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The role of the microenvironment and Th17 cells in the carcinogenesis of head and neck squamous cell carcinoma

by

Danielle Woodford

A thesis submitted to the faculty of the Medical University of South Carolina in partial fulfillment of the requirements for the degree Masters of Science in the College of Graduate Studies.

Department of Microbiology and Immunology

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Approved by:

M. Rita Young, PhD
Chair of Advisory Committee

Amanda C. LaRue, PhD

Terrence A. Day, MD

Julie A. Woolworth, PhD

Xian-Kui (John) Zhang, PhD

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ABSTRACT

Head and neck cancer is the sixth most common cancer worldwide. Despite advances in diagnosis and treatment, the survival rates for patients with head and neck cancer have remained relatively unchanged for the past 30 years. Head and Neck Squamous Cell Carcinoma is a highly aggressive malignancy with a 5-year survival rate of only 50%. Of the patients diagnosed every year, 5% of head and neck squamous cell carcinoma (HNSCC) patients develop additional primary tumors, an effect that is thought to be associated with the high degree of immune suppression induced by the tumor. During the premalignant stage of HNSCC, there is an increase in the number of helper T subset Th17 cells, which then decreases in fully established HNSCC. The focus of our laboratory is on delineating the role of Th17 cells in HNSCC tumor development. Thus, the goal of this study is to elucidate the role of the tumor microenvironment in the decrease in Th17 cells observed in established HNSCC. We hypothesize that the decrease in Th17 cells is due to changes that occur in the tumor microenvironment during the transition from premalignant tissue to established HNSCC. To examine this, we characterized the cytokine levels in normal, premalignant and HNSCC tongue tissues. We also investigated the role of the microenvironment in the decrease in Th17 cell numbers observed in fully developed HNSCC compared to the premalignant stages. Our data showed an inflammatory response at the microenvironment which included both an increase in inflammatory cytokines as well as an increase in Th17 cells. This response was later attenuated during the HNSCC stage. When incubated with supernatant from premalignant tissue, Th17 cells maintained a

higher level of IL-17 when compared to incubating with supernatant from control or HNSCC tissue. The opposite was found when splenocytes were incubated with these same supernatants. The levels of IL-17 dropped when incubated with supernatant from premalignant tissue when compared to control or HNSCC.

Chapter 1

Tumor environment and HNSCC

CHAPTER 1: TUMOR ENVIROMENT AND HEAD AND NECK SQUAMOUS CELL CARCINOMA

1.1 Clinical development of HNSCC

Despite advances in diagnosis and treatment, the survival rates for patients with head and neck cancer have remained relatively unchanged for the past 30 years [1]. HNSCC is a highly aggressive malignancy with a 5-year survival rate of only 50%. Of the patients diagnosed with HNSCC, 5% of HNSCC patients develop additional primary tumors [2-3]. This is thought to be associated with the high degree of immune suppression induced by the tumor [4-5]. Treatment of this malignancy is further complicated by the significant morbidity associated with surgery. Identification of the differences in the tumor microenvironment during the different stages of HNSCC pathogenesis would provide a foundation for the development of immunotherapeutic interventions for patients diagnosed with premalignant lesions or HNSCC.

HNSCC develops from keratinizing epithelial cells, most commonly at the lateral and ventral surfaces of the tongue, the anterior floor of the mouth, and the lip [6-7]. Initially asymptomatic, the first indication of disease is the appearance of thick adherent plaques, known as leukoplakias or erythroplakias [6]. If left untreated, these lesions can develop into

exophytic or ulcerative malignant tumors [6]. Though premalignant lesions may be removed, patients with high risk plaques have a significant risk of developing secondary premalignant lesions and HNSCC [6-8]. Local recurrence and regional lymph node spread are the predominant causes of the high mortality observed in patients with HNSCC [5-8]. Because it is believed that immune suppression in these patients contributes to morbidity and recurrence, attenuating this immune suppression represents a potential therapeutic option.

1.2 Immunosuppression in established HNSCC

Head and neck squamous cell carcinomas (HNSCCs) are associated with abnormal cell-mediated immunity at the primary tumor site [9]. The level of immunosuppression in patients with HNSCC varies widely. Immunosuppression in HNSCC has been postulated to occur in a hierarchical manner, in which the primary tumor region is the most affected site, followed by the draining lymph nodes [10]. This hierarchical phenomenon suggests that immunosuppression in HNSCC patients is mediated by a regional network of factors. It is likely that immune suppression at the site of the tumor and affected lymph nodes plays a role in limiting the efficacy of current immunotherapy protocols. It also likely that the immune system exerts a selective pressure that gives rise to the tumor variants that are then able to manipulate the immune

system [11-16]. A greater understanding of the mechanisms of local inhibition of immune function will aid in improving adoptive immunotherapy for the treatment of cancers [10].

While the onset of head and neck cancer is linked to environmental carcinogens (tobacco, alcohol), tumor progression itself appears to be linked to failure of the immune system [17]. In addition to escaping immune system surveillance, some head and neck cancers are also able to corrupt the antitumor response via several mechanisms [18]. These include targeting the antigen presenting machinery (APM) via the down regulation or loss of expression of human leukocyte antigen (HLA) class I molecules and/or other components of the APM [11,19]. Although effective antitumor immune responses likely involve multiple components of the immune system, T-cells are considered the most critical cells involved in antitumor immunity.

Recent evidence suggests that antitumor responses in HNSCC patients are compromised by functional defects or apoptosis of T-cells, both circulating and tumor-infiltrating [13-16]. Hoffmann et al. showed that the Fas/FasL pathway is involved in the spontaneous apoptosis of circulating Fas^+ T lymphocytes [16]. In fact, tumor-infiltrating lymphocytes (TILs) look like activated T cells but are functionally compromised. They can have absent or low expression of the CD3 zeta chain (a key signaling molecule) and they exhibit decreased proliferation in response to mitogens or IL-2 [11]. Some lack the ability to kill tumor cell targets [15-16] or demonstrate an imbalanced cytokine profile, with the striking absence of IL-2 and/or IFN-γ production [20]. There is also evidence of pronounced apoptotic features in a considerable proportion of TILs [11,21]. In addition,

HNSCC cells produce high quantities of TGF-β1, which reduces the expression of natural killer (NK) cell receptor NKG2D and CD16, which inhibits the biological function of NK cells and increases the level of Tregs in peripheral blood mononuclear cells in head and neck cancer patients [22].

1.3 Immunoediting

Immunoediting is a process that is responsible for both eliminating tumors and shaping the immunogenic phenotypes of tumors that eventually form in immunocompetent hosts. **Elimination** is the first stage of this process. Immune cells patrol the body, recognizing transformed cells and eliminating them in a mechanism involving Th1 cells and macrophages. Severe-combined immune-deficiency (SCID) mice or mice that lack recombination activating gene ($RAG^{-/-}$), leads to the absence of T cells, B cells, and Natural Killer cells, exhibit increased susceptibility to chemically-induced tumors [23-25]. Patients with immunodeficiencies are also at an increased risk for carcinogenesis, whether the cancers are of a viral or non-viral etiology [26-28]. In a response known as a type 1 response, T helper type 1 (Th1) lymphocytes secrete interleukin (IL)-2, interferon-γ, and lymphotoxin-α and stimulate type 1 immunity, which is characterized by intense phagocytic activity. This type 1 response was identified as the main effector of immunosurveillance after finding that mice with specific deletion of $CD4^+$ and/or $CD8^+$ cells, antibody mediated neutralization or knockout of IFN-γ, or

knockout of IL-12, perforin or TNF-related apoptosis-inducing ligand (TRAIL) all experienced an increased incidence and/or growth of several different types of cancer [29-33]. Conversely, a type 2 response involves Th2 cells that secrete IL-4, IL-5, IL-9, IL-10, and IL-13, and is characterized by high antibody titers. Type 1 and type 2 immunity are not strictly synonymous with cell-mediated and humoral immunity, because Th1 cells also stimulate moderate levels of antibody production, whereas Th2 cells actively suppress phagocytosis [34].

