2015

Immune Changes Associated with Progression of Premalignant Lesions to HNSCC

Sara D. Johnson
Medical University of South Carolina

Immune Changes Associated with Progression of Premalignant Lesions to HNSCC

by

Sara D. Johnson

A dissertation submitted to the faculty of the Medical University of South Carolina in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the College of Graduate Studies.

Department of Microbiology and Immunology

2015

Approved by:

M. Rita Young
Advisory Committee Chair

Carl Atkinson

Terry Day

Sebastiano Gattoni-Celli

Amanda LaRue

ACKNOWLEDGEMENTS

The work put into the scientific discovery and preparation of this dissertation could not have been completed without the love and constant encouragement of family and friends. The author would like to thank her parents, Clinton and Renee Johnson, for their love and support throughout her education, continuing through her time as a graduate student. Many thanks also go to her grandparents, Floyd and Louise Parrish, for supporting her dream to become a scientist. The author would also like to thank her sister, Lori Johnson, for always believing in her and her boyfriend, Ian Sidles, for giving her love and encouragement during the final stages of completing this work.

The author owes deepest gratitude to her mentor, Dr. Rita Young, Ph.D., for offering support and guidance throughout this project and for training her to become an independent thinker and researcher. Many thanks also go to Danielle Woodford and Corinne Levingston, for their technical assistance and friendship throughout the completion of this work.

The author would like to thank all members of her committee, Drs. Carl Atkinson, Terry Day, Sebastiano Gattoni-Celli and Amanda LaRue, whose assistance and advice have made this work possible.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

4-NQO	4-nitroquinoline 1-oxide
APC	allophycocyanin
CBA	cytometric bead array
CD	cluster of differentiation
CLN	cervical lymph node
COX-1/2	cyclooxygenase 1 / 2
DC	dendritic cell
DMEM	Dulbecco's modified Eagle medium
EGFR	epidermal growth factor receptor
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
G-CSF	granulocyte-colony stimulating factor
GM-CSF	granulocyte macrophage-colony stimulating factor
HBSS	Hank's buffered saline solution
HNSCC	head and neck squamous cell carcinoma
IFN-γ	interferon-gamma

IL-1α	interleukin-1 alpha
IL-2	interleukin-2
IL-4	interleukin-4
IL-6	interleukin-6
IL-10	interleukin-10
IL-17A	interleukin-17A
MCP-1	monocyte chemotactic protein-1 (CCL2)
MDSC	myeloid-derived suppressor cell
MHC I/II	major histocompatibility complex I/II
MIG	monokine induced by gamma interferon (CXCL9)
MIP-1α/β	macrophage inflammatory protein-1 alpha/beta
NK	natural killer
PerCP-Cy5.5	peridinin-chlorophyll protein complex - cy5.5 conjugate
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PD-1	programmed cell death protein (receptor)-1
PE	phycoerythrin

PGE_2	prostaglandin E_2
PMA	phorbol myristate acetate
RANTES	regulated on activation, normal T cell expressed and secreted (CCL5)
SCC	squamous cell carcinoma
SEM	standard error of the mean
Th1	T helper cell type 1
Th2	T helper cell type 2
Th17	T helper cell type 17
TGF-β	transforming growth factor-beta
TNF-α	tumor necrosis factor-alpha
TRAIL	TNF-related apoptosis-inducing ligand
VEGF	vascular endothelial growth factor

ABSTRACT

Head and neck squamous cell carcinoma (HNSCC) is characterized by immunosuppression, a state in which the established tumor evades the immune response. This presents a significant obstacle for treatment and the five-year survival rate for head and neck cancer patients remains at approximately 50%. While HNSCC causes profound immune inhibition, the immune changes that occur before HNSCC becomes established, at the premalignant lesion stage, are unknown. This study used the 4-nitroquinoline 1-oxide (4-NQO) mouse model of oral carcinogenesis to investigate how the immune environment changes as premalignant lesions progress to tumor. Premalignant lesion-bearing tongue tissue was found to release increased levels of Th1- and Th17-associated cytokines, including IL-2, IFN-γ, TNF-α and IL-17A, compared to control and HNSCC-bearing tongue tissue. Spleen cells and cervical lymph node cells produced increased levels of Th1- and Th2-associated cytokines in the presence of premalignant lesion-bearing tongue tissue compared to HNSCC-bearing tongue tissue. Premalignant lesion cells secreted significantly increased levels of proinflammatory G-CSF, RANTES, MCP-1, and PGE_2 compared to HNSCC cells. Premalignant lesion cell supernatant elicited increased production of innate proinflammatory mediators and Th1- and Th17-associated cytokines from spleen cells compared to HNSCC cell supernatant or media alone. An increased percentage of spleen cells expressed markers of activation in the presence of premalignant lesion cell supernatant compared to HNSCC cell supernatant or media alone. This data shows that premalignant lesion cells release increased levels of proinflammatory mediators compared to HNSCC cells, and

furthermore, elicit increased production of Th1- and Th17-associated cytokines and expression of activation markers in spleen cells compared to HNSCC cells. Compared to the HNSCC environment, the premalignant lesion environment is significantly more immune stimulatory, supporting a robust Th1- and Th17-associated immune response.

Immunosuppression is a significant obstacle to treatment for advanced HNSCC patients. The tumor itself plays a role in establishing this local and systemic immunosuppression, by secreting mediators such as PGE_2 that decrease T cell-mediated immunity and support immunosuppressive cells at the tumor site. This study aims to determine if inhibition of PGE_2 production at an earlier stage of carcinogenesis, the premalignant lesion stage, can restore a beneficial immune response and slow progression to tumor, in the 4-NQO mouse model of oral carcinogenesis. Initial studies showed that inhibition of PGE_2 production by premalignant lesion cells (but not HNSCC cells) induced increased production of the Th1-associated cytokine IL-2 and IFN-γ by spleen cells *in vitro*. To monitor the effects of inhibiting PGE_2 production *in vivo*, indomethacin, a COX (cyclooxygenase) inhibitor, was administered to mice bearing 4-NQO-induced premalignant lesions and progression to tumor was monitored by endoscopy. Mice receiving indomethacin at the premalignant lesion stage had significantly improved clinical outcomes compared to mice receiving diluent control treatment, as defined by lower lesion scores. Cervical lymph node analysis showed an increased percentage of $CD8^+$ T cells expressing IFN-γ at the endpoint of the study (20 weeks) in mice that had received indomethacin compared to mice that had received diluent control treatment, although the difference was not significant. Spleen cells from mice that had received

indomethacin secreted increased levels of IFN-γ at 6 weeks and 20 post-treatment compared to mice that had received control treatment, suggesting that indomethacin treatment induced an increased systemic Th1-type immune response. The data suggests that the premalignant state is characterized by an immune stimulatory and inflammatory milieu and that inhibiting PGE_2 production stimulates immune reactivity and slows progression to advanced HNSCC in the 4-NQO model.

Chapter 1

Introduction:

Immune Reactivity and HNSCC

CHAPTER 1: IMMUNE REACTIVITY AND HNSCC

1.1 An Introduction to Head and Neck Squamous Cell Carcinoma (HNSCC)

1.1.1 Development of HNSCC

Head and neck squamous cell carcinoma (HNSCC) accounts for over 90% of all head and neck cancers, with approximately 550,000 cases diagnosed each year worldwide [1, 2]. The major risk factors are tobacco and alcohol abuse, which have a synergistic effect on disease progression. HNSCC arises from keratinizing epithelial cells in the mucosa throughout the head and neck region, including the lip, oral cavity, nasal cavities, paranasal sinuses, pharynx and larynx. The development of HNSCC begins with the formation of leukoplakias or erythroplakias which, if left untreated, progress to carcinoma in situ and, finally, invasive carcinoma [3]. Because of the aggressive nature of the disease, the five-year survival rate for head and neck cancer patients remains around 50%, despite some advances in treatment over that last 30 years. A significant complication for treatment is that patients typically present with advanced tumors, which drastically reduces the effectiveness of traditional treatment strategies, including surgical resection and a combination of chemotherapy and radiation. In addition to having limited success, current treatment options greatly diminish patient quality of life, contributing to salivary gland hypofunction, xerostomia, bacterial infections, inflammation of the mucous membranes, and in some cases, malnutrition and dehydration due to the patient's inability to eat and drink [4]. A majority of patients presenting with advanced tumors relapse locoregionally and/or at distant sites after initial treatment, at which point treatment by surgery, if possible, and reirradiation are significantly less successful [5-7].

1.1.2 Complications to Current Treatment Options

Advanced HNSCC tumors use a variety of mechanisms to evade the host immune response and promote immunosuppression, presenting perhaps the most important obstacle for effective treatment and contributing to the high recurrence rate of this disease. HNSCC patients are characterized by systemic immunosuppression, exhibiting increased populations of regulatory T cells and $CD34^+$ progenitor cells, which suppress $CD8^+$ T cell- and $CD4^+$ helper T cell-mediated immunity at the primary tumor site and are associated with a poorer prognosis [8-11]. Increased levels of $CD127^{lo/-}$ regulatory T cells in HNSCC patients are associated with more advanced malignancies involving local lymph nodes [12]. Similarly, increased levels of $CD34^+$ progenitor cells are associated with increased tumor recurrence and metastasis [10]. While some immunotherapies, including IL-2/Interferon-gamma (IFN-γ) cytokine therapies and cetuximab/ nimotuzumab anti-epidermal growth factor receptor (EGFR) therapies have been shown to increase lymphocyte infiltration, prevent tumor progression and increase long-term survival when used in combination with standard treatment, responses vary greatly among patients and are often associated with significant toxicity [13-17]. Furthermore, some discrepancies in immune vs. clinical responses have been observed in trials with the anti-EGFR antibody nimotuzumab, in which clinical responses were not associated with significant lymphocyte activation [15]. A gap remains in our understanding of how HNSCC patients respond to immunotherapies since the immune changes that take place during the progression of HNSCC and how the tumor evades an effective immune response have not been completely mapped out.

1.2 Evasion of Immune Response by HNSCC Tumors

1.2.1 Immune Escape in HNSCC

It is well established that HNSCC tumors are associated with immune escape. The process of immunoediting is harnessed by many different types of tumors and generally begins with elimination, in which patrolling T helper cells recognize an invasive growth and send inflammatory signals to recruit natural killer (NK), NK T cells, macrophages, and dendritic cells to the site of the dysplastic tissue to mount an initial response [18]. During this stage, NK cells and antigen-presenting cells are transactivated by IFN-γ and IL-12 secretion and tumor cells are eliminated by TNF-related apoptosis-inducing ligand (TRAIL), perforin, and reactive nitrogen species. Tumor-specific $CD4^+$ and $CD8^+$ T cells begin to hone to the developing tumor as well, initiating the adaptive antitumor response [18]. The second stage of immunoediting is characterized by a dynamic equilibrium between the developing tumor bed and the host's immune system, and may last up to several years. During this stage, IFN-γ- producing $CD4^+$ and $CD8^+$ T cells are unable to completely eliminate the rapidly dividing/mutating HNSCC tumor cells. During the final stage of immunoediting, tumor escape, HNSCC tumor cells decrease antigen, major histocompatibility complex (MHC) I/II and costimulatory molecule expression to directly thwart an effective immune response [19-23]. In addition, by upregulating death ligands such as FasL and PD-L1, HNSCC tumors trigger apoptosis in infiltrating lymphocytes, further dampening the immune response at the tumor site [24, 25]. Infiltrating immunosuppressive populations, including myeloid-derived suppressor cells (MDSCs) and T regulatory cells, contribute to tumor-associated immunosuppression by directly inhibiting $CD8^+$ T cell-mediated immunity and promoting tumor growth [1, 12, 26].

1.2.2 Immune Environment of HNSCC

A significant complication of understanding how the tumor contributes to immunosuppression is the complex network of tumor cells, fibroblasts, carcinoma-associated fibroblasts (CAFs), smooth muscle cells, endothelial cells, and myriad of tumor-infiltrating immune populations including tumor-associated macrophages, B cells, T cells and antigen-presenting cells that make up the tumor microenvironment [27]. One of the major mechanisms by which HNSCC tumors are thought to evade host immune recognition is by modulating the cytokine environment at the tumor site. By secreting cytokines such as IL-6 and IL-10, HNSCC tumor cells promote a T helper cell type 2 (Th2)-skewed response, which is associated with decreased antitumor efficacy [20, 28]. This Th2-skewing is also evident systemically, as peripheral blood mononuclear cells (PBMCs) isolated from advanced HNSCC patients secrete abnormally high levels of Th2 cytokines [29, 30]. Along with Th2-skewed cytokines, HNSCC tumors secrete increased levels of immunosuppressive factors such as transforming growth factor-beta (TGF-β) that function to directly inhibit cytotoxic T cell-mediated immunity and recruit immunosuppressive cells, including MDSCs and M2-skewed macrophages, to the tumor site [31, 32]. Once at the tumor site, HNSCC tumor cells harness these immunosuppressive cells for a myriad of tumor-promoting functions, including increased growth and angiogenesis. HNSCC cells trigger increased IL-6 production from $CD34^+$ progenitor cells, for example, promoting angiogenesis in the tumor microenvironment [33].

HNSCC tumors also secrete factors that are typically associated with a proinflammatory response, harnessing these immune modulators to favor growth,

angiogenesis and paradoxically, immune escape. Several of these mediators have also been shown to recruit immunosuppressive cells to the tumor site. Granulocyte-macrophage colony-stimulating factor (GM-CSF) and prostaglandin E_2 (PGE_2), which have traditional proinflammatory roles that support the differentiation of macrophages and neutrophils in the early stages of inflammation, are significantly increased in the supernatant of primary SCC lines from patients with head and neck cancer [34]. Tumor-secreted GM-CSF has been shown to promote MDSC recruitment and differentiation, and high levels of GM-CSF in HNSCC patients are associated with a poorer prognosis [10, 35]. Increased levels of PGE_2 are associated with invasion and angiogenesis in aggressive early-stage tumors [36]. Other factors secreted by HNSCC tumors, including monocyte chemotactic protein-1 (MCP-1), have been shown to contribute to immunosuppression at the tumor site by recruiting a population of IL-10 and TGF-β-secreting M2 skewed tumor-associated macrophages [37]. By secreting a host of immune modulators, HNSCC tumors thwart an effective immune response and become increasingly difficult to treat.

1.3 Role of Prostaglandin E_2 in HNSCC

1.3.1 Role of PGE_2 in Cancer

The role of PGE_2 in tumor progression has not been clearly defined. One reason for this is that PGE_2 is produced by a myriad of cell types, including epithelial cells, fibroblasts, infiltrating immune cells and cancer cells, all of which comprise/surround a developing tumor [38]. Furthermore, the role of PGE_2 seems to shift during tumor progression. During early stages of inflammation, PGE_2 has been shown to promote the

infiltration of neutrophils, macrophages and mast cells [39-41]. However, PGE_2 has been shown to have an inhibitory effect on granulocyte function and macrophage phagocytosis, contributing to defective innate immunity in cancer patients and patients who have undergone bone marrow transplantation [38, 42-45]. PGE_2 also inhibits several arms of adaptive immunity. It has been shown to directly inhibit IL-2 production by T cells, decrease IL-2 responsiveness and suppress antigen-specific T cells [46, 47]. Several studies have shown that PGE_2 disrupts normal dendritic cell differentiation and suppresses the activity of T helper cell type 1 (Th1)-associated dendritic cells (DCs), resulting in decreased IL-12 production and Th2 skewing of mature T helper cells [48-51]. These PGE_2-skewed DCs, or MDSCs, have been shown to the suppress cytotoxic T cell responses, skew macrophage differentiation towards the pro-tumor M2 phenotype and contribute to overall dendritic cell dysfunction in the cancer setting [52-55]. However, the effect of PGE_2 on mature dendritic cells is more immune stimulatory. PGE_2 promotes the surface expression of chemokine receptor 7 (CCR7) and costimulatory molecules, contributing to DC migration to lymph nodes and their subsequent activation [49, 56-59]. All in all, the role of PGE_2 on immune function is complex and there remains a gap in our knowledge of how PGE_2 contributes to tumor progression.

1.3.2 PGE_2 and COX Inhibitors in HNSCC

It is well established that arachadonic acid metabolism is altered in established HNSCC tumors. COX-2, the inducible enzyme involved in PGE_2 production, is significantly upregulated in HNSCC tumors and increased levels of PGE_2 are associated with decreased levels of $CD8^+$ T cells and increased levels of suppressor cells at the

tumor site [20, 26, 28, 60]. Preliminary studies have shown that inhibiting COX-2 leads to increased apoptosis and decreased angiogenesis of HNSCC cells, both *in vitro* and *in vivo* [61]. In several murine and hamster models of head and neck cancer, administration of the COX-2 inhibitor celecoxib to animals with tumors resulted in decreased tumor expression of vascular endothelial growth factor (VEGF) and survivin, increased tumor cell apoptosis, and decreased tumor growth [61-63]. However, the impact of administering a COX inhibitor before tumors become established, at the premalignant lesion stage, has not been previously investigated.

COX-2 inhibitors are being explored as a possible adjuvant to traditional therapies in head and neck cancer treatment. In one study of head and neck cancer patients, preoperative administration of the COX-2 inhibitor Rofecoxib resulted in restored monocyte function and migration, suggesting that inhibiting COX-2 may be a key strategy in restoring immune function [64]. One retrospective study looking at the relationship between the use of nonselective cyclooxygenase inhibitors and overall survival in HNSCC patients found that median survival was increased among COX inhibitor users, although the regimen/type of COX inhibition was not controlled for in this study [65]. In several clinical trials, administration of celecoxib as an adjuvant therapy for head and neck cancer patients has been associated with significant response rates [66-68]. However, the mechanism by which COX-2 inhibition may be altering the immune response in HNSCC patients has not been extensively explored.

Some of the mechanisms by which PGE_2 may be modulating the immune response in other types of malignancies, including colorectal, lung, and breast cancers,

are starting to be defined. Several studies have shown that PGE_2 induces the production of IL-10 and directly suppresses the production of proinflammatory cytokines by $CD4^+$ T cells, implicating it as a key player in tumor-associated immunosuppression [26, 38, 69, 70]. In a model of lung carcinoma, inhibition of COX-2 resulted in increased antitumor reactivity by shifting the cytokine balance of resident immune cells towards a more proinflammatory, Th1-like phenotype, as characterized by increased IL-12 production and decreased IL-10 production [71]. The role of PGE_2 in modulating the immune response in head and neck cancer—and specifically what role PGE_2 plays as premalignant lesions progress to HNSCC tumors—has not been previously investigated and is the focus of Specific Aim 2 of this dissertation.

1.4 Premalignant Lesions

1.4.1 Immune Environment of Premalignant Lesions

A critical gap that remains in head and neck cancer research is the progression of premalignant lesions to established HNSCC. There has been little focus on the premalignant state of HNSCC, and the molecular and immunological conditions that foster development into established HNSCC. A major challenge is that patients typically present with more advanced tumors, so studies on the premalignant lesion state are less complete. In one study, premalignant lesion tissue extracted from patients secreted increased levels of Th1-associated IL-2 and IFN-γ and T helper cell type 17 (Th17)-associated IL-17A compared to HNSCC tissue and normal oral tissue [72]. Premalignant lesion tissue also released increased levels of pro-inflammatory IL-6 compared to HNSCC tissue and healthy oral tissue [72]. This study shows that

premalignant lesion tissue is more proinflammatory than HNSCC tissue and suggests that it may be more immune stimulatory compared to the established tumor. The immune changes observed at the site of the premalignant lesion or HNSCC tumor do not necessarily reflect changes in the immune response systemically. In the same study, levels of IL-2 and IFN-γ were not increased in the plasma of patients bearing premalignant lesions compared to HNSCC and control patients [72]. IL-17A, however, was increased in the plasma of patients bearing premalignant lesions compared to HNSCC and control patients, reflecting what was observed in premalignant lesion tissue [72]. Several studies have shown that IL-6 and tumor necrosis factor-alpha (TNF-α) were increased in the saliva of patients with premalignant oral lesions compared to control patients [73, 74], suggesting that a local inflammatory response occurs in the oral cavities of patients with developing lesions. Premalignant oral lesion epithelium has been shown to expresses several proinflammatory molecules, including TNF-α, stromal cell-derived factor 1 (SDF-1 or CXCL12), and the toll like receptors TLR4 and TLR9, which promote an inflammatory immune response [75-77].

Another difficulty in understanding the premalignant lesion environment is the complex network of epithelial cells, fibroblasts, endothelial cells, muscle cells, and infiltrating immune cells that make up the developing lesions. The contribution of inflammatory immune cells vs. resident cells such as endothelial cells and fibroblasts to the proinflammatory milieu of premalignant lesions is not always straightforward. Several studies have shown that premalignant lesions are characterized by an influx of proinflammatory immune cells, including NK cells, macrophages, and $CD8^+$ T cells [78, 79]. In these studies, the infiltration of NK cells and $CD8^+$ T cells into premalignant

lesions correlated with the degree of dysplasia [78, 79]. Dendritic Langerhans cells were also found to be increased in leukoplakias with dysplasia and were co-localized with T cells in many cases [78, 79]. There is evidence that $CD8^+$ T cells are more activated in premalignant lesions, as they have been shown to express increased levels of granzyme B and perforin in premalignant lesion tissue vs. in control tissue [80]. Endothelial cells also play a role in modulating the immune environment of premalignant lesions through their production of VEGF, PGE_2, IL-6, and TGF-β [81-83]. These factors recruit immune cells into premalignant lesions and also modulate their activity once at the site of the developing lesions. Because lesions are composed of such a complex network of cells and patients rarely present with such an early stage of disease, there remains a critical gap in our knowledge of the immune environment of premalignant lesions, and how premalignant lesion cells themselves, may be modulating the immune response.

1.4.2 4-NQO Mouse Model of Oral Carcinogenesis

The 4-NQO mouse model produces all stages of oral carcinogenesis and allows careful observation of the immune environment as premalignant lesions progress to HNSCC. This model is based on the carcinogenic effects of 4-NQO, which can be administered in the drinking water of C57BL/6 mice and mimics the effects of tobacco, a major risk factor for HNSCC [84, 85]. Other models of HNSCC, including the xenograft model in which human HNSCC cells are injected subcutaneously or into the mylohyoid muscle of the mouse oral cavity, do not allow for the study of multistep carcinogenesis or the contribution of the immune system on the progression of HNSCC from the premalignant state to established tumor. In addition, because injected tumor cells do not

originate from the epithelium of the head and neck region, the molecular changes that occur as normal epithelium becomes dysplastic cannot be tracked [86-88]. The 7,12-dimethylbenz[a]anthracene (DMBA) Syrian hamster cheek model induces premalignant oral lesions with genetic/epigenetic alterations that mimic human HNSCC, but because humans do not have an anatomic pouch, these tumors do not resemble human tumors anatomically or histologically [89-91].

The 4-NQO model of oral carcinogenesis induces the formation of premalignant oral lesions that progress to exophytic, HNSCC tumors. At the molecular level, 4-NQO causes the formation of reactive oxygen species (ROS), DNA adducts (adenosine to guanosine substitutions) and H-ras mutations, mimicking the DNA damage induced by tobacco and alcohol use, and therefore modeling human disease at the molecular level [92-94]. One of the strengths of the 4-NQO model is that 4-NQO is a water-soluble quinoline derivative that can be administered in the drinking water, so the mice are constantly exposed to the carcinogen, as a model human disease [95, 96]. The most relevant strength of the 4-NQO model is that it allows the immune response to be studied as premalignant lesions progress to HNSCC tumors. Administration of 4-NQO in the drinking water at a concentration of 50 μg/mL results in the formation of premalignant lesions on the tongue at 6-8 weeks, which progress to more exophytic, malignant tumors by 12-16 weeks. Because tumors formed in this model of oral carcinogenesis closely mimic HNSCC tumors in human patients at both the histological and molecular levels, the 4-NQO model is an ideal candidate for immunotherapy studies [95, 97-99].

1.4.3 Changes in the Immune Response as Premalignant Lesions Progress to HNSCC

Initial studies using the 4-NQO mouse model of oral carcinogenesis offer some insight into the immune changes that take place during the development of HNSCC. To investigate the immune response against developing oral tumors, these studies focused on immune cell infiltration into the tumor-draining, cervical lymph nodes of mice with 4-NQO-induced oral lesions and 4-NQO-induced established tumors. These studies showed that tumor-draining lymph nodes of HNSCC-bearing mice are characterized by an increase in the percentage and absolute number of $CD4^+Foxp3^+$ regulatory T cells compared to lymph nodes of premalignant lesion-bearing and control mice, demonstrating that there is increased infiltration of immunosuppressive cells in the established tumor state [100]. This mirrors the increase in T regulatory cells observed both in the periphery and in the tumors of HNSCC patients, supporting the 4-NQO model as a closely representative model of human disease [8-12]. In contrast, cervical lymph nodes of premalignant lesion-bearing mice are characterized by an increased percentage and absolute number of proinflammatory Th17 cells compared to lymph nodes of HNSCC-bearing mice and control mice, suggesting that the premalignant lesion state is proinflammatory but as the lesion progresses to tumor, the immune response wanes and immunosuppressive cells dominate. While the absolute numbers of conventional $CD4^+$ and $CD8^+$ T cells are increased in the cervical lymph nodes of HNSCC-bearing mice compared to that in premalignant lesion-bearing and control mice, conventional $CD4^+$ T cells isolated from the cervical lymph nodes of HNSCC-bearing mice were characterized by decreased proliferation and secreted decreased levels of IL-2 upon stimulation *in vitro*

compared to T cells isolated from control mice [100]. Taken together, these data showed that conventional $CD4^+$ T cells isolated from mice bearing established HNSCC tumors are functionally compromised compared to T cells isolated from control mice and, to some extent, premalignant lesion-bearing mice as well. These studies suggest that the tumor environment may be more immune inhibitory compared to the premalignant lesion environment. Future studies are necessary to determine how the immune response evolves from the premalignant lesion to established HNSCC and how this impacts on tumor progression.

