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Premalignant Oral Lesion Immunobiology: Immune Modulation and Vaccination

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by

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LIST OF ABBREVIATIONS

-/-	Homozygous genetic knockout
4NQO	4-nitroquinoline 1-oxide
4NQO Ctl	4NQO-treated control mice
ARG	Arginase
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid
BM	Bone marrow
CBA	Cytokine bead array
CD	Cluster of differentiation
CFSE	Carboxyfluorescein succinimidyl ester
COX-2	Cyclooxygenase-2
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T-lymphocyte antigen 4
DC	Dendritic cell
DCnt	Normal tongue epithelium lysate-pulsed dendritic cells or mice vaccinated with these cells
DCpm	Premalignant tongue epithelium lysate-pulsed dendritic cells or mice vaccinated with these cells
EGFR	Epidermal growth factor receptor
FasL	Fas ligand
FBS	Fetal bovine serum
Foxp3	Forkhead box protein 3
GAGE	G antigen
GM-CSF	Granulocyte macrophage colony stimulating factor

HBSS..... Hank's balanced salt solution

HNSCC..... Head and neck squamous cell carcinoma

HPV..... Human papillomavirus

IFN-γ..... Interferon-γ

IL..... Interleukin

KLRG1..... Killer cell lectin-like receptor subfamily G member 1

LN..... Lymph node

LPS..... Lipopolysaccharide

MDSC..... Myeloid-derived suppressor cell

MHC..... Major histocompatibility complex

MIP..... Macrophage inflammatory protein

MUC-1..... Mucin 1, cell surface associated

NK cell..... Natural killer cell

NY-ESO-1..... Cancer-testis antigen

PD-1..... Programmed death 1

PD-L1..... Programmed death ligand 1

PGE_2..... Prostaglandin E_2

PM..... Premalignant

PMA..... Phorbol 12-myristate 13-acetate

RAG..... Recombination activating gene

RAGE..... Renal tumor antigen

RANTES..... Regulated upon activation, normal T cell expressed, and secreted

SCID..... Severe-combined immune deficiency

SEM..... Standard error of the mean

TAA..... Tumor-associated antigen

TAM..... Tumor-associated macrophage

Tc1..... IFN-γ-producing cytotoxic T-lymphocyte

Tc17..... IL-17-producing cytotoxic T-lymphocyte

Tconv..... Conventional T cell

TGF-β..... Transforming growth factor-β

Th1..... Type 1 helper T-lymphocyte

Th2..... Type II helper T-lymphocyte

Th17..... IL-17-producing helper T-lymphocyte

TIL..... Tumor infiltrating lymphocyte

TNF..... Tumor necrosis factor

TRAIL..... Tumor necrosis factor-related apoptosis-inducing ligand

Treg..... Regulatory T cell

VEGF..... Vascular endothelial growth factor

WT..... Wild type

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ABSTRACT

While studies have indicated that squamous cell carcinoma of the head and neck (HNSCC) is associated with immune suppression, these studies did not analyze the immune response at the dysplastic stage. This study utilized a mouse model of 4-nitroquinoline 1-oxide (4NQO)-induced oral carcinogenesis to examine the alterations in immune phenotype at the premalignant and malignant stages of HNSCC. Cervical lymph nodes of HNSCC-bearing mice were found to contain a greater number of cells, including a greater number of conventional (Tconv) and regulatory (Treg) T cells, compared to lymph nodes of control and premalignant lesion-bearing mice. Premalignant lesion-bearing mouse lymph nodes consist of a greater percentage of Tconv cells expressing markers for activation, memory, and exhaustion compared to both control and HNSCC-bearing mice. Lymph nodes from both premalignant lesion-bearing and HNSCC-bearing mice include increased numbers of Th1, Tc1 and Th17 cells compared to control mice. The data show that while there is the expected increase in Tregs in lymph nodes when HNSCC is present, there is also an unexpected increase in immune populations usually associated with a beneficial anti-tumor response. In addition, the results demonstrate that the premalignant stage of HNSCC development is associated with a robust immune response involving an increase in inflammatory cells.

