17], are likely to still impart tolerance mechanisms [18,19]. Qualitative changes in the microenvironment are also evident [20]; expression of pro-inflammatory cytokines by the thymic stroma such as TGF-β and IL-6 are increased with age, possibly contributing to an increase in apoptosis of thymocytes [3]. In terms of T cell precursors, the early T lineage progenitors (ETPs) (Lin^- $CD44^+$ $CD25^-$ $CD117^{hi}$ $CD127^{low/neg}$), currently the earliest known committed T cell progenitor in the thymus, are reduced in both frequency and number with age in mice [21,22]. In addition, a recent study demonstrated ETPs from aged mice proliferate at a reduced rate when seeded into thymic lobes compared to those from young mice [22]. These alterations may be due to a reduction in both the number of progenitors entering the thymus, a loss in production of stromal derived cytokines and growth factors affecting proliferation, and/or intrinsic changes in the progenitor cells. The ultimate effect is that there is a decrease in the number of recent thymic emigrants, indicated by a reduction in expression of T cell receptor excision circles (TRECs), with age [3].

In the periphery, homeostatic mechanisms maintain total T cell numbers, which are kept relatively constant with age [23,24], however, there is a major shift towards a memory phenotype [25,26], their expansion facilitated by the decline in naïve T cell output [27,28]. As a consequence there is a reduced variability within the TCR repertoire in the elderly, resulting in compromised adaptive immune responses to newly encountered antigen [29]. In addition, $CD4^+$ T cells from aged mice have a reduced response to antibody stimulation compared to those from young mice [30–32]. These effects of aging upon the activation of T cells have been partly attributed to defects in the Raf-1 and JNK-dependent protein kinase pathways [32]. Overall, the age-related reduction in naïve cells and in functional capacity of existing cells, leads to an increased rate of mortality due to infectious diseases such as pneumonia and urinary tract infections in the elderly [7,9,33,34].

3. Understanding the endocrinology of thymic atrophy

The link between the reproductive system, its role in the production of certain endocrine hormones such as sex steroids, and immunity was first reported in 1904, in a study on castrated cattle, which were found to have enlarged thymi [35]. Both castration and ovariectomy-induced hypertrophy of the thymus, while testosterone re-administration suppressed this process. Subsequent investigations have revealed that imbalances in sex steroids may be detrimental too, with many immune abnormalities, such as an increase in autoimmune factors, occurring during periods of decreased fertility, spontaneous abortion, endometriosis and abnormal births as well as in hypogonadic patients [36–38].

3.1. Luteinizing hormone-releasing hormone and its receptor

The hypothalamic decapeptide LHRH (luteinizing hormone-releasing hormone) is an important focus of this review as it controls immune functions both directly and indirectly [36,39]. Cyclic impulses of LHRH are able to activate the hypothalamus–pituitary–adrenal/gonadal axis, resulting in synthesis and release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH), which then control production of gonadal sex steroids [36] (Fig. 1). However, LHRH may also form part of an intracellular signalling system in the thymus. LHRH binding to its receptors on thymocytes and thymic stromal cells (but also

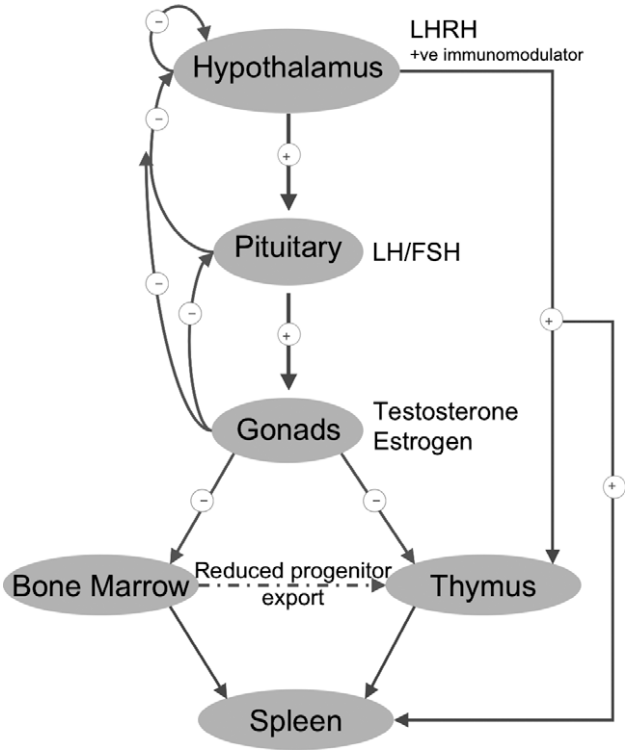

Fig. 1. Simplified diagram of the HPG axis and the immunomodulatory effects of its components. LHRH is produced by the hypothalamus and transported to the anterior pituitary where it controls the production and release of LH and FSH. These then govern production of gonadal steroids, which have negative effects on the thymus and bone marrow while LHRH itself is a positive immunomodulator. These effects are supported by experimental data obtained mainly from mice.

splenocytes and possibly NK cells) activates downstream protein kinase C translocation resulting in an upregulation of IL-2 receptor expression [40], potentially improving the proliferative capacity of these cells. However, studies on endometriosis patients treated with LHRH-agonists suggested LHRH could also have a negative functional impact by reducing the cytotoxicity of NK cells [41,42].

LHRH receptors have been observed on immune cells such as blood lymphocytes, mast cells, thymocytes and splenocytes [43–46] and are thought to play a role in the maturation of these cell types—suggesting a role for LHRH in early development as well as immune function and maintenance. A study involving treatment of male neonatal monkeys with an LHRH antagonist resulted in postnatal reduction in cortical cellularity and $CD8^+$ T cells within the thymus, comparative to vehicle-treated counterparts [47] while treatment of thymocytes with a LHRH-A reduced their proliferation [48] thus indicating a probable direct interaction between LHRH and the thymus. Similarly, neonatal ERα knockout mice have a reduced thymic cellularity also implicating estrogen to positively regulate early thymic development [49]. To date, however, LHRH has thus been shown more commonly to act as a positive immunomodulator in regards to the thymus [36] but clearly this could be age dependent. While generally considered to be very safe clinically, side effects reflecting its role in bone development were evident in studies of prostate cancer patients treated with LHRH analogues, where bone density was greatly reduced within a few months of treatment [50,51]. However, this is more likely to be due to the loss of androgen than a direct effect of LHRH.

