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The Role of Aging T-Lymphocytes in Prostate Cancer Development

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The Role of Aging T-lymphocytes in Prostate Cancer Development

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Dedication

This dissertation is dedicated to my dear family who has guided me and helped me achieve my goals.
To my dad who has not only given me unconditional support but has also taught me very valuable life skills, such as work ethic, critical thinking and the importance of a good sense of humor.
To my mom who has given me unconditional love and support throughout the years.
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The Role of Aging T-cells in Prostate Cancer Development

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The University of Texas at Austin, 2014

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Age is the single greatest factor associated with increased risk for prostate cancer development. Evidence implicates progressive age-related immune dysfunction with increased prostate cancer incidence. The aged T cell response is characterized by increased production of pro-inflammatory cytokines, which could significantly contribute to prostate tumorigenesis through induction of key pro-survival factors. The objective of these studies was to determine how age-related changes in T-lymphocyte function contribute to prostate tumorigenesis. The hypothesis that age-related changes in T-lymphocyte function to a pro-inflammatory phenotype promote prostate cancer development was tested using the glycerol-3-phosphate acyltransferase-1 (GPAT-1) knock-out mouse, which mimics many of the characteristics of an aged immune system. T cells from old (24-month) mice and aging-mimic T cell GPAT-1$^{-/-}$ mice generate more pro-inflammatory cytokines than T-lymphocytes from wild type mice. These cytokines turn on inflammatory pathways that stimulate proliferation, tissue disruption, and tumor growth. Initial studies showed that secreted factors from aging and T cell aging-

mimic GPAT-1$^{-/-}$ mice produce circulating factors that induce pro-inflammatory pathways in prostate cells, most notably the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB). Additionally, results from recent experiments demonstrate that serum from the GPAT-1 $^{-/-}$ mice induce protein expression of downstream targets of NF-κB in the prostate, most notably factors that induce macrophage infiltration and key pro-survival proteins. Furthermore, my experimental results suggest that the increased production of interleukin 17 (IL-17) by aged T cells play a role in the induction of pro-inflammatory pathways in the prostate. Based on these findings additional studies were design to determine if the increased production of pro-inflammatory cytokines by aging T-lymphocytes contributes to a more malignant phenotype in the prostate. Finally, the inter-relationship between an aging immune system and the aging tissues in the body was explored. Findings from these studies provide evidence that the dysregulation of cytokine production seen in aged T cells may directly contribute to the increased risk for prostate cancer in the elderly. This new perspective regarding the role of the aging immune system in cancer development opens new avenues for development of potential preventive interventions and screening biomarkers.

Table of Contents

List of Tables xii

List of Figures xiii

Chapter 1: Introduction 15

1.2 Aging, Immunity, and Prostate Cancer Risk 15

1.2 Inflammation and Prostate Carcinogenesis 16

1.3 Mouse Model of Aging T-lymphocytes 18

1.3.1 The Mouse as a Model Organism in Human Aging Research 18

1.3.2 Current Mice Models for Aging Research 19

1.3.3 The glycerol-3-phosphate acyltransferase-1 (GPAT-1) knock-out mouse of T-lymphocyte aging 23

1.4 Aging T-lymphocytes, Interlukin-17 and Prostate Tumorigenesis 24

1.5 Aging, Interlukin-6 and Prostate Tumorigenesis. 26

1.6 Dissertation Objectives 28

Chapter 2: Age-related Alterations in T-lymphocytes Modulate Key Pathways in Prostate Tumorigenesis 30

2.1 Introduction 30

2.2 Materials and methods 32

2.2.1 Murine Models of an Aging Immune System 32

2.2.2 T-lymphocyte isolation and stimulation 33

2.2.3 Cell Lines 34

2.2.4 Cytokine Array 34

2.2.5 Dual Luciferase assay 35

2.2.6 Immunofluorescence 35

2.2.7 Western blot analysis.. 36

2.2.8 Statistics 37

2.3 Results 37

2.3.1 Aging mimic GPAT-1 -/- T-lymphocytes have a pro-inflammatory cytokine profile 37

2.3.2 GPAT-1$^{-/-}$ and old wild type T-lymphocytes secrete factors that induce NF-κB activity 40

2.3.3 Circulating factors in the sera from mice with an aged immune system induce nuclear translocation of NF-κB 43

2.3.4 Factors secreted from aging T-lymphocytes induce NF-kB transcriptional activity 45

2.3.5 Factors secreted by aging T-lymphocytes upregulate the expression of key downstream targets of NF-**κ**B 49

2.4 Discussion.. 53

Chapter 3: Age-related Increase in IL-17 Activates Pro-inflammatory Signaling in Prostate Cells 56

3.1 Introduction 56

3.2 Materials and Methods.. 58

3.2.1 Murine Models of an Aging Immune System 58

3.2.2 T-lymphocyte isolation and stimulation 58

3.2.3 Cells and Cell Culture 59

3.2.4 Dual Luciferase assay 60

3.3.5 Western blot analysis.. 61

3.2.6 RT-qPCR.. 61

3.2.7 MTT Cell Proliferation Assay 62

3.2.8 Statistics 62

3.3 Results 63

3.3.1 IL-17 in the sera from Aging Mimic mice induces NF-κB activity in prostate epithelial cells 63

3.3.2 IL-17 secreted from aging T-lymphocytes induce NF-κB transcriptional activity 68

3.3.3 IL-17 secreted by aging T-lymphocytes regulates the expression of key downstream targets of NF-κB 70

3.3.4 The aging-associated increase in T-lymphocyte IL-17 secretion moderately mediates prostate cancer cell proliferation 78

3.4 Discussion 82

Chapter 4: The Role of IL-17 and IL-6 in Aging related Prostate Tumorigenesis .. 86

4.1 Introduction 86

4.2 Materials and Methods 88

4.2.1 Murine Models of an Aging Immune System 88

4.2.2 Cells and Cell Culture 89

4.2.3 Wound Healing Assay 91

4.2.4 Western blot analysis 91

analysis measured using GeneTools. 91

4.2.5 RT-qPCR 92

4.2.6 Statistics 92

4.3 Results 93

4.3.1 IL-17 and IL-6 in the sera from old and aging mimic mice induce activation of STAT3 93

4.3.2 Sera from old mice increase expression of the transcription factors Slug, Snail and Twist 95

4.3.3 Prostate epithelial cell migration and invasion induced by sera from OLD and AM mice 99

4.4 Discussion.. 102

Chapter 5: Concluding Remarks.. 106

5.1 Conclusions 106

5.2 Future Directions 111

Appendix 1: AM and OLD mice have PIN lesions and increased levels of prostate-infiltrating T_H17 cells 114

Appendix 2: Conditioned Media from Senescent Epithelial Cells Decreases T cell IL-2 production.. 115

Appendix 3: LNCaP cells treated with Conditioned Media from GPAT-1$^{-/-}$ T cells moderately induce recruitment of monocytes.. 116

References 117

Vita……………………………………………………………………………………………….125

List of Tables

Table 3.1 Sera from Aging Mimic mice differentially regulates the expression of downstream targets of NF-κB 74

List of Figures

Figure 2.1 Aging mimic GPAT-1 -/- T-lymphocytes have a pro-inflammatory cytokine profile. 39

Figure 2.2 Effect of circulating factors in the sera from the aged wild type and aging-mimic young GPAT-1 -/- mice on NF-κB transcriptional activity.42

Figure 2.3 Alteration of NF-kB subcellular localization induced by circulating factors in the sera from aged and young GPAT-1-/- mice. 44

Figure 2.4 Effect of isolated splenic T-lymphocyte-secreted factors from WT or young GPAT-1 -/- mice on NF-κB transcriptional activity..................... 47

48

Figure 2.5 Nuclear localization of NF-kB induced by factors secreted from stimulated aged T-lymphocytes. 48

Figure 2.6 Increased expression of gene targets of NF-κB in response to circulating factors secreted from aged-mimic T-lymphocytes. 51

Figure 2.7 Increased expression of gene targets of NF-κB in response to circulating factors secreted from aged-mimic T-lymphocytes 52

Figure 3.1 IL-17R expression in human prostate cells. 65

Figure 3.2 Expression levels of pSTAT3 in response to IL-17R knock-down. 66

Figure 3.3 NF-κB transcriptional activity is stimulated by IL-17 in the sera of Aging Mimic mice. 67

Figure 3.4 IL-17 secreted by isolated splenic T-lymphocytes from wild-type, aging-mimic or old mice induces NF-κB transcriptional activity 69

Figure 3.5 Sera from aging-mimic mice up-regulates the expression of key downstream targets of NF-κB 73

Figure 3.6 Effects of IL-17 in the sera from aging-mimic mice on expression of key downstream targets of NF-κB.. 76
Figure 3.7 Effects of IL-17 in the sera from aging-mimic mice on expression of key downstream targets of NF-κB. 77
Figure 3.8 Effect of IL-17 in the sera from Aging Mimic and wild-type mice on cellular proliferation. 80
Figure 3.9 Effect of isolated splenic T-lymphocyte-secreted IL-17 from wild-type, Aging Mimic or old mice on cell proliferation. 81
Figure 4.1 Effects of IL-6 and IL-17 in the sera from OLD and AM mice on expression of key downstream targets of STAT3.. 94
Figure 4.2 Effects of IL-6 and IL-17 in the sera from OLD and AM mice on expression of Slug.. 97
Figure 4.4 Circulating Factors in the sera from AM and OLD mice promote migration of prostate epithelial cells... 100
Figure 4.5 Circulating Factors in the sera from AM mice promote invasion of prostate epithelial cells 101
Figure 5.1 T-cell aging mimic mice have PIN lesions and increased levels of prostate-infiltrating TH17 cells. 114
Figure 5.2 IL-2 expression in human Jurkat T cells 115

Chapter 1: Introduction

1.2 Aging, Immunity, and Prostate Cancer Risk

Prostate cancer risk is strongly age dependent, rarely appearing before age 40 and typically affecting men around 70 years of age [1]. This association with age suggests that prostate cancer results from accumulation of genetic damage, perhaps due to oxidative stress or other endogenous or exogenous factors. During aging, there is a progressive accumulation of DNA damage and oxidative stress in most tissues, and this plays an important role in age-related tumorigenesis [2, 3]. In addition, there are significant changes in immune response regulation that accompany the aging process. These include increased pro-inflammatory cytokine production, as well as a decrease in appropriate immune surveillance. The importance of the immune system in preventing tumorigenesis has been supported by numerous research studies as well as epidemiological evidence [4]. Aging has multiple effects on the development, function and turnover of immune cells. In fact, aging is associated with low number of naïve T cells, decreased diversity and functionality of $CD4^+$ and $CD8^+$ T cells and inability to respond appropriately to antigens [5, 6]. Additionally, aging is associated with a low-grade chronic inflammation. Inflammation is critical for fighting infections in the elderly; a chronic low-grade inflammation is deleterious and highly correlated with prostate cancer risk [7, 8].

The retention of inflammatory responses in the elderly accompanied with the absence of counterbalancing and beneficial effects highly enhance the likelihood of developing prostate cancer.

1.2 Inflammation and Prostate Carcinogenesis

Chronic inflammation is believed to play a pathogenic role in age-related diseases, including prostate cancer. The role of inflammation in prostate cancer tumorigenesis has been well established. Most lesions that contain inflammatory infiltrates are associated with prostatic atrophy [7, 9].

Additionally, several key molecular pathways involved in prostate carcinogenesis have been associated with chronic or acute prostatic inflammation. Pro-inflammatory cytokines activate key transcription factors such as, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) or signal transducer and activator of transcription 3 (STAT3), in prostate epithelial cells. NF-κB and STAT3 control numerous pro-tumorigenic process, including survival, proliferation, growth, angiogenesis, and invasion. As part of a positive feedback loop, NF-κB and STAT3 induce production of additional chemokines that attract inflammatory cells to the prostate and help sustain an inflammatory environment [10].

Prostatic tissue normally contains endogenous immune cells, including T and B lymphocytes and macrophages. However, aging prostate tissue contains increased

inflammatory infiltrates. Aging prostate tissue has an increased number of macrophages and leukocytes. Studies by Steiner *et al.* demonstrated that most T cell in older prostate tissue expressed $CD4^+$ T cells; however non-inflamed younger prostate tissues expressed mostly $CD8^+$ T cells [11, 12]. Furthermore, Steiner *et al.* showed that activated T cells in chronically inflamed prostate tissue express high levels of IL-17 [13]. These diverse inflammatory infiltrates in the prostate communicate with each other by means of direct contact and cytokine production, and control prostate tumor initiation and promotion

Inflammation can contribute to prostate tumor initiation through increase in mutation rates, genomic instability and epigenetic modifications. Inflammation enhances the production of growth factors and cytokines that enhance the proliferation of mutated cells. Additionally, p53 mutations, caused by oxidative damage, were found in both cancer cells and inflamed epithelium, suggesting that chronic inflammation causes genomic mutations [14, 15]. Other findings, implicate that inflammation influences epigenetic mechanisms, including microRNA-based silencing and DNA methylation. One study demonstrated that inflammation plays a role in epigenetic reprogramming through regulation Jmjd3 which is encoded by a NF-kB target gene [16]. The connection between inflammations and prostate tumor initiation is not a one way street, studies have shown that DNA damage can lead to inflammation and thereby promote prostate tumorigenesis [16].

Aged related inflammation can affect every aspect of prostate tumor development and progression, via multiple mechanisms. Controlling inflammatory status may allow successful prevention and control of prostate tumorigenesis.

1.3 Mouse Model of Aging T-lymphocytes

1.3.1 The Mouse as a Model Organism in Human Aging Research

Aging is commonly defined as the accumulation of diverse deleterious changes occurring in cells and tissues. Aging is characterized by decrease in physiological capacity, reduced ability to respond adaptively to environmental stimuli and increased vulnerability to disease [17].

Using humans as subjects in aging research is complicated due to ethical issues, long life span, environmental influences, and various other limiting factors. Therefore, various animal models have been developed to study the biology of aging [17, 18]. Animal models used to investigate the human biology of aging and age-related diseases should mimic the biological changes that occur in humans while controlling for genetic background, diet, environmental changes and health status. Non-mammalian models, such as worms and fruit flies, have some advantages and have been useful in the study of aging-related genes. However, in order to understand the complexity of aging in humans, mammalian model organisms are indispensable. Primates should be ideal in that respect, but ethical issues and long life span make studies with primates

complicated. On the contrary, mice are good models for studying human aging. Not only can they be genetically modified but longitudinal studies are easy to conduct on mice because of their short life span [18].

1.3.2 Current Mice Models for Aging Research

Animal models use to study accelerated aging, aging biology and aging related diseases are generally grouped into four classes: experimentally induced models, gene-modified models, selection models, and spontaneous models. Researchers interested in the mechanisms of normal aging have to be prudent in their choice of animal models because early diseases leading to reduced life spans usually result from certain defects unrelated to mechanisms associated with normal aging. It can be difficult to distinguish between accelerated aging due to acceleration of the normal aging process and accelerated aging due to the manifestation of pathologies.

One model that has been used to study accelerated aging is the thymectomized lab mouse**.** When male mice are thymectomized their mean life span is reduced [19]. The reduced life span is thought to be due to accelerated aging of the immune system that involves a decline in spleen cell responsiveness to T cell mitogens [20]. Thymectomy reduces the number of peripheral $CD4^+$ T cells and affects the $CD4^+$ T cell population by changing it to a memory phenotype and removing their self-renewability. The thymectomized mice model can be useful for studying the effects of age related decline

in thymus function. The function of the thymus is known to decline in adults. In the elderly the thymus is barely identifiable, consisting mostly of fatty tissue [19, 21]. The thymectomized model of accelerated aging presents some limitations. First of all, the complete lack of a functional thymus is not representative of actual aging. Because despite the thymus involution that comes with age, the thymus continues its endocrine function. Additionally, Smolarchuk *et al*. found that thymectomized lab mice could generate T cells without contribution from the thoracic thymus. However, these mice had increased proportions of effector memory T cells and Regulatory T cells (Treg) phenotype cells, increased serum IgG1/2b, and increased frequency of T cells expressing IFN-γ, IL-17 or IL-10 [22]. Other studies have demonstrated that mice that received a thoracic thymectomy developed autoimmune gastritis [23]. Together the data from thymectomized lab mice suggests that this is not the best model for aging research.

Other mouse models for aging research are the *klotho* mouse model and the Senescence-accelerated mouse model (SAM). The *klotho* mouse model was generated by inserting a mutated transgene that disrupt the *klotho* gene locus. *Klotho* mice display various phenotypes resembling premature aging. Mice homozygous for the transgene show arteriosclerosis, osteoporosis, age-related skin changes, short lifespan and infertility, and growth retardation. The *klotho* gene encodes a membrane protein that shares a sequence similarity with the β-glucosidase enzymes and is expressed mainly in

the kidney and the brain. The *klotho* mice model is useful to study molecular-genetic mechanisms of both premature aging and accelerated aging. However, some common phenotypes seen in natural human aging are not present in *klotho* mice [24]. Additionally, *klotho* mice develop dramatic physiological changes that are not present in normal aging. Therefore, *klotho* mice are better suited as model for human progeroid syndrome rather than normal human aging.

The senescence-accelerated mouse (SAM) consists of fourteen senescence-prone inbred strains (SAMP) and four senescence-resistant inbred strains (SAMR). The manifestation of senescence in SAMP does not occur in the developmental stage, but it occurs in an accelerated manner following normal development, though there is no evidence of growth retardation, malformation, limb palsy, or other neurological signs, such as tremors and convulsions. The life span of SAMP is about 40 percent shorter than that of SAMR. Thus, accelerated senescence is considered to be a characteristic feature common to all SAMP mice. Both SAMP and SAMR strains manifest various aging related phenotypes, which are often characteristic enough to differentiate the strains. These phenotypes include impaired immune response, hyperinflation of the lungs, hearing impairment, deficits in learning and memory, cataracts, alveolar bone loss, degenerative joint disease, abnormality of circadian rhythms, emotional disorders, and brain atrophy [25, 26].

Senescence-accelerated mice (SAM) and their substrains have been developed for the study of human aging and are known to exhibit aging phenotypes. The pitfall of SAM mice as model for aging research is that the different aging phenotypes associated with SAM are distributed among the various SAM substrains and multiple gene mutations are implicated in causing the phenotypes observed in the SAM mouse model [24].

1.3.3 The glycerol-3-phosphate acyltransferase-1 (GPAT-1) knock-out mouse of T-lymphocyte aging

Glycerol-3-phosphotate acetyltransferases are rate limiting enzymes of triacylglycerol biosynthesis. There are four homologous isoforms of glycerol-3-phosphate acyltransferase (GPAT), each the product of a separate gene that catalyzes the synthesis of lysophosphatidic acid from glycerol-3-phosphate and long-chain acyl-CoA. GPAT-1 and 2 are found in the mitochondria and GPAT-3 and 4 are found in the micirosome [27]. GPAT-1 influences biological membrane composition which can have a profound effect on T cell function. While other tissues express both GPAT-1 and GPAT-2 on the mitochondria membrane, GPAT-1 is the only mitochondrial isoform expressed in T-lymphocytes [28].

The GPAT-1 knock-out mouse provides an unprecedented opportunity to investigate the effects of an aging T-lymphocyte population in the absence of other age related complications. Since GPAT-1 is the only mitochondrial isoform expressed in T-lymphocytes, GPAT-1 $^{-/-}$ mice display only minimal phenotype in other tissues. GPAT-1 catalytic activity is significantly down-regulated in aged rat-liver and T-lymphocytes, suggesting that impairment of the GPAT-1 mediated lipid biosynthetic pathway is a physiologically relevant event in the natural course of aging [29].

GPAT-1$^{-/-}$ mice T-lymphocytes possess multiple hallmarks of aging. Without GPAT-1, T cell proliferation is inhibited and activation induced apoptosis is increased. Importantly, consistent with an immune-senescent phenotype, T_H1 cytokine secretion is reduce in stimulated splenic T cells form GPAT-1$^{-/-}$ mice [30]. Additionally, T-lymphocytes from the GPAT-1$^{-/-}$ mouse up-regulate the production and secretion of pro-inflammatory cytokines [30, 31].

The GPAT-1$^{-/-}$ is the only animal model to mimic the membrane lipid and functional phenotype changes of an aged T cell with no other pathological conditions. Investigations on how specific aspects of the aging process contribute to prostate tumorigenesis are extremely difficult due to the multiple morbidities associated with aging. The GPAT-1 $^{-/-}$ mouse model provides an opportunity to investigate how T cell aging contributes to prostate cancer development.

1.4 Aging T-lymphocytes, Interlukin-17 and Prostate Tumorigenesis

It has been widely accepted that aging is characterized by a pro-inflammatory imbalance of T_H1/T_H2 cells [6]. A third subset of T helper cells, T_H17, has been implicated in the development of chronic inflammation in the elderly. Schmitt *et al.* demonstrated that, on a basal resting level, T_H17 cells were at a significantly increased level in older individuals. Other groups have also demonstrated that the proportion of T_H17 cells was garter in aged mice both in naïve and memory cell populations [32]. Some

investigators suggest that elevation of IL-1β and IL6 and reduction of IL-2 expression in aged mice promote T_H17 differentiation [33, 34].

T_H17 cells are characterized by producing pro-inflammatory cytokines IL-17, IL-21 and IL-22. IL-17 is a pro-inflammatory cytokine that is involved in recruiting inflammatory cells and induction of pro-inflammatory mediators [35]. As a pro-inflammatory cytokine, IL-17 has been implicated in a number of diseases including rheumatoid arthritis, asthmatic airways, allergic skin immune responses and the inflammatory process associated with strokes [36]. Additionally, IL-17 is produce by tumor infiltrating lymphocytes, and increases tumorigenicity in a number of cancers. IL-17 has also been implicated in tumor vascularization of cervix and ovarian cancer [13]. IL-17 exerts its effects through a family of receptors comprised of five members (IL17-RA, RB, RC, RD and RE). The roles of IL-17RD and IL-17RE are not clear, while IL-17B, IL17C and IL-17D are currently poorly studied with regards to their biological functions and receptors. IL-17RA forms a receptor heterodimer with IL-17RC. The receptor heterodimer is the preferred form by IL-17 ligands. When IL-17 binds to IL-17RA/C it activates NF-κB and MAPK signaling pathways [37] .

The contribution of IL-17 to prostate cancer has been reported by various investigators [11, 13, 38-45]. Isoforms of the IL-17 receptor have been detecte in certain prostate cancers [44]. Moreover, IL-17A expression is increase in 58% of prostate cancer biopsies and IL-17RA and IL-17RC receptors are expressed in aggressive forms of

prostate cancer **[13]**. It has also been reported that T_H17 cell number is increased in prostate cancer [45] and a higher percentage of T_H17 in the blood is correlated with a poorer outcome [38]. Recently, Zhang *et al.* also reported that IL-17 promotes the formation and growth of prostate adenocarcinoma in a mouse model of autochthonous prostate cancer and that IL-17 promotes development of castration resistant prostate cancer [42, 43]. Taken together the current knowledge indicates that there is a potential link between increased IL-17 expression in the elderly and prostate cancer development and progression.