The use of dendritic cell (DC) vaccines as treatment for malignancy is complicated by the immune evasion tactics often employed by carcinomas such as HNSCC. This study aims to determine if an immune response can be elicited by administering a DC vaccine during the premalignant stages of HNSCC, prior to

development of immune escape. Bone marrow-derived DCs were pulsed with premalignant lesion lysate (DCpm) and administered to 4NQO-treated mice exhibiting premalignant lesions. Endoscopic examination revealed that DCpm vaccination and control vaccination with dendritic cells pulsed with normal tongue epithelium lysate (DCnt) significantly decreased lesion burden. Analysis of lymph node cells revealed that while DCnt vaccination resulted in a rapid increase in total lymphocyte count, levels of activated Tconv cells, and Th1, Tc1, Th17, Tc17, and Th2 cells, DCpm vaccination results in a delayed, yet substantial, increase in these immune effector mechanisms. This suggests that dendritic cell vaccination may have a beneficial effect on clinical outcome regardless of type of antigenic stimulation, and DCs pulsed with premalignant lysate rather than normal tongue epithelium lysate result in a delayed immune effector response upon vaccination of premalignant lesion-bearing mice.

Chapter 1

Immunoediting and HNSCC

CHAPTER 1: IMMUNOEDITING AND HNSCC

1.1 An Introduction to Immunoediting

1.1.1 Elimination and Equilibrium

The classic theory of immunoediting describes the incredibly complex immune interactions with dysplastic tissue during and after the development of cancers. In the first stage of immunoediting known as elimination, immune cells patrol the body, recognizing transformed cells and eliminating them through a reaction that traditionally is described as involving Th1 cells which direct $CD8^+$ cytotoxic T cells (CTLs) and macrophages to clear the dysplastic tissue (1, 2). Evidence for this includes research showing that mice with either severe-combined immune-deficiency (SCID) or lack of recombination activating gene ($RAG^{-/-}$), leading to absence of T cells, B cells, and NK cells, are more susceptible to chemically-induced tumors than wild type (WT) controls (1, 3, 4). Humans with immunodeficiencies are also at an increased risk for carcinogenesis, whether the cancers are of a viral or non-viral etiology (5-9). The type 1 response was identified as the main effector mechanism of tumor immunosurveillance after it was found that mice with specific deletions of $CD4^+$ and/or $CD8^+$ cells, antibody-mediated neutralization or knockout of IFN-γ, or knockout of IL-12, perforin or TRAIL all have increased incidence and/or growth of several different types of cancer (2, 10-16).

Despite the evidence for beneficial immune responses against dysplastic tissue, sometimes the tissue is not sufficiently cleared. If the initial transformation results in weakly immunogenic tissue, then it may avoid detection, moving into the equilibrium phase of immunoediting. During this stage, immunogenic variants that

arise may be targeted and eliminated by the immune system, while less immunogenic variants that develop mutations making them more resistant to immune effector mechanisms survive to multiply. In effect, the combination of time and the selective pressure of the immune system on cells that are prone to accumulate mutations to a greater degree than healthy cells results in the emergence of tumor variants that have been shaped to completely avoid elimination by the immune system (17-19).

1.1.2 Immune Escape

Tumor variants emerging from the process of immune equilibrium lose all immunogenicity and develop methods of manipulating the immune system for the tumor's benefit. This last stage of immunoediting is referred to as escape. Established tumors have been shown to avoid immune recognition through downregulation of immunogenic markers and/or MHC class I molecules (20, 21). Malignancies can also combat immune destruction by sabotaging an antitumor immune response through direct and indirect upregulation of immunosuppressive cells and downregulation of immunostimulatory cells.