3.2. Sex steroids

The most compelling evidence for the role of sex steroids in thymic atrophy and diminished immune function with age, is that surgical or chemical gonadectomy (using LHRH analogues) reverses age-related thymic atrophy [52–54] and improves lymphocyte recovery following HSCT and chemotherapy [55,56] while the re-administration of androgen or estrogen prevents this reconstitution [57–59].

3.2.1. Androgens

The immunosuppressive role of testosterone is clearly evident in the rapid (within hours) induction of thymic atrophy after its administration and cessation of testosterone production by castration of males causing thymic hypertrophy [60–62]. Indeed, Olsen and colleagues have demonstrated the importance of both androgens and estrogens in the process of thymic involution [63]. Studies in transgenic mice that were defective in androgen expression on either the hematopoietic compartment or the stromal compartment, suggested that the direct interaction of sex steroids with their receptors on thymic stromal cells [63,64] were responsible for thymic degeneration. However, testosterone administration also causes apoptosis of $CD4^+CD8^+$ double positive (DP) thymocytes both *in vitro* and *in vivo* at least in part by inducing the release of TNF-α [61,65,66]. Thus, TNF-α is a major mediator of sex-steroid-induced apoptosis of DP thymocytes and possibly other thymocyte subsets to a lesser extent. The effects of enhanced androgen on the peripheral immune system have not been investigated as extensively as the thymus or bone marrow. However, while testosterone administration decreases total splenic cellularity, there is no obvious preferential depletion of a specific lymphoid subset [61].

3.2.2. Estrogens

Estrogen-induced thymic degeneration post-puberty in females occurs through a number of mechanisms [59] and is much more complex than that seen in males, not the least of which is the fact that estrogen is produced at multiple sites including the adrenals and adipose tissue. Increases in estrogens, such as during pregnancy, puberty or exogenous estrogen administration, enhance thymic atrophy [58,67] characterised by reduction in DP thymocytes and increases in the proportion of $CD4^+$ and $CD8^+$ single positive T cell subsets (SP) [68,69]. As with androgen administration in males, the SP T cells also decrease in number, however there is an opposing shift in the $CD4^+$ to $CD8^+$ ratio. With androgen replacement, $CD8^+$ T cells are increased relative to $CD4^+$ T cells, whereas this is the opposite with estrogen replacement [58,70–72] thus favouring a T helper cell phenotype, although it is unknown why this dimorphism occurs. In the bone marrow, elevated estrogen levels directly inhibit the production of thymic precursors such as $Flt3^+$ LSK ($Lineage^-$ $Sca\text{-}1^+$ $c\text{-}Kit^+$) and in the thymus—ETPs and $CD4^-CD8^-$ double negative (DN) subsets [73]. This implies that the rise in female sex steroids impacts not only on the thymus, but also the proliferation of hematopoietic lineage cells—including potential thymocyte progenitors in the bone marrow. For the B lymphoid compartment, estrogen causes a major loss of preB cells and their progeny [74,75]. Re-administration of estrogen to ovariectomised females also causes a decline in B lymphopoiesis [76]. Estrogen administration, however, does not seem to as robustly affect the peripheral immune organs as it does the thymus and bone marrow. Following even high-dose estradiol treatment of male and female rats, total splenic cellularity was only slightly reduced but this does not preclude functional loss [67,77]. There was, however, a significant decrease in total splenic T cells ($CD3^+$) in both sexes [77]. It is thus possible that T cells are the only major cell type negatively affected in the spleen by estrogen treatment, although destruction of B cell progenitors in the marrow ultimately leads to a loss of mature B cells [74,75,78].

3.3. Distribution of sex steroid receptors

It is clear that increases in circulating androgen and estrogen levels have major consequences on both central

and peripheral immune organs and their cellular subsets. Identifying sex steroid receptors on immune cells can help assess whether these effects are direct and/or indirect. Originally, the consensus was that androgen receptors (ARs) are only present on thymocyte and thymic stromal cell subsets [79,80] but not bone marrow or splenic lymphocytes. In the thymus, direct binding of androgen to ARs on DP thymocytes results in the apoptosis of these cells. However, what role these receptors play when in the presence of elevated androgen is still unclear as the only specific subset yet identified to decrease following androgen administration are DP thymocytes. With the advent of more sophisticated analytical techniques such as RT-PCR, further ARs have been described in the spleen and bone marrow [81,82]. In the bone marrow, androgens act via intracellular receptors in stromal cells to release TFG-β, which in turn suppresses B cell development [83]. In the spleen, both intracellular and "unconventional" surface ARs have been identified on both $CD4^+$ and $CD8^+$ T cells [81,82,84]. Thus, as testosterone administration promotes proliferation of these subsets, it can be assumed that there is a direct interaction with androgens.

Estrogens and estrogen receptors (ER) play seemingly conflicting, yet major roles in normal thymic development and maintenance in both males and females. ERs are present on many immune cell subsets including thymocytes, splenic T cells and splenic B cells [68,84]. In a study of ERα deficient male mice, the thymus and spleen did not develop to the same size as their wild type counterparts, indicating the importance of ERα in promoting initial thymus development [85]. The second ER (ERβ) was only discovered in 1995 and has since been shown to also be necessary for normal immune function in studies on ERβdeficient mice [86,87]. As with ovariectomised mice, ERβ deficient mice developed myelogenous hyperplasia and splenomegaly within the first 18 months post-partum. Post-puberty, however, activation of ERα and ERβ through exposure to estrogen induces significant atrophy of the thymus [58] and atrophy of myelogenous cells [88] implying both ERs (and estrogen) to be essential for homeostasis of the immune system. Estrogen thus seems to have both negative and positive influences on thymus development, differing according to the age of the individual—estrogen appears to promote thymus growth early in life but converts to a suppressive effect presumably around puberty. This suggests shifts in the signalling pathways with development since the receptors seem the same; this is an important issue to resolve particularly with the current endeavours to manipulate sex steroids to influence immune system status.