1.5 Aging, Interlukin-6 and Prostate Tumorigenesis.

IL-6 is the most prominent cytokine that is shared across age-related pathologies having a strong chronic inflammatory component. There is strong evidence that IL-6 serum concentration increases with age [46-49]. Maggio *et al.* reported that IL-6 mean values ranged from 1.4 pg/ml (men) and 1.1 pg/ml (women) in the 65–74 years age group to 3.5 pg/ml (men) and 2.1 pg/ml (women) in persons 85 years and older, and that the age trend is partially independent of major confounders [48]. Age-related increments in IL-6 are not explained by differential prevalence of IL-6 gene polymorphisms [50]. The etiology of chronically elevated IL-6 in older adults is likely multifactorial, with increased presence of disease states, declines in estrogen and

testosterone levels and changes in the immune system function and regulation all contributing to increases in IL-6 levels.

IL-6 expression is mainly modulated by the nuclear NF-κB. A number of different stimuli, including cytokines, infections, and toxins, induce IL-6 expression. The binding of IL-6 to its receptor activates two distinct signaling pathways: The Janus tyrosine family kinase (JAK)-signal transducer and activator of transcription (STAT) pathway and the extracellular signal-regulated kinase 1 and 2 (ERK1/2)-mitogen activated protein kinase (MAPK) pathway [51, 52]. The physiological role of IL-6 is to initiate and coordinate acute phase response, but there is strong evidence indicating that IL-6 also plays a central role in the pathogenesis of chronic disease.

In cancer, IL-6 is a growth/survival factor for a variety of tumor types. In prostate cancer, activation of STAT3 by IL-6 is correlated with increase proliferation, decreased apoptotic potential, regulation of epithelial-mesenchymal transition (EMT) and activation of androgen receptor genes [53]. An extensive literature suggests that IL-6 plays a critical role in prostate cancer initiation and progression [15, 39, 41, 53-57].

Overall, the retention of strong inflammatory responses accompanied with the absence of counterbalancing and beneficial effects of the immune system highly enhance the likelihood of developing prostate cancer in the elderly. A better understanding of the regulation and role of IL-6 and IL-17 in aging-related prostate cancer is required to develop effective therapeutic and prevention treatments.

1.6 Dissertation Objectives

Epidemiological studies suggest that after 50 years of age, men experience a progressively heightened risk of being diagnosed with prostate cancer with each subsequent year of life. This is due to several factors, including an increased duration of carcinogenesis, accumulation of DNA damage and an increased susceptibility of aging cells to environmental carcinogens. Another important mechanistic link between aging and prostate cancer is reduced immune function. As the immune system is strongly implicated in modulating systemic inflammation, and because the phenotypic functionality of immune-regulatory cells changes with age, immune dysfunction may actively promote prostate carcinogenesis in the elderly.

The objective of these studies was to determine how age-related changes in T-lymphocyte function contribute to prostate tumorigenesis. We hypothesized that age related changes in T-lymphocyte function, towards a pro-inflammatory phenotype may promote prostate cancer development. The rationale behind our hypothesis was largely based on preliminary data suggesting that, consistent with an aged phenotype, T-lymphocytes from the GPAT-1$^{-/-}$ mouse up-regulate the production and secretion of pro-inflammatory cytokines. Many of the pro-inflammatory cytokines produced by aged T-lymphocytes have been implicated in the promotion of prostate tumorigenesis, primarily through the induction of other pro-inflammatory pathways and the activation of transcription factors like NF-κB.

Given that preliminary data indicated that T-lymphocytes from aging mimic GPAT-1 $^{-/-}$ mice secrete high levels of IL-17, a key cytokine known to promote carcinogenesis, and that transcription factors like NF-κB are critical for both inflammation and tumor growth, we tested the role of IL-17 in GPAT-1$^{-/-}$-induced NF-κB activity.

After we had established that the aged T-cell response is in part characterized by increased production of cytokines that induce pro-inflammatory pathways in prostate cells, the next objective of my study was to determine if the increased production of pro-inflammatory cytokines by aging T-lymphocytes contributes to a more malignant phenotype in the prostate.

Given the prominent role of IL-6 in age-related pathologies with a chronic inflammatory component, it was also important to further understand the role of circulating levels of IL-6 in age-related prostate tumorigenesis.

This study is one of the first to explore the relationship between aging T-lymphocytes and prostate tumorigenesis. The research presented in this dissertation is highly relevant because the potential identification of pathways and biomarkers could help develop effective treatments for prostate cancer.

Chapter 2: Age-related Alterations in T-lymphocytes Modulate Key Pathways in Prostate Tumorigenesis

2.1 Introduction

Age is the single greatest factor associated with increased risk for prostate cancer development [1]. Epidemiological studies suggest that after the fifth decade of life, men experience a progressively heightened risk of being diagnosed with prostate cancer with each subsequent year of life until the age of 85 [58]. While localized events within the tissue microenvironment over time may influence prostate carcinogenesis, the contribution that cytokines impart on tumor initiation and development has been underexplored. As the immune system is strongly implicated in modulating systemic inflammation, and because the repertoire and phenotypic functionality of immune-regulatory cells changes with age, immune dysfunction may actively promote prostate carcinogenesis. To date, the limitation in delineating the precise contribution of aged immune cells to prostate cancer initiation has been a lack of appropriate models that recapitulate normal aging [30].

Recently we have developed a novel mouse model of accelerated T-lymphocyte aging, the glycerol-3-phosphate acyltransferase-1 (GPAT-1) knock-out mouse, in which young T lymphocytes possess multiple hallmarks of aging [28, 29, 59]. The GPAT-1 knock-out mouse provides an unprecedented opportunity to investigate the effects of

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Jolly, Christopher:
deGraffenried Linda: Principal Co-Investigator, study conception and design, critical revision

an aging T-lymphocyte population on prostate cancer development in the absence of other age related complications. GPAT-1 is an integral mitochondrial outer membrane protein that initiates the first and rate limiting step in *de novo* phospholipid and triacylglycerol biosynthesis [30]. GPAT-1 is one of four known isoforms (two on the mitochondria and two on the endoplasmic reticulum) and is the only mitochondrial isoform expressed in T-lymphocytes. Only a minimal phenotype is found in other tissues, which express both GPAT-1 and GPAT-2 on the mitochondrial membrane [28]. We have shown that GPAT-1 catalytic activity is significantly down-regulated in aged rat-liver and T-lymphocytes, suggesting that impairment of the GPAT-1 mediated lipid biosynthetic pathway is a physiologically relevant event in the natural course of aging [29, 59]. Consistent with an aged phenotype, T-lymphocytes from the GPAT-1$^{-/-}$ mouse up-regulate the production and secretion of pro-inflammatory cytokines [30]. Many of the pro-inflammatory cytokines produced by aged T-lymphocytes have been implicated in the promotion of prostate tumorigenesis, primarily through the induction of other pro-inflammatory pathways and by driving the activation of transcription factors such as NF-κB. Analogous engagement of these pathways is recapitulated in GPAT-1$^{-/-}$ T-lymphocytes, strongly suggesting that the aged immune system plays a critical role in promoting prostate carcinogenesis.

NF-κB, a key mediator of gene transcription during the immune response, becomes deregulated during carcinogenesis. NF-κB-mediated gene transcription drives

the expression of key survival factors that suppress apoptosis, promote cellular proliferation, and incite the inflammatory response [60]. The current emphasis in cancer immunology is to elucidate the biological pathways by which immune cells enhance inflammation to promote tumor development and progression.

We demonstrated that secreted cytokines from GPAT-1$^{-/-}$ T-lymphocytes closely mimic the cytokine array profile observed in normal aged T-lymphocytes. Treatment of normal, non-transformed or prostate cancer cells with serum derived from the GPAT-1$^{-/-}$ mice induced NF-κB transcriptional activity and nuclear localization. Aging and aging-mimic GPAT-1$^{-/-}$ T-lymphocytes secrete factors which, when compared to young wild-type T-lymphocytes, differentially induce NF-κB activation and promote cellular proliferation in prostate epithelial cells. These data strongly suggest that the aging immune system is not a passive component to tumorigenesis, but may actively promote initiation and progression through induction of inflammatory pathways.

2.2 Materials and methods

2.2.1 Murine Models of an Aging Immune System

C57BL/6 GPAT-1 +/- mice were obtained from Dr. Rosalind Coleman (University of North Carolina at Chapel Hill) and bred in our animal facilities to obtain homozygous knock-outs. Mice were fed a commercial chow diet (Prolab Rat/Mouse/Hamster 2000) provided by the animal facility. Offspring were numbered to monitor sex differences or

differences between litters. Aged (+22 months) C57BL/6 mice were purchased from the National Institute for Aging. Mice were housed on a 12:12-h light-dark cycle and had free access to commercial chow food and water.

2.2.2 T-lymphocyte isolation and stimulation

Splenic T-lymphocytes were isolated from young (6 month) wild-type (WT), young (6 month) GPAT-1 KO, and old (22 month) WT mice using negative selection (Miltenyi magnetic microbeads and antibody T-lymphocyte specific antibody combinations) as per the manufacturer's instructions, yielding a 95% pure splenic T-lymphocyte population. Isolation by negative selection prevents perturbation of the T-lymphocyte's receptor during the isolation procedure, as occurs with isolation via positive selection-lymphocytes were stimulated at 37°C in pre-warmed complete RPMI 1640 culture media (10% heat-inactivated fetal bovine serum plus 100 U/ml penicillin, 100 μg/ml streptomycin, 10 μM 2-mercaptoethanol, and 100 mM L-glutamine) with either 10 μg/ml plate-bound anti-CD3 and 1 μg/ml anti-CD28 or no stimulation as we have previously described (4). Anti-CD3 and CD28 antibodies are routinely used as polyclonal mitogens to mimic the *in vivo* T-cell response. After 24 hours of stimulation, the T-lymphocyte culture supernatant (conditioned media) was collected and used in subsequent experiments. All animal procedures used were approved by the University of Texas Animal Use and Care Committee.

2.2.3 Cell Lines

The LNCaP and PC-3 cell lines were purchased from the American Type Culture Collection (Rockville, MD) and grown in RPMI-1640 containing penicillin and streptomycin, supplemented with 10% fetal bovine serum (FBS) in a 5% (v/v) CO_2 humidified incubator at 37°C. The immortalized non-transformed RWPE-1 prostate epithelial cell line was purchased from American Type Culture Collection and grown in Keratinocyte Serum Free Medium (K-SFM) supplemented with bovine pituitary extract (BPE) and human recombinant epidermal growth factor (EGF) in a 5% (v/v) CO_2 humidified incubator at 37°C. The PrEC-human normal prostate epithelial cell line was purchased from Lonza (Basel, Switzerland) and grown in PrEGM Prostate Epithelial Cell Growth Medium containing a growth factor and cytokine-supplemented SingleQuots kit.

2.2.4 Cytokine Array

Conditioned media from splenic T-lymphocytes from wild-type young (6 week), old (22 months) and young GPAT-1$^{-/-}$ (6 week) mice were obtained by stimulating isolated T-cells with either 10 µg/ml plate-bound anti-CD3 and 1 µg/ml anti-CD28 in NaHCO3 or 10 nM phorbol myristate acetate (PMA) and 1 µM ionomycin. The cytokine profiles were performed using R&D Systems Mouse Cytokine Array, Panel A (R&D Systems Minneapolis, MN, USA) according to the manufacture's recommendations.

2.2.5 Dual Luciferase assay

LNCaP and RWPE-1 cells seeded in 6-well plates were grown to 60–80% confluence for transfection. 1µg NF-kB luciferase reporter plasmid (Stratagene, La Jolla, CA) and 20ng of control Renilla reporter plasmid were concurrently transfected together using FuGENE 6 (Roche, Valencia, CA, USA) transfection reagent, according to manufacturer's protocol. At 24 hr post-transfection, 10% sera or T-lymphocyte conditioned media was added directly to the cells. At 48 hr post-transfection, samples were harvested, washed in cold 1x PBS and lysed with 500µl of passive lysis buffer (Promega, Madison, WI, USA). 25 µl of cell extract was used in triplicate on white 96 well plates to measure NF-kB luciferase activity according to the dual-luciferase reporter assay system protocol (Promega). The NF-kB firefly luciferase activity was standardized to Renilla luciferase activity and represented as standardized luciferase units (SLU).

2.2.6 Immunofluorescence

LNCaP and RWPE-1 cells were cultured directly on an 8-chamber glass Lab Tek™ II Chamber Slide System in a density of 1 x 10^4 cells per chamber. Cells were exposed to 10% sera, conditioned media, or serum free media. Control cells were cultured in complete (10% FBS) RPMI or serum free RPMI. After recovery, slides were fixed in 4% paraformaldehyde/PBS, blocked in aldehyde blocking solution (100 mM glycine/PBS) and permeabilized in a 0.1% Trition X-100/PBS solution at room temperature for 5 min.

To determine localization of NF-κB, cells were probed with rabbit anti-p65 (Santa Cruz; sc-372). FITC and/or Cy3-conjugated donkey anti-rabbit secondary antibodies (Jackson ImmunoResearch Laboratories) allowed fluorescent detection of primary antibodies on a Zeiss Axiovert 40 inverted microscope. Cells were counterstained, with 200 ng/ml DAPI, for detection of cellular nuclei. Images of control and experimental cells were acquired under identical exposure conditions in three independent experiments to ensure reproducibility [61] .

2.2.7 Western blot analysis

Western blot analysis was performed as previously described [61]. Briefly, prostate cells were serum-treated directly or with conditioned media from stimulated T-lymphocytes, harvested and lysed in Laemmli lysis buffer for SDS-polyacrylamide gel electrophoresis and probed with: rabbit anti-BCL-Xl, rabbit anti-MCL-1, rabbit anti-GAPDH (all Cell Signaling, Boston, MA, USA), rabbit anti-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-Cyclin D1 (Millipore, Billerica, MA). Luminescent signal was detected on a Syngene imaging system and quantitative densitometric analysis measured using GeneTools.

2.2.8 Statistics

Values are presented as mean ± standard error of the mean (SEM). For the Western blot analyses and luciferase assays, means were compared across treatment groups using Student's t-test and $p \leq 0.05$ was considered statistically significant.

2.3 Results

2.3.1 Aging mimic GPAT-1 -/- T-lymphocytes have a pro-inflammatory cytokine profile

Previously we have demonstrated that GPAT-1 regulates proliferation and cytokine production in T-lymphocytes [30]. Without GPAT-1 T_H1 (IL-2 and IFN-gamma) cytokine secretion is reduced and T_H2 (IL-4 and IL-10) cytokine secretion is increased [30]. To further characterize and compare the relative cytokine expression profile from T-lymphocytes of young GPAT-1$^{-/-}$ (KO) mice to those derived from young and old (>22 month) wild-type (WT) mice, we utilized a cytokine immunoarray which is capable of simultaneously detecting different cytokines and chemokines. Consistent with our previous observation, GPAT-1$^{-/-}$ T-lymphocytes resembled the inflammatory phenotype that is characteristic of aged murine T-lymphocytes (**Fig 2.1**). While many of the cytokines and chemokines were at similar levels among the three groups (IL-1α and IL-1β, for example), the knock-out and old mice trended together, such as with MIP-1α and β, RANTES, IL-3 and I-309. Of note, compared to that found in the WT young mice, levels of IL-17 were 12-and 8-fold higher in the GPAT-1$^{-/-}$ (KO) and old mice, respectively.

Intriguingly, while IL-6 levels were 12-fold higher in the old mice, the levels were comparable between the WT and KO mice, suggesting that any observed phenotype with the KO is independent from IL-6 activity.

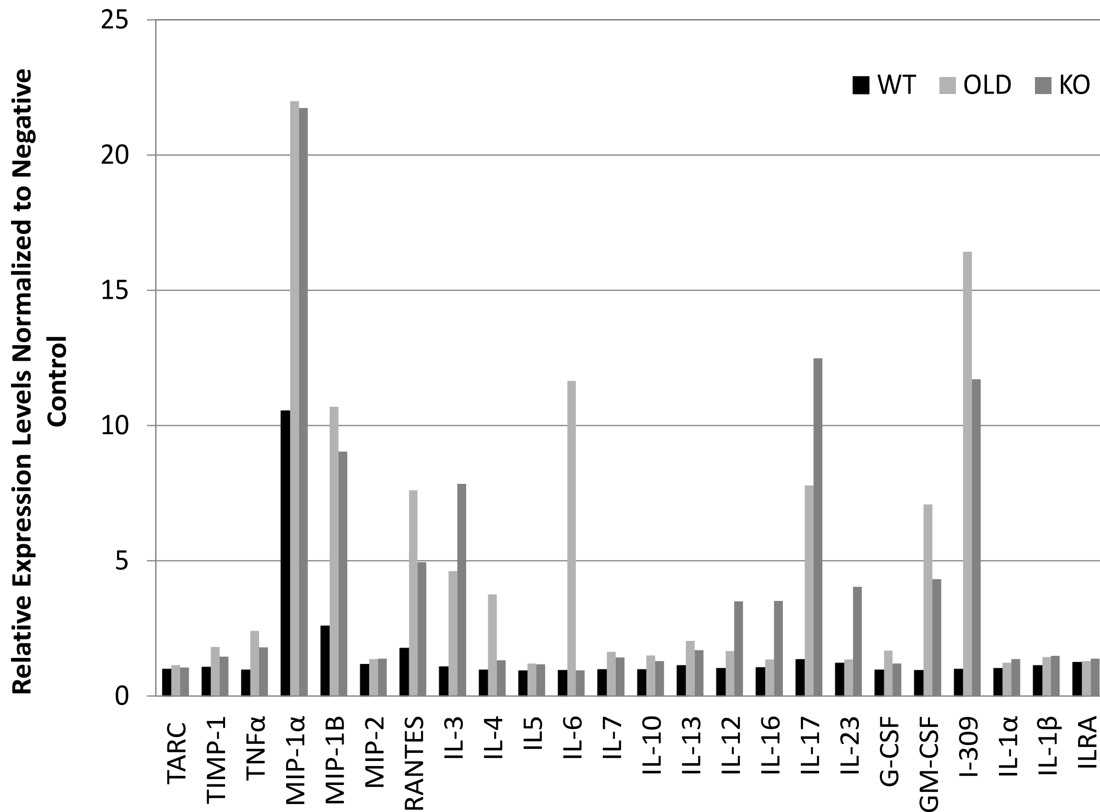


Figure 2.1 Aging mimic GPAT-1 -/- T-lymphocytes have a pro-inflammatory cytokine profile.

Secreted cytokine immunoarray profile of isolated and anti-CD3/CD-28 stimulated splenic T-lymphocytes derived from wild type (WT, black bars), old (light gray bars) and GPAT-1$^{-/-}$ (KO, dark gray bars) mice**.** After stimulation, T-lymphocytes were cultured in serum free media for 24 hr. Factors secreted into the serum free media (conditioned media) were incubated on the immunoarray to detect relative expression of indicated cytokines and chemokines. Levels were standardized relative to negative control.

2.3.2 GPAT-1$^{-/-}$ and old wild type T-lymphocytes secrete factors that induce NF-κB activity

NF-κB is a transcription factor that modulates the expression of many genes associated with inflammatory processes, cell adhesion, differentiation, proliferation, angiogenesis and apoptosis. Dysregulation of NF-κB has been implicated as a leading cause in the development of many diseases, including cancer [62-65]. In the prostate, upregulation of NF-κB is associated with increased inflammation, prostate carcinogenesis, progression to hormone independence and increased metastatic potential [66-68]. Therefore, to determine if circulating factors in the sera from aged or GPAT-1 $^{-/-}$ mice induce NF-κB activity in non-transformed prostate epithelial and prostate cancer cells, we exposed these cell lines to 10% sera from WT young, old or young GPAT-1$^{-/-}$ (KO) mice. An NF-κB luciferase reporter assay was used to measure NF-κB transcription activity (**Fig 2.2**). Exposure to either sera from old mice or GPAT-1 KO sera resulted in a significant increase in NF-κB activity in the PC-3 (50% and 100%, respectively) and LNCaP (75% and 400%, respectively) prostate cancer cell lines compared to sera from the young WT mice ($P<0.05$) (**Fig 2.2A**), suggesting that circulating factors in the sera of GPAT-1$^{-/-}$ mice mimic those in the aged mice in promoting NF-κB transcriptional activity. The same trend between the WT and KO mice was observed in the primary and non-transformed prostate cell lines PReC and RWPE-1 respectively (**Fig 2.2B**). These data indicate that circulating factors associated with an

aging T-cell induce activation of NF-κB, a critical step in prostate cancer initiation, not only in cancer cells but also in non-cancer cells.

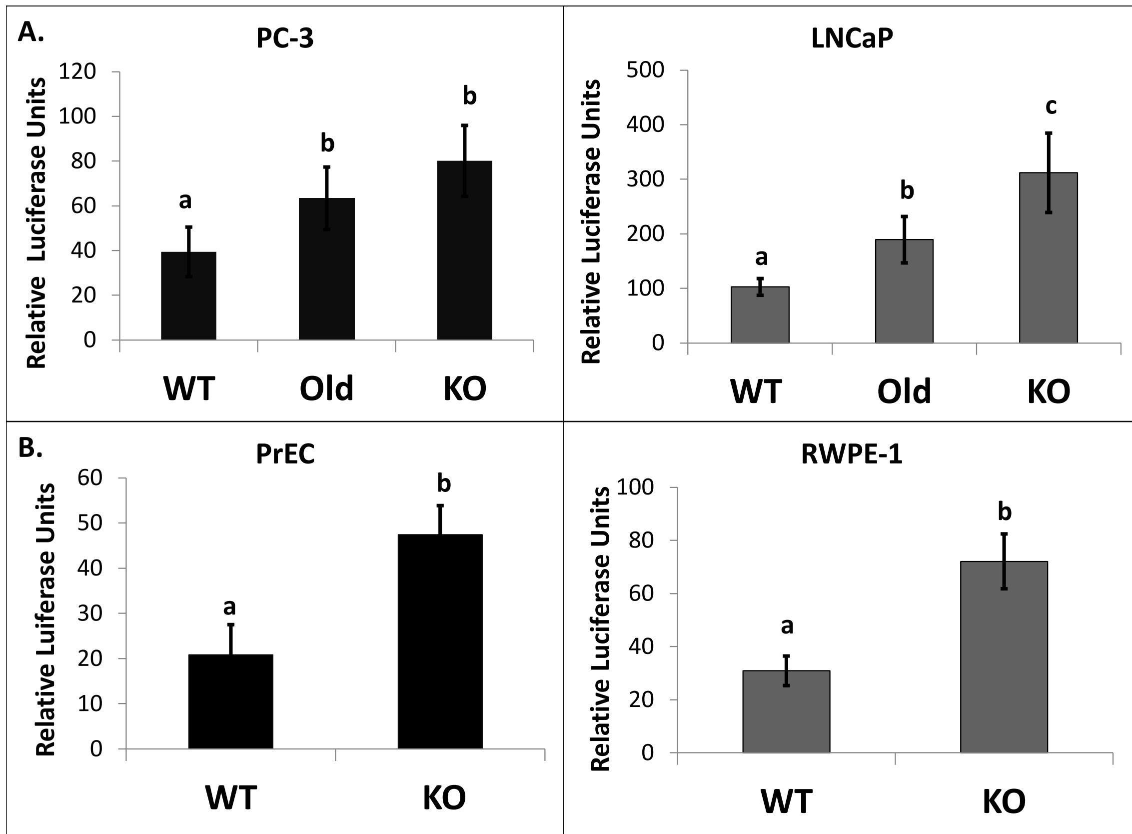


Figure 2.2 Effect of circulating factors in the sera from the aged wild type and aging-mimic young GPAT-1 -/- mice on NF-κB transcriptional activity.