As indicated before, a type I reaction, in which Th1 cells initiate cell-based immune responses involving various immune effectors, is considered necessary for a beneficial anti-tumor immune response to occur. In tumor escape, tumors develop the ability to downregulate the type I effector response and upregulate a type II effector response which, while beneficial under certain conditions, is most often associated with a poor prognosis in cancer patients. Interleukin-12 and IFN-γ, both

type I cytokines, have been found in reduced levels in multiple types of cancers (22-24). Reduced IL-12 in cancer patients has been associated with reduced numbers of CTLs, and reduced IFN-γ has been shown to reduce induction of MHC class I and class II and directly induce T cell anergy (25-27).

In contrast to type I cytokines, the type II cytokines IL-4, IL-10, and TGF-β have all been shown to be upregulated in patients with many types of advanced cancers (22-24, 28, 29). IL-4 directly inhibits Th1 cell differentiation, downregulates IFN-γ production, and upregulates the type II response (30-34). Like IL-4, IL-10 downregulates IFN-γ production (33). It has also been shown to reduce IL-12 production, downregulate MHC surface expression, and induce tumor-associated T cell tolerance (35-38). TGF-β affects the immune system in multiple ways: it has been shown to reduce differentiation, proliferation and functional capacity of T cells, including downregulation of a Th1 response by inhibition of IL-2 and IFN-γ production in Th1 cells and inhibition of FAS ligand and perforin expression in CTLs (39-45). TGF-β also inhibits costimulation, antigen presentation, maturation, and IL-12 production in antigen presenting cells, further downregulating a type I response (46, 47). Finally, TGF-β has been shown to increase tumor production of prostaglandin E_2 (PGE_2) and vascular endothelial growth factor (VEGF), which both promote tumor growth in multiple ways themselves (48-51). These changes in the cytokine milieu contribute a great deal to the blockade of a functional antitumor immune response in the microenvironment of many established tumors.

Many cancer types are also associated with an upregulation of immunosuppressive cells such as $CD34^+$ progenitor cells, myeloid-derived suppressor cells (MDSCs), tumor associated macrophages (TAMs), and regulatory T cells (Tregs). The $CD34^+$ progenitor cell refers to the earliest hematopoietic progenitor cell found in the circulation; these cells are found in low numbers in healthy individuals and in elevated numbers in both the circulation and tumor mass of cancer patients (52-55). These cells have been found to suppress T cell proliferation through the release of TGF-β (56, 57). MDSCs are less immature progenitor cells from the same lineage as $CD34^+$ progenitors; they too have been found to be elevated in various tissues of tumor-bearers, and their presence is associated with a poorer prognosis (58-60). These cells have been shown to directly suppress T cell responses and promote T cell apoptosis through production of ARG1 and nitric oxide synthase and indirectly suppress T cell responses through production of TGF-β and IL-10 (61-64). TAMs are mature myeloid cells that originated as circulating monocytes and have differentiated into type II macrophages in the tumor microenvironment. Like CD34+ progenitor cells and MDSCs, they are upregulated in multiple tumor types and their upregulation is associated with a poor prognosis (65-67). Also like MDSCs, TAMs suppress T cell function through the production of TGF-β and IL-10 (68-70). They also produce several growth factors, such as epithelial growth factor, platelet-derived growth factor, and basic fibroblast growth factor that can directly promote tumor growth (71, 72). Both $CD34^+$ progenitor cells and MDSCs appear to be mobilized from the bone marrow and recruited to the site of the tumor by a combination of tumor-

derived GM-CSF and VEGF (73-77). TAM differentiation is thought to be a result of the elevated levels of Type II cytokines such as IL-4, IL-6, IL-10, IL-13, PGE_2, and TGF-β in the tumor microenvironment, factors that also may contribute to MDSC mobilization (77-83).

Tregs are a heterogeneous population of either naturally-occurring or inducible T cells expressing the transcription factor forkhead box protein 3 (Foxp3) that act to suppress immune function and promote tolerance (84-86). Many of these cells also express the surface marker of T cell activation, CD25 (87). Elevated numbers of Tregs are found in the tumor microenvironment and periphery in multiple cancer types, and they most often correlate with a poor prognosis (88-96). Tregs suppress immune function through contact-dependent mechanisms, such as through membrane-bound TGF-β, CTLA-4 binding to B7 on target T cells or dendritic cells, and expression of FAS ligand or granzyme B, or through contact-independent mechanisms, such as through secretion of IL-10, TGF-β, and IL-4 (87, 97-101). Depletion of Tregs, through the use of antibodies targeting CD25 or antibodies targeting CTLA-4, has been shown to be beneficial in promoting tumor rejection (102-104).

