EFFECTS OF GENDER DIFFERENCES ON IMMUNE SENESCENCE MECHANISMS

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EFFECTS OF GENDER DIFFERENCES ON IMMUNE SENESCENCE MECHANISMS

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ABSTRACT

EFFECTS OF GENDER DIFFERENCES ON IMMUNE SENESCENCE MECHANISMS

Immunosenescence is thought as the main cause of higher incidence of infections and other diseases in aged people. Thymus is the maturation site of developing thymocytes and its size and function decrease during aging , thymic aging is called as thymic involution. Thymic involution casues decrease in naïve T cell output and adaptive immune response. Gender based difference is observed for thymic involution. Thymic cellularity and thymic output were maintained better in women for advancing ages. Incidence of autoimmune diseases is higher in women as a result. In this study, we had six mice experiment groups as letrozole or testosterone treated 3 monts old females for analysis of testosterone and estrogen effects on thymic involution in addition to 7 days old male, 7 days old female, 3 months old and 3 months old female groups. In bone marrow, hematopoietic stem and progenitor cells were analysed in bone marrow while thymocytes subpopulations, lymphoid tissue inducer (LTi) cells, myeloid derived suppressor cells (MDSC) and regulatory T (Treg) cells were analyzed for thymus. Thymic *ACTR2* (Arp2/3 component), *FGF7*, *FGF7RII*, *IL-7*, *IL-7R*, *IL-22* expressions were analyzed. Methylation analysis was made for ARP2 gene. Naive T cell output was analyzed with sjTREC copy number analysis. Numbers of LTi, double negative (DN) thymocytes which are positively correlated with thymic cellularity were significantly higher in 3 months old females while common lymphoid progenitors were significantly higher in 3 months old female bone marrow.. FGF7 gene expression was higher in 3 months old females. Cytoskeleton formation related *Arp2* gene in 3 months old mice thymus was found as lower than in 7 days old mice. Treg cells which may have negative effects on thymic stroma-thymocyte interactions was higher in 3 months old males. In conclusion, changes in bone marrow cells was correlated with changes in early thymocytes in thymus, gender seems as a determinant of changes in thymic cellularity related markers as FGF7. LTi and Treg cells may be important determinants of differences in thymic involution between genders.

ÖZET

CİNSİYET FARKLILIĞININ İMMÜN YAŞLANMA MEKANİZMALARI ÜZERİNE ETKİLERİ

İmmün yaşlanma ilerleyen yaşlarda enfeksiyon ve diğer hastalıkların daha sık görülmesinin sebebi olarak kabul edilmektedir. Timus T hücre gelişiminin gerçekleştiği yerdir ve yaşlanma ile birlikte boyutu küçülmekte ve fonksiyonunu kaybetmektedir. Timusun yaşlanması timik involüsyon olarak adlandırılmaktadır. Timik involüsyon sonucunda naif T hücre üretimi azalmakta ve adaptif immün yanıt zayıflamaktadır. Timik involüsyon sürecinde kadın ve erkek bireyler arasında farklılık gözlenmiştir, dişilerde timustaki hücresellik ve naif T hücre çıktısı ilerleyen yaşlara kadar daha iyi korunmaktadır. Bu farklılığın sonucu olarak dişilerde otoimmün hastalıklar daha sık görülmektedir. Bu çalışmada testosteron ve östrojenin timik involüsyonda etkisini anlamak üzere testosteron ve letrozol verilen 3 aylık dişiler, 7 günlük dişi ve erkek, 3 aylık dişi ve erkek olmak üzere 6 fare deney grubu, toplam 42 fare kullanılmıştır. Kemik iliğinde hematopoietik kök ve progenitör hücreler, timusta gelişen timositler ve lenfoid doku indükleyici (LTi), myeloid kökenli basklıyacı (MDSC) ve regülatör T (Treg) hücreler analiz edilmiştir. Gerçek zamanlı kantitatif PCR ile Arp 2/3 kompleks bileşeni *ARP2*, *IL-22*, *IL-7*, *IL-7R*, *FGF7* (KGF) *ve FGF7RII* genlerinin ekspresyon düzeyleri analiz edilmiştir. ARP2 geni için metilasyon analizi yapılmıştır. Naif T hücre çıktısının belirlenilmesi için sjTREC kopya sayısına bakılmıştır. Dişilerde kemik iliğinde ortak lenfoid progenitör hücre sayısı daha fazladır. 3 aylık dişi bireylerde timus hücreselliği ile pozitif korelasyon gösteren LTi, çiftli negatif timositlerin sayısı erkeklere göre daha yüksek, kemik iliğinde de lenfoid progenitör sayısı daha yüksektir. Timus hücreselliğini arttıran *FGF7* ekspresyonu 3 aylık dişilerde daha yüksektir. Hücre iskeletinin formasyonu ile ilgili *Arp 2* ekspresyonu 3 aylık farelerde 7 günlük farelerden daha düşük düzeydedir. Erkek bireylerde baskılayıcı etkisi olduğu düşünülen Treg hücre sayısı daha yüksektir. Sonuç olarak, kemik iliğindeki hücrelerdeki değişim timositlerdeki değişim ile uyumludur, timus selülaritesine etkisi olduğu bilinen FGF7 gibi belirteçlerde cinsiyete bağlı farklılık gözükmektedir. LTi ve Treg hücrelerinin de cinsiyete bağlı timik involüsyon farklılığında önemli role sahip olabileceği görülmüştür.

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3MF	3 months old females
3MFL	Letrozole treated 3 months old females
3MFT	Testosterone treated 3 months old females
3MM	3 months old males
7DF	7 days old females
7DM	7 days old males
A	Adipocytes
BMP2	Bone morphogenetic protein 2
BMP4	Bone morphogenetic protein 4
CK5	Cytokeratin 5
CK8	Cytokeratin 8
CJ	Coding joint
CLP	Common lymphoid progenitors
CMP	Common myeloid progenitors
cTECs	Cortical thymic epithelial cells
CTLA-4	Cytotoxic T lymphocyte-associated antigen 4
CTLs	Cytotoxic T lymphocytes
DC	Dendritic cells
DN	Double negative
DP	Double positive
DTR	Diphtheria toxinr eceptor
ETP	Early thymocyte progenitor
FGF	Fibroblast growth factor
FGF7	Keratinocyte growth factor
FOXN1	Forkhead box protein N1
FOXP3	Forkhead⁄ winged-helix transcription factor box P3
GITR	Glucocorticoid-induced tumour necrosis factor receptor family
GM-CSF	Granulocyte macrophage colony stimulator factor
GVHD	Graft-versus host disease
HSC	Hematopoietic stem cells
HT	Hydroxytestosterone

IL-2	Interleukin 2
IL-7	Interleukin 7
IL-7R	Interleukin 7 receptor
IL-7Rα	IL-7 receptor alpha chain
IL-22	Interleukin 22
IPEX	Immunodysregulation polyendocrinopathy enteropathy
X-linked syndrome	
JMY	Junction-mediating and regulatory protein
L	Lymphoid progenitor cells
LAG-3	Lymphocyteactivation gene-3
LTi	Lymphoid tissue Inducer
MDSC	Myeloid derived suppressor celll
MHC I	Major histocompatibility complex I
MHC II	Major histocompatibility complex II
MSC	Mesenchymal stem cells
mTECs	Medullary TECs
NPFs	Nucleation promoting factors
NWASP	Neural Wiskott-Aldrich syndrome protein
OB	Osteoblasts
SCF	Stem cell factor
SP	Single positive cells
STAT3	Signal transducer and activator of transcription 3
Tconv	Conventional T cells
TCR	T-cell receptor
TEC	Thymic epithelial cell
Tfh	T follicular helper
TGF-β	Transforming growth factor β
Th	T helper
TNF α	Tumor necrosis factor alpha
TRA	TR alpha locus
TRAC	TRA constant gene
TRD	TR delta locus
TREC	T Cell Receptor Excision Circle

Treg	Regulatory T cells
TSLP	Thymic stromal-derived lymphopoietin
WASP	Wiskott–Aldrich syndrome protein
WAVE	WASP family verprolin-homologous protein
ZAP70	Zeta-chain (TCR) associated protein kinase 70kDa

1. INTRODUCTION

1.1. HEMATOPOIESIS

Blood cell production in body is called as hematopoiesis, all committed mature blood cell types are produced from low numbers of hematopoietic stem cells at this process. Hematopoietic stem cells are multipotent stem cells which have self renewal ability and most of hematopoietic stem cells are located in bone marrow. At first step of hematopoiesis, hematopoietic stem cells are differentiated to multipotent progenitor cells in bone marrow [1]. These multipotent progenitors do not have self renewal ability and they differentiate to common lymphoid progenitors and common myeloid progenitors [3]. B lymphocytes, T lymphocytes and natural killer cells are originated from common lymphoid progenitors while granulocytes, macrophages, thrombocytes are originated from common myeloid progenitors [1]. Dendritic cells are an exception, both of common lymphoid progenitors and common lymphoid progenitors can differentiate to dendritic cells [1]. Myeloid cell production is called as myelopoiesis while lymphoid cell production is called as lymphopoiesis in nomenclature. Figure 1.1 gives summarized information about hematopoiesis hierarchy. Further myelopoiesis steps are not shown in Figure 1.1.

Four or five color antibody panels are used for immunophenotyping of the cells at different stages of hematopoiesis and/or lymphopoiesis. Hematopoietic stem cells are defined as $Lin^{neg}CD34^{+}CD38^{neg}CD90^{+}CD45RA^{neg}$ and $Lin^{neg/low}Sca1^{+}CD117^{+}CD135^{neg}$ for human and mouse respectively [1]. Multipotent progenitor cells are defined as $Lin^{neg}CD34^{+}CD38^{neg}CD90^{neg}CD45RA^{neg}$ cells for human while mouse multipotent progenitor cells are defined as $Lin^{neg/low}CD117^{high}CD135^{+}Sca1^{+}CD90^{neg}$ [1]. Human common lymphoid progenitor cells are defined as $Lin^{neg}CD34^{+}CD38^{+}CD10^{+}$ while $Lin^{neg}Sca1^{low}CD117^{low}CD127^{+}CD135^{+}$ are defined as mouse common lymphoid progenitor cells [1].

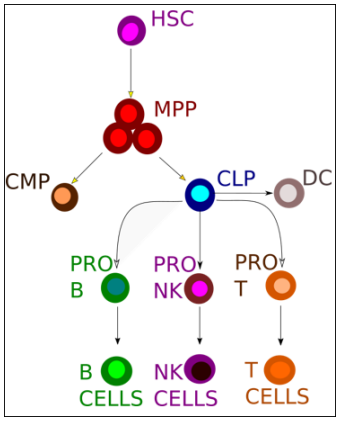

Figure 1.1 Scheme of Hematopoiesis.

Bone marrow is a part of lymphatic system, other organs of lymphatic system are appendix, lymph nodes, peyer patches, spleen, thymus and tonsils. The lymphatic system is an important part of the immunity [2]. Meaning of lymph is clear fluid and this name is related with high white blood cell content. Lymphatic system is a specialized vessel system, free cells and extracellular fluids are drained from tissue, they are transported through lymph vessels and discharges into blood circulation [3]. Bone marrow and thymus provide maturation sites for lymphocytes, both of B and T cells progenitors are produced in bone marrow, but only B cells mature in bone marrow, T cells have to migrate to thymus for further steps of maturation [2]. Lymphatic system organs are shown in Figure 1.2 [2].

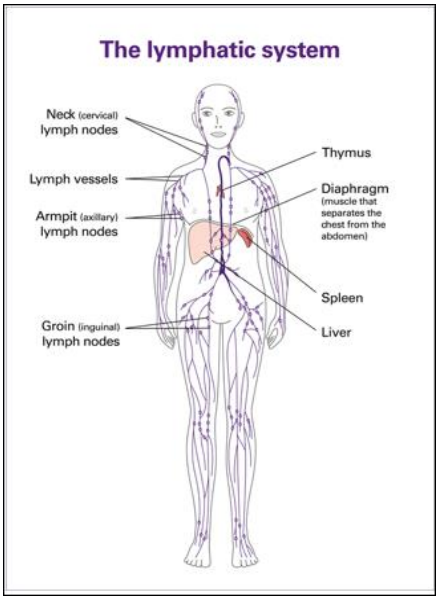

Figure 1.2. Lymphatic system [2]

1.2. STEM CELLS AND PROGENITOR CELLS IN BONE MARROW AND CHANGES IN THESE CELL POPULATIONS DURING AGING

Bone marrow is divided into two compartments as hematopoietic stem cell compartment and stromal compartment. Hematopoietic stem cell compartment contains hematopoietic stem cells and their differentiation products while stromal compartment contains mesenchymal stem cells and their differentiated stromal products, like adipocytes or osteoblasts.

Hematopoietic stem cells have self renewal ability and they can differentiate to all committed mature blood cell types [1]. Hematopoietic stem cells are spherical cells that have little cytoplasm, few organelles and they do not express any lineage markers such as monocytic, granulocytic, lymphocytic or erythrocytic cell surface markers [4]. Hematopoietic stem cells are rarely found in bone marrow, ratio of hematopoietic stem cells in total bone marrow cells change between 1/10000 to 1/100000 for humans [5]. In addition, fat, liver, spleen or muscle tissue contain less numbers of hematopoietic stem cells. Number of hematopoietic stem cells increases in blood after some conditions like post chemotherapy or irradiation, infections also increase the number of hematopoietic stem cells in circulation. They are in the G_0 phase of the cell cycle [6].

Depletion of lymphocyte production with aging is related with composition changes in these bone marrow compartments. Aging has different important effects on the deposit of hematopoietic stem cells. Numbers of hematopoietic stem cells within mice bone marrow seem to interestingly increase with aging, conversely capacity of regeneration depletes with aging [7–9]. Self renewal potential and function of hematopoietic stem cells decrease during aging, but old bone marrow produces higher numbers of myeloid cells than young bone marrow [10].

Expanded populations of aged hematopoietic stem cells consist of a large numbers of hematopoietic stem cells which are intrinsically shifted to myelopoiesis, therefore these are not truly uncommitted progenitor cells [10]. Myeloid shift of bone marrow hematopoietic stem cells have an important role in lymphoid aging. Increased expression of myeloid genes and downregulation of lymphoid genes in bone marrow are validated

with microarray analysis. Myeloid shift cause decreased production of common lymphoid progenitors (CLPs), pre-pro-B cells, pro-B cells, pre-B cells, and total B cells in bone marrow [10].

Decreased production capacity of pro and pre-pro B cells in bone marrow with aging is related with reduced common lymphoid progenitors proliferation [11]. However the decrease in the numbers of pre-B cells is associated with loss of expression of recombination activating gene two (RAG2) and activity of V(D)J recombinations [12]. Myeloid shift of bone marrow hematopoietic stem cells is a key initial step toward a series of changes that result in decreased lymphocyte production with senescence [13].

Decline in genomic longevity causes fail of hematopoietic production and hematopoietic production. Telomeres are shortened in hematopoietic stem cells after each cell division. The telomeric DNA loss in telomerase deficient mice is correlated with depletion in hematopoietic stem cell function [14, 15]. However, overexpression of telomerase does not enhance the HSCs fuction in old mice [16].

A hypothesis about thymic involution is proposed that thymus involutes because of the depletion of cell pool in bone marrow. Some scientists suggests that lymphoid progenitor cell production capacity of bone marrow decreases during aging and thymus tries to adapt this condition with decrease in size and capacity for prevention of a possible leukemia [17]. According to new studies, T cell leukemia can be developed as an answer for cell depletion in bone marrow [17].

Aging causes changes in bone marrow cell populations. For stromal compartment, Mesenchymal stem cells (MSC) differentiation skew from osteoblasts (OB) to adipocytes (A). In hematopoietic stem cell compartment, hematopoetic stem cells produce more myeloid cells (M) and less lymphoid progenitor cells (L) to B and T cell differentiation. [10].

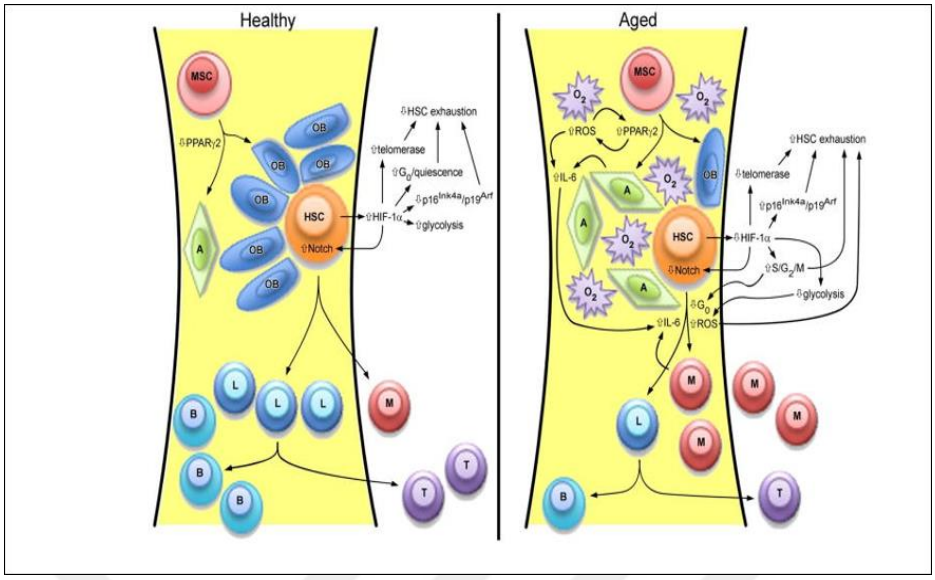

Figure 1.3. Aging related changes in bone marrow [10]

1.3. THYMUS AND AGING

1.3.1. Introduction to Thymus

Thymus is a specialized organ of the adaptive immune system, education, negative and positive s**e**lection of immature T cells was occurred in thymus [18]. Thymus has a bilobuar pyramid-like shape and it is located in front of heart and under the breastbone [19]. Two lobes of thymus are connected with connective tissue. Figure 1.4 shows the location and shape of human thymus.

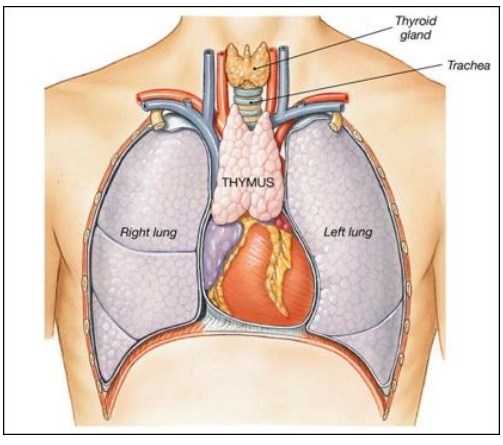

Figure 1.4. Anatomical location of thymus [3]

1.3.2. Development of Thymus

Thymic epithelial cells formation starts from two endodermal diverticula and these form third pharyngeal pouch and it into bordering mesoderm and neural crest originated mesenchyme ahead of ventral aorta [20]. First observation about third pharyngeal origin of thymus was made by Stieda in 1881 [21]. Previous discussions were made about contribution of surface ectoderm, but mouse studies in last decades shown that pharyngeal endoderm can form thymic cortex and medulla without pharyngeal ectoderm contribution [22]. Thymic epithelium is developed from third pharyngeal pouch endoderm [23]. Forkhead box protein N1 (FOXN1) is thought as the indicator of commitment of third pharyngeal pouch endoderm to thymic fate, but only FOXN1 can not drive cells to thymic fate [24]. Although skin keratinocytes produce high amount of FOXN1, they do not have thymopoietic ability. According to mouse experiments, FGF mediated signalling increased thymic epithelial cell numbers, but FGF is not a determinant for initialisation or differentiation [17].

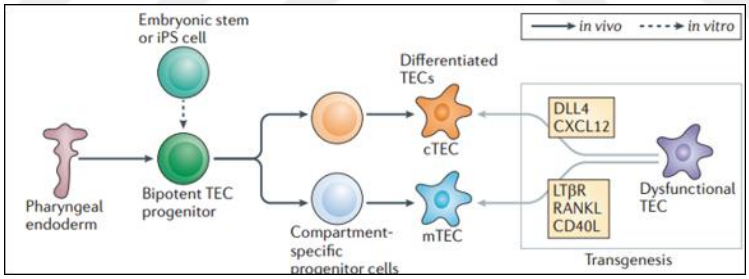

Figure 1.5. Thymic epithelial cell development [17]

Bipotent progenitors of TECs develop from third pharyngeal pouch endoderm; these cells are forkhead box protein N1 (FOXN1) positive. The bipotent progenitors are thought to give rise to mature cortical and medullary TECs through compartment-specific progenitor cells [17].

1.3.3. Thymus Structure and Cell Populations in Thymus

Thymus consists of four main parts and they can be listed as capsule, cortex, cortico-medullary junctions and medulla according to proximity to outer region of the thymus

[18]. Capsule covers each lobes of the thymus and it is built from thin connective tissue. Outer region of thymus is called as cortex and it is located under capsule and it contains immature developing lymphocytes, macrophages and epithelial cells [25].

Cortico-medullary junctions are located between cortex and medulla regions and contain high numbers of blood vessels. They consist of T lymphocytes, B lymphocytes, plasma cells, dendritic cells, connective tissue and blood vessels [25]. Inner regions of thymic lobes is called as medulla and it contains epithelial cells, epithelial reticular cells, more mature T lymphocytes, dendritic cells, B lymphocytes, few myoid cells and Hassal Corpuscles [25].

Hassal corpuscles are structural formations in thymic medulla. They are firstly identified by Arthur Hill Hassal in 1849 [26]. High numbers of reticular cells surround degenerated hyaline and formed Hassal corpuscles. Humans have well developed Hassal's Corpuscles, but mice and rats have poorly developed Hassal's Corpuscles [26]. Hassal's corpuscles are functional structures, they produce some cytokines and hormones as thymulin, thymopoietin, thymosin alpha 1 and thymosin beta 4 [26]. Thymulin is a nonapeptide hormone and it increases T cells functionality [27]. Thymopoietin is a polypeptide hormone that is found in thymus and periphery. It has a role in T cell maturation as an inducer for CD90 expression [26].

Thymic epithelium-thymocytes interactions are obligatory for appropriate thymocyte development and maintenance of thymus function [28]. Thymic epithelial cells are located in thymus and they build a complex network which is interacted with developing thymocytes [28]. They are named as cortical thymic epithelial cells (cTECs) and medullary thymic epithelial cells (mTECs) according to location in thymus. cTECs are located in outer cortex while mTECs are located in inner medulla. cTECs and mTECs are functionally different cells [28].

Thymic epithelial cells are located in thymic epithelial space for supporting T-cell maturation and development. Positive and negative selection of T lymphocytes are orchestrated by cortical and medullary thymic epithelial cells. TECs promote distinct stages of T cell development and repertoire selection. We basically know that thymic

epithelial cells are $CD45^{neg}$ cells and cytokeratins or keratins are mainly expressed by all TECs intracellularly [29].

While cytokeratin 5 (CK5) is predominantly expressed on mTECs but also minor part of mTECs express K8, vast part of cTECs don't express CK5 but express cytokeratin 8 (CK8) [29]. Figure 1.6 shows thymus sections. Green cells are $CD4^+$ cells and blue cells are $CD8^+$ cells. UEA1 (red) was used for staining of mTECs [28].

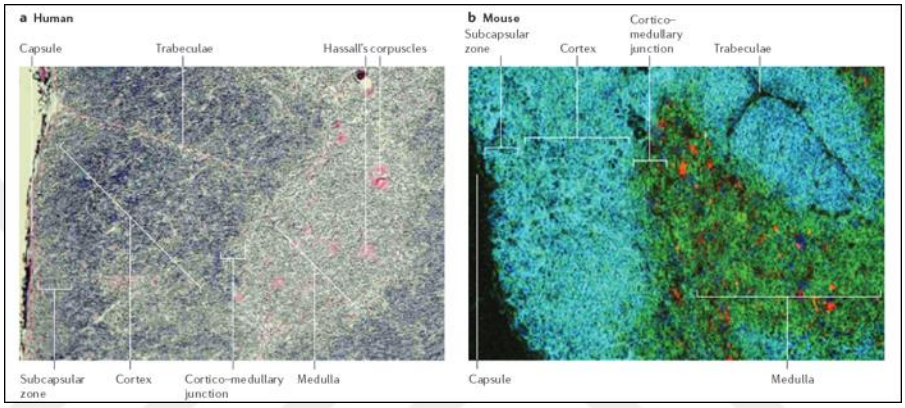

Figure 1.6. Thymus sections. (a) Hematoxylin-eosin staining of a new born child thymus section, (b) Thymus section of adult C57/BL6 mice [28]

1.3.4. Thymic Involution

Senescence is a slow and progressive process causes decreased fuction and malfunction of different organs and systems for both of humans and animals [19]. Normal function of immune system declines with aging and it is called as immunosenescence [19].

Immunosenescence is thought as main cause of increased incidence of infectious diseases, poor answer to vaccination and autoimmune diseases in aged people [19]. Mechanisms of immune sytem aging are not known exactly, anyway they affects adaptive and natural or innate immunity. Thymic involution or decrease in thymic cellularity or mass is a most typical and evolutionary conserved change of the aged immune system in all vertebrates [30].

The thymic epithelium starts to decrease in size as an essential feature of age-related thymic involution from the first years of human life at a rate of three per cent per year during adulthood [31, 32].

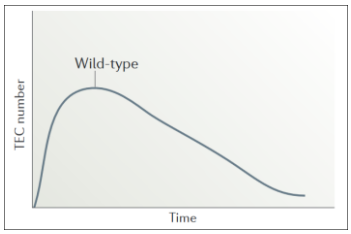

Figure 1.7. Changes in thymic epithelial cell number through the lifespan [17]

Thymic perivascular space fills with adipocytes and peripheral lymphocytes. Corticomedullary junctions are disorganized and medullary and cortex boundaries are lost in aged thymus [31, 33].

Thymic involution and related decline in adaptive immune response or reduced naive T cell production is assumed as an important cause of aging associated diseases and aging [34]. Thymic involution causes a decrease in T cell output and peripheral T cell diversity.

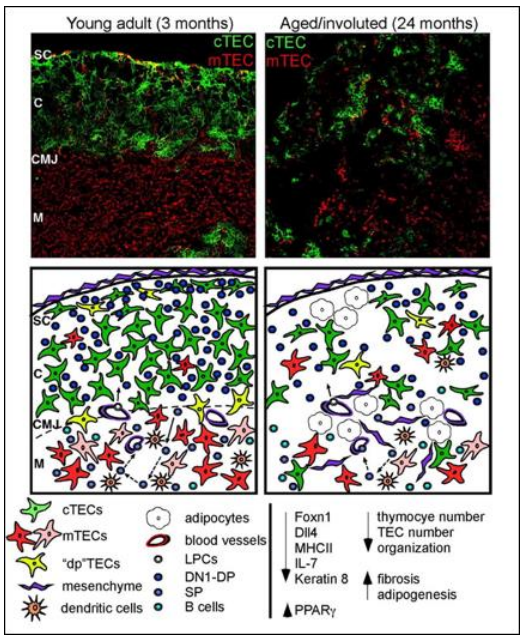

Figure 1.8. Composition of young and old thymus [10].

Figure 1.8 shows three months and twenty four months old mice thymuses were stained with Ly51 (green) for cTECs and cytokeratins 5 and 14 for mTECs (red). Young thymus has well structured cortical medullary junctions. Subcapsular (SC), cortical I and medullary (M) can be clearly observed. The involuted aged thymus demonstrates blended cTECs and mTECs, deprivation of TECs expressing both cTEC and mTEC markers (in yellow, double positive or "dp" TECs), and TEC-free regions. Dll4: delta-like 4, DN: double negative, DP: double positive, SP: single positive, LPC: lymphocyte progenitor cells [10].

1.3.5. Thymocyte Development and Effects of Immunosenescence on Thymocytes

Progenitor cells come out from bone marrow and enter thymus through blood circulation for selection and maturation. These cells are negative for both of CD4 and CD8 surface markers and they are called as double negative (DN). Double negative cells differentiate to CD4 and CD8 double positive cells and they are called as double positive thymocytes (DP). At the final maturation step, they lose one of them and express only one of CD4 or CD8 and they are called as single positive cells (SP). They are selected by negative and positive selection and go into blood circulation.

Initial committed T cells do not have the expression of T-cell receptor (TCR), CD8, CD4 and they are named as double negative thymocytes (DN, without CD8 or CD4 markers). According to immunophenotypes, double negative thymocytes can be subdivided into four differentiation stages (DN1, $CD44^{+}CD25^{-}$; DN2, $CD44^{+}CD25^{+}$; DN3, $CD44^{-}CD25^{+}$; and DN4, $CD44^{-}CD25^{-}$) [35].