Dual-Luciferase assay was used to measure NF-kB transcriptional activity in response to 10% FBS or sera from young wild-type, old or GPAT-1 $^{-/-}$ mice in A) PC3 and LNCaP prostate cancer cells or B) non-cancerous PrEC and RWPE-1 prostate epithelial cells. Presented is the combined average of three independent experiments and bars representing the standard error of the mean. Means with different letters are significantly different ($p < 0.05$).

2.3.3 Circulating factors in the sera from mice with an aged immune system induce nuclear translocation of NF-κB

NF-κB is a heterodimeric complex consisting of the p50 and p65 subunits. When bound by I-κB, NF-κB is excluded from the nucleus and remains in the inactive state. Engagement of membrane based receptors initiates activation of the I-κB kinase (IKK) complex which phosphorylates I-kB at two key serine residues, targeting it for proteasomal degradation through the ubiquitin ligase pathway. The liberated NF-κB complex is then free to translocate into the nucleus, and bind to NF-κB response elements, thereby modulating gene expression [69]. To determine the effect of sera from GPAT-1$^{-/-}$ (KO), young WT or old mice on the subcellular localization of NF-κB, immunofluorescence was used to visualize changes in p65 subcellular compartmentalization. As seen in **Figure 2.3**, sera from the young GPAT-1$^{-/-}$ mice (**iii and iv**) induced translocation of p65 into the nucleus in both the LNCaP cancer cell line (**Fig 2.3A**) and non-cancerous RWPE-1 cell line (**Fig 2.3B**), while the sera from WT young mice (**i and ii**) primarily showed cytosolic (inactive p65) staining. Importantly, results with the old WT mice (**v and vi**) in the LNCaP and RWPE-1 cells correlate with those obtained with the young GPAT-1$^{-/-}$ mice, strongly supporting the results obtained with the luciferase reporter studies.

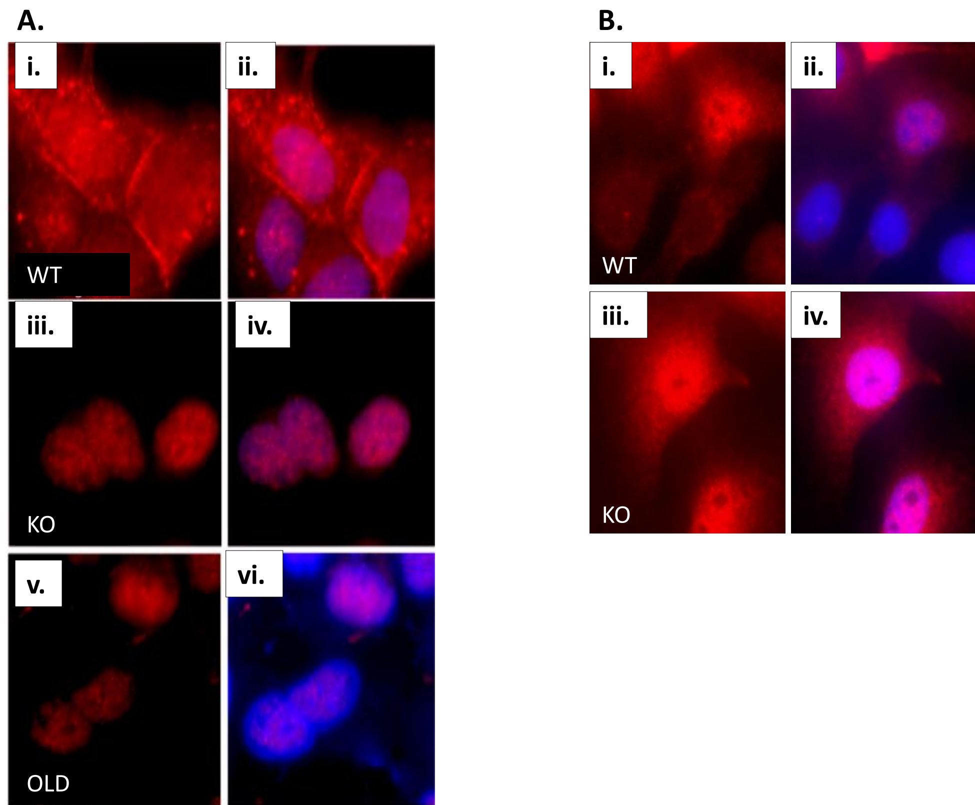


Figure 2.3 Alteration of NF-kB subcellular localization induced by circulating factors in the sera from aged and young GPAT-1-/- mice.

Immunofluorescence was used to visualize NF-κB localization (red) A) in LNCaP cells or B) non-cancerous RWPE-1 prostate epithelial cells exposed to sera from WT young (i), GPAT $^{-/-}$ (KO) (iii), old WT (V) mice. (ii), (iv) and (vi) are the images merged with the DAPI stain (blue), indicating cellular nuclei. Presented is a representative of three independent experiments.

2.3.4 Factors secreted from aging T-lymphocytes induce NF-kB transcriptional activity

Based on our observation that circulating factors from the sera of GPAT-1$^{-/-}$ mice induced NF-κB activation similar to that of old mice, we sought to determine whether factors originating from the T-lymphocytes alone were impacting NF-κB activity. Splenic T-lymphocytes were isolated from the respective mice then stimulated with or without anti-CD3/CD28 antibodies. After 24 hours stimulation, conditioned media from the T-lymphocytes was collected. The conditioned media from the stimulated and unstimulated T-lymphocytes was then used to culture primary non-transformed (RWPE-1) and prostate cancer (LNCaP) cell lines and NF-κB activity measured as in Figure 2 (**Fig 2.4**). Factors secreted into the conditioned media from GPAT-1$^{-/-}$ and old wild type T-lymphocytes induced a 3-fold and 2-fold induction of NF-κB, respectively, in the LNCaP cell line compared to those in the conditioned media from a young WT mice ($p < 0.05$) (**Fig 2.4A**) and more than 7-fold higher in the RWPE-1 cells exposed to conditioned media from GPAT-1$^{-/-}$ when compared to those exposed to conditioned from wild type young mice ($p< 0.05$) (**Fig 2.4B**). Notably, results with the old WT mice in the in RWPE-1 cells (**Fig 2.4B**) correlate with those obtained with the young GPAT-1$^{-/-}$ mice. This suggests that the aged and GPAT-1$^{-/-}$ T-lymphocytes directly modulate NF-κB activity in prostate epithelial and prostate cancer cells.

Additionally, fluorescence microscopy was performed to visualize p65 subcellular localization in response to exposure to the conditioned media. As seen in **Figure 2.5A**,

LNCaP cells display evidence of nuclear translocation of NF-κB in response to conditioned media from stimulated T-lymphocytes from young GPAT-1$^{-/-}$ (**Fig 2.5v**) and old wild-type mice (**Fig 2.5Avi**). Similar results were obtained with the non-cancerous RWPE-1 cells (**Fig 2.5B**). Nuclear translocation of NF-κB correlates with the strong induction of NF-κB transcriptional activity that was observed when LNCaP prostate cancer cells were exposed to conditioned media from stimulated aged wild type T-lymphocytes.

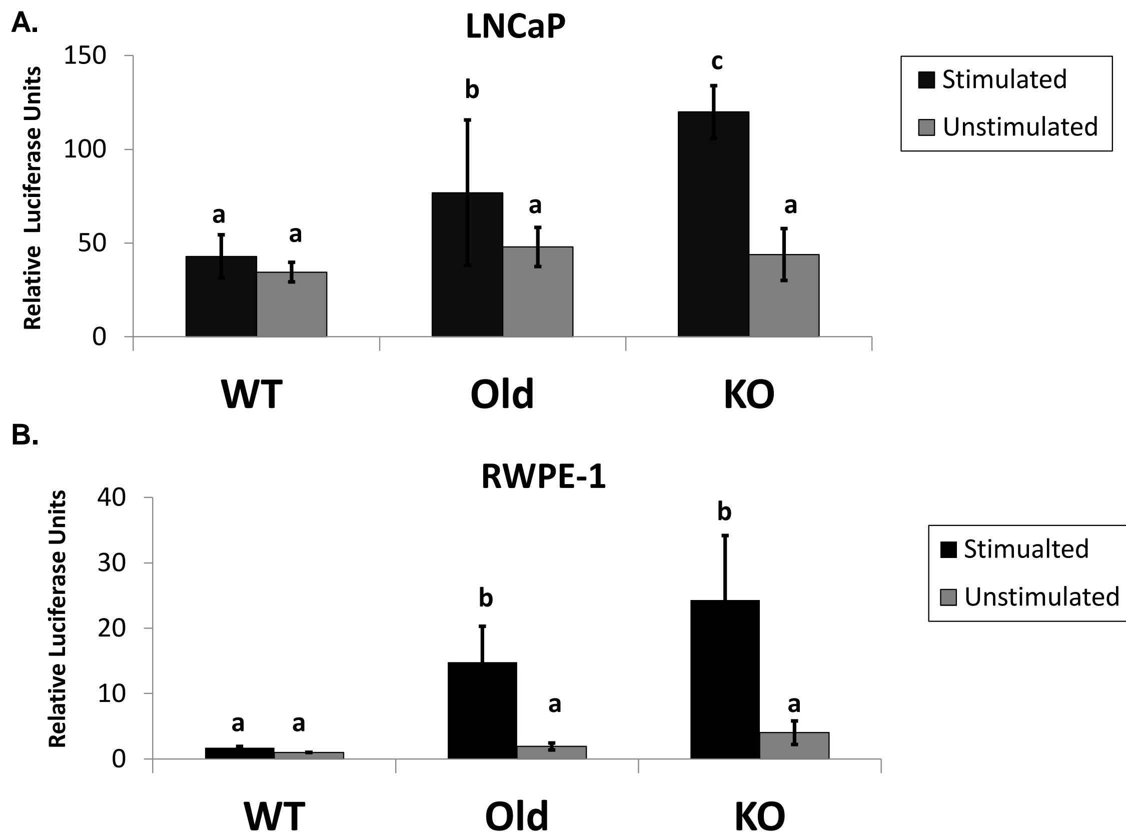
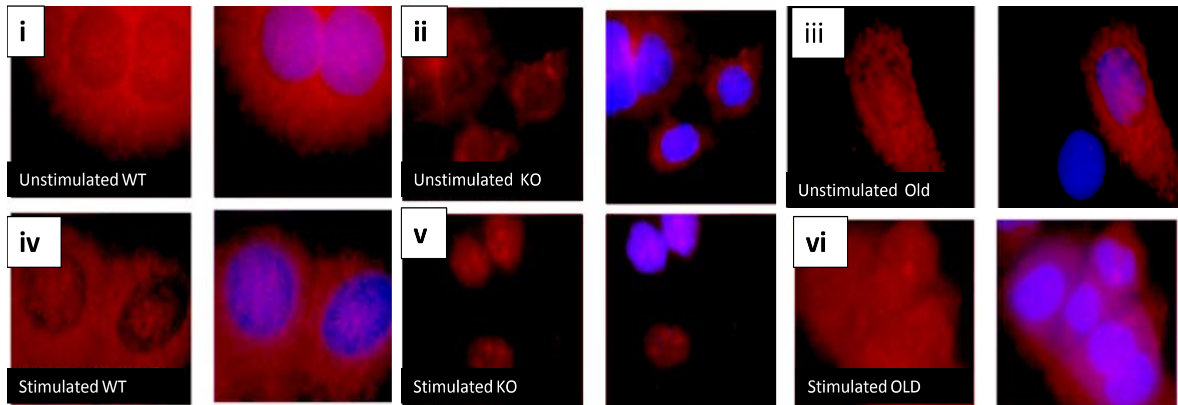


Figure 2.4 Effect of isolated splenic T-lymphocyte-secreted factors from WT or young GPAT-1 -/- mice on NF-κB transcriptional activity

LNCaP prostate cells (A) and RWPE-1 non-transformed prostate epithelial cells (B)were used. Dual-Luciferase assay was used to measure NF-kB transcriptional activity in response to conditioned media (CM) generated from anti-CD3 plus CD-28 stimulated cultured splenic T-lymphocytes from either young GPAT- $^{-/-}$ (KO), young wild-type (WT) or old wild type mice. Means with different letters are significantly different ($p < 0.05$).

A.



B.

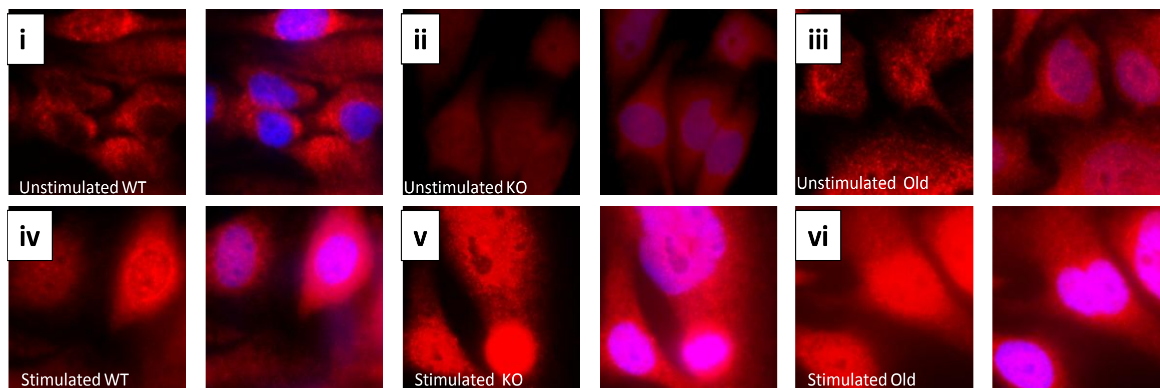


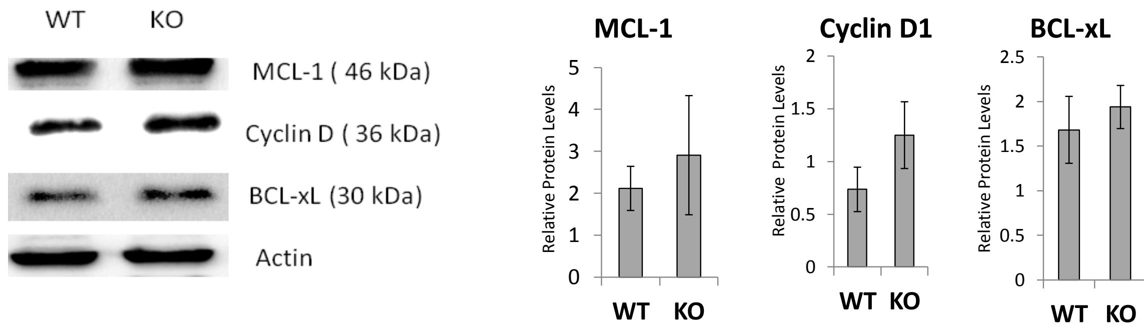
Figure 2.5 Nuclear localization of NF-kB induced by factors secreted from stimulated aged T-lymphocytes.

Immunofluorescence was used to visualize NF-kB localization (red) A) in LNCaP cell or B) non-cancerous RWPE-1 prostate epithelial cells in response to exposure to conditioned media generated from unstimulated (upper panel) and stimulated (lowerpanel) Tlymphocytes isolated from wild-type (WT Young) (i) and (iv), GPAT -/- (KO) (ii) and (v) and wild-type old (Old) (iii) AND (vi) mice. DAPI staining (blue) indicates cellular nuclei

2.3.5 Factors secreted by aging T-lymphocytes upregulate the expression of key downstream targets of NF-κB

NF-kB regulates the transcription of several genes involved in the suppression of cell death, proliferation and tumor promotion [38]. BCL-xL is one of the key downstream gene targets of NF-kB that promotes survival [70]. MCL-1, an important target gene of NF-kB, is an anti-apoptotic factor implicated in the survival of prostate cancer cells [70]. In addition to the regulatory role it plays in apoptosis, NF-κB is also capable of promoting cell cycle progression by modulating the expression of cell cycle specific genes, including cyclin D1[71]. Recent studies suggest that NF-κB-induced cyclin D1 expression is a key contributing element in mammary breast carcinogenesis [71-73] . Immunoblot analysis (**Fig 2.6**) shows that expression levels of key NF-κB genes (MCL-1, Cyclin D1, BCL-xL) are upregulated in LNCaP cells (30%, 40% and 10%) and RWPE-1 cells (15%, 35% and 60%, respectively) exposed to KO sera compared to levels observed with exposure to WT sera after treatment for 48 hours with 10% sera or conditioned media from the young GPAT-1$^{-/-}$ mice. Based on our observation that circulating factors from the sera of GPAT-1$^{-/-}$ mice induced activation of important targets of NF-κB, we sought to determine whether factors specifically originating from the T-lymphocytes impact regulation of key downstream targets of NF-κB. Splenic T-lymphocytes were isolated from the respective mice then stimulated with or without anti-CD3/CD28 antibodies. After 24 hours stimulation, conditioned media from the T-lymphocytes was collected.

The conditioned media from the stimulated and unstimulated T-lymphocytes was then used to culture primary non-transformed (RWPE-1) and prostate cancer (LNCaP) cell lines and western blot analysis was used to measure expression levels of key NF-κB genes. Factors secreted into the conditioned media from GPAT-1$^{-/-}$ mice up-regulate the expression of MCL-1, Cyclin D1 and BCL-xL in the LNCaP cells by 20%, 60% and 70% and by 80%, 66% and 50%, respectively in RWPE-1 cells by compared to those in the conditioned media from a young WT mice. Importantly, factors secreted into conditioned media from old WT T-lymphocytes also upregulated key NF-κB genes in similar ways that the conditioned media from the young GPAT-1$^{-/-}$ mice does (**Fig 2.7**).



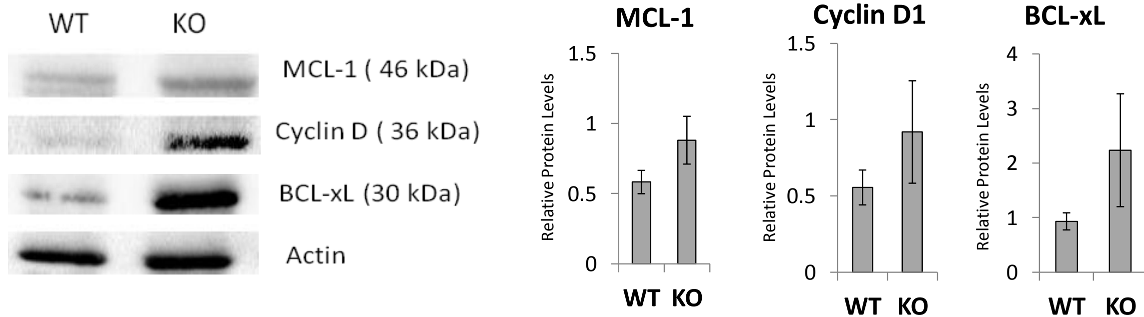


Figure 2.6 Increased expression of gene targets of NF-κB in response to circulating factors secreted from aged-mimic T-lymphocytes.

Immunoblot detection of MCL-1, Cyclin D1 and BCL-1-xL expression levels in LNCaP (A) and RWPE-1 (B) cells after exposure to 10% sera from young wild-type (WT) or GPAT-1$^{-/-}$ (KO) mice. Actin was used as a loading control. Graphs indicate the combined average relative densitometry values for three independent experiments, with bars indicating the SEM.

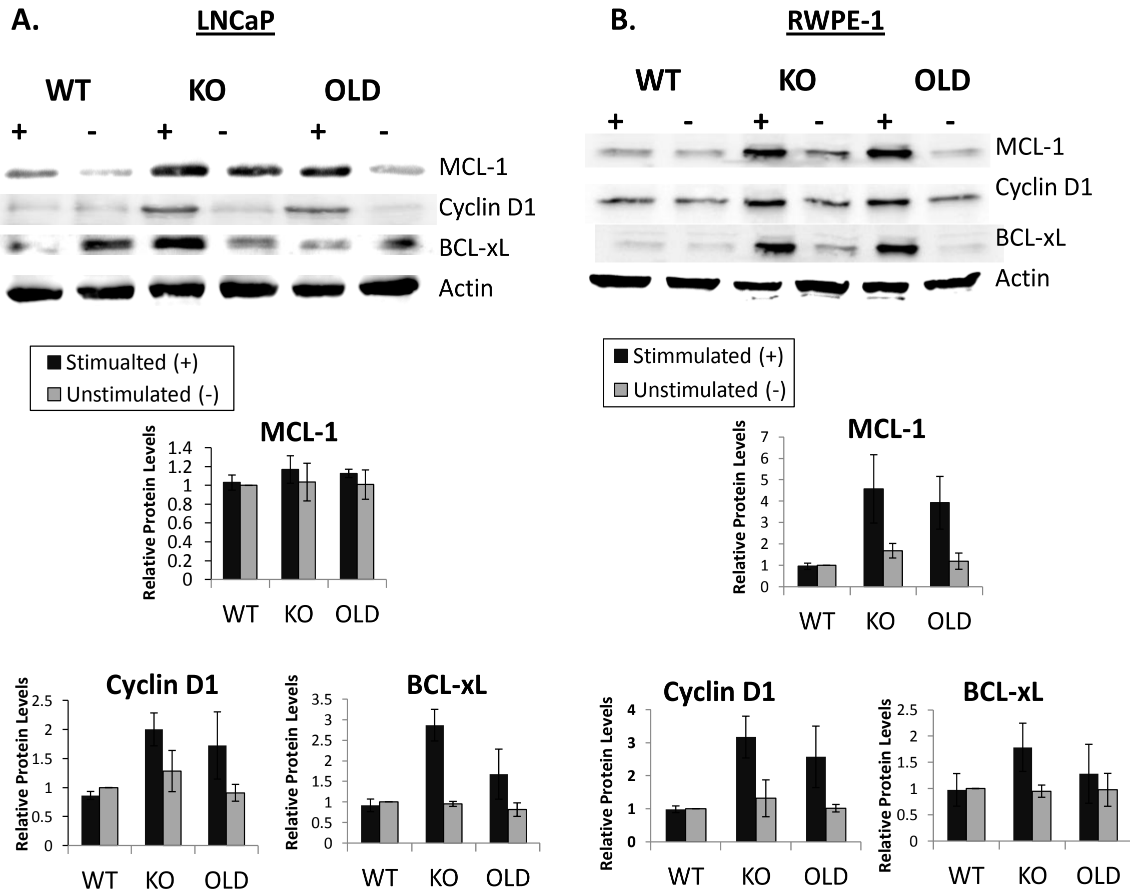


Figure 2.7 Increased expression of gene targets of NF-κB in response to circulating factors secreted from aged-mimic T-lymphocytes.