Once a solid tumor reaches a certain size, it begins to grow invasively and requires an enhanced blood supply, which is facilitated by the production of stromagenic and angiogenic proteins [35]. Invasive growth causes minor disruptions within the surrounding tissue that induce inflammatory signals leading to the recruitment of cells of the innate immune system; natural killer T cells (NKT), natural killer cells (NK), γδ T cells, macrophages and dendritic cells [36-38]. Molecules on the surfaces of transformed cells are recognized by infiltrating lymphocytes (NK, NKT, or γδ T cells), which are stimulated to produce IFN-γ [39-41]. The IFN-γ that is produced by these cells can cause small amounts of tumor death through anti-proliferative [42] and apoptotic [43] mechanisms. It also induces the production of chemokines from tumor cells and the normal surrounding tissue, some of which exert angiostatic effects leading to additional tumor death. Chemokines produced during the inflammation process also recruit NK cells and macrophages to the site. These tumor infiltrating NK cells and macrophages then transactivate one another by reciprocal IFN-γ and IL-12 production to eliminate additional tumor cells via tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), perforin, reactive nitrogen species and nitrogen intermediates [30]. Finally,

tumor-specific $CD4^+$ and $CD8^+$ T cells hone to the tumor site, where the cytolytic T cells destroy the remaining antigen-bearing tumor cells whose evasion ability has been enhanced by exposure to locally produced IFN-γ [23].

During **Equilibrium,** the host immune system and tumor cell variants that have survived the elimination process enter into a dynamic equilibrium. During this stage, lymphocytes and IFN-γ exert potent pressure on the tumor cells that is enough to contain, but not fully extinguish, a tumor bed containing many genetically unstable and rapidly mutating tumor cells. During this time period, although many of the original variants of the tumor are destroyed, new variants arise that contain mutations that provide them with increased resistance to immune attack. This process is likely the longest of the three, occurring possibly even over a period of years [44].

The last phase, **Escape,** involves the surviving tumor variants that have escaped immunologic detection and/or elimination through genetic or epigenetic changes and begin to expand in an uncontrolled manner. This results in a clinically observable malignant disease that, if left unchecked, results in the death of the host [44]. Established tumors have been shown to evade immune recognition through the downregulation of immunogenic markers and/or MHC class I molecules [45-46]. In escape, tumors develop the ability to down-regulate the type I effector response and upregulate the type II effector response, which, while beneficial under certain conditions, is most often associated with a poor prognosis in cancer patients. Reduced IL-12 levels in cancer patients has been shown to reduce the number of Cytotoxic T Lymphocytes (CTLs) and

reduced IFN-γ has been shown to reduce the induction of MHC class I and II and directly induce T cell anergy [47-49]. In contrast to type I cytokines, the type II cytokines IL-4, IL-10 and TGF-β have all been shown to be upregulated in patients with many types of advanced cancers [50-55]. IL-4 directly inhibits Th1 cell differentiation, downregulates IFN-γ production, and upregulates the type II response [56-60]. Like IL-4, IL-10 downregulates IFN-γ production [59]. It has also been shown to reduce IL-12 production, downregulate MHC surface expression, and induce tumor-associated T cell tolerance [61-64]. TGF-β affects the immune system in multiple ways. It has been shown to reduce the differentiation, proliferation and functional capacity of T cells, including downregulation of the Th1 response by inhibition of IL-2 and IFN-γ production by Th1 cells and inhibition of FAS ligand and perforin expression on CTLs [65-71]. TGF-β also inhibits co-stimulation, antigen presentation, maturation and IL-12 production by antigen presenting cells, further downregulating the type I response [72-73]. Finally, TGF-β has been shown to increase tumor production of prostaglandin E_2 (PGE_2) and vascular endothelial growth factor (VEGF), both of which promote tumor growth through multiple pathways [74-77]. These changes in the cytokine milieu contribute significantly to the blockade of a functional antitumor immune response in the microenvironment of many established tumors.

1.4 Th17 cells

Since their discovery only five years ago, Th17 cells have risen to prominence in studies of virology, autoimmune disease, inflammation, and immune responses to various

parasites and fungi. While their role in the pathogenesis of many of these conditions is well defined, their function in the context of tumor immunology remains controversial. Th17 cells are defined as $CD4^+$ helper T cells that secrete IL-17, and whose developmental program is controlled by multiple cytokines and the transcription factor RAR-related orphan receptor gamma T (RORγT) (78).

It has been shown that human tumor-associated Th17 cells express minimal levels of HLA-DR, CD25, and granzyme B, suggesting that they are not a "conventional" effector cell population [79]. Moreover, these cells also do not express programmed cell death 1 (PD-1) or forkhead box P3 (FoxP3), making it unlikely that they induce immune suppression through either pathway [79]. Tumor-associated Th17 cytokine products mimic those found in some instances of viral infection [80-81]. Tumor-associated Th17 cells highly express CXCR4 and CCR6, the c-type lectin receptor CD161, and the CD49 integrin isoforms c, d, and e. Although CCR2, CCR5, and CCR7 are not present on these cells [82], CCR6 and CD161 have been observed both on Th17 cells from healthy donors and on lymphocytes and dendritic cells in inflammatory environments [83-85]. Sharma *et al.* identified a similar Th17 phenotype in a mouse model of cancer [86]. Therefore, it appears that in both human and mouse malignancies, Th17 cells share the same effector cytokine profile.

In ovarian cancer, the prevalence of Th17 cells in tumor draining lymph nodes (TDLN) and blood is comparable to that of healthy donors. Although they make up only a small population of cells within the tumor microenvironment, they are proportionally

higher there in comparison to other immune cell subsets. Tumor-associated Th17 levels correlate positively with microenvironmental Th1 cells, cytotoxic $CD8^+$ T cells, and NK cells, and are inversely correlated with Tregs [82,87]. Su *et al.* also reported significantly higher numbers of Th17 cells expanded or induced from tumor infiltrating lymphocyte populations in cancer patients than from lymphocyte populations from non-tumor tissues [88].

The IL-17 cytokine family can induce the production of other proinflammatory cytokines, chemokines, and prostaglandins. There are six family members (A-F) that are expressed by a variety of innate and adaptive immune cell types. Zhang *et al.* examined IL-17^+ cells in patients with hepatocellular carcinoma (HCC) and suggested a potential pro-tumorigenic role for IL-17. Increased IL-17-producing cell density within HCC tumors correlated with both microvessel density and poor prognosis [89]. Notably, HCC is strongly associated with chronic viral hepatitis, and chronic viral infection may profoundly reshape the generation and function of Th17 cells in cancer patients. In non-small cell lung carcinoma (NSCLC) patients, higher levels of IL-17 within the tumor correlated with higher blood vessel density and shorter survival time [90].

In ovarian cancer patients, Th17 cells were the sole source of IL-17 in the ascites, and the level of IL-17 in this fluid correlated positively with patient survival. Even after debulking, tumor-associated IL-17 was a negative predictor of death hazard. The average survival rate of patients with levels of IL-17 greater than 220 pg/ml in ascites was 78 months, while that of patients with less than 220 pg/ml was 27 months. In the tumor

microenvironment, IL-17 synergized with IFN-γ to induce CXCL9 and CXCL10 production. These Th1-type chemokines recruit effector populations to the tumor itself, and ascites levels of CXCL9 and CXCL10 correlated directly with the prevalence of tumor-infiltrating NK and $CD8^+$ T cells [82]. In agreement with this, Sfanos *et al.* showed an inverse correlation between the levels of Th17 cells in prostate glands and tumor progression [91]. However, in another study examining patients with hormone resistant prostate cancer, Derhovanessian *et al.* demonstrated an inverse correlation between pre-treatment circulating levels of Th17 cells and time to disease progression [92]. A recent report by Zhang, *et al.* found no correlation between Th17 cells and clinicopathological characteristics or survival in patients with nasopharyngeal carcinoma [93]. Ye and colleagues investigated Th17 cells from 30 patients with lung adenocarcinoma or squamous cell carcinoma and found that a higher accumulation of Th17 cells in malignant pleural effusion (MPE) predicted improved patient survival [94]. In some types of epithelial cancer, Th17 cells constitute only a small fraction of the effector T cell population in the tumor microenvironment [78,82,95-96]. This has not been fully investigated in HNSCC.