1.1.3 Inflammation as a Tumor-Promoting Mechanism

Despite the increasing understanding of the traditional role of the immune system during tumor immunoediting, the role of the immune system during the development of cancer has proven to be much more complicated than previously thought. While directed inflammation in the form of a specific type I immune

response may help to eliminate growing and established tumors, unresolved chronic inflammation often serves to promote tumorigenesis. Around 20% of cancers are associated with chronic infections, such as *Helicobacter pylori* infection of the stomach and hepatitis B or C viral infection of the liver, while around 30% of cancers are attributed to tobacco use and 20% of cancers are attributed to obesity, both of which trigger chronic inflammation which has been linked to their tumor-promoting effects (105-110). The autoimmune conditions ulcerative colitis and Crohn's disease greatly increase the risk for intestinal cancers (111). In addition, use of anti-inflammatory agents such as COX2 inhibitors has been shown to reduce tumor incidence, especially for colon cancer, and possibly even help to reduce tumor burden in patients with certain cancers, such as breast and ovarian cancer, when used in combination with current treatment modalities (112-114). However, chronic inflammatory conditions such as rheumatoid arthritis and psoriasis do not increase the risk of cancers of the joints or skin (115). It is not well understood why chronic inflammation in some locations can contribute to cancer risk, while chronic inflammation in other locations does not.

While the relationship between tumor-promoting inflammation and antitumor immune responses is not fully elucidated, researchers have been able to identify inflammatory immune effectors that may at times promote rather than suppress tumorigenesis. As indicated above, a type II response has long been associated with a more tumor-promoting than antitumor response, mainly due to the downregulation of the type I response. However, type I responses have also been shown to contribute to tumor growth. While M1 macrophages produce IL-12,

helping to direct a Th1-type response, they are also responsible for the majority of the production of tumor-promoting cytokines such as IL-1β, IL-6, and TNFα (116, 117). IL-1 has been shown to be released in response to inhalation of asbestos or silica particles or in response to necrotic cell death as seen in a tumor after chemotherapeutic treatment or after the tumor's growth outpaces its blood supply, and it has been linked to enhanced tumor progression in multiple models (118, 119). IL-6 and TNFα have been shown to not only enhance tumor progression but also directly promote cancer cell growth and survival (117). Even IFN-γ producing Th1 cells and CTLs have been implicated in contributing to tumor promotion, progression, and metastasis in certain circumstances (120-122). Th17 cells have been linked to an improved prognosis in several cancer types and shown to result in a strong antitumor response when adoptively transferred into certain tumor models (123-128). Conversely, they have been shown to be associated with a poorer prognosis in patients with gastric cancer (129). Also, the main functions of the cytokines produced by Th17 cells, IL-17 and IL-23, include intense inflammation, angiogenesis, and possibly inhibition of effector CTL function, factors that promote tumorigenesis (130-133).

While it would certainly be preferential for one to be able to place specific immune effectors into the categories of "tumor-promoting" or "anti-tumor," the role of the immune system during the initiation and development of cancer is in fact much more complicated. The location, timing, duration, and intensity of the immune response are integral to determining the impact of this response. These factors and the overall balance of the various inflammatory and anti-inflammatory

signals are what determine if conditions are favorable for oncogenesis or tumor clearance. The elucidation of each of these individual factors in specific tumor types is integral to the development of immunotherapeutic techniques to prevent or combat those cancers.