Immunoblot detection of MCL-1, Cyclin D1 and BCL-xL expression levels in LNCaP (A) and RWPE-1 (B) cells after exposure to 10% conditioned media from stimulated wild-type (WT) T-lymphocytes (+), unstimulated wild-type (WT) T-lymphocytes (-) or stimulated (+) and unstimulated (-) GPAT$^{-/-}$ (KO) T-cells. Actin was used as a loading control. Graphs indicate the combined average relative densitometry values for three independent experiments, with bars indicating the SEM.

2.4 Discussion

The most established risk factors for prostate cancer development include age, race/ethnicity, and family history [58]. Longitudinal studies of aging men have revealed incidence curves indicating that prostate cancer risk begins to rise sharply after age 55 and peaks at age 70–74 [1]. Postmortem and histopathologic analysis of human prostate specimens provides further evidence of the link between aging and prostate cancer incidence [1, 74]. One widely accepted explanation for this association is that the slow growth characteristics observed within the prostatic tissue prolongs the time for acquired mutations to manifest [75] . To date, many studies have focused on distinguishing aggressive from indolent disease, or identifying prognostic biomarkers predictive of clinical outcome and survival; however, few studies have investigated the potential contribution that the normal aged immune system may have in the development of prostatic disease. While it is well established that age-related changes in immune surveillance and response occur, including loss of appropriate T-lymphocyte function and immunosenescence, this dysregulation has not yet been correlated with development of prostate cancer. In this investigation, we provide the first evidence that age-related T-lymphocyte dysfunction may promote prostate cancer cell survival through induction of NF-κB.

Pro-inflammatory cytokines can initiate and potentiate the activation of NF-κB, a nuclear transcription factor frequently dysregulated in cancer [9, 76]. NF-κB activity in turn up-regulates expression of pro-survival factors and cytokines such as survivin and interleukins 2, 6, 8 and 9 [77]. Building upon our previous studies demonstrating a critical role for NF-κB in prostate cancer cell survival [61], we sought to determine if one mechanism by which an aging immune system might contribute to prostate cancer initiation and progression is through induction of NF-κB. Intriguingly, circulating factors in the old WT and young GPAT-1$^{-/-}$ mouse sera both induced NF-κB transcriptional activity in the PC-3 and LNCaP prostate cancer cell lines, while the young WT mouse sera displayed no significant effect, suggesting that the age-specific shift in T lymphocyte function may be partly responsible for the increased NF-κB activity. Further, we observed a greater than 3-fold induction of NF-κB transcriptional activity in primary and immortalized, non-transformed (PrEC and RWPE-1) cells respectively, indicating that induction of NF-κB activity by elevated cytokines is not restricted to cancer cells. This is especially important since age is a risk factor for the development of prostate cancer, which occurs through transformation of normal epithelial cells.

The GPAT-1$^{-/-}$ model appears to strongly mimic the phenotypic changes observed in the aging immune system, particularly as it relates to T-cell function. T-lymphocytes from young GPAT-1$^{-/-}$ demonstrate reduced IL-2 production and subsequent proliferation, altered cytokine production, increased membrane cholesterol to

phospholipid ratio and increased activation-induced apoptosis. Additionally, our data indicate that T-lymphocytes from GPAT-1$^{-/-}$ mice also closely mimic aged T cells in secreted levels of key cytokines and chemokines known to promote tumorigenesis such as IL-17, RANTES (CCL5) and I-309 [42, 78, 79]. Intriguingly, while secreted IL-6 levels were elevated in the T-cells from the old mice, T-cells from the GPAT-1 $^{-/-}$ mice did not demonstrate significantly higher levels compared to the aged-matched wild type mice, suggesting that the observed induction of NF-κB activity was independent of IL-6 activity, possibly through IL-17.

These data have important implications for our current understanding of the contribution of the immune system to prostate cancer development. Rather than being merely a passive component to disease development, our data strongly support the concept that the aging immune system actively promotes cancer onset and progression, possibly through induction of chronic inflammation in the microenvironment resulting in upregulation of pathways leading to neoplastic changes, as has been proposed by Sfanos and De Marzo [80]. This change in perspective regarding the role of the aging immune system in cancer development opens new avenues for development of potential preventive interventions and screening biomarkers, and the results of our study suggest a focus on T cell-induced pathways.

Chapter 3: Age-related Increase in IL-17 Activates Pro-inflammatory Signaling in Prostate Cells

3.1 Introduction

Prostate cancer is mainly a disease of older men [81]. This is due to several factors, including an increased duration of carcinogenesis, accumulation of DNA damage and an increased susceptibility of aging cells to environmental carcinogens [2]. Another mechanism important to the link between aging and prostate cancer is reduced immune function in the elderly. Qualitative and quantitative changes in immune response, including profound changes in T cell function, are part of the aging process. Age-related alterations to T cell immunity include decreased T cell differentiation and increased pro-inflammatory cytokine secretion [82]. Age is positively correlated with increased circulating levels of many pro-inflammatory cytokines such as interleukin-1 beta (IL-1β), tumor necrosis factors alpha (TNFα), interleukin-6 (IL-6) and interleukin-17 (IL-17) [83] .

The inflammatory process is highly implicated in the pathogenesis of many common and severe age-related diseases, including prostate cancer [84]. Numerous studies have shown considerable evidence for inflammatory conditions being involved in the initiation and progression of prostate cancer [80, 85-87]. Furthermore, a large number of reports have specifically linked IL-17 to prostate cancer, with IL-17A expression increased in more than 50% of prostate cancers [13, 45, 88]. It has also been

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***The data from this chapter is published.**
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<u>**Author Contributions**</u>:
De Angulo, Alejandra: Study conception and design, acquisition of data, analysis and interpretation of data, drafting of manuscript.
Faris, Robert: Acquisition of data
Daniel, Benjamin: Study conception, critical revision
Jolly, Christopher: Principal Investigator, study conception and design, critical revision
deGraffenried Linda: Principal Investigator, study conception and design, critical revision

demonstrated that two IL-17 receptors (IL-17RA and IL-17RC) are expressed in prostate cancer [88], and T helper 17 (T_H17) cell number is increased in prostate cancer patients [45]. Recently, Zhang et al. reported that IL-17 promotes the formation and growth of prostate adenocarcinoma in a mouse model of autochthonous prostate cancer [42]. Taken together, the present literature suggests a strong link between IL-17 activity and prostate cancer development. However, little is known regarding the role of aged related increase in circulating IL-17 and regulation of pro-tumorigenic pathways in prostate epithelial cells.

We showed that, for the most part, the T cell cytokine profile of the aging-mimic T cell GPAT$^{-/-}$ (AM) mice mirrored that observed in aged wild-type (OLD) mice, including higher expression levels of IL-17. In order to dissect the relationship between aging T cells, IL-17 and pro-tumorigenic signaling in prostate cells, we used the young (6 month old) glycerol-3-phosphate acyltransferase-1 knock-out KO mouse, which T cells closely mimics the immune system of an aged (>22 month old) mouse. Serum and splenic T-lymphocytes from young (6 month old) wild-type (WT), AM and wild-type old (OLD) mice were collected to test the contribution of IL-17 in modulating key signaling pathways in prostate tumorigenesis.

3.2 Materials and Methods

3.2.1 Murine Models of an Aging Immune System

C57BL/6 GPAT-1 +/- mice were obtained from Dr. Rosalind Coleman (University of North Carolina at Chapel Hill) and bred in our animal facilities to obtain homozygous knock-outs. Mice were fed a commercial chow diet (Prolab Rat/Mouse/Hamster 2000) provided by the animal facility. Offspring were numbered to monitor sex differences or differences between litters. Aged (>22 month old) C57BL/6 mice were purchased from the National Institute for Aging. Mice were housed on a 12:12-h light-dark cycle and had ad libitum access to food and water.

3.2.2 T-lymphocyte isolation and stimulation

Splenic T-lymphocytes were isolated from 6 month old WT and KO, and 22 month old (OLD) wild-type mice using negative selection (Miltenyi magnetic microbeads and antibody T-lymphocyte specific antibody combinations) as per the manufacturer's instructions, yielding a 95% pure splenic T-lymphocyte population. Isolation by negative selection prevents perturbation of the T-lymphocyte's receptor during the isolation procedure, as occurs with isolation via positive selection. Lymphocytes were stimulated at 37°C in pre-warmed complete RPMI-1640 culture media (10% heat-inactivated fetal bovine serum (FBS) plus 100 U/ml penicillin, 100 µg/ml streptomycin, 10 µM 2-mercaptoethanol, and 100 mM L-glutamine) with either 10 µg/ml plate-bound anti-CD3

and 1 μg/ml anti-CD28 or no stimulation. Anti-CD3 and CD28 antibodies are routinely used as polyclonal mitogens to mimic the *in vivo* T cell response. After 24 hours of stimulation, the T lymphocyte culture supernatant (conditioned media) was collected and used in subsequent experiments. All animal procedures were approved by the University of Texas Institutional Animal Care and Use Committee.

3.2.3 Cells and Cell Culture

The LNCaP prostate cancer cell line was purchased from ATCC (Rockville, MD) and grown in RPMI-1640 containing 1% penicillin and streptomycin, supplemented with 10% FBS in a 5% (v/v) CO_2 humidified incubator at 37°C. The immortalized non-transformed RWPE-1 prostate epithelial cell line was purchased from ATCC and grown in Keratinocyte Serum Free Medium (K-SFM) supplemented with bovine pituitary extract (BPE) and human recombinant epidermal growth factor (EGF) in a 5% (v/v) CO_2 humidified incubator at 37°C. The IL-17R shRNA clones (designated shIL-17R1 and shIL-17R2) were generated by transfecting cells with the IL-17R shRNA Plasmid (h) sc-40037 (Santa Cruz Biotechnologies, Santa Cruz, CA), using FuGENE HD transfection reagent (Roche, Basel, Switzerland) per manufacturer's instructions. Control LNCaP and RWPE-1 cells (designated shControl) were generated by stably transfecting negative-control shRNA plasmids: Control Plasmid-B sc-108065 and Control Plasmid-C sc-108066 (Santa Cruz Biotechnologies, Santa Cruz, CA). Puromycin antibiotic (Santa Cruz

Biotechnologies, Santa Cruz, CA) was used to stably select transfected cells. All cells were maintained in selective RPMI-1640 or K-SFM media containing 4 µg/ml puromycin. RT-PCR was performed to monitor IL-17R gene expression knock-down using the IL-17R (h)-PR primer (Santa Cruz Biotechnologies, Santa Cruz, CA). In addition western blot analysis was used to confirm knock-down of the IL-17R gene. Antibodies against IL-17R antibody (Santa Cruz Biotechnologies, Santa Cruz, CA) and phospho- and total-STAT3 (Cell Signaling) were used.

3.2.4 Dual Luciferase assay

LNCaP and RWPE-1 shIL-17R1, shIL-17R2, and shControl cells were seeded in 6-well plates and grown to 60–80% confluence for transfection. 1µg NF-κB luciferase reporter plasmid (Stratagene, La Jolla, CA) and 20ng of control Renilla reporter plasmid were concurrently transfected using FuGENE HD (Roche, Basel, Switzerland) transfection reagent, according to manufacturer's protocol. 24 hr post-transfection, 5% sera or T-lymphocyte conditioned media was added directly to the cells. 48 hr post-transfection, samples were harvested, washed in cold 1x PBS and lysed with 500µl of passive lysis buffer (Promega, Madison, WI,). NF-κB luciferase activity was measured according to the dual-luciferase reporter assay system protocol (Promega, Madison, WI). The NF-κB luciferase activity was standardized to Renilla luciferase activity.

3.3.5 Western blot analysis

Prostate shIL-17R and shControl cells were exposed for 48 hours to sera or conditioned media from stimulated T-lymphocytes, then harvested and lysed in Laemmli lysis buffer for SDS-polyacrylamide gel electrophoresis. The lysates were probed with the following antibodies: VCAM, FAS, cAIP2 (all Cell Signaling, Boston, MA), cyclin D1 (Millipore, Billerica, MA), and actin (Santa Cruz Biotechnology, Santa Cruz, CA). Luminescent signal was detected on a Syngene (Frederick, MD) imaging system and quantitative densitometric analysis measured using GeneTools.

3.2.6 RT-qPCR

Prostate Cancer LNCaP cells were exposed to sera for 48 hours. Total RNA was isolated with the QIAGEN (Valencia, CA) RNA extraction system according to the manufacturer's instructions and transcribed into complementary DNA (cDNA). Gene expression of 84 genes from the QIAGEN NF-κB Signaling Targets RT^2 Profiler PCR Array (Valencia, CA) was quantified by QIAGEN SYBR green real-time PCR on an Eppendorf instrument (Santa Clara, CA). Nonspecific signals caused by primer dimers were excluded by dissociation curve analysis and use of non-template controls. To normalize for loaded cDNA, actin was used as an endogenous control.

3.2.7 MTT Cell Proliferation Assay

Cells were seeded in at a density of 8×10^3 in 96-well plates. After 24 hours of growth in 10% FBS media, the cells were exposed experimental conditions for 96 hours. MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide MTT reagent in PBS (5 mg/ml) was then added to each well to a final concentration of 0.5 mg/ml. After three hours of incubation in a CO_2 humidified incubator at 37°C, the MTT containing media was removed and 200 ul dimethyl sulfoxide (DMSO) was added. Absorbance was read at 570 nm on a FLUOstar Omega Spectrometer (BMG Labtech, Offenberg, Germany). Relative cell proliferation was calculated by dividing each absorbance value by the absorbance for shControl cells grown in young WT sera or CM.

3.2.8 Statistics

Values are presented as mean ± standard error of the mean (SEM). For the Western blot analyses, MTT assays and Luciferase assays, means were compared across treatment groups using Student's t-test and one-way ANOVA Multiple Comparison was used for comparing more than two conditions. $P \leq 0.05$ was considered statistically significant

3.3 Results

3.3.1 IL-17 in the sera from Aging Mimic mice induces NF-κB activity in prostate epithelial cells

NF-κB is a transcription factor that modulates the expression of many genes associated with inflammatory processes, cell adhesion, differentiation, proliferation, angiogenesis and apoptosis. In the prostate, upregulation of NF-κB is associated with increased inflammation, prostate carcinogenesis and increased metastatic potential [63]. We determined that circulating factors in the sera from OLD or AM mice induce NF-κB activity in non-transformed RWPE-1 prostate epithelial and LNCaP prostate cancer cells (**Fig.2.2)**. To confirm that IL-17 in the sera from AM mice was in part responsible for the observed activation of NF-κB in prostate epithelial cells, shRNA was used to knock down the IL-17 receptor (IL-17R) in RWPE-1 and LNCaP cells and make stable IL-17R knock-down clones (shIL-17R1 and shIL-17R2). Clones of RWPE-1 and LNCaP cells transfected with a control shRNA (shControl) were also generated to serve as the negative control for comparison. IL-17R knock-down was confirmed by western blot analysis of IL-17R protein expression levels **(Fig 3.1A**), and qPCR determination of IL-17R mRNA levels (**Fig 3.1B**).

To further characterize our IL-17R shRNA clones, we assessed whether IL-17R knock-down in human prostate cells inhibits the activation STAT3, its downstream target. In the absence of IL-17R, IL-17-induced STAT3 activity was diminished by 3- and

6-fold in RWPE-1 cells and LNCaP cells respectively. However, addition of interleukin-6 (IL-6), which has been shown to activate STAT3, did not diminish STAT3 activity in the IL-17R shRNA clones, demonstrating the specificity of the knock-down **(Fig 3.2)**.

Next, the IL-17R shRNA clones were exposed to sera from WT, AM or OLD mice and an NF-κB luciferase reporter assay was used to measure NF-κB transcriptional activity (**Fig 3.3**). In chapter 2, I established that exposure to sera from KO versus young WT mice results in a significant increase in NF-κB activity in LNCaP and RWPE-1 cells **(Fig 2.1)**. Importantly, in the absence of IL-17R (shIL-17R1 and shIL-17R2), NF-κB activity was significantly diminished by 47% in LNCaP cells exposed to sera from AM mice (**Fig 3.3A**). In the absence of IL-17R (shIL-17R1 and shIL-17R2) OLD sera was still able to promote NF-κB activity. Sera form OLD mice have additional cytokines that could be promoting NF-κB activity in the absence of IL-17. A similar trend was observed in the RWPE-1 cell shIL-17R clones, where NF-κB activity was reduced by 50% when IL-17R was absent in the cells exposed to sera from AM mice (**Fig 3.3B**). Exposure to sera from OLD versus young WT mice increases NF-κB activity in RWPE-1 cells by 1.5-fold. In the absence of IL-17R (shIL-17R1 and shIL-17R2), NF-κB activity was significantly diminished by 20% in RWPE-1 cells exposed to sera from AM mice. Taken together, these data indicate that the aged-T cell-related up-regulation of IL-17 can promote NF-κB activation in prostate cancer cells as well as in non-cancerous prostate epithelial cells.

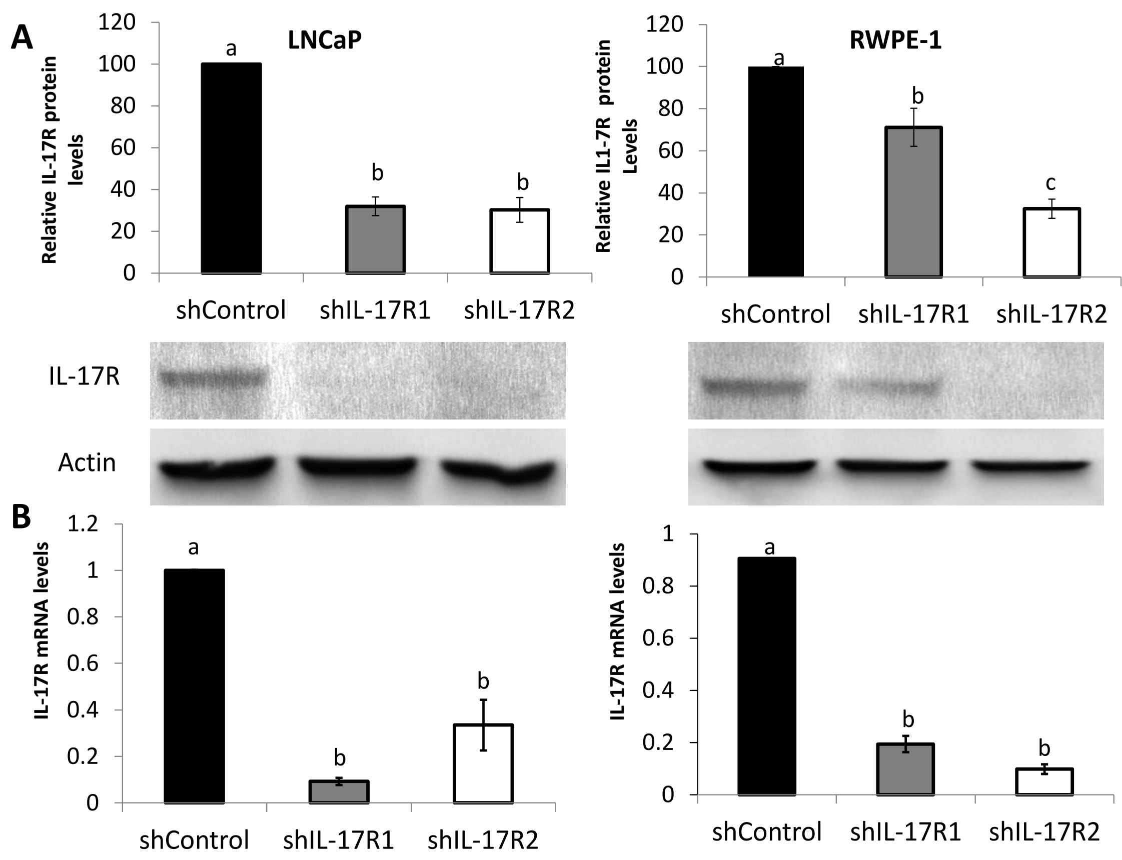

Figure 3.1 IL-17R expression in human prostate cells.

IL-17R protein expression levels were determined by western blot analysis (A) in LNCaP prostate cancer cells transfected with IL-17R shRNA (designated shIL-17R1 and shIL-17R2) or control shRNA (shControl) (left) and RWPE-1 prostate epithelial cells transfected with IL-17R shRNA or control shRNA (right). Graphs indicate the average relative densitometry values from three independent experiments. IL-17R mRNA levels was determined by qPCR (B). Different letters indicate statistically significant differences ($p<0.05$)

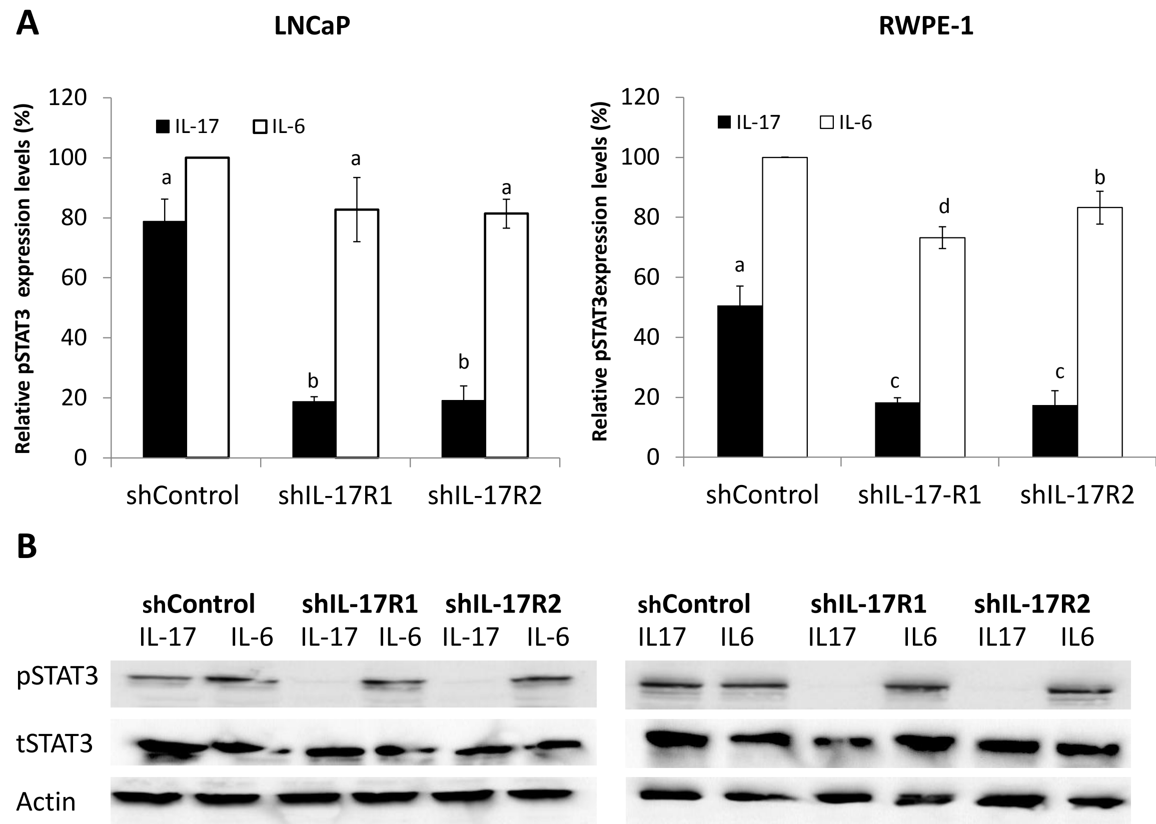

Figure 3.2 Expression levels of pSTAT3 in response to IL-17R knock-down.