Because few studies have focused on primary Th17 cells in the tumor microenvironment, it is difficult to predict the exact role(s) they may play in cancer progression. In patients with epithelial cancer, Th17 levels are an indicator of improved patient survival and reduced tumor progression. In mice with established tumors, studies have documented the potent antitumor efficacy of both Tc17 and Th17 populations. It is possible that Th17 function may vary dependent on the cause, type and location of the

cancer [97]. For example, accumulated data suggest that Th17 cells and/or IL-17, along with other factors, induce inflammation and promote the initiation and early growth of tumors in three different murine models of cancer: immune-deficient mice, mice with chemical carcinogen-induced tumors, and mice with pathogen-induced tumors [79]. This occurs despite high levels of IL-6, TGF-β, and IL-1, factors that promote mouse Th17 cell development [98-100], suggesting that Th17 cell development may be suppressed in the tumor microenvironment. In support of this, Th17 cells are tightly regulated by the local cytokine environment [78] and Treg cells inhibit Th17 cell expansion [82,95].

Th17 differentiation is potently driven by TGF-β and IL-6 [101-102] and is reinforced by IL-23 [103-104]. The Th17-specific master transcription factors retinoic acid-related orphan receptor (ROR)γt and RORα, two orphan nuclear receptors, have been recently shown to regulate Th17 differentiation [105-106]. It has been thought that effector differentiation is a "terminal" process for naive Th cells; however, recent results suggest the plasticity of Th subsets. For example, it has been reported that $Foxp3^+$ regulatory T cells can be reprogrammed to become Th17-like cells by IL-6 and other proinflammatory cytokines [107]

Chapter 2

Rationale, Hypothesis, and Specific Aims

CHAPTER 2: RATIONALE, HYPOTHESIS, AND SPECIFIC AIMS

2.1. Rationale

The National Cancer Institute predicted that more than 52,000 men and women in the United States would be diagnosed with head and neck cancers in 2011 [1]. Despite advances in diagnosis and treatment, the survival rates for patients with head and neck cancer have remained relatively unchanged for the past 30 years [1]. HNSCC is a highly aggressive malignancy with a 5-year survival rate of only 50% and in addition, every year, 5% of HNSCC patients develop additional primary tumors [2-3]. HNSCC is associated with the high degree of immune suppression induced by the tumor [4-5], which could be the cause for the level of recurrence. While systemic immune suppression is a hallmark of HNSCC, immune suppression during the premalignant stage is believed to be restricted to the local environment. By identifying the changes that occur in the microenvironment during the transition from normal tissue to premalignant tissue to HNSCC we may better understand the role of immunosuppression and better yet, understand how to prevent it. Because few studies have focused on the role of Th17 cells in the tumor microenvironment, it is difficult to deduce the exact role(s) they may play in cancer progression.

In some cancer types, increased Th17 cells and elevated IL-17 is an indicator of poor prognosis, while in other types of cancer it is indicative of a positive outcome [79]. In mice with established tumors, studies have documented the potent antitumor efficacy of both Tc17 and Th17 populations. Thus, Th17 function may vary dependent on the cause, type and location of the cancer [79].

2.1.1 Hypothesis

To understand the role of the tumor microenvironment in immune suppression, it is crucial to understand the phenotypic differences and cytokine variations that occur during the different stages of HNSCC development. Our laboratory has previously shown that IL-17A levels are decreased in cervical lymph nodes of mice with fully established HNSCC compared to premalignant or control. **We hypothesized that there will be a decrease in IL-17A in the microenvironment resulting from changes that occur during the transition from premalignant tissue to established HNSCC.**

2.1.2. Specific Aims

This hypothesis was tested through the following specific aims:

Aim 1. *Characterization of the cytokine levels in normal, premalignant and HNSCC tongue tissues from both human samples and a mouse model.* 4-nitroquinoline-1-oxide (4NQO) was used to generate premalignant lesions or HNSCC in a mouse model. Tongue tissue was then enzymatically digested and stimulated with PMA and Ionomycin. Using a cytometric bead array, we analyzed the cytokine levels secreted from tissue from normal, premalignant and HNSCC-bearing tongue tissue. Human premalignant and HNSCC tissue was collected through one of the IRB approved protocols and normal adjacent tissue was collected from a tumor bank. The tissue was lysed and the cytokine levels were analyzed using the same cytometric bead array.

Aim 2. *Investigation of the role of the microenvironment in the decrease in Th17 cell numbers observed in HNSCC tissue compared to premalignant tissue.* Polarized mouse Th17 cells were exposed to supernatant from normal, premalignant or HNSCC tongue tissue to determine the effects of the tumor microenvironment on the Th17 cell population. Supernatants from this co-incubation were collected and cytokine levels were analyzed by cytometric bead array. The same supernatants were then co-incubated with splenocytes and the cytokine levels from this were also analyzed by cytometric bead array.

2.2. Importance

This aim of this study is to further define the role of the microenvironment in the progression of HNSCC. HNSCC is the sixth most common cancer worldwide, with few treatment options and a high mortality rate. A better understanding of the mechanisms by which this disease progresses will aid in the development of more effective treatments.

Chapter 3

Characterization of Cytokines in the Tumor Microenvironment

CHAPTER 3: CHARACTERIZATION OF CYTOKINES IN THE TUMOR MICROENVIRONMENT

Aim 1: ***To characterize the cytokine levels in normal, premalignant and HNSCC tongue tissues from both human samples and a mouse model.***

3.1 Introduction

The National Cancer Institute predicted that more than 52,000 men and women in the United States would be diagnosed with head and neck cancers in 2011 [1]. Despite advances in diagnosis and treatment, the survival rates for patients with head and neck cancer have remained relatively unchanged for the past 30 years [1]. HNSCC is a highly aggressive malignancy with a 5-year survival rate of only 50%. In addition, every year, 5% of HNSCC patients develop additional primary tumors [2-3], an event thought to be associated with the high degree of tumor-induced immune suppression [4-5]. While systemic immune suppression is a hallmark of HNSCC, immune suppression during the premalignant stage of HNSCC is believed to be restricted to the local environment. By identifying the changes that occur in the microenvironment during the transition from normal tissue to premalignant tissue to HNSCC, we may better understand the role of immunosuppression. More importantly, this knowledge may allow us to prevent it. A

greater understanding of the mechanisms of local inhibition of immune function will aid in improving adoptive immunotherapy for the treatment of cancers [10]. Here, we characterize the microenvironments found in normal, premalignant and HNSCC tissue to better understand the mechanisms by which the local immune system is suppressed and which cell types are affected.

3.2 Materials and Methods

Oral HNSCC carcinogenesis

Carcinogen-induced oral premalignant lesions and HNSCC was established in mice by the administration of 4NQO at 5 mg/ml in propylene glycol stock in the drinking water (diluted to 50 μg/ml) of 2-month old (at start) female C57BL/6 mice. 4NQO was left in their water for 8 weeks, to induce the development of premalignant oral lesions detectable on the tongue, or for 16 weeks to establish HNSCC. To monitor the development of premalignant oral lesions and HNSCC, the oral cavities of the mice were examined by endoscopy using a Stryker 1.9 mm x 30° endoscope and a Stryker 1088 HD camera. The mice were sedated with inhaled isoflurane (Piramal Healthcare) during this procedure.