4. Sexual dimorphism in the regulation of the immune system

Autoimmune diseases are a major health issue as they are estimated to afflict 5% of the population [89]. Despite the fact that it is well-known that females are more affected than males [90] to date there has been no consensus hypothesis as to why this is so. There are, however, two plausible explanations: that estrogen is less immunosuppressive than testosterone (which may be directly at the level of the effectors, or through lower levels of T regulatory cells being generated), or that estrogen positively influences autoreactive cells. Neither of these is conclusive yet. Furthermore, during pregnancy and post-partum, disease severity is reported to fluctuate with hormone levels, with symptoms of pre-existing autoimmune diseases such as multiple sclerosis subsiding during pregnancy, only to return post-partum [91] while in men with rheumatoid arthritis, elevated estrogen levels within the synovial fluid may be the cause of the inflammatory state [92,93]. However, this is not true for all kinds of autoimmune diseases [94]. Animal studies have shown that in the presence of estrogen, serum IgG levels are higher in females [95] compared to pre-pubertal individuals. Furthermore, estrogen has been reported to have stimulating effects on the immune system [96]; females produce more vigorous cellular and humoral immune reactions and are more resistant to certain infections compared to males [97]; again this would be consistent with fewer T regulatory cells in females but this is still unresolved (see below).

Alternatively in the absence of LHRH or sex steroids, such as female hypogonadal (HPG) mice, when estrogen was reintroduced, serum IgG levels were suppressed [98]. Thus, the immunomodulatory effect of estrogen in potentially increasing autoantibodies may be linked to the presence of LHRH but it is more likely than in this instance of hypogonadism, the immunosuppressive function of (exogenous) estrogen is simply being more easily revealed. Alternatively gender differences in autoimmune susceptibility maybe due to increased extrathymic T cell proliferation in response to elevated estrogen levels, to possibly counter the decline in intrathymic T cell proliferation. This has been shown to occur in the liver of young male mice administered estrogen [99]. Hence, this highlights a sexual dimorphism in the immune response and implies a role for sex hormones in immune regulation and autoimmunity and for estrogen at least in both a stimulatory and inhibitory capacity.

The increase in the frequency of immune disorders and autoimmune diseases with age has been linked closely with quantitative and/or qualitative defects of cells within the regulatory arm of the immune response. One major player is a subset of $CD4^+$ T cells; the $CD4^+CD25^+$ T regulatory cells (Tregs), which express the forkhead/winged helix transcription, factor Foxp3 [100–102]. Tregs follow the normal path of thymic development and are selected as part of the natural $CD4^+$ T cell repertoire. However, they appear late in ontogeny and are evident in the periphery around 3 days after birth in mice and humans [103]. With the thymus being integral to Treg generation, it is conceivable that thymic atrophy will lead to similar age-related changes as we see with conventional T cells.

There is little evidence on the effects of immunosenescence and sex steroids on Tregs. Contrary to expectations,

human studies assessing Tregs have reported an increase in peripheral blood $CD4^+CD25^+$ and $CD4^+CD25^{hi}$ Tregs in the elderly compared to young adults [104]. This numerical increase does not explain why the elderly have an increased risk of autoimmune disease and therefore raises the issue of whether such cells are still functionally suppressive in older age. Similar to that observed with conventional T cells during age-related thymic involution, the Treg TCR repertoire could also be severely restricted due to homeostatic proliferation of pre-existing Tregs [105–108].

Sex steroids have been implicated to directly influence Treg number and function. As mentioned earlier, androgen and estrogen receptors are expressed in primary lymphoid organs and on mature peripheral B and T cells in mice and humans [109]. It was unknown whether these receptors are present on Tregs, however indirect evidence suggests they do possess estrogen receptors, with increase in circulating estrogen either during pregnancy [110] or experimentally administered [111,112], leading to the expansion and increased functional suppression of $CD4^+CD25^+$ Tregs. It cannot be excluded that this is an indirect effect, however a more recent study confirmed the presence of ERα by immunoblotting of resting human Treg cell lysates [113]. This was quite a significant finding as it explains at least in part the mechanisms by which some autoimmune diseases regress during times of increased sex hormone production.

5. Pregnancy and thymic atrophy

Atrophy of the maternal thymus during pregnancy has been explained as an evolutionary phenomenon designed to prevent rejection of the foetus [114]. Maximal involution occurs around gestational day 18.5 in mice, indicated by a fivefold reduction in thymocyte cellularity [115]. These studies show a block in T cell development evident at the early pre-T cell/DN2 ($CD3^-CD44^+CD25^+$) stage in mice, with all thymocyte subsets (ETP, DN2-4, DN, DP, $CD4^+$ and $CD8^+$) reduced in number. Since there are no major shifts in proportions of the subsets, the reduced thymopoiesis would be consistent with inhibition at the level of either reduced immigrant thymocyte progenitors of their intrathymic proliferation. We have also found a proportional loss in thymic stromal cells, including cortical and medullary epithelial cells, yet no proportional loss on the immature $CD4^+CD8^+$ thymocytes. (Sakkal et al., in preparation). This contrasts the well-known effect of corticosteroids to be primarily on DP thymocytes [116,117], which we have confirmed (Sakkal et al., in preparation). Thus from this we can infer that glucocorticoids are perhaps not primarily responsible for pregnancy related thymic involution or are not produced at levels that affect the most glucocorticoid sensitive population of cells in the thymus [118]. We have also shown that corticosteroids reduce both the cortical and medullary thymic epithelium in a manner that is different to pregnancy (Sakkal et al., manuscript in preparation).

Whilst, we would expect similar results during pregnancy in humans, this has not been established given the difficulties in obtaining maternal thymi. Indeed, there is a great paucity in our understanding of thymopoiesis in the human maternal thymus with the only reliable clinical data assessing leukocyte numbers in blood, which only provide detail about thymic output, and not early thymocyte development or stromal cell interactions.