While cells proceed through the stages of DN2 to DN4, the pre-TCR is expressed which consists of a rearranged TCR β-chain and non-rearranging pre-Tα chain [36]. True expression of pre-TCR causes important cell proliferation in the course of the DN4-double positive transition and changing of the pre-TCR α-chain with TCR α-chain which is newly rearranged, which provides a complete αβ TCR.

After that, thymocytes which are $\alpha\beta\text{-}TCR^{+}CD4^{+}CD8^{+}$ double positive interplay with cortical epithelial cells which express a MHC class I and MHC class II molecules related

to self-peptides. Double positive thymocytes fate is attached to signalling mediated through interplay of the TCR with that high density of MHC ligands which are with self-peptides [37, 38].

Delayed apoptosis is encountered after too little signalling, that is death by neglect. Too much signalling is able to stimulate acute apoptosis, that is negative selection. This is the most common phenomenon in the medulla, encountering with vigorously activated self-ligands on hematopoietic cells, especially dendritic cells. TCR signalling with proper intermediate level starts efficient maturation, that is positive selection. Thymocytes with TCRs expression which bind MHC class II ligands with self peptide begin to be $CD4^+$ T cells, then they are available for exportation from the medulla into the sites of peripheral lymphoid. SP, single positive.

Thymic epithelial cells (TECs) and thymocytes interaction is obligatory for T cell selection, positive selection of T cells is mediated by cortical thymic epithelial cells (cTECs) while negative selection of T cells is mediated by medullary thymic epithelial cells (mTECs) [39]. T cells that are able to interact with major histocompatibility complex (MHC) are selected by positive selection, cTECs present self antigens on MHC and only MHC I and MHC II interacting T cells receive survival signals [40]. Negative selection eliminates self antigen reacting T cells by self antigen presenting on the MHCs of medullary epithelial cells, thymocytes which have strong interaction with self antigens receive apoptotic signal and die [40].

Thymus needs the continuous input of bone marrow progenitors, any age-related changes in the function of hematopoietic stem cells could help to bring about thymic involution. Experiments showed that old hematopoietic stem cells seem to display an augmented tendency to myeloid differentiation together with a decreased capability for lymphoid maturation; which has been demonstrated in human and mice [41, 42]. Suchlike changes in the function of hematopoietic stem cells may show within the activity of early thymocyte progenitors (ETP). Old mice possess decreased number of early thymocyte progenitor with decreased potential of differentiation and proliferation [43, 44].

Early thymocyte progenitors of young mice can differentiate into every stages of T cell development in the fetal thymic organ culture, contrarily old ETP demonstrated a decrease in activity of T cell differentiation [44].

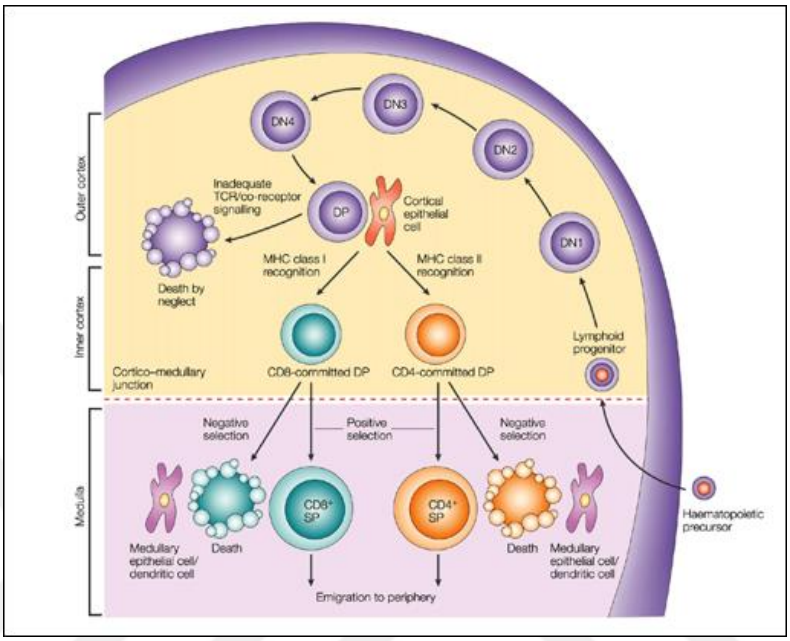

Figure 1.9. Stages of thymocyte development [45]

Moreover, early thymocyte progenitors of old mice demonstrate enhanced frequency in the apoptotic cells and decreased number of $Ki67^{+}$ cells [44].

Early thymocyte progenitors are included within the initial phases of DN thymocytes, aging causes a reduction in the proportion of $CD44^{-}CD25^{+}$ (DN3) and $CD44^{+}CD25^{+}$ (DN2) $CD44^{-}CD25^{+}$ (DN3) cells [46, 47]. Moreover, $CD44^{+}CD24^{-}CD3^{+}$ DN population has been demonstrated [31, 48]. Similar population has been detected in adult murine bone marrow, that seems to be related to a function in decreasing hematopoiesis [49], bring about the chance that gathering of these cells in the aging thymus may effect thymopoiesis negatively, therefore it may contribute to the thymic involution.

Further phases of thymocyte maturation display phenotypical changes along with aging, specially, researchers have showed age-related decrease in CD3 expression on SP thymocytes and DP [31, 36]. These alterations can cause distrupted TCR-dependent stimulation. It is also showed that old thymocytes demonstrate decreased Concanavalin

A-stimulated proliferation [36, 50]. It was observed that old cells could not enter into the G_2M phase of the cell cycle [35].

The age-related alterations in thymopoiesis are probably gained by recent thymic emigrants, it is possible that such cells may display decreased immunocompetence. Recent thymic emigrants of old people have phenotypic maturation with belated kinetics, they show reduced capacity of proliferation, disabled calcium signaling after TCR induction and decreased memory and helper activity [51]. Thymocytes and peripheral T cells from more older mice showed augmented resistance towards apoptosis [52]. The deterioration of old recent thymic emigrants can not be acquired in the periphery, however it is imprinted throughout their developement within the old thymus and this condition suggests that these damaged cells have tendency towards further contribution to peripheral immunosenescence. If the development of T cells is functionally active in old mice is a question which needs further studies [31].

1.3.6. T Cell Receptor Excision Circle (TREC)

Atrophy of thymus occurs with increasing age, resulting decrease in activity of thymus. Fat acquires an increasing number or quantity in human thymus during life, leading to decrease in the T cell output, because of being progressively blunt for thymopoiesis [53]. Assigning the number of this decrease brought into open new aspects of it. It was showed by several studies that a decrease with mathematical exponent in T cell output by age. It was pointed out that there was an exponential decrease in T cell output starting early in life and predicted to end at around seventy five years [54].

A study showed that the T cell number in the every microliter of blood does not alter significantly throughout the age range, but copy numbers of sjTRECs in each microliter of blood demonstrate wide change and this cause a decrease in thymic output in age-associated manner, in two hundred people between the ages of fifty eight and one hundred four years who participated in this study. It was also demonstrated that an important decrease in sjTREC/T cell levels in individuals with more than ninty years in both females and males. These results provided knowledge for potential thymic output

end-point and pointed out that analysis of sjTREC may become a suitable marker for ageing studies [55].

Thymus is the primary anatomic location for new T cell generation from undifferentiated hematopoietic precursors resulting generation of cells answering many types of antigenic stimuli. T cell receptor and the CD4 and CD8 co-receptors are important receptors for progression of T-cell maturation into discrete stages. To express distinct cell surface markers and specific gene rearrangements, thymocytes experience different maturation steps, before coming to the end stage [56-58].

Lymphoid progenitors move to the thymus and increasingly rearrange the genes of the T-cell receptor. The *TR* delta locus, located within the *TR* alpha locus have to be removed via DNA recombinations, before *TRA* locus rearrangements. Nearly seventy per cent of thymocytes have got recombination of δREC–ψJα, causing the formation of a δREC–ψJα coding joint in the chromosome and formation of a δREC–ψJα signal joint in a circular DNA and this is called as T cell receptor excision circle. Thymocytes undergoing maturation divide three or four times in the thymus, in which the duplication of TRECs do not occur. Solely, a part (around one per eight to one per sixteen) of the originally $TREC^+$ cells leave the thymus as $TREC^+$ recent-thymic emigrants. Further dilution of TREC will be determined by peripheral proliferation [59-60]. Accordingly, TRECs are the excised DNA circles generated in the duration of the TRA chain *VJ* recombination process.

Quantification assays of TRECs in peripheral blood are carried out in the research and clinical laboratories to analyze naive T cells as immigrants from the thymus. Even though TRECs do not represent recent thymic emigrants as markers, on account of a small fraction persists in the peripheral blood, called as “old” thymic emigrants. Various characteristics describe them as “bona fide” utile markers for thymic output. They are diluted during the progeny, because upon cell division they do not replicate, do not and they are stable, do not deteriorate with ease, are entirely of thymic origin, free from extrathymic origins of *TR* rearrangements [60].

Douek DC et al. first proposed the TREC quantitative assay, and then it is changed in various approaches and TREC number has been detected in various biological samples, and also it was calculated by various ways, this caused scarcely comparable results [61]. TREC calculation has been achieved more correctly once the measurement was done relatively to a control gene, including *TRA* constant (*TRAC*) gene, such as chemokine (C-C motif) receptor five, albumin [62, 63]. TREC quantity was stated as relatively to peripheral blood mononuclear cells. It was also measured within the sorted different T-cell subsets [62, 64]. Moreover, number of T cell receptor excision circles was determined as absolute number of TRECs per μg of total DNA in T lymphocytes or PBMCs [65, 66]. The number of TREC is determined for one million cells [67]. Measurement of TREC per 1 million PBMCs can cause wrong evaluation because of dilution of the divisions of peripheral T-cell, but it was overcomed by defining TREC as per ml of blood [68]. Despite lack of knowledge about half-life of TRECs, many mathematical models to find out the actual thymic output for the TREC number have been suggested [59, 68].

Children with primary immunodeficiencies have been analysed for their TREC level. Children who have severe combined immunodeficiency and Omenn syndrome which is another form of immunodeficiency demonstrated highly low or undetectable levels of TREC [69, 70]. Although circulating T cells are in normal numbers, very low levels of TREC were demonstrated in patients with the 22q11.2 deletion syndrome and patients with zeta-chain (TCR) associated protein kinase seventy kDa (ZAP70) deficiency. TREC quantification provides an improvement in our understanding the pathophysiology of other primary immunodeficiencies including $CD4^+$ T lymphocytopeniase which had correlated TREC levels with the severity of the T-cell immunodeficiency. Overall, TREC assay are utilized to increase the understanding of abnormalities in T-cells, but its exact role is not completely clear yet [71-73].

1.3.7. Gender based differences in thymic involution and thymocyte development

Gender based differences in immune system are observed in all of the vertebrates. In general, females have more robust immune response to infections and vaccination prevelance of autoimmune diseases in females was higher than males [74]. Innate and

adaptive immune system are affected directly or indirectly by sex hormones but we have not adequaate information about mechanisms under this difference [75].

For many years, it is known that sex hormones affect development of lymphocytes. Increasing body of reports have provided new evidences that sex hormones have crucial roles on the immune response and onset and/or perpetuation of autoimmune diseases [76]. Steroid hormones have been shown as important contributors to immune response for example as enhancers. Estrogen is a good example, autoimmune diseases are diagnosed in women with higher incidence [77]. Because, women have lower levels of testosterone which has protective/inhibitory effect.

As animal models, Swiss Jim Lambert (SJL) mice showed increased level of severity in experimental autoimmune encephalomyelitis. Non-obese diabetic (NOD) mice after prepubertal castration had augmented incidence of immunologically mediated diabetes. However after implantation of testesteron pellets in female SJL and NOD mice, the severity of experimental autoimmune encephalomyelitis and prevalence of diabetes decreased, respectively [78, 79].

The severity and prevalence of adjuvant arthritis and thyroid disease increased after castration, but testosterone treatment protected mice from these conditions. The incidence of the later onset of multiple sclerosis in males increases after decrease in testosterone in the body compared with females. This may contribute to protection for young men who have genetic susceptibility to multiple sclerosis. 5α-dihydrotestosterone treatment increased IL-10 production in $CD4^+$ T lymphocytes. Testosterone treatment decreases IFN-γ expression and increases IL-10 expression by autoantigen-specific T lymphocytes. The whole mechanism is still not clear yet [80].

Testosterone supplementation plays versatile roles during development of experimental autoimmune orchitis via inhibition of Th1-specific cytokine production in testicular draining to lymph nodes. Testosterone seems to stimulate an expansion of suppressive Tregs from naive T cells, causing an increase in the Tregs within the $CD4^+$ T cell population meantime simultaneously blocking the synthesis of proinflammatory mediators MCP-1, TNF-a, and IL-10 in the testis [81].

Another sex hormone as estradiol has important effects on thymus. Estradiol stimulates thymic atrophy through blocking the development of thymocyte at various phases without a detectable apoptosis and it is suggested that this process is caused by inhibition of production of DP thymocytes, not a direct killing. Increased estradiol levels block thymopoiesis at various stages of development [82]. Another study showed that estrogen but not progesterone inhibits development of T cell in the thymus, but estrogen depletion via oophorectomy does not stimulate the development of T cell [83].

Cytochrome P450 monooxygenase is an enzyme which has a important function in metabolism of sex steroids [84]. Andro-stenedione, hydroxytestosterone (HT), 7HT, 2-HT and 16-HT were found in thymus as common testosterone metabolites [84]. Level of these metabolites is highly correlated with expression level of cytochrome P450 and adult thymus contains eight fold higher expression of cytochrome P450 and three fold higher expression of testosterone receptor than fetal thymus [84].

Epithelial cells have known as androgen receptor positive since earlier studies while these earlier studies pointed thymocytes as androgen receptor negative cells [85]. But later immunoblotting, flow cytometry and ligand binding assays shown thymocytes are androgen receptor positive cells [85]. Testosterone injection increases thymic involution while cutting out of testosterone undos involution of thymus [86]. Level of sex steroids affects on thymic involution and thymic cells as thymic epithelial cells, thymocytes and mesenchymal cells is not known. Female thymic epithelial cells proliferation rate is faster than males and testosterone treated females [87]. Recent findings show that androgens suppress Dll4 expression of thymic epithelial cells [88]. Dll4 is a Notch receptor ligand and it is indispensable for maturation, commitment of thymocytes [88]. Cutting out of sex hormones increased Dll4 expression. Over expression of Dll4 by cultured stromal cells promote *ex vivo* thymocyte development. Cutting out of sex hormones increases lymphopoiesis in bone marrow, thymus and it encourages recovery in autologous and allogeneic hematopoietic stem cell transplantation [89].

Cellularity is increased in cortical thymus especially as an answer to estrogen depletion and estrogen injection reverses increased cell numbers to normal numbers [90]. 17-estradiol treatment decreases proliferation of thymus in culture, but 17-estradiol

treatment has not any direct effect on thymocyte apoptosis. Thus, estrogen effects on thymocyte apoptosis seems as indirect and it has thymic epithelial cell origin [90]. Furthermore, same research group cocultured thymocytes with thymic epithelial cells rich thymic stromal cells and they administrated 17-estradiol on these cells [90]. Thus, estrogen effects on thymocyte apoptosis seems as indirect and it has thymic epithelial cell origin [90]. They found that 17-estradiol treatment increases fragmentation of DNA and decreases DNA content in addition to plasma membrane depolarization. 17-estradiol treated thymic epithelial cells conditioned medium induced apoptosis [90]. Thus, estrogen effects on thymocyte apoptosis seems as indirect and it has thymic epithelial cell origin [90]. Apoptotic effect of estrogen was found as a caspase dependent process and this is an genomic pathway and tamoxifen, inhibitors of transcription and translation halted apoptotic action of estrogen [91].

1.3.8. The Cells Responsiple or Possibly Responsible for Thymic Involution

1.3.8.1. Regulatory T Cells and Their Roles in Thymic Function and Involution

$CD4^+$ T cells are classified as conventional T helper and regulatory T cells. Regulatory T cells constitute almost 10% of peripheral $CD4^+$ T cells. They have immunosuppressive activities and suppress detrimental activities of effector T cells to maintain immunhomeostasis. Treg cells express CD25, CD4 and FOXP3. Regulatory T cells are crucial for autoimmune and autoinflammatory disorders, cancer, allergy, acute and chronic infections, immune-mediated inflammation and metabolic inflammation [92]. CD4 expressing Treg cells were discovered in 1990s.

The primary role of Tregs is suppression of autoimmune diseases through maintaining immunologic self-tolerance, T cell activation initiated by weak stimulus [93]. The other functions of these cells are suggested as suppression of asthma, allergy, pathogen-induced immunologic pathology, stimulation of maternal tolerance towards fetus, tolerance against dietary antigens, organization of the effector type of the immune response, feedback control of the magnitude of the immune response through effector Th cells, conservation of commensal bacteria from removal by the immune system,

protection of T cells that are activated by their true high-affinity agonist ligand from killing cells that solely express low-affinity T-cell receptor ligands including the self peptide-major histocompatibility complex molecule that positively selected the T cells [93]. All of these functions of Treg need further investigations to prove.

The mechanisms responsible for Treg suppression is not clear. Contradictions may arise from the differences in *in vivo* and *in vitro* models. There are three types of suppressive mechanisms: local secretion of inhibitory cytokines, local competition for growth factors and cell–cell contacts [94].

The mission for inhibition of autospecific T cells' harmful effects belongs to regulatory T cells (Treg cells). Both Treg and Tconv cells are generated in the thymus, they are originated from a common hematopoietic precursor. Treg cells are enriched for autospecific cells, but not that of conventional T cells (Tconv cells) do not enrich. The development of this different populations seems to be controlled by different mechanisms. The development of Tregs are governed by the cognition of agonist ligands including major histocompatibility complex and peptide molecules, causing negative selection of Tconv cells. In addition, transforming growth factor β (TGF-β) and the cytokines interleukin 2 and co-stimulation through CD28 are necessary for Treg cell development, not necessary for the development of Tconv cells. These diversities would lead differential transition of Tconv cells development versus Treg cells development in the thymus [95].

Recent study shown that peripheral Treg cells can migrate to the thymus and they block *de novo* development of its precursors in mouse. Number of recirculating Treg cells increased with age, leading to progressively decreased output of newly developed Treg cells. It is also found that recirculating cells with both differentiated and activated phenotype exist in the human thymus, suggesting negative feedback loop in the development of Treg cells looking like in mouse might be found in humans [96].

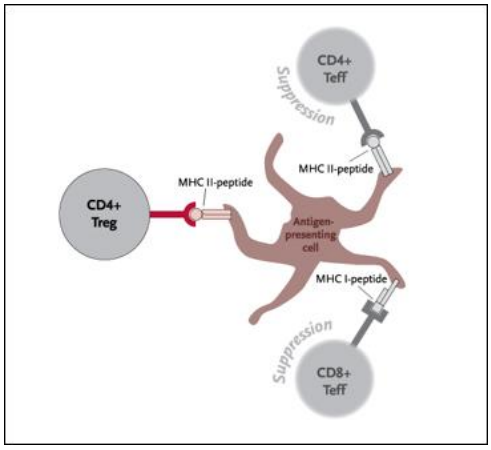

Figure 1.10. Immune responses of adjacent T cells are supressed by Treg cells [97].

A regulatory T cell is responsive to a specific antigen and it suppresss immune reactions of T cells which are responsive to antigens from same antigen presenting cells. Thus, regulatory T cells provide a tolerance for some antigens and maintain immune homeostasis [97].

The markers which are commonly used for identification of Treg cells are CD127, CD25, lymphocyte activation gene-3 (LAG-3), glucocorticoid-induced tumour necrosis factor receptor family-related gene (GITR), cytotoxic T lymphocyte-associated antigen 4 (CTLA-4), forkhead⁄ winged-helix transcription factor box P3 (Foxp3) [93]. But these markers are not exactly specific for Tregs.

Once activated, CD25, is alpha chain of interleukin-2 (IL-2) receptor which is receptor of a T-cell growth factor IL-2, it is expressed by all T cells. After two or three days of activation, CTLA-4 is increased in all CD8+ and CD4+ T cells, works as a supressive regulator for T-cell activation. GITR and LAG-3 are started to be expressed in T cells following induction. CD127 which is alpha chain of IL-7 receptor is important marker to distinguish Treg and Th cells as having low and high expression in human, respectively. But there is some discrepancy that T follicular helper (Tfh) cells which help B cells show no CD127 expression and many CD4+ T cells have decreased expression of CD127 after activation. Even though FOXP3 is important marker for Tregs, it can be expressed temporary by many $CD4^+$ and $CD8^+$ T cells in humans. Treg markers which

are currently used are not specific for Treg cells, they are not trustworthy to discriminate Tregs and activated conventional T helper cells [93, 98].

FOXP3 gene is necessary for development and function of Tregs. Mutated *FOXP3* gene causes IPEX syndrome which is an multi organ autoimmune disease; immunodysregulation, polyendocrinopathy, enteropathy, X-linked syndrome [99]. $FOXP3^+$ Tregs depletion caused devastating autoimmunity and then death within weeks in healthy adult mice [100]. Tregs are crucial for maintenance of immunotolerance.

Tregs are formed in the thymus, thymus removal on 3rd day after birth caused development of many autoimmune diseases. But removal of thymus on 7th day did not cause autoimmune diseases showing Tregs derived from thymus are necessary for prevention of autoimmunity [101].

$FOXP3^+CD4^+$ Treg cells derived from thymus are also called as natural Treg cells. FOXP3 expressing Tregs can be generated from differentiation of naive CD4+ T cells without *FOXP3* expression in the periphery, called as induced Treg cells [102]. The development of natural and induced Tregs are probably occured by different mechanisms.

To study the role of Tregs in immune homeostasis, Treg cells are set with the human diphtheria toxin receptor (DTR) in Foxp3 DTR knock-in and Foxp3-DTR BAC transgenic mice [100, 103]. Adult healthy Foxp3 DTR mice died after chronic surgical removal of Tregs, because of furious myelo-and lympho proliferative diseases. It shows that Treg cells are indispensable for mice health. The study performed using germ free Foxp3 DTR mice shown rampant myelo and lympho proliferative disease after Tregs removal indicating important role of Tregs in immune homeostasis in normal animals and Tregs are necessary for constriction of self-MHC-restricted T cells in any case of the existence of the commensal microbiota [100, 104].

Lack of Foxp3 cause deficiency of Tregs and it causes mortal autoimmunity. Treg transfer ameliorated neonatal Foxp3 deficient mice [105]. T cell specific removal of Foxp3 is enough for stimulation of the similar early onset of lympho proliferative

syndrome. Deletion of Foxp3 allele in thymic epithelial cells or dendritic cells did not cause immune abnormality or any difference in differentiation of T cells [106, 107]. Increasing number of evidences suggest that the reason of the disease in the Foxp3 mutant mice is only related with lack of FOXP3 expression in regulatory T cells .

Recent studies on regulatory T cells enlighten many unknown sides of its roles, differentiation, heterogeneity and behaviour in various tissue environments. Further studies are necessary for answering of remaining questions about them.

1.3.8.2. Lymphoid Tissue Inducer (LTi) Cells and Their Function in Thymus

Lymhoid tissue inducer cells are innate lymphoid cells and these cells are essential for development of lymphoid tissues. In embriyonic development, lymphoid tissue development is initiated by lymphoid tissue inducer (LTi) cells migration from fetal liver to the periphery. LTi cells initiate the formation of Peyer's patches and lymph nodes [108]. In addition, lymphoid tissue inducer cells in thymus is thought as an important factor for thymic repair mechanism. At first, $Lin^{neg}CD4^{neg}CD117$(c-Kit)$^{+}CD127^{+}CD25$(IL-2Rα)$^{+}CD90$(Thy-1)$^{+}$ cells were accepted as LTi cells, but $CD4^{neg}$ LTi cells were found in later studies.

Their origin is $Lin^{neg}CD4^{neg}CD117^{int}$Sca-$1^{+}CD127^{+}$ fetal liver lymphoid progenitor cells. They are dependent on the transcription factors RORγ and Id2 for differentiation, and their activity and survival were determined by IL-7 [109-111]. Peyer's patches and lymph nodes anlagens contain LTi cells [109-114]. And these cells induce lymph nodes and Peyer's patches formation during fetal development [115]. Lymph nodes and peyer patches do not form after RORγ depletion [113, 116].

Tertiary lymphoid tissues maturation after birth and repair of secondary lymphoid tissues after viral infections are promoted by adult $CD4^{+}$ LTi cells [117, 118]. Adult LTi cells are Notch signal dependent for differentiation [119].

According to findings of a study in 2012, thymic epithelial cells survival and function is promoted by interleukin-22 production of lymphoid tissue inducer cells in irradiated

mice. Interleukin 22 production by lymphoid tissue inducer cells is promoted by interleukin 23 release from dendritic cells [120].

Thymic lymphoid tissue inducer cells are found in thymus since embriyonic day fourteen and they express same markers as lymphoid tissue inducer cells in secondary lymphoid organs [121]. Interactions of thymic lymphoid tissue inducer cells with $RANK^+$ mTECs are occurred via TRANCE, this interaction is important for development of secondary lymphoid tissues [121]. RANK is the receptor of TRANCE and it induce AIRE expression [121]. AIRE or autoimmune regulator is a transcription factor and it controls the expression of self antigens on mTECs [121]. Thus, self reactive thymocytes can be eliminated with negative selection. AIRE is obligatory for negative selection, lack of AIRE causes multiorgan autoimmunity in mice [121]. mTECs' central tolerance needs AIRE expression and it is modulated by thymic lymphoid tissue inducer cells [121].

1.3.8.3. Myeloid Derived Suppressor Cells and Their Function in Thymus

Myeloid derived suppressor cells or MDSCs suppress T cell response in animal models and humans. MDSCs can accumulate in tumor tissue, circulation and lymphoid organs [122]. As a result of this accumulation, tumor development, angiogenesis and metastasis are promoted. Myeloid derived cells in tumor bearing mice have increased levels of signal transducer and activator of transcription 3 (STAT3). Additionaly, suppression of STAT3 expression suppress the MDSC poliferation and increase the level of T cell activation. Thus, phosphorylated STAT3 level is used as a marker of MDSCs activity [123].

MDSCs' arginase 1 production is increased by cytokines which are originated from T helper 2 cells. Arginase 1 is interacted with nitric oxide and produce peroxynitrite, therefore T cell receptor CD3ζ tyrosines are nitrated, here after TCR can not be phosphorylated for T cell activation. Numbers of regulatory T cells also increase and consequently T cells are directed to apoptosis [124, 125]. MDSCs and $CD4^+CD25^+$ cells interaction is required for suppression effect, hence CD80 expression increase on MDSCs for interact with CD152 on regulatory T cells [126].

MDSC cells differentiate into dendritic cells and macrophages and expression of costimulatory molecules and major histocompatibility complex 2 increase in the presence of granulocyte macrophage colony stimulator factor (GM-CSF) and interleukin 4 or GM-CSF and tumor necrosis factor alpha. MDSCs interaction with other cells decrease when MDSCs are directed to differentiation.

A model for T-cell tolerance in cancer induced by MDSC is shown in Figure 1.11. Tumor-derived factors induce the production of MDSC from hematopoietic stem cells (HSC) in the bone marrow. MDSCs go into peripheral lymphoid organs or migrate to the tumor site or peripheral lymphoid organs. They have high arginase I activity and they produce reactive oxygen species as peroxynitrite. They present antigen to T cells and nitrate their CD8 and T cell receptor, thus T cell can not interact with MHC molecules and they become unresponsive to presented antigens [125]. MDSC production from HSC pool is promoted by tumor originated factors [125].