1.2 Immune Reactivity Potential Toward HNSCC

Head and neck squamous cell carcinomas (HNSCC) are aggressive malignancies, and treatment options have traditionally involved surgery or, more recently, also radiation plus chemotherapy. Despite advances in treatment options and attempts at organ preservation, the success rate of treatment has not improved significantly, and current treatments have typically resulted in debilitating effects and reduced quality of life (134). An alternative or adjuvant treatment approach being tested for various malignancies is immunotherapy, although studies testing effectiveness of novel immunotherapeutic approaches for HNSCC are lagging.

As with other malignancies, the rationale for considering immunotherapy for HNSCC is based on the expression of potentially immunogenic antigens that are either selectively expressed on malignant versus normal tissues or expressed in increased levels, or tumor-associated antigens (TAAs). These antigens include the mucin MUC-1, epidermal growth factor receptor (EGFR), the RAGE and GAGE families of tumor antigens, NY-ESO-1, and others (135-138). To increase the effectiveness of immunotherapeutic strategies, efforts are underway to improve the identification of candidate tumor antigens that cause T cell responses. One such effort involves the use of a newly developed, automated, two-dimensional

chromatography system PF2D that fractionates the proteome of human tumor tissues (137). Advances in this area have the potential to reveal more immunogenic proteins to target, leading to stronger and more enduring responses to therapy.

Immunotherapeutic approaches hinge on the ability of the immune system to recognize these tumor antigens as foreign and develop a response, humoral and/or cellular, against the malignant tissue. Patients with HNSCC have been shown to mount antibody responses to antigens that are expressed on the tumor tissue. Reactivity includes antibody responses to the mucin MUC-1, with increased serum levels of MUC-1 and antibodies to MUC-1 in patients with more advanced disease and nodal involvement (139). Antibodies to p53, which is frequently mutated in HNSCC, are also detectable in serum of HNSCC patients and are indicative of nodal disease involvement (140). In addition to humoral responses to HNSCC, patients can mount cellular immune responses to the tumor. Increased intraepithelial $CD8^+$ tumor-infiltrating lymphocytes (TILs) in HNSCC metastases, as well as increased numbers of $CD20^+$ B cells in involved lymph nodes, are associated with a better prognosis (141). Also, both $CD4^+$ and $CD8^+$ T cells isolated from patients with HNSCC can be activated in response to tumor antigens (137). Antigen processing and cross-presentation by dendritic cells to $CD8^+$ T cells can stimulate their reactivity to tumor antigens. Less well studied has been the potential of γ/δ T cells to react to tumor. Recently, a subset of γ/δ T cells expressing the NK cell-associated molecule CD56 has been shown to exhibit cytolytic reactivity to HNSCC (142). These studies indicate that HNSCC is a good candidate for the development of

immunotherapeutic approaches, as HNSCC expresses tumor antigens and the immune system has the potential to react against these antigens.

1.3 HNSCC-Induced Defects in Immune Function

1.3.1 Avoiding Immune Detection and Destruction

Despite the above-described potential reactivity toward tumor antigens, these immune responses have not proven to be sufficient to prevent tumor progression. This is likely due to the many mechanisms by which established HNSCC tumors achieve immune escape. This includes the development of ways by which tumors can avoid detection by the immune system or decrease vulnerability to immune effector mechanisms. HNSCC cells have been found to decrease surface expression of MHC class I molecules, thereby making them less susceptible to detection by tumor specific CTLs (143-145). Further research shows that HNSCC cells also downregulate surface expression of costimulatory B7 molecules that are necessary for proper T cell signaling (146, 147). HNSCC has also been shown to avoid immune effector mechanisms by upregulation of surface expression of Fas ligand and PD-L1, both of which can induce apoptosis in activated lymphocytes (148, 149).