In addition to testosterone and estrogen, thymic involution has been associated with elevated levels of two other major hormones—progesterone [115,119,120], and corticotropic-releasing hormone (CRH) [121,122]. Further, it has also been described that glucocorticoids regulate hormonal expression by suppressing the production of pituitary luteinizing hormone and ovarian progesterone and estrogen, resulting in estradiol resistance by target tissues [121], presumably also limiting the extent of thymic atrophy. Since there was no proportional loss of DP cells, this again contrasts to the effects of glucocorticoids such as dexamethasone [123]; Sakkal et al., in preparation). Thus the role of glucocorticoids in pregnancy-induced thymic involution appears to be more of a regulatory one, rather than direct. Experiments using progesterone receptor deficient mice found that its expression on stromal cells was essential for thymic involution [120]. In this study, progesterone receptor deficient thymi grafted under the kidney capsule of normal pregnant mice showed no involution. Whilst progesterone has clearly been implicated in maternal thymic involution, estrogen has also been suggested to contribute. Injection of 17-beta estradiol causes a reduction in ETP and DN2-4 populations [73] associated with reduced proliferation [115], suggesting the rising estrogen levels during pregnancy impact on the early thymic precursors.

Whilst many of the studies which assess thymocyte and stromal involution during pregnancy are primarily in rodent models [124,125], they may not be directly applicable to humans although given the highly conserved nature of these hormonal interactions it is very likely to be equivalent. It is important to note that the pregnancy-induced thymic involution is reversible, with complete regeneration observed at four weeks post-partum [126], thus making pregnancy a useful model of thymic regression and regeneration. The major changes in thymic function may also be linked to the need to prevent fetal immune rejection although this is still a poorly understood connection, which requires rigorous experimentation.

6. Other reproductive hormones

Other hormones associated with the reproductive system such as LH and FSH also play small but significant roles in immune maintenance. The presence of LH receptors on T cells has been documented [127] but in regards to direct interaction of LH and FHS with the thymus and/or other lymphoid components, no significant studies have been published.

Post-menopausal women suffer long-term withdrawal of progesterone and estrogens. Although there are other hormonal pathways affected during this stage of life, it has been suggested that the decline in total lymphocytes is due to the lack of progesterone/estrogen [128]. While female hypogonadal (HPG) mice do not show a significant decline in thymic cellularity with age [129,130], the absence of LHRH as well as estrogen in these mice suggests that the inhibitory effect of estrogen post-puberty relies on other members of the gonadal axis such as LHRH. There are conflicting reports on the role of sex steroids and LHRH in age-related thymic atrophy in males. In male HPG mice there is evidence of an increase in total thymic cellularity compared to their wild type counterparts [129]. Another group, however, observed no difference in thymic cellularity in both young and old HPG mice [131] although it was not stated what gender of mice was used in this case. Interestingly, it does appear that thymic atrophy still occurs in the HPG male mouse regardless of the absence of sex steroids [129,131] supporting the involvement of other age related mechanisms, such as a decline in bone marrow derived progenitor cells or loss of LHRH which may have a direct stimulatory effect on the thymus and immune system. This is in fact a very important observation because it has great relevance to defining the approaches necessary to reverse thymic atrophy clinically.

7. Other mechanisms of thymic involution with age

Sex steroids are unlikely to be solely responsible for the global immune degradation with age. Changes in the production of other hormones and growth factors, as well as intrinsic changes to HSCs that impact on their proliferative and differentiation potential, are likely to be involved.

7.1. Alterations in the bone marrow niche

Age-related deterioration of lymphopoiesis is not restricted to the thymus with significant effects also observed in the bone marrow. Deterioration in the ability of the bone marrow stroma to support lymphopoiesis has been reported in mice, particularly concerning the reduced production of IL-7 [132] and using in vitro B-cell culturing techniques, it was found that B-cell progenitors have a significantly reduced responsiveness to IL-7 with age [133]. Similarly within the committed lymphoid progenitors, there is a large reduction in the number and frequency of the common lymphoid progenitor-1 (CLP1) as well as a decline in their proliferative potential and their ability to respond to IL-7 [134,135]. Further studies have shown enhanced mobilisation of stem cells in response to granulocyte-colony stimulating factor (G-CSF) in old mice compared to young mice, which may be due to a reduced adhesion of these stem cells to the bone marrow stroma with age [136]. Long term hematopoietic stem cells (LT-HSCs), defined as LSK flk2^{-}CD34^{-}, increase in frequency with age [137] and undergo progressive loss of differentiation and maturation potential [137–139]. With each self-renewal, HSCs undergo replicative stress and their reconstitution ability is reduced as shown in serial transplantation experiments of purified LSK cells, which in some ways mimic the natural aging process [138]. Changes in signalling with the stromal microenvironment may contribute to an apparent, but still controversial, bias away from the lymphoid lineage towards the myeloid lineage as well as age related intrinsic changes [137] and this could lead to the reduced frequency and number of ETPs evident with age [21].

Translating these findings into the human setting, it has been found that, in contrast to the mouse, there are fewer circulating CD34^{+} HSCs [140] and following administration of G-CSF, aged individuals mobilise fewer CD34^{+} cells into the periphery [141]. Functionally it has been found that CD34^{+} cells from aged individuals have a reduced ability to develop into T-cells [142] as well as reduced self-renewal [143] and proliferative potential [144]. Therefore the impact of aging in the bone marrow can result in a reduction in progenitor proliferation, lymphoid lineage differentiation and export of hematopoietic progenitor cells, very likely contributing to the loss in total thymic cellularity.

7.2. Changing profiles of cytokines and growth factors

Changes in cytokine profiles in the thymic microenvironment have been associated with both a reduction in thymopoiesis and productive rearrangement of the TCR. This is most likely due to a reduction with age in the transcriptional regulator, E2A, which is involved in the rearrangement of the TCRβ genes thus leading to incorrect selection [145,146]. Indeed there are major changes in expression of growth factors produced by thymic epithelial cells such growth hormone (GH) and IGF-1, which promote thymopoiesis as well as the cytokines IL-13, IL-2, IL-9, IL-10, IL-14 and IL-7 [145–147]. In addition there is a reduction in the transcription factor Foxn1, an essential regulator of cortical thymic epithelial cell development [145,148]. In contrast, there are increases in suppressive cytokines such as LIF, OSM and IL-6, all of which induce thymic atrophy following their exogenous administration, which admittedly may be supra physiological [146,149]. Together, these results indicate that thymic atrophy with increasing age could also be due to both a reduction in the promotion of thymocyte maturation as well as an increase in T-cell apoptosis, which has been observed by many [150–152]. It is still unclear, however, whether these changes are completely independent of sex steroids or are a secondary effect from a functional impact of sex steroids on the stromal cells themselves.