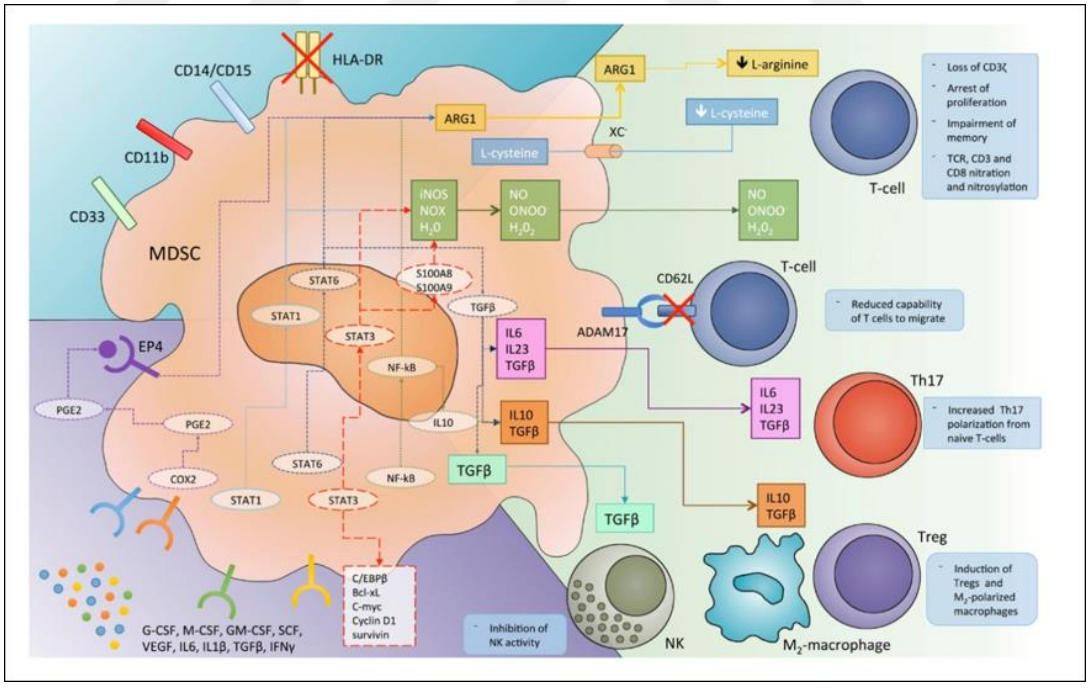

Figure 1.11. MDSC mediated immune supression pathways [127]

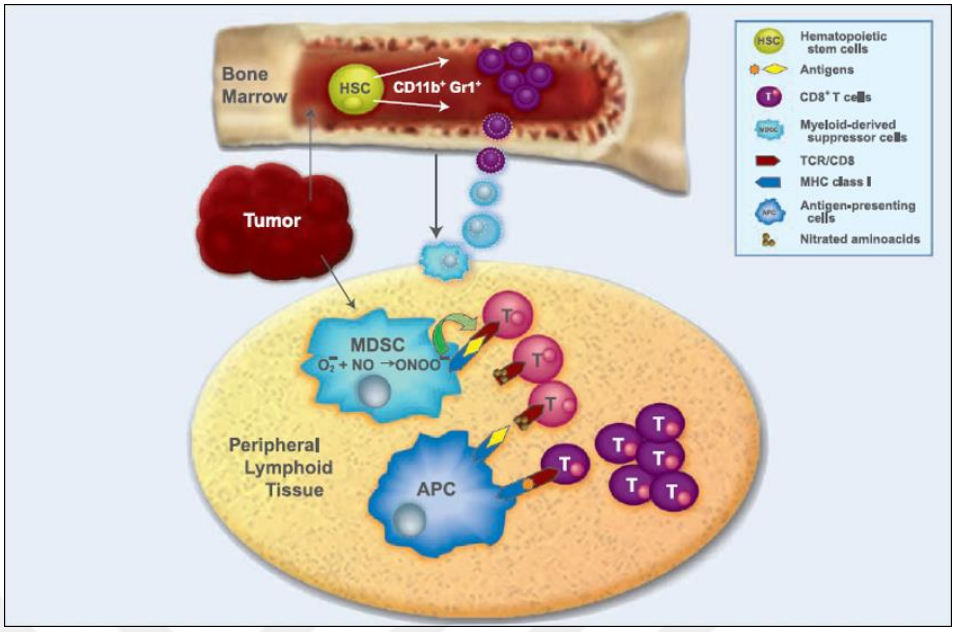

Figure 1.12. MDSC induced tolerance of T cells [125]

1.3.9. Receptors or Ligands Which are Responsible or Possibly Responsible for Thymic Involution

1.3.9.1. Keratinocyte Growth Factor and Its Receptor

Some cytokines and signal molecules behave like a stimulant for aged thymus. Keratinocyte growth factor is one of them and produced by mesenchymal origin cells and has a mitogenic effect on epithelial cells [128]. In thymus, thymocyte KGF production capacity increase during thymocytes differentiation and thymic fibroblasts are main KGF producers in thymic stroma [128]. Epithelial cells are main cells that express KGF receptors FGFR2IIIB. KGF treatment increase thymus cellularity in immune ablated young and old mice [128]. Aging related microenvironment damage can be repaired with KGF treatment.

Keratinocyte growth factor is encoded by the FGF7 gene. FGF7 belongs to heparin-binding fibroblast FGF family whose members play role in biological processes such as cell growth, embriyonic development, morphogenesis, tumor development and invasion. It is specifically effective on epithelial cells, mitogenically [129]. It is found in the epithelialization phase of wound healing. Mesenchymal originated cells produce FGF7,

indicating a possible paracrine intermediary for mesenchymal-epithelial communication. FGF7 receptor, KGFR/FGFR2-IIIb is encoded by *FGFR-2* gene, which represents tyrosine kinase isoform, is found in epithelial cells of various organs [130]. FGF1, FGF3, FGF7, FGF10, and FGF22 are ligands for KGFR. Mesenchymal cells express alternatively spliced isoform, FGFR2IIIc [131].

FgfR2IIIb deficient mice stops growth of thymus after twelve and half embryonic day, a stage that takes precedence of its detection in thymic epithelial cells, causes a very hypoplastic thymus compared to those of normal mice [132]. Disruption of *FGFR2IIIb* gene caused developmental defects in various organs including hypoplastic thymus [132, 133]. Postnatal thymus has FGFR2IIIb, FGF7, and FGF10, it shows that this signalling pathway remains for the maintainance of thymus environment. FGF7 treatment to adult mice transiently changes the appearance of thymic epithelial cells, plays role in differentiation of thymic epithelium through increasing postnatal T cell development by inducing expansion and differention of both mature and immature thymic epithelial cells [134].

FGF7 are mainly produced by fibroblasts and mature $CD4^+$ and $CD8^+$ thymocytes in the thymus and effects on thymocytes and thymic epithelial cells to induce their function and proliferation [129-134].

FGF7 decreased thymic aging in aged murine models, it induced T cell production and protected medullary construction. It adjusts T cell development and TEC function related genes, such as Wnt5b, Wnt10b, BMP2 and BMP4 through p53 and NF-κB signaling pathways [129-134].

FGF7-/-, wildtype, and FGF7+/- mice shown similar thymic cellularity and distribution of thymocyte subset [129-134]. FGF7-/- were more indefensible to sublethal irradiation and they had a significant diminish in thymic cellularity after irradiation. Disabled peripheral T-cell reconstitution and thymopoiesis were seen in FGF7-/- recipients of syngeneic or allogeneic bone marrow transplant, however usage of FGF7-/- mice as donor did not affect the development of T-cells. FGF7 caused a retardation in the early development in the thymus, FGF7 treatment in young and old mice stimulated

thymopoiesis and quickened the healing of thymus after irradiation, administration of cyclophosphamide, and dexamethasone. Middle-aged recipients of an allogeneic bone marrrow transplant shown increased numbers of T cells and thymopoiesis after FGF7 treatment. Overall, FGF7 has important function in postnatal thymic recovery [132]. FGF7 treatment before bone morrow transplantation stimulated the ability of generation of thymocytes derived from donor. FGF7 pre-treatment provided normalization of the proportion of thymic subpopulations, augmented the naive T cells in the periphery and increased the response towards neoantigen immunization. It was shown that intrathymic IL-7 production was increased after FGF7 treatment and IL-7 signaling pathway is necessary for thymopoietic function of FGF7 [132]. FGF7 treatment improved thymic T cell reconstitution and repaired thymic construction before $CD34^+$ peripheral blood progenitor transplant in rhesus macaques and the numbers of naive T cell and sjTREC and T cell function in the periphery were augmented [130,132].

1.3.9.2. ARP 2/3 Complex

Arp 2/3 complex is built from seven protein subunits and it behaves as a regulator in actin polymerization. Arp 2/3 complex contribute into the fibroblast monolayer migration and cell polarity according to findings from wound healing model with mice embriyonic fibroblast cells.

Actin cytoskeleton is very important in the lifetime of the eukaryotic organisms. The movement of the cells depends on the dynamism of actin cytoskeleton originated from actin filaments which are in continual turn over individually. The formation of new actin filaments starts with nucleation. The nucleation from monomers needs actin-nucleating proteins which consists of the Arp2/3 complex and its nucleation promoting factors, formins, and tandem-monomer-binding nucleators.

Actin is found as the most plenty and highly conserved protein in the eukaryotic cells. It is the main component of cell cytoskeleton. G-actin as actin monomers form F-actin filaments by assembling. These filaments are polarized, having fast growing barbed ends and slow growing pointed end. The formation of a stable multimer of actin monomers is the rate limiting process during polymerization because of the instability of actin dimer

intermediates and the suppression of spontaneous nucleation by actin monomer sequestering proteins. Arp2/3 complex, formins and tandem-monomer binding nucleators play role in the solution of the kinetic problem for nucleation.

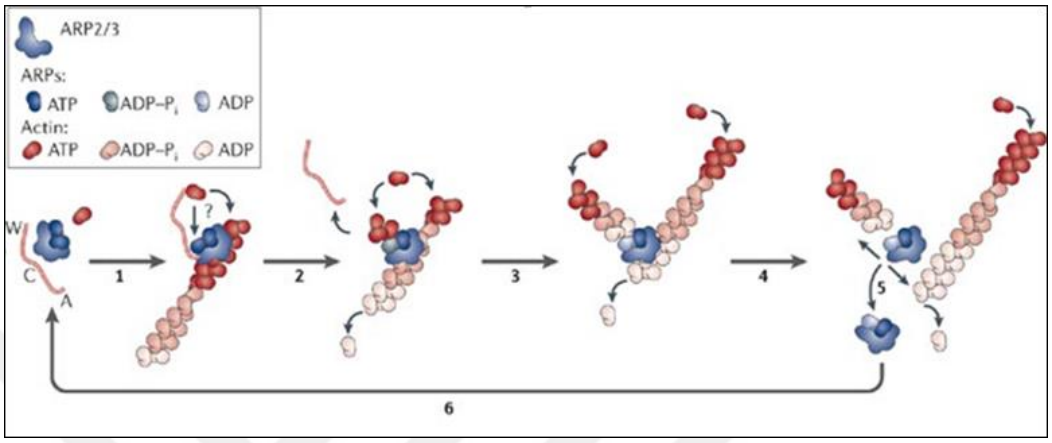

Figure 1.13. Actin nucleation by Arp 2\3 [135]

In Figure 1.13, ARPs: ARP2/3 ATP, blue ARP2/3 ADP inorganic phosphate, grey; ARP2/3 ADP, light grey. Actin: actin ATP, red; actin ADP P_i, pink; actin–ADP, light pink. At first step of actin nucleation**,** the ARP2/3 complex begins in an open conformation as an inactive form. Domain of Wiskott Aldrich syndrome protein homology 2 stimulates a conformational alteration which makes ready the complex for activation, happening upon the WCA actin ARP2/3 assembly binding to the mother filament, preferably close to barbed end. At second step of actin nucleation, WCA domain gives an ATP actin monomer to the probably to the barbed end of the mother filament and/or to the complex. At third step of actin nucleation, even though the stimulator is unknown, The WCA separates. ARP2 release phosphate. At forth step of actin nucleation, daughter and mother filaments extend and age via ATP hydrolysis and release of phosphate. At fifth step of actin nucleation, Release of phosphate from ARP2 and filament aging reduce the interplay between mother and/or daughter filament and ARP2/3. At sixth step of actin nucleation, letting disassembly of branch and ARP2/3 complex release, probably ADP bound conformation as inactive. Cycle restart with the exchange of nucleotide on ARP2 and probably on ARP3 [135].

Arp2/3 complex is an actin nucleator, consists of evolutionary conserved subunits including Arp2 ans Arp3 as the actin-related proteins and ARPC1 5 as five other subunits [135]. For activation of Arp2/3 complex, it is necessary to bind to actin filaments and nucleation promoting factors which possess catalytic domain have G-actin binding Wiskott-Aldrich homology 2 domain and Arp2/3 binding central/acidic sequences. When the Arp2/3 complex is activated, it nucleates the formation of new filaments that stretch out from existing filaments at a seventy degree angle for the formation of a Y branched network [135].

Arp2/3 complex is not able to nucleate alone. Cellular cofactors are necessary for the ability of nucleating and branching of Arp2/3 complex. Many NPFs of Arp2/3 can be divided into 2 different classes. Type I NPFs VCA domain consist of three conserved motifs which causes G-actin binding and ARP2/3 via V and CA motif, respectively. This group of NPFs contain Wiskott-Aldrich syndrome protein, WASP family verprolin-homologous protein, WASP and SCAR homologue, neural WASP and junction-mediating and regulatory protein [136].

Type II NPFs do not have complete VCA domains, instead possess acidic domains at the amino terminal site and tandem repeat domains which bind F- actin that is able to bind the ARP2/3 complex and F-actin, respectively [135, 136]. Cortactin which is a type II NPFs can faintly acivate ARP2/3 complex and bind to the branches of ARP2/3-nucleated filament for inhibition of debranching [135,136].

It is hard to study the function of ARP2/3 complex *in vivo*, because its distruption becomes lethal. The knowledge about its structure and biochemistry comes from *in vitro* studies. The cellular function was interpretated from its localization status [136]. In recent years, it becomes possible to study the function of ARP2/3 complex thanks to generating tools such as some inhibitors that helps the investigation of ARP2/3 function in various cell types [136].

One of these generating tools is CK 666 which is a small molecule, inhibitor for ARP2/3 complex. It can reversibly inhibit ARP2/3 function and ARP2/3 was shown to play role

in cytoplasmic streaming of actin and asymmetric cell division in oocytes and actin retrograde flow in neuron through this inhibitor [136].

A study shown that Arp2/3 complex inhibition using CK 666 caused disruption of actin veils, a decrease in barbed end actin assembly site density at the leading edge, and eventuated veil retraction [137]. Inhibition of Arp 2/3 complex increased rates of retrograde actin flow. But, inhibition of myosin II and Arp2/3 complex induced retardation of retrograde actin flow and veils no longer drawn back. If the Arp2/3 complex and myosin II are spatially separated, actin nets gathered by the Arp2/3 complex can limit myosin II dependent contractile ability with resultant effects on growth cone movement ability [138].

Another study shown that disruption of Arp2/3 using CK 666 and Arpc2 and Arpc3 RNAi caused defects in spindle migration, asymmetric division, and oocyte cytokinesis [139]. It suggested that the Arp2/3 complex may regulate oocyte polarization via its effects on spindle migration, asymmetric division and cytokinesis during mouse oocyte meiotic maturation [139]. An other study in mouse oocytes shown that inhibition of Arp2/3 complex reduced flow and cytoplasmic streaming and make possible a reverse streaming driven by myosin II based cortical contraction, moving the spindle away from the cortex. Arp2/3 complex directs balanced forces which maintain the asymmetric MII spindle position [136].

Loss-of-function genetics is another strategy to study ARP2/3 comlex *in vivo.* Cell lines which ARP2/3 knockdown and knockout with maintaining proliferative ability *in vitro* [140, 141]. Owing to these cell lines, the effect to ARP2/3 complex on cell motility was studied.

ARPC3$^{-/-}$ fibroblasts could not extend lamellipodia but formed dynamic leading edges consisted of primarily of filopodia-like protrusions. ARPC3$^{-/-}$ fibroblasts shown deficiency in the coordination of the protrusive activities at the leading edge indicating the important role of Arp2/3 complex in lamellipodia extension and directional fibroblast migration [141]. The different results were demonstrated for the role of ARP2/3 complex on chemotaxis. One report shown no impact on chemotaxis along platelet-derived

growth factor gradients for the cells which had RNAi mediated depletion of the ARP2/3 complex or inhibited by CK666. Another report shown that *Arpc3*−/− cells had disabled migration along epidermal growth factor gradients. The difference may be caused by different methodologies which needs further investigation [140, 141].

The ARP2/3 complex was demonstrated to be necessary for haptotaxis, the ability of sensing extracellular matrix gradients on the substrate. ARP2/3 complex deficient cells failed in sensation or response to gradients of fibronectin, laminin or vitronectin, while control cells in the same microfluidic chamber could migrate into the higher concentrations of the extracellular matrix [140]. Focal adhesions, structures that mediate extracellular matrix cell interactions were not alligned well in the ARP2/3 deficient cells in comparison to control cells, indicating its role in haptotaxis.

Thymosin β 4 make a complex with profilin and actin at higher thymosin β 4 concentrations (>5-10μM) and this condition suppresses actin polymerization and causes a large increase in unpolymerized actin amount in cells [141]. Wise et al. shown that thymosin β expression in rats was affected by sex hormones, thymosin β level was changed in castrated females and castrated androgenized females. Changes in ACTR2 seems in correlation with changes in thymosine β [142]. ACTR2 level may change as a cell answer to changed thymosine β levels or ACTR2 level may decrease as an answer to decreased thymosine β levels [143].

Testosterone levels affect Arp 2 gene promoter methylation in hippocampus, thus testosterone has a role in control of Arp 2/3 complex production [142]. We can propose that testosterone related Arp 2 methylation or expression change as a determinant for repair differences between three months old female and male mice. Arp2 methylation may suppresses the expression of Arp2 in thymus like hippocampus and as a result of this less amount of Arp2 protein is produced. Because of that, decrease in formation capacity of Arp2/3 complex may cause a defect in thymus repair mechanism.

1.3.9.3. Interleukin-7 and Its Receptor

Interleukin-7 is mainly produced in bone marrow and thymic stroma [128]. Interleukin 7 receptor is expressed by diverse cell types. IL-7 is a necessary factor for T and B cell development in mice, but not necessary for B cell development in humans. Thymic cellularity decrease in IL-7 and IL-7R knockout mice. Different results are obtained from the studies about IL-7 effect on thymic involution [128].

IL-7, a hematopoietic growth factor secreted by stromal cells in the bone marrow and thymus [143, 144], it is included in the cytokines family and exerts its signaling through the cytokine gamma chain (γc), also through the IL-7 receptor alpha chain. IL-7 plays a key role in the regulation of lymphopoiesis which is directed by predominant cell which produces IL-7 in the thymus is major histocompatibility complex class II^+ epithelial cell [145]. Fetal and adult liver cells, keratinocytes, dendritic cells, hepatocytes, neurons, and epithelial cells also secrete IL-7. Normal lymphocytes do not secrete it.

Fibronectin and heparan sulfate are glycosaminoglycans of extracellular matrix which bind to IL-7 and this is important in IL-7 induced signaling within the microenvironment [145].

Thymic stromal-derived lymphopoietin is found in the cytokine family which shares overlapping and also different biological activities with IL-7. The overlapping properties are originated from the use of common receptor components, IL-7Rα which is necessary component for TSLP signalization through TSLPR complex, while γc is not necessary [146].

IL-7Rα is included in the family of hematopoietin receptors and a type I membrane glycoprotein. Its extracellular domain consists of two hundred twenty amino acids, shows high homology with other family members. Transmembrane region and cytoplasmic tail consists of twenty five and one hundred ninety five amino acids, respectively, which are crucial in recruitment of signalling molecules including kinases which are necessary for signal transduction whereat intrinsic tyrosine kinase activity is not found in intracellular part of IL-7Rα [147]. So, tyrosine phosphorylation by IL-7 is

entirely via the recruitment of intracellular kinases. IL-7 receptor activation may stimulate some kinase members of PI3-kinase, Janus and Src families. NFAT, STATs, c-myc and AP-1 are some important transcription factors which are latter activated [148].

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IL-7 has many common functions with other cytokines such as IL-2. It operates on lymphocytes like IL-2, utilizing receptor part γc chain. IL-7 plays important function particularly in the early phases of T cell development. It has also important roles in the development of the immune system. IL-7 stimulates some intracellular proteins to be phosphorylated in pre-B cells, leukemic T cells and thymocytes [148].

Mutated γc and Jak3 cause SCID syndrome which show defects in T- and natural killer (NK) cells similar to that of γc-deficient mice [149]. IL-7R α mutations in humans caused T cell deficiency, impairments in the function of B cells and alterations in the number of NK cells [150].

Binding of IL-7 to IL-7Rα leads to dimerize with γc [151]. Jak3 which is connected to γc phosphorylates tyrosine residues of IL-7Rα in the cytoplasmic part. This recruits Jak1 and STAT molecules. The requirement of γc for IL-7 signalling is only because of the absence of intrinsic tyrosine kinase activity of IL-7Rα, so Jak3 is required for stimulation of phosphorylation [151].

IL-7 has function in B cell development. It has roles in the specification and commitment into the B lineage, the survival and proliferation of B cell progenitors; and maturation while transition of the pro-B into pre-B cell [152]. While inhibition in the pre–B cell development from pro-B cell was seen in IL-$7^{-/-}$ mice, inhibiton in the pre–pro–B cell as earlier phase was reported n IL-$7R\alpha^{-/-}$mice [153]. It may regulate development of B cells in the pre–pro–B cell stage. Administration of IL-7 stimulates the increase in the pre-B cells and mature B cells in both lymphocyte deficient and normal mice [153].

Interleukin-7 also plays roles in the development of T cells, also. The supportive cells in the thymus, such as epithelial cells, fibrobalsts allow for the formation of the microenvironment with growth factors and the others. IL-7 can be identified within the

thymus of thirteen-day murine embryos can have IL-7, suitable with the first thymocyte expansion [154]. A subset of MHC class II^+ epithelial cells have been reported to express IL-7 along with SCF which is another important factor for thymocyte development [155], they act together for proliferation of thymocyte. IL-7 has been found to stimulate immature T cell proliferation and survival. The studies using IL-$7^{-/-}$ and IL-$7R\alpha^{-/-}$ mice have shown that other molecules utilizing the IL-7Rα chain was crucial for the development of early T-cells. Thymic cellularity decreased to 0.01 per cent to ten per cent in IL-$7R\alpha^{-/-}$ mice and decreased to twenty fold in IL-$7^{-/-}$ mice compared to normal mice. Developed αβ T cells did not act normal in a part of IL-$7R\alpha^{-/-}$ mice. Triple negative immature thymocytes were fractionally inhibited in IL-$7^{-/-}$ mice [156]. 12 weeks treatment of anti–IL-7 monoclonal antibody led to a dramatic decrease (more than 99% of normal) and an arrest before $CD44^+CD25^+$ phase with an increase in the $CD4^-CD8^-$ cells [157]. IL-7 overexpression augments in the numbers of T-cells, namely T cell output at least partially [158]. IL-7 has function in early thymocytes survival in the TN developmental phase via the members of bcl-2 family [158]. IL-7 plays role in differentiation of thymocytes. IL-7 was reported to have function in the inhibiton of the CD4 expression transcriptionally and it CD8 single positive cells indicating an important role for differentiation from double positive to single positive [159].

IL-7 has function in thymic aging. While IL-7Rα is necessary for survival, proliferation and RAG expression in adult thymocytes, it is not necessary for survival and RAG expression in fetal thymocytes, but required for proliferation [160]. IL-7 treatment of elderly mice causes marked increase in TN thymocytes without any important alteration in the relative ratio of the cells in each groups. IL-7R expression and IL-7 mRNA did not decrease with age [161]. Even though IL-7 increases thymic function throughout aging, another factors may be more important in thymic ageing than IL-7 alone.

IL-7 also regulates mature T-cell function. It increases proliferation and cytokine production of T cell [162]. It increases CXCR4 chemokine receptor expression, CXCR4 is a potential marker for T-cell homing to lymphoid tissues and found in a subset of memory $CD4^+$ T cells [153]. IL-7 inhibits programmed cell death and maintains mature T cells probably via lung Kruppel-like factor and T-cell survival factor [163]. It also induces lytic activity of classical $CD8^+CD3^+$ cytotoxic T lymphocytes (CTLs), NKT

cells, NK lytic effectors and $CD4^{-}CD8^{-}$ γδ T cells [164]. IL-7 treatment increases antitumor and antiviral activity of T cells compared to IL-2 or IL-4 treatment [165].

1.3.9.4. Interleukin-22

Interleukin-22 is a cytokine which is encoded by IL22 gene in humans, it is found in the IL-10 superfamily [166]. Its heterodimeric receptors constitute of IL-10R2 and IL-22R1 subunits, IL-22R is expressed by tissue cells and not by immune cells [167]. It plays important roles in host defense at mucosal surfaces, chronic inflammatory diseases and tissue repair. It is produced by immune cells such as T-helper (Th) cells and innate lymphocytes including LTi, LTi-like cells, NK cells and distinctive for the property of acting solely on non-hematopoietic stromal cells, including hepatocytes, epithelial cells and keratinocytes [168].

IL-22 cytokine stimulates Stat3 signaling cascades, mitogen-activated protein kinase and Akt pathways and then activation of some tissue specific genes, such as serum amyloid A, mucins and antimicrobial proteins including b-defensin, Reg3c, lipocalin-2 [169]. It may play as a survival factor through activating anti-apoptotic pathways and proliferation for tissue protection [170].

IL-22 expression is extremely encountered in various chronic inflammatory disease such as rheumatoid artiritis and psoriasis [171]. IL-22 can have either protective and inflammatory functions. IL-22 overexpressing transgenic mice shown some skin properties like psoriasis, it was also shown that it is crucial for epidermal remodeling [172]. It was demonstrated that IL-22 was inflammatory during a T-cell-mediated model of psoriasis [172]. Keratinocyte proliferation and epithelial hyperplasia were increased by IL-22 *in vitro* [173]. Thus, IL-22 is inflammatory during skin inflammation and it is very important in the thickening of epidermis through induction of keratinocyte proliferation and migration.

IL-22 has important inflammatory function in rheumatoid arthritis. IL-22 deficient mice showed lower incidence of pannus and arthritis generation [174]. IL-22 has also protective and therapeutic functions during inflammation including hepatitis and

inflammatory bowel disease. Injection of IL-22 expressing Th17 cells to mice before stimulation of acute liver inflammation limited the tissue damage [175]. IL-22 gene therapy into mice colons heal inflammation [175]. IL-22 is a protective factor for hepatocytes exerting this survival role via inducing expression of STAT3, ergo expression of various anti-apoptotic and mitogenic proteins [176]. Clinical studies of genome-wide association shown that inflammatory bowel disease patients had mutations in the genes of IL-22 and IL-10Rb subunit of its receptor [176]. It is important in inducing liver lipogenesis, liver regeneration and hepatic steatosis [176].

IL-22 is protective in fibrosis development. IL-22 expressing gammadelta T cells with IL-22 expression are crucial for protection from inflammation-induced pulmonary fibrosis. It was shown that inhibition of IL-22 expression resulted in an increase in lung fibrosis and treatment of IL-22 inhibited lung fibrosis through using a mouse model of lung fibrosis [177]. IL-22 has important roles in wound healing. *In vitro* assays have shown that administration of IL-22 into keratinocytes accelerated healing after damage [178]. IL-22 deficient mice shown a retardation in recovery of mucosal colonic biopsies [178]. It has also curative roles for alcohol-induced injury and liver regeneration after hepatectomy [178].

IL-22 is important in thymic regeneration. Owing to its role in inducing and decreasing autoimmune pathology within epithelial parts, IL-22 could have effect in epithelial regeneration after thymic damage. Thymic amelioration was stimulated by depletion of $CD4^{+}CD8^{+}$ double positive cells, the regeneration was disrupted in IL-22 deficient mice and IL-22 levels were significantly enhanced in the thymus of normal mice after thymic damage [179]. Furthermore, IL-22 treatment increased thymic amelioration after following total body irradiation. Radio-resistant $ROR\gamma(t)^{+}CCR6^{+}NKp46^{-}$ lymphoid tissue-inducer cells upregulated IL-22 expression after thymic damage in an IL-23-dependent way.

An endogenous pathway for thymic regeneration was revealed showing that DP thymocytes decrease and this decrease induces IL-23 upregulation which stimulates IL-22 production by thymic innate lymphoid cells. This network gives rise to recovery of the epithelial compartment which has supportive roles and then rejeneration of

thymopoiesis [180]. Another study which aimed to reveal the role of IL-22 on the recovery and function of thymus from graft-versus host disease mice after allogeneic bone marrow transplantation shown that IL-22 quickened the recovery of thymus and improved the IFN-γ-producing ability of thymus $CD4^+$ and $CD8^+$ T cells from GVHD mice [181]. IL-22 could exert its effects through thymic epithelial cells and also probably non-epithelial cells including endothelium.

1.4. AIM OF THE STUDY

The specific objectives of the study are:

- determine the effect of Arp 2/3 complex production on gender related differences in thymus repair
- determine the effect of sex hormones or gender differences on MDSC and Treg related T cell supression
- determine the effect of sex hormones or gender differences on stem cells and progenitor cells in bone marrow
- determine the effect of sex hormones or gender differences on KGF, IL-7 and their receptors expression in thymus.
- determine the effect of sex hormones or gender differences on thymocyte development
- determine the effect of sex hormones on naive T cell production
- determine the effect of sex hormones on thymic LTi cells in thymus

2. MATERIALS AND METHODS

In this study, five different group of experiments were realized.