Chapter 2

Rationale, Hypothesis, and Specific Aims

CHAPTER 2: RATIONALE, HYPOTHESIS, AND SPECIFIC AIMS

2.1 Rationale

It is well established that advanced HNSCC is associated with local and systemic immunosuppression. Previous studies have shown that HNSCC tumors produce several immune inhibitory factors, including TGF-β and PGE_2, which recruit immunosuppressive cells and directly inhibit T cell function at the tumor site [8, 10, 11, 19, 20, 28, 31, 32, 70]. However, much less is known about factors produced by premalignant lesions, and how the immune environment shaped by premalignant lesions impacts on the immune response as lesions progress to tumor. A few patient studies have suggested that premalignant lesions are more proinflammatory, characterized by increased production of IL-6 and IL-17A, compared to HNSCC tumors [72-74]. Preliminary studies using the 4-NQO mouse model of oral carcinogenesis have shown that immune cells infiltrating the lymph nodes draining premalignant lesions are more activated and produce higher levels of proinflammatory cytokines (IL-17A) compared to immune cells infiltrating the cervical lymph nodes of HNSCC-bearing mice [100]. Taken together, these data suggest that immune environment of the premalignant lesions may be more immune stimulatory than in the tumor environment. But little is known about the immune mediators produced by premalignant lesion cells themselves, and how these mediators impact on immune cell function at this stage.

Traditional treatment options for HNSCC patients, including surgery and chemotherapy/radiation, are not successful in the majority of cases and the 5 year survival rate for this disease remains less than 50% [2]. A major obstacle to treatment is

the immunosuppressed state of HNSCC patients. While immunotherapies have been successful in some cases, overall these therapies are not widely effective and many are associated with considerable toxicity. Our lab is interested in modulating the immune response at the premalignant lesion stage as a means to stimulate the antitumor response. One mediator that is known to promote immunosuppression in the HNSCC environment is PGE_2. HNSCC tumors directly secrete PGE_2 and while the immune effects of PGE_2 in the tumor environment are not completely mapped out, increased levels of PGE_2 have been correlated with decreased T cell function, increased infiltration of MDSCs, and a worse prognosis in patients [20, 26, 28, 60, 69, 70]. The role of PGE_2 in the premalignant environment remains unknown. Our preliminary studies have shown that premalignant lesion cells produce PGE_2 in significantly higher levels than HNSCC cells [101]. This suggests that PGE_2 may be modulating the immune response at a much earlier stage of disease than has been previously appreciated and inhibition of PGE_2 at this stage may lead to a greater immunity against tumor. After defining the immune milieu of premalignant lesions, our goal is to understand how PGE_2 impacts on the immune response in the premalignant lesion environment and then to inhibit PGE_2 production at the premalignant lesion stage to sustain a Th1-type response and slow progression to tumor.

2.1.1 Hypothesis

Our hypothesis is that the premalignant lesion environment is highly proinflammatory, which supports a robust immune response, but as the tumor becomes established, the immune environment becomes less immune stimulatory, resulting in decreased

Th1-mediated immune responses. We also hypothesize that PGE_2 is a key player in skewing the immune stimulatory environment of the premalignant lesion to the immunosuppressive environment of the HNSCC tumor and COX inhibition at the premalignant lesion stage will sustain Th1-mediated responses and slow progression to tumor.

2.1.2 Specific Aims

This hypothesis will be tested through the following specific aims:

Aim 1: To investigate how the immune environment changes as premalignant lesions progress to tumor and how the evolution of the immune environment impacts on immune cell activation and function in the 4-NQO mouse model of oral carcinogenesis.

HNSCC tumors are known to secrete factors such as TGF-β and PGE_2 that dampen the immune response at the site of the tumor, and contribute to the immunosuppression of established HNSCC. Considerably less is known about the environment of premalignant lesions. The focus of this aim is to define the premalignant lesion environment in the 4-NQO model and to determine how factors produced by premalignant lesion cells impact on immune cell cytokine production and activation.

Aim 2: To prevent the immune skewing and tumor progression in 4-NQO-treated mice by inhibiting PGE_2 production at the premalignant lesion stage.

In the tumor environment, PGE_2 is known to inhibit an effective antitumor response in a number of ways, including recruitment of immune inhibitory cells and

inhibition of conventional T cell function. Inhibition of PGE_2 production has been shown to promote a more active immune response and lead to increased survival in HNSCC patients. Our preliminary studies show that premalignant lesion cells produce high levels of PGE_2. The focus of this aim is to understand how PGE_2 modulates the immune response in the premalignant lesion environment and how inhibiting PGE_2 production at this stage impacts on immune cell function and tumor progression.

2.2 Significance

Head and neck cancer is the sixth most common cancer worldwide, with an overall survival rate that remains below 50%. Patients often present with advanced tumors and systemic immunosuppression, making treatment very challenging. Many studies have focused on factors secreted by HNSCC tumors and how they contribute to immunosuppression, both at the tumor site and systemically. Factors such as TGF-β and PGE_2 lead to decreased T cell reactivity at the tumor site and also modulate the activity of systemic immune cells, skewing them to a Th2 phenotype and inhibiting an effective antitumor response. It is evident that mediators produced by tumor cells play a considerable role in controlling immunity. Therefore, therapies that activate the immune response in patients at high risk of developing HNSCC may prove to be more effective as adjuvant treatments to surgery or photodynamic therapies, or even as primary treatments to prevent progression of lesions to cancer.

What is not evident is how mediators produced by premalignant lesion cells impact on the immune response. One reason for this is a lack of studies focusing on patients with premalignant lesion disease and the other is a focus on animal models that

have primarily advanced disease. Our lab is interested in the immune response at the premalignant lesion stage, and how the response changes as premalignant lesions progress to HNSCC. The 4-NQO mouse model of oral carcinogenesis is ideal because it produces all stage of oral carcinogenesis and is based on the effects of 4-NQO, a carcinogen that induces DNA damage and models the effects of tobacco at the molecular and histological levels. Our primary focus is to isolate premalignant lesion cells and evaluate how factors produced by these cells impacts on immune cell activation and function, compared to HNSCC cells. This model allows us to directly compare the premalignant lesion and HNSCC environments, and tease apart the contribution of each cell type to immune reactivity. Preliminary studies have shown that premalignant lesion cells themselves produces high levels of PGE_2. Although PGE_2 is known to contribute to immunosuppression in established HNSCC, its role in the premalignant lesion environment is not defined. The 4-NQO model allows us to administer the COX inhibitor indomethacin at the premalignant lesion stage to better understand the role of PGE_2 in modulating the immune response at this stage and to track how COX inhibition impacts on tumor progression. By understanding how the immune response changes as premalignant lesions progress to tumor, we can better tailor immunotherapies to promote an effective antitumor response.

Chapter 3

Effect of the Premalignant Lesion and HNSCC Environments on Immune Cell Reactivity

CHAPTER 3: EFFECT OF THE PREMALIGNANT LESION AND HNSCC ENVIRONMENTS ON IMMUNE CELL REACTIVITY

Aim 1: To investigate how the immune environment changes as premalignant lesions progress to tumor and how the evolution of the immune environment impacts on immune cell activation and function in the 4-NQO mouse model of oral carcinogenesis.

3.1 Introduction

HNSCC accounts for over 90% of all head and neck cancers and, despite some advances in treatment over the last 30 years, the five-year survival rate remains around 50% [1, 2]. HNSCC patients are characterized by systemic immunosuppression, exhibiting increased populations of regulatory T cells and $CD34^+$ progenitor cells, which suppress $CD8^+$ T cell- and $CD4^+$ helper T cell-mediated immunity at the primary tumor site and are associated with a poorer prognosis [8-12]. By evading the immune response, HNSCC tumors become increasingly difficult to treat and are also characterized by a high recurrence rate.

Several of the ways in which HNSCC tumors evade the host immune response have been defined. Over the course of immune escape, HNSCC cells lose MHC and costimulatory molecule expression and secrete immune inhibitory factors such as TGF-β and PGE_2, inhibiting an effective cytotoxic T cell response and allowing the tumor to persist [19-22, 26, 31, 32, 60]. HNSCC tumors also secrete factors that are typically

associated with a proinflammatory response, harnessing these immune modulators to favor growth, angiogenesis and, paradoxically, immune escape. GM-CSF and MCP-1, which have traditional proinflammatory roles that support the differentiation of macrophages and neutrophils in the early stages of inflammation, are significantly increased in the supernatant of primary SCC lines from patients with head and neck cancer and have been shown to promote MDSC recruitment and differentiation as well as M2 macrophage polarization [10, 35, 37]. Secretion of these factors by HNSCC tumors has been correlated with patient prognosis. High levels of GM-CSF in HNSCC patients are associated with a poorer prognosis and increased levels of PGE_2 are associated with invasion and angiogenesis in aggressive early-stage tumors [10, 20, 36, 60]. These studies indicate that factors secreted by HNSCC tumors play a direct role in modulating a patient's immune response, which plays a critical role in their prognosis and treatment options.

There remains a critical gap in our understanding of the immune changes that occur before HNSCC becomes established, at the premalignant lesion stage. One study analyzing tissue from patients with premalignant oral lesions, showed that premalignant lesion tissue releases increased levels of several proinflammatory cytokines, including IL-2, IFN-γ, and IL-17A, compared to HNSCC tissue [72]. Preliminary studies using the 4-NQO mouse model of oral carcinogenesis have shown that the cervical lymph nodes of premalignant lesion-bearing mice are characterized by an increased percentage of activated T cells and increased levels of proinflammatory cytokines, including IL-17A

[100]. These data suggest that an active immune response is taking place in the preneoplastic stage and that this response is thwarted once the tumor becomes established. However, the mediators released directly by premalignant lesion cells themselves and how they impact on the immune response as the tumor becomes established remains unknown.

The current study uses the 4-NQO mouse model to define the immune environment of premalignant lesions at both the tissue and cellular levels, and to determine how soluble mediators from cells established from carcinogen-induced oral premalignant lesions and HNSCC tumors impact on immune cell activation and function. Premalignant lesion-bearing tongue tissue was defined by increased Th1- and Th17-associated cytokines compared to both HNSCC-bearing and control tongue tissue. Premalignant lesion cells, themselves, secreted high levels of several proinflammatory mediators, including G-CSF, RANTES, MCP-1, and PGE_2. To determine how the premalignant lesion and HNSCC environments modulate immune cell function and activation, spleen cells from control C57BL/6 mice were cultured with media conditioned by premalignant lesion cell-conditioned media or HNSCC cell-conditioned media. Results of these studies showed that in the presence of premalignant lesion cell-conditioned media, spleen cells produced significantly increased levels of Th1-, Th2- and Th17-associated cytokines and an increased percentage of $CD8^+$ T cells expressed the activation marker $CD69^+$, compared to spleen cells in the presence of HNSCC cell-conditioned media or media alone. Premalignant lesion cell-conditioned media also

elicited increased production of Th1-, Th2-, and Th17-associated cytokines from cervical lymph node cells compared to HNSCC cell-conditioned media. Overall, these studies showed that the premalignant lesion environment was more proinflammatory and elicited a more activated immune response compared to the HNSCC environment.

3.2 Materials and Methods

Oral HNSCC carcinogenesis

Five mg/mL 4-NQO in propylene glycol stock was diluted into the drinking water at a final concentration of 50 µg/mL and administered to 2-month-old female C57BL/6 mice (Charles Rivers Laboratory, Wilmington, MA, USA) until the development of premalignant oral lesions (6–8 weeks) or HNSCC (12–16 weeks). To monitor lesion development, mice were examined endoscopically weekly using a Stryker 1.9 mm × 30° endoscope and images were taken using a Stryker 1088 camera (Kalamazoo, MI, USA). During the procedure, mice were sedated with inhaled isoflurane (Piramal Healthcare, Boise, ID, USA).

Tongue processing and culture

After extraction, each tongue was minced and enzymatically digested using 100 µg/mL of complete liberase cocktail (Roche, Indianapolis, IN, USA) in 10 mL Hank's Buffered Saline Solution (HBSS, Life Technologies, Grand Island, NY, USA) at 37º C for 4 h. Tissue was plated overnight in 12-well tissue culture plates (one dissociated tongue/well) in Dulbecco's Modified Eagle Media (DMEM) supplemented with 10% FBS and penicillin/streptomycin/amphotericin B at 37° C. Tissue was restimulated with 2 µl/mL Cell Stimulation Cocktail (eBioscience) consisting of phorbol myristate acetate (PMA) and ionomycin for the last 4-6 h of culture at 37º C and supernatant was collected for cytokine/chemokine analysis. For each dissociated tongue, cytokine/chemokine levels were standardized to total protein. To measure total protein, tissue was collected, washed

with Dulbecco's phosphate buffered saline (DPBS, Life Technologies, Grand Island, NY, USA), resuspended in 1 mL phosphate buffered saline (PBS) and lysed using an ultrasonic processor for 2 min. on ice. Total protein was quantitated using a BCA protein assay kit (Thermo Scientific, Rockford, IL, USA).

Premalignant lesion and HNSCC cell lines

Primary cell lines were established by excising the tongues of premalignant or HNSCC-bearing mice at the appropriate stage, as defined through histopathological analysis by the oral pathology section in the Center for Oral Health Research at the Medical University of South Carolina. Premalignant lesions or HNSCC were excised from the tongues of 4-NQO treated mice. To establish cell lines, premalignant lesions or HNSCC tumors were washed several times with PBS and antibiotic/antimycotic solutions containing 100 mg/mL penicillin, 100 mg/mL streptomycin, and 200 mg/mL neomycin. Premalignant lesions or HNSCC tumors were plated on 60 mm tissue culture dishes in DMEM media supplemented with 10% FBS and penicillin/streptomycin/amphotericin B and cultured for two weeks at 37º C, replacing the media twice per week. After this period, adhering premalignant lesion or carcinoma cells were removed and cultured in 75 cm^2 flasks in fresh media at 37° C, replacing the media twice per week. Prior to defining cells as premalignant or HNSCC, their epithelial phenotype was confirmed as well as uniformity of their microscopic and growth characteristics. To detach adherent cells, they were trypsinized, washed, and resuspended in fresh medium. After cell lines were established,

supernatants were collected after 48 h of culture (when cells reached 80% confluence) for cytokine analysis.

Cytokine bead array

All reagents used for the cytokine bead array are from BD Biosciences unless otherwise specified. The levels of IFN-γ, IL-2, IL-17A, IL-4, IL-6 and IL-10 in cell or tissue cultures were determined using a mouse cytometric bead array (CBA) Th1/Th2/Th17 cytokine kit. Levels of IL-1α, IL-1β, IL-13, IL-12p70, IL-9, GM-CSF, G-CSF, MIG, MCP-1, MIP-1α, MIP-1β and RANTES in cell or tissue cultures were determined using cytometric bead array flex sets for the individual cytokines according to the manufacturer's instructions. A FACS Canto (BD Biosciences, San Jose, CA, USA) flow cytometer was used to quantify cytokine profiles and relative amounts of each cytokine were analyzed using FCAP Array Software (manufactured by Soft Flow Hungary Ltd. for BD Biosciences, San Jose, CA, USA). In experiments where premalignant lesion or HNSCC supernatants were added to spleen cell cultures, cytokine levels in total spleen cell supernatants were adjusted for baseline levels in premalignant or HNSCC supernatants.

TGF-β1 ELISA

To quantify TGF-β1 in cell supernatants, a sandwich ELISA was performed according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA). Before analysis, latent TGF-β1 was activated to immunoreactive TGF-β1 by addition of HCl solution, as described by the manufacturer. Premalignant lesion and HNSCC cell

supernatants were diluted 1:5 in sample diluent for analysis. Optical density at 450 nm was measured using a Spectra Max M2 plate reader. Concentration of TGF-β1 was determined using SoftMax Pro 5.4.2 plate reader software. A standard concentration curve was generated for each independent experiment. Samples were analyzed in duplicate for each of three independent experiments.

Prostaglandin E_2 ELISA

To quantify PGE_2 in cell supernatants, a competitive ELISA was performed according to the manufacturer's instructions (Pierce Biotechnology, Rockford, IL, USA). Cell supernatants were diluted 1:2 for analysis. Optical density at 405 nm was measured using a Spectra Max M2 plate reader. Concentration of PGE_2 was determined using SoftMax Pro 5.4.2 plate reader software. A standard concentration curve was generated for each independent experiment. Samples were tested in duplicate for each of three independent experiments.

Spleen processing

Spleens were harvested from control C57BL/6 mice and homogenized using a glass homogenizer. Cells were passed through a 70 μm cell strainer (BD Falcon, San Jose, CA, USA) and rinsed with HBSS. Red blood cells were lysed by adding ACK Lysing Buffer (Lonza, Walkersville, MD, USA) for 3 min. Splenocytes were then washed twice with HBSS. Cell number was determined by counting cells excluding trypan blue using a hemocytometer.

Cervical lymph node processing

Cervical lymph nodes were harvested from control C57BL/6 mice and homogenized using a Stomacher 80 homogenizer (Seward) set on high for 90 sec. Cells were then passed through a 70 μm cell strainer (BD Falcon, San Jose, CA, USA) and rinsed with HBSS. Cell number was determined by counting cells excluding trypan blue using a hemocytometer.

Culture medium

Cell culture media consisted of 1X DMEM (Life Technologies, Grand Island, NY, USA) containing 4.5 g/L D-glucose and L-glutamine, supplemented with 10% fetal bovine serum (FBS, Sigma, St. Louis, MO, USA) and 1X antibiotic antimycotic solution (Sigma, St. Louis, MO, USA) containing penicillin, streptomycin and amphotericin B. To establish cell lines from premalignant lesions and HNSCC tumors, culture media was supplemented with 2X antibiotic antimycotic solution for the first 2 weeks of culture.

Spleen cell cultures

Spleen cells were cultured in 12-well anti-CD3-coated tissue culture plates at $1X10^6$ cells/well in fresh media or media conditioned by premalignant lesions or HNSCC with IL-2 (R&D Systems, Minneapolis, MN, USA) for 72 hr at 37º C. Premalignant lesion cell and HNSCC cell supernatants were diluted 1:2 in fresh media for culture with spleen cells. Premalignant lesion-bearing and HNSCC tongue tissue supernatants were diluted 1:10 in fresh media for culture with spleen cells. For spleen cell cytokine analysis, cells were

restimulated with 2 µL/mL Cell Stimulation Cocktail (eBioscience, PMA/ionomycin) for the last 4-6 h of culture at 37º C and supernatants were collected for analysis by CBA. For analysis of surface marker expression by flow cytometry, cells were gently collected from tissue culture plates with 25 cm cell scrapers (Sarstedt, Inc., Newton, NC, USA), washed once with stain buffer, and transferred to polystyrene tubes at $1X10^6$ cells/tube for analysis by flow cytometry. For analysis of intracellular cytokine expression by flow cytometry, cells were restimulated with 2 µL/mL Cell Stimulation Cocktail (eBioscience, PMA/ionomycin) and 0.6 µL/mL GolgiStop (BDBiosciences) for the last 4-6 h of culture at 37º C. Cells were gently collected from tissue culture plates with 25 cm cell scrapers (Sarstedt, Inc., Newton, NC, USA), washed once with stain buffer, and transferred to polystyrene tubes at $1X10^6$ cells/tube for analysis by flow cytometry.

Cervical lymph node cell cultures

Cervical lymph node cells were cultured in 12-well anti-CD3-coated tissue culture plates at $1X10^6$ cells/well in fresh media or media conditioned by premalignant lesions or HNSCC, with IL-2 (R&D Systems, Minneapolis, MN, USA) for 72 hr at 37º C. Premalignant lesion cell and HNSCC cell supernatants were diluted 1:2 in fresh media for culture with lymph node cells. For cytokine analysis, cells were restimulated with 2 µL/mL Cell Stimulation Cocktail (eBioscience, PMA/ionomycin) for the last 4-6 h of culture at 37º C and supernatants were collected for analysis by CBA.

Flow cytometric analysis of spleen cells: surface markers

All antibodies and reagents for this section are from BDBiosciences unless otherwise stated. Spleen cells were gently collected from tissue culture plates with 25 cm cell scrapers (Sarstedt, Inc., Newton, NC, USA), washed once with stain buffer, and transferred to polystyrene tubes at $1X10^6$ cells/tube for flow cytometric analysis. Cells were resuspended in 300 µL FACS block buffer, containing 2% FBS in sterile 1X PBS, at 4º C for 15 min and centrifuged at 1000 rpm for 5 min. Cells were then incubated in 10 µL Fc block, containing anti-CD16/32 antibody at a 1:100 dilution in sterile 1X PBS, at 4º C for 10 min to block nonspecific antibody binding to the cell surface. Cells were stained with the following antibodies: FITC CD4, PE/APC CD8a, PE CD69, APC CD25 (eBioscience) and APC PD-1 (eBioscience). A tube of cells stained with the appropriate isotype controls was included. Cells were washed twice in 1 mL stain buffer (BD Biosciences) and resuspended in 400 µL stain buffer for analysis of extent and frequency of positive cells on a FACS Canto flow cytometer.

Flow cytometric analysis of spleen cells: cytokine expression

All antibodies and reagents for this section are from BDBiosciences unless otherwise stated. For cytokine staining, spleen cells were restimulated with 2 µL/mL Cell Stimulation Cocktail (eBioscience, PMA/ionomycin) and 0.6 µL/mL GolgiStop for the last 4-6 h of culture at 37º C. Cells were then gently collected from tissue culture plates with 25 cm cell scrapers (Sarstedt, Inc., Newton, NC, USA), washed once with stain buffer, and transferred to polystyrene tubes at $1X10^6$ cells/tube for flow cytometric analysis.

Cells were resuspended in 300 µL FACS block buffer, containing 2% FBS in sterile 1X PBS, at 4ºC for 15 min and centrifuged at 1000 rpm for 5 min. Cells were then incubated in 10 µL Fc block, containing anti-CD16/32 antibody at a 1:100 dilution in sterile 1X PBS, at 4º C for 10 min to block nonspecific antibody binding to the cell surface. Cells were washed twice in stain buffer, resuspended in 1 mL cold Cytofix (BD Biosciences), and incubated for 20 min at 4ºC. Cells were centrifuged, washed twice in 1 mL stain buffer (BD Biosciences), resuspended in 1 mL Perm/Wash Buffer (BD Biosciences) and incubated for 15 min at room temperature. Cells were then centrifuged, resuspended in 50 µL BD Perm/Wash Buffer and incubated for 30 min at room temperature with equal concentrations of antibodies or appropriate isotype controls: FITC CD4, APC/PE CD8a, PerCP-Cy5.5 IFN-γ, and PE-Cy7 IL-17A. Cells were washed twice in 1 mL BD Perm/Wash Buffer and resuspended in 400 µL stain buffer for analysis of extent and frequency of positive cells on a FACS Canto flow cytometer.

Statistical analyses

Data were reported using the mean as a measure of central tendency $\pm$ standard error of the mean. To compare one variable condition between groups, a one-way ANOVA analysis was initially performed (GraphPad Prism version 6.03 for Windows, GraphPad Software, La Jolla, CA, USA). If differences were identified by the ANOVA analysis, a two-tailed Student's *t* test was then performed to determine significance of differences between each of two groups (ex.: control *vs.* premalignant, control *vs.*

HNSCC, premalignant *vs.* HNSCC) using GraphPad Prism version 6.03. Significance was reported in the 95% confidence interval.

3.3 Results

Premalignant lesion-bearing tongues release increased levels of Th-1 and Th17-associated cytokines and chemokines compared to HNSCC-bearing and control tongues

Previous studies have shown that immune cells infiltrating the draining lymph nodes of premalignant lesion-bearing mice are more activated and release increased levels of proinflammatory cytokines compared to HNSCC-bearing and control mice, suggesting that the premalignant lesion environment supports a more activated, Th1-type response [100]. However, the immune response at the site of the developing premalignant lesion of the tongue has not been extensively studied. To define the immune environment of premalignant lesions as they progress to HNSCC, tongues were extracted from mice bearing premalignant lesions or HNSCC tumors and tissue was analyzed for levels of a spectrum of proinflammatory cytokines and chemokines. These studies showed that premalignant lesion-bearing tongue tissue releases increased levels of Th1- and Th17-associated cytokines and other proinflammatory mediators compared to HNSCC-bearing and control tongue tissue. As shown in Figure 3.1 below, supernatant collected from premalignant lesion-bearing tongue tissue cultures contained significantly increased levels of Th1-associated IL-2, IFN-γ, and TNF-α compared to HNSCC-bearing and control tongue tissue. In addition, the levels of Th17-associated IL-17A were increased in supernatants collected from premalignant lesion-bearing tongue tissue cultures compared to supernatants from HNSCC-bearing or control tongue tissue cultures, but variability of values among samples precluded statistical significance. Other proinflammatory

mediators, including GM-CSF and G-CSF (granulocyte-colony stimulating factor), were reduced in HNSCC-bearing tongue tissue supernatants compared to premalignant lesion-bearing and control tongue tissue supernatants but, unlike the increased levels of other cytokines shown in Figure 3.1, are not increased in supernatants of premalignant lesion-bearing tongue tissue vs. supernatants of control tongue tissue. In addition to increased Th1- and Th17-associated cytokines, premalignant lesion-bearing tongue tissue released increased levels of Th2-asociated cytokines, including IL-4, IL-6 and IL-10 compared to control tongue tissue, although these differences were not as significant as Th1-associated cytokines (data not shown). The levels of immune inhibitory TGF-β were not significantly different in supernatants collected from control tongue, premalignant lesion-bearing tongue, and HNSCC tongue cultures (data not shown).