8. Sex steroid ablation to reverse thymic aging

Numerous studies over the years have demonstrated enhanced thymic growth and immune reconstitution

following sex steroid ablation, be it surgical or chemical-induced androgen or estrogen deprivation. As mentioned earlier, surgical castration studies began with Henderson [35] where castrated cattle were shown to have significantly larger thymic size and weight compared to bulls and heifers [35]. Many studies have since confirmed this effect in several animal models along with an increase in thymopoiesis [153–159]. It is unclear what long-term health benefits this may translate to but eunuchs have been reported to live up to 12 years longer than non-castrated people [160].

8.1. Sex steroid ablation in males—surgical castration

Castration by both surgical and chemical means has been shown to reverse age-related thymic atrophy (Table 1) [161]. In particular we have shown it enhances immune reconstitution in young (4–6 weeks), young-adult (3 months), middle-aged (9 months) and aged male mice (18–24 months) in several immunocompromised models including chemotherapy and allogeneic and autologous HSCT [22,56,162,163]. Following castration, we have shown an increase in proliferation and cellularity of early thymocyte subsets such that by 14 days post-castration, the atrophic thymus resembles that of a young thymus [56,162–164]. Reversal of age related alterations in the architectural organization between medullary and cortical regions follow. Importantly castration also induces an increase in immature cell types in the bone marrow (Fig. 2), such as LSKs, HSCs and CLPs, which result in an increase in all immature B cell subsets [56,162] and may contribute to the increase along the thymocyte development pathway [68,162,163] (Dudakov et al., in preparation).

Following castration, there is also an increase in the lymphoid cellularity of the peripheral organs, such as the spleen and lymph nodes [61,165,166]. Along with an increase in $CD4^+$ and $CD8^+$ T cells resulting from an increase in thymic output [163,166], lymphocytes from castrated mice are more sensitive to antigen-mediated stimulation as demonstrated by ConA. Low ConA concentrations were able to induce proliferation of thymocytes from castrated male rats but not from T cells from their intact age-matched counterparts [157]. This was also evident in castrated mice vaccinated with OVA or TRAMP-C1 cells where peripheral T lymphocytes taken from the mice proliferated more readily when re-exposed to the nominated antigen than cells taken from sham-castrated mice [166]. Splenocytes from castrated male mice when co-stimulated with anti-CD3 and CD28, showed increased responsiveness to TCR stimulation above young intact counterparts [163].

Hence, surgical castration is able to enhance both the proliferative and functional capacities of thymocytes and their progenitors. It is extremely important that the effects of thymic regeneration following sex steroid ablation on the immune system encompasses all immune subsets in order to maintain immune balance as an imbalance of one and not the other type of cell could lead to the increased risk of infections, cancer or autoimmune diseases.

In terms of immune regulation, reports on the effects of castration on Tregs are scarce. The first study reported an androgen dependent maintenance of Tregs in humans, where chemical castration (see next section), using the LHRH antagonist Acyline, reduced the percentage of peripheral blood $CD4^+CD25^+$ Tregs. This decline normalised during recovery with the return of normal hormone levels [167]. More recently, however, the effect of neonatal castration at post-natal day 3 in rats was assessed [165]. In this case there was no significant difference in the percentage of thymic $CD4^+CD25^+Foxp3^+$ Tregs, although the absolute number was increased in castrated rats than in sham castrated rats due to thymic hypercellularity [165]. The differing reports here could obviously be due species differences but also to the timing of when sex steroids are removed. This issue of the effects of sex steroids and their removal on Tregs requires more detailed study as it could prove useful in the establishment of new treatments for autoimmune disease. In this regard, we have recently found that Tregs return to significantly higher levels in cancer chemotherapy patients undergoing HSC transplantation treated with LHRH, compared to the non-LHRH control group (Sutherland et al. submitted).

Table 1
Effects of aging upon the immune system and the aspects that can be corrected with surgical castration

Aging on the immune system	Effect of castration
Increased memory:naïve T cell (Reduced peripheral CD4+ and CD8+ T cell numbers)[5,24]	Increase in CD4+ and CD8+ peripheral T cells; reduced proportion and number of memory T cells [163]
Reduced TRECs in peripheral blood [3]	Increase in TREC levels with chemical castration [162]
Reduced proportion and proliferation of RTEs in the peripheral T cell pool [3]	Increase in RTE numbers [163]
Loss of functional tissue: cortical thymocytes [20]	Increase in absolute numbers of DN, SP CD4+, SP CD8+ and DP thymocytes [22,161–163]*
Reduced thymic stroma [20]	Increase in thymic stromal cell numbers [63]
Increase in PVS: accumulation of adipocytes and connective tissue [11]	Reorganization of the thymic microenvironment/architecture [155]
Reduced ability to respond to sudden loss of peripheral T cells with an increase in T cell output [10]	Increased recovery rate of peripheral naïve T cell numbers following HSCT and chemotherapy [162]
Reduced proliferation of progenitors ie ETP and LSK [21,31]	Increased number of ETP and LSK (reduced apoptosis of ETP) [22,162,163]

* Effect also seen following Ovariectomy[53].