1.3.2 HNSCC-Derived Soluble Immune Inhibitory Mediators

Another way by which HNSCC tumors can achieve immune escape is through the induction of profound immune suppression that is characteristic for HNSCC patients. In fact, a prospective study analyzing risk factors for HNSCC patient

survival showed that among the top 4 risk factors was the extent of immune suppression of the HNSCC patients (150). The mechanisms of immune suppression are multifocal. HNSCC produces several immune inhibitory factors including PGE_2, TGF-β, VEGF, IL-6, and IL-10 (151-153), mediators long known to be inhibitory toward T-cell functions (154-156). The release of TGF-β and PGE_2 from HNSCC has been shown to correlate with reduced intratumoral levels of T-cells, in particular CTLs (157). More recently, dendritic cells of HNSCC patients have been shown to be defective in maturation and functionally impaired (158, 159). This impairment is attributed in part to the production by HNSCC of VEGF, PGE_2 and TGF-β. These mediators induce a shift in dendritic cell cytokine production to contribute to a more tolerogenic phenotype. They also alter expression of the chemokine receptors that are important in allowing dendritic cell migration to tumor-draining lymph nodes. In these studies, the dendritic cell dysfunction induced by HNSCC was overcome by blocking PGE_2 and TGF-β. Investigation into additional strategies aimed at diminishing or avoiding the effects of immune suppressive mediators produced by HNSCC may help to enhance the effectiveness of immune stimulatory treatment.

1.3.3 HNSCC Induction of Immune Suppressive Cells

In addition to producing soluble mediators that inhibit immune reactivity of HNSCC patients, HNSCC also induces immune inhibitory cells. The levels of Treg cells within HNSCC patients is greater than levels in healthy controls (160, 161). A lower ratio of CTLs to Tregs is associated with decreased survival (162). In addition

to secreting the immunosuppressive cytokines TGF-β and IL-10, Tregs hydrolyze ATP to a greater extent and, in turn, cause increased levels of adenosine-mediated suppression of effector T cells. However, Bergmann found that Tregs in the peripheral blood and tumor were elevated even further in patients with no evident disease after oncologic therapy compared to patients with active disease, and Badoual found that Treg infiltration into the tumor was actually associated with improved locoregional control (163, 164). This association of Treg infiltration with an improved prognosis may possibly be attributed to Treg-induced suppression of tumor-associated cells producing inflammatory mediators, growth factors, and angiogenic factors linked to promotion of advanced HNSCC tumors. This indicates that the impact of elevated Treg levels in HNSCC may be more complicated than previously believed.

Another immune suppressive cell that our laboratory has shown to be in increased levels in the peripheral blood of HNSCC patients is the immature $CD34^+$ progenitor cell. These cells are mobilized from the bone marrow by tumor-derived GM-CSF and are chemoattracted into the tumor by VEGF. They mediate their immune inhibitory activity by production of TGF-β (55, 76, 165, 166). Depending on the cytokine milieu, the tumor-mobilized $CD34^+$ cells can differentiate into granulocytes, monocytes or dendritic cells (55, 165, 167). Studies in animal tumor models have shown that these cells can also differentiate into endothelial cells (52). In order to alleviate the level of immune suppression in HNSCC patients, our studies used the strategy of taking advantage of the immature status of these $CD34^+$ cells and driving the differentiation of these inhibitory cells into stimulatory dendritic

cells. This was accomplished with treatment of HNSCC patients with the active hormone 1,25-dihydroxyvitamin D_3 (168, 169). Such a strategy not only resulted in a decrease in levels of intratumoral $CD34^+$ suppressor cells and an increase in numbers of mature dendritic cells and T cells expressing markers of activation within the HNSCC, but it also significantly prolonged the time between surgical treatment and cancer recurrence. Others have shown that HNSCC $CD34^+$ progenitor cells are induced to produce increased levels of the immune inhibitory mediator, IL-6 (170). These $CD34^+$ cells are likely to be an earlier precursor within a spectrum of immune inhibitory cells in various stages of differentiation that include MDSCs. MDSCs, which are found in a multitude of tumor types including HNSCC, mediate their immune suppressive activity mainly through the induction of reactive oxygen species production (171). Their suppressive activity can be blocked by inhibiting NADPH oxidase. A newly recognized tumor-induced immune suppressive population that has been described for HNSCC patients is the immune inhibitory endothelial cell (172). The suppressive activity of endothelial cells is induced by tumor-derived VEGF. These HNSCC-induced immune inhibitory endothelial cells mediate their immune suppressive activity through production of PGE_2 which, in turn, blocks T-cell proliferation, production of IFN-γ, perforin and granzyme B, suggesting inhibition of both T-cell helper and cytolytic functions. Blockade of VEGF through the use of bevacizumab could potentially prevent the induction of both $CD34^+$ progenitor cell and suppressive endothelial cell populations.