Oral carcinogenesis classification

To monitor the development of premalignant oral lesions and HNSCC, the oral cavities of the mice were examined by endoscopy using a Stryker 1.9 mm x 30° endoscope and a Stryker 1088 HD camera. The mice were sedated with inhaled

isoflurane (Piramal Healthcare) during this procedure. Classification was done based on the size and type of lesion. For those only classified as premalignant and HNSCC, the lesions are considered premalignant when leukoplakias are present that are flush with the tongue (Fig. 3.1.B), the lesions are considered HNSCC once they become exophytic (Fig. 3.1.D) For those considered early and late premalignant or HNSCC, early premalignant is the first sign of a leukoplakia (Fig. 3.1.B), late premalignant is when there are extremely large or multiple leukoplakias (Fig. 3.1.C). Early HNSCC is the first appearance of an exophytic lesion (Fig. 3.1.D), late HNSCC is presence of an extremely large or multiple exophytic lesions (Fig.3.1E).

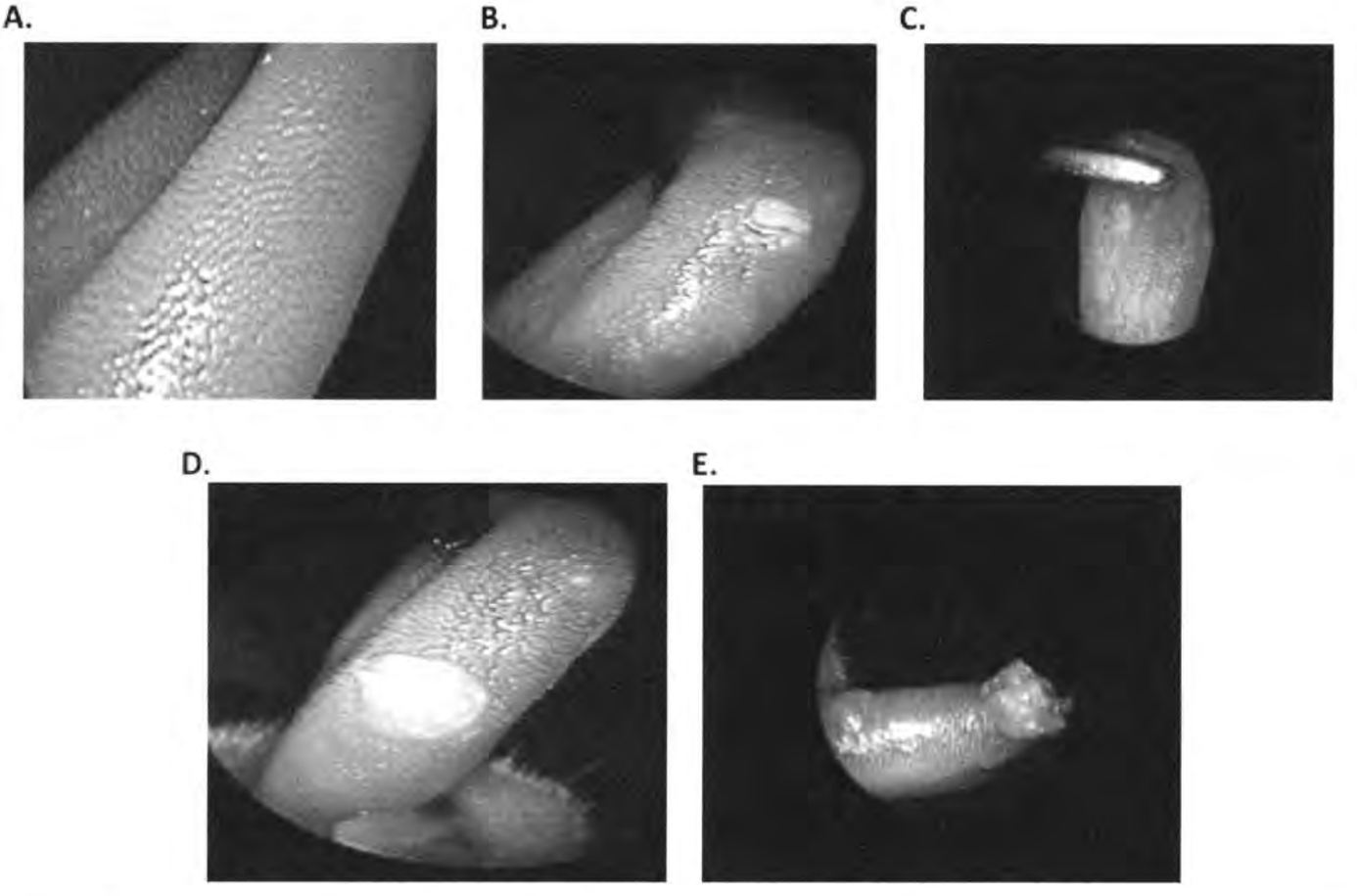

Figure 3.1. Levels of IL-6, IL-2, and TGF-β change in the microenvironment during the progression of HNSCC. Pictorial representation of the stages of carcinogenesis resulting from 4NQO treatment. Normal tongue (A), Stage is considered premalignant when leukoplakias are present that are flush with the tongue (B), Stage is considered late premalignant when there are extremely large or multiple leukoplakias (C), Stage is considered HNSCC once the lesions become exophytic (D), Stage is considered late HNSCC when the exophytic lesions are extremely large or there are multiple exophytic lesions (E).

Tongue tissue processing

Control, premalignant, and HNSCC tongues were harvested from C57BL/6 mice and minced using a scalpel. The tissue was then enzymatically digested at 37˚C with 0.23U of Liberase (Roche) for 4 hrs. The tissue was washed with PBS and allowed to recover overnight.

Human samples

Recruitment of patients into this study was approved by the Institutional Review Board of record. Tissue samples were collected from patients bearing premalignant lesions or HNSCC tumors. Normal, non-carcinogenic oral tissue was procured from the adjacent areas bordering the cancerous tissue, and these tissues were deemed pathologically normal with no microscopic evidence of invasive carcinogenic disease. These samples were cryopreserved at -80°C until they were used for cytokine analysis.

Cytokine bead array

All reagents used in this assay were from BD unless otherwise specified. The day following the enzymatic digestion, the tongue tissue was stimulated for 4 hours at 37°C with 50 ng/ml PMA and 1 μg/ml ionomycin without the addition of Brefeldin A. Both mouse and human tissues were lysed through sonication and protein concentrations were determined by BCA protein assay (Pierce). Final cytokine levels in tissue were expressed as pg/100 μg of protein. The levels of IFN-γ, IL-2, IL-17 (IL-17A), IL-4 and IL-10 in tissue lysate or supernatant were determined using a cytometric bead array Th1/Th2/Th17 cytokine kit, while the levels of active TGF-β in tissue lysate or supernatant were determined using cytometric bead array flex sets according to the manufacturer's instructions. Relative amounts of each cytokine were analyzed using FCAP Array software.

Statistical analysis

Data are reported as the measure ± standard deviation of the value. To compare one variable condition between groups, the two-tailed Student's *t*-test was used. Significance was reported in the 95% confidence interval.

3.3 Results

Changes in the tumor microenvironment during the progression of HNSCC.

Head and neck squamous cell carcinomas (HNSCCs) are associated with abnormal cell-mediated immunity at the primary tumor site [9]. The level of immunosuppression in patients with HNSCC varies widely. Immunosuppression in HNSCC has been postulated to occur in a hierarchical manner, in which the primary tumor region is the most affected site, followed by the draining lymph nodes [10]. This hierarchical phenomenon suggests that immunosuppression in HNSCC patients is mediated by a regional network of factors. It is likely that suppression at the site of the tumor and the affected lymph nodes plays a role in limiting the efficacy of current immunotherapy protocols [11-16]. Although effective antitumor immune responses likely involve multiple components of the immune system, T-cells are considered the critical cells involved in antitumor immunity.