- Animal studies
- Gene expression analysis of *ACTR2, IL7, IL7R, FGF7, FGFRII, IL22* with quantitative real time PCR
- Methylation analysis of *ACTR2* gene promoter region with conventional PCR
- sjTREC copy number analysis with quantitative real time PCR
- Immunophenotyping of bone marrow hematopoietic stem and progenitor cells, thymic lymphoid tissue inducer cells, myeloid derived suppressor cells, regulatory T cells and thymocytes in different stages of development.

2.1. ANIMAL EXPERIMENTS

2.1.1. Experiment Groups

Total 42 C57BL/6 mice were used for experiments. Their gender and age are shown in Table 2.1

Table 2.1. List of animal groups

Animal Groups				
Species	**Strain**	**Gender**	**Age**	**Number**
Mus musculus	C57BL/6	female	3 month	21
Mus musculus	C57BL/6	male	3 month	7
Mus musculus	C57BL/6	female	7 day	7
Mus musculus	C57BL/6	male	7 day	7

2.1.2. Animal Care, Feeding, Treatments and Sample Collection

C57BL/6 strain mice were fed with standard chow under suitable ventilation and sterile conditions. Seven mice in twenty one female mice group were injected with a

compatible synthetic aromatase inhibitor (subcutaneous Letrozole ten micrograms per day) for supression of estrogen synthesis for three months, thus effects of of estrogen on immunesenescence was determined [182]. In addition, other seven female mice were treated with subcutaneous testosterone in corn oil (five mg per kg, once in three days) for three months, effect of testosterone supplement on thymic involution determined in this way [183]. Other seven female mice were were only fed with normal chow. All of the other experimental groups were only fed with normal chow.

Seven day or three month old mice were killed with exsanguniation under anesthesia (50mg/kg pentobarbital intravenous). Blood was collected in BD Vacutainer® blood collection tubes (with EDTA) (Becton Dickinson, USA). Thymus, two tibia and femur were dissected and transferred into 10% FBS (Gibco, USA), 1% penicillin-streptomycin (Sigma, USA) RPMI (Gibco, USA). All samples were stored and transferred to laboratory in special sample transfer boxes.

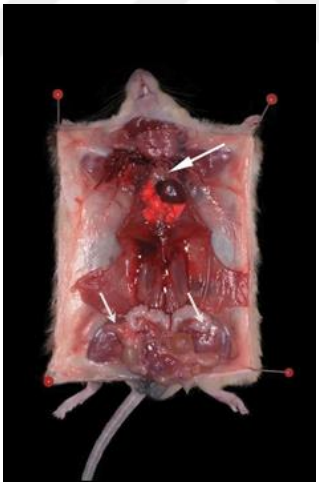

Figure 2.1. Mice thymus and tibia femur bones [184]

2.2. DETERMINATION OF ARP2 EXPRESSION DIFFERENCES IN DIFFERENT EXPERIMENT GROUPS

RNA, DNA and protein are isolated from thymus simultaneously with DNA/RNA/Protein Allprep Mini Kit (Qiagen, Germany) according to manufacturer's protocol. 260/280nm ratio and concentration is measured for with Biotek Epoch ELISA Reader TAKE3 apparatus (Biotek, Germany).

Complementary DNA (cDNA) is reverse transcribed from mRNA with SuperScript III First Strand Synthesis Kit according to manufacturer's protocol. cDNA is used for *Arp2* gene expression analysis with quantitative Realtime Polymerase Chain Reaction (qRT-PCR). TaqMan gene expression assays were used as predesigned TaqMan assays (Life Technologies, USA).

TaqMan Reference (housekeeping) gene and Assay number:
TBP (TATA Box binding protein) Mm00446971_m1

TBP gene was selected according to its stable expression under different conditions [185]. Figure 2.2 shows the changes in Ct value of 13 housekeeping genes in 16 different tissues (thymus, spleen, etc).

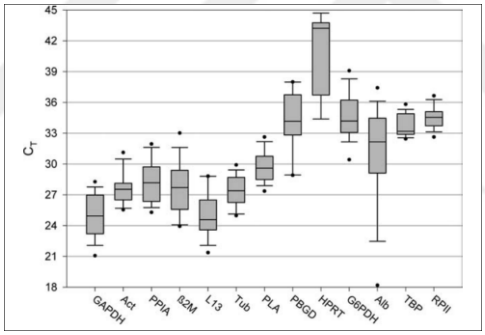

Figure 2.2. Changes in Ct values of 13 housekeeping genes in 16 different tissues [185]

Pre-designed *ACTR2* Gene Expression Assay (Mm00773842_m1) and TaqMan Universal Master Mix with UNG (Life Technologies, USA) were used for qRT PCR reactions. StepOne Plus (Applied Biosystems, USA) was used as Real Time PCR system.

Table 2.2. List of components and volumes in reaction mix for single reaction.

Component	**Volume (µl)**	**Final Concentration**
TaqMan Universal Master Mix II with UNG	10	1x
TaqMan Gene Expression Assay	1	1x
cDNA template+PCR grade water	9	100 ng

Table 2.3. PCR Program for qRT PCR Experiment

	Step 1	**Step 2**	**Step 3**	**Step 4**
Time (mm:ss)	50	95	95	60
Temperature (ºC)	2	10	00:15	1:00
No. of Cycle	1	1	40	

Negative controls were used for false positives. Amplification curves were analysed for appropriate amplification. Calibrations of Real Time PCR instrument were made with StepOne Plus Spectral Calibration Kit (Applied Biosystems, USA) before real time PCR experiments.

2.3. DETERMINATION OF PROMOTER METHYLATION OF THYMIC ACTR2 GENE

ACTR2 gene promoter region in mice was determined with bioinformatics softwares and possible methylation sites on promoter region were determined through Swiss Institute of Bioinformatics-Eukaryotic Promoter Database website. Methylation analysis of *ACTR2* gene promoter region is performed with thymic DNA and Promoter Methylation PCR Kit (Affymetrix, USA). Primers were designed with Primer3 software and analyzed with Integrated DNA Technologies Oligo Analyzer for hairpin, primer dimers. Forward and reverse primer specificity were analyzed with National Center for Biotechnology Information (NCBI) Primer Blast.

Promoter methylation analysis procedure consists of three main steps. MseI restriction enzyme is used for fragmentation of genomic DNA. DNA fragments are incubated with methylation binding protein (MBP) for formation DNA/protein complex. Methylated DNA fragments are isolated and multiplying with PCR. PCR products run on agarose gel. If promoter region is methylated, bands can be seen on agarose gel.

Protocol of Promoter Methylation PCR Kit:

- 10µl of isolated thymic DNA was fragmented with 2 hours incubation in MseI enzyme, NE Buffer and distilled water mix at 37°C.
- 100µl purification column binding (PB) buffer was added into the fragmented DNA and all of the solution was transferred into the DNA purification column. Column was centrifuged at 10000g for 60 seconds for binding of DNA. Flow-through was discarded.
- 750µl of PE Buffer was added into the column and column was centrifuged at 10000g for 60 seconds. Flow through was discarded and column was centrifuged at maximum speed for 1 minute.
- 10µl of distilled water was added into the column and column was incubated for 5 minutes. Fragmented DNA was eluted with centrifugation at maximum speed for 1 minutes.
- For formation of MBP and DNA complex, 2µl MBP, 6µl purified DNA, 4µl binding buffer, 8µl dH_2O mixed and incubated for 30 min.
- Separation column was washed with chilled 500µl column incubation buffer
- Incubated MBP-DNA complex was mixed with 20µl column incubation buffer. This mixture was transferred into separation column and column was incubated on ice for 30 minutes. The column was centrifuged at 7000RPM at 4ºC for 30 seconds, flow through was discarded.
- Column was washed with 600µl of column wash buffer and it was centrifuged at 7000RPM at 4ºC for 30 seconds. This step was repeated for 3 times. An additional centrifugation was made at 7000RPM at 4ºC for 30 seconds.
- 10µl column elution buffer was added into separation column and column was incubated for 5 minutes at room temperature.
- Separation column was inserted in a new sterile 1,5ml tube and it was centrifuged at 10000 RPM at room temperature for 1 minute.
- Isolated methylated DNA was used for PCR amplification. For PCR reaction, 1,25µl 10µM forward primer, 1,25µl 10µM reverse primer, 12,5µl 2x Phusion High Fidelity Master Mix (New England Biolabs, UK), 5µl PCR grade dH_2O, 5µl isolated DNA were mixed on ice. PCR Program: 98ºC for 30 seconds, 35x

(98ºC for 5 seconds, 64ºC for 10 seconds, 72ºC for 10 seconds), 4º for ∞. PCR products ran on 2% agarose gel.

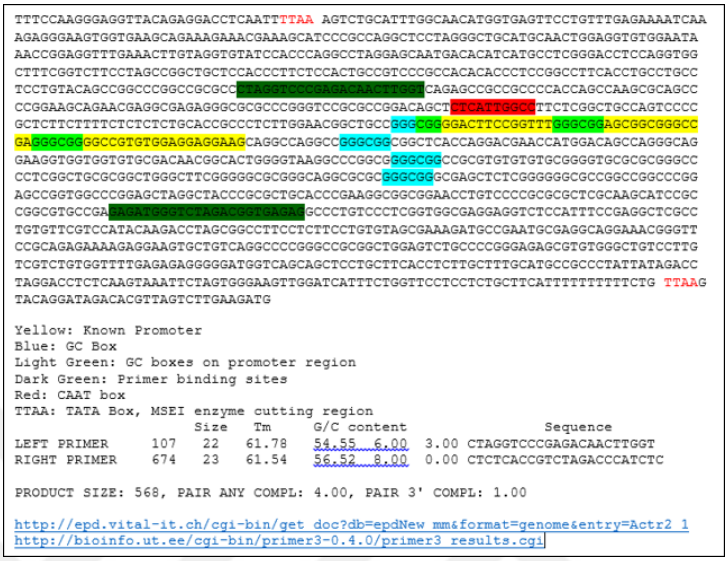

Figure 2.3. MseI enzyme cutting regions, and primer binding sites on *Arp2 (Actr2*) gene promoter region

2.4. DETERMINATION OF IL-7, IL-7R, IL-22, KGF (FGF7) AND FGFR2 EXPRESSIONS IN THYMUS

cDNA was synthesized from RNA of mice thymic tissues by SuperScript III First Strand Synthesis Super Mix (Invitrogen, USA). 5µL of RNA, 1µl oligodT primer, 1µl annealing buffer and 1µl PCR grade water were mixed on ice. Mix was incubated at 65ºC for 5 minutes and it was chilled on ice for 1 min. 10µl 2x first strand reaction mix and 2µl Superscript III/RNAse out enzyme mix were added into the tube. Mix was incubated at 50ºC for 50min, reaction was terminated with incubation at 85ºC for 5min.

100ng cDNA was used for each qPCR reaction, and two replicates were performed for every primers and probes. $2^{-\Delta\Delta Ct}$ method was used for calculation of relative gene expression. Seven days old female group was used for normalisation, the relative expression levels were calculated according to this group. One-Way ANOVA test was used for statistical analysis for groups. P<0,05 was accepted as statistically significant.

Pre-designed TaqMan probe and primers assays, master mix, plates and plate films are bought from Life Technologies (USA) for gene expression analysis. Applied Biosystems (USA) StepOne Plus is used as Realtime PCR System. Assays are listed below.

Reference (housekeeping) gene and Assay Number:

TBP (TATA Box binding protein) Mm00446971_m1

Target Genes and Assay Numbers:

- *IL7* Mm01295803_m1
- IL7R Mm00434295_m1
- *IL22* Mm01226722_g1
- KGF Mm00433291_m1
- KGFR (FGFR2IIIB) Mm01269930_m1

Table 2.4. List of components and volumes in reaction mix for single reaction.

Component	**Volume (µl]**	**Final Concentration**
TaqMan Gene Expression Assay	1	1x
cDNA template+PCR grade water	9	100ng

Table 2.5. PCR Program for qRT PCR Experiment

	Step 1	**Step 2**	**Step 3**	**Step 4**
Time (mm:ss)	50	95	95	60
Temperature (ºC)	2	10	00:15	1:00
No. of Cycle	1	1	40	

All qRT PCR experiments were carried on StepOne Plus Real Time PCR System and StepOne Software Ver. 2.2.2 was used. Standard ramp speed was used. Analysis was made with Comparative ΔΔCt method.

2.5. ABSOLUTE QUANTIFICATION OF sjTREC COPY NUMBERS IN PERIPHERAL BLOOD AND THYMUS WITH QUANTITATIVE REAL TIME PCR

Total DNA was isolated from blood with Qiagen Qiaamp DNA Mini Kit according to manufacturer protocol. DNA from peripheral blood were used for Zen probe based absolute quantification of copy number of sjTRECs. Copy standard is obtained from Duke University (USA) stored as pure plasmid in -20^0C . Zen probes are a special kind of qRT-PCR probes which contain double quencher and have both a lower backgound fluorescence and higher signal. Zen probes and HPLC grade primers were obtained from Integrated DNA Technologies (USA).

Mouse forward sjTREC primer: 5'- CAT TGC CTT TGA ACC AAG CTG -3'.
Mouse reverse sjTREC primer: 5'- TTA TGC ACA GGG TGC AGG TG -3'.
Mouse probe: 5'- /56-FAM/CA GGG CAG G/ZEN/T TTT TGT AAA GGT GCT CAC TT/3IABkFQ/ -3'.

Total reaction volume: 20µl
10µl TaqMan Universal Master Mix II with UNG
2 µl genomic DNA (50-100 ng/ml)
0.9 µl 20µM forward primer
0.9 µl 20µM reverse primer
1 µl 5µM Zen Probe
5.2 µL pcr grade water

PCR Reaction:
50ºC 2 minutes
95ºC 10 minutes
95ºC 15 seconds
60ºC 1 minute
} 45 cycles

All qRT PCR experiments were carried on StepOne Plus Real Time PCR System and StepOne Software Ver. 2.2.2 was used. Standard ramp speed was used. Analysis was made with absolute quantification method according to dilutions of plasmid from 10^7 to 10^2 copy number. All results were compared with results of 7 days old female mice.

2.6. ANALYSIS OF THYMOCYTE DEVELOPMENT, MYELOID DERIVED SUPPRESSOR CELLS (MDSC), LYMPHOID TISSUE INDUCER (LTi) CELLS AND REGULATORY T CELLS (Treg) IN THYMUS

Thymus was divided into two equal parts for immunophenotyping with flow cytometry and DNA/RNA/protein isolation. For flow cytometry, fat and connective tissues were removed and thymus was homogenized with mechanical aggregation. Homogenate was transferred into 3-4ml disruption medium (37ºC) and pipetting was applied. Disruption medium is 0,15% collagenase D (Gibco, USA), 0,01% DNAse I (Gibco,USA), 15% FBS supplemented RPMI 1640 medium (Gibco,USA). Thymus homogenate was incubated in disruption medium at 37ºC for 15 minutes. Incubation in disruption medium was repeated for complete disruption of thymus. Centrifuge was applied at 300g for 5 min. Pellet was resuspended and used for immunophenotyping.

Sample preparation steps for flow cytometry are listed below.

i. Optimized amounts of antibodies were added into round bottom poystrene tubes.
ii. 100µl of sample was added into the tube (1 million cell) and mixed with antibodies with fingertapping. Sample was incubated with antibodies for 15 minutes.
iii. Sample-antibody mix was treated with 2ml lysis buffer for 10 minutes.
iv. Centrifuge was applied at 300g for 5 minutes.
v. Supernatant was discarded and pellet was resuspended in 2ml PBS.
vi. Centrifuge was applied at 300g for 5 minutes.
vii. Supernatant discharged and pellet was resuspended in 2ml PBS.
viii. Centrifuge was applied at 300g for 5 minutes.
ix. Supernatant was discharged and pellet was resuspended in 500µl PBS and it was used for flow cytometric analysis.

Cell populations in thymus were analysed with flow cytometry. $Lin^{neg}CD90^{+}$ $CD117^{+}CD127^{+}$ cells were defined as LTi cells [186]. $CD11b^{+}Ly\text{-}6G^{+}$ cells were defined as MDSCs [124]. $CD4^{+}CD25^{+}CD127^{neg/low}$ cells were defined as Treg cells. In addition, thymocyte development is analyzed with specific antibodies with five color flow cytometer analysis. Antibodies are listed below.

- 15-0041-81 Anti Mouse CD4 PE-Cy5 50µg (eBioscience, USA)
- 25-0251-81 Anti-Mouse CD25 PE-Cyanine7 50µg (eBioscience, USA)
- 12-1271-81 Anti-Mouse CD127 PE 50µg (eBioscience, USA)
- 25-1171-82 Anti-Mouse CD117 (c-Kit) PE-Cyanine7 100µg (eBioscience, USA)
- 15-0900-82 Anti-Mouse/Rat CD90 PE-Cyanine5 100µg (eBioscience, USA)
- 11-0112-41 Anti-Mouse CD11b FITC (eBioscience, USA)
- 15-5931-81 Anti-Mouse Ly-6G (Gr-1) PE-Cyanine5 (eBioscience, USA)
- 22-7770-72 Mouse Hematopoietic Lineage FITC Cocktail (eBioscience, USA)

Before they are used, all antibodies were validated and optimized.

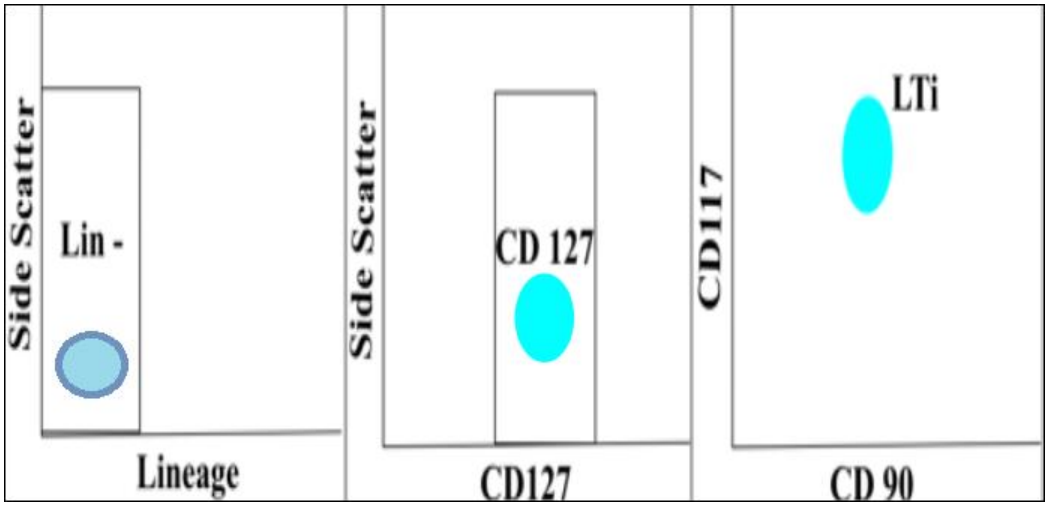

Figure 2.4. Gating strategy for LTi cells

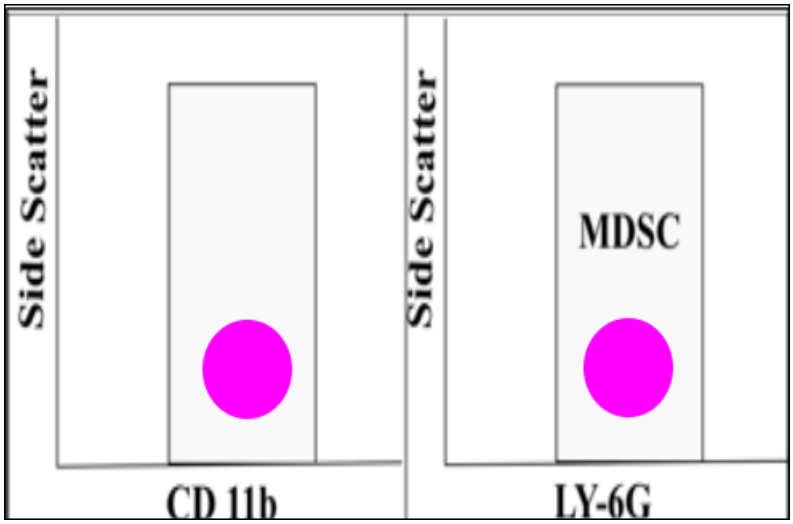

Figure 2.5. Gating strategy for MDSC

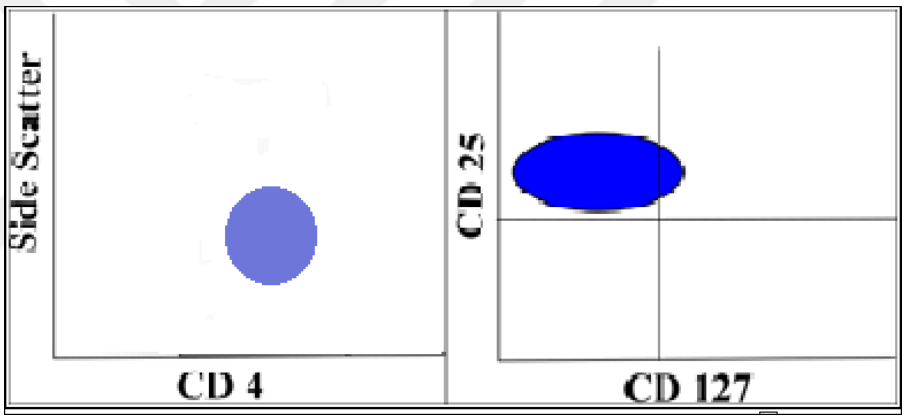

Figure 2.6. Gating strategy for Treg

Another panel of antibodies were used for immunphenotyping of T cells in different stages of development (Table 2.6).

Table 2.6. Stages of Thymocyte Development and their markers

THYMOCYTE -> T Cell Development Stages	
Triple Negative Cells	CD45+ $CD3^-$ $CD4^-$ $CD8^-$
Double Negative 1 Cells	$CD3^+$ $CD4^-$ $CD8^-$ $CD44^+$ $CD25^-$
Double Negative 2 Cells	$CD3^+$ $CD4^-$ $CD8^-$ $CD44^+$ $CD25^+$
Double Negative 3 Cells	$CD3^+$ $CD4^-$ $CD8^-$ $CD44^-$ $CD25^+$
Double Negative 4 Cells	$CD3^+$ $CD4^-$ $CD8^-$ $CD44^-CD25^-$
Double Positive Cells	$CD3^+$ $CD4^+$ $CD8^+$
Single Positive Cells	$CD3^+$ $CD4^-$ $CD8^+$, $CD3^+$ $CD4^+$ $CD8^-$

Catalog number, conjugation and vendors of antibodies are listed below.

- 11-0032-80 Anti-Mouse CD3 FITC (eBioscience, USA)
- 15-0041-81 Anti Mouse CD4 PE-Cy5 50µg (eBioscience, USA)
- 12-0081-81 Anti Mouse CD8 PE 50µg (eBioscience, USA)
- 25-0251-81 Anti-Mouse CD25 PE-Cyanine7 50µg (eBioscience, USA)
- 61-0441-82 Anti-Human/Mouse CD44 PE-eFluor® 610 100µg (eBioscience, USA)
- 61-0451-80 Anti-Mouse CD45 PE-eFluor® 610 (eBioscience, USA)

Before they are used, all antibodies were validated and optimized.

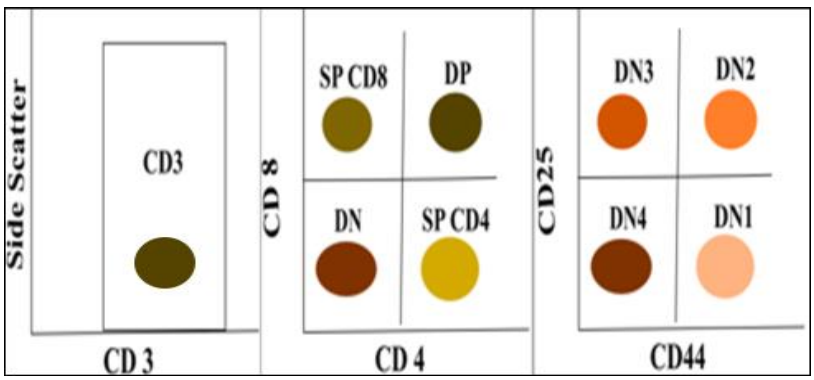

Figure 2.7. Gating strategy for thymocytes

2.7. DETERMINATION OF STEM CELLS AND PROGENITOR CELLS IN ASPIRATED CELLS FROM BONE MARROW

For extraction and preparation of bone marrow cells,

i. All connective tissue and muscles were removed from dissected tibias and femurs.
ii. Cleaned bones were crushed in 2% FBS supplemented PBS with mortar.
iii. Supernatant were collected after crushing and passed through 40µm pore sized cell strainer (Becton Dickinson Biosciences, USA)
iv. Remained bone parts in mortar washed with equal volume of 2% FBS supplemented PBS (Gibco, USA) and passed through 40µm pore sized cell strainer and transferred into same tube.
v. Last remained bone parts were treated with 5mg/ml collaganenase/dispase and mixed well. Continue mixing for 15 minutes.
vi. 15ml PBS was added and shaked.
vii. Supernatant was passed through 40 µm pore sized cell strainer.
viii. Tubes were centrifuged 400g at 4ºC for 8minutes. Supernatant was discharged.
ix. Cells were resuspended with appropriate amount of PBS.

Standard cell immunophenotyping protocol was used. Catalog numbers, conjugations and vendors of antibodies were listed below.

Antibodies for Hematopoietic Stem Cells ($Lin^{neg/low}Sca1^{+}CD117^{+}CD135^{neg}$) [187]:

- 22-7770-72 Mouse Hematopoietic Lineage FITC Cocktail (eBioscience, USA)
- 12-5981-81 Anti-Mouse Ly-6A/E (Sca-1) PE (eBioscience, USA)
- 25-1171-82 Anti-Mouse CD117 (c-Kit) PE-Cyanine7 (eBioscience, USA)
- 562537 PE-CF594 Rat Anti-Mouse CD135 (FLT3) (Becton Dickinson, USA)

Antibodies for Multipotent Progenitors ($Lin^{neg/low}CD117^{high}CD135^{+}Sca1^{+}CD90^{neg}$) [188]:

- 25-1171-82 Anti-Mouse CD117 (c-Kit) PE-Cyanine7 (eBioscience, USA)
- 562537 PE-CF594 Rat Anti-Mouse CD135 (FLT3) (Becton Dickinson, USA)
- 22-7770-72 Mouse Hematopoietic Lineage FITC Cocktail (eBioscience, USA)

- 12-5981-81 Anti-Mouse Ly-6A/E (Sca-1) PE (eBioscience, USA)
- 15-0900-82 Anti-Mouse/Rat CD90 PE-Cyanine5 (eBioscience, USA)

Antibodies for common Lymphoid Progenitors ($Lin^{neg}Sca1^{low}CD117^{low}CD127^{+}CD135^{+}$) [189]:

- 22-7770-72 Mouse Hematopoietic Lineage FITC Cocktail (eBioscience, USA)
- 12-5981-81 Anti-Mouse Ly-6A/E (Sca-1) PE (eBioscience, USA)
- 25-1171-82 Anti-Mouse CD117 (c-Kit) PE-Cyanine7 (eBioscience, USA)
- 15-1271-81 anti-mouse CD127 PE-Cyanine5 (eBioscience, USA)
- 562537 PE-CF594 Rat Anti-Mouse CD135 (FLT3) (Becton Dickinson, USA)

All antibodies were used after validation and optimization.