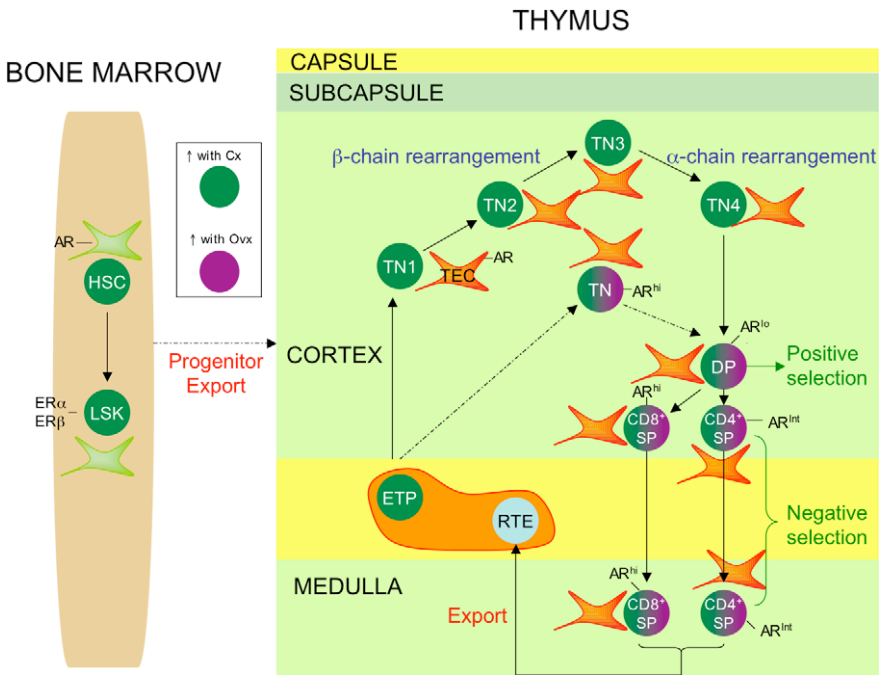

Fig. 2. Distribution of androgen and estrogen receptors in the thymus and bone marrow. Expression of the estrogen receptor (ER) has been shown on total thymocytes, but it is unclear on which particular subsets. Cessation of androgen or estrogen production by gonadectomy (Cx = male, Ovx = female) improves all thymocyte cell subsets, with those more susceptible depicted in the figure; ovariectomy results in the increased proportion of predominantly the DP thymocyte subset.

8.2. Sex steroid ablation in females—surgical ovariectomy

Ovariectomy of female mice and rats has also been shown to cause enlargement of the thymus [59,168] however to a lesser degree than that seen with castration in males [169]. The hormonal response to ovariectomy is, of course, more complex than castration of males as it causes a decrease in estrogens as well as prolactin, progesterone and dehydroepiandrosterone, which also have immunomodulatory effects [170]. It is further complicated by the production of estrogen by the adrenal glands by the conversion of local testosterone [171], which increases with age and can compromise the impact on thymus rejuvenation. Adrenalectomy of female (and male) mice results in thymic hypertrophy but this also involves a loss of glucocorticoids [172]. The impact of ovariectomy on thymocyte subsets also differs to that of castration in males. Although there is an increase in all thymocyte subsets (Fig. 2) [53], thymic hypertrophy does not obviously include increased proliferation of progenitors as is seen with castration, but rather an expansion of pre-existing subsets and mature subsets in the periphery [206]. Within the bone marrow compartment, ovariectomy of young-adult mice leads to an increase in total bone marrow cellularity reflected by an increase in total B cells ($CD19^{+}$) [76]. There is little change in total splenic cellularity or in either splenic T or B cell subsets however, similarly to castration, splenocytes taken from ovariectomised mice showed an increased proliferative capacity when introduced to LPS *in vitro* [168].

The long-term effects of sex steroid ablation on the immune system are of particular interest. While castration and ovariectomy appear to be long-term in respect to enhanced immune reconstitution (enhanced thymic size is evident for at least a year) it is unknown how this translates in the human setting. We have found that the thymus of castrated male mice is still enlarged (2–3-fold) twelve months post-surgery (Sutherland, Dudakov unpublished observations). Interestingly we have also found that ovariectomy is very much age dependent: in young mice there is a profound increase in thymic size, as for males but there is little effect on female mice 6–12 months of age (Hince, Vlahos unpublished observations). This is possibly due to the contribution of adrenal and fat metabolism derived estrogen—indeed the serum of the ovariectomised older mice still contained levels of estrogen comparable to sham-ovariectomised control mice (Hince et al., in preparation). Clinically, the treatment of females should therefore incorporate the use of ER blockers to eliminate the effect of peripheral estrogens. Long-term studies by others have shown that the thymus returns to age-matched controls by 20 months post-ovariectomy in C57Bl/6 female mice [169] again presumably due to the gradual return of circulating estrogens [171] or as mentioned previously the potential reduction in supply of bone marrow progenitors.

8.3. Chemical castration: LHRH-agonists

One very obvious major advantage of chemical castration is that it is completely reversible and has a very high

safety record clinically. Most commonly it is affected by LHRH (otherwise referred to as gonadotrophin releasing hormone or GnRH) analogues. LHRH agonists are the most effective and are given in constant, supra-physiological levels to achieve sex steroid deprivation by first over-stimulating the LHRH receptors in the pituitary. These then become desensitised to LHRH and stop production of LH and FSH, which results in the cessation of gonadal hormone production and spermatogenesis [173]. This treatment has the advantage of being completely reversible with fertility returning to normal within weeks after cessation of treatment although closer to 1–2 months in man, [173]. It is obviously less invasive than the surgical alternative. LHRH has been used as the "standard of care" for over 25 years for millions of patients with sex steroid exacerbated clinical conditions. The most prominent group are prostate cancer patients who, having received LHRH-agonist, have an increased rate of remission as well as an improved quality of life [174–177]. The most likely effect on these patients is the simple reduction of testosterone to deprive the cancer cells of this growth factor but they also have improved T cells [178], which very likely contribute to the cancer control and may well be a "hidden jewel" in the treatment strategy. We have shown that these patients have increased levels of naïve T cells (both $CD4^+$ and $CD8^+$) [163,178] and the Kwon group has found an increase in T cell infiltration of the prostate following androgen reduction [179].

The use of LHRH-agonist has shown a reversal as well as a delay of thymic atrophy in aged mice and rats [163,180,181]. Although the seminal vesicles do not reduce to the size seen with castration, thymus hypertrophy is greater with LHRH than that following surgical castration [180]. One likely explanation for this is the direct stimulatory effect of LHRH on the thymus itself since there are LHRH-Rs on thymic cells and indeed the thymus produces its own LHRH the level of which is upregulated by castration [57]. There is, however, an initial thymic atrophy caused by the agonist [181] most likely due to an indirect influence following the surge in sex steroids caused by the initial hyperstimulation of the pituitary prior to desensitising of the LHRH-R. Following this there is an increase in LHRH binding sites within the thymus [182] indicating a possible compensation mechanism with the lack of available LHRH in the thymus.