Recent studies have shown that some of these cells lack the ability to kill tumor cell targets [15-16] or demonstrate an imbalanced cytokine profile, with a striking absence of IL-2 and/or IFN-γ production [20]. HNSCC cells also produce high quantities of TGF-β1, which reduces the expression of the natural killer (NK) cell receptor NKG2D and CD16, inhibits the biological function of NK cells, and increases the level of Tregs in peripheral blood mononuclear cells in head and neck cancer patients [22].

To determine the composition of cytokines in the tumor microenvironment, tongue tissue samples taken from 4NQO-treated mice at the premalignant stage or with fully established HNSCC were processed and analyzed. Cytokine levels were determined by flow cytometric analysis using a cytometric bead array. The premalignant mice showed a marked increase in both IL-2 (Fig 3.2.a) and IL-6 (Fig 3.2.b) compared to control mice and mice with established HNSCC. They also showed a decrease in TGF-β during the early premalignant stage, which increased during the late premalignant stage (Fig 3.1.c). This data indicated that the early premalignant stage is marked by an inflammatory response at lesion site that decreases once the premalignant lesions develop into fully established HNSCC tumors.

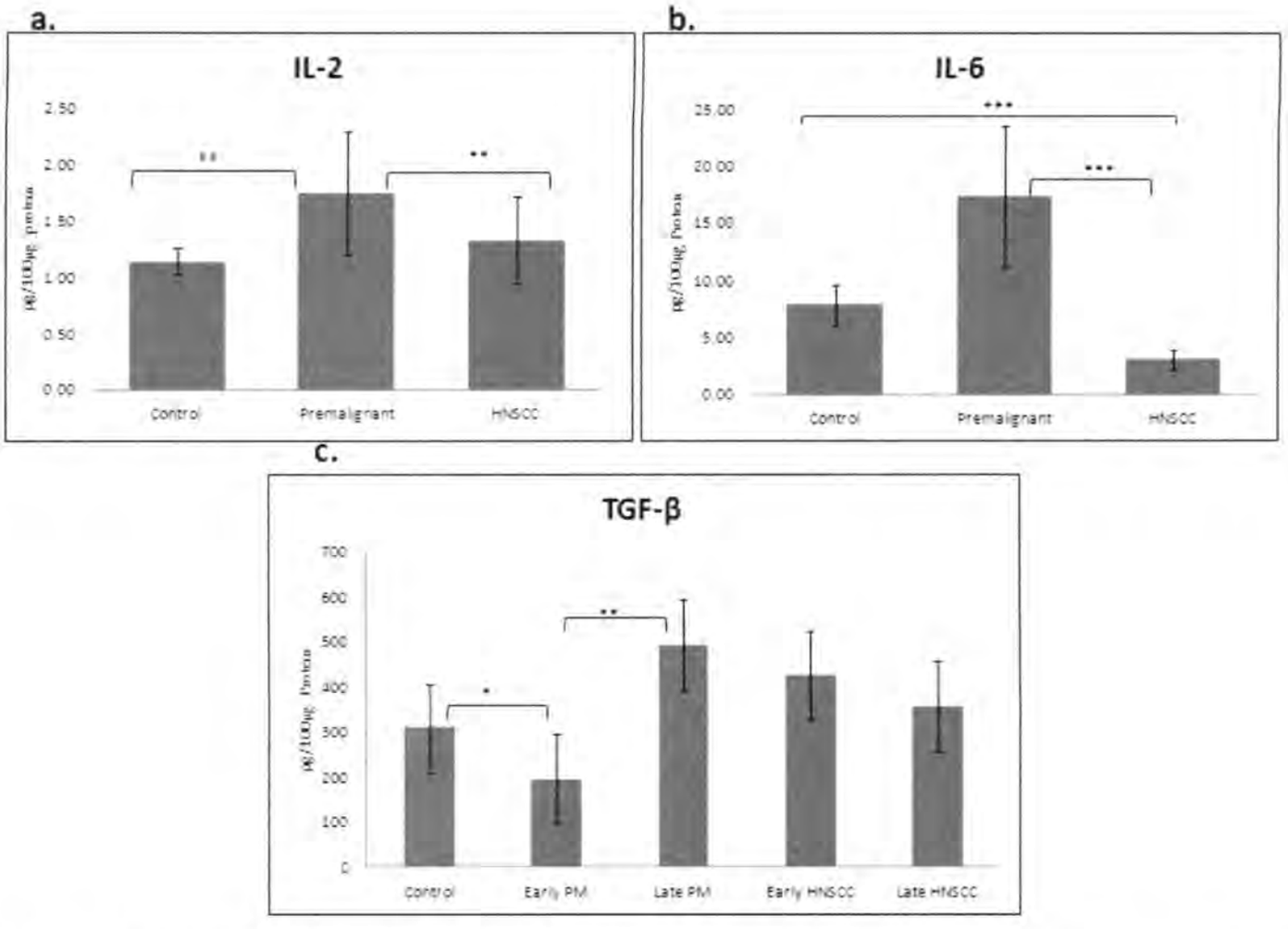

Figure 3.2. Levels of IL-6, IL-2, and TGF-β change in the microenvironment during the progression of HNSCC. Graphical representation of the results of cytometric bead array analysis of control, 4NQO-treated premalignant, and 4NQO-treated HNSCC tissues. For graph A and B; control (n= 7), premalignant (n=6), HNSCC (n=16). For graph C; control (n= 7), early premalignant (n=5), late premalignant (n=3), early HNSCC (n=4), late HNSCC (n=13). Data are presented as the mean ± standard deviation. *, $p < 0.05$; **, $p < 0.01$; *** $p < 0.001$ (Two-tailed Student's t-test).

IL-17A levels are increased in premalignant stage compared to control and HNSCC in human samples and the 4NQO mouse model.

Patient samples of normal/adjacent, premalignant, and HNSCC tissue were collected and analyzed by cytometric bead array after being lysed. Control, premalignant, and HNSCC tongue tissues were collected from 4NQO treated mice. The tissue was stimulated with PMA and ionomycin after enzymatic digestion and the supernatants were was also analyzed by cytometric bead array. In both the human samples and in the mouse tissue the premalignant stage showed a significant increase in IL-17 when compared to

control and HNSCC. The mouse model was further broken down into early and late stages of premalignant and HNSCC to further elucidate the time at which this increase and decrease occurs. It was found that the late premalignant stage is when the increase in IL-17A levels occurs and that the levels significantly decrease during the early stage of HNSCC.