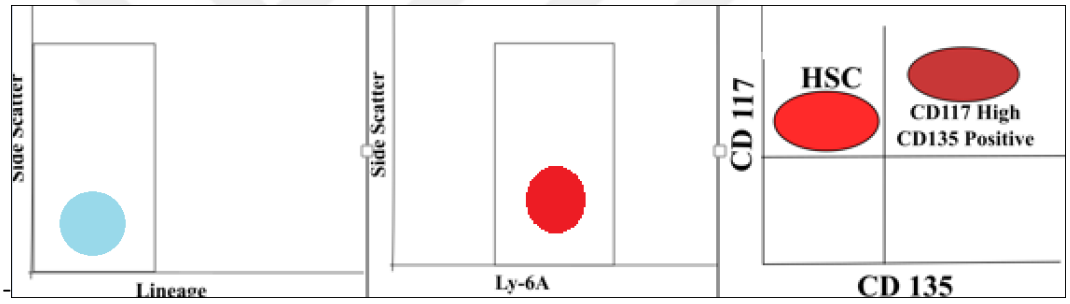

Figure 2.8. Gating strategy for HSC

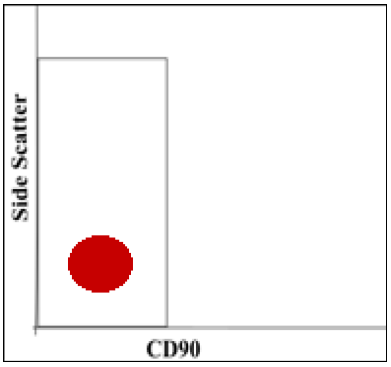

Figure 2.9. Gating strategy for MPP (from $CD117^{high}$ and$CD135^{+}$ gate)

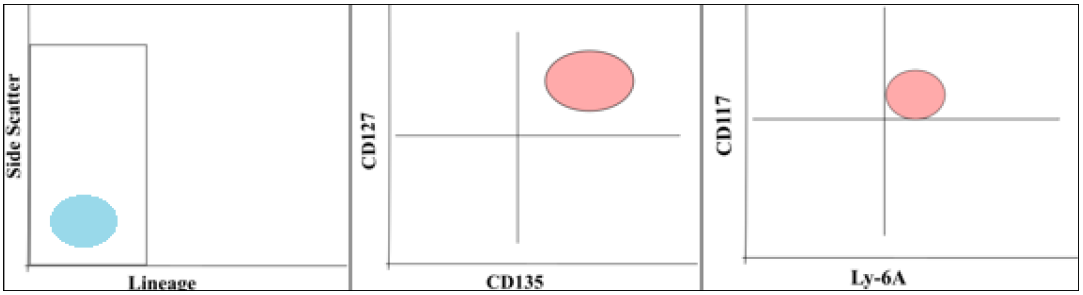

Figure 2.10. Gating strategy for CLP

3. RESULTS

Results are given according to sequence of analysis. Results of gene expression analysis of *ACTR2, IL-7, IL-7R, FGF7, FGFRII, IL22* genes and analysis of *ACTR2* gene promoter methylation in thymus and copy number analysis of *sjTREC* in peripheral blood were given at first. Secondly, results from immunophenotyping of bone marrow hematopoietic stem, progenitor cells and thymic LTi, MDSC, Treg and thymocytes at different developmental stages are given in details.

3.1. THE ANALYSIS OF TARGET GENES EXPRESSIONS IN THYMUS TISSUE THROUGH REAL TIME PCR

The expression levels of *ACTR2, IL7, IL7R, IL22, FGF7, FGFR2* genes were analysed with quantitative RT PCR. One way ANOVA test was used for statistical analysis, $p<0,05$ is accepted as statistically significant. All results are shown in Table 3.1 and p values are listed in Table 3.2.

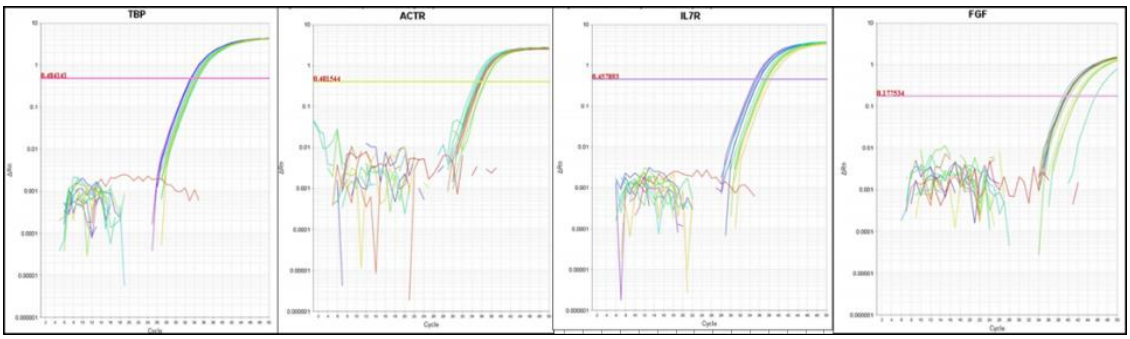

Figure 3.1. RT PCR Curves of *ACTR2, IL7R, FGF7, TBP*

ACTR2 expression decreased from seventh day to third month of mice life for females and males. One point eight (p=0,049) and one point six (p=0,008) fold decrease were found for females and males respectively. The results for *ACTR2* gene are shown in Figure 3.2.

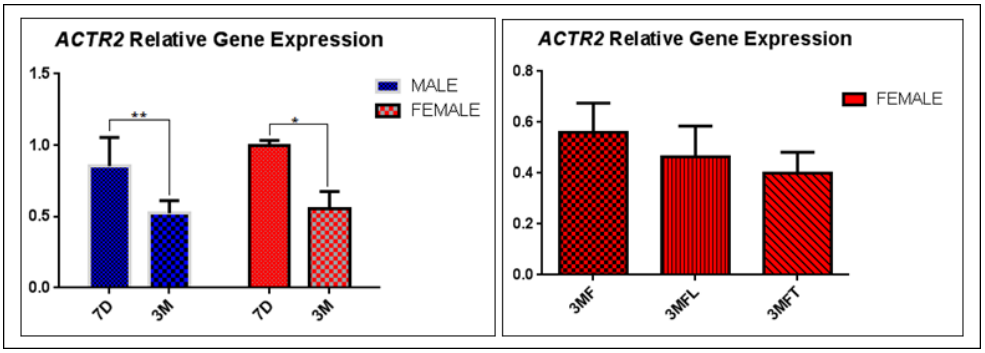

Figure 3.2. The relative expression of *ACTR2* gene in experiment groups.

In Figure 3.2, y axis shows the relative expression levels according to seven days old female group. One-way ANOVA test was used for statistical analysis. $p<0.05$ is accepted as statistically significant. p=0,008 for 7DM vs. 3MM and p=0,049 for 7DF vs. 3MF. 7D: 7 days old, 3M: 3 months old, 3MFL: Letrozole treated 3 months old, 3MFT: Testosterone treated 3 months old, F: Female, M:Male

Expression of *FGF7* gene in three months old females nearly four fold higher than in three months old males (p=0,014). Three months old females had ten and half fold higher expression than seven days old females (p=0,008) while three months old males had two and half fold higher levels of *FGF7* (p=0,046). Inhibition of estrogen synthesis increased *FGF7* expression two point seven fold (p=0,023) while testosterone treatment caused seven point six fold decrease (p=0,035) for three months old females. Changes in *FGF7* expression between experimental groups are shown in Figure 3.3.

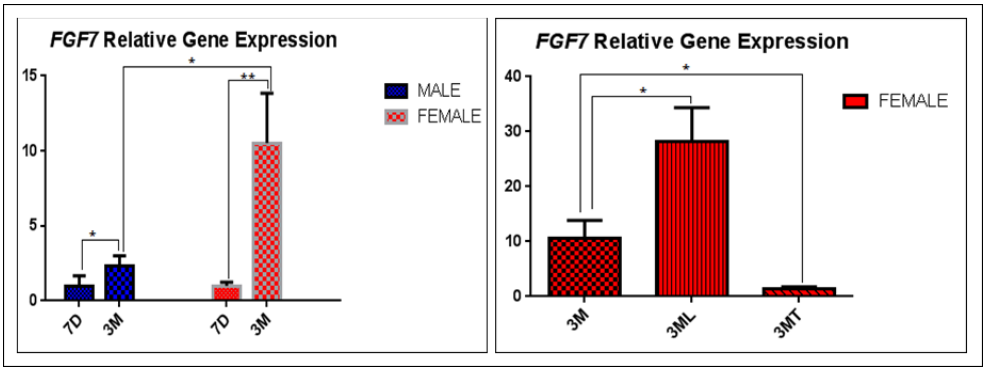

Figure 3.3. The relative expression of *FGF7* (KGF) gene in experiment groups.

In Figure 3.3, Y axis shows the relative expression levels according to seven days old female group. One way ANOVA test was used for statistical analysis. p<0.05 is accepted as statistically significant. p= 0,046 for 7DM vs. 3MM, p=0,008 for 7DF vs. 3MF, p=0,014 for 3MF vs. 3MM, p=0,023 for 3MF vs. 3ML and p=0,035 for 3MF vs. 3MT. 7D: 7 days old, 3M: 3 months old, 3ML: Letrozole treated 3 months old female, 3MT: Testosterone treated 3 months old female, F: Female, M:Male

Seven days old males have one point six fold higher expression of *IL7R* gene than seven days old females (p<0,001). *IL7R* expression decreased from seventh day to third month of mice life for females (p=0,013) and males (p<0,001). Letrozole treatment increased IL7R expression two point five fold in three months old females (p=0,032). Results are shown in Figure 3.4.

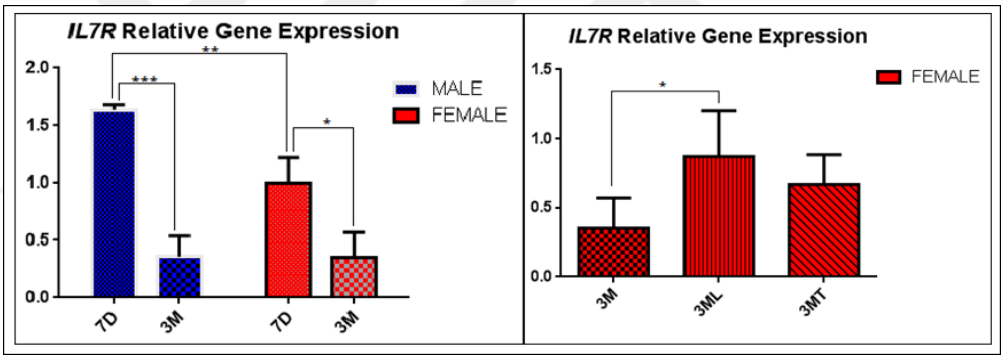

Figure 3.4. The relative expression of *IL7R* gene between experiment groups.

In Figure 3.4, y axis shows the relative expression levels according to seven days old female group. One way ANOVA test was used for statistical analysis. p<0.05 is accepted as statistically significant. p<0,001 for 7DF vs. 7DM, p<0,001 for 7DM vs. 3MM, p=0,013 for 7DF vs. 3MF and p=0,032 for 3MF vs. 3ML. 7D: 7 days old, 3M: 3 months old, 3ML: Letrozole treated 3 months old female, 3MT: Testosterone treated 3 months old female, F: Female, M:Male

For *FGFR2* gene, no significant differences between groups were found.

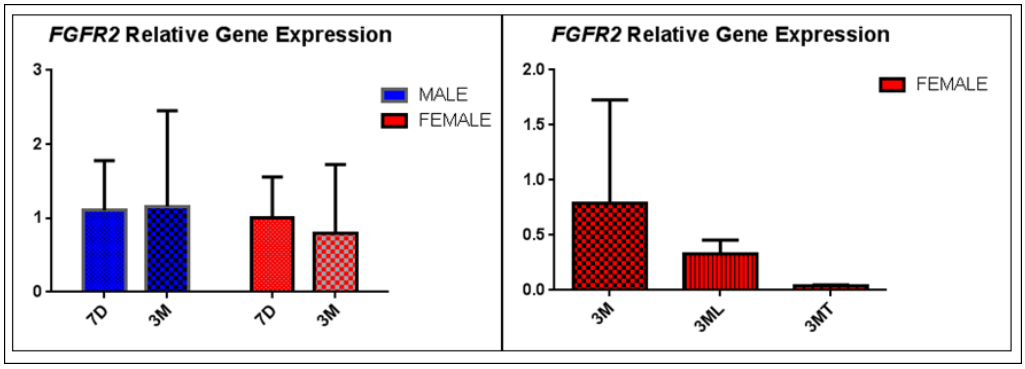

Figure 3.5. The relative expression of *FGFR2* gene between experiment groups.

In Figure 3.5, y axis shows the relative expression levels according to seven days old female group. One way ANOVA test was used for statistical analysis. $p<0.05$ is accepted as statistically significant. Abbreviations: 7D: 7 days old, 3M: 3 months old, 3ML: Letrozole treated 3 months old female, 3MT: Testosterone treated 3 months old female, F: Female, M: Male

Females had two point one fold higher expression of *IL7* gene than males for seven days old mice (p=0,038). Three fold decrease in *IL7* expression was observed from seventh day to third month of male mice life (p=0,047).

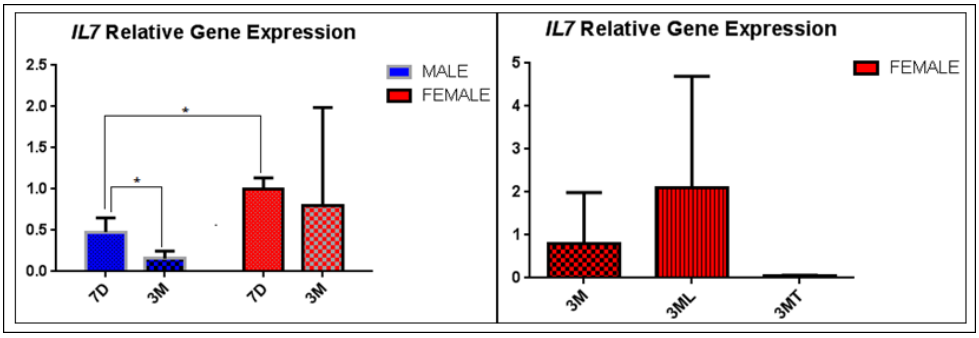

Figure 3.6. The relative expression of *IL7* gene in experiment groups.

In Figure 3.6, y axis shows the relative expression levels according to seven days old female group. One way ANOVA test was used for statistical analysis. $p<0.05$ is accepted as statistically significant. p=0,038 for 7DF vs. 7DM and p=0,047 for 7DM vs. 3MM. Abbreviations: 7D: 7 days old, 3M: 3 months old, 3ML: Letrozole treated 3 months old female, 3MT: Testosterone treated 3 months old female, F: Female, M: Male

For *IL22* gene, no statistical significant difference between groups were found.

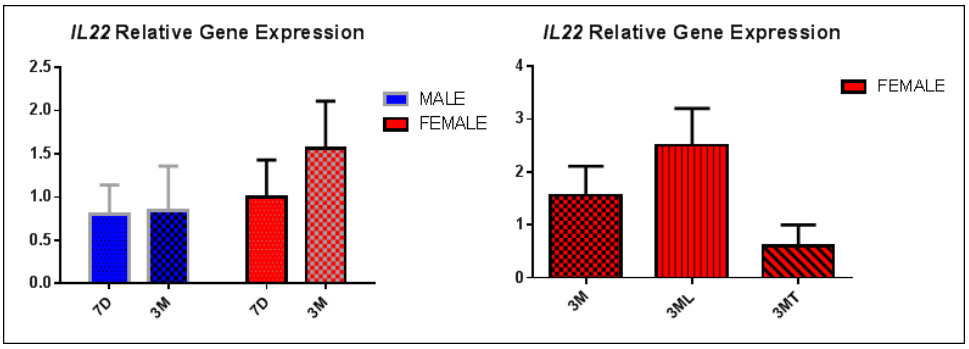

Figure 3.7. The relative expression of *IL22* gene in experimental groups.

In Figure 3.7, y axis shows the relative expression levels according to seven days old female group. One way ANOVA was used for statistical analysis. $p<0.05$ was accepted as statistically significant. Abbreviations: 7D: 7 days old, 3M: 3 months old, 3ML: Letrozole treated 3 months old female, 3MT: Testosterone treated 3 months old female, F: Female, M:Male

Table 3.1. Changes in genes expressions according to experimental groups

Changes in Thymic Gene Expression (Fold Change)						
	7DF	7DM	3MF	3MM	3MFL	3MFT
ACTR2	1	0,855	0,558	0,524	0,465	0,401
FGF7	1	1,001	10,513	2,538	28,184	1,383
FGFR2	1	1,105	0,792	1,160	0,329	0,036
IL7	1	0,478	0,803	0,157	2,102	0,039
IL7R	1	1,631	0,352	0,357	0,873	0,668
IL22	1	0,803	1,561	0,842	2,502	0,604

Table 3.2. p values for gene expression difference between different groups

	7DF vs. 7DM	**3MF vs. 3MM**	**7DF vs. 3MF**	**7DM vs. 3MM**	**3MF vs. 3MFL**	**3MF vs. 3MFT**
ACTR2	0,280	0,384	0,049	0,008	0,617	0,605
FGF7	0,965	0,014	0,008	0,046	0,023	0,035
FGFR2	0,988	0,710	0,652	0,951	0,233	0,671
IL7	0,038	0,400	0,835	0,047	0,474	0,327
IL7R	0,010	0,971	0,013	<0,001	0,032	0,107
IL22	0,214	0,102	0,09	0,146	0,236	0,173

Abbreviations: 7DF: 7 days old female, 7DM: 7 days old male, 3MF: 3 months old female, 3MM: 3 months old male, 3ML: 3 months old letrozole treated female, 3MT: 3 months old testosterone treated male

3.2. ANALYSIS OF METHYLATION ON THE PROMOTER REGION OF ACTR2 GENE

Affymetrix Promoter PCR Methylation Kit was used for analysis of methylation on the promotor region of *ACTR2* or *Arp2* gene which is a component of Arp 2/3 complex. For all experiment groups, no bands of methylated Arp2 promoter was observed on agarose gel. This may be due to deterioration or malfunction of PCR Methylation Kit components, further studies should be designed for Arp2 methylation analysis. Lack of bands may also caused by no statistical change in gene expression levels of *ACTR2* genderwise. Further exploration could not be realized due to limited financial resources.

3.3. DETERMINATION OF NAIVE T CELL OUTPUT USING ALTERATION OF SJTREC COPY NUMBER THROUGH REAL-TIME PCR

Naive T cell output or the production capacity of thymus was measured through analysis of sjTREC copy number using DNA from mouse blood. Two hundred nanograms DNA was used for the reaction. Results and p values are shown in Table 3.3 and 3.4.

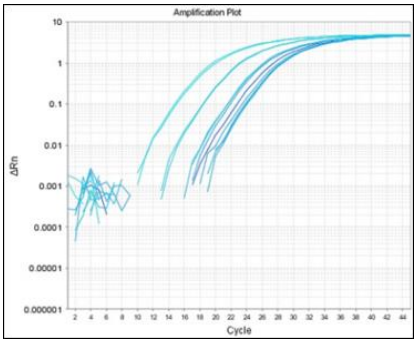

Figure 3.8. RT PCR Curves of sjTREC

The level of T cell output was one point fifteen fold higher in seven days old male mice than those of seven days old female mice. T cell output was increased in three months old mice compared to seven days old mice. There was no important difference between the genders within the groups with same age. T cell output was one point five fold higher in three months old females than in seven days old female mice (p=0,033). T cell output in letrozole and testosterone treated three months old females have fifty nine (p=0,011) and five hundred ten (p=0,011) fold lower T cell output than in three months old female mice as respectively. The results are shown in Figure 3.9.

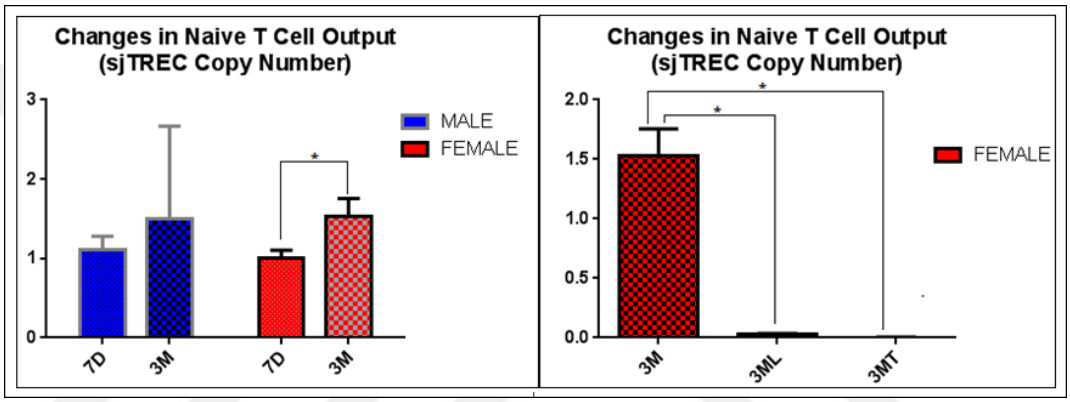

Figure 3.9. The alteration in the sjTREC copy number in experiment groups

In Figure 3.9, sjTREC copy numbers in seven days old female mice was used for normalisation of results. One Way ANOVA test was used for statistical analysis, all p values are given in Table 3.4. $p<0.05$ is accepted as statistically significant. p=0,035 for 7DF vs. 3MF, p=0,011 for 3MF vs. 3MFL and p=0,011 for 3MF vs. 3MFT Abbreviations: 7D: 7 days old, 3M: 3 months old, 3ML: Letrozole treated 3 months old female, 3MT: Testosterone treated 3 months old female, F: Female, M:Male

Table 3.3. Changes in TREC copy number in experiment groups.

Naïve T Cell Output-sjTREC copy number (Fold Change)						
	7DF	**7DM**	**3MF**	**3MM**	**3ML**	**3MT**
sjTREC	1	1,115	1,532	1,507	0,026	0,003

Abbreviations: 7D: 7 days old, 3M: 3 months old, 3ML: Letrozole treated 3 months old female, 3MT: Testosterone treated 3 months old female, F: Female, M:Male

Table 3.4. p values for difference in TREC copy numbers between different groups

	7DF vs. 7DM	**3MF vs. 3MM**	**7DF vs. 3MF**	**7DM vs. 3MM**	**3MF vs 3ML**	**3MF vs. 3MT**
p	0,367	0,979	0,033	0,574	0,011	0,011

Abbreviations: 7DF: 7 days old female, 7DM: 7 days old male, 3MF: 3 months old female, 3MM: 3 months old male, 3ML: 3 months old letrozole treated female, 3MT: 3 months old testosterone treated male

3.4. DETERMINATION OF THE ALTERATION IN TARGET CELL TYPES IN THYMUS AND BONE MARROW THROUGH FLOW CYTOMETRY

3.4.1. Lymphoid tissue-inducer cells, Myeloid Derived Suppressor Cells, Regulatory T Cells

The presence of LTi, MDSC, Treg cells in the thymus which are suggested as important determinants of gender related differences in thymus repair capacity were analzed using flow cytometry. Examples of immunophenotyping of LTi, MDSC and Treg cells are shown in Figure 3.10. All results are shown in Table 3.5 and P values are shown in Table 3.6.

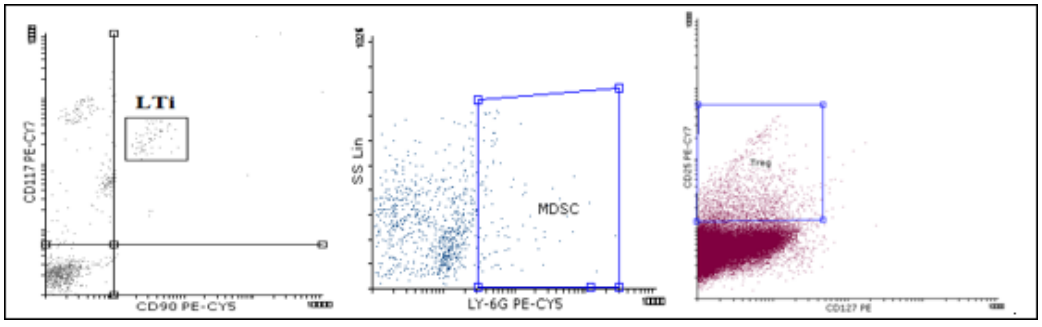

Figure 3.10. Immunophenotyping of LTi, MDSC and Treg cells

Seven days old males had one point two fold higher percentage of Treg cells than in seven days females (p=0,009). The percentage of Treg cells within the thymus cells was

significantly higher in three months old male (p=0,002) and three months old female (p=0,008) experiment groups compared to seven days old experiment groups. It was significantly higher in the three months old males (p=0,049). Testosterone treated three months old female group have higher values than normal three months old females. Testosterone treated three months old female group have two fold higher percentage of T regs while three months old males have two point five fold higher percentage of Tregs than three months old females. Letrozole treatment in three months old female group slightly decreased percentage of Treg cells to ninety four per cent of untreated three months old females. The results are shown in Figure 3.11

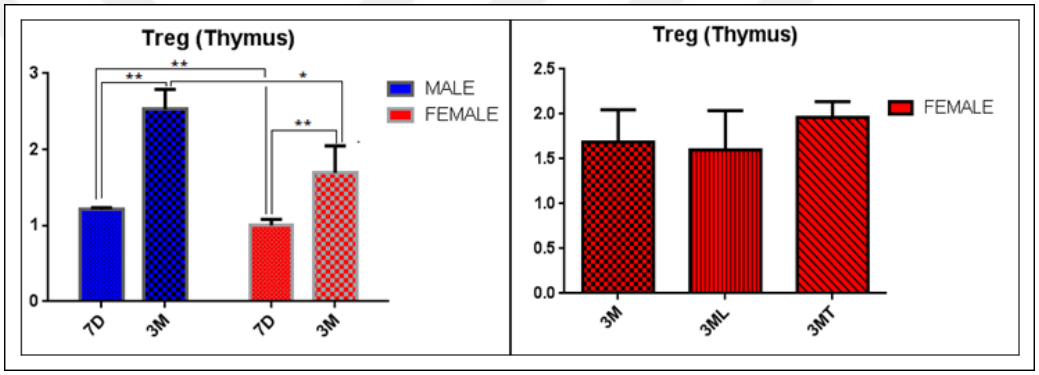

Figure 3.11. Changes in percentage of Treg cells in thymus in experiment groups

In Figure 3.11, Percentage of Treg cells in 7 days old female thymus was used as reference and Treg in other experiment groups were shown as a ratio to this reference. One way ANOVA test was used for statistical analysis and p<0,05 is accepted as statistically significant. p=0,009 for 7DF vs. 7DM, p=0,049 for 3MF vs. 3MM, p=0,008 for 7DF vs. 3MF and p=0,002 for 7DM vs. 3MM. Abbreviations: 7D: 7 days old, 3M: 3 months old, 3ML: Letrozole treated 3 months old female, 3MT: Testosterone treated 3 months old female, F: Female, M:Male

Percentage of myeloid derived suppressor cells seemed as sixty four, sixty five and ninety per cent higher than three months old female group for three months old male group, letrozole treated three months old female group and testosterone treated three months old female group respectively. And percentage of MDSCs in three months old

groups was found higher than seven days old group except three months old females. Significant difference between groups were not found.

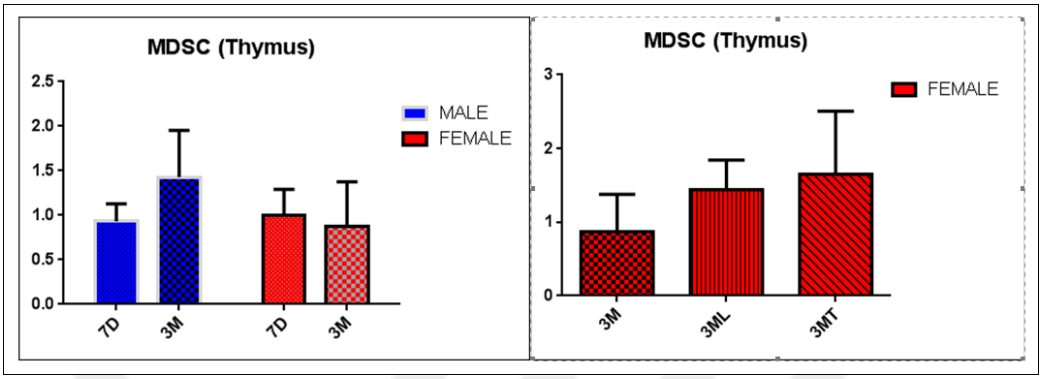

Figure 3.12. Changes in MDSCs in thymus between experiment groups

In Figure 3.12, Y axis shows the ratio of percentage of MDSC cells to the percentage of the same cell type in seven days old mice thymus One way ANOVA test was used for statistical analysis and P<0,05 is accepted as statistically significant. Abbreviations: 7D: 7 days old, 3M: 3 months old, 3ML: Letrozole treated 3 months old female, 3MT: Testosterone treated 3 months old female, F: Female, M:Male

Three months old females have 1.6 fold higher percentage of LTi cells than in three months old males (p=0,048) and testosterone treatment significantly decreased percentage of LTi cells in three months old female thymus (p=0,001).