Many studies have focused on determining the mechanisms by which established HNSCC escapes an anti-tumor response. However, these studies are

complicated by the complex nature of the relationship of the immune system with cancer. It is clear that further investigation into the mechanisms by which HNSCC manipulates the immune system and strategies to block or reverse the consequent immune suppression may greatly assist the efficacy of attempts to stimulate immune rejection of HNSCC. Investigation into the origin of these mechanisms by examining the immune responses during HNSCC development may help in understanding HNSCC-associated immune escape, and such research may uncover potential therapeutic avenues by which one may target dysplastic tissue before immunosuppression arises.

Chapter 2

Rationale, Hypothesis, and Specific Aims

CHAPTER 2: RATIONALE, HYPOTHESIS, AND SPECIFIC AIMS

2.1 Rationale

Tumor-induced immunosuppression is utilized by many solid malignancies to avoid detection by the immune system as well as to avoid the effects of an anti-tumor immune response. However, many malignancies arise out of and even promote smoldering inflammation concurrently with the induction of immunosuppression. The promotion of oncogenesis versus tumor clearance depends on a multitude of factors including the mediators involved, the location of the tumor, and the intensity and timing of the response. Therefore, investigation into each of these factors is necessary to determine the appropriate targets for immunotherapeutic intervention to combat carcinogenesis.

HNSCC is a highly aggressive malignancy that has proven difficult to treat, with a 5-year survival rate of about 50%, due in part to the many immune evasive tactics employed by the tumor (28, 150, 163, 164, 173-177). While many of the aspects of immune escape utilized by HNSCC have been studied, some of the data has been contradictory, such as the clinical implications of the increase in Tregs seen in HNSCC (163). Development of oral HNSCC usually begins with the appearance of thick adherent plaques, known as leukoplakias and erythroplakias, on the floor of mouth, the lip, and the lateral tongue (173). Very little is known about the immune response at the premalignant stages. One way to clarify the role of immune effectors in established HNSCC is to investigate the immune responses elicited during the early stages of dysplasia leading up to HNSCC. In addition,

exposure of immune competency at the premalignant stage of HNSCC may provide clinicians with an avenue of immunotherapeutic delivery that avoids the complications of immunosuppression seen in established HNSCC.

Novel treatment approaches for HNSCC patients are essential due to the minimal level of improvement in patient survival over the last few decades, and immunotherapy could be a strong candidate for one such approach. However, some approaches to actively stimulate immune reactivity have not lived up to expectations in the past, likely due to the multitude of immune inhibitory mechanisms that are induced by HNSCC (178, 179). It is possible that initiating immunotherapy prior to the development of immune escape may prove more successful at preventing the establishment of HNSCC. HNSCC is somewhat unique in that it may be diagnosed in its premalignant stages. Also, HNSCC and oral premalignant lesions have been shown to share overexpression of certain antigens that may be utilized as markers to allow specific recognition of both types of tissues by immune effectors (136, 180). Dendritic cell-based vaccines have been used in multiple studies as a means to stimulate antitumor immune reactivity through the use of primed, activated dendritic cells to activate tumor antigen-specific T cells (181-185). The development of premalignant lesion antigen-pulsed dendritic cell vaccines, administered at the premalignant stage of HNSCC, may be utilized to stimulate protective immunity against further development of premalignant oral lesions and progression to HNSCC.

2.1.1 Hypothesis

"We hypothesize that the premalignant stage of HNSCC development will be associated with a local increase in inflammatory immune effector mechanisms and lack of immunosuppression while established HNSCC will be associated with local and systemic immunosuppression. Furthermore, we hypothesize that administration of a premalignant lesion lysate-pulsed dendritic cell vaccine to mice bearing premalignant lesions will induce immunity against development of premalignant lesions and HNSCC."