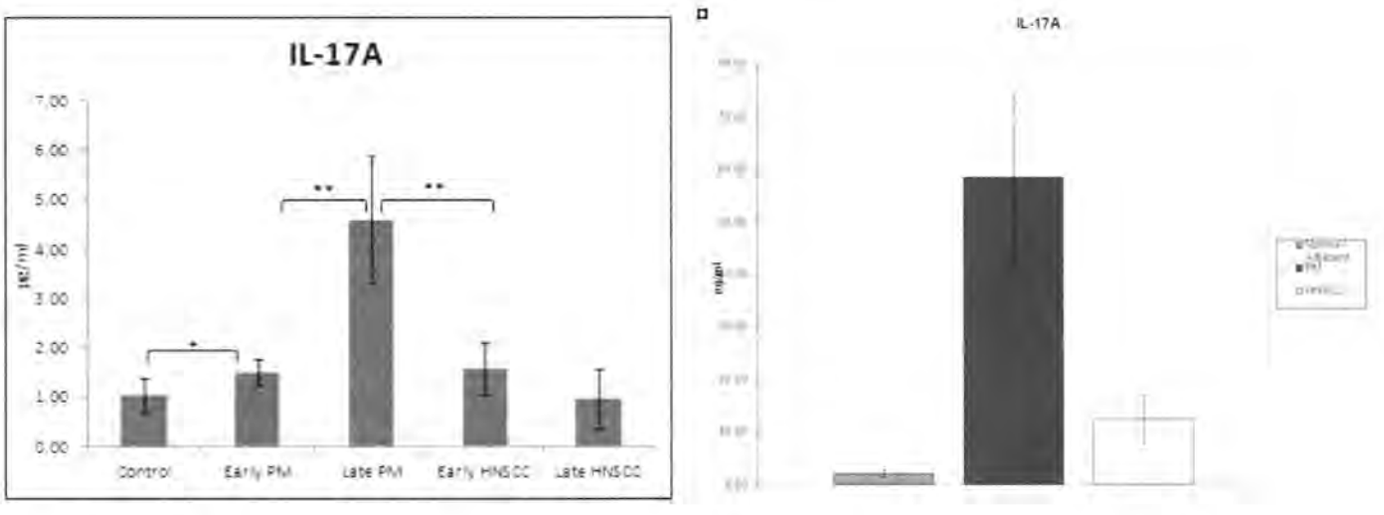

Figure 3.3. Both human and mouse samples show an increase in IL-17A in premalignant tissue compared to control/normal and HNSCC tissues. Graphical representation of the results of cytometric bead array analysis of control, 4NQO-treated premalignant, and 4NQO-treated HNSCC tissues from mice (left panel) or normal/adjacent, premalignant, and HNSCC human tissue (right panel). For human data; normal/adjacent (n=10), premalignant (n=6), HNSCC (n=25). For mouse data; control (n=5), early premalignant (n=6), late premalignant (n=5)m early HNSCC (n=7), late HNSCC (n=12). Data are presented as the mean ± standard deviation. *, $p < 0.05$; **, $p < 0.01$; *** $p < 0.001$ (Two-tailed Student's t-test).

3.4 Discussion

Depending on the microenvironment, as well as many other factors, the immune system can either mount an immune response against dysplastic cells or contribute to tumor development and progression. Because each component of the immune system can be both beneficial and detrimental, it is important to evaluate the changes that occur between the normal, premalignant, and established stages of HNSCC development. This study investigated the changes that occur in various cytokine levels in the

microenvironment during the carcinogenesis of HNSCC using both samples from human patients and a chemically-induced mouse model of HNSCC. Previously, our lab has shown that mice that have developed premalignant lesions exhibit an increased number of Th17 cells in the spleen and cervical lymph nodes compared to control mice and mice with HNSCC[108]. The data presented above show that the amount of IL-17 is increased in the microenvironment of both human and mouse premalignant tissues. To further elucidate the changes that occur during the pathogenesis of HNSCC, the mouse model was broken down into the early and late premalignant stages and HNSCC, and the IL-17 levels were calculated for each of these stages. The results showed that IL-17 levels increase as the premalignant stage progresses and then decrease in established HNSCC, with the lowest levels of expression occurring during late-stage HNSCC. This data is supported by the marked increase in IL-6 observed in the microenvironment during the premalignant stage, as IL-6 and TGF-β are necessary for the differentiation of Th17 cells. There was also an increase in the levels of IL-2, which is necessary for the maintenance and stability of effector T cells. This data indicates that during the premalignant stage of HNSCC, an immune response is mounted. Once the lesion is allowed to progress to established HNSCC, this immune response is decreased. In addition, the number of Tregs in the spleen and cervical lymph nodes has been shown to be increased in mice with fully established HNSCC when compared to control and mice at the premalignant stage of HNSCC[108]. As Tregs and Th17 cells have been shown to have an inversely proportional relationship, the decrease in IL-17 observed in this study may be due to an increase in the Treg population.

Chapter 4

Effect of the Microenvironment on the $CD4^+$ Population

CHAPTER 4: MICROENVIRONMENT EFFECT ON THE CD4$^+$POPULATION

Aim 2: ***Investigation of the role of the microenvironment in the decrease IL-17 in HNSCC tissue compared to premalignant tissue.***

4.1 Introduction

Despite advances in diagnosis and treatment, the survival rates for patients with head and neck cancer have remained relatively unchanged for the past 30 years [1]. HNSCC is a highly aggressive malignancy with a 5-year survival rate of only 50%. Of the patients diagnosed with HNSCC, 5% will develop additional primary tumors [2-3]. This effect is believed to be associated with the high degree of immune suppression induced by the tumor [4-5]. Treatment of this malignancy is further complicated by the significant morbidity associated with surgery. Identification of differences in the tumor microenvironment during the different stages of HNSCC and the role of Th17 cells in disease progression will provide a foundation for the development of immunotherapeutic interventions for patients with premalignant lesions or HNSCC.

Premalignant oral lesions likely induce local immune suppression, rather than systemic immune suppression, as is observed in HNSCC [4,109]. The current study was designed to investigate the mechanisms that mediate this difference in immune suppression. Because few studies have focused on the function of Th17 cells in the tumor

microenvironment, it is difficult to deduce the exact role(s) they play in cancer progression. While their role in the pathogenesis of a number of conditions is well defined, their function in the context of tumor immunology remains controversial. To further elucidate the role of the microenvironment on the increase/decrease of certain cell phenotypes, we manipulated the culture conditions of established Th17 cells. By exposing splenocytes, which are roughly 10% $CD4^+$ cells, and polarized Th17 cells to the microenvironment of control, premalignant, and HNSCC tissues, we were able to evaluate the effects of these microenvironments on the $CD4^+$ population. Based on data obtained previously from cervical lymph nodes, we expected to see a decrease in Th17 cell numbers and an increase in Tregs when polarized Th17 cells were exposed to the HNSCC microenvironment, while Th17 cell numbers would remain constant or possibly increase when exposed to the microenvironment found in premalignant tissue.

4.2 Materials and Methods

Oral HNSCC carcinogenesis

Carcinogen-induced oral premalignant lesions and HNSCC were established in mice by administration of 4NQO at 5 mg/ml in propylene glycol stock in the drinking water (diluted to 50 μg/ml) of 2 month old (at start) female C57BL/6 mice for 8 weeks, resulting in the development of premalignant oral lesions, or for 16 weeks to establish HNSCC. To monitor the development of premalignant oral lesions and HNSCC, the oral

cavities of the mice were examined by endoscopy using a Stryker 1.9 mm x 30° endoscope and a Stryker 1088 HD camera. The mice were sedated with inhaled isoflurane (Piramal Healthcare) during this procedure.

Tongue tissue processing

Control, premalignant, and HNSCC tongues were harvested from C57BL/6 mice and minced using a scalpel. The tissue was then enzymatically digested at 37°C with 0.23 U of Liberase (Roche) for 4 hrs. The dissociated tissue was then washed with PBS and allowed to recover overnight.

Generation of Th17 cells

Polarized Th17 cells were obtained from Dr. Chrystal Palous' lab. The cells were produced by stimulating murine $CD4^+$ cells from the spleen with TRP peptide (1 μg/mL) along with incubation in polarizing media containing human IL-1β (10 ng/ml), human IL-21 (100 ng/ml), human IL-6 (100 ng/ml), human TGF-β (30 ng/ml), anti-IL-4 (10ug/ml) and anti-IFN-γ (10 μg/ml) for 5 days. The cells were cultured for 5 days with the addition of human IL-2 (20 IU/ml) on day two.

Treatment of splenocytes with supernatant

Splenocytes were plated 100,000 per well in 48 well dish in 500μl of 10% RPMI media along with 500μl of of supernatant from either control, premalignant, or HNSCC tissue was then added. The supernatant was formed after the enzymatically digested tongue tissue was stimulated for 4 hours at 37°C with 50 ng/ml PMA and 1 μg/ml

ionomycin without the addition of Brefeldin A. The cells incubated with the supernatant for 36 hours and supernatant was collected.

Treatment of Th17 cells with supernatant

Th17 cells were plated 100,000 per well in a 96 well plate in 50µl of their polarization media as previously described. Then 150µl of supernatant from either control, premalignant, or HNSCC tissue was then added. The supernatant was formed after the enzymatically digested tongue tissue was stimulated for 4 hours at 37°C with 50 ng/ml PMA and 1 µg/ml ionomycin without the addition of Brefeldin A. The cells incubated with the supernatant for 36 hours and supernatant was collected.