Protein expression levels of pSTAT3, relative to tSTAT3, were detected by immunoblot in LNCaP (left) and RWPE-1 (right) cells transfected with IL-17R shRNA (shIL-17R1 and shIL-17R2) or control shRNA (shControl) after exposure to 100ng/ml IL-17 or IL-6. Graphs indicate the combined average relative densitometry values from three independent experiments, with different letters indicating statistically significant differences ($p<0.05$).

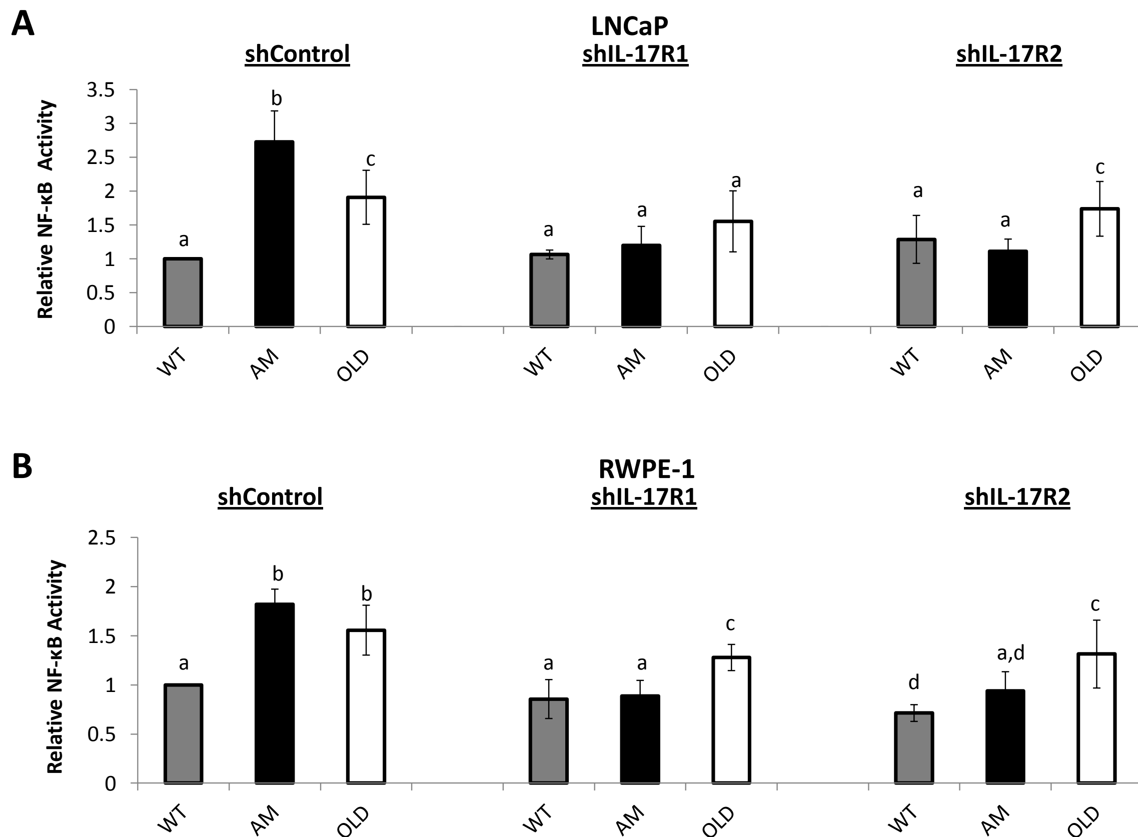

Figure 3.3 NF-κB transcriptional activity is stimulated by IL-17 in the sera of Aging Mimic mice.

NF-κB transcriptional activity was measured by dual luciferase assay in response to sera from young wild-type (WT), old wild-type (OLD) or Aging Mimic GPAT -1 $^{-/-}$ (AM) in LNCaP (A) or RWPE-1 (B) cells transfected with IL-17R shRNA (shIL-17R1 and shIL-17R2) or control shRNA (shControl). Presented is the average of three independent experiments standardized to WT shControl. Different letters indicate statistically significantly differences ($p<0.05$).

3.3.2 IL-17 secreted from aging T-lymphocytes induce NF-κB transcriptional activity

Based on our observation that IL-17 in the sera of AM mice induced NF-κB activation we sought to determine whether IL-17 originating from the T-lymphocytes alone was impacting NF-κB activity. Splenic T-lymphocytes isolated from young WT and AM mice as well as old mice were stimulated with anti-CD3/CD28 antibodies, and conditioned media (CM) from the T-lymphocytes was collected after 24 hours. NF-κB activity was then measured in RWPE-1 and LNCaP cell IL-17R shRNA clones (shIL-17R1 and shIL17-R2) and control shRNA clones (shControl) following exposure to this CM (**Fig 3.4**). LNCaP shIL-17R2 clone exposed to AM or Old T-lymphocyte CM induced almost 50% less NF-κB activity as compared to the LNCaP shControl clones under the same treatment conditions (**Fig 3.4A**). RWPE-1 shIL-17R2 clones also exhibited around 20% less NF-κB activity in comparison to RWPE-1 shControl clones after exposure to AM T-lymphocyte CM (**Fig 3.4B**). This suggests that IL-17 secreted from the AM –or OLD T-lymphocytes directly modulates NF-κB activity in prostate cells.

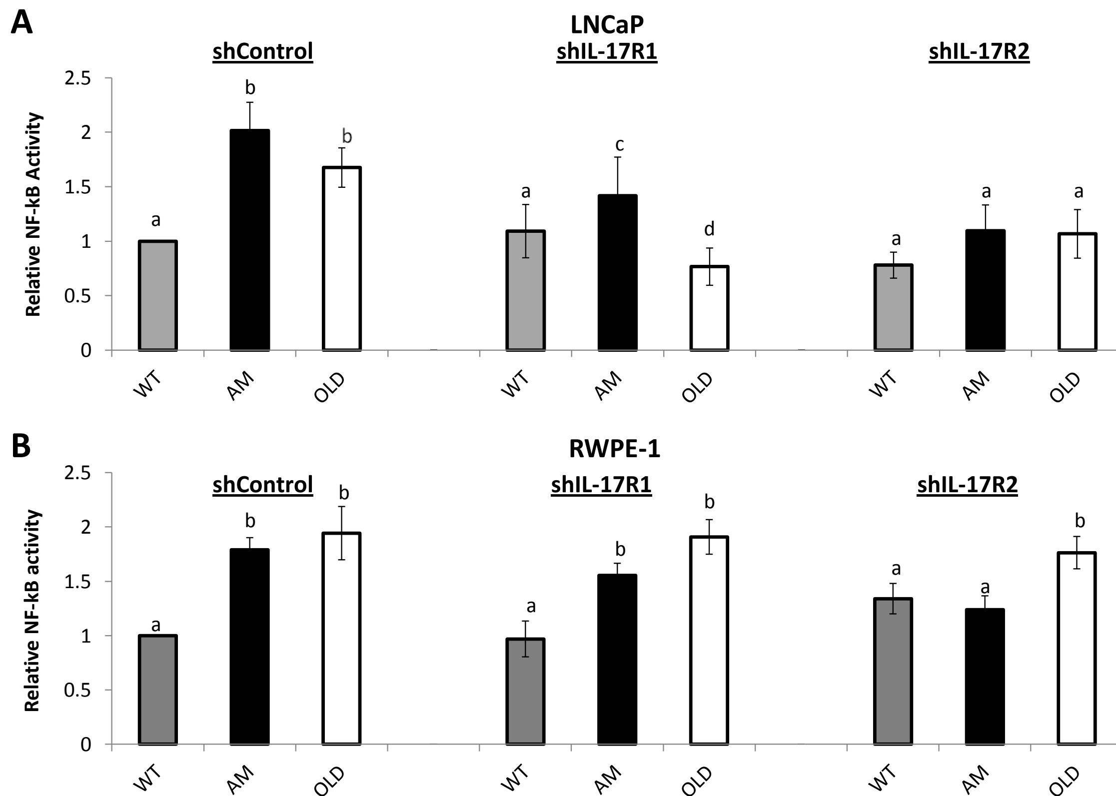

Figure 3.4 IL-17 secreted by isolated splenic T-lymphocytes from wild-type, aging-mimic or old mice induces NF-κB transcriptional activity.

Conditioned media (CM) was generated from anti-CD3 plus CD-28 stimulated splenic T-lymphocytes from young Aging Mimic (AM), young wild-type (WT) or old wild-type mice (OLD). Dual luciferase assay was then used to measure NF-κB transcriptional activity in LNCaP (A) or RWPE-1 (B) cells expressing IL-17R shRNA (shIL-17R1 and shIL-17R2) or Control shRNA (shControl) following exposure to this CM. Presented is the average of three independent experiments standardized to WT shControl. Different letters indicate statistically significantly differences ($p < 0.05$).

3.3.3 IL-17 secreted by aging T-lymphocytes regulates the expression of key downstream targets of NF-κB

NF-κB PCR arrays were used to demonstrate the impact of circulating factors in the AM versus WT mouse sera on NF-κB target gene expression in LNCaP cells (**Fig 3.5A**). NF-κB regulates the transcription of several genes involved in tumor promotion. Notably, several NF-κB target genes involved in the suppression of cell death, promotion of inflammatory processes and regulation cell adhesion were upregulated by the sera from the AM mouse **(Table 3.1**). Baculoviral IAP repeat-containing protein 3 (cIAP2), a key gene NF-κB target gene that promotes survival of prostate cancer cells by interfering with the activation of caspases [89], was upregulated in LNCaP cells exposed to sera from the AM mouse (**Fig 3.5B**). In prostate cancer, increased expression of cIAP2 has been found in biopsy specimens from all stages of the disease, suggesting an important role in development and progression [90]. In addition to the regulatory role it plays in apoptosis, NF-κB is also capable of promoting cell cycle progression by modulating the expression of cell cycle specific genes, including cyclin D1 [71, 91] . Cyclin D1 gene expression was also upregulated in LNCaP cells exposed to sera from AM mouse (**Fig 3.5B).** Various studies have suggested that NF-κB-induced cyclin D1 expression is a key contributor in prostate carcinogenesis [71-73, 91] . NF-κB also regulates the expression of vascular cell adhesion protein 1 (VCAM-1), an important mediator of cell adhesion in many tumors, including prostate carcinomas [92]. Certain tumor cells can use VCAM-1

to adhere to the endothelium and recruit monocytes, aiding tumor growth [92-94]. Exposure to sera from AM mouse up-regulated the expression of VCAM-1 in LNCaP cells (**Fig 3.5B**). FAS gene expression was also up-regulated in LNCaP cells exposed to sera from AM mice. FAS is a type-II transmembrane protein that belongs to the tumor necrosis factor (TNF) family. Binding of FAS to its receptor induces apoptosis.

Immunoblot analysis (**Fig 3.6**) confirmed that expression levels of key NF-κB genes (VCAM-1 and cIAP2) were upregulated in LNCaP cells (by 9% and 40%, respectively) when exposed to AM versus WT mouse sera. In contrast, the pro-apoptotic protein FAS was downregulated by 29% after treatment with AM mouse sera. A similar trend was observed when sera from OLD mice were exposed to LNCaP cells. Immunoblot analysis (**Fig 3.6**) showed that expression levels VCAM-1 and cIAP2 were upregulated in LNCaP cells (by 29% and 66%, respectively) when exposed to OLD versus WT mouse sera. Exposure to sera from AM, WT or OLD mice did not have a significant effect on regulation of Cyclin D1 protein levels in LNCaP cells. Based on our previous observation that IL-17 in the AM mouse sera induces NF-κB activation, we examined whether IL-17R knock-down impacts the expression of key NF-κB target genes. Our results showed that IL-17R knock-down in LNCaP cells decreased expression levels of VCAM-1 and cIAP2 (by 26% and 53%, respectively) following exposure to AM mouse sera. Knocking-down IL-17R in LNCaP cells did not modulate VCAM-1 expression in LNCaP cells exposed to sera from OLD mice. However, IL-17R knock-down in LNCaP cells

decreased expression levels of cIAP2 by 66% following exposure to OLD mouse sera. Interestingly, FAS expression levels were increased by 8% in LNCaP cell IL-17R shRNA clones (shIL-17R1 and shIL17-R2) exposed to OLD mouse sera (**Fig 3.6B**). Our results showed that expression levels of key NF-κB genes (VCAM-1 and cIAP2) were also upregulated in RWPE-1 cells (by 28% and 19%, respectively) when exposed to AM versus WT mouse sera **(Fig 3.7).** IL-17R knock-down in RWPE-1 cells decreased expression levels of VCAM-1 and cIAP2 (by 75% and 73%, respectively) following exposure to AM mouse sera (**Fig 3.7)**. FAS was down-regulated in RWPE-1 cell by 63% after treatment with AM mouse sera. IL-17R knock-down in RWPE-1 cells increased expression levels of FAS by 57% following exposure to AM mouse sera. A similar trend was observed when RWPE-1 cells were exposed to sera form OLD mice. However, IL-17R knock-down in RWPE-1 cells did not decrease the expression levels of VCAM-1 after exposure to sera from OLD mice (**Fig 3.7)**. Taken together, our results suggest that IL-17 in the sera from AM mice is playing an important role in the regulation of key NF-κB target genes involved in prostate tumorigenesis.

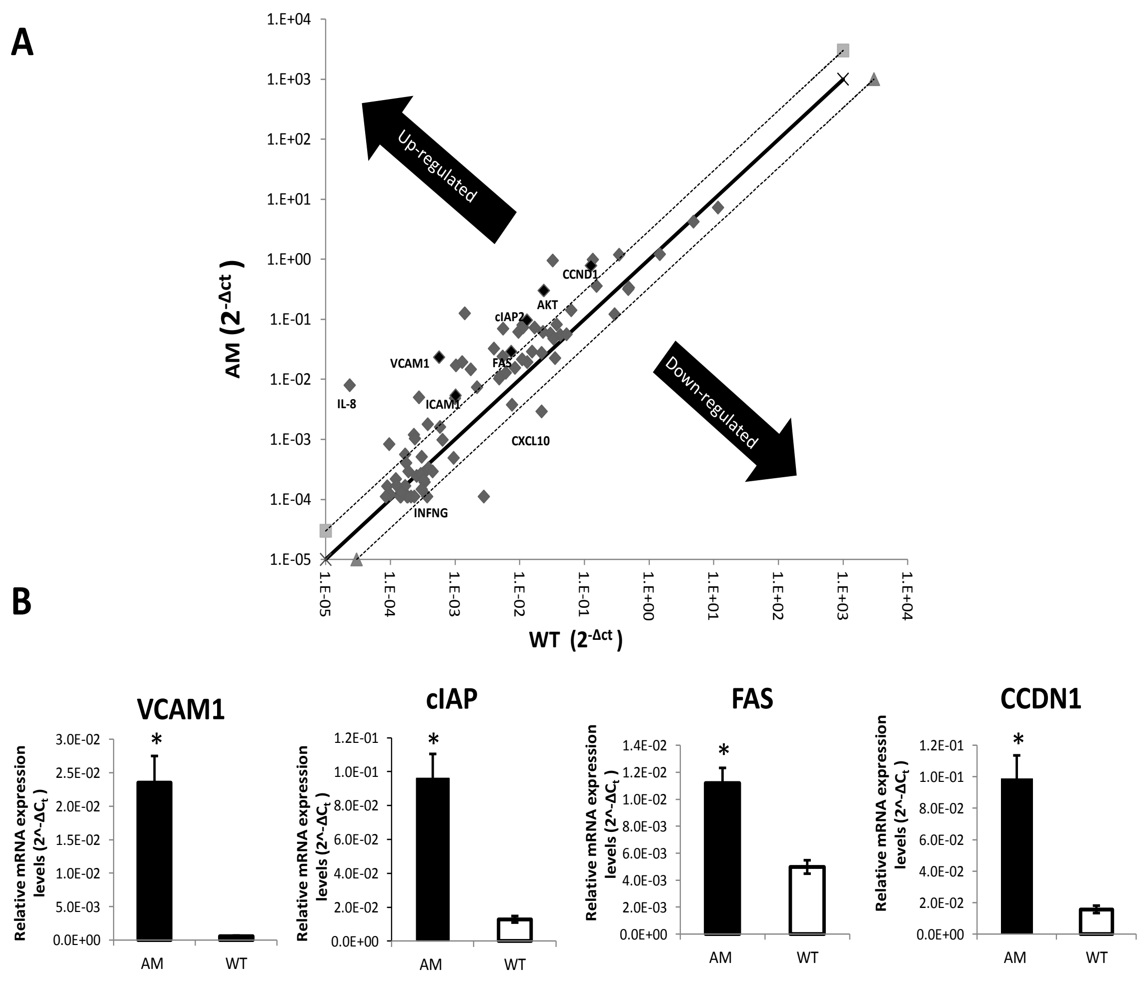

Figure 3.5 Sera from aging-mimic mice up-regulates the expression of key downstream targets of NF-κB.

NF-κB PCR arrays revealed NF-κB gene targets that are differentially regulated in LNCaP cells in response to circulating factors in the sera from Aging Mimic (AM) versus wild-type (WT) mice. The relative expression level for each gene in the two samples is plotted against each other in the scatter plot (A). Graphs show the relative mRNA levels of key NF-kB gene targets (IAP2, CyclinD1, VCAM-1 and FAS) (B)

Table 3.1 Sera from Aging Mimic mice differentially regulates the expression of downstream targets of NF-κB

Gene Name	*Fold Up or Down regulation (AM/WT)*
ADM	-1.04
AGT	**4.72**
AKT1	**12.64**
ALDH3A2	**89.26**
BCL2A1	2.31
BCL2L1	1.87
BIRC2	1.29
BIRC3	**7.46**
C3	**5.17**
CCL11	**N/A**
CCL2	**8.40**
CCL22	-1.87
CCL5	**7.26**
CCND1	**6.23**
CCR5	-1.16
CD40	1.54
CD69	-1.67
CD80	-1.21
CD83	2.14
CDKN1A	-1.44
CFB	**6.45**
CSF1	**4.32**
CSF2	-2.10
CSF2RB	1.85
CSF3	-1.03
CXCL1	**N/A**
CXCL10	**-7.46**
CXCL2	1.80
CXCL9	-1.54
EGFR	1.95
EGR2	**-24.93**
F3	-1.01
F8	2.25
FAS	**3.92**
FASLG	-1.30
IL1RN	**12.64**
ICAM1	**5.24**
IFNB1	**N/A**
INFNG	**-3.32**
IL12B	-1.62
IL15	**3.39**
IL1A	-1.11
IL1B	**8.69**
IL1R2	-1.93

IL2	1.22
IL2RA	**N/A**
IL4	1.68
IL6	**N/A**
IL8	**340.14**
INS	1.31
IRF1	**8.17**
LTA	-1.74
LTB	**18.13**
MAP2K6	-2.01
MMP9	-1.54
MYC	-2.41
MYD88	1.43
NCOA3	1.48
NFKB1	**6.50**
NFKB2	**4.29**
NFKBIA	**29.45**
NQO1	-1.49
NR4A2	**4.86**
PDGFB	1.54
PLAU	-1.27
PTGS2	-2.07
REL	2.11
RELA	2.69
RELB	**16.56**
SELE	2.75
SELP	1.38
SNAP25	-1.11
SOD2	**7.31**
STAT1	1.06
STAT3	2.33
STAT5B	1.97
TNF	**15.03**
TNFRSF1B	**3.32**
TNFSF10	1.27
TP53	2.27
TRAF2	**4.41**
VCAM1	**41.36**
XIAP	2.20
B2M	-1.15
HPRT1	**3.46**
RPL13A	-1.21
GAPDH	-1.56
ACTB	-1.60

Table 3.1 Effects of sera from aging-mimic mice on the expression of downstream targets of NF-κB. LNCaP cells were exposed to sera from Aging Mimic (AM) or wild-type (WT) mice and the transcription of downstream targets of NF-κB was analyzed by qPCR. The table shows fold-change differences between the AM and WT samples in expression of 86 different NF-κB target genes. Statistically significant difference are indicated in bold ($p<0.05$).

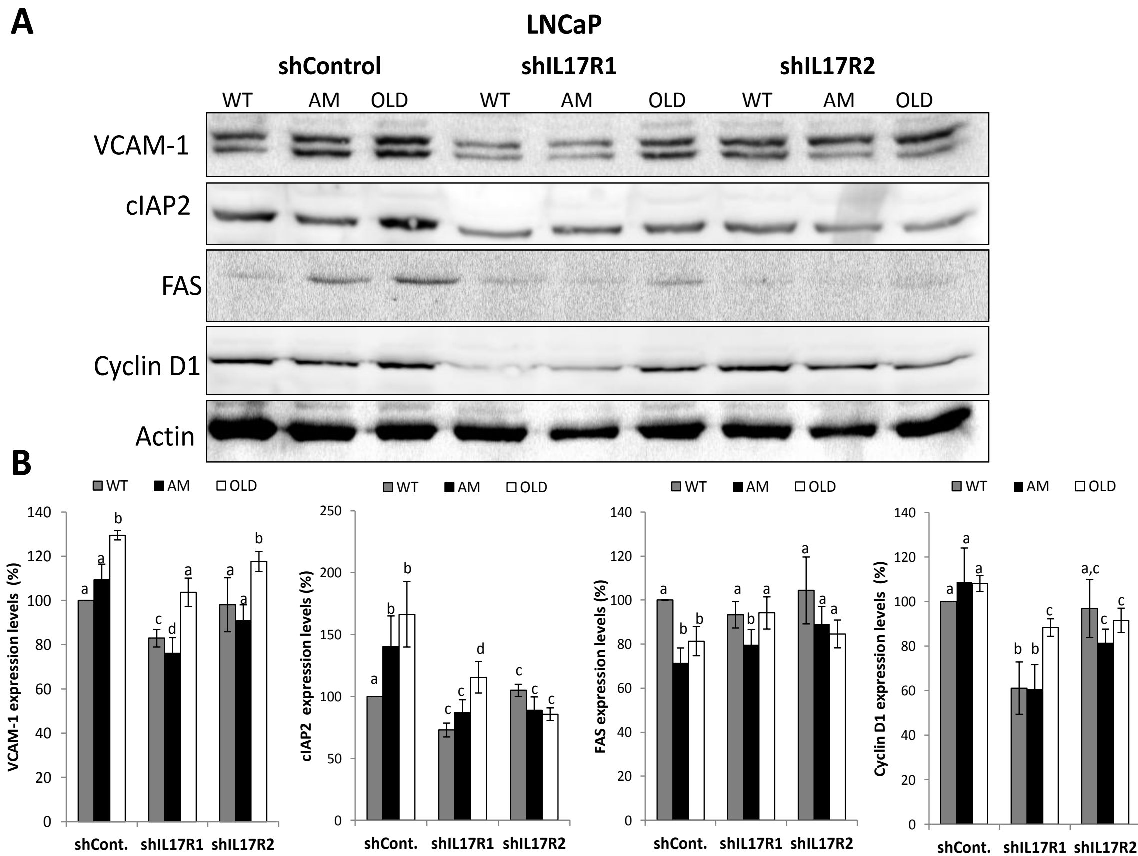

Figure 3.6 Effects of IL-17 in the sera from aging-mimic mice on expression of key downstream targets of NF-κB.