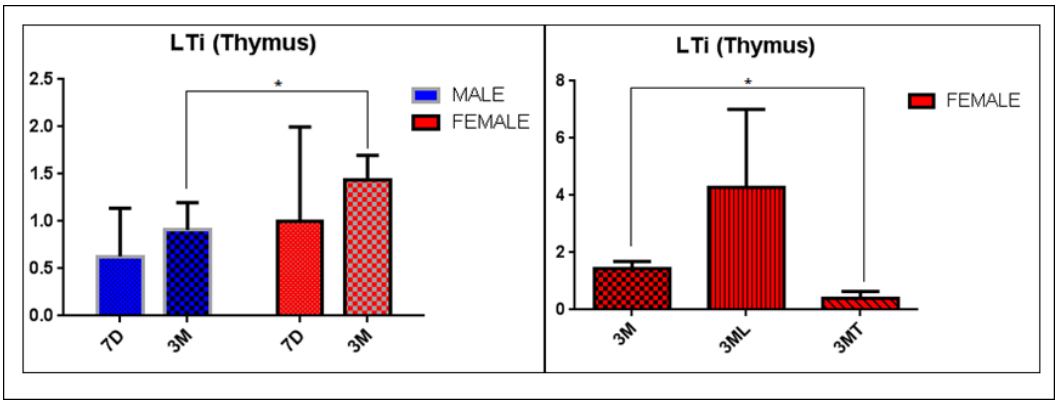

Figure 3.13. Analysis of thymic LTi cells in experiment groups

In Figure 3.13, y axis shows the ratio of percentage of LTi cells to the percentage of the same cell type in seven days old mice thymus. One-way ANOVA test was used for statistical analysis. $p<0,05$ is accepted as statistically significant. $p=0,048$ for 3MF vs. 3MM and $p=0,001$ for 3MF vs. 3MT. Abbreviations: 7D: 7 days old, 3M: 3 months old, 3ML: Letrozole treated 3 months old female, 3MT: Testosterone treated 3 months old female, F: Female, M:Male.

In Table 3.5, Changes in thymic lymphoid tissue inducer cells, myeloid derived precursor cells, regulatory T cells are given as fold change according to 7 days old females. Statistical calculations were made for different group sets and P values are listed in Table 3.6.

Table 3.5. Changes in numbers of thymic cells according to experiment groups

Thymus-LTi, MDSC and Treg Cells (Fold Change)						
	7DF	7DM	3MF	3MM	3MFL	3MFT
LTi	1	0,630	1,444	0,910	4,275	0,401
MDSC	1	0,933	0,870	1,429	1,435	1,653
Treg	1	1,209	1,692	2,530	1,595	1,966

Table 3.6. p values for difference in thymic LTİ, MDSC and Treg cells between different groups

	7DF vs. 7DM	**3MF vs. 3MM**	**7DF vs. 3MF**	**7DM vs. 3MM**	**3MF vs. 3MFL**	**3MF vs. 3MFT**
LTi	0,597	0,048	0,361	0,437	0,506	0,001
MDSC	0,756	0,255	0,719	0,244	0,188	0,248
Treg	0,009	0,049	0,008	0,002	0,786	0,410

Abbreviations: 7DF: Seven days old females, 7DM: Seven days old males, 3MF: Three months old females, 3MM: Three months old males, 3MFL: Letrozole treated three months old females, 3MFT: Testosterone treated three months old females, 7D: 7 days old, 3M: 3 months old, F: female, M: male

3.4.2. Thymocytes (Thymus)

The thymocyte populations in the different developmental stages in thymus were analyzed. Results were analyzed using One-way ANOVA test. p<0,05 is accepted as statistically significant. All results are listed in Table 3.7 and P values are listed in Table 3.8.

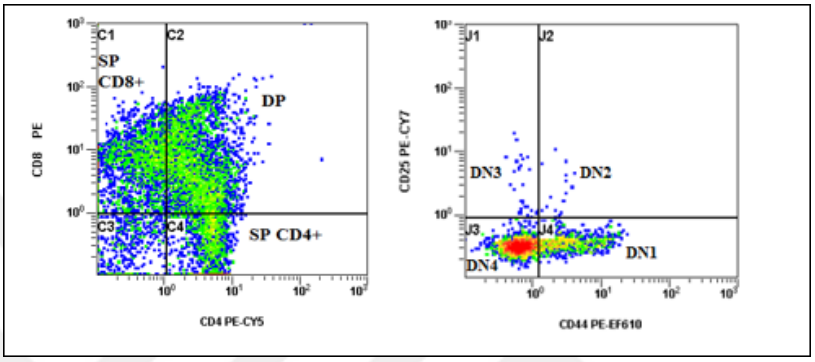

Figure 3.14. Immunophenotyping of thymocytes

The percentage of TN cells in three months old male mice shown ninety one per cent lower than in seven days old female mice (p=0,001). Similarly, it was four point six fold lower in three months old females than in seven days old females (p=0,002). Percentage of TN cells in three months old females had two and half fold higher than in three months old males (p=0,042).

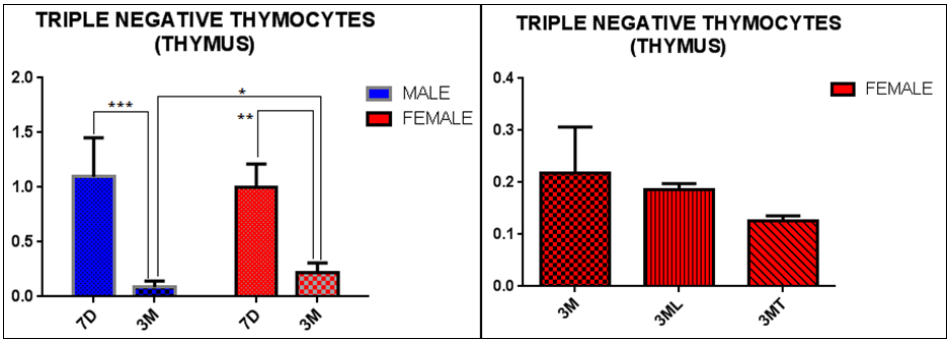

Figure 3.15. Changes in percentage of triple negative cells in experiment groups

In Figure 3.15, y axis shows the ratio of percentage of triple negative cells to the percentage of the same cell type in seven days old mice thymus. One-way ANOVA test was used for statistical analysis. p<0,05 is accepted as statistically significant. p=0,002

for 7DF vs. 3MF, p=0,001 for 7DM vs. 3MM and p=0,042 for 3MF vs. 3MM Abbreviations: 7D: 7 days old, 3M: 3 months old, 3ML: Letrozole treated 3 months old female, 3MT: Testosterone treated 3 months old female, F: Female, M:Male.

Three months old males had two point one fold higher percentage of DN1 cells than in seven days old males (p=0,001) while three months old females had two point seven fold higher percentage DN1 cells than in seven days old females (p<0,001). Letrozole and testosterone treatment decreased percentage of DN1 cells as one point two (p=0,002) and one point eight fold (p<0,001) respectively. Three months old females had three point seven fold higher percentage of DN2 cells than in seven days old females (p<0,001) while three months old males had three point eight fold higher percentage of DN2 cells than in seven days old males (p=0,005). Letrozole treatment increased DN2 percentage as one and half fold (p=0,035) while testosterone treatment decreased percentage of DN2 cells as one point eight fold in thymus of three months old females (p=0,033). Three months old females had two point two fold higher percentage of DN4 thymocytes than in three months old males (p=0,002) and seven days old females (p<0,001).

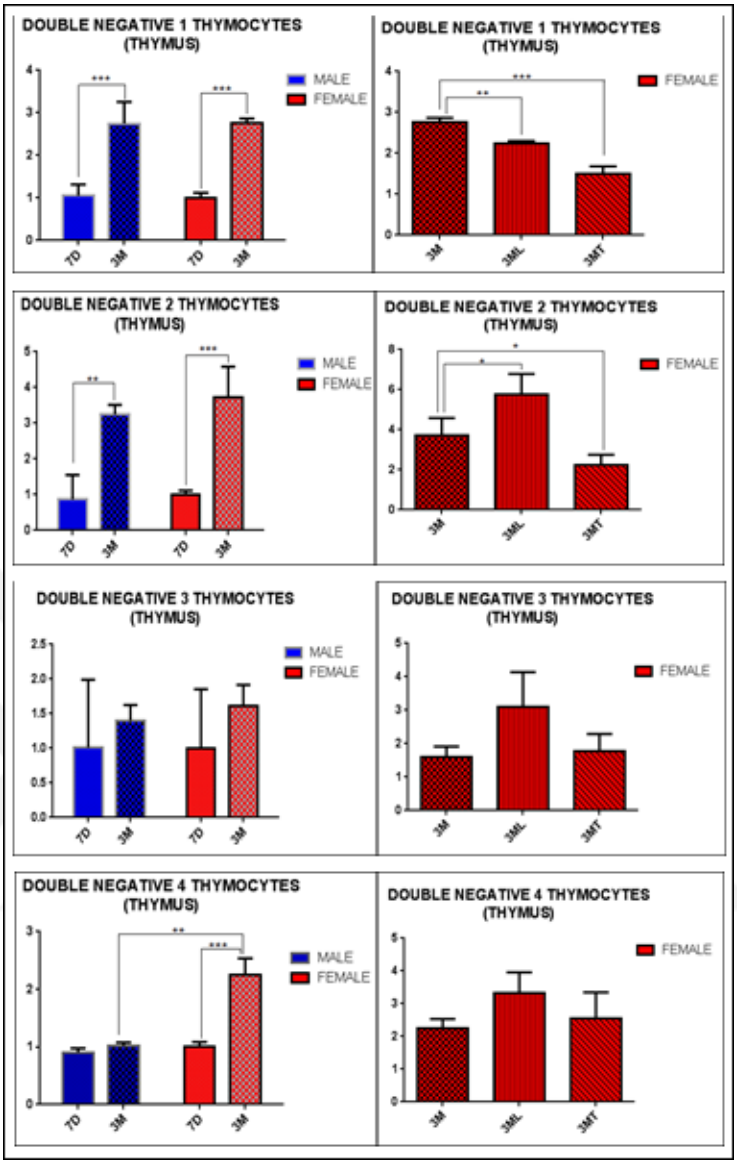

Figure 3.16. Changes in double negative thymocytes between experiment groups.

In Figure 3.16, y axis shows the ratio of percentage of DN1, DN2, DN3 and DN4 cells to the percentage of the same cell type in seven days old mice thymus. One-way ANOVA test was used for statistical analysis. $p<0,05$ is accepted as statistically significant. For DN1 cells, $p<0,001$ for 7DF vs. 3MF, $p=0,001$ for 7DM vs. 3MM, $p=0,002$ for 3MF vs. 3ML and $p<0,001$ for 3MF vs. 3MT. For DN2 cells, $p<0,001$ for 7DF vs. 3MF, $p=0,005$ for 7DM vs. 3MM, $p=0,035$ for 3MF vs. 3ML and $p=0,033$ for 3MF vs. 3MT. For DN4 cells, $p=0,002$ for 3MF vs. 3MM and $p=0,001$ for 7DF vs. 3MF. Abbreviations: 7D: 7 days old, 3M: 3 months old, 3ML: Letrozole treated 3 months old female, 3MT: Testosterone treated 3 months old female, F: Female, M:Male.

The percentage of DP cells in thymus was one point two fold higher in seven days old female mice compared to seven days old male mice (p=0,034). Three months old females had three point eight fold lower percentage of DP cells than in seven days old females (p<0,001) while three months old males had two points three fold lower percentage of DP cells than in seven days old males (p=0,002). The percentage of SP $CD4^+$ cells was significantly higher in three months old female individiuals than three months old male inividuals (p=0,049), conversely the percentage of SP $CD8^+$ cells was higher in three months old male individuals than those of three months old female individuals (p=0,047). Testosterone (p=0,036) or letrozole treatment increased the percentage of SP $CD8^+$ cells in thymus of three months old females, conversely percentage of SP $CD4^+$ cells in total thymus cells was significantly decreased with testosterone (p=0,048) and letrozole (p=0,046) treatment. Number of $CD4^+$ and $CD8^+$ SP (p<0,001) cells decreased from seventh day to third month of mice life.

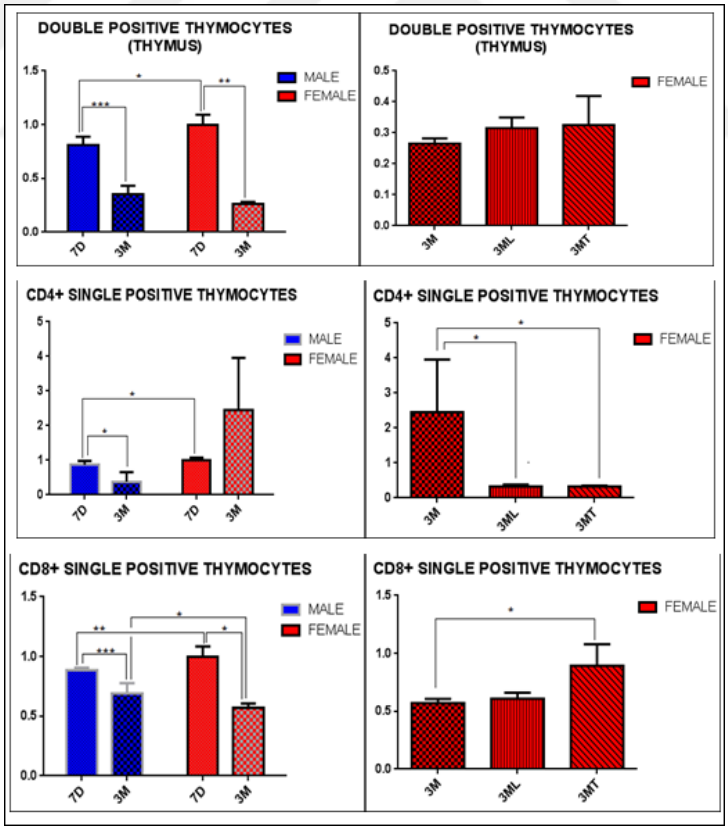

Figure 3.17. Changes in double and single positive thymocyte populations between experiment groups.

In Figure 3.17, Y axis shows the ratio of percentage of DP, $CD4^+$ SP or $CD8^+$ SP cells to percentage of same cell type in 7 days old female thymus. One-way ANOVA test was used for statistical analysis. $p<0,05$ is accepted as statistically significant. For DP cells, $p=0,034$ for 7DF vs. 7DM, $p<0,001$ for 7DF vs. 3MF and $p=0,002$ for 7DM vs. 3MM. For SP $CD4^+$ cells, $p=0,019$ for 7DM vs 3MM, $p=0,049$ for 3MF vs. 3MM, $p=0,046$ for 3MF vs. 3ML and $p=0,048$ for 3MF vs. 3MT. For SP $CD8^+$ cells, $p=0,002$ for 7DF vs. 7DM, $p=0,047$ for 3MF vs. 3MM, $P<0,001$ for 7DF vs. 3MF, $p=0,025$ for 7DM vs. 3MM and $p=0,036$ for 3MF vs. 3MT Abbreviations: 7D: 7 days old, 3M: 3 months old, 3ML: Letrozole treated 3 months old female, 3MT: Testosterone treated 3 months old female, F: Female, M:Male.

Table.3.7. Changes in thymic thymocyte subpopulations

Thymocytes (Fold Change)						
	7DF	**7DM**	**3MF**	**3MM**	**3MFL**	**3MFT**
TN	1	1,096	0,218	0,088	0,185	0,125
DN1	1	1,051	2,767	2,747	2,231	1,494
DN2	1	0,863	3,720	3,241	5,746	2,232
DN3	1	1,011	1,612	1,399	3,105	1,763
DN4	1	0,902	2,255	1,019	3,314	2,559
DP	1	0,808	0,265	0,356	0,315	0,326
SP CD4+	1	0,871	2,456	0,383	0,331	0,317
SP CD8+	1	0,884	0,572	0,687	0,610	0,892

Table 3.8. p values for differences in thymocytes between different groups

	7DF vs. 7DM	**3MF vs. 3MM**	**7DF vs. 3MF**	**7DM vs. 3MM**	**3MF vs. 3MFL**	**3MF vs. 3MFT**
TN	0,659	0,042	0,002	0,001	0,670	0,144
DN1	0,729	0,553	<0,001	0,001	0,002	<0,001
DN2	0,702	0,412	<0,001	0,005	0,035	0,033
DN3	0,988	0,386	0,297	0,541	0,639	0,688
DN4	0,171	0,002	<0,001	0,097	0,482	0,561
DP	0,034	0,229	<0,001	0,002	0,592	0,249
SP CD4+	0,118	0,049	0,102	0,019	0,046	0,048
SP CD8+	0,002	0,047	<0,001	0,025	0,927	0,036

Abbreviations: 7DF: Seven days old females, 7DM: Seven days old males, 3MF: Three months old females, 3MM: Three months old males, 3MFL: Letrozole treated three

months old females, 3MFT: Testosterone treated three months old females, 7D: 7 days old, 3M: 3 months old, F: female, M: male.

3.4.3. Analysis of Hematopoietic Stem Cells, Multipotent Progenitor Cells and Common Lymphoid Progenitor Cells in The Bone Marrow

Flow cytometry was used for analysis of the bone marrow cells, results are shown in Table 3.9. One-way ANOVA test was used for statistical analysis and p<0,05 is accepted as statistically significant . P values are shown in Table 3.10.

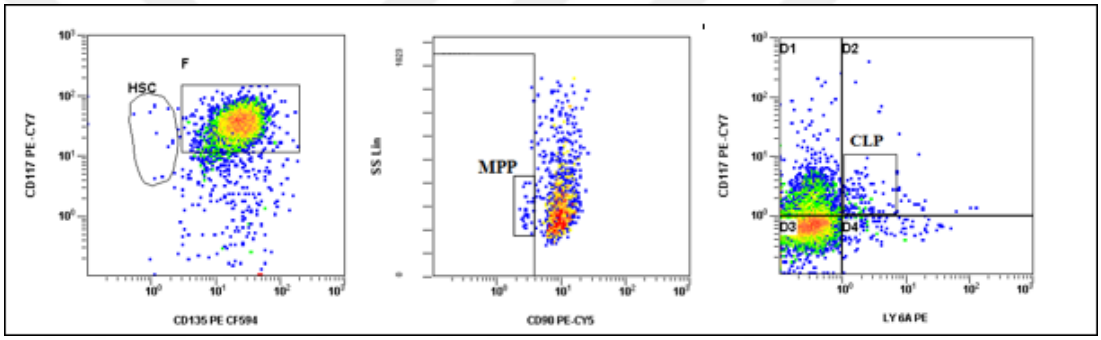

Figure 3.18. Immunophenotyping of bone marrow stem and progenitor cells

Hematopoietic stem cells (p=0,042) and common lymphoid progenitor cells (p<0,001) numbers significantly decreased from seventh day to third month of mice life for both of females and males. Testosterone treatment increased number of hematopoietic stem cells in three months old females bone marrows (p=0,009). Three months old females had three point two fold higher numbers of common lymphoid progenitors than in three months old males (p=0,013). Letrozole treatment caused three point two fold decrease in number of common lymphoid progenitors (p=0,030). Seven days old females had eight fold higher numbers of multipotent progenitors than in seven days old males (<0,001). Testosterone treatment increased number of multipotent progenitors (p=0,005) while letrozole treatment decreased number of multipotent progenitors (p=0,046).

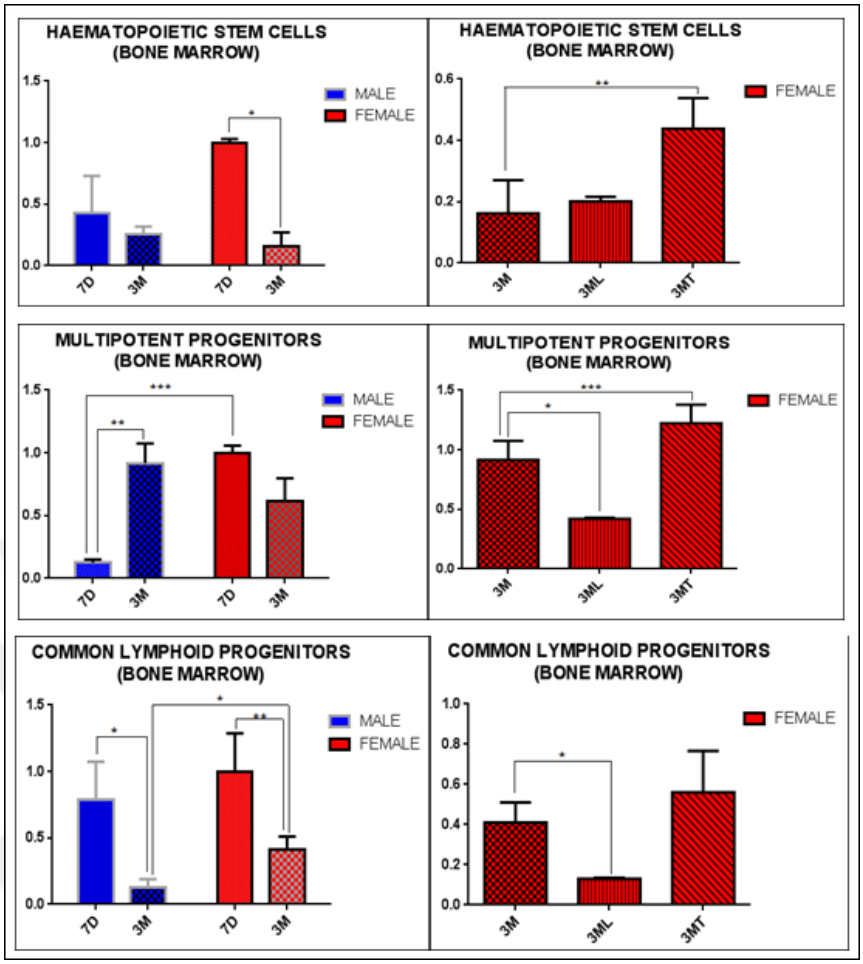

Figure 3.19. Changes in haematopoietic stem and progenitor cells in bone marrow between experiment groups.

In Figure 3.19, Y axis shows the ratio of percentage of HSC, MPP and CLP cells to percentage of same cell type in 7 days old female thymus. One-way ANOVA test was used for statistical analysis. For HSCs, p=0,048 for 7DF vs. 3MF, p=0,009 for 3MF vs. 3MT. For MPPs, p<0,001 for 7DF vs. 7DM, p=0,010 for 7DM vs. 3MM, p=0,046 for 3MF vs. 3ML and p=0,005 for 3MF vs. 3MT. For CLPs, p=0,012 for 3MF vs. 3MM, p=0,002 for 7DF vs. 3MF, p=0,018 for 7DM vs. 3MM and p=0,030 for 3MF vs. 3ML. p<0,05 is accepted as statistically significant Abbreviations: 7D: 7 days old, 3M: 3 months old, 3ML: Letrozole treated 3 months old female, 3MT: Testosterone treated 3 months old female, F: Female, M:Male.

Table 3.9. Changes in bone marrow hematopoietic stem cells, multipotent progenitors and common lymphoid progenitors numbers as fold change according to 7DF.

BONE MARROW- HSC, MPP, CLP Cells (Fold Change)						
	7DF	7DM	3MF	3MM	3MFL	3MFT
HSC	1	0,431	0,161	0,253	0,202	0,439
MPP	1	0,124	0,917	0,614	0,419	1,226
CLP	1	0,785	0,412	0,128	0,128	0,559

Table 3.10. p values for stem cells, progenitor cells in bone marrow between different groups

	7DF vs. 7DM	**3MF vs. 3MM**	**7DF vs. 3MF**	**7DM vs. 3MM**	**3MF vs. 3MFL**	**3MF vs. 3MFT**
HSC	0,189	0,253	0,048	0,976	0,643	0,009
MPP	<0,001	0,095	0,366	0,010	0,046	0,005
CLP	0,289	0,013	0,002	0,018	0,030	0,332

Abbreviations: 7DF: 7 days old females, 7DM: 7 days old males, 3MF: 3 months old females, 3MM: 3 months old males, 3MFL: Letrozole treated 3 months old females, 3MFT: Testosterone treated 3 months old females, 7D: 7 days old, 3M: 3 months old, F: female, M: male.

4. DISCUSSION AND CONCLUSION

Arp 2/3 complex takes place in actin polymerization which is an important function of cytoskeleton. In the wound healing models using mouse embryonic fibroblast cells, it was shown that Arp2/3 complex has function in migration of monolayer of fibroblasts and maintaining cell polarity. Arp2 or ACTR2 is a component of Arp2/3 complex, its expression in nervous system is affected by testosterone. When the differences in the expression level of *ACTR2* were analyzed, it is thought that the rebuilding of cytoskeleton and cell migration to damaged areas in thymus to repair negatively affected by testosterone. Thymosin β make a complex with profilin and actin at higher thymosin β concentrations (>5-10μM) and this condition suppresses actin polymerization and causes a large increase in unpolymerized actin amount in cells [190]. Wise et al. shown that thymosin β expression in rats was affected by sex hormones, thymosin β level was altered in castrated females and castrated androgenized females [191]. Changes in *ACTR2* may be due to changes in thymosine β as suggested by other researchers. ACTR2 level may alter as a cell answer to altered thymosine β levels or ACTR2 level may changes as an answer to decreased thymosine β levels [190]. In this study, lower mean value for *ACTR2* was observed in males and testosterone and letrozole treated groups had lower *ACTR2* expression than untreated females. Unfortunately, these results are not statıstically significant according to compared groups. We did not find any significant relation between *ACTR2* level and gender or sex hormones. But, *ACTR2* expression significantly decreased from 7^{th} day to 3^{rd} month of mice life for both of males and females, this finding support the hypothesis about role of *ACTR2* in thymic involution. Aging related thymic involution may be related with decreased repair capacity as a result of depleted actin polymerisation capacity. Further animal models should be designed for role of ACTR2 in thymic damage repair.

Our results show that there is a repressive effect of sex hormones on the expression of *FGF7* gene in thymus. It is known that keratinoytes growth factor positively effects thymus cellularity and increases the repair of thymus injury [192]. In mice mammary gland, estrogen has been shown to increase the FGF7 levels [193]. In thymus, the condition seems different from mammary gland. Expression of *FGF7* gene in three

months old females is four fold higher than in three months old males. Three months old females had ten and half fold higher expression than seven days old females while three months old males had two fold higher levels of *FGF7*. Inhibition of estrogen synthesis significantly increased *FGF7* expression (2.7 fold) while testosterone treatment caused seven point fold decrease for three months old females. Estrogen and testosterone were found as *FGF7* suppressor, but females have higher expression of *FGF7* and fold change in testosterone treatment is higher than the letrozole treatment. Testosterone seems as stronger than estrogen for FGF7 suppression. The highest *FGF7* expression was found in the letrozole group, FGF7 treatment was shown to increase the number of DN2, DN3 and DN4 cells in the literature, our study shows that the highest mean of DN2, DN3 and DN4 cells were in the letrozole treated group between three months old mice, but results are only statistically significant for DN1 cells [172].

Triple negative or TN cells are at the first step of thymocyte development in thymus and changes in their number give information about numbers of progenitor cells that enter thymus [156]. Results of thymocyte immunophenotyping shown that TN numbers dramatically and statistically significantly decreased from seventh day to third month of mice life. This finding support the idea of aging studies should be designed for early ages for understanding the beginning of the process. Three months old females had two and half fold higher percentage of TN than three months old males. This finding indicates that higher number of progenitor cells come to thymus for young adult females than males. According to previous literature, number of TN cells decreased after IL7 depletion [156]. Our results show that mean of IL7 expression was higher in 3 months old males, but this finding is not statistically significant. But, it indicates that changes in TN number between males and females may be linked to IL7 expression levels. Further studies should be planned for relation between IL7 and gender based differences in number of early thymocytes. The positive effect of estrogen was seen on DN1 cells as thymocytes at early stages of development in thymus, inhibition of estrogen synthesis decreased DN1 numbers in thymus. Testosterone treatment decreased number of DN1 and DN2 cells in thymus. Therefore testosterone may seem as an important suppressor of early thymocyte development. The number of DP cells in thymus was 1.25 fold higher in seven days old female mice compared to seven days old male mice. Three months old females had three point eight fold lower number of DP cells than in seven

days old females while three months old males had two point three fold lower number of DP cells than in seven days old males. Decreased numbers of DP cells in three months old mice may indicate decrease in thymocyte development. The percentage of $CD4^+$ cells from SP cells in the thymic cells were markedly higher in the three months old female mice than three moths old male mice, this was possibly a result of the repressive effect of androgens on the differentiation of helper T cells [194]. Three months old male mice had higher number of $CD8^+$ SP cells and this finding is correlated with previous literature. Testosterone treatment increased the number of $CD8^+$ SP cells in female thymus while testosterone treatment or estrogen synthesis inhibition with letrozole decreased the number of $CD4^+$ thymocytes in thymus. Our findings indicates that testosterone's suppressor effect on $CD4^+$ T cell development is stronger than positive effect of estrogen. Because, testosterone treatment could suppress $CD4^+$ SP cell development in spite of the existence of estrogen. Testosterone seems as more dominant determinant for DP to SP cell commitment.