Cytokine bead array

All reagents used for this assay were from BD unless otherwise specified. After 36 hours the supernatant from the incubation of the Th17 cells with the tissue supernatants and splenocytes with supernatant were collect and analyzed. The levels of IL-17 were determined using a cytometric bead array Th1/Th2/Th17 cytokine kit. The supernatants alone were also run to normalize the results. The average of the supernatants alone contained only 2.2 pg/ml. Relative amounts of each cytokine were analyzed using FCAP Array software.

Statistical analysis

Data are presented as mean ± standard deviation of the value. To compare one variable condition between groups, the two-tailed Student's *t*-test was used. Significance was reported in the 95% confidence interval.

4.3 Results

IL-17A secretion from splenocytes decreases in the control microenvironment but with a subsequent increase in premalignant followed by an increasing trend again for HNSCC.

In this study, we examined how the tumor microenvironment affected immune cells (splenocytes) and their secretion of IL-17. IL-17 secretion from cells exposed to control, premalignant or HNSCC microenvironments were compared to the levels of IL-17 secreted from cells incubated with 10% RPMI media alone. When compared to the media alone cells, IL-17 secretion decreased significantly when Th17 cells were co-incubated with supernatant from control tongue, increased significantly when they were co-incubated with the supernatant from premalignant tongue, and saw a rising trend once again when co-incubated with supernatant from HNSCC tongue (Fig 4.1). Based on the data from the tongue lysate we were expecting to see an increase in IL-17 when treated with the premalignant supernatant and a decrease when treated with HNSCC supernatant. Contradictory to what we predicted, the levels of IL-17 did not decrease when the splenocytes were incubated with supernatant from HNSCC tissue. This could allude to the fact that the response at the tumor or lesion site is different than the systemic response. Once again, for example, Th17 cells and/or IL-17, along with other factors,

have been reported to induce inflammation and promote the initiation and early growth of tumors in three different murine models of cancer: immune-deficient mice, mice with chemical carcinogen-induced tumors, and mice with pathogen-induced tumors [79]. This occurs despite high levels of IL-6, TGF-β, and IL-1, factors that promote mouse Th17 cell development [98-100], suggesting that Th17 cell development may be suppressed in the tumor microenvironment.

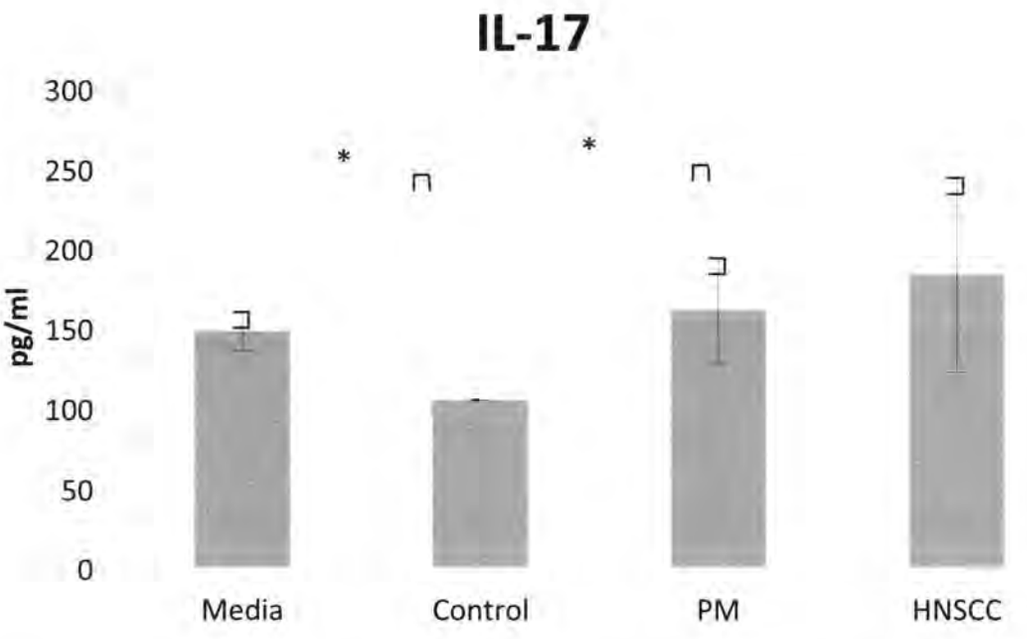

Figure 4.1 Graphical representation of the results of cytometric bead array analysis of 100,00 splenocytes cells co-incubated with control, 4NQO-treated premalignant, and 4NQO-treated HNSCC tongue supernatants for 36 hours. Data are presented as the mean ± standard deviation. *, $p < 0.05$; **, $p < 0.01$; *** $p < 0.001$ (Two-tailed Student's t-test).

IL-17A secretion from Th17 cells decreases in the control or HNSCC microenvironment but remains constant in the premalignant microenvironment.

Because the response from the splenocytes was not what we expected, we decided to focus on the specific effect the microenvironment would have on the Th17 cell population. Few studies have focused on the function of primary Th17 cells in the tumor microenvironment, the precise role(s) they may play in cancer progression remained unknown. In patients with epithelial cancer, Th17 levels are an indicator of improved patient survival and reduced tumor progression. In mice with established tumors, studies have documented the potent antitumor efficacy of both Tc17 and Th17 populations. It is possible that Th17 function may vary dependent on the cause, type and location of the cancer [97]. For example, Th17 cells and/or IL-17, along with other factors, have been reported to induce inflammation and promote the initiation and early growth of tumors in three different murine models of cancer: immune-deficient mice, mice with chemical carcinogen-induced tumors, and mice with pathogen-induced tumors [79]. This occurs despite high levels of IL-6, TGF-β, and IL-1, factors that promote mouse Th17 cell development [98-100], suggesting that Th17 cell development may be suppressed in the tumor microenvironment. In support of this, Th17 cells are known to be tightly regulated by the local cytokine environment [78], and Treg cells have been shown to inhibit Th17 cell expansion [82,95].

In this study, we examined how the tumor microenvironment affected the secretion of IL-17. IL-17 secretion from cells exposed to control, premalignant or HNSCC microenvironments were compared to the levels of IL-17 secreted from cells incubated with media alone. When compared to the media alone cells, IL-17 secretion

decreased significantly when Th17 cells were co-incubated with supernatant from control tongue, increased significantly when they were co-incubated with the supernatant from premalignant tongue, and decreased significantly once again when co-incubated with supernatant from HNSCC tongue (Fig 4.1). This data suggests that the premalignant microenvironment facilitates the maintenance and function of Th17 cells compared to the control and HNSCC microenvironments.