These data show that the immune environment of tongue tissue bearing 4-NQO-induced premalignant lesions is more proinflammatory than the immune environment of tongue tissue bearing established HNSCC, characterized by increased levels of Th1- and Th17-associated cytokines. This suggests that an active, Th1- and Th17-associated immune response occurs at the premalignant lesion stage, but as the lesion progresses to HNSCC, the response wanes significantly, possibly contributing to the immunosuppression evident in established HNSCC.

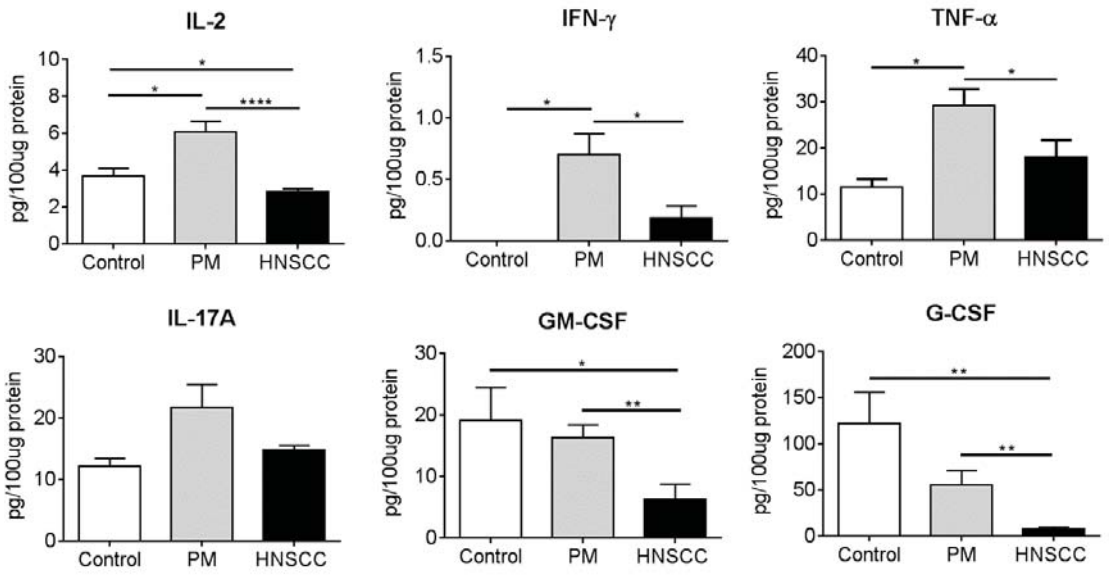


Fig. 3.1 Premalignant lesion-bearing tongues release increased levels of Th1- and pro-inflammatory mediators compared to HNSCC-bearing and control tongues. Tongues were excised from control, premalignant lesion (PM)-bearing, and HNSCC-bearing mice and mechanically and enzymatically digested in liberase. Tongue tissue was cultured for 24 h, restimulated with PMA/ionomycin for the last 4 h of culture, and supernatants were collected for cytokine analyses by cytokine bead array (CBA). Data shown represent mean ± standard error of the mean (SEM) of three independent experiments, each performed in duplicate. *$p<0.05$ **$p<0.01$ ***$p<0.001$ ****$p<0.0001$ (2-tailed Student's *t* test).

Spleen cells produce increased levels of Th1- and Th2-associated cytokines in the presence of premalignant lesion-bearing tongue supernatant compared to spleen cells exposed to HNSCC-bearing tongue supernatant

The premalignant and HNSCC environments are complex and are composed of preneoplastic and neoplastic cells, as well as infiltrating immune cells. To investigate how the environments shaped by premalignant lesions vs. HNSCC tumors impact on immune cell reactivity, supernatants from tongue tissue cultures from control, premalignant lesion-bearing and HNSCC-bearing mice were cultured with spleen cells from control C57BL/6 mice. In the presence of premalignant lesion-bearing tongue tissue supernatant, spleen cells produced increased levels of Th1-associated IL-2 compared to spleen cells in the presence of HNSCC-bearing tongue tissue supernatant (Figure 3.2). Upon re-stimulation with PMA/ionomycin, spleen cells produced comparable levels of IL-2 in the presence of control, premalignant lesion-bearing, and HNSCC-bearing tongue tissue supernatant, demonstrating that spleen cells are capable of producing IL-2 in all conditions, but they produce increased levels of IL-2 in the premalignant lesion-bearing tongue tissue environment without stimulation. This supports the hypothesis that the premalignant lesion environment is inherently more immune stimulatory than both the control and HNSCC environments. The level of IFN-γ produced by spleen cells in the premalignant lesion-bearing tongue tissue environment was increased compared to spleen cells in the control tongue tissue environment ($p=0.053$) and, to a lesser extent, spleen cells in the HNSCC tongue tissue environment ($p=0.078$). Re-stimulation with

PMA/ionomycin resulted in a slight increase of IFN-γ production by spleen cells in the premalignant lesion tongue tissue environment and a significant increase of IFN-γ production by spleen cells in the HNSCC tongue tissue environment. As with IL-2, these data show that spleen cells were capable of producing IFN-γ in all conditions, but they produce increased levels of IFN-γ in the premalignant lesion-bearing tongue tissue environment, without re-stimulation. In other words, HNSCC tongue tissue supernatant alone was not enough to induce maximal IFN-γ production, but spleen cells could be restimulated to produce levels of IFN-γ comparable to premalignant lesion-bearing tongue tissue conditions.

A similar trend was evident for the production of Th2-associated cytokines by spleen cells in the presence of premalignant lesion-bearing tongue tissue supernatant, although overall the levels of these cytokines were lower compared to Th1-associated cytokines (Figure 3.2). Spleen cells produced an increased level of IL-4 in the presence of premalignant lesion-bearing tongue tissue supernatant compared to HNSCC-bearing tongue tissue supernatant. However, the level of IL-4 produced by spleen cells in the presence of control tongue tissue supernatant was not significantly different that the level produced by spleen cells in premalignant lesion-bearing tongue tissue supernatant. After re-stimulation, the level of IL-4 produced by spleen cells in the presence of HNSCC-bearing tongue tissue supernatant was comparable to the level produced by spleen cells in the premalignant lesion tongue tissue environment, indicating that spleen cells were capable of producing IL-4 in the HNSCC environment after PMA/ionomycin

stimulation. Spleen cells produced increased levels of IL-10 in the presence of premalignant lesion-bearing tongue tissue supernatant compared to spleen cells in the presence of both HNSCC-bearing tongue tissue supernatant and control tongue tissue supernatant. After re-stimulation with PMA/ionomycin, the level of IL-10 produced by spleen cells in the premalignant lesion tongue tissue environment was increased compared to the level of IL-10 produced by spleen cells in the control tongue tissue environment and HNSCC tongue tissue environment, although the difference between the premalignant lesion tongue tissue environment and HNSCC tongue tissue environment was not significant.

The levels of the proinflammatory mediators IL-17A and TNF-α produced by spleen cells were comparable in the premalignant lesion tongue tissue and HNSCC tongue tissue environments. Upon restimulation with PMA/ionomycin, the level of IL-17A produced by spleen cells in the presence of premalignant lesion-bearing tongue tissue supernatant was decreased compared to spleen cells in the presence of HNSCC tongue tissue supernatant and control tongue tissue supernatant. A similar trend was evident for spleen cell production of TNF-α, although after re-stimulation with PMA/ionomycin, the level of TNF-α produced by spleen cells in the premalignant lesion-bearing tongue tissue environment was not significantly different than the level produced by spleen cells in the HNSCC tongue tissue environment.

These data support the hypothesis that the immune environment of premalignant lesion-bearing tongue tissue elicits increased production of Th1-associated cytokines, and

to a lesser degree, Th2-associated cytokines, by spleen cells compared to the immune environment of HNSCC-bearing tongue tissue. These data suggest that the premalignant lesion environment may support a more robust immune response, compared to the established tumor environment.

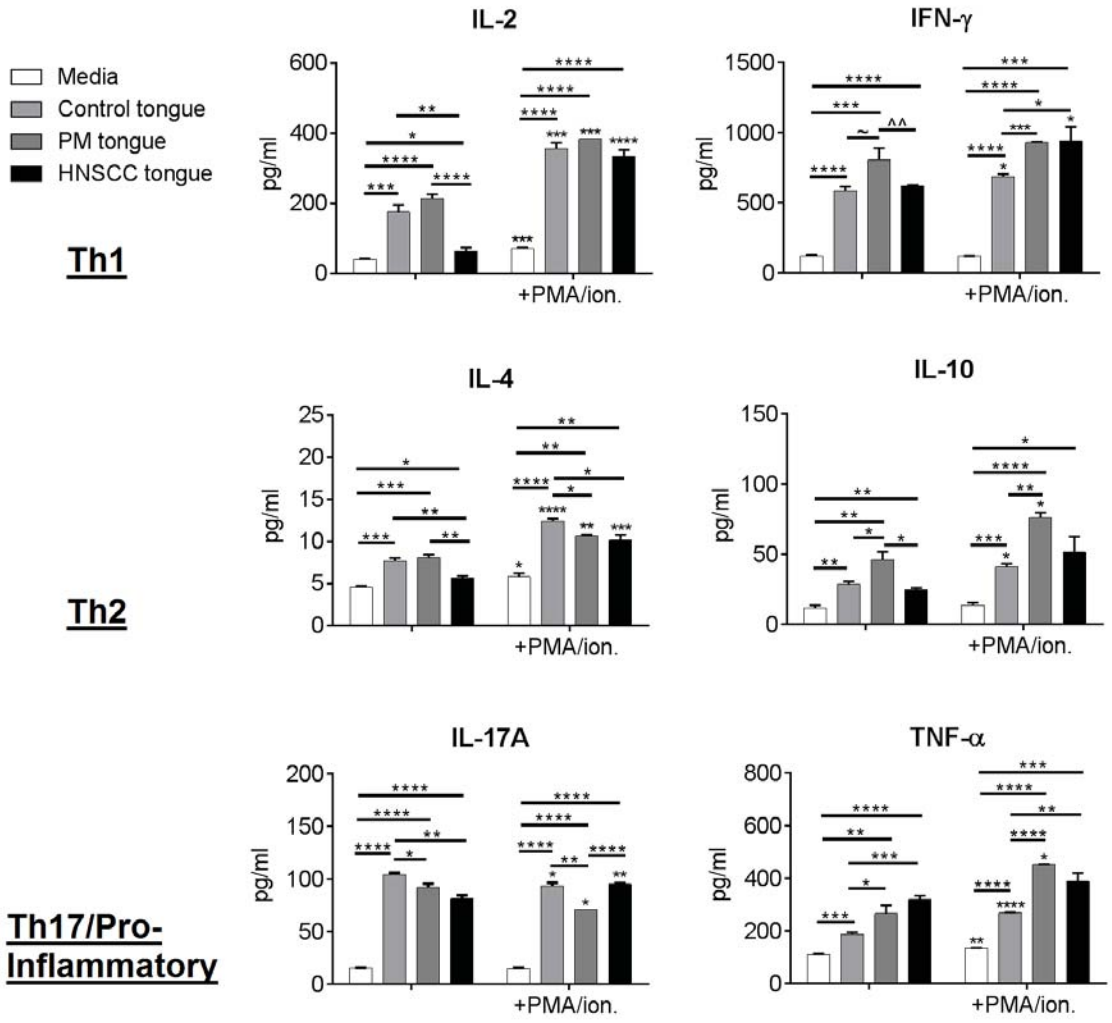


Fig. 3.2 Spleen cells stimulated to produce increased levels of Th1- and Th2-associated cytokines in the presence of premalignant lesion-bearing tongue tissue. Spleen cells from control C57BL/6 mice were cultured with media conditioned by tongue tissue from control, premalignant lesion (PM)-bearing, or HNSCC-bearing mice at a 1:10 dilution in fresh media for 72 h. Spleen cells remained unstimulated or were restimulated with PMA/ionomycin for the last 4 h of culture and supernatants were collected for cytokine analysis by cytokine bead array (CBA). Data shown represent mean ± SEM of three independent experiments, each performed in duplicate. *p<0.05 **p<0.01 ***p<0.001 ****p<0.0001 ~p=0.053 ^^p=0.078 (2-tailed Student's *t* test).

Premalignant lesion cells release increased levels of proinflammatory mediators compared to HNSCC cells

While the above-described studies showed the premalignant lesion environment to be proinflammatory and capable of stimulating immune cell cytokine production, the present study aimed to determine the contribution of premalignant lesion cells vs. the complex premalignant lesion milieu to the immune stimulatory environment. Previous studies have shown that both murine and human SCC cell lines and freshly isolated primary HNSCC tumors secrete increased levels of a panel of immune mediators, including IL-1α, IL-6, GM-CSF, TGF-β and PGE_2 [20, 31, 34, 60, 102-104]. There has been little to no focus on immune mediators released by premalignant lesion cells, and how this compares to HNSCC cells in the 4-NQO model. To investigate this question, tongues were extracted from premalignant lesion-bearing and HNSCC-bearing mice to establish primary cell lines so that the levels of immune mediators released by the cells themselves could be analyzed. It was not feasible to include comparison to control tongue cells because normal epithelial cells do not grow in culture without added growth factor, which would alter the properties of the cells. Analysis of supernatants collected from the cell cultures showed that premalignant lesion cells secrete significantly increased levels of several proinflammatory mediators compared to HNSCC cells (Figure 3.3). The levels of G-CSF, RANTES (regulated on activation and normal T cell expressed and secreted), and MCP-1, while detectable in HNSCC cell supernatant, were detected in much higher levels in premalignant lesion cell supernatant. Of note, premalignant lesion cells released

significant levels of PGE_2 compared to HNSCC cells. In contrast, HNSCC cell supernatant was characterized by increased levels of immune inhibitory TGF-β compared to premalignant lesion cells. These data show that the levels of immune mediators secreted by premalignant lesion cells vs. HNSCC cells are quite different and suggest that the immune environment changes as premalignant lesions progress to tumor. Further, these data show that premalignant lesion cells secrete significantly higher levels of several proinflammatory mediators compared to HNSCC cells *in vitro*, supporting the contribution of premalignant lesion cells to the immune stimulatory environment of tongue lesions.

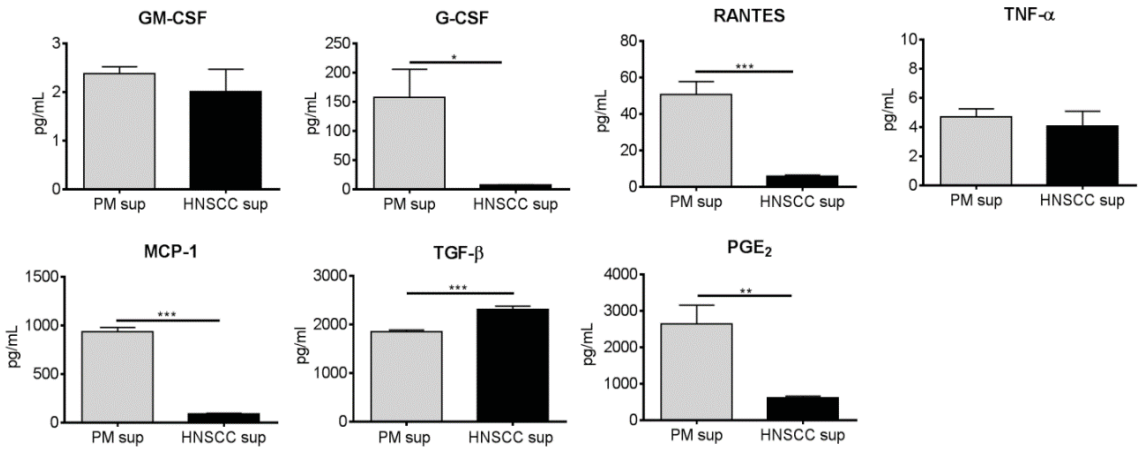


Fig. 3.3 Premalignant lesion cells release increased levels of pro-inflammatory mediators compared to HNSCC cells *in vitro*. Cell lines were established from 4-NQO-induced premalignant lesions or HNSCC tumors and grown to confluency. Supernatants were collected after 48 h for cytokine and chemokine analyses. Data shown represent 3 independent experiments performed in duplicate. *p<0.05 **p<0.01 ***p<0.001 ****p<0.0001 (2-tailed Student's *t* test).

Spleen cells produce increased levels of innate proinflammatory mediators in the presence of premalignant lesion cell supernatant compared to spleen cells exposed to HNSCC cell supernatant or media alone

The results in Figure 3.2 show the stimulatory effect of premalignant tongue tissue on normal spleen cell cytokine production, but not known is the contribution of premalignant lesion cells to this inflammatory immune stimulation. To investigate how factors produced by premalignant lesion and HNSCC tumor cells modulate the production of innate proinflammatory mediators by immune cells, splenocytes were cultured with premalignant or HNSCC cell-conditioned media, and supernatants were collected for cytokine and chemokine analyses. To simplify visualization of the effects of premalignant lesion cells vs. HNSCC cells on spleen cell cytokine production, data were normalized to cytokine levels secreted by spleen cells cultured in media alone (control cultures) and presented as fold change/control values. As shown in Figure 3.4, the levels of proinflammatory IL-1α and Th1-associated RANTES secreted by splenocytes in the presence of premalignant lesion cell supernatant were significantly increased compared to levels secreted by spleen cells that were incubated with HNSCC cell supernatant or media alone, with and without stimulation with PMA/ionomycin. The level of IL-1α secreted by splenocytes in the presence of premalignant lesion cell supernatant increased from about 40-fold/control to almost 70-fold/control after stimulation, whereas the level of RANTES secreted by splenocytes was about 12-fold/control with and without stimulation, respectively, *in vitro*. There were no significant differences in the levels of

macrophage inflammatory protein-1 alpha (MIP-1α) and macrophage inflammatory protein-1 beta (MIP-1β) secreted by splenocytes in the presence of premalignant and HNSCC supernatant, respectively, compared to control (data not shown).

In addition to stimulating increased production of IL-1α and RANTES, premalignant lesion cell supernatant elicited increased production of granulocyte and macrophage-associated monokine induced by gamma interferon (MIG), G-CSF and GM-CSF from splenocytes compared to HNSCC supernatant or media alone, with and without stimulation. The amount of MIG secreted by splenocytes in the presence of premalignant cell supernatant was about 15-fold the level secreted by splenocytes in the presence of HNSCC cell supernatant or media alone, with and without stimulation. Similarly, the level of G-CSF secreted by splenocytes in the presence of premalignant cell supernatant was over 275-fold the level secreted by splenocytes in the presence of HNSCC cell supernatant or media alone. The level of GM-CSF secreted by splenocytes in the presence of premalignant cell supernatant was 88-fold/control, increasing to about 140-fold/control after stimulation *in vitro*, whereas the level secreted by splenocytes in the presence of HNSCC cell supernatant was 2-fold/control, increasing to only 3-fold/control after stimulation.

These data show the contribution of premalignant lesion cells to inflammatory and immune stimulatory environment of the premalignant lesion. While premalignant cells stimulate a significant proinflammatory innate response, HNSCC cells are

significantly less immune stimulatory. This may be a key mechanism by which the HNSCC tumor escapes an effective immune response.

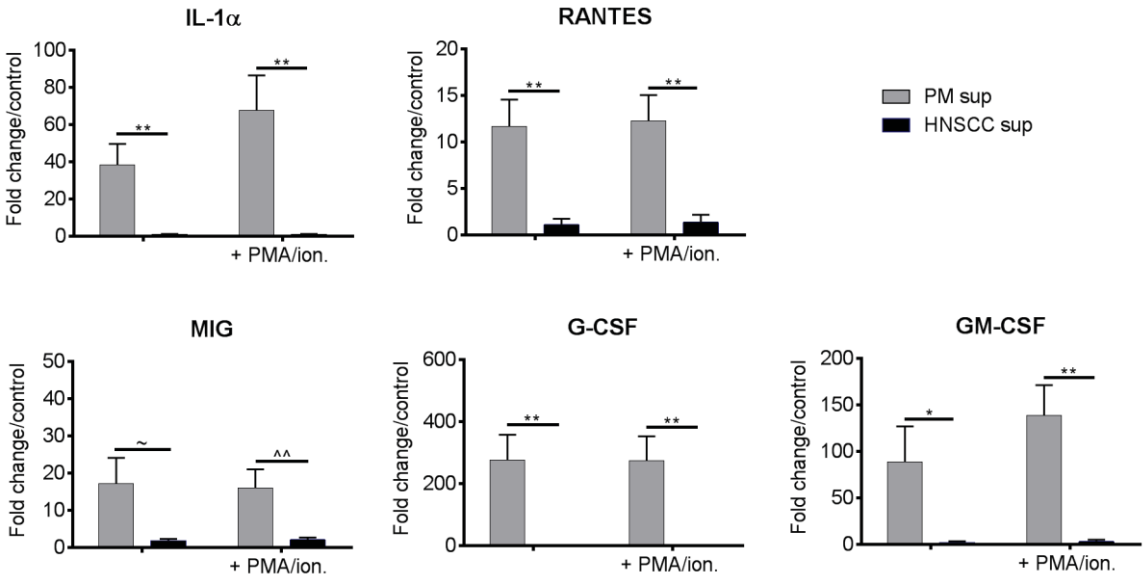


Fig. 3.4 Splenocytes cultured with premalignant cell-conditioned media release increased levels of innate proinflammatory mediators compared to splenocytes cultured with HNSCC cell-conditioned media or fresh media alone. Splenocytes from control C57BL/6 mice were incubated with fresh medium or media conditioned by premalignant lesion cells or HNSCC cells, with or without PMA/ionomycin stimulation for the last 4 h of culture. Supernatants were collected from spleen cell cultures for cytokine and chemokine analyses after 72 h. Cytokine and chemokine levels were standardized to levels produced by spleen cells in media alone and data is shown as fold change/control. Data represent 3 independent experiments, each performed in duplicate**.** *$p<0.05$ **$p<0.01$ ~$p=0.051$ ^^$p=0.064$ (2-tailed Student's *t* test).

Spleen cells produce increased levels of Th1- and Th17-associated cytokines in the presence of premalignant lesion supernatant compared to spleen cells exposed to HNSCC supernatant or media alone

The above studies showed that premalignant lesion cells elicit increased production of innate proinflammatory mediators from immune cells compares to HNSCC cells. The present study was conducted to determine the impact of premalignant lesion cells on stimulating the production of Th1-, Th2- and Th17-type cytokines by immune cells. To investigate how immune mediators produced by premalignant lesion cells and HNSCC cells alters T cell-associated cytokines, spleen cells were cultured with media conditioned by premalignant lesion cells or HNSCC cells and stained intracellularly for IFN-γ expression. As shown in Figure 3.5a,b below, an increased percentage of $CD4^+$ T cells expressed IFN-γ in the presence of premalignant lesion cell-conditioned media compared to HNSCC cell-conditioned media or fresh media alone. More prominent was the increased percentage of $CD8^+$ T cells that expressed IFN-γ in response to premalignant lesion cell-conditioned media compared to HNSCC cell-conditioned media or fresh media alone.

In addition to analysis by flow cytometric staining, supernatants were collected from spleen cell cultures to investigate how premalignant lesion cell or HNSCC cell supernatants impact on the secretion of a panel of Th1-, Th2-, and Th17-associated cytokines. As described above, to simplify visualization of the effects of premalignant lesion cells vs. HNSCC cells on spleen cell cytokine production, data were normalized to

cytokine levels secreted by spleen cells cultured in media alone (control cultures) and presented as fold change/control values. As shown in Figure 3.5c, we found that the level of IL-2 secreted by splenocytes cultured with premalignant supernatant was significantly higher (20-fold/control) than splenocytes cultured with HNSCC supernatant (3-fold/control), after stimulation *in vitro*. Similarly, the levels of Th1-associated IFN-γ and TNF were significantly higher in the supernatant of splenocytes cultured with premalignant lesion cell supernatant compared to HNSCC cell supernatant, with and without stimulation. This data further supports the hypothesis that the premalignant environment supports a Th1-type response, whereas the tumor microenvironment does not.

Similarly, splenocytes cultured with premalignant lesion cell supernatant secreted significantly higher levels of IL-17A (78-fold/control) than splenocytes cultured with HNSCC supernatant (1.2-fold/control), even without re-stimulation. After stimulation with PMA/ionomycin, the amount of IL-17A secreted by splenocytes in the presence of premalignant supernatant increased to over 120-fold/control, whereas the amount of IL-17A released in the presence of HNSCC supernatant increased only slightly to 1.4-fold/control. This suggests that while the premalignant environment elicits a significant Th17-type response, the HNSCC environment does not.