Expression levels of cIAP2, VCAM-1, Cyclin D1 and FAS in LNCaP cells expressing IL-17R shRNA (shIL-17R1 and shIL-17R2) or Control shRNA (shControl) were detected by immunoblot after exposure to sera from young wild-type (WT), old wild-type (OLD) or Aging Mimic (AM) mice (A). Graphs show the average relative densitometry values for three independent experiments. Different letters indicate statistically significant differences ($p<0.05$) (B)

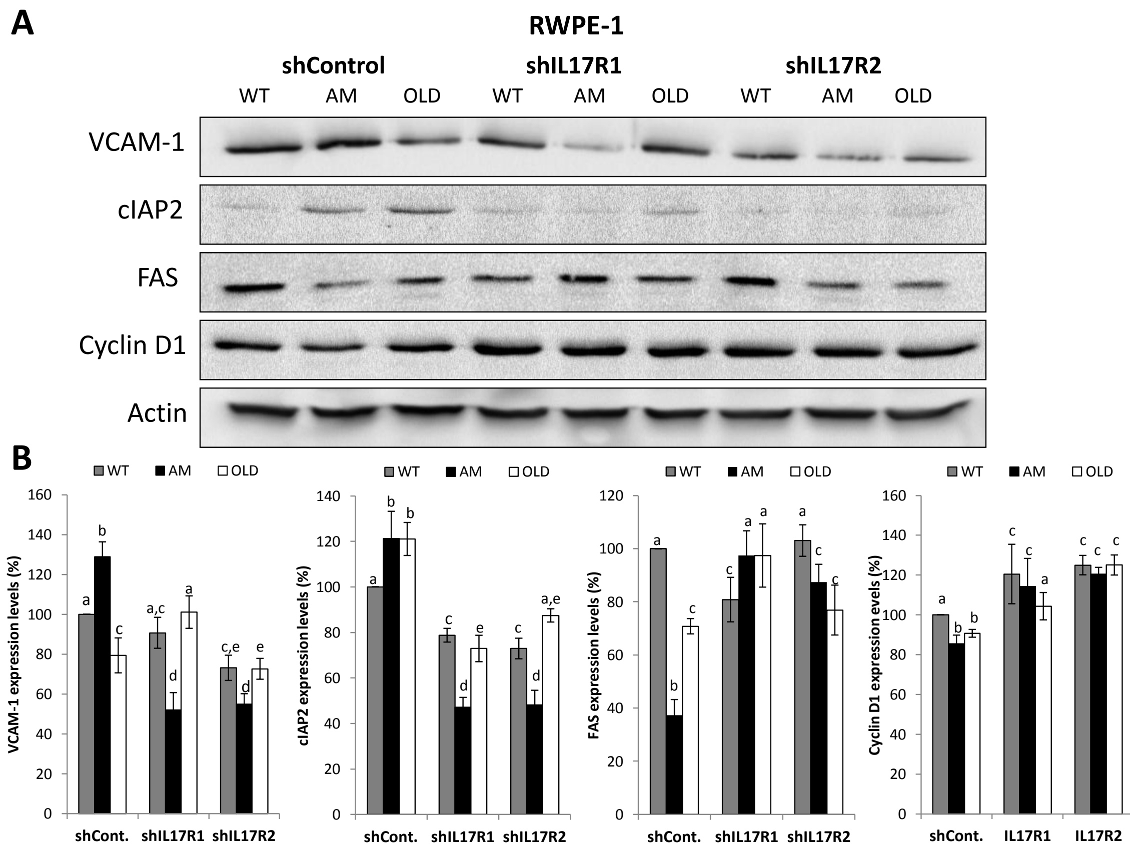

Figure 3.7 Effects of IL-17 in the sera from aging-mimic mice on expression of key downstream targets of NF-κB.

Expression levels of cIAP2, VCAM-1, Cyclin D1 and FAS in RWPE-1 cells expressing IL-17R shRNA (shIL-17R1 and shIL-17R2) or Control shRNA (shControl) were detected by immunoblot after exposure to sera from young wild-type (WT), 22-month old wild-type (OLD) or Aging Mimic (AM) mice (A). Graphs show the average relative densitometry values for three independent experiments. Different letters indicate statistically significant differences ($p<0.05$) (B).

3.3.4 The aging-associated increase in T-lymphocyte IL-17 secretion moderately mediates prostate cancer cell proliferation

After observing that IL-17 in the AM mouse sera regulates the transcription of several genes involved in proliferation and tumor promotion, we examined the effect of aging-associated circulating IL-17 on prostate cell proliferation. MTT assay was used to measure proliferation of the prostate cell lines. Exposure to AM mouse sera increased proliferation in LNCaP (**Fig 3.8A**) and RWPE-1 (**Fig 3.8B**) cells by 20% and 15%, respectively, in comparison to WT mouse sera. However, AM mouse sera-induced proliferation was decreased by 40% in shIL-17R1, 20% in shIL-17R2 and 18% in shIL-17R1, 13% in shIL-17R2 in LNCaP and RWPE-1 cells, respectively (**Fig 3.8**). Collectively, these results suggest that IL-17 in the AM mouse sera promotes prostate cancer cell proliferation, but has only minor effect on normal prostate epithelial cell proliferation.

Based on our observation that IL-17 in the AM T-lymphocyte CM induced NF-κB activation (**Fig 3.4**), we also investigated whether IL-17 specifically originating from T-lymphocytes promotes prostate cell proliferation. LNCaP and RWPE-1 cells transfected with IL-17R shRNA or control shRNA were exposed to CM from stimulated T-lymphocytes from young WT and AM as well as old mice. AM and old CM stimulated greater proliferation in the shControl LNCaP cells in comparison to WT CM. This enhanced proliferation was slightly reduced by IL-17R knock-down in the LNCaP (**Fig 3.9A**). In contrast, AM and old CM did not induce significantly greater proliferation in

the shControl RWPE-1 cells in comparison to WT CM, and IL-17R knock-down had no significant effect on RWPE-1 cell proliferation (**Fig 3.9B**). Collectively, these results suggest that IL-17 originating from AM and Old mouse T-lymphocytes promotes the proliferation of prostate cancer cells, but not of non-transformed prostate epithelial cells.

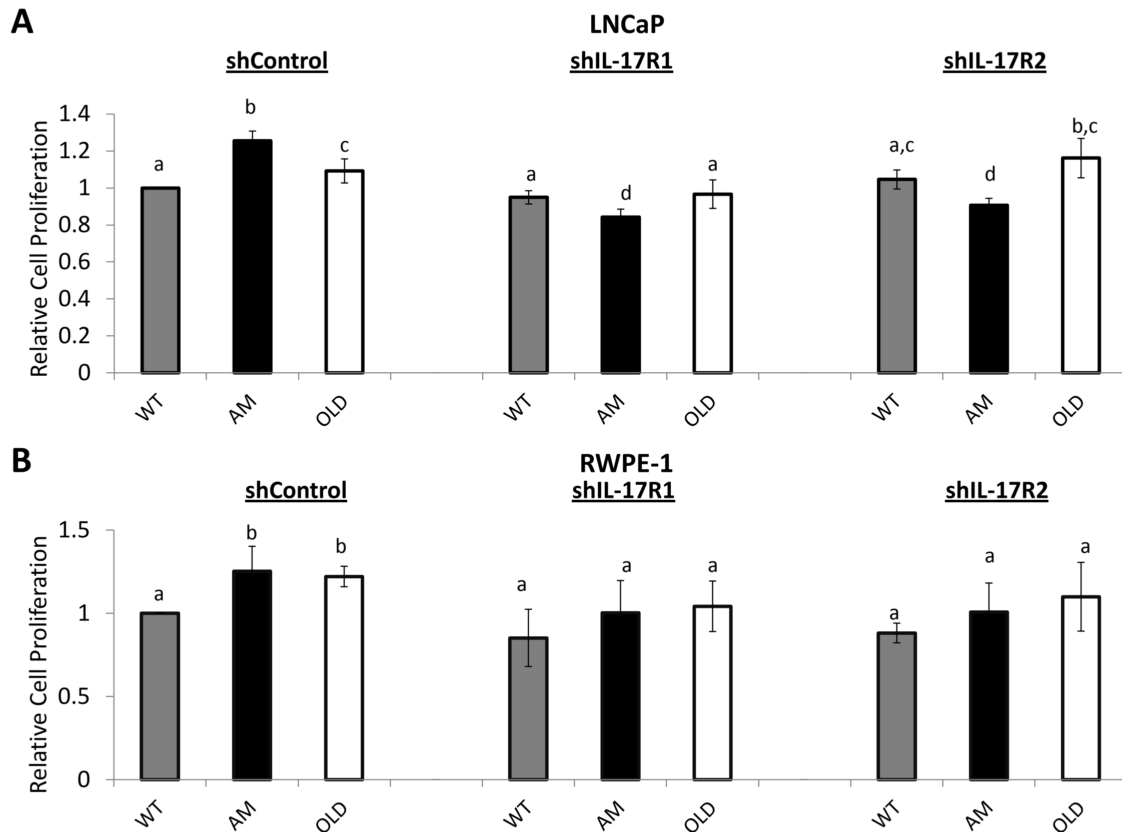

Figure 3.8 Effect of IL-17 in the sera from Aging Mimic and wild-type mice on cellular proliferation.

MTT assay was used to measure cell proliferation in response to sera from GPAT-1$^{-/-}$ Aging Mimic (AM), young wild-type (WT) or old wild-type (OLD) in LNCaP (A) and RWPE-1 (B) cells expressing IL-17R shRNA (shIL17-R1 and shIL-17R2) or Control shRNA (shControl). Presented is the average of three independent experiments standardized to WT shControl. Different letters indicate statistically significant differences ($p<0.05$).

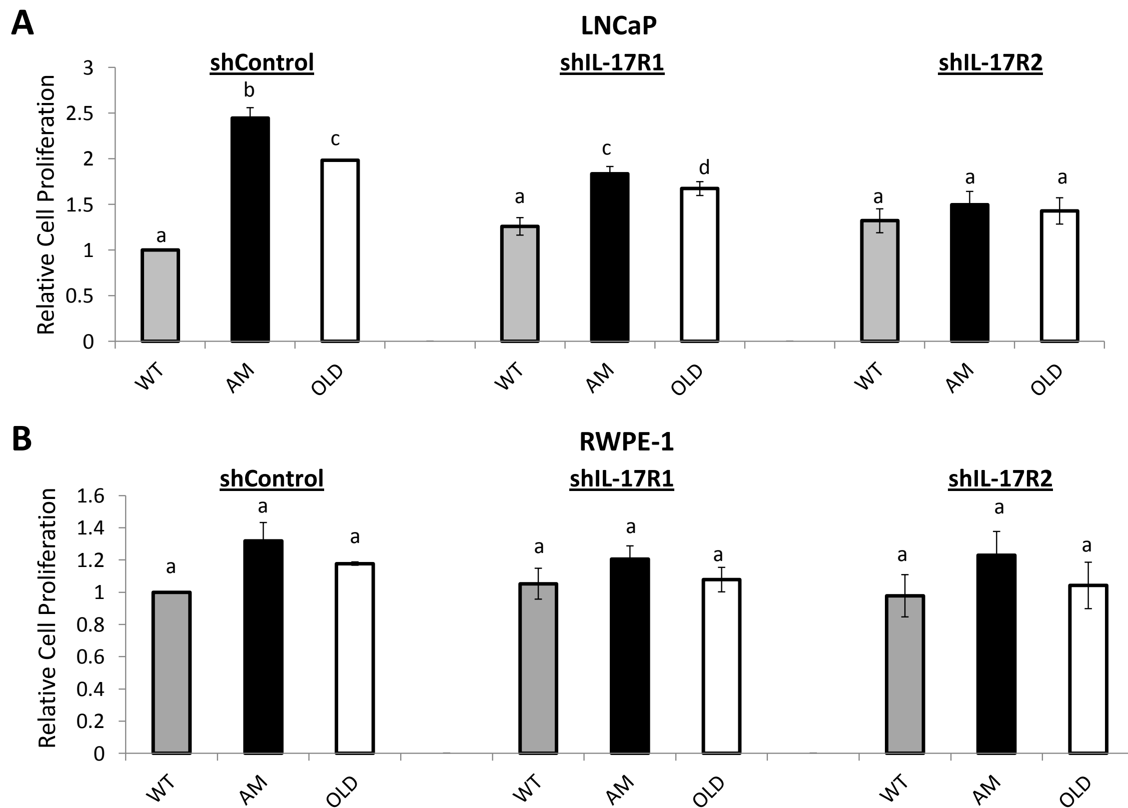

Figure 3.9 Effect of isolated splenic T-lymphocyte-secreted IL-17 from wild-type, Aging Mimic or old mice on cell proliferation.

MTT assay was used to measure cell proliferation in response to conditioned media generated from anti-CD3 plus CD-28 stimulated splenic T-lymphocytes from either young GPAT-1 $^{-/-}$ Aging Mimic (AM), young wildtype (WT) or old wild-type (OLD) mice. Proliferation was assessed in LNCaP (A) and RWPE-1 (B) cells expressing IL-17R shRNA (shIL-17R1 and shIL-17R2) or Control shRNA (shControl). Presented is the average of three independent experiments standardized to WT shControl.. Different letters indicate significant differences ($p<0.05$).

3.4 Discussion

One of the most significant risk factors for prostate cancer development is age [81]. One widely accepted explanation for the association between age and prostate cancer is the fact that prostate carcinogenesis is a characteristically long process [75] . Another possible mechanism responsible for prostate tumorigenesis in the elderly is aging-associated dysregulation of immune function. To date, multiple studies have shown the importance of the immune system in preventing tumor formation [4, 95]. However, few studies have investigated the potential contribution of the retention of strong inflammatory responses to age-related prostate tumorigenesis. Retention of strong inflammatory responses with age, in the absence of counterbalancing and beneficial responses from the immune system, may dramatically enhance prostate tumorigenesis. Inflammation is well established to be an amplificatory factor in prostate tumorigenesis [80]. Our data suggest that an aging immune system possibly promotes prostate cancer onset through induction of chronic inflammation, specifically via enhanced NF-κB signaling. In this chapter, I presented data showing the influence of one specific cytokine, IL-17, on age-related induction of pro-inflammatory pathways in prostate cells. Recent studies by other investigators have demonstrated that IL-17 becomes dysregulated with age and that the proportion of IL-17 producing cells is higher in aged mice than in young ones [80]. Furthermore, various researchers have shown that IL-17 promotes the formation and growth of prostate cancer [43, 96, 97].

Therefore, we hypothesized that age-related changes in IL-17 could potentially initiate prostate tumorigenesis through the activation of NF-κB [42, 98], which up-regulates the expression of pro-tumorigenic factors [42].

Building upon our data that demonstrated that aging-related factors induce NF-κB activity and its pro-survival downstream targets, we examined whether an aging immune system may contribute to the induction of pro-inflammatory pathways in the prostate thought the increased production of IL-17. Intriguingly, knocking down the IL-17R in LNCaP prostate cancer cells exposed to sera from GPAT-1$^{-/-}$Aging Mimic (AM) mice markedly diminished NF-κB transcriptional activity. This suggests that the aging-related increase in IL-17 production may be partly responsible for the observed induction of NF-κB activity in prostate cancer cells exposed to AM sera. We also observed a significant reduction in NF-κB transcriptional activity in non-transformed RWPE-1 prostate epithelial cells transfected with IL-17R shRNA and exposed to AM mouse sera, indicating that this phenomenon is not restricted to cancer cells, and may play a role even during the very early stages of prostate carcinogenesis. We also examined the effect of sera from old mice on induction of NF-κB in the prostate. We found numerous similarities between the T cell aging-mimic and old mice in terms of regulation of NF-kB in prostate epithelial cells. Intriguingly, there were some key differences, which may reflect the more systemic events associated with the aging process as a whole and the inter-relationship between an aging immune system and the

aging tissues. The impact that the sera from old mice have on prostate cancer cells represents the actual aging process as a whole, not just the aging T cell population, as with our aging-mimic transgenic mice.

One mechanism by which IL-17 may initiate prostate cancer progression is through the regulation of NF-κB target genes that control cancer cell proliferation and tumor growth. Our results show that knock-down of the IL-17R in prostate cells significantly reduces the aging-induced expression of two key NF-κB target genes, cIAP2 and VCAM. Moreover, expression of the pro-apoptotic protein FAS is upregulated in prostate cancer cells lacking IL-17R. These results have important implications for our current understanding of IL-17's contribution to induction of pro-tumorigenic pathways in the prostate, particularly given the changes in pro-inflammatory signaling found in non-transformed prostate epithelial cells exposed to AM mouse sera and T-lymphocyte CM. Furthermore, the observed increase in prostate cancer cell proliferation upon exposure to AM sera was significantly reduced when the IL-17 receptor was knocked down, demonstrating that the aging-related up-regulation of T-lymphocyte IL-17 secretion may play a key role in prostate cancer progression.

In summary, the present study provides evidence that the aging-associated increase in circulating IL-17 promotes pro-inflammatory signaling in prostate epithelial cells. T cells from our aging-mimic mice secrete elevated levels of IL-17, possibly due to an imbalance in the T Helper 17(T_H17)/Regulatory T (T_{reg}) cell ratio, commonly

associated with aging, making this an excellent model for the study of age-related changes in T-lymphocyte function [45]. The change toward a pro-inflammatory phenotype and increased production of IL-17 during aging may be actively promoting prostate cancer development via recruitment of inflammatory cells and induction of pro-inflammatory mediators.

Better understanding of the immune dysfunction associated with aging will increase our ability to restore appropriate immune function and alleviate the burden of prostate cancer in the elderly. Future novel immunotherapies for prostate cancer could target the T_H17/Treg imbalance associated with aging. Further investigation in this area is warranted.

Chapter 4: The Role of IL-17 and IL-6 in Aging related Prostate Tumorigenesis

4.1 Introduction

Aging is positively correlated with increased circulating levels of many pro-inflammatory cytokines such as interleukin-1 beta (IL-1β), tumor necrosis factors alpha (TNFα), interleukin-6 (IL-6) and interleukin-17 (IL-17) [83]. IL-6 is the most prominent cytokine shared across age-related pathologies having a strong chronic inflammatory component. There is strong evidence demonstrating that IL-6 serum concentration increases with age [46-49]. Maggio *et al.* reported that IL-6 mean values ranged from 1.4 pg/ml (men) and 1.1 pg/ml (women) in the 65–74 years age group to 3.5 pg/ml (men) and 2.1 pg/ml (women) in persons 85 years and older, and that the age trend is partially independent of major confounders [48]. The etiology of chronically elevated IL-6 in older adults is likely multifactorial, with increased presence of disease states, declines in estrogen and testosterone and changes on the immune system function and regulation, all contributing to IL-6 levels increase.

In cancer, IL-6 is a growth/survival factor for a variety of tumor types. In prostate cancer, activation of STAT3 by IL-6 is correlated with increase proliferation, decreased apoptotic potential, regulation of epithelial-mesenchymal transition (EMT) and activation of androgen receptor genes [53]. A great number of reports have shown that elevated levels of circulating IL-6 are critical for prostate cancer development and progression [55, 56, 99, 100]. Human prostate cancer cell lines as well as clinical

prostate cancer specimens show constitutive expression of IL-6 and its receptor [101]. Studies have also demonstrated that IL-6 is elevated in sera of patients with castration resistant prostate cancer compared to normal controls, benign prostatic hyperplasia, and localized prostate cancer [102]

Tumorigenesis involves the acquisition of genetic and epigenetic changes that cause the aberrant loss or gain of functions by cellular proteins. The consequences include the ability of tumor cells to proliferate, resist apoptosis, demonstrate angiogenic potential, migrate, and invade, as well as the ability of these cells to evade immune surveillance. IL-6 can induce tumor initiation through activation of STAT3. Activated STAT3 has been linked to tumor initiation in part through the transcriptional regulation of critical target genes, including those for c-myc, c-fos, cyclin D1, matrix metalloproteinase 9 (MMP-9), MMP-2, vascular endothelial growth factor, Bcl-x_L, Mcl-1, survivin, , as well as the epithelial-mesenchymal transition-related proteins – Snail, Slug and Twist [40, 103-105].

Key features of early tumorigenesis include morphology changes, loss of cell contact inhibition and loss of cell polarity [106] . These early changes in cell morphology are widely associated with epithelial-mesenchymal transition (EMT), which also plays key roles in normal physiological processes such as embryogenesis, wound repair, and tissue remodeling [107-110]. The molecular hallmarks for EMT are down-regulation of epithelial markers, such as E-cadherin,, and up-regulation of mesenchymal markers

[109]. The induction of EMT can be triggered by transcription factors such as Snail, Slug, and Twist, which simultaneously repress the expression of genes that are required for the epithelial phenotype and induce the expression of genes required for mesenchymal properties [110]. The expression of these transcription factors is modulated by a number of signaling molecules, including STAT3 [111].

The objective of the following study was to better understand the connection between IL-6 and IL-17 in promoting prostate tumorigenesis. We previously demonstrated that IL-17 and IL-6 coming from aging T cells activate pro-inflammatory signaling in prostate epithelial cells, our next objective was to determine the role of IL-6 and IL-17 in induction of age related prostate tumorigenesis.

4.2 Materials and Methods

4.2.1 Murine Models of an Aging Immune System

C57BL/6 GPAT-1 +/- mice were obtained from Dr. Rosalind Coleman (University of North Carolina at Chapel Hill) and bred in our animal facilities to obtain homozygous knock-outs. Mice were fed a commercial chow diet (Prolab Rat/Mouse/Hamster 2000) provided by the animal facility. Offspring were numbered to monitor sex differences or differences between litters. Aged (>22 month old) C57BL/6 mice were purchased from the National Institute for Aging. Mice were housed on a 12:12-h light-dark cycle and had ad libitum access to food and water.