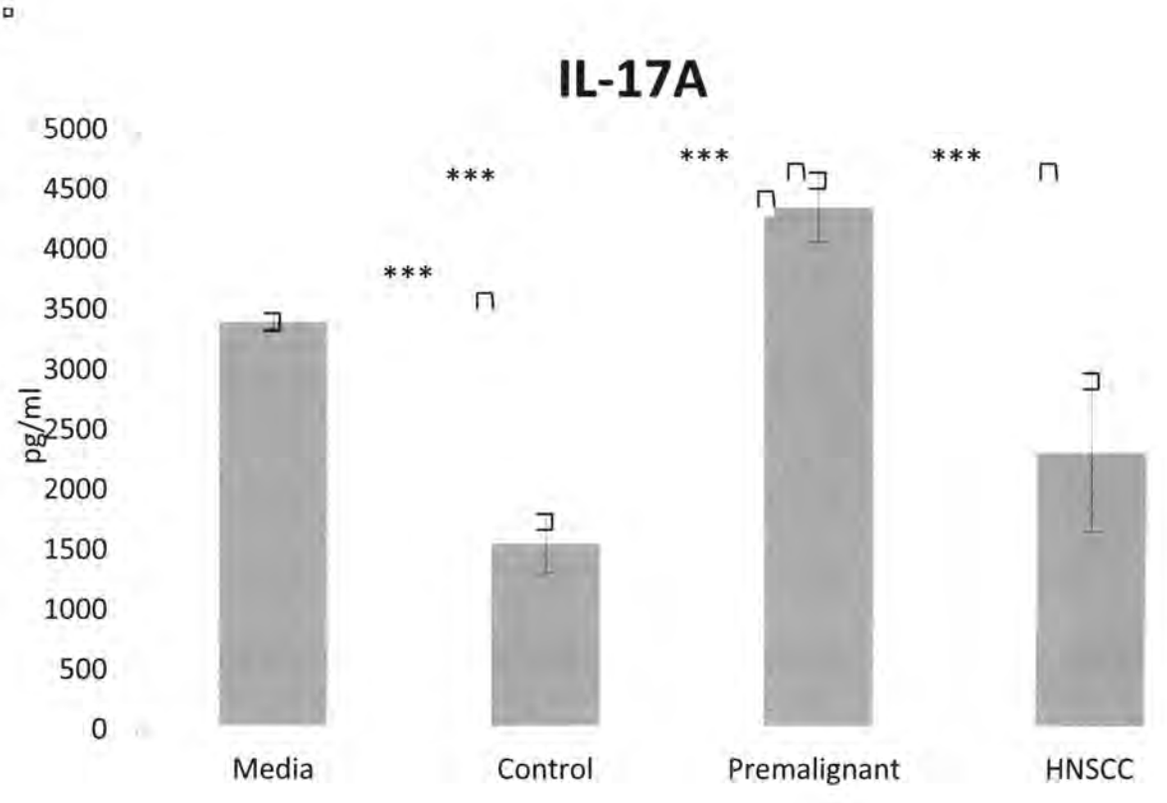

Figure 4.2 Graphical representation of the results of cytometric bead array analysis of 100,00 Th17 cells co-incubated with control, 4NQO-treated premalignant, and 4NQO-treated HNSCC tongue supernatant for 36 hours. Data are presented as the mean ± standard deviation. *, $p < 0.05$; **, $p < 0.01$; *** $p < 0.001$ (Two-tailed Student's t-test).

4.4 Discussion

Since their discovery only five years ago, Th17 cells have risen to prominence in studies of virology, autoimmune disease, inflammation, and immune responses to various parasites and fungi. While their role in the pathogenesis of many of these conditions is well defined, their function in the context of tumor immunology remains controversial. In ovarian cancer, the prevalence of Th17 cells in tumor draining lymph nodes (TDLN) and blood is comparable to that of healthy donors. Although they make up only a small population of cells within the tumor microenvironment, they comprise a higher proportion than other immune cell subsets. In ovarian cancer patients, Th17 cells are the sole source of IL-17 in the ascites, and the level of IL-17 in this fluid correlated positively with patient survival. Even after debulking, tumor-associated IL-17 was a negative predictor of death. The average survival rate of patients with ascites levels of greater than 220 pg/ml of IL-17 was 78 months, while that of patients with less than 220 pg/ml was 27 months. In some types of epithelial cancer, Th17 cells constitute only a small fraction of the effector T cell population in the tumor microenvironment [78,82,95-96]. This has not been fully investigated in HNSCC because few studies have focused on Th17 cells in the tumor microenvironment; therefore, it is difficult to predict the exact role(s) they may play in cancer progression.

When we treated Th17 cells with the supernatant from control, premalignant or HNSCC tissues, a significant increase in IL-17A was observed only when the cells were co-cultured with supernatant from premalignant tissue. In contrast, a significant decrease was observed when the cells were co-cultured with supernatant from HNSCC tissue. This data further supports our findings of increased levels of IL-17A in the microenvironment of premalignant tissue, as well as our previous findings showing an increase in IL-17A and Th17 cells in the cervical lymph nodes of mice bearing premalignant lesions.

Chapter 5

General Discussion

CHAPTER 5: GENERAL DISCUSSION

Head and neck squamous cell carcinomas (HNSCCs) are associated with abnormal cell-mediated immunity at the primary tumor site [9], the levels of which vary widely from patient to patient. Immunosuppression in HNSCC has been postulated to occur in a hierarchical manner, in which the primary tumor region is the most affected site, followed by the draining lymph nodes [10]. This hierarchical phenomenon suggests that immunosuppression in HNSCC patients is mediated by a regional network of factors. It is likely that suppression at the site of the tumor and the affected lymph nodes plays a role in limiting the efficacy of current immunotherapy protocols. It also likely that the immune system exerts a selective pressure that gives rise to tumor variants that are able to manipulate the immune system. [11-16]. A greater understanding of the mechanisms the mediate local inhibition of immune function will aid in improving the effectiveness of adoptive immunotherapy for the treatment of cancers [10]. While we did see evidence of immune suppression in the microenvironment in fully established HNSCC, elevated levels of inflammatory cytokines were observed primarily during the premalignant stage. These premalignant tissues had significantly higher levels of IL-17, which is indicative of a heightened TH17 cell response. Significantly higher levels of both IL-6 and IL-2 were also observed during this stage. IL-6 is required for Th17 differentiation, and the levels of both IL-6 and IL-17 were decreased during HNSCC. IL-2 also plays an important role in the immune response as it is needed for the maintenance and expansion of effector T cells. There was also an increase in TGF-β observed during the late premalignant stage,

which remained elevated through the late stage of HNSCC. This increase in TGF-β, along with a decrease in IL-6, creates an ideal environment for Tregs. If there is an increase in the Treg population during the late premalignant stage, this may be indicative of the onset of immune suppression. If the timing and cause of this suppression can be identified, treatments able to halt or attenuate this process can be developed, allowing the effectiveness of current immunotherapeutic intervention to be enhanced.

Since their discovery only five years ago, Th17 cells have risen to prominence in studies of virology, autoimmune disease, inflammation, and immune responses to various parasites and fungi. While their role in the pathogenesis of many of these conditions is well defined, their function in the context of tumor immunology remains controversial. In ovarian cancer, the prevalence of Th17 cells in tumor draining lymph nodes and blood is comparable to that of healthy donors. Although they make up only a small population of cells within the tumor microenvironment, they are present in proportionally higher numbers compared to other immune cell subsets. In ovarian cancer patients, Th17 cells were found to be the sole source of IL-17 in the ascites, the levels of which correlated positively with patient survival. Even after debulking, tumor-associated IL-17 was a negative predictor of death hazard. The average survival rate of patients with levels of IL-17 greater than 220 pg/ml in ascites was 78 months, while that of patients with less than 220 pg/ml was 27 months. In some types of epithelial cancer, Th17 cells constitute only a small fraction of the effector T cell population in the tumor microenvironment [78,82,95-96].

Because few studies have focused on primary Th17 cells in the tumor microenvironment in HNSCC, it is difficult to predict the exact role(s) they may play in

cancer progression. When splenocytes were incubated with control, premalignant, and HNSCC supernatant we saw a decrease in IL-17 levels with the control and an increase with both premalignant and HNSCC supernatant. When Th17 cells were cultured with the supernatant from control, premalignant, and HNSCC tissues, only exposure to the premalignant microenvironment induced a significant increase in IL-17. In contrast, a significant decrease was observed when Th17 cells were co-cultured with supernatant from HNSCC tissue. This data further supports our finding of increased levels of IL-17 in the microenvironment of premalignant tissue, as well as previous findings by our laboratory showing an increase in IL-17 and Th17 cells in the cervical lymph nodes of mice bearing premalignant lesions. If the timing and cause of this suppression can be identified, treatments able to halt or attenuate this process can be developed, allowing the effectiveness of current immunotherapeutic intervention to be enhanced.

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