A similar trend was discovered with Th2-associated cytokines, although overall, the levels of Th2-associated cytokine were lower compared to Th1-associated cytokines.

The levels of IL-4 and IL-6 secreted by splenocytes cultured with premalignant lesion cell supernatant were significantly higher than splenocytes cultured with HNSCC cell supernatant or media alone. Before stimulation, the levels of IL-4 and IL-6 secreted by splenocytes in the presence of premalignant supernatant were increased approximately 2-fold/control and over 150-fold/control, respectively. After stimulation *in vitro*, these levels rose to over 4-fold/control for IL-4 and decreased slightly to 130-fold/control for IL-6. The level of IL-10 was increased in the supernatant of splenocytes co-cultured with premalignant lesion cell supernatant (about 124-fold/control) compared to HNSCC cell supernatant (5-fold/control), although the difference was not statistically significant ($p=0.094$). After restimulation with PMA/ionomycin, the level of IL-10 produced by spleen cells in the presence of premalignant lesion cell supernatant (165-fold/control) was significantly increased compared to spleen cells in the presence of HNSCC cell supernatant (4-fold/control). Overall, these data indicate that premalignant lesion supernatant elicits a significant Th1-, Th2-, and Th17-associated response, whereas HNSCC supernatant is significantly less immune stimulatory.

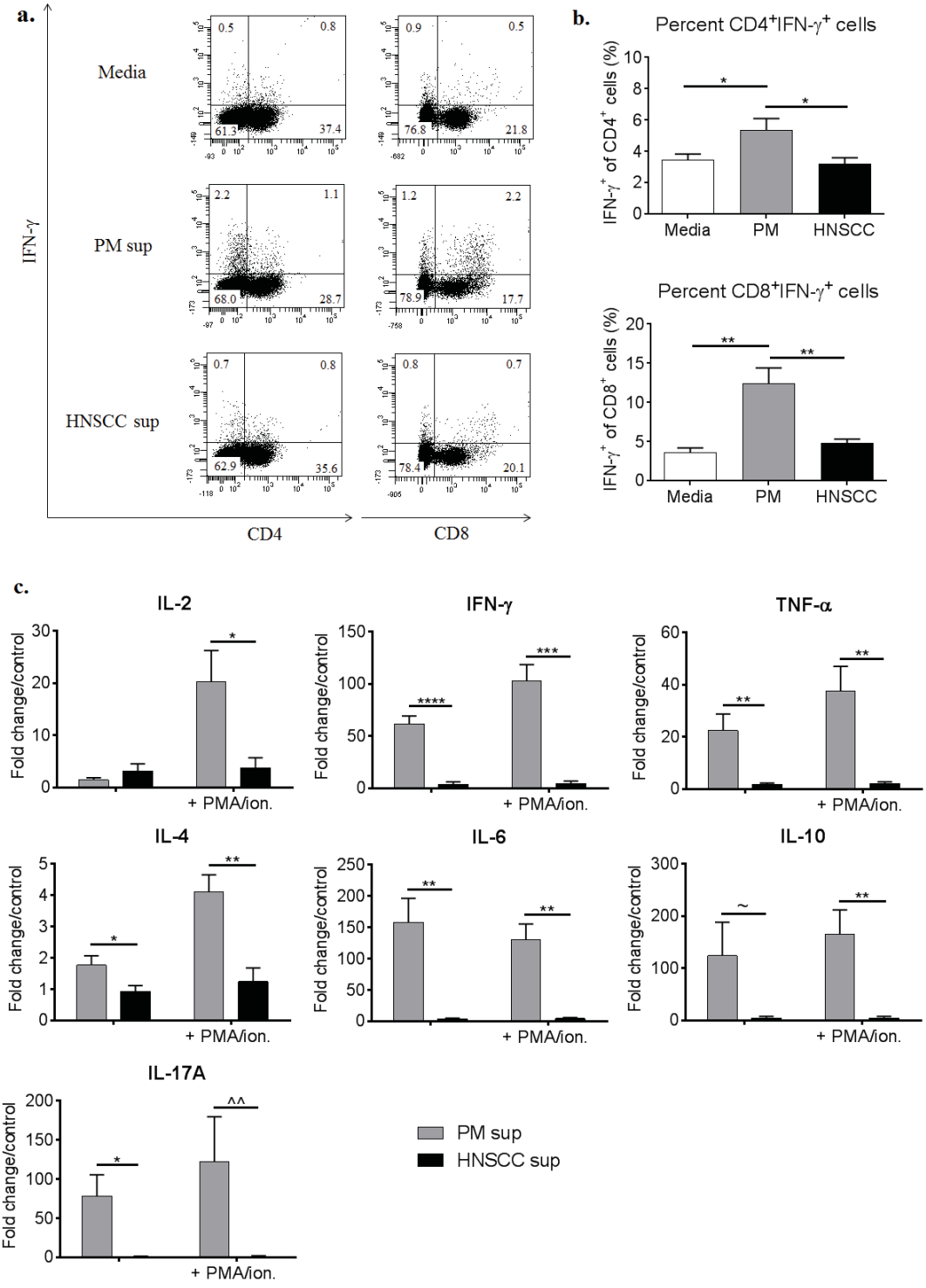
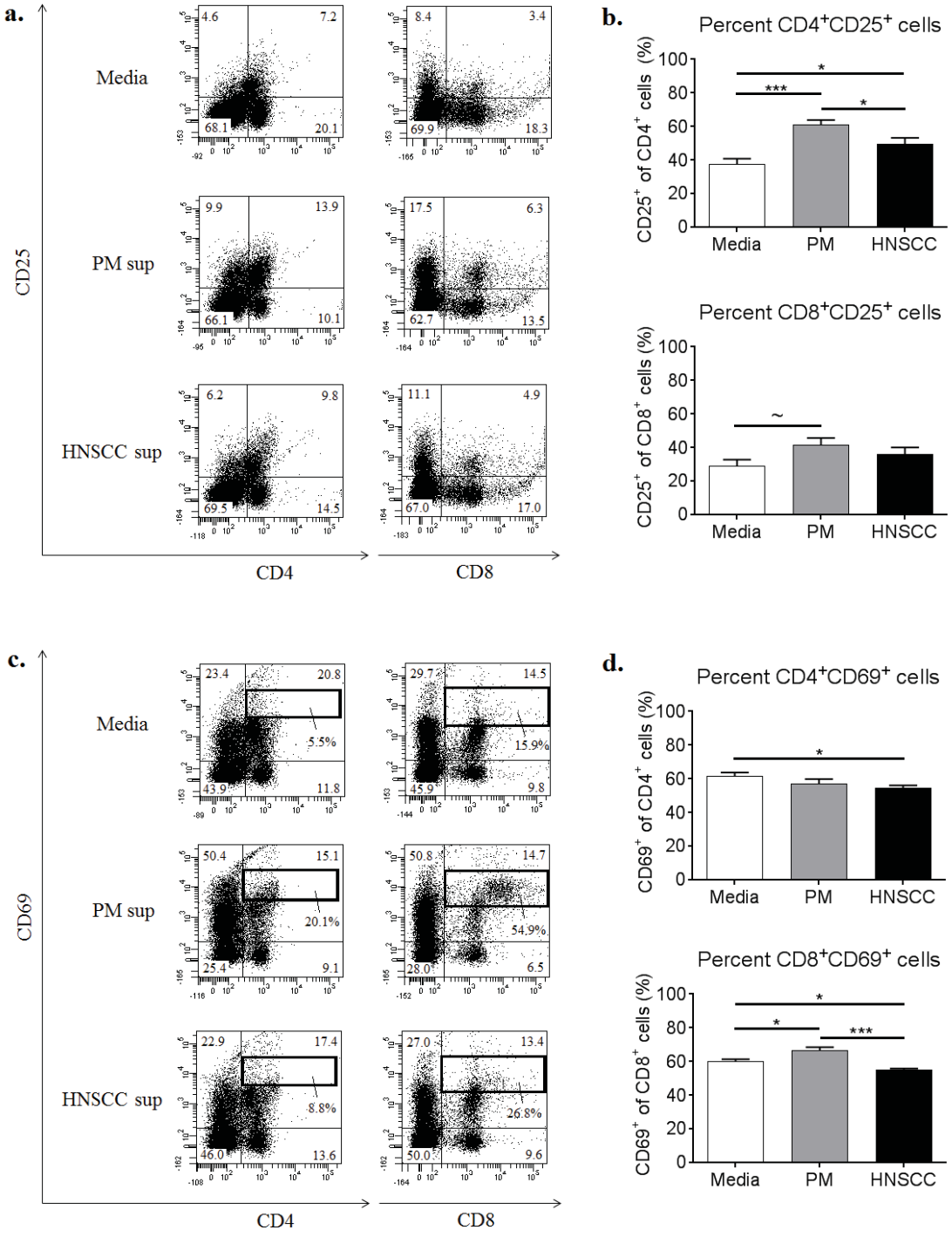

a.
Media
PM sup
HNSCC sup
IFN-γ
CD4
CD8
0.5
0.8
61.3
37.4
0.9
0.5
76.8
21.8
2.2
1.1
68.0
28.7
1.2
2.2
78.9
17.7
0.7
0.8
62.9
35.6
0.8
0.7
78.4
20.1
b.
Percent CD4+IFN-γ+ cells
IFN-γ+ of CD4+ cells (%)
Media
PM
HNSCC
Percent CD8+IFN-γ+ cells
IFN-γ+ of CD8+ cells (%)
c.
IL-2
IFN-γ
TNF-α
IL-4
IL-6
IL-10
IL-17A
Fold change/control
+ PMA/ion.
PM sup
HNSCC sup

Fig. 3.5 Premalignant lesion cell-conditioned media elicits increased expression of IFN-γ in $CD4^+$ and $CD8^+$ T cells and increased secretion of Th1-, Th2-, and Th17-associated cytokines from spleen cells compared to cytokines produced in HNSCC cell-conditioned media or media alone. Representative results (a) and graphical representation (b) of flow cytometric staining of spleen cells from control C57BL/6 mice cultured with media alone, premalignant lesion cell-conditioned media, or HNSCC cell-conditioned media for 72 h. Spleen cells were restimulated with PMA/ionomycin for the last 4 h of culture. Data show staining of spleen cells from 3 independent experiments, run in duplicate. For cytokine analyses (c), supernatants were collected from spleen cell cultures after 72 h and analyzed by CBA. Cytokine levels were standardized to levels produced by spleen cells in media alone and data is shown as fold change/control. Data represent mean ± SEM. *$p<0.05$ **$p<0.01$ ***$p<0.001$ ****$p<0.0001$ ~$p=0.094$ ^^$p=0.061$ (2-tailed Student's *t*-test).

Increased percentage of $CD4^+$ T cells express IL-2 receptor CD25 and $CD8^+$ T cells express activation marker CD69 in the presence of premalignant lesion cell supernatant compared to expression in the presence of HNSCC cell supernatant

The immune environment shaped by HNSCC has been shown to impact on immune cell activation and function [10, 20, 100, 105, 106]. Previous studies, as well as the current study, suggest that immune cells are more activated in the premalignant lesion environment [72, 100, 101]. The direct impact of immune mediators released by premalignant lesion cells on the activation status of immune cells, however, is not well defined. While the above studies showed premalignant lesion cell stimulation of spleen cell cytokine production, an alternate approach was used to further demonstrate T-cell stimulation. To further analyze how immune mediators released by premalignant lesions cells and HNSCC cells impact on immune cell function, the expression of IL-2 receptor CD25 and activation marker CD69 on spleen cells in the presence of supernatant collected from premalignant lesion cell and tumor cell cultures was analyzed. In the presence of premalignant lesion cell supernatant, an increased percentage of $CD4^+$ T cells expressed CD25 compared to HNSCC cell supernatant or media alone. This increase in CD25 expression could reflect an increase in the percentage of T regulatory cells induced in the premalignant lesion environment or it could be an indicator of increased T cell activation. A similar trend was observed for $CD8^+$ T cells, in which a greater percentage expressed CD25 in the presence of premalignant lesion cell supernatant compared to what was expressed in media alone, although the difference was not significant. There

was no difference in the expression of CD25 in the $CD8^+$ T cell compartment between the premalignant lesion cell and HNSCC cell environments. In the $CD4^+$ T cell compartment, the percentage of cells expressing the early activation marker CD69 in cultures with HNSCC cell-conditioned media was significantly decreased compared to cultures in media alone. In the $CD8^+$ T cell compartment, the percentage of $CD69^+$ cells was significantly increased after culture with premalignant lesion cell-conditioned media compared to culture with HNSCC cell-conditioned media or media alone. Further analysis of CD69 expression revealed an increased percentage of $CD69^{hi}$-expressing cells among $CD4^+CD69^+$ and $CD8^+CD69^+$ T cells in cultures with premalignant lesion cell supernatant compared to cultures with HNSCC cell supernatant or media alone, suggesting that the premalignant lesion environment induces a population of highly activated cells in both the $CD4^+$ and $CD8^+$ T cell compartments (gating strategy and graphical results shown in Figure 3.7c,e). These data confirm the above described cytokine analyses by showing that the immune environment shaped by premalignant lesion cells is more stimulatory compared to the environment shaped by HNSCC cells and induces a greater percentage of activated, $CD4^+$ T cells expressing CD25 and a greater percentage of $CD4^+$ and $CD8^+$ T cells expressing high levels of CD69.


a.
Media
PM sup
HNSCC sup
CD25
CD4
CD8
4.6
7.2
68.1
20.1
8.4
3.4
69.9
18.3
9.9
13.9
66.1
10.1
17.5
6.3
62.7
13.5
6.2
9.8
69.5
14.5
11.1
4.9
67.0
17.0
b.
Percent CD4+CD25+ cells
CD25+ of CD4+ cells (%)
Media
PM
HNSCC
Percent CD8+CD25+ cells
CD25+ of CD8+ cells (%)
c.
CD69
23.4
20.8
5.5%
43.9
11.8
29.7
14.5
15.9%
45.9
9.8
50.4
15.1
20.1%
25.4
9.1
50.8
14.7
54.9%
28.0
6.5
22.9
17.4
8.8%
46.0
13.6
27.0
13.4
26.8%
50.0
9.6
d.
Percent CD4+CD69+ cells
CD69+ of CD4+ cells (%)
Percent CD8+CD69+ cells
CD69+ of CD8+ cells (%)

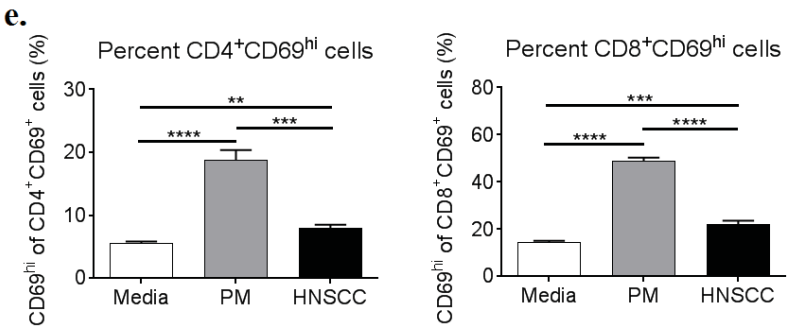


Fig. 3.6 Premalignant lesion cell-conditioned media elicits increased expression of CD25 on $CD4^+$ T cells and CD69 on $CD4^+$ and $CD8^+$ T cells, compared to HNSCC cell-conditioned media or media alone. Representative results (a,c) and graphical representation (b,d) of flow cytometric staining of spleen cells from control C57BL/6 mice cultured with media alone, premalignant lesion cell-conditioned media, or HNSCC cell-conditioned media for 72 h. Data show staining of spleen cells from 3 independent experiments, run in duplicate. Graphical results of the percentage of $CD69^{hi}$ populations among $CD4^+CD69^+$ cells and $CD8^+CD69^+$ cells gated in (d) are shown in (e). Data represent mean $\pm$ SEM. *p<0.05 **p<0.01 ***p<0.001 ~p=0.056 (2-tailed Student's *t*-test).

Cervical lymph node cells produce increased levels of Th1-, Th2-, and Th17-associated cytokines and proinflammatory mediators in the presence of premalignant lesion cell supernatant

In the 4-NQO model, cervical lymph nodes drain the oral cavity and therefore house immune cells that directly infiltrate the developing lesions and tumors [95, 97, 98, 100]. To investigate how immune mediators produced by premalignant lesion cells and HNSCC cells impact on cytokine production by immune cells in the draining lymph nodes of the developing lesions or tumors, cervical lymph node cells were cultured with media conditioned by premalignant lesion cells or HNSCC cells and supernatants were collected for analysis by CBA. As shown in Figure 3.7, in the presence of premalignant lesion cell supernatant, cervical lymph node cells produced increased levels of Th1-associated cytokines compared to cervical lymph node cells cultured in the presence of HNSCC cell supernatant or media alone. This included increases in cervical lymph node cell production of IL-2, IFN-γ, and TNF-α in the presence of premalignant lesion cell supernatant compared to lymph node cell production of these Th1-type cytokines in the presence of HNSCC cell supernatant or media alone. A similar trend was found for Th2-associated cytokines, although the overall levels of Th2-associated cytokines were lower than Th1-associated cytokines. In the presence of premalignant lesion cell-conditioned media, cervical lymph node cells secreted increased levels of IL-4 and IL-6 compared to cervical lymph node cells in the presence of HNSCC cell-conditioned media or media alone.

Similarly, cervical lymph node cells produced increased levels of the Th17 cell-associated cytokine IL-17A and other proinflammatory mediators in the presence of premalignant lesion cell-conditioned media compared to levels produced by lymph node cells cultured with HNSCC cell-conditioned media or fresh media alone (Figure 3.7). The level of IL-17A produced by spleen cells in the premalignant lesion cell environment was higher than the level produced by spleen cells in the HNSCC cell environment or media alone. Cervical lymph node cells produced significantly higher levels of the chemokines MIP-1α and MIP-1β in the presence of premalignant lesion cell-conditioned media compared to when cultured in HNSCC cell-conditioned media or media alone, suggesting the premalignant lesion environment supports a more proinflammatory macrophage response compared to the HNSCC environment. The level of macrophage-supporting G-CSF was also higher in the supernatant of cervical lymph node cells cultured with premalignant lesion cell-conditioned media compared to when cultured with HNSCC cell-conditioned media or media alone. Overall, premalignant lesion cell supernatant elicits increased production of proinflammatory mediators from cervical lymph node cells compared to what is elicited by HNSCC cell supernatant.

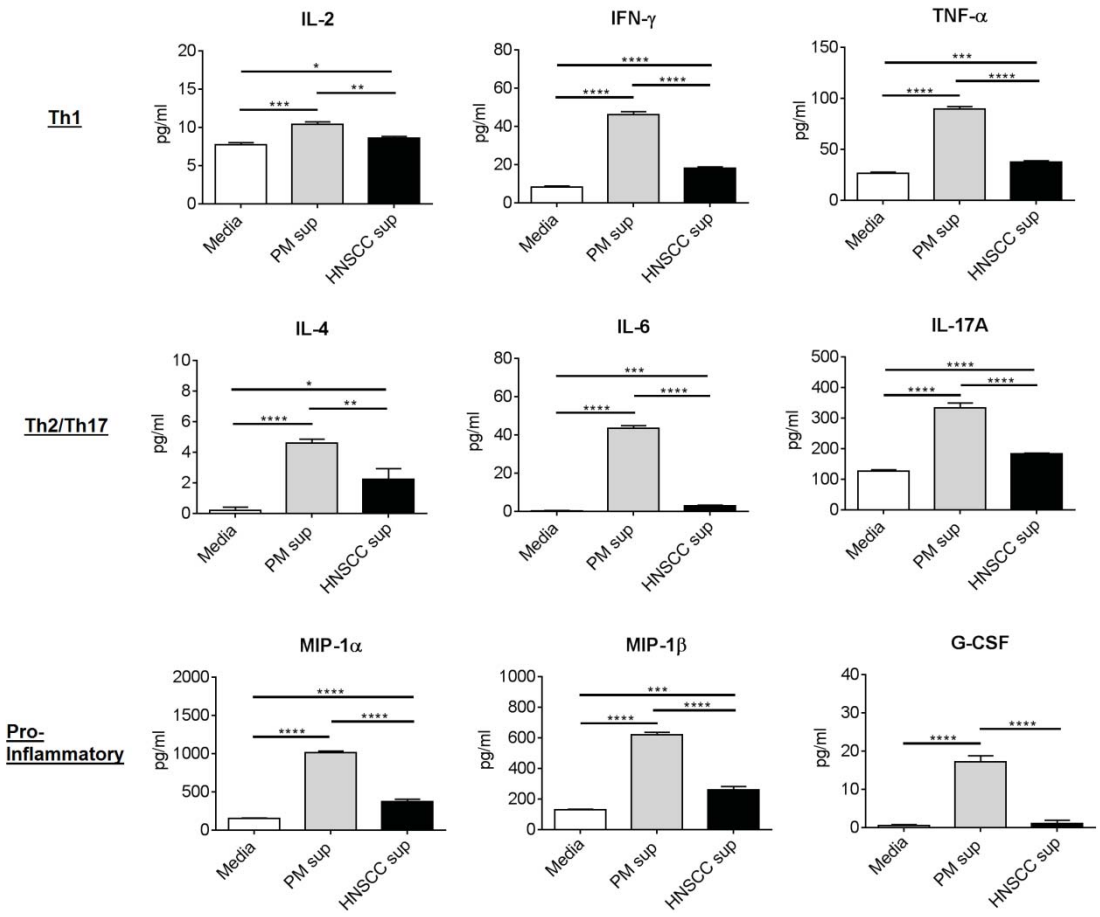


Fig. 3.7 Cervical lymph node cells release increased levels of Th1-, Th2- and Th17-associated cytokines and proinflammatory mediators in the presence of premalignant cell-conditioned media compared levels released by lymph node cells in the presence of HNSCC cell-conditioned media or fresh media alone. Cervical lymph node cells were extracted from control C57BL/6 mice and cultured with premalignant lesion cell-conditioned media, HNSCC cell-conditioned media or media alone for 72 h. Cell cultures were restimulated with PMA/ionomycin for the last 4 h of culture and supernatants were collected for cytokine analyses by CBA. Data shown represent mean ± SEM of three independent experiments, each performed in duplicate. *p<0.05 **p<0.01 ***p<0.001 ****p<0.0001 (2-tailed Student's *t* test).

Chapter 4

Administration of Indomethacin to Premalignant Lesion-bearing Mice

CHAPTER 4: ADMINISTRATION OF INDOMETHACIN TO PREMALIGNANT-LESION BEARING MICE

Aim 2: To prevent the immune skewing and tumor progression in 4-NQO-treated mice by inhibiting PGE_2 production at the premalignant lesion stage.

4.1 Introduction

The five-year survival rate for HNSCC patients remains at 50% [2]. Treatment for HNSCC patients is significantly complicated by immunosuppression and, in many cases, the traditional routes of therapy, including surgical resection and chemo-radiation, are ineffective at completely eliminating the tumor [1, 2, 20, 26, 28]. Furthermore, due to the location of primary tumors on the head and neck region and the effect of major surgery in this region, patient quality of life is often significantly decreased. There exists a critical need for more effective therapies, and focus has recently shifted to immunotherapies to reduce the burden of primary tumors and prevent recurrence.

The goal of any effective immunotherapy is to activate the patient's own immune system to eradicate tumor. HNSCC tumors use a variety of mechanisms to evade the immune response, including recruitment of immunosuppressive cells, inhibition of cytotoxic T cells, and secretion of factors such that dampen the immune response at the tumor site [10, 28, 31, 32, 35, 106]. One of the factors that is upregulated in the HNSCC tumor environment is PGE_2. While PGE_2 is a proinflammatory mediator, it has also been

shown to directly inhibit T cell function, recruit immunosuppressive cells to the tumor site, skew dendritic cells toward myeloid-derived suppressor cells, and increase tumor growth [26, 46-49, 52, 53, 55, 60, 61]. Increased expression of COX-2, the enzyme responsible for increased PGE_2 production by HNSCC tumors, is correlated with a worse prognosis in patients, suggesting that PGE_2's role in dampening the immune response at the tumor site plays a role in a patient's clinical status [20, 26, 28, 60]. Previous studies have shown that treating HNSCC cells with COX inhibitors results in increased apoptosis and decreased growth of tumor cells [61]. In multiple models of murine and hamster head and neck cancer, administration of the COX-2-specific inhibitor celecoxib resulted in decreased tumor cell growth and angiogenesis [61-63]. These studies suggest that COX inhibition may be beneficial as a therapy for HNSCC patients. One retrospective study showed that HNSCC patients who had been on a regimen of COX inhibitors prior to surgery had increased survival rates compared to patients who had not [65], although neither the type of COX inhibitor nor the dosage was controlled for in this study. Several clinical trials have shown that administration of celecoxib to HNSCC patients as an adjuvant therapy resulted in significantly improved response rates [66-68]. The impact of COX inhibition on the immune response in HNSCC patients, however, has not been extensively studied. One study showed that preoperative administration of Rofecoxib, another COX-2-specific inhibitor, resulted in improved monocyte function, but the impact on T cell function or overall immune status, was not investigated [64].