IL-7 and IL-7R interaction effects thymus cellularity and the decrease in the thymus cellularity was shown in the literature when *IL7* and *IL7R* genes were knocked out [128]. IL7 was produced by thymic stroma while IL7R is expressed by thymocytes. For seven days old mice, *IL7* expression was significantly higher in females than in males. This finding is correlated with higher level of *IL7R* in seven days old males. In general, receptor numbers increase as an answer to decreased amount of ligand. Developing thymocytes in 7 days old male thymus seem to increase their receptor number as an answer to low level of *IL7*. These data support males and females thymic expression patterns show some differences before sexual maturation. *IL7* and *IL7R* expression levels significantly decreased from seventh day to third month of male mice life while only *IL7R* expression level significantly decreased in same period of life. This findings indicates that *IL7-IL7R* interactions maybe significantly depleted in thymus since early adulthood. Letrozole treatment caused a significant increase in *IL7R* expression and this shows the suppressive effect of estrogen on *IL7R* expression.

Thymus is the organ which is a site of development and selection of T cells. Before coming to the thymus, cells undergo various development stages in the bone marrow [3]. Thymus is known as getting smaller and losing its function together with aging. It can be

thought that the cells coming from bone marrow decrease in course of time and this situation may effect the thymic involution [17]. The difference in the male and female individuals can come up as a result of the cell population change in the bone marrow. In this situation, it should be taken into consideration that the production of the cells like HSC, MPP or CLP may be higher in the bone marrow of females. But previous studies in mice shown that male mice have higher numbers of HSCs, but these cells shift to myelopoiesis, we found similar results in our study [195, 196]. Testosterone treatment significantly increased HSC and MPP numbers in bone marrow, however females had higher numbers of CLP than males. Most of these HSCs and MPPs may be committed to myeloid lineage [195, 196].

The percentage of the cells in the early stage of thymocyte development such as TN and DN1 was the highest within the thymus cells in the three months old females, this is correlated with results from bone marrow.

LTi cells increase the survival and proliferation of thymic epithelial cells [120]. LTi cells is important for lymphoid tissue development and they have a function in thymic repair [117]. The percentage of thymic LTi cell in the three months old females was significantly higher than those of three months old males. In addition, testosterone treatment significantly decreased the number of LTi cells in thymus of female mice. These findings are correlated. Testosterone acts as a suppressor of LTi cells. Changes in LTi cell numbers between genders may be an important determinant of gender based differences in thymic involution.

Interaction of thymocyte and thymus epithelial cells is necessary for maintaining of thymus development and function. When the repressive effect of Treg cells on immunity is considered, Treg cells in thymus may negatively affect the interaction between thymocytes and the thymus [93]. And, previous studies showed that testosterone treatment increases Treg numbers in interstitial compartment [197]. Therefore, the analysis of thymic Treg cells was included in this study. It was found that number of Treg cells increased from seventh day to third month of mice life. Number of Treg cells in thymuses of three months old females was significantly lower than three months old males in this study. Higher number of Treg cells in male thymus may be an important

actor of gender based differences in thymic involution via inhibition of thymocyte-thymic stroma interaction.

Previous studies shown that MDSCs were increased with aging in the lymphoid tissues [198]. During designing of this study, MDCSs were included in the study because of the supposed repressive effect on thymic cells interactions and propagative effect on thymic involution. The difference between the groups for myeloid derived suppressor cells was not statistically significant, but the change in the percentage of MDSCs between groups was as expected. The percentage of MDSCs in the thymus was markedly lower in the three months old females than those of three month old males, testosterone and letrozole treated groups. Thus, it can be thought that testosterone treatment or suppression of estrogen synthesis increased the percentage of thymic MDSCs and increase in the percentage of MDSCs can negatively effect thymic repair. To prove this, the significant results must be acquired via increasing the sample size.

Testosterone treatment or suppression of estrogen synthesis was seen to decrease the number of naive T cells in the peripheral blood. The lowest TREC copy number was observed in the testosterone treated female group is consistent with the literature. This finding indicates that suppressor effect of androgen may be more effective than estrogen. Because, testosterone could suppress T cell output in spite the existence of estrogen. After testosterone replacement treatment on hypogonadal males, TREC level decreased as eighty per cent in peripheral blood [199]. This result is concordant with our findings.

When all the data were analyzed together, it was determined that the difference in the repair capacity between adult females and males was originated from quantitative changes in the various cell types and the alteration in the expression of various genes. The results supported the hypothesis that the difference in the repair capacity of thymus between adult females and males was determined by the difference in the production capacity of MPP and CLP progenitor cell populations in the bone marrow. Higher number of CLP in the bone marrow of mature females leads to higher number of early stage thymocytes. Analysis of TN cells supported this finding. As a result, the interaction between thymocytes-thymic stroma may be better in adult females, thymus cellularity can be protected better than those of males. The cells which affect the survival

of thymic epithelial cells such as LTi have higher percentage within the thymic cells in the adult females. Conversely, the percentage of the cells such as Treg which suppress and negatively affect the thymocyte-thymus interaction is higher in the thymus for adult males. Whereas, it was found that the difference in the percentage of the cells such as LTi and Treg were effective on higher thymus repair capacity in the females. In addition, Arp2 expression maybe an important factor for age related thymic involution through the role in the cytosekeleton formation and cell migration to damaged areas. But, gender did not affect the Arp2 expression. IL-7R and FGF7 may have roles in thymic involution, but only FGF7 shown gender based difference. Testosterone seems as the main actor in male thymmic involution via its suppressor function.

Limited published data are available on the effect of testosterone and estrogen on thymic involution. Studies about function of lymphoid tissue inducer cells, myeloid derived suppressor cells and Treg cells, ACTR2, KGF, KGFR, IL-7, IL-7R in thymic involution are absent or very limited and existed studies are not interested with gender based differences in thymic involution. Our data are genuine on this aspect.

The comprehensive studies need to be planned for identification of the pathways for the cells and genes which have roles in the process. Interaction of different cells and their molecular activity levels need to be explored. In our future studies, we plan to concentrate on studying Arp 2/3 complex in thymic cell culture and LTi cell function in thymic involution.

REFERENCES

1. J. Seita, and I. Weissman. Hematopoietic Stem Cell: Self Renewal Versus Differentiation. *Wiley Interdisciplinary Review*, 2:640-653, 2010.

2. Lymphoma Association, "An Overview of The Lymphatic System", http://www.lymphomas.org.uk/about-lymphoma/what-lymphoma/overview-lymphatic-system [retrieved 15 May 2016].

3. K. Murphy, and C. Weaver. *Janeway's Immunobiology, 9th edition*. Garland Science, New York, 2016.

4. D. Wisniewski, M. Affer, J. Willshire, and B. Clarkson. Further Phenotypic Characterization of The Primitive Lineage-$CD34^{+}CD38^{-}CD90^{+}CD45RA^{-}$ Hematopoietic Stem Cell/Progenitor Cell Sub-Population Isolated From Cord Blood, Mobilized Peripheral Blood And Patients With Chronic Myelogenous Leukemia. *Blood Cancer Journal*, 1:36, 2011.

5. Y. Ng, and M. Baert. Isolation Of Human and Mouse Hematopoietic Stem Cells. *Genetics Modification of Hematopoietic Stem Cells*, 506:13-21, 2009.

6. A. N. Ishizu, H. Takizawa, and T. Suda. The Analysis, Roles and Regulation of Quiescence in Hematopoietic Stem Cells. *Development*, 141:4656–66, 2014.

7. S. Tuljapurkar, and T. McGuire. Changes in Human Bone Marrow Fat Content Associated with Changes in Hematopoietic Stem Cell Numbers and Cytokine Levels with Aging. *Journal of Anatomy*, 219:574-581, 2011.

8. T. Hotta, N. Hirabayashi, and M. Utsumi. Age Related Changes in The Function of Hemopoietic Stroma in Mice. *Experimental Hematology*, 8:933-936, 1980.

9. S. Chambers, C. Shaw, and C. Gatza. Aging Hematopoietic Stem Cells Decline in

Function and Exhibit Epigenetic Dysregulation. *PLoS Biology*, 5:201, 2007.

10. I. K. Chinn, C. C. Blackburn, N. R. Manley, and G. D. Sempowski. Changes in Primary Lymphoid Organs with Aging. *Seminars in Immunology*, 24:309–320, 2012.

11. S. J. Morrison, A. M. Wandycz, K. Akashi, A. Globerson, and I. L. Weissman. The Aging of Hematopoietic Stem Cells. *Nature Medicine*, 2:1011–1016, 1996.

12. H. Min. Effects of Aging on The Common Lymphoid Progenitor to Pro B Cell Transition. *Journal of Immunology*, 176:1007-1012, 2006.

13. R. Cho, H. Sieburg, and C. M. Sieburg. A New Mechanism for The Aging of Hematopoietic Stem Cells: Aging Changes The Clonal Composition of The Stem Cell Compartment But Not Individual Stem Cells. *Blood*, 111:5553-5561, 2008.

14. J. Labrie, A. Sah, and D. Allman. Bone Marrow Microenvironmental Changes Underlie Reduced RAG Mediated Recombination and B Cell Generation in Aged Mice. *Journal of Experimental Medicine*, 200:211-223, 2004.

15. D. J. Rossi, D. Bryder, J. Seita, A. Nussenzweig, J. Hoeijmakers and I. L. Weissman. Deficiencies in DNA Damage Repair Limit The Function of Haematopoietic Stem Cells with Age. *Nature*, 447:725–729, 2007.

16. H. Vaziri, and W. Dragowska. Evidence for A Mitotic Clock in Human Hematopoietic Stem Cells: Loss of Telomeric DNA with Age. *Proceedings of National Academy of Science*, 91:9857-9860, 1994.

17. T. Boehm, and J. B. Swann. Thymus Involution and Regeneration: Two Sides of The Same Coin?. *Nature Reviews Immunology*, 13:831–838, 2013.

18. G. Hollaender, and B. Wang. Developmental Control Point in Induction of Thymic Cortex Regulated by A Subpopulation of Prothymocytes. *Nature*, 373:350-353, 1995.

19. R. Rezzani, L. Nardo, G. Favero, M. Peroni, and L. Rodella. Thymus and Aging: Morphological, Radiological, and Functional Overview. *Age,* 36:313-351, 2014.

20. B. H. Waksman. Role of The Thymus in Immune Reaction in Rats: Changes in The Lymphoid Organs of Thymectomized Rats. *Journal of Experimental Medicine*, 116: 187–206, 1962.

21. "Thymus Development - Embryology.", https://embryology.med.unsw.edu.au/embryology/index.php/Thymus_Development#cite_note-PMID15098031-2 [retrieved 15 May 2016].

22. J. Gordon, V. A. Wilson, N. F. Blair, J. Sheridan, A. Farley, L. Wilson, N. R. Manley, and C. C. Blackburn. Functional Evidence for A Single Endodermal Origin for The Thymic Epithelium. *Nature Immunology*, 5:546–553, 2004.

23. J. Gordon, A. R. Bennett, C. C. Blackburn and N. R. Manley. Gcm2 and Foxn1 Mark Early Parathyroid and Thymus Specific Domains in The Developing Third Pharyngeal Pouch. *Mechanisms of Development*, 103:141–143, 2001.

24. J. Dooley, M. Erickson, and A. G. Farr. Lessons from Thymic Epithelial Heterogeneity: Foxn1 and Tissue-Restricted Gene Expression by Extrathymic, Endodermally Derived Epithelium. *Journal of Immunology*, 183:5042–5049, 2009.

25. G. Pearse. Normal Structure, Function and Histology of The Thymus. *Toxicological Pathology*, 34: 504–514, 2006.

26. W. Savino, and M. Dardenne. Neuroendocrine Control of Thymus Physiology. *Endocrinology Reviews*, 21:412–443, 2000.

27. J. F. Bach, and M. Dardenne. Thymulin, A Zinc-Dependent Hormone. *Medical Oncology Tumor Pharmacotherapy*, 6:25–29, 1989.

28. Y. Takahama. Journey through The Thymus: Stromal Guides for T-Cell

Development and Selection. *Nature Reviews Immunology*, 6:127–135, 2006.

29. D. H. Townson, A. N. Putnam, B. T. Sullivan, L. Guo, and H. F. I. Rodgers. Expression and Distribution of Cytokeratin 8/18 Intermediate Filaments in Bovine Antral Follicles and Corpus Luteum: An Intrinsic Mechanism of Resistance to Apoptosis?. *Histological Histopathology*, 25:889–900, 2010.

30. D. B. Palmer. The Effect of Age on Thymic Function. *Frontiers in Immunology*, 4:316, 2013.

31. D. Aw, F. Taylor-Brown, K. Cooper, and D. Palmer. Phenotypical and Morphological Changes in The Thymic Microenvironment from Ageing Mice. *Biogerontology*, 10:311-322, 2009.

32. J. Gui, L. M. Mustachio, D.-M. Su, and R. W. Craig. Thymus Size and Age-Related Thymic Involution: Early Programming, Sexual Dimorphism, Progenitors and Stroma. *Aging Disease*, 3:280–290, 2012.

33. D. D. Taub, and D. L. Longo. Insights into Thymic Aging and Regeneration. *Immunology Reviews*, 205:72–93, 2005.

34. J. Gui, X. Zhu and J. Dohkan. The Aged Thymus Shows Normal Recruitment of Lymphohematopoietic Progenitors But Has Defects in Thymic Epithelial Cells. *International Immunology*, 19:1201-1211, 2007.

35. D. Aw, A. B. Silva, and D. B. Palmer. The Effect of Age on The Phenotypea Function of Developing Thymocytes. *Journal Comparative Pathology*, 142:45–59, 2010.

36. L. L. Lau, and L. M. Spain. Altered Aging-Related Thymic Involution in T Cell Receptor Transgenic, MHC Deficient, and CD4 Deficient Mice. *Mechanisms of Ageing and Development*, 114:101–121, 2000.

37. A. J. T. George, and M. A. Ritter. Thymic Involution with Ageing: Obsolescence or Good Housekeeping?. *Immunology Today*, 17:267–272, 1996.

38. G. G. Steinmann, B. Klaus, and H. K. M. Hermelink. The Involution of The Ageing Human Thymic Epithelium is Independent of Puberty. *Scandinavian Journal of Immunology*, 22:563–575,1985.

39. K. Alexandropoulos, and N. Danzl. Thymic Epithelial Cells: Antigen Presenting Cells That Regulate T Cell Repertoire and Tolerance Development. *Immunological Research*, 54:177-190, 2012.

40. T. K. Starr, S. C. Jameson, and K. A. Hogquist. Positive and Negative Selection of T Cells. *Annual Reviews Immunology*, 21:39–76, 2003.

41. G. Van Zant, and Y. Liang. Concise Review: Hematopoietic Stem Cell Aging, Life Span, and Transplantation. *Stem Cells Translational Medicine*, 1:651–657, 2012.

42. I. Beerman, W. J. Maloney, I. L. Weissmann, and D. J. Rossi. Stem Cells and The Aging Hematopoietic System. *Current Opinion in Immunology*, 22:500–506, 2010.

43. H. Min. Reduction in The Developmental Potential of Intrathymic T Cell Progenitors with Age. *Journal of Immunology*, 173:245-250, 2004.

44. T. Heng, and G. Goldberg. Effects of Castration on Thymocyte Development in Two Different Models of Thymic Involution. *Journal of Immunology*, 175:2892-2893, 2005.

45. R. N. Germain. T Cell Development and The CD4-CD8 Lineage Decision. *Nature Reviews Immunology*, 2:309–322, 2002.

46. M. Thoman. The Pattern of T Lymphocyte Differentiation is Altered During Thymic Involution. *Mechanisms of Ageing and Development*, 82:155-170, 1995.

47. R. Aspinall. Age Associated Thymic Atrophy in The Mouse is Due to A Deficiency

Affecting Rearrangement of The TCR During Intrathymic T Cell Development. *Journal of Immunology*, 158:3037-3045, 1997.

48. B. Fowlkes, and D. Pardoll. Molecular and Cellular Events of T Cell Development. *Advance in Immunology* 44:207-264, 1989.

49. M. Sykes. Unusual T Cell Populations in Adult Murine Bone Marrow. Prevalence of CD3+CD4-CD8- and Alpha Beta TCR+ NK1.1+ Cells. *Journal of Immunology*, 145: 3209-3215, 1990.

50. L. Li, H. C. Hsu, G. E. William, C. R. Stockard, K. J. Ho, P. Lott, P. A. Yang, H. G. Zhang, and J. D. Mountz. Cellular Mechanism of Thymic Involution. *Scandinavian Journal of Immunology*, 57:410–422, 2003.

51. J. S. Hale, T. E. Boursalian, G. L. Turk, and P. J. Fink. Thymic Output in Aged Mice. *Proceedings in National Academy of Science*, 103:8447–8452, 2006.

52. M. Provinciali, G. Di Stefano, and S. Stronati. Flow Cytometric Analysis of CD3/TCR Complex, Zinc, and Glucocorticoid-Mediated Regulation of Apoptosis and Cell Cycle Distribution in Thymocytes from Old Mice. *Cytometry*, 32:1–8, 1998.

53. M. D. Kendall, H. R. Johnson, and J. Singh. The Weight of The Human Thymus Gland at Necropsy. *Journal of Anatomy*, 131:483–497, 1980.

54. J. Loeffler, R. Bauer, H. Hebart, D. C. Douek, G. Rauser, P. Bader, and H. Einsele. Quantification of T Cell Receptor Excision Circle DNA Using Fluorescence Resonance Energy Transfer and The Lightcycler System. *Journal of Immunological Methods*, 271: 167–175, 2002.

55. W. A. Mitchell, P. O. Lang, and R. Aspinall. Tracing Thymic Output in Older Individuals. *Clinical Experimental Immunology*, 161:497–503, 2010.

56. G. J. V. Nossal. Negative Selection of Lymphocytes. *Cell*, 76:229–239, 1994.

57. A. Fry, L. Jones, A. Kruisbeek, and L. Matis. Thymic Requirement for Clonal Deletion During T Cell Development. *Science*, 246:1044–1046, 1989.

58. W. A. Dik, K. P. Overzet, F. Weerkamp, D. de Ridder, E. F. E. de Haas, M. R. M. Baert, P. van der Spek, E. E. L. Koster, M. J. T. Reinders, J. J. M. van Dongen, A. W. Langerak, and F. J. T. Staal. New Insights on Human T Cell Development by Quantitative T Cell Receptor Gene Rearrangement Studies and Gene Expression Profiling. *Journal of Experimental Medicine*, 201:1715–1723, 2005.

59. M. Hazenberg, and M. Verschuren. T Cell Receptor Excision Circles as Markers for Recent Thymic Emigrants: Basic Aspects, Technical Approach, and Guidelines for Interpretation. *Journal of Molecular Medicine,* 79:631-640, 2001.

60. F. Serana, M. Chiarini, C. Zanotti, A. Sottini, D. Bertoli, A. Bosio, L. Caimi, and L. Imberti. Use Of V(D)J Recombination Excision Circles to Identify T and B Cell Defects and to Monitor The Treatment in Primary and Acquired Immunodeficiencies. *Journal of Translational Medicine*, 11:119, 2013.

61. D. C. Douek, R. D. McFarland, P. H. Keiser, E. A. Gage, J. M. Massey, B. F. Haynes, M. A. Polis, A. T. Haase, M. B. Feinberg, J. L. Sullivan, B. D. Jamieson, J. A. Zack, L. J. Picker, and R. A. Koup. Changes In Thymic Function with Age and During The Treatment of HIV Infection. *Nature*, 396:690–695, 1998.

62. M. D. Hazenberg, S. A. Otto, J. W. Cohen Stuart, M. C. Verschuren, J. C. Borleffs, C. A. Boucher, R. A. Coutinho, J. M. Lange, T. F. Rinke de Wit, A. Tsegaye, J. J. van Dongen, D. Hamann, R. J. de Boer, and F. Miedema. Increased Cell Division But Not Thymic Dysfunction Rapidly Affects The T-Cell Receptor Excision Circle Content of The Naive T Cell Population in HIV-1 Infection. *Nature Medicine*, 6:1036–1042, 2000.

63. D. Zubakov, F. Liu, M. C. van Zelm, J. Vermeulen, B. A. Oostra, C. M. van Duijn, G. J. Driessen, J. J. M. van Dongen, M. Kayser, and A. W. Langerak. Estimating Human Age from T Cell DNA Rearrangements. *Current Biology*, 20:970–971, 2010.

64. F. Ponchel, C. Toomes, K. Bransfield, F. T. Leong, S. H. Douglas, S. L. Field, S. M. Bell, V. Combaret, A. Puisieux, A. J. Mighell, P. A. Robinson, C. F. Inglehearn, J. D. Isaacs, and A. F. Markham. Real Time PCR Based on SYBR-Green I Fluorescence: An Alternative to The Taqman Assay for A Relative Quantification of Gene Rearrangements, Gene Amplifications and Micro Gene Deletions. *BioMed Central Biotechnology*, 3:18, 2003.

65. A. Hug, M. Korporal, I. Schroder, J. Haas, K. Glatz, B. Storch-Hagenlocher, and B. Wildemann. Thymic Export Function and T Cell Homeostasis in Patients with Relapsing Remitting Multiple Sclerosis. *Journal of Immunology*, 171:432–437, 2003.

66. M. Nobile, R. Correa, J. A. M. Borghans, C. D'Agostino, P. Schneider, R. J. De Boer, and G. Pantaleo. De Novo T-Cell Generation in Patients at Different Ages and Stages Of HIV-1 Disease. *Blood*, 104:470–477, 2004.

67. M. D. Hazenberg. T Cell Receptor Excision Circle and T Cell Dynamics after Allogeneic Stem Cell Transplantation are Related to Clinical Events. *Blood*, 99:3449–3453, 2002.

68. R. M. Ribeiro, and A. S. Perelson. Determining Thymic Output Quantitatively: Using Models to Interpret Experimental T-Cell Receptor Excision Circle (TREC) Data. *Immunological Reviews*, 216:21–34, 2007.

69. J. L. G. Thompson, J. F. Wilkey, J. C. Baptiste, J. S. Navas, S. Y. Pai, K. A. Pass, R. B. Eaton, and A. M. Comeau. High-Throughput Multiplexed T-Cell-Receptor Excision Circle Quantitative PCR Assay with Internal Controls For Detection of Severe Combined Immunodeficiency in Population Based Newborn Screening. *Clinical Chemistry*, 56:1466–1474, 2010.

70. Y. Morinishi, K. Imai, N. Nakagawa, H. Sato, K. Horiuchi, Y. Ohtsuka, Y. Kaneda, T. Taga, H. Hisakawa, R. Miyaji, M. Endo, T. Oh-Ishi, Y. Kamachi, K. Akahane, C. Kobayashi, M. Tsuchida, T. Morio, Y. Sasahara, S. Kumaki, K. Ishigaki, M. Yoshida, T. Urabe, N. Kobayashi, Y. Okimoto, J. Reichenbach, Y. Hashii, Y. Tsuji, K. Kogawa, S.

Yamaguchi, H. Kanegane, T. Miyawaki, M. Yamada, T. Ariga, and S. Nonoyama. Identification of Severe Combined Immunodeficiency by T-Cell Receptor Excision Circles Quantification Using Neonatal Guthrie Cards. *Journal of Pediatrics*, 155:829–833, 2009.

71. C. M. Roifman, R. Somech, F. Kavadas, L. Pires, A. Nahum, I. Dalal, and E. Grunebaum. Defining Combined Immunodeficiency. *Journal of Allergy Clinical Immunology*, 130: 177–183, 2012.

72. K. Lima, T. G. Abrahamsen, I. Foelling, S. Natvig, L. P. Ryder, and R. W. Olaussen. Low Thymic Output in The 22q11.2 Deletion Syndrome Measured by CCR9+CD45RA+ T Cell Counts and T Cell Receptor Rearrangement Excision Circles. *Clinical Experimental Immunology*, 161:98–107, 2010.

73. N. Amariglio, A. Lev, A. Simon, E. Rosenthal, Z. Spirer, O. Efrati, A. Broides, G. Rechavi, and R. Somech. Molecular Assessment of Thymus Capabilities in The Evaluation of T-Cell Immunodeficiency. *Pediatric Researches*, 67:211–216, 2010.

74. J. G. Markle, and E. N. Fish. SeXX Matters in Immunity. *Trends in Immunology*, 35:97–104, 2014.

75. J. S. Danska. Sex Matters for Mechanism. *Science Translational Medicine*, 6:258, 2014.

76. G. Feigen, R. Fraser, and N. Peterson. Sex Hormones and The Immune Response. *International Archives of Allergy and Applied Immunology* , 57:488-497, 1978.

77. P. Duquette, and J. Pleines. The Increased Susceptibility of Women to Multiple Sclerosis. *Canadian Journal of Neurological Science*, 19:466-471, 1992.

78. F. Fitzpatrick. Influence of Castration, Alone or Combined With Thymectomy, on The Development of Diabetes in The Nonobese Diabetic Mouse. *Endocrinology*, 129:1382-1390, 1991.

79. H. Fox. Androgen Treatment Prevents Diabetes in Nonobese Diabetic Mice. *Journal of Experimental Medicine*, 175:1409-1412, 1992.

80. M. Dalal, S. Kim, and R. R. Voskuhl. Testosterone Therapy Ameliorates Experimental Autoimmune Encephalomyelitis and Induces A T Helper 2 Bias in The Autoantigen-Specific T Lymphocyte Response. *Journal of Immunology*,159:3–6, 1997.

81. M. Fijak, E. Schneider, and J. Klug. Testosterone Replacement Effectively Inhibits The Development of Experimental Autoimmune Orchitis in Rats: Evidence for A Direct Role of Testosterone. *Journal of Immunology*, 186:5162-5172, 2011.

82. A. Zoller, and G. Kersh. Estrogen Induces Thymic Atrophy by Eliminating Early Thymic Progenitors and Inhibiting Proliferation of B-Selected Thymocytes. *Journal of Immunology*, 176:7371-7378, 2006.

83. A. G. Rijhsinghani, K. Thompson, S. K. Bhatia, and T. J. Waldschmidt. Estrogen Blocks Early T Cell Development in The Thymus. *American Journal of Reproductive Immunology*, 36:269–277, 1996.

84. J. Borlak, I. Schulte, and T. Thum. Androgen Metabolism in Thymus of Fetal And Adult Rats. *Drug Metabolism and Disposition*, 32:675–679, 2004.

85. N. J. Olsen, G. Olson, S. M. Viselli, X. Gu, and W. J. Kovacs. Androgen Receptors in Thymic Epithelium Modulate Thymus Size and Thymocyte Development. *Endocrinology*, 142:1278–1283, 2001.

86. J. S. Sutherland, G. L. Goldberg, M. V Hammett, A. P. Uldrich, S. P. Berzins, T. S. Heng, B. R. Blazar, J. L. Millar, M. A. Malin, A. P. Chidgey, and R. L. Boyd. Activation of Thymic Regeneration in Mice and Humans Following Androgen Blockade. *Journal of Immunology*, 175:2741–2753, 2005.

87. M. D. Lagacé, S. Brochu, C. St. Pierre, and C. Perreault. Adult Thymic Epithelium Contains Nonsenescent Label Retaining Cells. *Journal of Immunology*, 192:2219–2226,

2014.

88. E. Velardi, J. J. Tsai, A. M. Holland, T. Wertheimer, V. W. C. Yu, J. L. Zakrzewski, A. Z. Tuckett, N. V Singer, M. L. West, O. M. Smith, L. F. Young, F. M. Kreines, E. R. Levy, R. L. Boyd, D. T. Scadden, J. A. Dudakov, and M. R. M. van den Brink. Sex Steroid Blockade Enhances Thymopoiesis by Modulating Notch Signaling. *Journal of Experimental Medicine*, 211: 2341–2349, 2014.

89. G. Goldberg, and Ö. Alpdogan. Enhanced Immune Reconstitution by Sex Steroid Ablation Following Allogeneic Hemopoietic Stem Cell Transplantation. *Journal of Immunology*, 78:7473-7484, 2007.

90. B. Hareramadas, and U. Rai. Cellular Mechanism of Estrogen-Induced Thymic Involution in Wall Lizard: Caspase-Dependent Action. *Journal of Experimental Zoology Part A Comparative Experimental Biology*, 305: 396–409, 2006.