8.4. Chemical castration: LHRH-antagonists

Whereas LHRH-agonists cause initial over-stimulation of LHRH receptors resulting in a surge of LH and FSH and subsequently a "flare" in gonadal steroid levels, LHRH-antagonists competitively bind the LHRH receptors in the pituitary causing an immediate suppression of LH and FSH release [183]. LHRH-antagonists require constant binding with the LHRH receptors and if natural LHRH were to displace this event, sex steroids are produced once again. Such "leakage" together with the induction of some hypersensitivity, can occur clinically with some antagonists precluding them as the drug of choice for sex steroid suppression for prolonged periods. Clinically this can have very detrimental effects because it can allow promoted growth of AR^+ prostate cancer cells. LHRH-agonists, on the other hand, despite the initial "flare" are clinically safer because they maintain desensitised LHRH receptors for a period of time even after contact with the receptor is lost [174]. Another disadvantage of LHRH-antagonist treatment to enhance immune recovery is that even with the suppression of sex steroid production it actually suppresses thymocyte proliferation in response to ConA treatment as well as reduce the number of lymphocytes in the thymus and spleen [47,184]. This may be in part due to blocking of the stimulatory effects of LHRH binding to LHRH-R on lymphoid cells. In this regard, LHRH-agonist treatment has been shown to improve T cell status in humans undergoing HSC transplantation following myeloablative chemotherapy (see below; Sutherland et al. submitted manuscript; interim data presented at ASH 2003). Thus, further investigation into the comparative functional impact of LHRH antagonists on the thymus and immune responsiveness is required if it is to be considered an effective treatment to enhance immune recovery following chemotherapy or HSCT.

8.5. Androgen and estrogen receptor blockers

As both ARs and ERs are present on thymocytes, thymic stromal cells and most other immune cell subsets, it is likely that the use of receptor blocking agents could prevent the ablative action of sex steroids on the thymus as well as the initial ablative action of sex steroid flaring with LHRH-agonists. This would not replace any beneficial effects of LHRH-agonist binding directly to its receptor on thymic and other lymphoid cells. Few studies have investigated the effect of these ER and AR blocking agents on the immune system, although they are routinely used in the treatment of breast, prostate and other sex steroid related cancers [175,185,186]. Use of Flutamide, an androgen receptor antagonist, has shown increases in spleen cellularity and thymic weight [67], although the thymic/splenic subsets affected by this treatment were not analysed. It could be assumed, however, that any subsets expressing the androgen receptor would benefit from the Flutamide treatment, as it would restrict the negative impact of testosterone. Similarly, Tamoxifen, an anti-estrogen receptor agent, has been shown to increase relative total thymic weight [172,187] as well as decrease circulating levels of testosterone and LH [188]. Thus, the action of this agent in males may be to simply decrease the inhibitory effects of testosterone and LH on the thymus whereas in females it prevents the suppressive action of total (both gonadal and adrenal) estrogens on the thymus.

9. Proposed mechanisms of immune recovery

The mechanisms involved in the enlargement of the thymus following castration are not yet precisely known, but

several likely theories have been proposed [165]. A direct impact of LHRH may be involved as intrathymic levels of LHRH are increased following surgical castration [57], which may be from the lack of negative feedback from the gonads to the hypothalamus, and lymphoid cells express receptors for LHRH (Fig. 1). There is only scant evidence, however, for a functional impact of LHRH on T cells. When treated with LHRH, Jurkat cells, a human mature leukemic cell line that closely resembles mature T cells, had an enhanced proliferative activity [189]. Therefore, increased LHRH in the periphery may directly stimulate mature T cells and enhance proliferation, contributing to the increase in T cells in the spleen following castration but prior to the export from the thymus of newly derived naïve T cells. This ability of LHRH to directly impact on the immune system is of great functional relevance because it would provide reason to treat patients in whom sex steroids are already in part reduced such as menopausal women or following chemotherapy, which can damage gonadal function.

Following castration of male neonatal rats, a decrease in the number of apoptotic cells within the thymus is evident, aligning thymic recovery following castration with increased survival. Furthermore, increased thymocyte proliferation, shown by an increase in $BrdU^+$ cells, was seen in thymocyte cultures taken from castrated male rats [164,165]. These both suggest an increased production of cytokines and growth factors, although we have shown that mRNA levels of IL-7, NGF, IGF-1, SCF or Fgf7 (KGF) in total thymic stromal cells did not change significantly after castration compared to sham-castrated controls [163]. Although KGF, for example, is required for thymic recovery following sublethal irradiation [190] we have shown that castration of KGF−/− mice still results in thymic enlargement [56]. Subtle changes may not have been revealed in the heterogeneous population of cells in these early experiments and so this needs to be confirmed on purified stromal cell subsets. Indeed, Olsen and colleagues have demonstrated the importance of androgen receptors present on thymic stromal cells for the inhibitory effect of androgen on the thymus; chimeric mice deficient in AR expression on non-hematopoietic stromal cells did not undergo involution with androgen treatment [63]. It is also possible that the increase in thymic cellularity following castration is due to an increase in thymocyte progenitor entry.

10. Clinical application: sex steroid ablation to enhance HSCT engraftment and thymic recovery

It is well-documented that the recovery period for T cells, particularly naïve cells, from treatments such as chemotherapy, radiotherapy and that associated with HIV infection is severely delayed with age, resulting in a long period of immunosuppression and subsequent high risk of opportunistic infections. In children (pre-pubertal) the recovery of $CD4^+$ T cells to protective (i.e., normal) levels takes ~6 months whereas in post-pubertal adults, with an atrophic thymus, these cells take years to recover and in some cases, never return to pre-treatment numbers [191]. Thus any method to enhance T cell-based immune reconstitution in these circumstances will be of major clinical benefit.