There remains a gap in our knowledge of how COX inhibition affects the immune response in HSNCC patients, and specifically, how COX inhibition impacts on immune cell reactivity as premalignant lesions progress to tumor. Our preliminary studies in the 4-NQO mouse model of oral carcinogenesis have shown that, despite the broad-range immune stimulatory and inflammatory environment of premalignant oral lesions, PGE_2 is produced in much higher levels by premalignant lesion cells compared to what is produced by HNSCC cells This suggests that PGE_2 may be playing a previously unappreciated immune-modulatory role as premalignant lesions progress to tumor. There may be a critical window, such as during the premalignant lesion stage, in which COX inhibitors can be administered to activate and direct a patient's immune system to eradicate the developing tumor or to slow tumor progression. The hypothesis of the current study is that inhibiting PGE_2 production at the premalignant lesion stage will skew immune cells to a Th1-associated phenotype, leading to improved clinical outcomes and slowing progression to tumor.

The focus of this study is to investigate whether inhibiting PGE_2 production by premalignant lesions results in increased activation of immune cells and if this plays a role in the clinical outcome of mice with 4-NQO-induced oral lesions. Treating premalignant lesion cells with indomethacin, a pan-COX inhibitor, resulted in increased induction of immune cell production of Th1-associated cytokines *in vitro*. To advance studies to be clinically relevant, indomethacin was administered to mice with 4-NQO-induced premalignant lesions and the phenotype of immune cells infiltrating the cervical

lymph nodes and progression to tumor were observed (timeline shown in Figure 4.1). Mice receiving indomethacin at the premalignant lesion stage had significantly improved clinical outcomes compared to mice receiving diluent control treatment. The cervical lymph nodes and spleens of mice receiving indomethacin were characterized by increased IFN-γ expression compared to what was seen in mice receiving control treatment, suggesting that indomethacin plays a role in skewing immune cells to a Th1-associated phenotype, which are part of a more effective antitumor response.

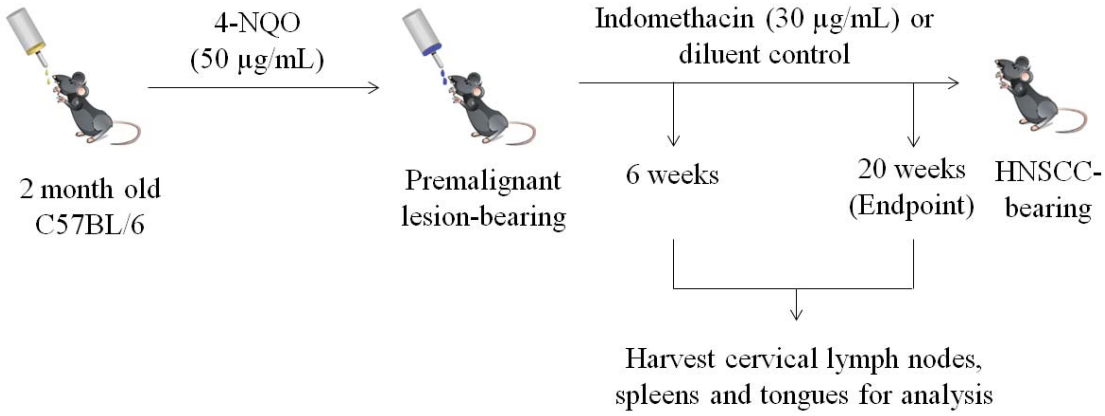


Figure 4.1 Timeline of indomethacin administration

4.2 Materials and Methods

Oral HNSCC carcinogenesis

Five mg/mL 4-NQO in propylene glycol stock was diluted into the drinking water at a final concentration of 50 μg/mL and administered to 2-month-old female C57BL/6 mice (Charles Rivers Laboratory, Wilmington, MA, USA) until the development of premalignant oral lesions (6–8 weeks) or HNSCC (12–16 weeks). To monitor lesion development, mice were examined endoscopically weekly using a Stryker 1.9 mm × 30° endoscope and images were taken using a Stryker 1088 camera (Kalamazoo, MI, USA). During the procedure, mice were sedated with inhaled isoflurane (Piramal Healthcare, Boise, ID, USA).

Culture medium

Cell culture media consisted of 1X DMEM (Life Technologies, Grand Island, NY, USA) containing 4.5 g/L D-glucose and L-glutamine, supplemented with 10% fetal bovine serum (FBS, Sigma, St. Louis, MO, USA) and 1X antibiotic antimycotic solution (Sigma, St. Louis, MO, USA) containing penicillin, streptomycin and amphotericin B. To establish cell lines from premalignant lesions and HNSCC tumors, culture media was supplemented with 2X antibiotic antimycotic solution for the first 2 weeks of culture.

Premalignant lesion and HNSCC cell lines

Primary cell lines were established by excising the tongues of premalignant or HNSCC-bearing mice at the appropriate stage, as defined through histopathological analysis

by the oral pathology section in the Center for Oral Health Research at the Medical University of South Carolina. Premalignant lesions or HNSCC were excised from the tongues of 4-NQO treated mice. To establish cell lines, premalignant lesions or HNSCC tumors were washed several times with PBS and antibiotic/antimycotic solutions containing 100 mg/mL penicillin, 100 mg/mL streptomycin, and 200 mg/mL neomycin. Premalignant lesions or HNSCC tumors were plated on 60 mm tissue culture dishes in DMEM media supplemented with 10% FBS and penicillin/streptomycin/amphotericin B and cultured for two weeks at 37º C, replacing the media twice per week. After this period, adhering premalignant lesion or carcinoma cells were removed and cultured in 75 cm^2 flasks in fresh media at 37° C, replacing the media twice per week. Prior to defining cells as premalignant or HNSCC, their epithelial phenotype was confirmed as well as uniformity of their microscopic and growth characteristics. To detach adherent cells, they were trypsinized, washed, and resuspended in fresh medium.

Spleen processing

Spleens were harvested from control C57BL/6 mice and homogenized using a glass homogenizer. Cells were passed through a 70 μm cell strainer (BD Falcon, San Jose, CA, USA) and rinsed with HBSS. Red blood cells were lysed by adding ACK Lysing Buffer (Lonza, Walkersville, MD, USA) for 3 min. Splenocytes were then washed twice with HBSS. Cell number was determined by counting cells excluding trypan blue using a hemocytometer.

Indomethacin treatment of premalignant lesion cells and HNSCC cells

Premalignant lesion cells and HNSCC cells were cultured for 24 h at 37° C at 1×10^6 cells/well in 12-well tissue culture plates in fresh media with or without 6 μg/mL indomethacin, as previously described [107]. Supernatants were collected for culture with spleen cells. To serve as a control for indomethacin treatment, spleen cells were cultured in fresh media with 6 μg/mL indomethacin.

Spleen cell cultures with indomethacin-treated premalignant lesion and HNSCC cell supernatants

Spleen cells from control C57BL/6 mice were cultured for 72 h at 37° C with supernatants collected from indomethacin-treated premalignant lesion cells and HNSCC cells, respectively, at 1×10^6 cells/well in 12-well anti-CD3-coated tissue culture plates at a dilution of 1:2 in fresh media containing 30 IU recombinant mouse IL-2. Controls consisted of splenocytes cultured for 72 h at 37° C in fresh medium, with or without 6 μg/mL indomethacin. Supernatants were collected and the levels of cytokines, chemokines, or prostaglandins produced by spleen cells were quantitated using CBAs and ELISA. The cytokine profile and activation status of spleen cells was analyzed by CBA and flow cytometric staining.

Cytokine bead array

All reagents used for the cytokine bead array are from BD Biosciences unless otherwise specified. The levels of IFN-γ, IL-2, IL-17A, IL-4, IL-6 and IL-10 in cell cultures

were determined using a mouse cytometric bead array (CBA) Th1/Th2/Th17 cytokine kit. Levels of IL-1α, IL-1β, IL-13, IL-12p70, IL-9, GM-CSF, G-CSF, MIG, MCP-1, MIP-1α, MIP-1β and RANTES in cell cultures were determined using cytometric bead array flex sets for the individual cytokines according to the manufacturer's instructions. A FACS Canto (BD Biosciences, San Jose, CA, USA) flow cytometer was used to quantify cytokine profiles and relative amounts of each cytokine were analyzed using FCAP Array Software (manufactured by Soft Flow Hungary Ltd. for BD Biosciences, San Jose, CA, USA). In experiments where premalignant lesion or HNSCC supernatants were added to spleen cell cultures, cytokine levels in total spleen cell supernatants were adjusted for baseline levels in premalignant or HNSCC supernatants.

Indomethacin treatment of 4-NQO-treated mice

Two-month-old female C57BL/6 mice (Charles Rivers Laboratory, Wilmington, MA, USA) were administered 50 μg/mL 4-NQO in their drinking water until the development of premalignant oral lesions (8 weeks), as determined by weekly endoscopy of the oral cavities. At this time point, one group of C57BL/6 control mice (5 mice) and one group of premalignant lesion-bearing mice (5 mice) were euthanized and tongues and cervical lymph node cells were harvested for analysis. To the remaining premalignant lesion-bearing mice, 4-NQO water was removed and replaced with drinking water containing either 30 μg/mL indomethacin (10 mice) or diluent (1% ethanol) control (10 mice) [108]. Oral cavities were endoscopically monitored weekly for the development of HNSCC using a Stryker 1.9 mm × 30° endoscope. Lesions were counted and scored

blindly every 10 days and the development of tumors was documented using a Stryker 1088 camera. Six and twenty weeks after beginning indomethacin or diluent control treatment one group of mice receiving indomethacin (5 mice) and one group of mice receiving diluent control (5 mice) were sacrificed and tongues and cervical lymph nodes were harvested for analysis. A group of age-matched untreated C57BL/6 control mice were also sacrificed at these time points and tongues and cervical lymph nodes harvested for analysis.

Lesion scoring

As part of the endoscopic exam performed every 10 days, the total number and gross pathologic score of tongue lesions in 4-NQO-treated mice receiving either indomethacin or diluent control were blindly assessed and recorded as previously described [100, 109]. Lesions were scored on a 1-4 scale, with 1 indicating a flat macule, 2 indicating a raised papule, 3 indicating a raised plaque, and 4 indicating a grossly exophytic lesion.

Cervical lymph node processing and culture

Cervical lymph nodes were harvested from experimental and control C57BL/6 mice at designated time points, as described in individual experiments. Cervical lymph nodes were homogenized using a Stomacher 80 homogenizer (Seward) set on high for 90 sec. Cells were then passed through a 70 μm cell strainer (BD Falcon, San Jose, CA, USA) and rinsed with HBSS. Cell number was determined by counting cells excluding trypan blue using a hemocytometer. Cells were cultured in 12-well anti-CD3-coated

tissue culture plates at $1X10^6$ cells/well in fresh media for 72 h at 37º C. Supernatants were collected (500 µL) from each well for analysis by CBA. Cells were then restimulated with 2 µL/mL Cell Stimulation Cocktail (eBioscience, PMA/ionomycin) and 0.6 µL/mL GolgiStop (BD Biosciences) for the last 4-6 h of culture at 37º C. Cells were gently collected from tissue culture plates with 25 cm cell scrapers (Sarstedt, Inc., Newton, NC, USA), washed once with stain buffer, and transferred to polystyrene tubes at $1X10^6$ cells/tube for flow cytometric analysis.

Flow cytometric analysis of cells

All antibodies and reagents for this section are from BDBiosciences unless otherwise stated. Spleen cells or cervical lymph node cells were processed to a single cell suspension in polystyrene tubes at $1X10^6$ cells/tube and were resuspended in 300 µL FACS block buffer, containing 2% FBS in sterile 1X PBS, at 4º C for 15 min and centrifuged at 1000 rpm for 5 min. Cells were then incubated in 10 µL Fc block, containing anti-CD16/CD32 antibody at a 1:100 dilution in sterile 1X PBS, at 4º C for 10 min to block nonspecific antibody binding to the cell surface. Cells were washed twice in stain buffer (BD Biosciences), resuspended in 1 mL cold BD Cytofix, and incubated for 20 min at 4º C. Cells were centrifuged, washed twice in 1 mL stain buffer, resuspended in 1 mL BD Perm/Wash Buffer and incubated for 15 min at room temperature. Cells were then centrifuged, resuspended in 50 µL BD Perm/Wash Buffer and incubated for 30 min at room temperature with equal concentrations of antibodies or appropriate isotype controls: FITC CD4, APC CD8a, PerCP-Cy5.5 IFN-γ and PE CD69. Cells were washed twice in

1 mL BD Perm/Wash Buffer and resuspended in 400 µL stain buffer for analysis of extent and frequency of positive cells on a FACS Canto flow cytometer.

Tongue processing and culture

Three tongues per group of mice (control untreated mice or premalignant lesion-bearing mice treated with diluent control or indomethacin) were harvested for analysis of PGE_2 levels by ELISA at the designated timepoints. After extraction, each tongue was minced and enzymatically digested using 100 µg/mL of complete liberase cocktail (Roche, Indianapolis, IN, USA) in 10 mL HBSS (Life Technologies, Grand Island, NY, USA) at 37º C for 4 h. Tissue was resuspended in 1 mL sterile PBS and lysed using an ultrasonic processor for 2 min. on ice. For each dissociated tongue, PGE_2 levels were standardized to total protein. Total protein was quantitated using a BCA protein assay kit (Thermo Scientific, Rockford, IL, USA).

Prostaglandin E_2 ELISA

To quantify PGE_2 in tongue tissue, a competitive ELISA was performed according to the manufacturer's instructions (Pierce Biotechnology, Rockford, IL, USA). Tongue tissue lysates were diluted 1:50 for analysis. Optical density at 405 nm was measured using a Spectra Max M2 plate reader. Concentrations of PGE_2 were determined using SoftMax Pro 5.4.2 plate reader software. A standard concentration curve was generated for each independent experiment. Tongue lysate samples were tested in duplicate for 3 mice/group.

Statistical analyses

Data were reported using the mean as a measure of central tendency ± standard error of the mean. To compare one variable condition between groups, a one-way ANOVA analysis was initially performed (GraphPad Prism version 6.03 for Windows, GraphPad Software, La Jolla, CA, USA). If differences were identified by the ANOVA analysis, a two-tailed Student's *t* test or Mann-Whitney U test was then performed to determine significance of differences between each of two groups (ex.: control *vs.* premalignant, control *vs.* HNSCC, premalignant *vs.* HNSCC, indomethacin *vs.* diluent control) using GraphPad Prism version 6.03. Significance was reported in the 95% confidence interval.

4.3 Results

Indomethacin-treated premalignant lesion cells are increased in their capacity to skew spleen cell cytokine production

One mechanism by which PGE_2 impacts on immune reactivity in the tumor environment is by inhibiting the production of Th1-associated cytokines by T cells [46-49]. Inhibiting PGE_2 production in a model of lung carcinoma resulted in a skewing of cytokine production by resident immune cells towards a more Th1-type phenotype characterized by increased IL-12 production, leading to decreased tumor growth [71]. To analyze how inhibiting PGE_2 production impacts on cytokine production by immune cells in the premalignant lesion environment, and how this compares to the HNSCC environment, spleen cells from control C57BL/6 mice were cultured with media conditioned by supernatants collected from indomethacin-treated premalignant lesion cells or HNSCC cells.

As shown in Figure 4.1 below, treatment of both premalignant lesion cells and HNSCC cells with indomethacin, a pan-COX inhibitor, significantly decreased PGE_2 in cell culture supernatant after 24 hours. This demonstrates that indomethacin effectively inhibited COX enzymes in these cell cultures. Indomethacin treatment of premalignant lesion cells significantly increased their induction of spleen cell production of Th1-associated IL-2, whereas indomethacin treatment of HNSCC cells had no effect on the induction of spleen cell production of IL-2. Indomethacin treatment of premalignant lesion cells also increased their induction of spleen cell production of IFN-γ and IL-6, although the differences were not significant. Similar to what was observed with IL-2,

indomethacin treatment of HNSCC cells has no effect on the induction of IFN-γ or IL-6 production by spleen cells. These data suggest that inhibiting PGE_2 production by premalignant lesion cells may elicit increased production of Th1-type cytokines by immune cells. Interestingly, supernatants from indomethacin-treated premalignant lesion cells also led to increased spleen cell production of the Th2-associated cytokine IL-10 compared to exposure to supernatants from premalignant lesion cells that were not treated with indomethacin, suggesting that PGE_2 might be playing a role in triggering Th2-mediated immunity as well as Th1-mediated immunity. However, the pro-tumoral vs. anti-tumoral implications of indomethacin are not clear. Indomethacin treatment of both premalignant lesion cells and HNSCC cells resulted in decreased production of IL-17A by spleen cells, suggesting that in both the premalignant lesion and tumor environments, PGE_2 plays a role in maintaining Th17 cells or directly promotes IL-17A production. Treatment of premalignant lesion cells with indomethacin did not alter spleen cell expression of the IL-2 receptor CD25 or the early activation marker CD69 (data not shown). The given data show that indomethacin treatment of premalignant lesion cells skewed cytokine production by spleen cells towards a Th1-type response, which has been shown to be more effective against tumor.

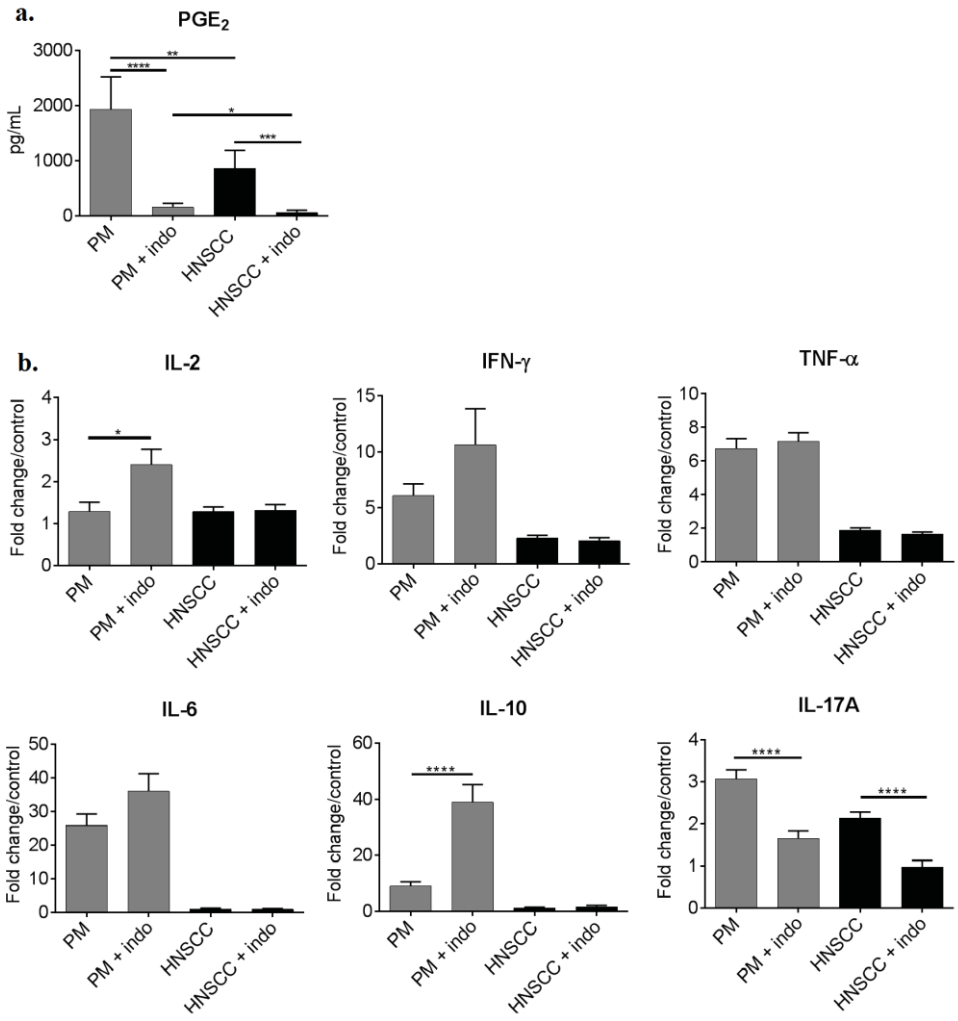


Fig. 4.2 Pretreatment of premalignant lesion cells with indomethacin skews cytokine production by splenocytes, leading to increased production of Th1-associated IL-2 and decreased production of Th17-associated IL-17A. (a) Premalignant lesion cells and HNSCC cells were plated at $1X10^6$ cells/well and treated with 6 µg/mL indomethacin for 24 h. The levels of PGE_2 in cell supernatants were analyzed by ELISA. (b) Spleen cells from control C57BL/6 mice were cultured for 72 h with media conditioned by supernatants from premalignant lesion cell or HNSCC cell cultures that remained untreated or were previously treated with 6 µg/mL indomethacin. Supernatants were collected for cytokine analysis by CBA. Cytokine levels were standardized to levels produced by spleen cells in media alone and data is shown as fold change/control. Data represent 5 independent experiments, each run in duplicate. Data represent mean ± SEM. *p<0.05 **p<0.01 ***p<0.001 ****p<0.0001 (2-tailed Student's *t*-test).

Administration of indomethacin to mice with 4-NQO-induced premalignant lesions results in an improved clinical response compared to diluent control treatment

The goal of any effective immunotherapy is to improve clinical outcome. The current study aimed to stimulate immune cell reactivity and slow progression to tumor by administering indomethacin to mice at the premalignant lesion stage. To monitor the development of premalignant lesions to HNSCC in mice treated with indomethacin vs. diluent control, mice were endoscoped every 10 days, beginning at the premalignant lesion stage. The lesions were scored on a 1-4 scale, with 1 representing a flat macule, and 4 representing a grossly exophytic tumor. As shown in Figure 4.3a below, mice receiving indomethacin treatment beginning at the premalignant lesion stage had a significantly better overall clinical outcome compared to diluent control mice. At the endpoint of the study (day 139, about 20 weeks post-initiation of treatment), the average lesion score for mice receiving indomethacin was 1.9, whereas the average lesion score for mice receiving diluent control was 3.4. Differences in clinical outcome were more striking at later time points, as shown in Figure 4.3. There were no significant differences in lesion stage between the diluent control and indomethacin groups until the last time point (day 139 post-initiation of treatment), although by day 104, the average lesion score for mice receiving diluent control treatment (2.5) was higher than mice receiving indomethacin treatment (2.1). By the endpoint of the study (day 139) two mice in the diluent control group progressed to advanced HNSCC (4), bearing exophytic tumors, whereas no mice in the indomethacin group progressed to this stage during the study.

Furthermore, 4 out of 5 mice in the diluent control group had lesions scores of 3 or higher at the endpoint of the study, compared to one mouse in the indomethacin group. These data show that administering indomethacin to mice with 4-NQO-induced premalignant lesions improves clinical outcome and slows progression to HNSCC.