4.2.2 Cells and Cell Culture

The LNCaP prostate cancer cell line was purchased from ATCC (Rockville, MD) and grown in RPMI-1640 containing 1% penicillin and streptomycin, supplemented with 10% FBS in a 5% (v/v) CO_2 humidified incubator at 37°C. The immortalized non-transformed RWPE-1 prostate epithelial cell line was purchased from ATCC and grown in Keratinocyte Serum Free Medium (K-SFM) supplemented with bovine pituitary extract (BPE) and human recombinant epidermal growth factor (EGF) in a 5% (v/v) CO_2 humidified incubator at 37°C. The IL-17R shRNA clones (designated shIL-17R1 and shIL-17R2) were generated by transfecting cells with the IL-17R shRNA Plasmid (h) sc-40037 (Santa Cruz Biotechnologies, Santa Cruz, CA), using FuGENE HD transfection reagent (Roche, Basel, Switzerland) per manufacturer's instructions. Control LNCaP and RWPE-1 cells (designated shControl) were generated by stably transfecting negative-control shRNA plasmids: Control Plasmid-B sc-108065 and Control Plasmid-C sc-108066 (Santa Cruz Biotechnologies, Santa Cruz, CA). Puromycin antibiotic (Santa Cruz Biotechnologies, Santa Cruz, CA) was used to stably select transfected cells. All cells were maintained in selective RPMI-1640 or K-SFM media containing 4 µg/ml puromycin. RT-PCR was performed to monitor IL-17R gene expression knock-down using the IL-17R (h)-PR primer (Santa Cruz Biotechnologies, Santa Cruz, CA). In addition western blot analysis was used to confirm knock-down of the IL-17R gene. Antibodies against IL-17R

antibody (Santa Cruz Biotechnologies, Santa Cruz, CA) and phospho- and total-STAT3 (Cell Signaling) were used.

4.2.3 Wound Healing Assay

Cells were seeded into 6-well flat-bottomed plates at a density of 5×10^5 cells per well and allowed to grow to 90% confluence. After aspirating the medium, the monolayer was scratched with a sterile 100 µl pipette tip to create a denuded zone (gap) of constant width. The scratched areas were photographed at 0, 12, 24 and 48 hours after wounding using a phase-contrast microscopy. Cell migration was calculated as percentages of cell coverage to the initial cell-free zone. The values are the means of three independent experiments.

4.2.4 Western blot analysis

Prostate shIL-17R and shControl cells were exposed for 48 hours to sera or conditioned media from stimulated T-lymphocytes, then harvested and lysed in Laemmli lysis buffer for SDS-polyacrylamide gel electrophoresis. The lysates were probed with the following antibodies: pSTAT3, STAT3, E-cadherin, pSMAD2/3, VCAM, FAS, cAIP2 (all Cell Signaling, Boston, MA), cyclin D1 (Millipore, Billerica, MA), and cMYC, ICAM, Vimentin, Survivin and Actin (Santa Cruz Biotechnology, Santa Cruz, CA). Luminescent signal was detected on a Syngene (Frederick, MD) imaging system and quantitative densitometric analysis measured using GeneTools.

4.2.5 RT-qPCR

Prostate Cancer LNCaP cells were exposed to sera for 48 hours. Total RNA was isolated with the QIAGEN (Valencia, CA) RNA extraction system according to the manufacturer's instructions and transcribed into complementary DNA (cDNA). Gene expression of Tenascin, Fibronectin and Intering β6 was measure using the respecting primers. Expression was quantified by QIAGEN SYBR green real-time PCR on an Eppendorf instrument (Santa Clara, CA). Nonspecific signals caused by primer dimers were excluded by dissociation curve analysis and use of non-template controls. To normalize for loaded cDNA, actin was used as an endogenous control.

4.2.6 Statistics

Values are presented as mean ± standard error of the mean (SEM). For the Western blot analyses and Luciferase assays, means were compared across treatment groups using Student's t-test and one-way ANOVA Multiple Comparison was used for comparing more than two conditions. $P \leq 0.05$ was considered statistically significant

4.3 Results

4.3.1 IL-17 and IL-6 in the sera from old and aging mimic mice induce activation of STAT3

Immunoblot analysis (**Fig 4.1**) confirmed that expression levels of phospho-STAT3 and key STAT3 target genes were upregulated in LNCaP cells when exposed to AM and OLD versus WT mouse sera for 24 hours. IL-17R knock-down in LNCaP cells decreased activation of STAT3 and expression of its gene targets following exposure to AM mouse sera. Knocking-down IL-17R in LNCaP cells does not modulate pSTAT3 expression in LNCaP cells exposed to sera from OLD mice. However, IL-17R knock-down in LNCaP cells significantly decreased expression levels of pSTAT3 following exposure to OLD mouse sera plus IL-6 depleting antibody.

Our results showed that expression levels of key STAT3 genes were also upregulated in RWPE-1 cells when exposed to OLD versus young WT mouse sera IL-17R knock-down in RWPE-1 cells decreased expression levels of pSTAT3 and its target genes following exposure to AM mouse sera (**Fig 4.1)**. IL-17R knock-down in RWPE-1 cells did not decrease the expression levels of pSTAT3 after exposure to sera from OLD mice. However, when IL-6 was depleted from the sera from OLD mice, activation of STAT3 diminished completely. Overall these data suggests that IL-6 and IL-17 in the OLD and AM mice are essential for STAT3 activation in prostate epithelial cells.

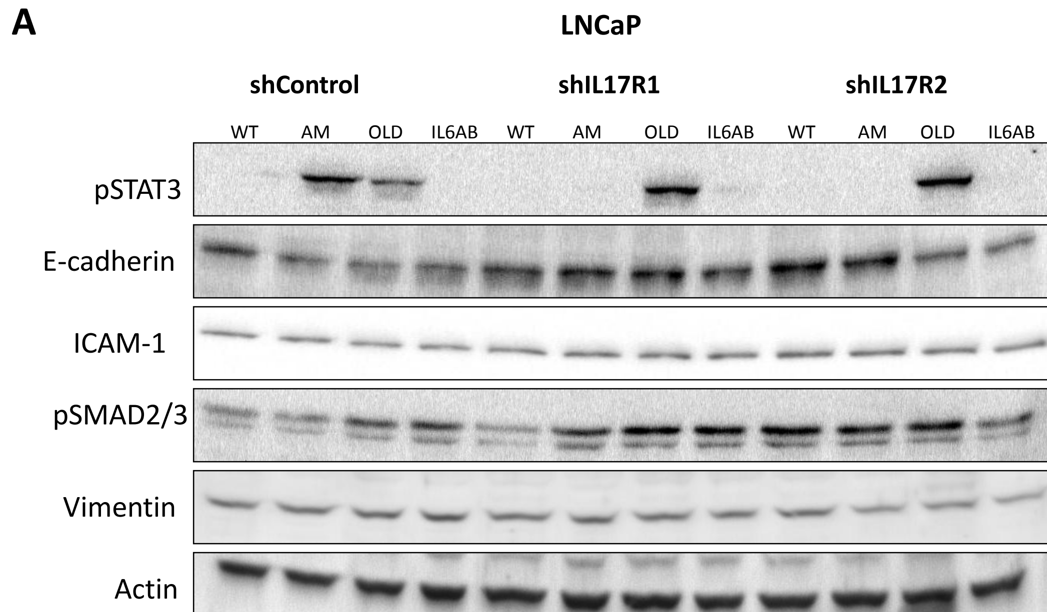

Figure 4.1 Effects of IL-6 and IL-17 in the sera from OLD and AM mice on expression of key downstream targets of STAT3.

Expression levels of pSTAT3, E-cadherin, ICAM-1, pSMAD2/3 and Vimetin LNCaP and RWPE cells expressing IL-17R shRNA (shIL-17R1 and shIL-17R2) or Control shRNA (shControl) were detected by immunoblot after 24 hour exposure to sera from young wild-type (WT), old wild-type (OLD) or Aging Mimic (AM) mice or sera from old wild-type mice plus 10ug/ml IL-6 depleting antibody (O+IL6AB) (A). Graphs show the average relative densitometry values for three independent experiments. Different letters indicate statistically significant differences ($p<0.05$) (B).

4.3.2 Sera from old mice increase expression of the transcription factors Slug, Snail and Twist

Transcription factors, such as Snail, Slug, and Twist, are pivotal activators of EMT [106]. Snail, Slug and Twist have been reported to mediate EMT, resulting in tumor progression, and poor survival in patients with prostate cancer. Several transcription factors have been reported to be involved in EMT via repression of E-cadherin, and some of these include Twist, Snail, and Slug [110, 112, 113]. Since Slug, Snail and Twist are important targets of STAT3 implicated in tumorigenesis , we focused our attention on these three proteins.

After establishing that IL-6 and IL-17 in the sera from old and AM mice is important for activation of STAT3 in prostate epithelial cells, we sought to determine whether this activation of STAT3 results in enhance expression of key regulators of prostate tumorigenesis. We used qPCR to measure expression levels of Snail, Slug and Twist. Twist mRNA levels were upregulated in RWPE-1 non-transformed cells when exposed to OLD versus WT or AM mouse sera **(Fig 4.2-4.3).** Expression levels of Slug were upregulated in RWPE-1 non-transformed cells when exposed to OLD and AM versus WT sera. IL-17R knock-down in RWPE-1 cells significantly decreased expression of Twist (**Fig 4.3**) and Slug (**Fig 4.2**) following exposure to AM mouse sera. Knocking-down IL-17R in RWPE-1 cells moderately modulated expression of Slug **(Fig 4.2)** in RWPE-1

cells exposed to sera from OLD mice. However, IL-17R knock-down in RWPE-1 cells significantly decreased expression levels of Slug following exposure to OLD mouse sera plus IL-6 depleting antibody **(Fig 4.2)**.

Overall these data suggests that IL-6 and IL-17 in the sera from OLD and AM mice play important role on expression of proteins involved in tumor initiation.

A **RWPE-1**

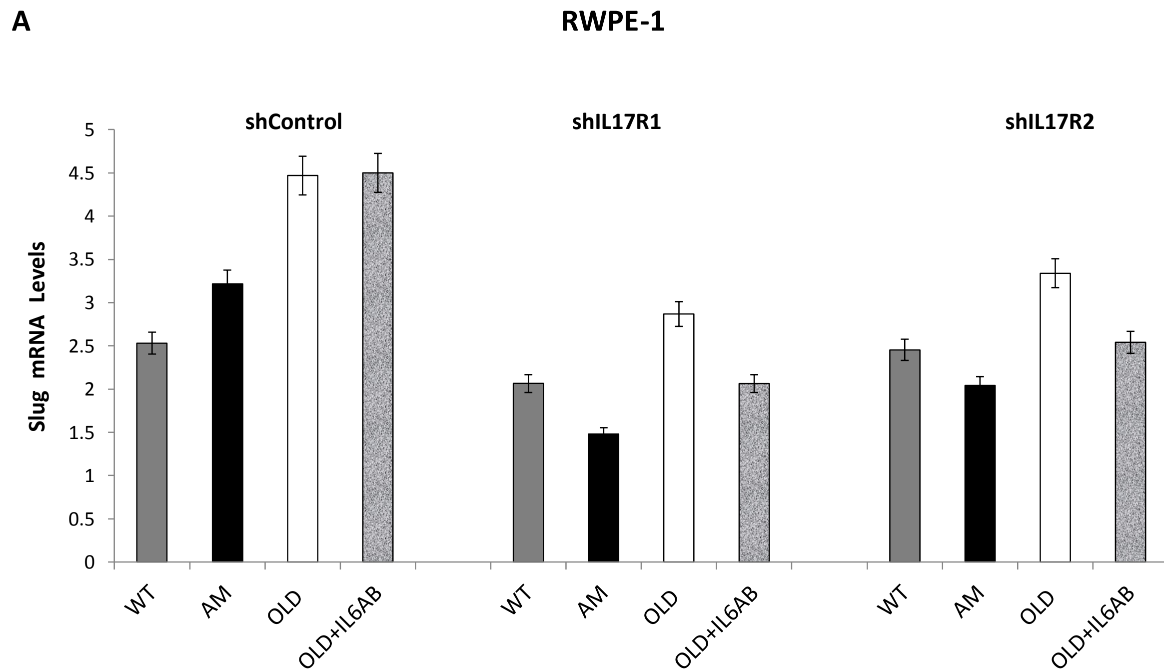

Figure 4.2 Effects of IL-6 and IL-17 in the sera from OLD and AM mice on expression of Slug

Slug mRNA levels in RWPE-1 cells expressing IL-17R shRNA (shIL-17R1 and shIL-17R2) or Control shRNA (shControl) were detected by qPCR after exposure to sera from young wild-type (WT), old wild-type (OLD) or Aging Mimic (AM) or to sera from old wild-type mice plus 10ng/ml of IL-6 depleting antibody (OLD +IL6AB) (A).

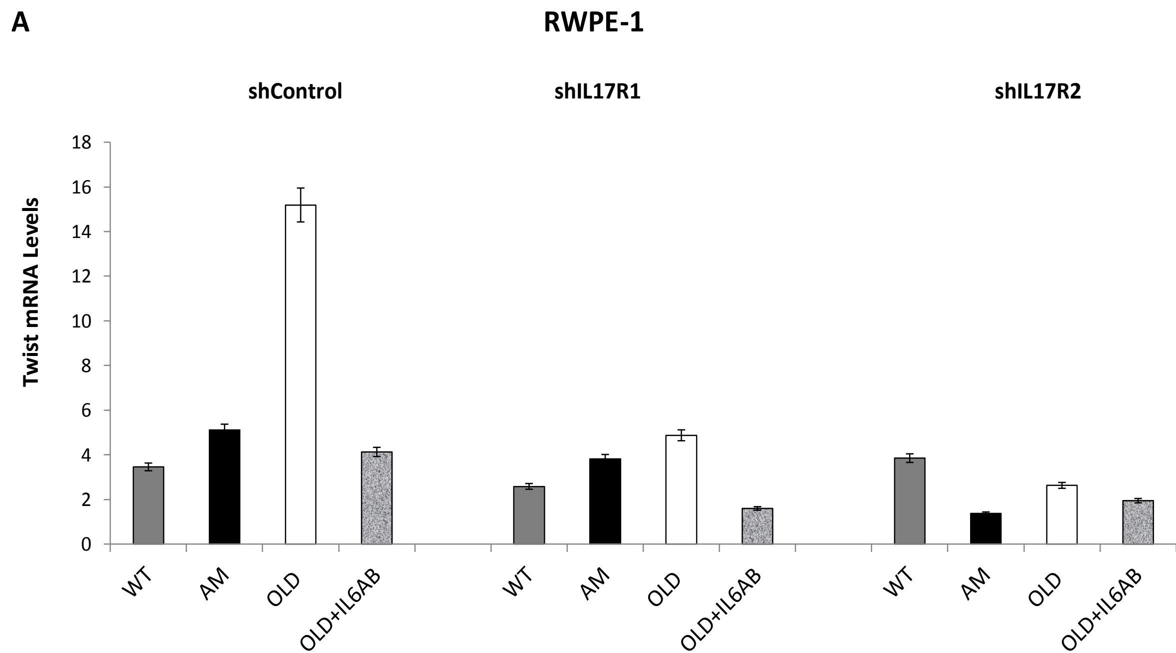

Figure 4.3 Effects of IL-6 and IL-17 in the sera from OLD and AM mice on expression of Twist

Twist mRNA levels in RWPE-1 cells expressing IL-17R shRNA (shIL-17R1 and shIL-17R2) or Control shRNA (shControl) were detected by qPCR after exposure to sera from young wild-type (WT), old wild-type (OLD) or Aging Mimic (AM) or to sera from old wild-type mice plus 10ng/ml of IL-6 depleting antibody (OLD +IL6AB) (A)

4.3.3 Prostate epithelial cell migration and invasion induced by sera from OLD and AM mice

To determine whether the aging-associated induction of EMT could result in phenotypical changes in prostate epithelial cells, we assessed the impact of sera from OLD and AM mice on prostate epithelial cell migration **(Fig 4.4)** and prostate epithelial cell invasion (**Fig 4.5**). After 24 hour exposure to sera, we observed that sera from AM and OLD mice moderately increased migration of prostate epithelial cells. Furthermore, exposure to sera from AM mice increased prostate epithelial cell invasion by 1.8 fold (**Fig 4.5)**. A similar trend was seen when we examined the impact of exposure to OLD sera on prostate epithelial cell migration. OLD sera stimulated greater cell migration in comparison to WT sera. Overall, these preliminary results strongly suggest that aging-associated circulating factors may promote early phenotypical chances related to prostate tumorigenesis.

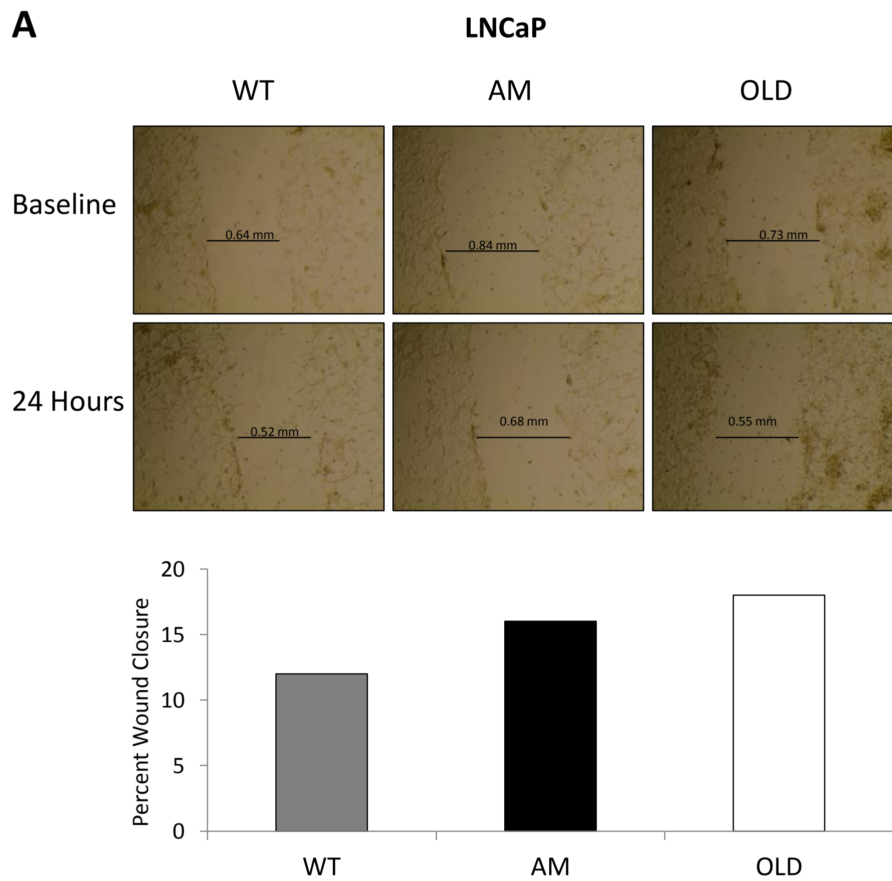

Figure 4.4 Circulating Factors in the sera from AM and OLD mice promote migration of prostate epithelial cells

Wound Healing Assay was used to measure cell migration in response to sera from GPAT-1$^{-/-}$ Aging Mimic (AM), young wild-type (WT) or old wild-type (OLD) in LNCaP (A). Cells were exposed to sera for 24 hours and pictures were taken at 0, 12, 24 and 48 hours after wounding. Bars represent cell coverage percentages.

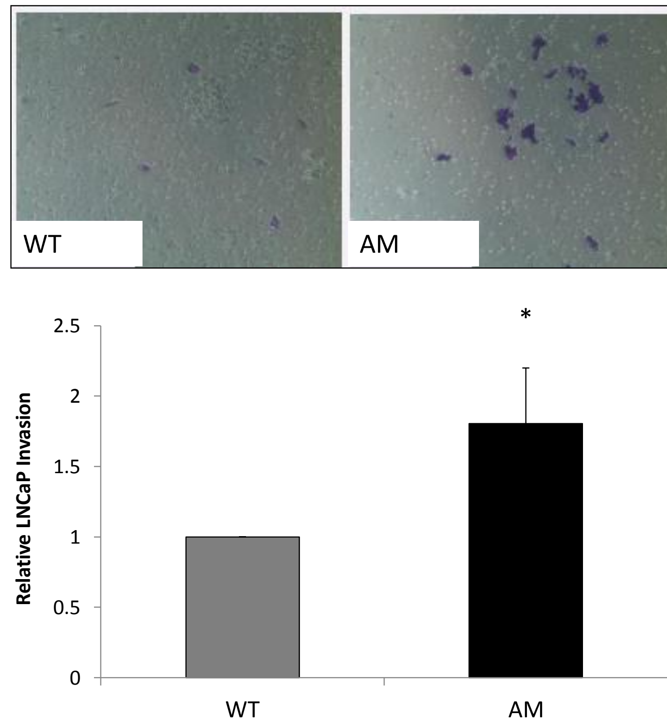

Figure 4.5 Circulating Factors in the sera from AM mice promote invasion of prostate epithelial cells

Cell invasion assay using 24- trans well plate was used to measure cell migration in response to sera from GPAT-1$^{-/-}$ Aging Mimic (AM) or young wild-type (WT) in LNCaP (A) cells. Presented is the average of three independent experiments standardized to WT. Different letters indicate significant differences ($p<0.05$).

4.4 Discussion

One of the most significant risk factors for prostate cancer development is age [81]. One widely accepted explanation for the association between age and prostate cancer is the fact that prostate carcinogenesis is a characteristically long process [75] . Another possible mechanism responsible for prostate tumorigenesis in the elderly is aging-associated dysregulation of immune function. Aging is positively correlated with increased circulating levels of many pro-inflammatory cytokines such as interleukin-1 beta (IL-1β), tumor necrosis factors alpha (TNFα), interleukin-6 (IL-6) and interleukin-17 (IL-17) [83].

Tumorigenesis involves the acquisition of genetic and epigenetic changes that cause the aberrant loss or gain of functions by cellular proteins. The consequences include the ability of tumor cells to proliferate, resist apoptosis, demonstrate angiogenic potential, migrate, and invade, as well as the ability of these cells to evade immune surveillance. IL-6 can induce tumor initiation through activation of STAT3. Activated STAT3 has been linked to tumor initiation in part through the transcriptional regulation of critical target genes involve in epithelial-mesenchymal transition [40, 103-105].

Data presented in previous chapters demonstrated that IL-6 and IL-17 in the AM and OLD mice activate pro-inflammatory signaling in prostate epithelial cells that could lead to initiation of prostate tumorigenesis. In this chapter I presented data showing the influence of IL-6 and IL-17 on activating targets of STAT3 that induce aged related

tumorigenesis. The activation of STAT3 in combination with the previously observed activation of NF-κB could be crucial for mediation of tumor initiation in non-transformed prostate epithelial cells. Tumor initiation is a process in which normal cells acquire the first mutational hit that sends them on the tumorigenic track by providing growth and survival advantages over their neighbors [114, 115]. Various investigators have previously demonstrated that constitutively activation of STAT3 can transform immortalized fibroblasts and breast epithelial cells, in part as a consequence of increased cyclin D1 and MMP-9 expression [105]. However, the levels of neither of these targets were increased in non-transformed RWPE-1 cells expressing constitutively active STAT3 [104], suggesting that there are additional STAT3 targets which are important for tumor initiation rather than tumor progression.

Azare *et al.* demonstrated that the introduction of an activating mutant form of STAT3 into non-tumorigenic RWPE-1 prostate epithelial cells resulted in tumorigenesis. Constitutively activated STAT3 decreased E-cadherin levels, increased numbers of lamellipodia and stress fibers, and enhanced migratory capacities, with an associated increase in the expression of EMT markers [104].