91. B. Afzali, G. Lombardi, RI. Lechler, and GM. Lord. The Role of T Helper 17 and Regulatory T Cells (Treg) in Human Organ Transplantation and Autoimmune Disease. *Clinical Experimental Immunology*, 148: 32-46, 2007.

92. S. Sakaguchi, K. Wing, and M. Miyara. Regulatory T Cells A Brief History and Perspective. *European Journal of Immunology*, 37: 116-123, 2007.

93. A. Corthay. How Do Regulatory T Cells Work?. *Scandinavian Journal of Immunology*, 70 (4): 326–336, 2009.

94. A. Scheffold, K. M. Murphy, and T. Höfer. Competition for Cytokines: T(Reg) Cells Take All. *Nature Immunology*, vol. 8: 1285–1287, 2007.

95. D. K. Sojka, Y. H. Huang, and D. J. Fowell. Mechanisms of Regulatory T-Cell Suppression - A Diverse Arsenal for A Moving Target. *Immunology*, 124: 13–22, 2008.

96. N. Thiault, J. Darrigues, V. Adoue, M. Gros, B. Binet, C. Perals, B. Leobon, N.

Fazilleau, O. P. Joffre, E. A. Robey, J. P. M. van Meerwijk, and P. Romagnoli. Peripheral Regulatory T Lymphocytes Recirculating to The Thymus Suppress The Development of Their Precursors. *Nature Immunology*, 16: 628–634, 2015.

97. Harvard Medical School, "Immune System Taught to Play Nice with Unrecognized Protein", https://hms.harvard.edu/news/immune-system-taught-play-nice-unrecognized-protein-3-21-08 [retrieved 14 May 2016].

98. S. Allan, and S. Crome. Activation Induced FOXP3 in Human T Effector Cells Does Not Suppress Proliferation or Cytokine Production. *International Immunology*, 19:345-354, 2007.

99. E. d'Hennezel, and M. B. Shoshan. FOXP3 Forkhead Domain Mutation and Regulatory T Cells in The IPEX Syndrome. *New England Journal of Medicine,* 361: 1710-1713, 2009.

100. J. Kim, J. Rasmussen, and A. Rudensky. Regulatory T Cells Prevent Catastrophic Autoimmunity throughout The Lifespan of Mice. *Nature Immunology*, 8:191-197, 2007.

101. Y. Nishizuka, and T. Sakakura. Thymus and Reproduction: Sex-Linked Dysgenesia Of The Gonad After Neonatal Thymectomy in Mice. *Science,* 166:753-755, 1969.

102. I. Apostolou, and H. Von Boehmer. In Vivo Instruction of Suppressor Commitment in Naive T Cells. *Journal of Experimental Medicine*, 199:1401-1408, 2004.

103. K. Lahl, and C. Loddenkemper. Selective Depletion of Foxp3+ Regulatory T Cells Induces A Scurfy-Like Disease. *Journal of Experimental Medicine*, 204:57-63, 2007.

104. T. Chinen, and P. Volchkov. A Critical Role for Regulatory T Cell Mediated Control of Inflammation in The Absence of Commensal Microbiota. *Journal of Experimental Medicine*, 207:2323-2230, 2010.

105. J. Fontenot, M. Gavin, and A. Rudensky. Foxp3 Programs The Development and

Function of CD4+ CD25+ Regulatory T Cells. *Nature Immunology*, 4:330-336, 2003.

106. J. Fontenot, J. Rasmussen, and L. Williams. Regulatory T Cell Lineage Specification by The Forkhead Transcription Factor Foxp3. *Immunity*, 22:309-341, 2005.

107. A. Liston, A. Farr and Z. Chen. Lack of Foxp3 Function, and Expression in The Thymic Epithelium. *Journal of Experimental Medicine*; 204:475–480, 2007.

108. R. Mebius. Educating Stroma. *Immunology and Cell Biology*, 85:79-80, 2007.

109. G. Eberl, S. Marmon, M. J. Sunshine, P. D. Rennert, Y. Choi, and D. R. Littman. An Essential Function for The Nuclear Receptor Rorgamma in The Generation of Fetal Lymphoid Tissue Inducer Cells. *Nature Immunology*, 5: 64–73, 2004.

110. D. Meier, C. Bornmann, S. Chappaz, S. Schmutz, L. A. Otten, R. Ceredig, H. Acha-Orbea, and D. Finke. Ectopic Lymphoid-Organ Development Occurs through Interleukin 7 Mediated Enhanced Survival of Lymphoid-Tissue-Inducer Cells. *Immunity*, 26:643–654, 2007.

111. Y. Yokota, A. Mansouri, S. Mori, S. Sugawara, S. Adachi, S. Nishikawa, and P. Gruss. Development of Peripheral Lymphoid Organs and Natural Killer Cells Depends on The Helix-Loop-Helix Inhibitor Id2. *Nature*, 397:702–706, 1999.

112. R. E. Mebius, P. Rennert, and I. L. Weissman. Developing Lymph Nodes Collect $CD4^{+}CD3^{-}$ $LT\beta^{+}$ Cells That Can Differentiate to APC, NK Cells, and Follicular Cells but Not T or B Cells. *Immunity*, 7:493–504, 1997.

113. Z. Sun. Requirement for RORgamma in Thymocyte Survival and Lymphoid Organ Development. *Science*, 288:2369–2373, 2000.

114. H. V. Fernandes, M. C. Coles, K. E. Foster, A. Patel, A. Williams, D. Natarajan, A. Barlow, V. Pachnis, and D. Kioussis. Tyrosine Kinase Receptor RET is A Key

Regulator of Peyer's Patch Organogenesis. *Nature*, 446:547–551, 2007.

115. R. E. Mebius. Organogenesis of Lymphoid Tissues. *Nature Reviews Immunology*, 3: 292–303, 2003.

116. S. Kurebayashi, E. Ueda, M. Sakaue, D. D. Patel, A. Medvedev, F. Zhang, and A. M. Jetten. Retinoid Related Orphan Receptor Gamma is Essential for Lymphoid Organogenesis and Controls Apoptosis During Thymopoiesis. *Proceedings in National Academy of Science*, 97:10132–10137, 2000.

117. E. Scandella, B. Bolinger, E. Lattmann, S. Miller, S. Favre, D. R. Littman, D. Finke, S. A. Luther, T. Junt, and B. Ludewig. Restoration of Lymphoid Organ Integrity through The Interaction of Lymphoid Tissue-Inducer Cells with Stroma of The T Cell Zone. *Nature Immunology*, 9:667–675, 2008.

118. M. Tsuji, K. Suzuki, H. Kitamura, M. Maruya, K. Kinoshita, I. I. Ivanov, K. Itoh, D. R. Littman, and S. Fagarasan. Requirement for Lymphoid Tissue-Inducer Cells in Isolated Follicle Formation and T Cell-Independent Immunoglobulin A Generation in The Gut. *Immunity*, 29:261–271, 2008.

119. C. Possot, S. Schmutz, S. Chea, L. Boucontet, A. Louise, A. Cumano, and R. Golub. Notch Signaling is Necessary for Adult, But Not Fetal, Development of Rorγt^{+} Innate Lymphoid Cells. *Nature Immunology*, 12:949–958, 2011.

120. J. A. Dudakov, A. M. Hanash, R. R. Jenq, L. F. Young, A. Ghosh, N. V Singer, M. L. West, O. M. Smith, A. M. Holland, J. J. Tsai, R. L. Boyd, and M. R. M. van den Brink. Interleukin-22 Drives Endogenous Thymic Regeneration in Mice. *Science*, 336:91–95, 2012.

121. M. Y. Kim. Roles Of Embryonic and Adult Lymphoid Tissue Inducer Cells in Primary and Secondary Lymphoid Tissues. *Yonsei Medical Journal*, 49:352–356, 2008.

122. N. Monu, and A. Frey. Myeloid Derived Suppressor Cells, and Anti Tumor T Cells:

Complex Relationship. *Immunological Investigations*, 4:595-613, 2012.

123. M. Kortylewski, M. Kujawski, T. Wang and S. Wei. Inhibiting Stat3 Signaling in The Hematopoietic System Elicits Multicomponent Antitumor Immunity. *Nature Medicine*, 11:1314-1321, 2005.

124. N. Dilek, and R. De Silly. Myeloid Derived Suppressor Cells: Mechanisms of Action and Recent Advances in Their Role in Transplant Tolerance. *Frontiers in Immunology*, 3:9, 2012.

125. S. Nagaraj, and D. I. Gabrilovich. Tumor Escape Mechanism Governed by Myeloid Derived Suppressor Cells. *Cancer Research*, 68:2561–2563, 2008.

126. R. Yang, Z. Cai, Y. Zhang, and W. Yutzy. CD80 in Immune Suppression by Mouse Ovarian Carcinoma Associated Gr-1+ CD11b+ Myeloid Cells. *Cancer Research*, 66: 6807-6815, 2006.

127. C. Botta, A. Gullà, P. Correale, P. Tagliaferri, and P. Tassone. Myeloid Derived Suppressor Cells in Multiple Myeloma: Pre-Clinical Research and Translational Opportunities. *Frontiers in Oncology*, 4:348, 2014.

128. H. Lynch, and G. Goldberg. Thymic Involution and Immune Reconstitution. *Trends in Immunology*, 30:366-373, 2009.

129. M. G. Mattei, O. de Lapeyrire, J. Bresnick, C. Dickson, D. Birnbaum, and I. Mason. Mouse Fgf7 (Fibroblast Growth Factor 7) and Fgf8 (Fibroblast Growth Factor 8) Genes Map to Chromosomes 2 and 19 Respectively. *Mammalian Genome*, 6:196–197, 1995.

130. J. Rubin. Keratinocyte Growth Factor. *Cell Biology International*, 19:399–412, 1995.

131. A. O. Urtreger, M. T. Bedford, T. Burakova, E. Arman, Y. Zimmer, A. Yayon, D. Givol, and P. Lonai. Developmental Localization of The Splicing Alternatives of

Fibroblast Growth Factor Receptor-2. *Developmental Biology*, 158:475–486, 1993.

132. J. M. Revest, R. K. Suniara, K. Kerr, J. J. T. Owen, and C. Dickson. Development of The Thymus Requires Signaling through The Fibroblast Growth Factor Receptor R2IIIb. *Journal of Immunology*, 167:1954–1961, 2001.

133. L. De Moerlooze, B. S. Dene, J. Revest, M. Hajihosseini, I. Rosewell, and C. Dickson. An Important Role for The IIIb Isoform Of Fibroblast Growth Factor Receptor 2 in Mesenchymal-Epithelial Signalling During Mouse Organogenesis. *Development*, 127:483–492, 2000.

134. S. W. Rossi, L. T. Jeker, T. Ueno, S. Kuse, M. P. Keller, S. Zuklys, A. V Gudkov, Y. Takahama, W. Krenger, B. R. Blazar, and G. A. Holländer. Keratinocyte Growth Factor Enhances Postnatal T-Cell Development via Enhancements in Proliferation and Function Of Thymic Epithelial Cells. *Blood*, 109:3803–3811, 2007.

135. E. D. Goley, and M. D. Welch. The ARP2/3 Complex: An Actin Nucleator Comes of Age. *Nature Reviews Molecular Cell Biology*, 7:713–726, 2006.

136. K. Yi, J. Unruh, and M. Deng. Dynamic Maintenance of Asymmetric Meiotic Spindle Position through Arp2/3-Complex-Driven Cytoplasmic Streaming in Mouse Oocytes. *Nature Cell Biology*, 13:1252-1258, 2011.

137. J. E. S. M. Ruiz, and P. C. Letourneau. The Role Of Arp2/3 in Growth Cone Actin Dynamics and Guidance is Substrate Dependent. *Journal of Neuroscience*, 34:5895–5908, 2014.

138. Z. Yang, and H. Xiong. Culture Conditions and Types of Growth Media for Mammalian Cells. *Biomedical Tissue Culture*, 1:3–18, 2012.

139. S. C. Sun, Z. B. Wang, Y. N. Xu, S.E. Lee, X.-S. Cui, and N.H. Kim. Arp2/3 Complex Regulates Asymmetric Division and Cytokinesis in Mouse Oocytes. *PLoS One*, 6:18392, 2011.

140. C. Wu, S. B. Asokan, M. E. Berginski, E. M. Haynes, N. E. Sharpless, J. D. Griffith, S. M. Gomez, and J. E. Bear. Arp2/3 is Critical for Lamellipodia and Response to Extracellular Matrix Cues But is Dispensable for Chemotaxis. *Cell*, 148:973–987, 2012.

141. P. Suraneni, B. Rubinstein, J. R. Unruh, M. Durnin, D. Hanein, and R. Li. The Arp2/3 Complex is Required for Lamellipodia Extension and Directional Fibroblast Cell Migration. *Journal of Cell Biology*, 197:239–251, 2012.

142. L. Zhou, X. Zhang, X. Wu, H. Yang, K. Zhong, H. Wang, T. Zhou, T. Sheng, Y. Tong, D. Fan, and D. Chui. Testosterone Regulates Arp2/3 Expression by DNA Methylation in Hippocampus. *Molecular Neurodegeneration*, 7:27, 2012.

143. M. V Wiles, P. Ruiz, and B. A. Imhof. Interleukin-7 Expression During Mouse Thymus Development. *European Journal of Immunology*, 22:1037–1042, 1992.

144. T. Sakata, S. Iwagami, Y. Tsuruta, H. Teraoka, Y. Tatsumi, Y. Kita, S. Nishikawa, Y. Takai, and H. Fujiwara. Constitutive Expression of Interleukin-7 mRNA and Production of IL-7 by A Cloned Murine Thymic Stromal Cell Line. *Journal of Leukocyte Biology*, 48:205–212, 1990.

145. D. Clarke, O. Katoh, R. V. Gibbs, S. D. Griffiths, and M. Y. Gordon. Interaction of Interleukin 7 (IL-7) with Glycosaminoglycans and Its Biological Relevance. *Cytokine*, 7:325-330, 1995.

146 L. S. Park, U. Martin, K. Garka, B. Gliniak, J. P. Di Santo, and W. Muller. Cloning of the Murine Thymic Stromal Lymphopoietin (Tslp) Receptor: Formation of a Functional Heteromeric Complex Requires Interleukin 7 Receptor. *The Journal of Experimental Medicine*, 192:659-670, 2000.

147. Y. W. He, and T. R. Malek. The Structure and Function of γc-Dependent Cytokines and Receptors: Regulation of T Lymphocyte Development and Homeostasis. *Critical Reviews in Immunology*, 18:503-524, 1998.

148. R. Hofmeister, A. Khaled, N. Benbernou, E. Rajnavolgyi, K. Muegge, and S. Durum. Interleukin-7: Physiological Roles and Mechanisms of Action. *Cytokine and Growth Factor Reviews*, 10:41-60, 1999.

149. P. Macchi, A. Villa, S. Giliani, M. G. Sacco, A. Frattini, and F. Porta. Mutations of Jak-3 gene in Patients with Autosomal Severe Combined Immune Deficiency (SCID). *Nature*, 377:65-68, 1995.

150. A. Puel, S. F. Ziegler, R. H. Buckley, and W. J. Leonard. Defective IL7R Expression in T-B+ NK+ Severe Combined Immunodeficiency. *Nature Genetics*, 20:394-397, 1998.

151. S. Y. Lai, W. Xu, S. L. Gaffen, K. D. Liu, G. D. Longmore, and W. C. Greene. The Molecular Role of The Common Gamma C Subunit in Signal Transduction Reveals Functional Ssymmetry within Multimeric Cytokine Receptor Complexes. *Proceedings of the National Academy of Sciences*, 93:231-235, 1996.

152. S. A. Corfe, and C. J. Paige. The Many Roles of IL-7 in B cell Development; Mediator of Survival, Proliferation and Differentiation. *Seminars in immunology*, 24:198-208, 2012.

153 P. J. Morrissey, P. Conlon, S. Braddy, D. E. Williams, A. E. Namen, and D. Y. Mochizuki. Administration of IL-7 to Mice with Cyclophosphamide Induced Lymphopenia Accelerates Lymphocyte Repopulation. *The Journal of Immunology*, 146:1547-1552, 1991.

154. M. A. Oosterwegel, M. C. Haks, U. Jeffry, R. Murray, and A. M. Kruisbeek. Induction of TCR Gene Rearrangements in Uncommitted Stem Cells by a Subset of IL-7 Producing, MHC Class II–Expressing Thymic Stromal Cells. *Immunity*, 6:351-360, 1996.

155. T. A. Moore, J. U. von Freeden, R. Murray, and A. Zlotnik. Inhibition of Gamma Delta T cell Development and Early Thymocyte Maturation in IL-$7^{-/-}$ Mice. *The Journal of Immunology*, 157:2366-2373, 1996.

156. S. Bhatia, L. T. Tygrett, K. H. Grabstein, and T. J. Waldschmidt. The Effect of *in Vivo* IL-7 Deprivation on T cell Maturation. *The Journal of Experimental Medicine*, 181:1399-1409, 1995.

157. E. Mertsching, C. Burdet, and R. Ceredig. IL-7 transgenic mice: Analysis of The Role of IL-7 in The Differentiation of Thymocytes *in Vivo* and *in Vitro*. *International Immunology*, 7:401-414, 1995.

158. U. V. F. Jeffry, N. Solvason, M. Howard, and R. Murray. The Earliest T Lineage–Committed Cells Depend on IL-7 for Bcl-2 Expression and Normal Cell Cycle Progression. *Immunity*, 7:147-154, 1997.

159. E. Brugnera, A. Bhandoola, R. Cibotti, Q. Yu, T. I. Guinter, and Y. Yamashita. Coreceptor Reversal in The Thymus: Signaled $CD4^{+}$ 8^{+} Thymocytes Initially Terminate CD8 Transcription Even When Differentiating into $CD8^{+}$ T Cells. *Immunity*, 13:59-71, 2000.

160. T. Crompton, S. V. Outram, J. Buckland, and M. J. Owen. Distinct Roles of The Interleukin-7 Receptor α Chain in Fetal and Adult Thymocyte Development Revealed by Analysis of Interleukin-7 Receptor α Deficient Mice. *European Journal of immunology*, 28:1859-1866, 1998.

161. G. D. Sempowski, L. P. Hale, J. S. Sundy, J. M. Massey, R. A. Koup, and D. C. Douek. Leukemia Inhibitory Factor, Oncostatin M, IL-6, and Stem Cell Factor mRNA Expression in Human Thymus Increases with Age and Associated with Thymic Atrophy. *The Journal of Immunology*. 164:2180-2187, 2000.

162. G. D. Chazen, G. Pereira, G. LeGros, S. Gillis, and E. M. Shevach. Interleukin 7 is A T Cell Growth Factor. *Proceedings of the National Academy of Sciences*, 86(15):5923-5927, 1989.

163. P. Jourdan, J. P. Vendrell, M. F. Huguet, M. Segondy, J. Bousquet, and J. Pène. Cytokines and Cell Surface Molecules Independently Induce CXCR4 Expression on $CD4^+$ $CCR7^+$ Human Memory T Cells. *The Journal of Immunology*, 165:716-724, 2000.

164. S. L. Schober, C. T. Kuo, K. S.. Schluns, L. Lefrancois, J. M. Leiden, and S. C. Jameson. Expression of The Transcription Factor Lung Krüppel-like Factor is Regulated by Cytokines and Correlates with Survival of Memory T cells *in Vitro and in Vivo*. *The Journal of Immunology*, 163:3662-3667, 1999.

165. P. Wiryana, T. Bui, C. R. Faltynek, and R. J. Ho. Augmentation of Cell Mediated Immunotherapy Against Herpes Simplex Virus by Interleukins: Comparison of *in Vivo* Effects of IL-2 and IL-7 on Adoptively Transferred T Cells. *Vaccine*, 15:561-563, 1997.

166. M. H. Xie, S. Aggarwal, W. H. Ho, J. Foster, Z. Zhang, and J. Stinson. Interleukin (IL)-22, A Novel Human Cytokine that Signals through The Interferon Receptor Related Proteins CRF2–4 and IL-22R. *The Journal of Biological Chemistry*, 27: 31335-31339, 2000.

167. B. C. Jones, N. J. Logsdon, and M. R. Walter. Structure of IL-22 Bound to Its High-Affinity IL-22R1 Chain. *Structure*, 16:1333-1144, 2008.

168. S. Rutz, C. Eidenschenk, and W. Ouyang. IL-22, not Simply a Th17 Cytokine. *Immunological Reviews*, 252:116-132, 2013.

169. D. Lejeune, L. Dumoutier, S. Constantinescu, W. Kruijer, J. J. Schuringa, and J. C. Renauld . Interleukin-22 (IL-22) activates the JAK/STAT, ERK, JNK, and p38 MAP Kinase Pathways in A Rat Hepatoma Cell Line Pathways That are Shared with and Distinct from IL-10. *Journal of Biological Chemistry*, 277:33676-3382, 2002.

170. S. Radaeva, R. Sun, H. Pan, F. Hong, and B. Gao. Interleukin 22 (IL-22) Plays a Protective Role in T cell Mediated Murine Hepatitis: IL-22 is A Survival Factor for Hepatocytes via STAT3 Activation. *Hepatology*, 39:1332-1342, 2004.

171. K. Wolk, E. Witte, E. Wallace, W. D. Döcke, S. Kunz, and K. Asadullah. IL-22 Regulates The Expression of Genes Responsible for Antimicrobial Defense, Cellular Differentiation, and Mobility in Keratinocytes: A Potential Role in Psoriasis. *European Journal of Immunology*, 36:1309-1323, 2006.

172. H. L. Ma, S. Liang, J. Li, L. Napierata, T. Brown, and S. Benoit. IL-22 is Required for Th17 Cell Mediated Pathology in A Mouse Model of Psoriasis Like Skin Inflammation. *The Journal of Clinical Investigation*, 118:597-607, 2008.

173. L. Geboes, L. Dumoutier, H. Kelchtermans, E. Schurgers, T. Mitera, and J. C. Renauld. Proinflammatory Role of The Th17 Cytokine Interleukin-22 in Collagen Induced Arthritis in C57BL/6 Mice. *Arthritis and Rheumatism*, 60:390-395, 2009.

174. L. A. Zenewicz, G. D. Yancopoulos, D. M. Valenzuela, and A. J. Murphy, M. Karow, R. A. Flavell. Interleukin-22 But Not Interleukin-17 Provides Protection to Hepatocytes During Acute Liver Inflammation. *Immunity*, 27:647-659, 2007.

175. K. Sugimoto, A. Ogawa, E. Mizoguchi, Y. Shimomura, A Andoh, and A. K. Bhan. IL-22 Ameliorates Intestinal Inflammation in A Mouse Model of Ulcerative Colitis. *The Journal of Clinical Investigation*, 118:534-544, 2008.

176. M. S. Silverberg, J. H. Cho , J. D. Rioux, D. P. McGovern, J. Wu, and V. Annese Ulcerative Colitis–Risk Loci on Chromosomes 1p36 and 12q15 Found by Genome-Wide Association Study. *Nature Genetics*, 41:216-220, 2009.

177. L. Yang, Y. Zhang, L. Wang, F. Fan, L. Zhu, and Z. Li. Amelioration of High Fat Diet Induced Liver Lipogenesis and Hepatic Steatosis by Interleukin-22. *Journal of Hepatology*, 53:339-347, 2010.

178. S. Eyerich, K. Eyerich, D. Pennino, T. Carbone, F. Nasorri, and S. Pallotta. Th22 Cells Represent a Distinct Human T cell Subset Involved in Epidermal Immunity and Remodeling. *The Journal of Clinical Investigation*, 119:3573-3585, 2009.

179. G. Pickert, C. Neufert, M. Leppkes, Y. Zheng, N. Wittkopf, and M. Warntjen. STAT3 Links IL-22 Signaling in Intestinal Epithelial Cells to Mucosal Wound Healing. *The Journal of Experimental Medicine*, 206:1465-1472, 2009.

180. S. H. Ki, O. ParK, M. Zheng, O.Ibanez, J. Kolls, and R. Bataller. Interleukin-22 Treatment Ameliorates Alcoholic Liver Injury in A Murine Model of Chronic-Binge Ethanol Feeding: Role of Signal Transducer and Activator of Tanscription. *Hepatology*, 52:1291-1300, 2010.

181. J. A. Dudakov, A. M. Hanash, R. R. Jenq, L. F. Young, A. Ghosh, and N. V. Singer. Interleukin-22 Drives Endogenous Thymic Regeneration in Mice. *Science*, 336:91-95, 2012.

182. B. Long, and D. Jelovac. Therapeutic Strategies Using The Aromatase Inhibitor Letrozole and Tamoxifen in A Breast Cancer Model. *Journal of National Cancer Institute*, 96:456-465, 2004.

183. J. A. Rettew, Y. M. Huet-Hudson, and I. Marriott. Testosterone Reduces Macrophage Expression in The Mouse of Toll-Like Receptor 4, A Trigger For Inflammation and Innate Immunity. *Biology of Reproduction*, 78:432–437, 2008.

184. V. Covelli. Guide to the Necropsy of The Mouse Chapter 5: Thoracic Cavity http://www.pathbase.net/Necropsy_of_the_Mouse/index.php?file=Chapter_5.html#2 [retrieved 02 June 2016]

185. A. Radonic, S. Thulke, I. M. Mackay, O. Landt, W. Siegert, and A. Nitsche. Guideline to Reference Gene Selection for Quantitative Real-time PCR. *Biochemical and Biophysical Research Communications*, 313: 856-862, 2004.

186. S. Koyasu, and K. Moro. Role of Innate Lymphocytes in Infection and Inflammation. *Frontiers in Immunology*, 3:1-13, 2012.

187. S. Fahl, and R. Crittenden. C-Myb Is Required for Pro-B Cell Differentiation. *Journal of Immunology*, 183:5582-5592, 2009.

188. A. Y. Lai, S. M. Lin, and M. Kondo. Heterogeneity of Flt3-Expressing Multipotent Progenitors in Mouse Bone Marrow. *Journal of Immunology*, 175:5016–5023, 2005.

189. R. Kumar, V. Fossati, M. Israel and H. W. Snoeck. Lin-Sca1+Kit- Bone Marrow Cells Contain Early Lymphoid-Committed Precursors That are Distinct from Common Lymphoid Progenitors. *Journal of Immunology*, 181:7507–7513, 2008.

190. E. G. Yarmola, and M. R. Bubb. Profilin: Emerging Concepts and Lingering Misconceptions. *Trends in Biochemical Science*, 31:197–205, 2006.

191. T. Wise, and J. Klindt. Thymic Weight Changes and Endocrine Relationships During Maturation in Cattle: Effects of Age, Sex, and Castration. *Growth, Developmental and Aging.* 59:139-148, 1995.

192. B. B. Maoz, E. M. Rodriguez, R. A. J. Signer, and K. Dorshkind. Fibroblast Growth Factor-7 Partially Reverses Murine Thymocyte Progenitor Aging by Repression of Ink4a. *Blood*, 119:5715–5721, 2012.

193. Y. Cui, and Q. Li. Effect of Mammogenic Hormones on The Expression of FGF7, FGF10 and Their Receptor in Mouse Mammary Gland. *Science in China Series C Life Sciences.* 51: 711-717, 2008.

194. H. T. Kissick, M. G. Sanda, L. K. Dunn, K. L. Pellegrini, S. T. On, J. K. Noel, and M. S. Arredouani. Androgens Alter T-Cell Immunity by Inhibiting T-Helper 1 Differentiation. *Proceedings in National Academy of Science*, 111:9887–9892, 2014.

195 V. A. Kozlov, I. G. Tsyrlova, and I. N. Zhuravkin. Different Effect of Testosterone

on Polypotential Stem Hematopoietic Stem Cells and Immunocompetent B Lymphocytes. *Zhurnal Mikrobioligii, Epidemilogii, Immunobiologii*. 9: 72-76, 1979.

196. S. W. Kim, J. H. Hwang, J. M. Cheon, N. S. Park, S. E. Park, S. J. Park, and H. J. Yun. Direct and Indirect Effects of Androgens on Survival of Hematopoietic Progenitor Cells *In Vitro*. *Journal of Korean Medical Science.* 20: 409-416, 2005.

197. M. Fijak, E. Schneider, and L. Klug. Testosterone Replacement Effectively Inhibits the Development of Experimental Autoimmune Orchitis in Rats: Evidence for a Direct Role of Testosterone on Regulatory T Cell Expansion. *The Journal of Immunology*. 186:5162-5172, 2011.

198. D. M. Bowdish. Myeloid Derived Suppressor Cells, Age and Cancer. *Oncoimmunology*, 2:247-254, 2013.

199. N. J. Olsen, and W. J. Kovacs. Evidence That Androgens Modulate Human Thymic T Cell Output. *Journal of Investigation in Medicine*, 59:32–35, 2011.