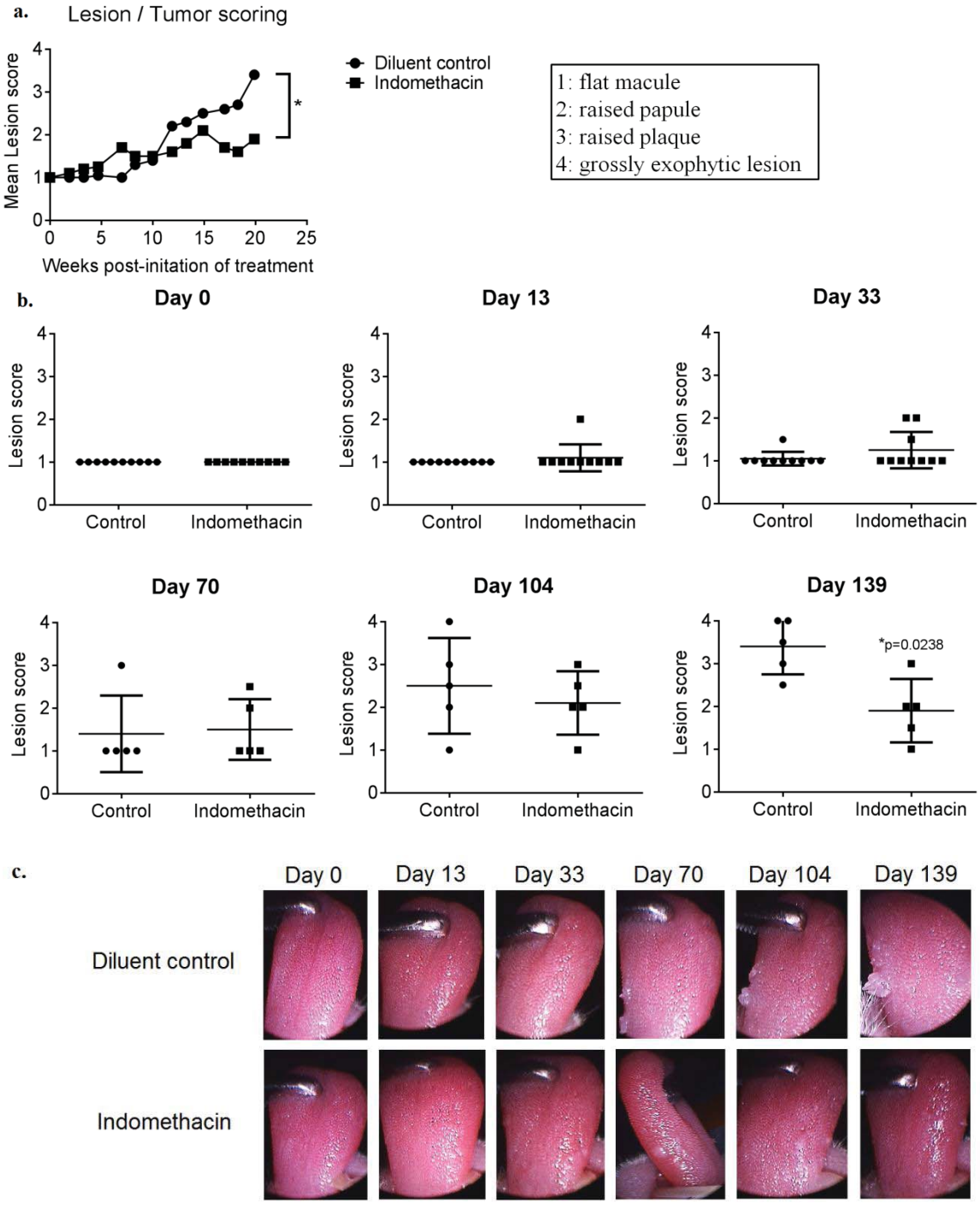

a.
Lesion / Tumor scoring
Mean Lesion score
Weeks post-initation of treatment
Diluent control
Indomethacin
*
1: flat macule
2: raised papule
3: raised plaque
4: grossly exophytic lesion
b.
Day 0
Day 13
Day 33
Day 70
Day 104
Day 139
Lesion score
Control
Indomethacin
*p=0.0238
c.
Day 0
Day 13
Day 33
Day 70
Day 104
Day 139
Diluent control
Indomethacin

Fig. 4.3 Administering indomethacin to mice with 4-NQO-induced premalignant lesions improves clinical outcome and slows progression to HNSCC. (a) Indomethacin or diluent control was administered in the drinking water of mice with 4-NQO-induced premalignant oral lesions. Oral cavities of mice were endoscoped every 10 days and lesions were scored 1-4. Lesion scores for each mouse at representative time points are shown in (b). Images of the oral cavity of a representative mouse from each group are shown in (c). Lesion score data represent mean $\pm$ SEM. Shown in (b) and (c) are scores for mice endoscoped at baseline and at days 13 (1.9 wks), 33 (4.7 wks), 70 (10 wks), 104 (14.9 wks) and 139 (19.9 wks) after initiating indomethacin treatment. *$p<0.05$ (Mann-Whitney U test).

Administration of indomethacin to mice with 4-NQO-induced premalignant lesions leads to increased activation of cervical lymph node cells 20 weeks post-onset of treatment, compared to that of diluent control treatment

Since the above studies showed an improved clinical response in mice receiving indomethacin beginning at the premalignant lesion stage and other studies have shown that inhibiting PGE_2 leads to increased immune reactivity in the tumor environment [71], we sought next to investigate whether indomethacin treatment impacted on immune cell reactivity in our model. To investigate whether indomethacin treatment of mice with premalignant lesions skews cytokine production by immune cells *in vivo*, cervical lymph node cells were extracted from 4-NQO-treated mice receiving indomethacin or diluent control treatments 6 weeks and 20 weeks post-onset of treatment and immunostained by flow cytometry. As shown in Figure 4.4a below, the percentage and total number of T cells expressing IFN-γ was not significantly different between the indomethacin and diluent control treatment groups 6 weeks post-onset of treatment, although the total number of $CD8^+IFN\text{-}\gamma^+$ T cells was increased in cervical lymph nodes of mice receiving indomethacin compared to control C57BL/6 mice ($p=0.059$). The percentage of T cells expressing the activation marker CD69 was not significantly different between the indomethacin and diluent control treatment groups. At 20 weeks post-initiation of treatment, the percentage of $CD8^+$ T cells expressing IFN-γ in the cervical lymph nodes of mice receiving indomethacin was increased compared to mice receiving diluent control treatment, although the difference was not significant. The total number of

$CD8^+IFN\text{-}\gamma^+$ T cells was increased in the cervical lymph nodes of mice receiving indomethacin (p=0.066) and mice receiving diluent control treatment (p=0.034), compared to control mice. Although the percentage of T cells expressing CD69 was not different between the indomethacin and diluent control groups, the total numbers of $CD4^+CD69^+$ and $CD8^+CD69^+$ T cells in the cervical lymph nodes of mice in the indomethacin-treated group was increased compared to that in the untreated control group, although the differences were not statistically significant (p=0.087, p=0.099). While indomethacin treatment of mice with 4-NQO-induced premalignant lesions did not result in statistically significant changes in the number of $CD4^+$ or $CD8^+$ cells expressing IFN-γ, it caused a progressive tendency toward an increase in the number of $CD8^+$ T cells expressing IFN-γ and this tendency became more prominent with a more prolonged period of indomethacin treatment. This suggests that indomethacin may play a role in skewing cytokine production towards a Th1-type response by immune cells as premalignant oral lesion progress to HNSCC.

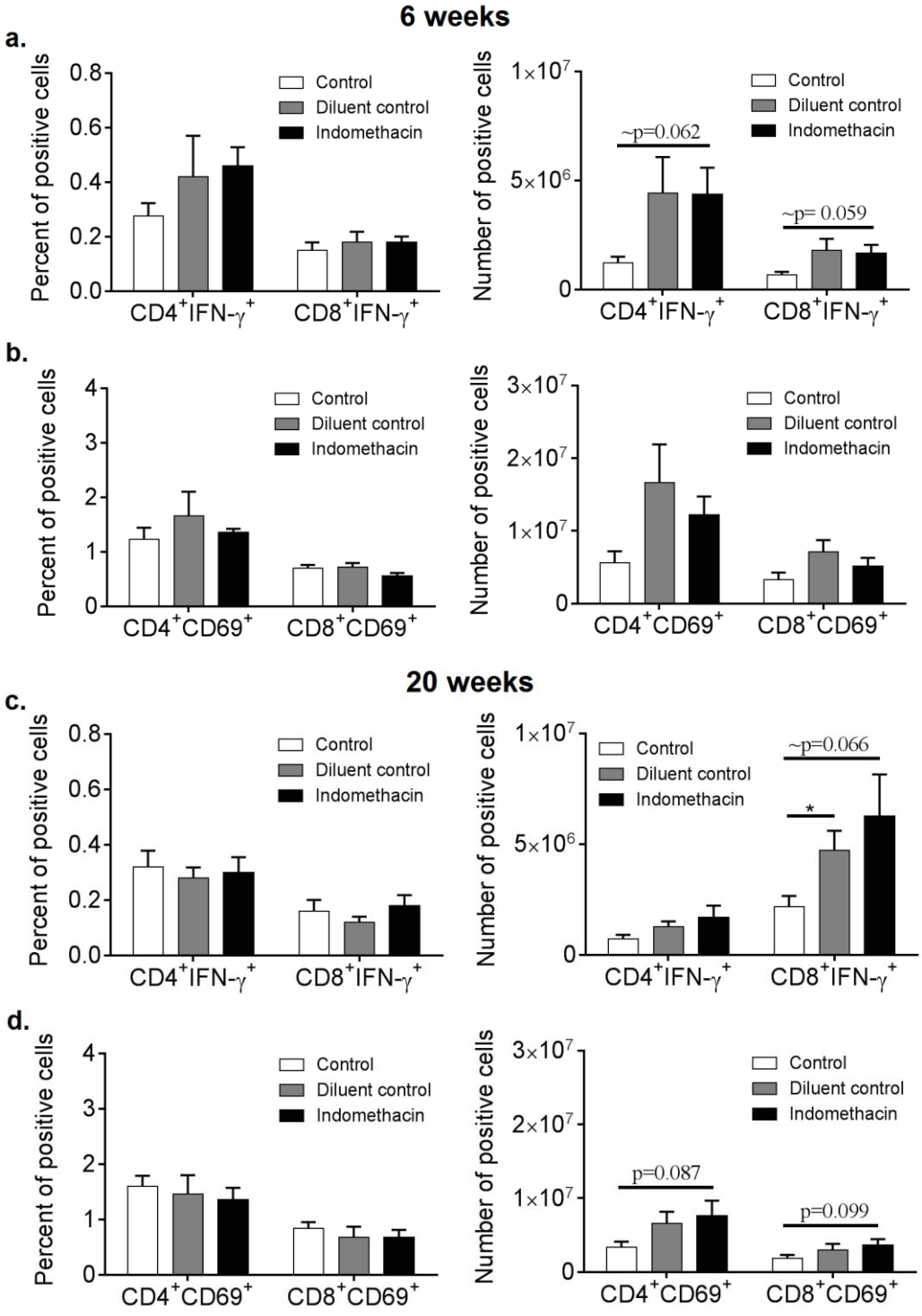

6 weeks
a.
Percent of positive cells
0.8
0.6
0.4
0.2
0.0
Control
Diluent control
Indomethacin
CD4+IFN-γ+
CD8+IFN-γ+
Number of positive cells
1×10^7
5×10^6
0
~p=0.062
~p= 0.059
b.
Percent of positive cells
4
3
2
1
0
CD4+CD69+
CD8+CD69+
Number of positive cells
3×10^7
2×10^7
1×10^7
20 weeks
c.
~p=0.066
*
d.
p=0.087
p=0.099

Fig. 4.4 Administering indomethacin to mice with 4-NQO-induced premalignant lesions tends to increase the number of IFN-γ-expressing $CD8^+$ T cells and CD69-expressing $CD4^+$ and $CD8^+$ T cells at 20 weeks post-onset of treatment. Graphical representation of flow cytometric staining of cervical lymph node cells extracted from mice with 4-NQO-induced oral lesions receiving indomethacin or diluent control treatment 6 weeks post-onset of treatment (a,b) and 20 weeks post-onset of treatment (c,d). For intracellular cytokine staining, cells were stimulated with PMA/ionomycin for the last 4 h of culture. Five mice were examined for each group per time point. Total number of positive cells was calculated by multiplying percent positive cells by the number of cervical lymph node cells for each mouse. Data represent mean $\pm$ SEM. *$p<0.05$ (2-tailed Student's *t*-test).

Administration of indomethacin to mice with 4-NQO-induced premalignant lesions results in increased production of IFN-γ by spleen cells at 6 and 20 weeks post-onset of treatment

Inhibiting PGE_2 production has been shown to have immune stimulatory effects in several animal models of cancer and chronic infection [71, 110-112]. The above studies suggested a shift toward Th1-skewed immune cells in the cervical lymph nodes of premalignant lesion-bearing mice treated with indomethacin. To determine if indomethacin treatment induces a systemic Th1-type response in mice with 4-NQO-induced premalignant lesions, cytokine production by spleen cells from mice receiving indomethacin or diluent control treatments was analyzed by cytokine bead array. IL-2 production by spleen cells was not significantly affected by indomethacin or diluent control treatments 6 weeks or 20 weeks post-onset of treatment (shown in Figure 4.5 below). However, the level of IFN-γ produced by spleen cells in mice receiving indomethacin was increased compared to the level produced by spleen cells of diluent control-treated mice. Importantly, while the level of IFN-γ produced by spleen cells in mice receiving diluent control treatment was lower than the level produced by spleen cells of untreated control mice, the level of IFN-γ produced by spleen cells in mice receiving indomethacin was actually greater than control values, suggesting that indomethacin does play a role in inducing a systemic, Th1-type response. The level of IL-17A produced by spleen cells of mice receiving indomethacin was decreased compared to the level produced by spleen cells of mice receiving diluent control and

untreated control mice both 6 weeks and 20 weeks post-onset of treatment. The level of proinflammatory TNF-α produced by spleen cells of indomethacin-treated mice receiving indomethacin was increased compared to the levels produced by spleen cells of either diluent-treated mice or from untreated mice 6 weeks post-onset of treatment ($p=0.073$), although this difference was not evident 20 weeks post-onset of treatment. Overall, these data suggest that administering indomethacin to mice with 4-NQO-induced oral lesions can skew immune responses toward a systemic Th1-type response.

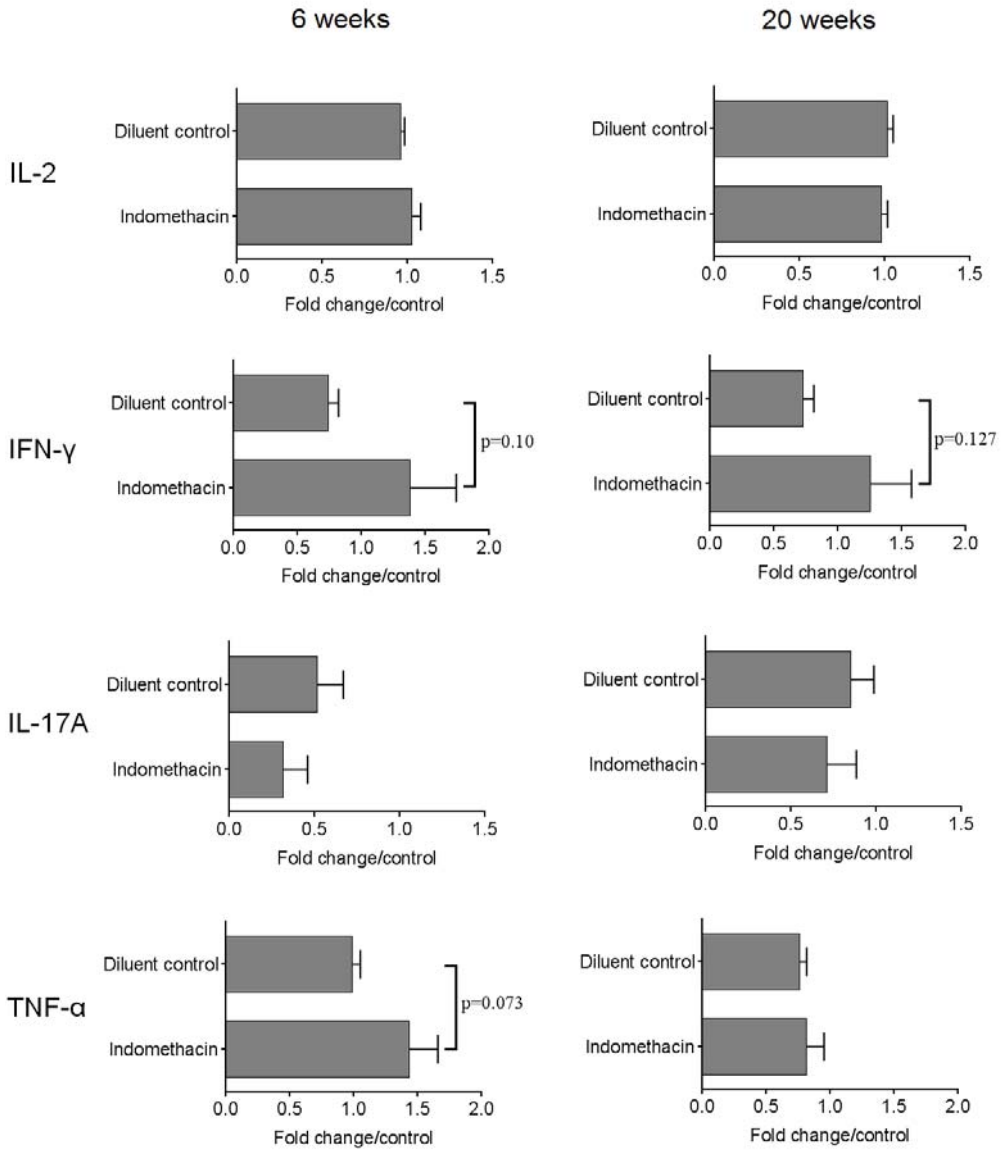


Fig. 4.5 Administering indomethacin to mice with 4-NQO-induced premalignant lesions increases spleen cell production of IFN-γ 6 weeks and 20 weeks post-onset of treatment. Spleens from mice receiving indomethacin or diluent control treatments were extracted at 6 weeks and 20 weeks post-onset of treatment. Spleen cells were cultured for 72 h at $1X10^6$ cells/well and supernatants were collected for analysis by CBA. Five mice were examined for each group per time point. Spleen cells from untreated C57BL/6 mice served as controls. Cytokine levels were analyzed as fold change from values for controls. Data represent mean ± SEM. *$p<0.05$ (2-tailed Student's *t*-test).

APPENDIX

Cervical lymph nodes of mice with 4-NQO induced premalignant lesions contain an increased percentage of $CD8^+CD69^+$ cells compared to levels in control mice

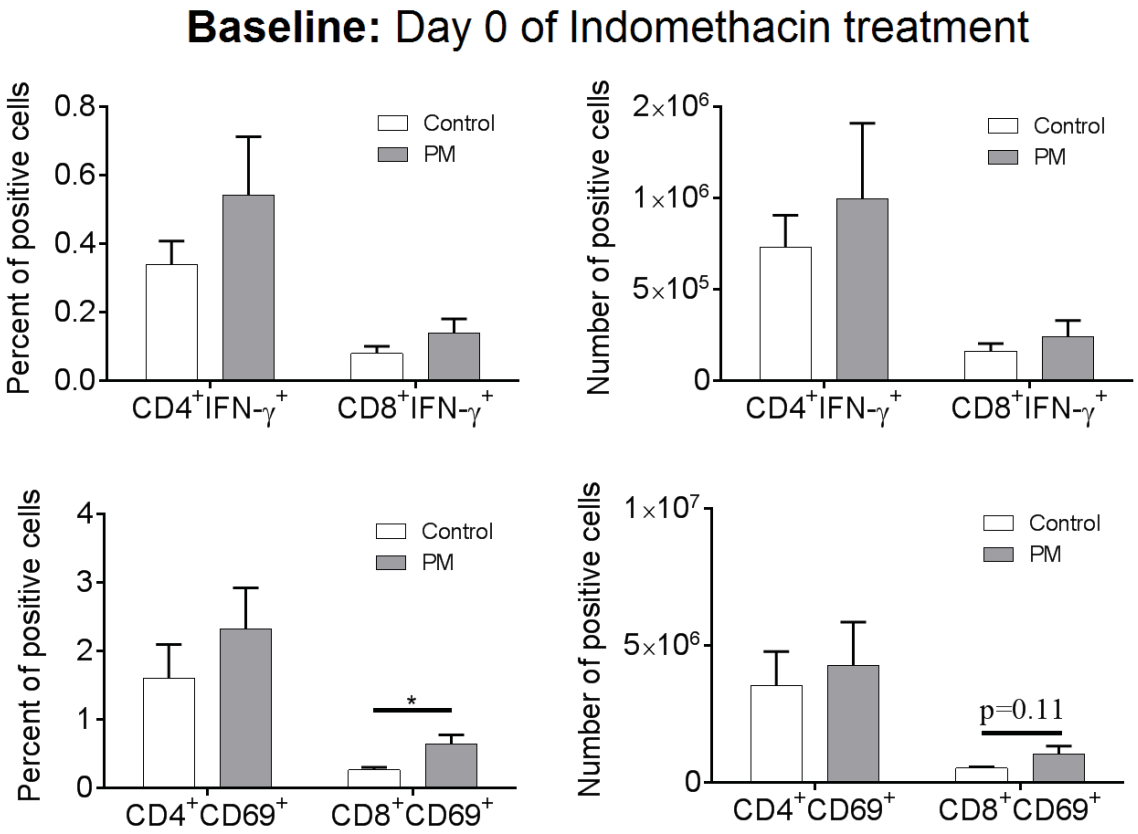


Fig. S4.1 Cervical lymph nodes of mice with 4-NQO induced premalignant lesions contain an increased percentage of $CD8^+CD69^+$ cells. Graphical representation of flow cytometric staining of cervical lymph node cells extracted from mice with 4-NQO-induced premalignant oral lesions. For intracellular cytokine staining, cells were stimulated with PMA/ionomycin for the last 4h of culture. Five mice were examined for each group per time point. Total number of positive cells was calculated by multiplying percent positive cells by the number of cervical lymph node cells for each mouse. Data represent mean ± SEM. *$p<0.05$ (2-tailed Student's *t*-test).

Administration of indomethacin to mice with 4-NQO-induced premalignant lesions decreases levels of PGE_2 in tongue tissue compared to levels in diluent control-treated mice

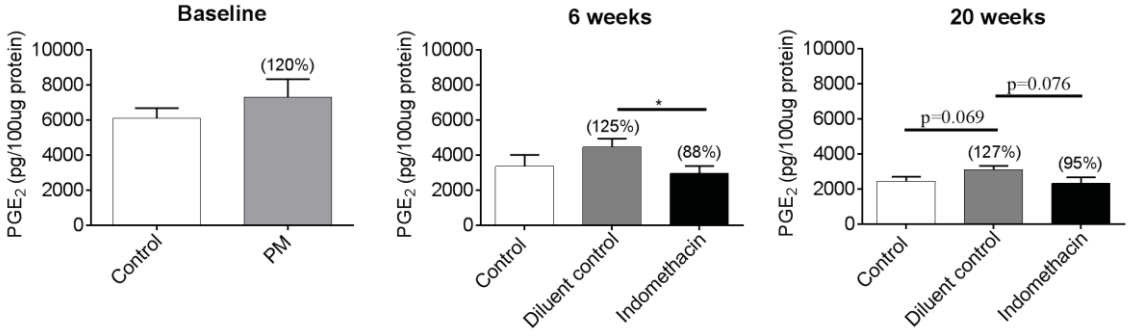


Fig. S4.2 Administering indomethacin to mice with 4-NQO-induced premalignant lesions decreases levels of PGE_2 in tongue tissue 6 weeks post-onset of treatment. Tongues from mice receiving indomethacin or diluent control treatments were extracted at baseline, 6 weeks and 20 weeks post-onset of treatment. Tongue tissue was mechanically and enzymatically digested using a liberase cocktail. Tongue tissue was lysed and levels of PGE_2 in lysates were determined by ELISA after diluting samples 1:50. Levels of PGE_2 were normalized to tissue protein levels. Data represent 3 mice per group per time point, run in duplicate. Data represent mean $\pm$ SEM. *$p<0.05$ (2-tailed Student's *t*-test).

Administration of indomethacin to mice with 4-NQO-induced premalignant lesions results in an increase in total cervical lymph node cell count 6 weeks and 20 weeks post-onset of treatment

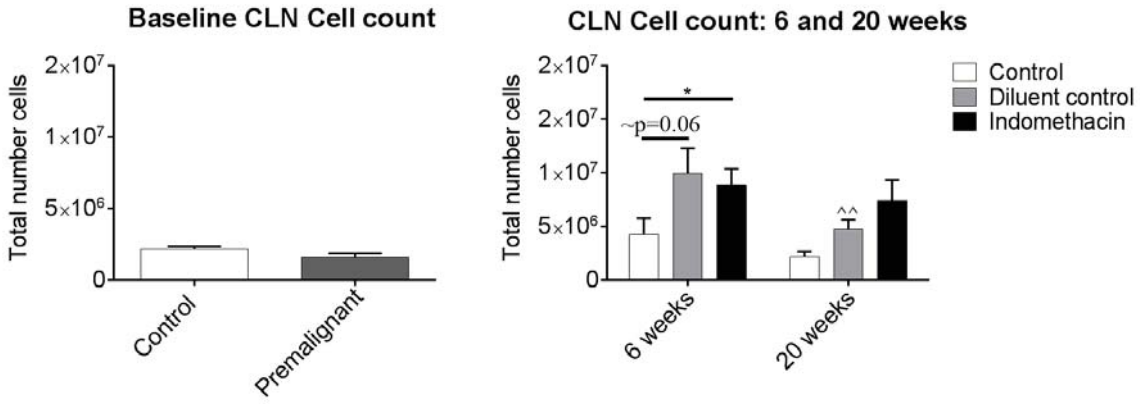


Fig. S4.3 Administering indomethacin to mice with 4-NQO-induced premalignant lesions increases the total number of cervical lymph node cells 6 and 20 weeks post-initiation of treatment. Cervical lymph nodes from mice receiving indomethacin or diluent control treatments were extracted at baseline, 6 weeks and 20 weeks post-initiation of treatment. Cells were processed to single cell suspensions and total number of trypan blue-excluding cells were counted. Data represent 5 mice per group per time point. Data represent mean $\pm$ SEM. *$p<0.05$ (2-tailed Student's *t*-test). The total number of cervical lymph node cells in mice receiving diluent control treatment decreased from 6 to 20 weeks post-initiation of treatment (^^$p=0.07$).