We have studied in detail the impact of either surgical or chemical (LHRH) castration in immunodepleted settings such as following chemotherapy and HSCT. When surgical castration was performed prior to autologous HSCT in mice, total LSK numbers were significantly increased by 2 weeks post-transplant, and by 4 weeks the majority of LSKs were donor-derived [163]. In the thymus, there was a marked increased in all thymocyte subsets of both donor- and host-origin [162]. We have also shown castration-induced increased immune recovery in mice undergoing myeloablative radiation followed by allogeneic HSCT [56]. An important finding in that study was the graft versus host disease (GvHD) was not exacerbated by the loss of sex steroids but Graft versus Leukaemia (GvL) was retained. In both the autologous and allogeneic transplants, there were increased levels of stem cells in the bone marrow and more efficient engraftment as reflected by the total cellularity of the bone marrow, thymus and spleen [56,162]. We further showed that prostate cancer patients treated for 4 months with an LHRH agonist, relative to pre-treatment controls, showed increased levels of total and naïve $CD4^+$ T cells; $CD8^+$ T cells were also increased [163]. This is also associated with increased T cell infiltration of the prostate [163,179]. On the basis of extensive pre-clinical studies and these data on prostate cancer patients using an approved drug with a very high safety profile from over 25 years of clinical use, we undertook a pilot clinical trial on cancer patients that had undergone myeloablative chemotherapy and allogeneic or autologous HSCT with LHRH. The primary endpoint was increased levels of naïve $CD4^+$ T cells, given that this is normally "flat-line" for such adult patients even after ~2 or more years. Although the patient numbers are small, and not all responded, there was clearly a significant increase in $CD4^+$ T cells (naïve and total) from 9 months compared to ~6 months for children. In a similar setting, children regain their T cells by ~6 months. These cells also had a broad TCR repertoire as determined by spectratyping (Sutherland et al. submitted manuscript; interim data analysis presented at ASH 2003). These highly promising but preliminary findings are now being explored in greater detail in Phase II, double-blinded placebo controlled clinical trials on autologous HSCT patients. Sex steroid ablation, by chemical means, is thus a valuable tool to enhance both immune recovery and transplant engraftment following HSCT. LHRH potentially represents a major new tool in the clinical management of diseases of T cell origin, in particular immunodeficiency states. Such conditions are not restricted to cancer or HIV patients, however; it could also be used for corrective therapy of the immune system in autoimmune disease.

Another possible clinical application of sex steroid ablation therapy is during the lead up to major surgery

and following major burn injury. In burn injuries, the cell-mediated response is suppressed with the stress involved in bodily damage. As males are more at risk of mortality from infection following such injury, androgen deprivation by Flutamide (androgen-receptor antagonist) treatment has been tested in mice. Mice castrated 2 weeks prior to trauma haemorrhage prevented the depression of MHCII expression on peritoneal macrophages as occurred in their intact counterparts [192]. Similarly, Flutamide treatment of male mice following burn injury enhanced the delayed-type hypersensitivity response [193]. Thus these treatments would presumably lead to a decreased risk of developing sepsis in burn patients or those undergoing major surgery.

11. Other models/clinical strategies to reactivate the thymus

While sex steroids are clearly linked to thymic atrophy with age, there are many other contributing factors. In the context of reversing thymic atrophy clinically, it is important to define these as alternatives to sex steroid disruption, or be incorporated as "adjuvants," enhancing the effectiveness of LHRH. Many growth factors effecting thymus growth are being investigated in pre-clinical studies. Hepatocyte growth factor (HGF) has been shown to both promote T cell reconstitution and inhibit chronic GVHD following HSCT in mice [194]. IL-7, which has the ability to enhance peripheral T cell proliferation in several species including primates [195] and increase TRECs in humans [196] is a promising therapeutic for immune deficiency and low-doses may reduce the occurrence of GVHD [197]. KGF has also been shown to protect epithelial cells from GVHD and to enhance thymic recovery after atrophy induced by aging [198] irradiation treatment and chemotherapy [190] while increasing naïve T cell proliferation as indicated by TREC levels [199]. GH also promotes immune reconstitution; in low doses it enhances immune recovery following HSCT as well as increasing total thymic size in mice and rats which is linked to a reduction in thymic adipocyte numbers which accumulate with aging [200–202]. However, in humans, there are very few reports on thymic reconstitution. Low doses of rhGH appear to enhance recovery of haematopoietic cell numbers following chemotherapy, and increase T cell recovery in HIV patients [203], but there were significant side effects in the latter study and the drug had the practical issue of having to be administered daily [204,205]. Thus it seems likely that to effectively enhance thymus recovery in a raft of clinical conditions (e.g., in the aged and following chemotherapy or radiation), a combination of treatments may be required, with sex steroid ablation as the foundation platform, and the administration of one or more growth factors including IL-7.

12. Conclusions and future directions

It has been long recognised that there is a profound impact of sex steroids on the immune system in general and the thymus in particular. In this context, the effects of sex steroids—androgens and estrogens, are complex and wide reaching. In the neonatal period, estrogen at least appears to be essential for normal immune development, while post-pubertal increases in sex steroid levels suppress stem and progenitor cells and downstream T and B cell differentiation and maturation, associated with a profound reduction in thymic output and an eventual increased risk of opportunistic infections. During periods of severe immunosuppression best typified by chemotherapy and radiotherapy, both surgical and chemical ablation of sex steroids improves immune recovery of the thymus and bone marrow and subsequently T and B lymphoid compartments. There is increased lymphocyte reactivity to antigen stimulation in both males and females post-castration, although the improvement in females is not as impressive. While LHRH variants (particularly agonists) are shown to be very effective at enhancing immune recovery, their use in combination with either estrogen or androgen receptor blockers could prevent the initial surge in hormone/sex steroid levels and further increase thymic rebound. Thus, temporary sex steroid ablation has the potential to become an important tool for improving immune recovery following severe immunodepletion. In doing so, the ability to enhance thymus and bone marrow function provides a realistic platform for not only increasing T cell output, but also for manipulating the types and function of the T cells produced including the generation of T regulatory cells and thymic uptake of donor HSC to create tolerance in the recipient to transplants from that donor.

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ERRATA AND ADDENDUM

Chapter 1 – Literature Review

p. 21, para 2, line 11: replace comma with a full stop.

Chapter 3 – Experimental Parameters governing kinetics and magnitude of the thymopoietic response to LHRH-A treatment

Clarification of apparent discrepancy of control data in Figure 3.12a and Figure 3.8a – Reviewer 1.

It was noted that total thymic numbers in Figure 3.12a for the control group were smaller than the equivalent group in Figure 3.8a. This only occurred once and was not due to a calculation error, but rather it should be made clear that mice used within Figure 3.8 were housed in stressful conditions due to renovations of the animal facility during that time and were thus beyond our control. Reduced control thymic numbers were observed by many researchers during this period. While we cannot directly compare the two experiments in this case, we were still able to observe significant changes relative to the suitable controls.