In the study described in this chapter we focused our attention on the role of IL-6 and IL-17, in the sera from OLD and AM mice, in inducing expression of STAT3 and its key downstream targets. We specifically focused on Slug, Snail and Twist, given their known roles in early stages of tumorigenesis [116, 117]. We demonstrated that IL-6 and

IL-17 in the sera from OLD and AM mice are essential for activating STAT3 in prostate epithelial cells. Our results showed that STAT3 activation by circulating factors in the sera from OLD and AM mice led to significant increases in the expression of Slug and Twist. Our findings strongly suggested that IL-6 and IL-17 in the sera from AM and OLD mice induced EMT through up-regulation of Slug and Twist, thereby favoring prostate epithelial cell migration and invasion. Furthermore, our *in vitro* experiments showed that stimulation with sera from OLD and AM mice could markedly increase migratory and invasive ability of prostate epithelial cells. Transcription factors, such as Snail, Slug, and Twist, are pivotal activators of EMT [110]. Despite lack of direct evidence, our findings strongly suggested that IL-6 and IL-17 in the sera from OLD and AM induced EMT through up-regulation of Slug and Twist, thereby favoring prostate epithelial cell migration and invasion.

In summary, the present study provides evidence that the aging-associated increase in circulating IL-6 and IL-17 promotes activation of STAT3 signaling in prostate epithelial cells. Previously we demonstrated that T cells from our aging-mimic mice secrete elevated levels of IL-17, which in turn activate NF-κB. STAT3 and NF-κB have crucial and integrated roles in inflammatory responses that promote prostate cancer development and growth [111]. The elevated levels of circulating IL-6 and increased production of IL-17 during aging may be actively promoting prostate cancer development via activation of NF-κB and STAT3. Here we showed preliminary evidence

that demonstrates that circulating factors in the sera from AM and OLD mice induce EMT, prostate epithelial cell migration and invasion, possibly via activation of the NF-κB and STAT3 pathways.

Chapter 5: Concluding Remarks

5.1 Conclusions

The population of the United States is getting older, due not only to aging boomers but also to an increase in life expectancy [118]. An aging population means increased diagnosis of prostate cancer. In a society where life expectancy has increased, it is important to determine how aging is related to prostate cancer risk. The goal of my thesis was to better understand the mechanism by which age-related changes of the immune system may contribute to increased susceptibility to prostate cancer. The aging process affects the adaptive cell-mediated immune response while causing a shift to a more inflammatory cytokine profile. I hypothesize that the age-related shift in T-lymphocyte cytokine profile is a significant contributing factor to the association between age and prostate tumorigenesis.

To test my hypothesis I used the GPAT-1$^{-/-}$ mice model of aging T cells. The GPAT-$^{-/-}$ mouse serves as a novel model of accelerated T-lymphocyte aging since its T-lymphocytes possess multiple hallmarks of aging [28-31, 59]. The GPAT-1$^{-/-}$ mouse provides a unique opportunity to investigate the effects of aging T-lymphocytes and inflammation on prostate cancer development in the absence of other age-related complications. Our data first demonstrated that T-lymphocytes from the GPAT-1$^{-/-}$ mouse up-regulate the production and secretion of pro-inflammatory cytokines. Many of the pro-inflammatory cytokines produced by aged T-lymphocytes have been

implicated in the promotion of prostate tumorigenesis, primarily through the induction of other pro-inflammatory pathways and the activation of transcription factors like nuclear factor-kappa B (NF-κB). Analogous engagement of these pathways is recapitulated in the GPAT-1$^{-/-}$ mice model, strongly suggesting that the aged immune system plays a critical role in promoting prostate carcinogenesis. To further characterize and compare the relative cytokine expression profile from T-lymphocytes from young GPAT-1$^{-/-}$ mice to those derived from young and old (>22 month) wild-type (WT) mice, a cytokine immunoarray was used. Consistent with our previous observation, the inflammatory phenotype of GPAT-1$^{-/-}$ T-lymphocytes resembled that which is characteristic of aged murine T-lymphocytes (**Fig 2.1**). While the three groups had similar levels of several of the cytokines and chemokines (IL-1α and IL-1β, for example), the knock-out and old mice trended together for many others, including MIP1α and β, RANTES, IL-3 and I-309. Of note, compared to that found in the wild-type young mice, levels of IL-17 were 12- and 8-fold higher in the GPAT-1 $^{-/-}$ and old mice, respectively. Levels of IL-6 were more than ten times higher in the old mice compared to the GPAT-1$^{-/-}$ and WT mice, suggesting that in old mice the presence of aged cells and tissue induce the further secretion of IL-6.

Cytokines secreted by aging T-lymphocytes can induce pro-inflammatory signaling that promotes activation of the NF-κB pathway, which has previously been shown to be critical for prostate cancer progression [62-66, 77]. Therefore, to determine

how cytokines from the aged and aging-mimic GPAT-1$^{-/-}$ T-lymphocytes affect NF-κB transcriptional activity, non-transformed prostate epithelial cells and prostate cancer cells were exposed to sera from young WT, old WT or young GPAT-1$^{-/-}$ aging mimic mice (AM) (**Fig 2.2)**, as well as to conditioned media from young WT, old WT or GPAT-1$^{-/-}$ splenic T-lymphocytes **(Fig 2.3).** Circulating factors in the old WT and young GPAT-1$^{-/-}$ mouse sera both induced significantly greater NF-κB transcriptional activity in the PC-3 and LNCaP prostate cancer cell lines (**Fig 2.2A)** in comparison to the young WT mouse sera, suggesting that the age-specific shift in T-lymphocyte function may be partly responsible for the increased NF-κB activity. Further, we observed a greater than 3-fold induction of NF-κB transcriptional activity in the non-transformed prostate epithelial cells (PrEC and RWPE-1) after exposure to young GPAT-1$^{-/-}$ mouse sera (**Fig 2.2B),** indicating that induction of NF-κB activity by the age-related elevation in circulating cytokines is not restricted to cancer cells. This age-related NF-κB induction in the non-transformed epithelial cells could play a role in their transformation to prostate cancer cells, suggesting one mechanistic link to explain why age is a risk factor for the development of prostate cancer. In addition, we observed that factors secreted specifically by GPAT-1$^{-/-}$ and old WT T-lymphocytes also induced NF-κB in the LNCaP prostate cancer cells, as well as in the non-transformed RWPE-1 cells, suggesting that the aged and GPAT-1$^{-/-}$ T-lymphocytes directly modulate NF-κB activity in prostate epithelial and prostate cancer cells.

Since the initial data indicated that T-lymphocytes from GPAT-1 $^{-/-}$ secrete high levels of IL-17, a key cytokine known to promote carcinogenesis, and that transcription factors like NF-κB are critical for both inflammation and tumor growth, we sought to determine the role of IL-17 in GPAT-1$^{-/-}$-induced NF-κB activity (**Fig 3.3)**. shRNA was used to knockdown the receptor for IL-17 (IL-17R) in prostate cancer cells (LNCaP) and non-transformed prostate epithelial cells (RWPE-1). NF-κB activation was assessed in the IL-17R shRNA clones exposed to sera from the experimental and control mice. In the absence of IL-17R, there was a significant reduction in NF-κB transcriptional activity induce by old WT and GPAT-1$^{-/-}$ mouse, in both LNCaP and RWPE-1 cells **(Fig 3.3).** This data indicates that the increased secretion of IL-17 from the GPAT-1$^{-/-}$ T-lymphocytes is playing a crucial role in the activation of pro-inflammatory signaling in prostate cancer and normal epithelial cells.

Since our data demonstrated that sera from old mice has high levels of IL-6, an important cytokine that promotes pro-oncogenic inflammatory pathways. We sought to determine the role of age related IL-6 and IL-17 expression in inducing pro-tumorigenic mediators in prostate epithelial cells. Our results demonstrate that increased production of IL-6 and IL-17 in the old mice induce the STAT3 signaling pathway. (**Fig 4.1**) IL-17 coming from aging T-cells, in combination with elevated IL-6 levels, induces pro-inflammatory pathways that promote prostate tumor initiation and progression. The elevated levels of circulating IL-6 and increased production of IL-17 during aging may be

actively promoting prostate cancer development via activation of NF-κB and STAT3. Preliminary evidence demonstrates that circulating factors in the sera from AM and OLD mice induce EMT, prostate epithelial cell migration and invasion, possibly via activation of the NF-κB and STAT3 pathways.

In summary, the results from this thesis indicate that aged T cells produce circulating factors that induce pro-inflammatory pathways such as NF-κB and STAT3, leading to induction of factors that promote EMT changes, proliferation and further inflammation. Furthermore, results from these studies suggest that the increased production of IL-17 by aged T-cells in combination with aged-related increase in IL-6 secretion induces pro-inflammatory pathways in prostate cells that in turn contribute to a more malignant phenotype in the prostate.

This study has important implications for our current understanding of the contribution of the immune system to prostate cancer development. The data presented in this dissertation strongly supports the concept that the aging immune system actively promotes cancer development and progression, possibly due to the induction of chronic inflammation in the microenvironment that results in upregulation of pathways leading to neoplastic changes. This contribution to the understanding of the role of the aging immune system in cancer development opens new avenues for development of potential preventive interventions as well as screening biomarkers.

5.2 Future Directions

In our second chapter we used a cytokine array to characterize and compare the relative cytokine expression profile from T-lymphocytes of young GPAT-1$^{-/-}$ (AM) mice to those derived from young and >22 month old (OLD) and wild-type (WT) mice. We demonstrated that the AM T-lymphocytes as well as the OLD T-lymphocytes secrete increased amount of pro-inflammatory cytokines, such as IL-17, IL-6, MIP-1 and I-309 (**Fig 2.1**). On chapter three and four we focused our attention on the role that IL-17 and IL-6 play in inducing prostate tumorigenesis. For future studies it is important to study the impact of MIP-1 and I-309 in prostate tumorigenesis.

MIP1α and β levels are 20-fold and 10-fold higher in the sera from OLD mice versus WT mice. MIP-1 proteins are major factors produced by macrophages after stimulation from bacterial infection. They are crucial for immune responses towards infection and inflammation. MIP-1 proteins also induce the synthesis and release of other pro-inflammatory cytokines such as interleukin 1 (IL-1), IL-6 and TNF-α from fibroblasts and macrophages [119]. Since these proteins are potent inducers of inflammation and they are highly elevated in AM and OLD mice, it is important for future studies to examine the role of MIPα and β in prostate cancer development. Knocking-down the receptor for IL-17R strongly diminished the activation of NF-κB and STAT3 by sera from OLD and AM mice, suggesting that IL-17 in the sera is mainly responsible for activating these pro-inflammatory pathways in prostate epithelial cells.

It is important to understand why MIP-1 is not playing such an important role in activating NF-κB in prostate epithelial cells. It is also interesting to notice that I-309 expression levels are 16- and 11 fold higher in the OLD and AM mice compared to the WT mice. I-309 is secreted by activated T cells and attracts monocytes, NK cells, and immature B cells. Since elevated amounts of I-309 in the sera from AM and OLD mice may further increase inflammation, it is important to investigate to role of I-309 in prostate cancer initiation and progression.

For future studies is also imperative to understand the interaction between aging prostate epithelial cells and aging T cells. In order to understand the interaction between aging prostate epithelial cells and aging T cells in the prostate microenvironment, we first sought to determine the type of T cells that were infiltrating the prostate from AM and OLD mice. Preliminary results indicate that there are more T_H17 T cells in the prostate from AM and OLD mice versus the WT mice **(Appendix 1).**

We also did a series of preliminary *in-vitro* studies where we exposed T cells from human to conditioned media from senescent prostate epithelial and fibroblast cells and measured cytokine expression on the T cells. For these studies we used Jurkat T cells. Jurkat cells are an immortalized line of human T cells that are used to study acute T cell signaling. Jurkat cells are also useful for T cell signaling studies because of their ability to produce interleukin 2. Our preliminary studies demonstrated that conditioned media from senescent prostate epithelial cells was able to regulate Jurkat T

cell IL-2 expression (**Appendix 2**). Suggesting that aged prostate cells can potentially affect the phenotype of T cells in the prostate microenvironment.

We also studied the potential effect of condition media from aging T cells on recruitment of inflammatory cells to the prostate. Our results demonstrated that conditioned media from aging T cells induce monocyte recruitment to the prostate (**Appendix 3**). This data indicates that the enhanced cross-talk between the immune cells and the epithelial cells in an aging environment can mediate tumor-promoting inflammation. Additional studies need to be done to further characterize the interaction between aging prostate epithelial cells and the aging immune system.

It is also important to further understand the mechanism by which aged T cells become dysregulated. Better understanding of the mechanisms by which the immune system deteriorates and contributes to prostate cancer development may lead to better interventions in the elderly.

Appendix 1: AM and OLD mice have PIN lesions and increased levels of prostate-infiltrating T_H17 cells

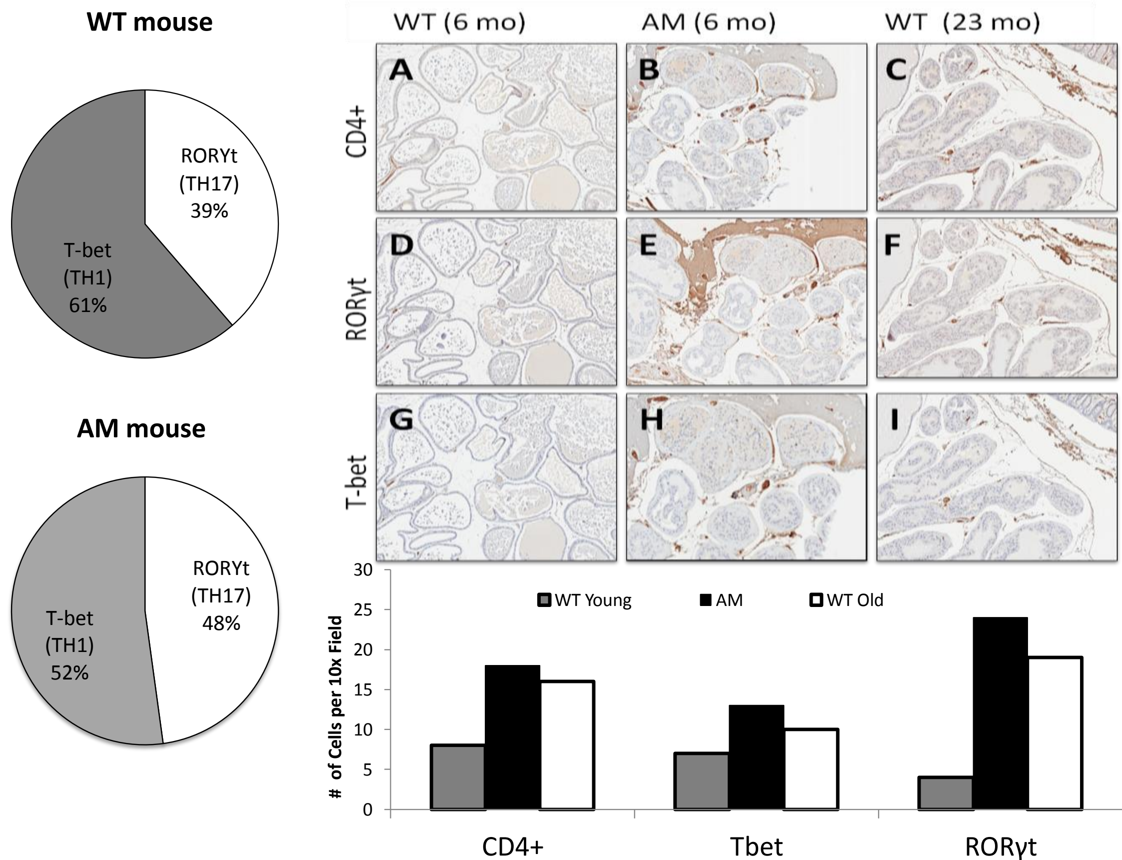

Figure 5.1 T-cell aging mimic mice have PIN lesions and increased levels of prostate-infiltrating TH17 cells.

(A -C) $CD4^+$ Immunohistochemistry staining of prostate tissue from AM, WT and OLD mice. (D-F) RORγt (T_H17 marker) immunohistochemistry staining of prostate tissue from AM, WT and OLD mice. (G-I) T-bet (T_H1 marker) immunohistochemistry staining of prostate tissue from AM, WT and OLD mice. Bar graph shows number of CD4+, T_H1 (Tbet) and T_H17 (RORγt) T cells per 10x field. Pie graph shows the proportion of T_H1 versus T_H17 cells in AM mice versus WT mice.

Appendix 2: Conditioned Media from Senescent Epithelial Cells Decreases T cell IL-2 production

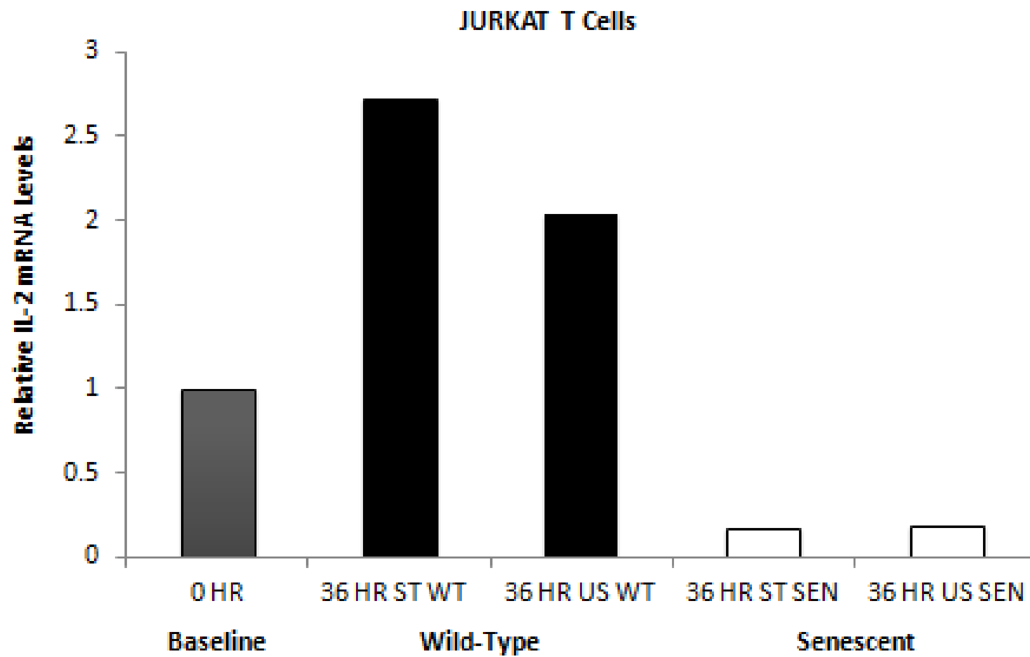

Figure 5.2 IL-2 expression in human Jurkat T cells

IL-2 mRNA levels in stimulated and unstimulated Jurkat T cells were detected by qPCR after exposure to conditioned media from senescent and wild-type prostate epithelial RWPE-1 cells. IL-2 expression levels were 2.5-fold lower in the stimulated Jurkat T cells exposed to conditioned media from senescent RWPE-1 cells versus cells exposed to conditioned media from wild-type RWPE-1 cells

Appendix 3: LNCaP cells treated with Conditioned Media from GPAT-1$^{-/-}$ T cells moderately induce recruitment of monocytes

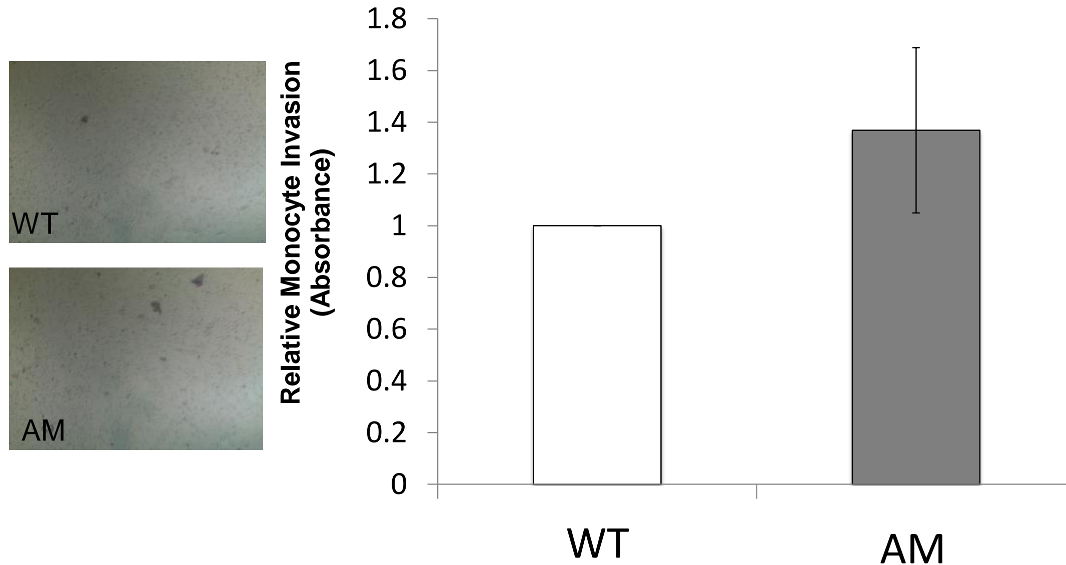

Figure 5.3 LNCaP cells treated with Conditioned Media from GPAT-1$^{-/-}$ T cells moderately induce recruitment of monocytes.

To determine if circulating factors coming from aging T-cells induce the production of specific proteins that recruit monocytes to the prostate, we prepared conditioned media by exposing LNCaP prostate cancer cells to sera from AM mice and preformed an invasion assay to see if agents released in the conditioned media attract monocytes to prostate cancer cells. The conditioned media from LNCaP cells exposed to sera from the GPAT-1 KO mice induced monocyte invasion by 37% as compared to control (WT).

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Vita

Alejandra De Angulo was born in Bogota, Colombia. She graduated from the University of Texas in 2010 with a degree in nutrition and began graduate school the next semester. Alejandra has worked as a Teaching Assistant for several undergraduate nutrition courses. In 2010 Alejandra was awarded the AACR- Bristol-Myers Squib Oncology Scholar-in-Training Award for her poster presentation at the American Association of Cancer Research annual meeting. In 2012 she was awarded two travel awards: Keystone Symposia Scholarship to attend a symposium on The Role of Inflammation during Carcinogenesis in Dublin, Ireland and The AACR Minorities in Cancer Research Award to attend the American Association of Cancer Research annual meeting. Alejandra demonstrated strong leadership skills as President for the Nutrition Graduate Student Association and was an outstanding ambassador for the Department of Nutritional Sciences as the Jean Andrews Centennial Fellow in Human Nutrition. Her service to the Department and University were evident with the numerous positions she filled during her time as both an undergraduate and graduate student. Next year, Alejandra will be a postdoctoral fellow at the Children's Nutrition Research Center at Baylor College of Medicine. Eventually, Alejandra plans to become a professor and a nutrition researcher.

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