Administration of indomethacin to mice with 4-NQO-induced premalignant lesions does not impact on total number of CD4$^+$ and CD8$^+$ T cells within the cervical lymph nodes

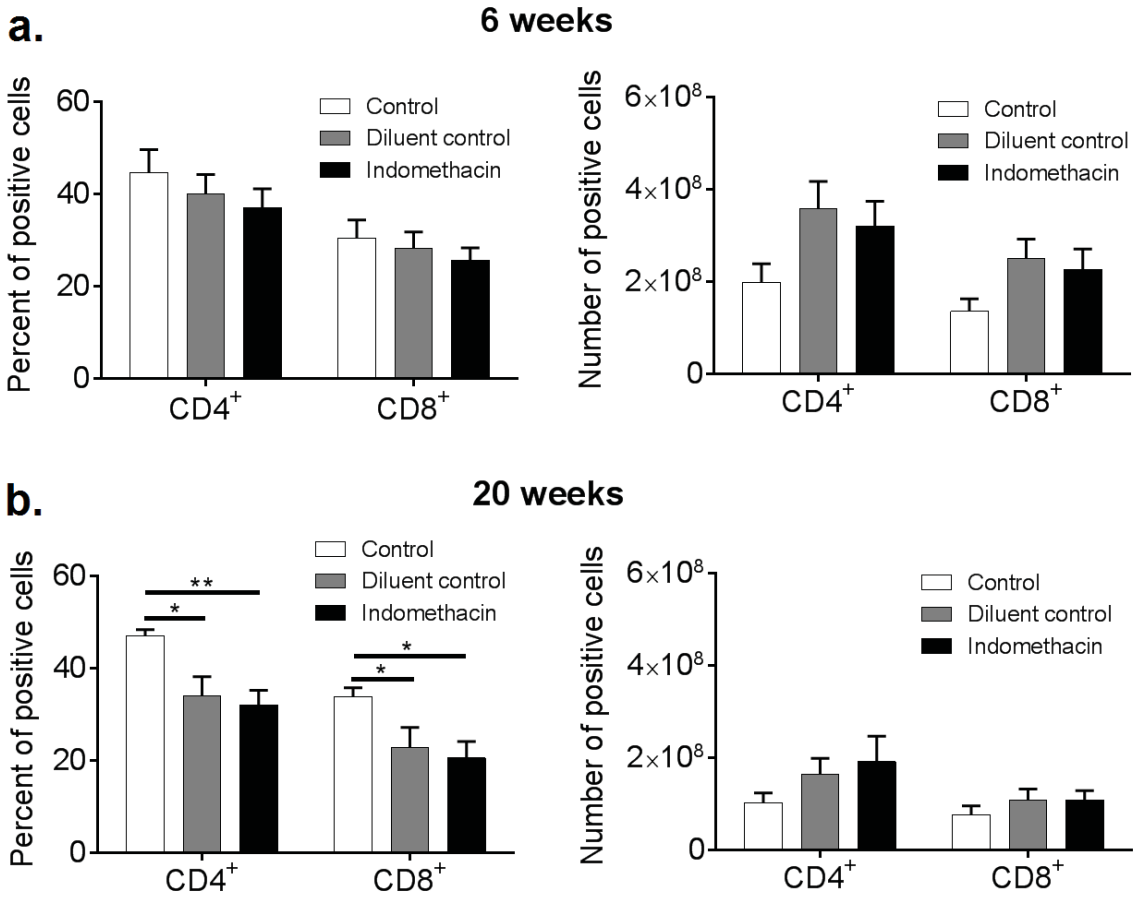


Fig. S4.4 Administering indomethacin to mice with 4-NQO-induced premalignant lesions does not impact on the total number of CD4$^+$ or CD8$^+$ T cells within cervical lymph nodes. Graphical representation of flow cytometric staining of cervical lymph node cells extracted from mice with 4-NQO-induced oral lesions receiving indomethacin or diluent control treatment 6 weeks post-onset of treatment (a) and 20 weeks post-onset of treatment (b). Five mice were examined for each group per time point. Total number of positive cells was calculated by multiplying percent positive cells by the number of cervical lymph node cells for each mouse. Data represent mean $\pm$ SEM. *p<0.05 **p<0.01 (2-tailed Student's *t*-test).

Chapter 5

General Discussion

CHAPTER 5: GENERAL DISCUSSION

5.1 HNSCC Tumors Suppress Immune Cell Reactivity

HNSCC patients are characterized by immunosuppression, both systemically and at the site of the tumor [8-12]. This remains one of the most challenging obstacles for treatment and research has turned toward the development of immunotherapies aimed at reactivating a patient's immune system against the tumor. A critical part of developing an effective immunotherapy is understanding the mechanisms by which developing HNSCC tumors evade the immune response, which is the focus of the current study.

The process of tumor escape can be divided into three major phases [18, 113]. In the first stage, elimination, transformed cells are recognized by circulating immune cells and an inflammatory response is initiated to eliminate the dysplastic tissue. If any remaining malignant cells persist, the equilibrium stage begins, which can last for many years [18]. Though infiltrating immune cells recognize the dysplastic tissue and mount a robust proinflammatory response, the tumor persists. The final stage, tumor escape, is characterized by a loss of MHC expression by HNSCC cells and direct secretion of immune-modulating factors [19-23]. Tumor-infiltrating T cells exhibit a number of functional defects, including decreased proliferation in response to IL-2, decreased expression of the CD3 ζ chain, and an imbalanced cytokine profile, marked by significantly decreased IL-2 and IFN-γ secretion; in short, they exhibit a less activated Th1 phenotype that is not effective against tumor [105, 106].

Primary tumors from HNSCC patients have been shown to secrete a panel of immune-modulating factors, including IL-6, IL-10, GM-CSF, TGF-β and PGE_2 [10, 20,

28, 31, 34-36, 103, 104]. The role of each of these factors in the tumor environment is complex, as factors typically associated with a proinflammatory immune response are harnessed by the tumor for growth and angiogenesis. Tumor-secreted IL-6 and IL-10 promote a Th2-type response at the site of the tumor, which has been shown to be less effective at eliminating a growing tumor and actually contributes to tumor growth by recruiting a population of immunosuppressive T regulatory cells and skewing the environment away from a more proinflammatory, antitumor Th1-type response [20, 28-30]. Increased levels of GM-CSF at the site of HNSCC tumors have been associated with an increased infiltration of MDSCs and a worse prognosis in patients [10, 35]. HNSCC tumors also harness factors secreted by infiltrating immune cells. MDSC-derived IL-6 has been shown to promote tumor angiogenesis [33]. Even factors such as IFN-γ, secreted by T cells, have been shown to contribute to Th17 cell differentiation and increased tumor growth [114, 115]. Increased expression of COX-2, the enzyme responsible for increased PGE_2 production by HNSCC tumors, is also associated with increased tumor growth and a poorer prognosis in patients [20, 26, 28, 36, 60]. Although PGE_2 contributes to neutrophil recruitment during the early stages of inflammation and infection, it has been shown to directly inhibit $CD8^+$ T cell function in the tumor environment [39-41, 46-48]. It is well established that factors secreted by HNSCC cells themselves play a critical role in shaping the immune response both at the tumor site and systemically.

5.2 Inflammatory Environment of Premalignant Lesions

While many studies have focused on the mechanisms by which established HNSCC tumors evade an effective immune response, less focus has been given to what the present study has shown to be a stage in which an inflammatory immune response is active, and yet unsuccessful at eliminating, the dysplastic tissue, i.e. the premalignant lesion stage. At what point does the inflammatory environment shaped by a robust immune response turn towards a tumor-promoting mechanism? Is there a point, before the tumor becomes established, that the immune response can be rescued to slow progression to tumor?

A few studies have shown that premalignant oral lesions in patients are characterized by increased levels of proinflammatory mediators, including IL-6 and IL-17A, compared to HNSCC tumor tissue, but these studies are based on a low number of patients and do not delve into the mechanism by which mediators released by the lesions themselves impact on immune cell reactivity [72-74]. The current study uses the 4-NQO mouse model of oral carcinogenesis to investigate how the immune environment of premalignant lesions differs from that of established HNSCC, and more specifically, how factors secreted by premalignant lesion cells modulate the immune response and how this interaction leads to persistence of the tumor vs. eradication of the dysplastic tissue. Preliminary studies in the 4-NQO model have shown that the cervical lymph nodes of premalignant lesion-bearing mice are characterized by increased numbers of proinflammatory T cells (Th17 cells) and proinflammatory cytokines and chemokines (IL-17A, MIP-1α, MIP-1β) compared to that seen in HNSCC-bearing and control mice, suggesting that immune cells are more activated in the premalignant lesion environment

[100]. The current study shows that tongue tissue from mice with 4-NQO-induced premalignant lesions releases increased levels of Th1-and Th17-associated cytokines, including IL-2, IFN-γ, TNF-α, and IL-17A compared to what is released from tongue tissue from HNSCC-bearing and control mice. This indicates that the environment of premalignant lesions, at the site of the tongue itself, is more proinflammatory compared to the environment of established tumors, and suggests that the premalignant lesion environment supports a more robust, Th1-type response compared to the HNSCC environment.

When spleen cells from control C57BL/6 mice were cultured with media conditioned by premalignant lesion-bearing tongue tissue, they produced significantly increased levels of Th1- and Th2-associated cytokines, including IL-2, IL-4, and IL-10, compared to spleen cells cultured with media conditioned by HNSCC-bearing tongue tissue or media alone. This data indicates that the environment of premalignant lesion-bearing tongue tissue is more immune stimulatory than HNSCC-bearing tongue tissue. An interesting observation from this study is that after re-stimulation with PMA/ionomycin, spleen cells produced comparable levels of both Th1- and Th2-associated cytokines in both the premalignant lesion and HNSCC environments, demonstrating that 1) Premalignant lesion-bearing tongue tissue is inherently more immune stimulatory than HNSCC-bearing tongue tissue and 2) Spleen cells can be stimulated to produce increased levels of these cytokines in the HNSCC environment, so the cells are not inherently defective. It is somewhat perplexing that premalignant lesion-bearing tongue tissue elicited increased production of both Th1-associated cytokines such

as IL-2 and Th2-associated cytokines such as IL-10, which typically play a more immunosuppressive role. This could suggest that the environment shaped by premalignant lesions stimulates both Th1-type and Th2-type cells. Alternatively, the increase in Th2-type cytokines may instead be the result of an initial dramatic increase in Th1-type proinflammatory cytokines as a mechanism to "turn off" the cycle of inflammation. In this way, the premalignant lesion state represents a battle of immune cells secreting proinflammatory mediators to eliminate the dysplastic tissue against immunosuppressive cells secreting immune inhibitory molecules to dampen inflammation, which ultimately can lead to the establishment of HNSCC. As the current study demonstrates, HNSCC-bearing tongue tissue does not elicit a significant Th1- or Th2-type response from immune cells because by the time the tumor is established, the battle has essentially been lost.

For the current study, a holistic *in vitro* approach was chosen to investigate how mediators released by premalignant lesion-bearing vs. HNSCC-bearing tongue tissues impact on spleen cell cytokine production. Because of the muscular nature of the tongue and the mechanical and enzymatic methods of tongue digestion, harvesting a sufficient population of live immune cells from tongue tissue for analysis of cytokine production is not feasible. To account for secretion of cytokines and other immune mediators during the digestion process, cytokine levels in spleen cell cultures were adjusted for baseline levels in tongue tissue and all tongues were digested by identical means, so the results could be interpreted clearly. Overall, the data demonstrate that the environment of a developing premalignant lesion is more proinflammatory and elicits increased production

of cytokines by immune cells compared to the environment of an established HNSCC tumor. To further delve into the question of how the premalignant lesion vs. HNSCC environment impacts on immune cell cytokine production, future studies could culture isolated T cell subsets ($CD4^+$, $CD8^+$, $Foxp3^+$) with media conditioned by tongue tissue from premalignant lesion-bearing and control mice. This could more precisely tease out the effects of the premalignant lesion environment skewing of $CD4^+$ vs. $CD8^+$ T cells and offer insight into how the premalignant lesion environment impacts on proinflammatory vs. immunosuppressive immune cells.

5.3 Mediators Secreted by Premalignant Lesion Cells

Dissecting how premalignant lesion cells and HNSCC cells themselves contribute to the immune environment, and how this impacts on infiltrating immune cells, is difficult for many reasons, one of which is the complexity of a developing tumor. Premalignant lesions, as they develop into tumors, consist of a complex network of cells, including epithelial cells, endothelial cells, fibroblasts, stromal cells, smooth muscle cells, infiltrating immune cells, and cells that are transforming or have become malignant [27].

The present study initiated the dissection of the premalignant lesion by establishing primary cultures of premalignant lesion cells and HNSCC cells. These cells were then used to define the immune mediators produced directly by premalignant lesion and HNSCC cells as well as their impact on immune cells, as they also contribute to the lesion and tumor environments. This study shows that premalignant lesion cells established from mice with 4-NQO-induced lesions secrete significantly higher levels of

several immune mediators, including MCP-1, RANTES, G-CSF, and PGE_2, compared to HNSCC cells. While the levels of these mediators were detectable in the supernatant of HNSCC cells, as a few previous studies using human and murine HNSCC cell lines have indicated [60, 102-104], they were detected in much higher levels in the supernatant of premalignant lesion cells. These data bring to light a new observation: premalignant lesion cells themselves produce high levels of several proinflammatory mediators. The data suggest that the proinflammatory environment of premalignant lesion-bearing tongues and increased infiltration of proinflammatory cells into the draining lymph nodes of premalignant lesion-bearing mice observed in the 4-NQO model may be a direct consequence of the environment set up by the premalignant lesion cells themselves.

To address the question of how proinflammatory mediators produced by premalignant lesion cells might impact on immune cell cytokine production, spleen cells or cervical lymph node cells were cultured with media conditioned by premalignant lesion cells, HNSCC cells, or media alone. Premalignant lesion cell-conditioned media elicited significantly increased production of innate proinflammatory mediators as well as Th1-, Th2-, and Th17-associated cytokines by spleen cells and cervical lymph node cells compared to HNSCC cell-conditioned media or media alone. Analysis by flow cytometric staining showed that an increased percentage of $CD4^+$ and $CD8^+$ T cells expressed IFN-γ and an increased percentage of $CD8^+$ T cells expressed the early activation marker CD69 in the presence of premalignant lesion-conditioned media compared to what was expressed in the presence of HNSCC cell-conditioned media or media alone. Furthermore, among both $CD4^+$ and $CD8^+$ T cells, a striking

$CD69^{hi}$- expressing population was observed in the presence of premalignant lesion-conditioned media compared to what was observed in the presence of HNSCC cell-conditioned media or media alone. The data shows that mediators released by premalignant lesion cells skew immune cells toward a more activated, Th1-like phenotype and also skew secretion of cytokines. This suggests that, at the cellular level, premalignant lesion cells promote an activated, Th1-type response and set up a cycle of inflammation at the site of the developing lesion.

The current study is based on cell cultures established from 4-NQO-induced premalignant lesions or HNSCC tumors. Therefore, one consideration is whether culturing the cells *in vitro* may lead to the production of a different panel of cytokines or chemokines, which may not reflect the *in vivo* phenotype. To account for changes in phenotype over the periods of culture and ensure consistency, the baseline levels of cytokines and chemokines in collected supernatants were analyzed for each experiment. The strength of establishing cell cultures from premalignant lesions and HNSCC tumors, is that the cytokine and chemokine environment set up by the cells themselves (vs. surrounding tissue), can be defined, which offers valuable insight into how premalignant lesions might be modulating the immune response as they progress to tumor.

5.4 Role of PGE_2 in the Progression of Premalignant Lesions to HNSCC

While the role of several immune mediators, such as PGE_2, has been studied in the HNSCC environment, their role in the premalignant lesion environment has not been defined. Previous studies have shown that PGE_2 is upregulated in HNSCC tumors, and increased levels of PGE_2 have been associated with decreased levels of cytotoxic T cells

increased levels of immunosuppressive cells at the tumor site [20, 28, 36, 60]. Increased levels of COX-2, the inducible enzyme responsible for the increased PGE_2 production, in HNSCC tumors has also been associated with a worse prognosis in patients [26, 36]. In other models of cancer, PGE_2 has been shown to play an immunosuppressive role, by promoting the secretion of Th2-associated IL-10, inhibiting production of IL-2 by T cells, and supporting a population of immunosuppressive cells at the site of the tumor [46-53, 55]. In a model of lung carcinoma, inhibiting PGE_2 production led to a shift in cytokine production by T cells towards a more Th1-type response and decreased tumor burden [71].

In the current study, it was found that premalignant lesion cells secrete significantly increased levels of PGE_2 compared to HNSCC cells. This is paradoxical given the immune stimulatory and inflammatory environment of premalignant lesions. The current study aimed to determine the pro-tumor vs. anti-tumor role of PGE_2 as premalignant lesions progress to HNSCC. We hypothesized that inhibition of PGE_2 production at the premalignant lesion stage may skew T cell cytokine production towards a more Th1-type response, rather than a broad state of activation and inflammation, and lead to improved clinical outcomes. *In vitro*, pretreatment of premalignant lesion cells with indomethacin, a pan-COX inhibitor, resulted in increased induction of spleen cell production of Th1-associated IL-2. Indomethacin treatment of HNSCC cells had minimal effect on their induction of spleen cell cytokine production, suggesting that treatment at the premalignant stage may be optimal to reactivate a Th1-type immune response. Administration of indomethacin in the drinking water of mice with 4-NQO-induced premalignant lesions resulted in reduced tumor burden and significantly improved

clinical outcomes compared to that seen for diluent control-treated mice. At the endpoint of the study, 4 out of 5 mice in the diluent control group had progressed to lesion scores of 3 or higher (bearing raised plaques or grossly exophytic lesions or tumors) whereas only one mouse had progressed to a lesion score of 3 in the indomethacin group. Analysis of cervical lymph nodes revealed that $CD8^+$ T cells in mice that had received indomethacin expressed increased levels of IFN-γ compared to levels expressed by lymph node cells of mice that had received diluent control treatment at 20 weeks post-onset of treatment, although the results were not significant. Interestingly, the levels of IFN-γ secreted by spleen cells were also significantly increased in mice that had received indomethacin treatment compared to what was secreted by spleen cells of mice that had received diluent control treatment, suggesting that indomethacin may play a role in activating a systemic, Th1-type response that slows progression to tumor.

The results of this study offer insight into how PGE_2 modulates the immune response at the premalignant lesion stage. Previous studies have looked at the effect of inhibiting PGE_2 production in tumors and have suggested that PGE_2 plays a role in supporting T regulatory cells and promoting a Th2-type response [26, 38, 69-71]. The current study suggests that PGE_2 may also be playing an immunosuppressive role before a tumor becomes established, as inhibiting PGE_2 production by premalignant lesion cells resulted in increased production of IL-2 by spleen cells *in vitro*. However, inhibiting premalignant lesion cell production of PGE_2 also led to an increased production of IL-10 *in vitro*, so the effect of indomethacin treatment cannot be classified singularly as "pro-Th1." The concurrent increase in IL-10 may be explained as a consequence of an

initial increase in Th1-type cytokines, similar to what was observed with spleen cells cultured with premalignant lesion cell-conditioned media alone. *In vivo*, administration of indomethacin to mice beginning at the premalignant lesion stage resulted in significantly improved clinical outcomes and increased production of IFN-γ by immune cells, compared to what was seen for diluent control treatment. Interestingly, spleen cells from mice that had received indomethacin secreted higher levels of IFN-γ at both 6 and 20 weeks post-onset of treatment compared to what was produced by spleen cells of mice that had received diluent control treatment, suggesting that indomethacin could promote a systemic, Th1-type response as lesions progress to tumor. Overall, the results suggest that inhibiting PGE_2 production at the premalignant lesion stage is advantageous to an improved clinical outcome.

The current study adds valuable insight into the role of IL-17A, and Th17 cells, as premalignant lesions progress to HNSCC. The role for Th17 cells in HNSCC carcinogenesis, as with many other cancers, has not been completely defined. Some studies have indicated they play a pro-tumoral role by promoting tumor angiogenesis and growth in models of head and neck cancer as well as pancreatic neoplasia and skin cancer [114, 116-118]. Other studies in both head and neck cancer and models of pancreatic cancer suggest that they are part of a beneficial, antitumor response and promote tumor cell apoptosis [116, 119]. PGE_2 has been shown to play a role in sustaining Th17 cells in the tumor environment through the IL-23 axis [120]. But how PGE_2 impacts on Th17 cells in the premalignant environment has not been defined. By inhibiting PGE_2 production at the premalignant lesion stage, the current study offers insight into the

PGE_2-Th17 cell axis as premalignant lesions progress to HNSCC. *In vitro*, inhibiting the production of PGE_2 by premalignant lesion cells and HNSCC cells led to decreased production of IL-17A by spleen cells. This supports previous work implicating PGE_2 as a key player in sustaining Th17 cells in the HNSCC environment, but also suggests a novel role for PGE_2 in promoting the secretion of IL-17A by Th17 cells, in both the premalignant lesion and HNSCC environments. Furthermore, spleen cells from mice that had received indomethacin at the premalignant lesion stage produced decreased levels of IL-17A *ex vivo* compared to IL-17A production by spleen cells of mice that had received diluent control treatment, suggesting that a decrease in PGE_2 in the lesion and developing tumor environment may lead to a systemic decrease in IL-17A secretion by Th17 cells. The current study offers evidence to support the hypothesis that Th17 cells play a pro-tumoral role as premalignant lesions progress to tumor, as inhibiting PGE_2 production at the premalignant lesion stage reduced production of IL-17A by spleen cells and led to significantly improved clinical outcomes.

Future studies with larger cohorts of mice will be necessary to investigate the role of PGE_2 in modulating Th17 cells as premalignant lesions progress to tumor. In the current study, the number of cervical lymph node cells was not sufficient to detect levels of cytokine section by CBA. To investigate the secretion of IL-17A by cells closer to the site of the lesion and developing tumor, future studies could focus on the intracellular expression of IL-17A by cervical lymph node cells over the course of indomethacin treatment. Future studies might also investigate the role of IL-23 as a downstream mediator of PGE_2 in sustaining Th17 cells in the premalignant lesion environment by

administering an IL-23 inhibitor along with a COX inhibitor to mice with 4-NQO-induced premalignant lesions.

One weakness of the current study is that the treatment groups consisted of five mice each, making statistical analysis more difficult. Future studies based on larger cohorts of mice will more clearly delineate differences in cytokine production between treatment groups. This may also allow pooling of cervical lymph node cells among treatment groups for analysis by CBA. In the current study, indomethacin, a pan-COX inhibitor, was administered to mice through their drinking water, a mode of COX inhibition that has been established in other mouse models of cancer [108, 121]. This method is convenient, and in the case of oral cancer, allows treatment to be administered at the site of the lesion and developing tumor. However, as shown in Supplementary Figure S4.1, the levels of PGE_2 in the supernatants of tongue tissue from mice that had received indomethacin were moderately decreased 6 weeks post-onset of treatment compared to mice that had received diluent control treatment and only minimally decreased 20 weeks post-onset of treatment. This may be a consequence of the digestion method, which damages the tissue and may promote the release of PGE_2 from endothelial cells, fibroblasts, lesion cells, tumor cells and other cells in the tongue. Another explanation is that because a majority of mice had progressed past the premalignant lesion stage by the endpoint of the study and the level of PGE_2 produced by HNSCC tumor cells is significantly reduced compared to the level produced by premalignant lesion cells, differences induced by indomethacin treatment may not be as striking in the later stages of tumor development. In the future, analyzing PGE_2 levels in the plasma

may offer insight into how indomethacin impacts systemic levels of PGE_2 as premalignant lesions progress to tumor. Future studies might also investigate how COX-2-specific inhibition, through administration of celecoxib, impacts on immune cell reactivity and clinical outcome as premalignant lesions progress to tumor. In this way, the role of PGE_2 in skewing the immune response at the premalignant lesion stage can be further delineated.

5.5 Closing Remarks

Oral cancer is a unique malignancy, in that it can be diagnosed when tissue first becomes dysplastic and presents as leukoplakias or erythroplakias developing on the tongue and floor of mouth. By the time HNSCC becomes established, patients exhibit local and systemic signs of immunosuppression, making treatment more difficult and increasing the chance of recurrence. By diagnosing oral lesions before they develop into established HNSCC and administering immunotherapies to activate a patient's immune response, treatment may become more effective for these patients.

The current study offers evidence that significant immune changes are occurring before HNSCC becomes established, at the premalignant lesion stage. Premalignant lesion cells established from mice with 4-NQO-induced lesions secrete a host of mediators, including proinflammatory chemokines and PGE_2, and elicit increased production of proinflammatory cytokines by immune cells compared to HNSCC cells. Overall, the premalignant lesion environment is more immune stimulatory than the HNSCC environment. The role of PGE_2 in the premalignant lesion environment, however, has not been previously defined. Previous studies have shown that HNSCC

tumors are characterized by increased levels of PGE_2, which has been associated with a decreased infiltration of T cells at the site of the tumor and a worse prognosis in patients. Administration of COX inhibitors as adjuvant therapy to HNSCC patients has resulted in improved clinical outcomes. However, the current study shows that PGE_2 is produced in much higher levels by premalignant lesion cells compared to HNSCC cells, suggesting it may be playing an immune-modulating role well before HNSCC is established. Therefore, intervening with treatment to skew the response towards a more effective Th1-type antitumor response may be more beneficial at the premalignant lesion stage, when the battle against the dysplastic tissue is first being fought. The current study offers evidence to support this view, as administration of indomethacin, a COX inhibitor, to mice with 4-NQO-induced premalignant lesions led to increased production of Th1-type cytokines by T cells, significantly improved clinical outcomes and slowed progression to tumor. Overall, the current study shows that significant immune changes are occurring much earlier in the progression of HNSCC than has been previously appreciated and suggests that administration of immunotherapies, such as COX-2 inhibitors, at the premalignant lesion stage may boost the immune response against tumor and improve clinical outcomes for patients.

LIST OF REFERENCES

1. Duray, A., et al., *Immune suppression in head and neck cancers: a review.* Clin Dev Immunol, 2010. **2010**: p. 701657.

2. Grandis, J.R., et al., *Head and neck cancer: meeting summary and research opportunities.* Cancer Res, 2004. **64**(21): p. 8126-9.

3. Klein, J.D. and J.R. Grandis, *The molecular pathogenesis of head and neck cancer.* Cancer Biol Ther, 2010. **9**(1): p. 1-7.

4. Jensen, S.B., et al., *A systematic review of salivary gland hypofunction and xerostomia induced by cancer therapies: management strategies and economic impact.* Support Care Cancer, 2010. **18**(8): p. 1061-79.

5. Vermorken, J.B. and P. Specenier, *Optimal treatment for recurrent/metastatic head and neck cancer.* Ann Oncol, 2010. **21 Suppl 7**: p. vii252-61.

6. Tanvetyanon, T., et al., *Prognostic factors for survival after salvage reirradiation of head and neck cancer.* J Clin Oncol, 2009. **27**(12): p. 1983-91.

7. Gilbert, H. and A.R. Kagan, *Recurrence patterns in squamous cell carcinoma of the oral cavity, pharynx, and larynx.* J Surg Oncol, 1974. **6**(5): p. 357-80.

8. Bergmann, C., et al., *T regulatory type 1 cells in squamous cell carcinoma of the head and neck: mechanisms of suppression and expansion in advanced disease.* Clin Cancer Res, 2008. **14**(12): p. 3706-15.

9. Badoual, C., et al., *Prognostic value of tumor-infiltrating CD4+ T-cell subpopulations in head and neck cancers.* Clin Cancer Res, 2006. **12**(2): p. 465-72.

10. Young, M.R., et al., *Increased recurrence and metastasis in patients whose primary head and neck squamous cell carcinomas secreted granulocyte-macrophage colony-stimulating factor and contained CD34+ natural suppressor cells.* Int J Cancer, 1997. **74**(1): p. 69-74.

11. Chikamatsu, K., et al., *Relationships between regulatory T cells and CD8+ effector populations in patients with squamous cell carcinoma of the head and neck.* Head Neck, 2007. **29**(2): p. 120-7.

12. Drennan, S., et al., *Increased frequency and suppressive activity of CD127(low/-) regulatory T cells in the peripheral circulation of patients with head and neck squamous cell carcinoma are associated with advanced stage and nodal involvement.* Immunology, 2013. **140**(3): p. 335-43.

13. De Stefani, A., et al., *Improved survival with perilymphatic interleukin 2 in patients with resectable squamous cell carcinoma of the oral cavity and oropharynx.* Cancer, 2002. **95**(1): p. 90-7.

14. Whiteside, T.L., et al., *Evidence for local and systemic activation of immune cells by peritumoral injections of interleukin 2 in patients with advanced squamous cell carcinoma of the head and neck.* Cancer Res, 1993. **53**(23): p. 5654-62.

15. Rojo, F., et al., *Pharmacodynamic trial of nimotuzumab in unresectable squamous cell carcinoma of the head and neck: a SENDO Foundation study.* Clin Cancer Res, 2010. **16**(8): p. 2474-82.

16. Mehra, R., R.B. Cohen, and B.A. Burtness, *The role of cetuximab for the treatment of squamous cell carcinoma of the head and neck.* Clin Adv Hematol Oncol, 2008. **6**(10): p. 742-50.

17. Ho, C., et al., *Escalating weekly doses of cetuximab and correlation with skin toxicity: a phase I study.* Invest New Drugs, 2011. **29**(4): p. 680-7.

18. Schreiber, R.D., L.J. Old, and M.J. Smyth, *Cancer immunoediting: integrating immunity's roles in cancer suppression and promotion.* Science, 2011. **331**(6024): p. 1565-70.

19. Badoual, C., et al., *Better understanding tumor-host interaction in head and neck cancer to improve the design and development of immunotherapeutic strategies.* Head Neck, 2010. **32**(7): p. 946-58.

20. Young, M.R., *Protective mechanisms of head and neck squamous cell carcinomas from immune assault.* Head Neck, 2006. **28**(5): p. 462-70.

21. Ferris, R.L., T.L. Whiteside, and S. Ferrone, *Immune escape associated with functional defects in antigen-processing machinery in head and neck cancer.* Clin Cancer Res, 2006. **12**(13): p. 3890-5.

22. Ogino, T., et al., *HLA class I antigen down-regulation in primary laryngeal squamous cell carcinoma lesions as a poor prognostic marker.* Cancer Res, 2006. **66**(18): p. 9281-9.

23. Grandis, J.R., et al., *Human leukocyte antigen class I allelic and haplotype loss in squamous cell carcinoma of the head and neck: clinical and immunogenetic consequences.* Clin Cancer Res, 2000. **6**(7): p. 2794-802.

24. Zeng, X., Q. Chen, and M. Nie, *[The relationship of Fas and Fas L protein expression in oral carcinogenesis].* Hua Xi Kou Qiang Yi Xue Za Zhi, 2003. **21**(4): p. 259-62.

25. Cho, Y.A., et al., *Relationship between the expressions of PD-L1 and tumor-infiltrating lymphocytes in oral squamous cell carcinoma.* Oral Oncol, 2011. **47**(12): p. 1148-53.

26. Young, M.R., et al., *Mechanisms of immune suppression in patients with head and neck cancer: influence on the immune infiltrate of the cancer.* Int J Cancer, 1996. **67**(3): p. 333-8.

27. Koontongkaew, S., *The tumor microenvironment contribution to development, growth, invasion and metastasis of head and neck squamous cell carcinomas.* J Cancer, 2013. **4**(1): p. 66-83.

28. Jewett, A., C. Head, and N.A. Cacalano, *Emerging mechanisms of immunosuppression in oral cancers.* J Dent Res, 2006. **85**(12): p. 1061-73.

29. Bose, A., et al., *Dysregulation in immune functions is reflected in tumor cell cytotoxicity by peripheral blood mononuclear cells from head and neck squamous cell carcinoma patients.* Cancer Immun, 2008. **8**: p. 10.

30. Lathers, D.M., N.J. Achille, and M.R. Young, *Incomplete Th2 skewing of cytokines in plasma of patients with squamous cell carcinoma of the head and neck.* Hum Immunol, 2003. **64**(12): p. 1160-6.

31. Lu, S.L., et al., *Overexpression of transforming growth factor beta1 in head and neck epithelia results in inflammation, angiogenesis, and epithelial hyperproliferation.* Cancer Res, 2004. **64**(13): p. 4405-10.

32. Wahl, S.M., J. Wen, and N. Moutsopoulos, *TGF-beta: a mobile purveyor of immune privilege.* Immunol Rev, 2006. **213**: p. 213-27.

33. Nitsch, S.M., R. Pries, and B. Wollenberg, *Head and neck cancer triggers increased IL-6 production of CD34+ stem cells from human cord blood.* In Vivo, 2007. **21**(3): p. 493-8.

34. Chen, Z., et al., *Expression of proinflammatory and proangiogenic cytokines in patients with head and neck cancer.* Clin Cancer Res, 1999. **5**(6): p. 1369-79.

35. Bronte, V., et al., *Unopposed production of granulocyte-macrophage colony-stimulating factor by tumors inhibits CD8+ T cell responses by dysregulating antigen-presenting cell maturation.* J Immunol, 1999. **162**(10): p. 5728-37.

36. Hambek, M., et al., *Inverse correlation between serum PGE2 and T classification in head and neck cancer.* Head Neck, 2007. **29**(3): p. 244-8.

37. Liss, C., et al., *Paracrine angiogenic loop between head-and-neck squamous-cell carcinomas and macrophages.* Int J Cancer, 2001. **93**(6): p. 781-5.

38. Kalinski, P., *Regulation of immune responses by prostaglandin E2.* J Immunol, 2012. **188**(1): p. 21-8.

39. Yu, Y. and K. Chadee, *Prostaglandin E2 stimulates IL-8 gene expression in human colonic epithelial cells by a posttranscriptional mechanism.* J Immunol, 1998. **161**(7): p. 3746-52.

40. Nakayama, T., et al., *Prostaglandin E2 promotes degranulation-independent release of MCP-1 from mast cells.* J Leukoc Biol, 2006. **79**(1): p. 95-104.

41. Weller, C.L., et al., *Chemotactic action of prostaglandin E2 on mouse mast cells acting via the PGE2 receptor 3.* Proc Natl Acad Sci U S A, 2007. **104**(28): p. 11712-7.

42. Smith, R.J., *Modulation of phagocytosis by and lysosomal enzyme secretion from guinea-pig neutrophils: effect of nonsteroid anti-inflammatory agents and prostaglindins.* J Pharmacol Exp Ther, 1977. **200**(3): p. 647-57.

43. Aronoff, D.M., C. Canetti, and M. Peters-Golden, *Prostaglandin E2 inhibits alveolar macrophage phagocytosis through an E-prostanoid 2 receptor-mediated increase in intracellular cyclic AMP.* J Immunol, 2004. **173**(1): p. 559-65.

44. Canetti, C., et al., *Activation of phosphatase and tensin homolog on chromosome 10 mediates the inhibition of FcgammaR phagocytosis by prostaglandin E2 in alveolar macrophages.* J Immunol, 2007. **179**(12): p. 8350-6.

45. Serezani, C.H., et al., *Prostaglandin E2 suppresses bacterial killing in alveolar macrophages by inhibiting NADPH oxidase.* Am J Respir Cell Mol Biol, 2007. **37**(5): p. 562-70.

46. Walker, C., et al., *Lymphokine regulation of activated (G1) lymphocytes. I. Prostaglandin E2-induced inhibition of interleukin 2 production.* J Immunol, 1983. **130**(4): p. 1770-3.

47. Rincon, M., et al., *Prostaglandin E2 and the increase of intracellular cAMP inhibit the expression of interleukin 2 receptors in human T cells.* Eur J Immunol, 1988. **18**(11): p. 1791-6.

48. Kalinski, P., et al., *IL-12-deficient dendritic cells, generated in the presence of prostaglandin E2, promote type 2 cytokine production in maturing human naive T helper cells.* J Immunol, 1997. **159**(1): p. 28-35.

49. Kalinski, P., et al., *Prostaglandin E2 induces the final maturation of IL-12-deficient CD1a+CD83+ dendritic cells: the levels of IL-12 are determined during the final dendritic cell maturation and are resistant to further modulation.* J Immunol, 1998. **161**(6): p. 2804-9.

50. Kalinski, P., et al., *T-cell priming by type-1 and type-2 polarized dendritic cells: the concept of a third signal.* Immunol Today, 1999. **20**(12): p. 561-7.

51. Gustafsson, K., et al., *Recruitment and activation of natural killer cells in vitro by a human dendritic cell vaccine.* Cancer Res, 2008. **68**(14): p. 5965-71.

52. Sharma, S., et al., *Tumor cyclooxygenase 2-dependent suppression of dendritic cell function.* Clin Cancer Res, 2003. **9**(3): p. 961-8.

53. Sombroek, C.C., et al., *Prostanoids play a major role in the primary tumor-induced inhibition of dendritic cell differentiation.* J Immunol, 2002. **168**(9): p. 4333-43.

54. Heusinkveld, M., et al., *M2 macrophages induced by prostaglandin E2 and IL-6 from cervical carcinoma are switched to activated M1 macrophages by CD4+ Th1 cells.* J Immunol, 2011. **187**(3): p. 1157-65.

55. Obermajer, N., et al., *Positive feedback between PGE2 and COX2 redirects the differentiation of human dendritic cells toward stable myeloid-derived suppressor cells.* Blood, 2011. **118**(20): p. 5498-505.

56. Jonuleit, H., et al., *Pro-inflammatory cytokines and prostaglandins induce maturation of potent immunostimulatory dendritic cells under fetal calf serum-free conditions.* Eur J Immunol, 1997. **27**(12): p. 3135-42.

57. Rieser, C., et al., *Prostaglandin E2 and tumor necrosis factor alpha cooperate to activate human dendritic cells: synergistic activation of interleukin 12 production.* J Exp Med, 1997. **186**(9): p. 1603-8.

58. Luft, T., et al., *Functionally distinct dendritic cell (DC) populations induced by physiologic stimuli: prostaglandin E(2) regulates the migratory capacity of specific DC subsets.* Blood, 2002. **100**(4): p. 1362-72.

59. Scandella, E., et al., *Prostaglandin E2 is a key factor for CCR7 surface expression and migration of monocyte-derived dendritic cells.* Blood, 2002. **100**(4): p. 1354-61.

60. Abrahao, A.C., et al., *A role for COX2-derived PGE2 and PGE2-receptor subtypes in head and neck squamous carcinoma cell proliferation.* Oral Oncol, 2010. **46**(12): p. 880-7.

61. Kim, Y.Y., et al., *Anti-cancer effects of celecoxib in head and neck carcinoma.* Mol Cells, 2010. **29**(2): p. 185-94.

62. Feng, L. and Z. Wang, *Chemopreventive effect of celecoxib in oral precancers and cancers.* Laryngoscope, 2006. **116**(10): p. 1842-5.

63. Wang, Z., C.F. Fuentes, and S.M. Shapshay, *Antiangiogenic and chemopreventive activities of celecoxib in oral carcinoma cell.* Laryngoscope, 2002. **112**(5): p. 839-43.

64. Lang, S., et al., *Impaired monocyte function in cancer patients: restoration with a cyclooxygenase-2 inhibitor.* FASEB J, 2003. **17**(2): p. 286-8.

65. Gillespie, M.B., et al., *Head and neck cancer recurrence and mortality in nonselective cyclooxygenase inhibitor users.* Arch Otolaryngol Head Neck Surg, 2007. **133**(1): p. 28-31.

66. Shin, D.M., et al., *Chemoprevention of head and neck cancer by simultaneous blocking of epidermal growth factor receptor and cyclooxygenase-2 signaling pathways: preclinical and clinical studies.* Clin Cancer Res, 2013. **19**(5): p. 1244-56.

67. Saba, N.F., et al., *Chemoprevention of head and neck cancer with celecoxib and erlotinib: results of a phase ib and pharmacokinetic study.* Cancer Prev Res (Phila), 2014. **7**(3): p. 283-91.

68. Wirth, L.J., et al., *Phase I study of gefitinib plus celecoxib in recurrent or metastatic squamous cell carcinoma of the head and neck.* J Clin Oncol, 2005. **23**(28): p. 6976-81.

69. Bao, Y.S., et al., *The regulation of CD4+ T cell immune responses toward Th2 cell development by prostaglandin E2.* Int Immunopharmacol, 2011. **11**(10): p. 1599-605.

70. MacKenzie, K.F., et al., *PGE(2) induces macrophage IL-10 production and a regulatory-like phenotype via a protein kinase A-SIK-CRTC3 pathway.* J Immunol, 2013. **190**(2): p. 565-77.

71. Stolina, M., et al., *Specific inhibition of cyclooxygenase 2 restores antitumor reactivity by altering the balance of IL-10 and IL-12 synthesis.* J Immunol, 2000. **164**(1): p. 361-70.

72. Woodford, D., et al., *An Inflammatory Cytokine Milieu is Prominent in Premalignant Oral Lesions, but Subsides when Lesions Progress to Squamous Cell Carcinoma.* J Clin Cell Immunol, 2014. **5**(3).

73. Juretic, M., et al., *Salivary levels of TNF-alpha and IL-6 in patients with oral premalignant and malignant lesions.* Folia Biol (Praha), 2013. **59**(2): p. 99-102.

74. Rhodus, N.L., et al., *The feasibility of monitoring NF-kappaB associated cytokines: TNF-alpha, IL-1alpha, IL-6, and IL-8 in whole saliva for the malignant transformation of oral lichen planus.* Mol Carcinog, 2005. **44**(2): p. 77-82.

75. Younes, F., et al., *Expression of TNF and the 55-kDa TNF receptor in epidermis, oral mucosa, lichen planus and squamous cell carcinoma.* Oral Dis, 1996. **2**(1): p. 25-31.

76. Xia, J., et al., *Expressions of CXCL12/CXCR4 in oral premalignant and malignant lesions.* Mediators Inflamm, 2012. **2012**: p. 516395.

77. Kotrashetti, V.S., et al., *Immunohistochemical expression of TLR4 and TLR9 in various grades of oral epithelial dysplasia and squamous cell carcinoma, and their roles in tumor progression: a pilot study.* Biotech Histochem, 2013. **88**(6): p. 311-22.

78. Bondad-Palmario, G.G., *Histological and immunochemical studies of oral leukoplakia: phenotype and distribution of immunocompetent cells.* J Philipp Dent Assoc, 1995. **47**(1): p. 3-18.

79. Ohman, J., et al., *Langerhans cells and T cells sense cell dysplasia in oral leukoplakias and oral squamous cell carcinomas--evidence for immunosurveillance.* Scand J Immunol, 2012. **76**(1): p. 39-48.

80. Costa, N.L., et al., *Distinct expression of perforin and granzyme B in lip and oral cavity squamous cell carcinoma.* J Oral Pathol Med, 2011. **40**(5): p. 380-4.

81. Dalrymple, N.A. and E.R. Mackow, *Endothelial cells elicit immune-enhancing responses to dengue virus infection.* J Virol, 2012. **86**(12): p. 6408-15.

82. Mulligan, J.K. and M.R. Young, *Tumors induce the formation of suppressor endothelial cells in vivo.* Cancer Immunol Immunother, 2010. **59**(2): p. 267-77.

83. Vielma, S.A., et al., *Skewing of immune cell cytokine production by mediators from adipocytes and endothelial cells.* Adipocyte, 2014. **3**(2): p. 126-31.

84. Forastiere, A., et al., *Head and neck cancer.* N Engl J Med, 2001. **345**(26): p. 1890-900.

85. Schoop, R.A., M.H. Noteborn, and R.J. Baatenburg de Jong, *A mouse model for oral squamous cell carcinoma.* J Mol Histol, 2009. **40**(3): p. 177-81.

86. Somers, K.D., M. Koenig, and G.L. Schechter, *Growth of head and neck squamous cell carcinoma in nude mice: potentiation of laryngeal carcinoma by 17 beta-estradiol.* J Natl Cancer Inst, 1988. **80**(9): p. 688-91.

87. O'Malley, B.W., Jr., et al., *A new immunocompetent murine model for oral cancer.* Arch Otolaryngol Head Neck Surg, 1997. **123**(1): p. 20-4.

88. Simon, C., et al., *An orthotopic floor-of-mouth cancer model allows quantification of tumor invasion.* Laryngoscope, 1998. **108**(11 Pt 1): p. 1686-91.

89. Gimenez-Conti, I., *The hamster cheek pouch carcinogenesis model.* Acta Odontol Latinoam, 1993. **7**(1): p. 3-12.

90. Shklar, G., *Development of experimental oral carcinogenesis and its impact on current oral cancer research.* J Dent Res, 1999. **78**(12): p. 1768-72.

91. Zenklusen, J.C., et al., *Transforming growth factor-beta 1 expression in Syrian hamster cheek pouch carcinogenesis.* Mol Carcinog, 1994. **9**(1): p. 10-6.

92. Hecht, S.S., *Tobacco carcinogens, their biomarkers and tobacco-induced cancer.* Nat Rev Cancer, 2003. **3**(10): p. 733-44.

93. Phillips, D.H., *Smoking-related DNA and protein adducts in human tissues.* Carcinogenesis, 2002. **23**(12): p. 1979-2004.

94. Yuan, B., et al., *Harvey ras (H-ras) point mutations are induced by 4-nitroquinoline-1-oxide in murine oral squamous epithelia, while squamous cell carcinomas and loss of heterozygosity occur without additional exposure.* Cancer Res, 1994. **54**(20): p. 5310-7.

95. Hawkins, B.L., et al., *4NQO carcinogenesis: a mouse model of oral cavity squamous cell carcinoma.* Head Neck, 1994. **16**(5): p. 424-32.

96. Smith, L.P. and G.R. Thomas, *Animal models for the study of squamous cell carcinoma of the upper aerodigestive tract: a historical perspective with review of their utility and limitations. Part A. Chemically-induced de novo cancer, syngeneic animal models of HNSCC, animal models of transplanted xenogeneic human tumors.* Int J Cancer, 2006. **118**(9): p. 2111-22.

97. Kanojia, D. and M.M. Vaidya, *4-nitroquinoline-1-oxide induced experimental oral carcinogenesis.* Oral Oncol, 2006. **42**(7): p. 655-67.

98. Vitale-Cross, L., et al., *Chemical carcinogenesis models for evaluating molecular-targeted prevention and treatment of oral cancer.* Cancer Prev Res (Phila), 2009. **2**(5): p. 419-22.

99. Czerninski, R., et al., *Targeting mammalian target of rapamycin by rapamycin prevents tumor progression in an oral-specific chemical carcinogenesis model.* Cancer Prev Res (Phila), 2009. **2**(1): p. 27-36.

100. De Costa, A.M., et al., *Characterization of the evolution of immune phenotype during the development and progression of squamous cell carcinoma of the head and neck.* Cancer Immunol Immunother, 2012. **61**(6): p. 927-39.

101. Johnson, S.D., A.M. De Costa, and M.R. Young, *Effect of the premalignant and tumor microenvironment on immune cell cytokine production in head and neck cancer.* Cancers (Basel), 2014. **6**(2): p. 756-70.

102. Pries, R., et al., *Secretion of tumor-promoting and immune suppressive cytokines by cell lines of head and neck squamous cell carcinoma.* In Vivo, 2006. **20**(1): p. 45-8.

103. Smith, C.W., et al., *The host environment promotes the development of primary and metastatic squamous cell carcinomas that constitutively express proinflammatory cytokines IL-1alpha, IL-6, GM-CSF, and KC.* Clin Exp Metastasis, 1998. **16**(7): p. 655-64.

104. Mann, E.A., et al., *Cytokine expression by head and neck squamous cell carcinomas.* Am J Surg, 1992. **164**(6): p. 567-73.

105. Whiteside, T.L., *Immunobiology of head and neck cancer.* Cancer Metastasis Rev, 2005. **24**(1): p. 95-105.

106. Reichert, T.E., et al., *Mechanisms responsible for signaling and functional defects.* J Immunother, 1998. **21**(4): p. 295-306.

107. Battersby, S., et al., *Expression and localization of endothelial monocyte-activating polypeptide II in the human endometrium across the menstrual cycle: regulation of expression by prostaglandin E(2).* J Clin Endocrinol Metab, 2002. **87**(8): p. 3928-35.

108. Hashimoto, K., et al., *Cyclooxygenase inhibition augments allergic inflammation through CD4-dependent, STAT6-independent mechanisms.* J Immunol, 2005. **174**(1): p. 525-32.

109. De Costa, A.M., et al., *Administration of a vaccine composed of dendritic cells pulsed with premalignant oral lesion lysate to mice bearing carcinogen-induced premalignant oral lesions stimulates a protective immune response.* Int Immunopharmacol, 2012. **13**(3): p. 322-30.

110. Hubbard, L.L., et al., *A role for IL-1 receptor-associated kinase-M in prostaglandin E2-induced immunosuppression post-bone marrow transplantation.* J Immunol, 2010. **184**(11): p. 6299-308.

111. Stables, M.J., et al., *Priming innate immune responses to infection by cyclooxygenase inhibition kills antibiotic-susceptible and -resistant bacteria.* Blood, 2010. **116**(16): p. 2950-9.

112. Olesch, C., et al., *MPGES-1-derived PGE2 suppresses CD80 expression on tumor-associated phagocytes to inhibit anti-tumor immune responses in breast cancer.* Oncotarget, 2015. **6**(12): p. 10284-96.

113. Dunn, G.P., L.J. Old, and R.D. Schreiber, *The three Es of cancer immunoediting.* Annu Rev Immunol, 2004. **22**: p. 329-60.

114. Xiao, M., et al., *IFNgamma promotes papilloma development by up-regulating Th17-associated inflammation.* Cancer Res, 2009. **69**(5): p. 2010-7.

115. Roberts, S.J., et al., *Characterizing tumor-promoting T cells in chemically induced cutaneous carcinogenesis.* Proc Natl Acad Sci U S A, 2007. **104**(16): p. 6770-5.

116. Kesselring, R., et al., *Human Th17 cells can be induced through head and neck cancer and have a functional impact on HNSCC development.* Br J Cancer, 2010. **103**(8): p. 1245-54.

117. McAllister, F., et al., *Oncogenic Kras activates a hematopoietic-to-epithelial IL-17 signaling axis in preinvasive pancreatic neoplasia.* Cancer Cell, 2014. **25**(5): p. 621-37.

118. Wang, L., et al., *IL-17 enhances tumor development in carcinogen-induced skin cancer.* Cancer Res, 2010. **70**(24): p. 10112-20.

119. Gnerlich, J.L., et al., *Induction of Th17 cells in the tumor microenvironment improves survival in a murine model of pancreatic cancer.* J Immunol, 2010. **185**(7): p. 4063-71.

120. Qian, X., et al., *Increased Th17 cells in the tumor microenvironment is mediated by IL-23 via tumor-secreted prostaglandin E2.* J Immunol, 2013. **190**(11): p. 5894-902.

121. Chinen, T., et al., *Prostaglandin E2 and SOCS1 have a role in intestinal immune tolerance.* Nat Commun, 2011. **2**: p. 190.