2.1.2 Specific Aims

This hypothesis will be tested through the following specific aims:

Aim 1: ***To investigate the alterations in immune phenotype at the premalignant and malignant stages of HNSCC within a 4NQO carcinogen-induced mouse model and in human patients.***

While HNSCC is associated with widespread immune manipulation, it is likely that this phenomenon has not yet developed or has developed to a lesser degree at the premalignant stage. Aim 1 analyzes the immune responses at the local and systemic levels in mice treated with the carcinogen 4NQO until the development of premalignant lesions or HNSCC and humans with premalignant lesions or HNSCC.

Aim 2: ***To use premalignant lesion-pulsed dendritic cells in vivo to skew the immune phenotype within mice bearing premalignant lesions towards immunity against further development of premalignant oral lesions and progression to HNSCC.***

Initiation of immunotherapy at the premalignant stage of HNSCC may have the increased success because of the avoidance of the widespread immunosuppression seen in HNSCC. As both premalignant lesions and HNSCC share overexpression of common tumor antigens, dendritic cell vaccination using premalignant lesion lysate as an antigen source may be able to promote an anti-tumor response. Aim 2 investigates if premalignant lesion lysate-pulsed dendritic cell vaccination of mice bearing premalignant lesions can stimulate protective immunity against the further development of premalignant lesions and progression to HNSCC.

2.2 Significance

Many studies have explored the local and systemic immune evasive factors employed by established HNSCC. However, it is still unclear what the roles of each cell type are in the HNSCC microenvironment. In addition, this immunosuppression has impeded attempts at the immunotherapeutic stimulation of an anti-tumor response. A goal of this study is to further investigate the immune alterations in the setting of HNSCC as well as to, for the first time, investigate the immune alterations during the development of HNSCC. Not only will this help to understand the roles of

the immune system in HNSCC, it will also help in identifying specific targets and possible avenues for the development of immunotherapeutic techniques to either prevent or treat HNSCC.

This study also aims to further explore the feasibility of using premalignant lesion-pulsed dendritic cells as a preventative treatment for HNSCC, a disease that presently has few effective treatments and a high mortality rate. Optimally, the initiation of this treatment at the premalignant stage will allow for the development of an effective anti-tumor response without the obstacle of the extensive immunosuppression seen with HNSCC. This will ideally lead to the identification of individual antigens in these tissue types responsible for eliciting immunity, allowing for the development of an antigen-specific vaccine that could be applied to multiple patients. The long-term goal is to use vaccination to prevent recurrence in patients who have developed oral premalignant lesions and had them surgically removed, as these patients would still have a high risk of developing more premalignant lesions and HNSCC if left untreated.

Chapter 3

Immune Response to HNSCC Development

CHAPTER 3: IMMUNE RESPONSE TO HNSCC DEVELOPMENT

Aim 1: ***To investigate the alterations in immune phenotype at the premalignant and malignant stages of HNSCC within a 4NQO carcinogen-induced mouse model and in human patients.***

3.1 Introduction

Head and neck squamous cell carcinoma (HNSCC) is an aggressive malignancy that has proven quite difficult to treat. Despite advances in care over the past 30 years, the five-year survival rate remains around 50% (173), with a five-year survival rate of 28% for patients with advanced HNSCC (186). In addition to the high mortality rate, surgical treatment of advanced HNSCC is associated with significant morbidity due to the major disfiguring consequences and functional defects that can result (173, 186). However, this malignancy is somewhat unique in that, with proper care and attention, it can be diagnosed in its premalignant stages as white or red patches on the oral mucosa referred to as leukoplakias or erythroplakias. If diagnosed at this stage, prognosis is considerably improved, though another hallmark of oral premalignancy and HNSCC is a high likelihood of recurrence (173). Therefore, our focus is shifting towards identifying this disease in its premalignant stages and ultimately developing treatment strategies to halt progression towards established HNSCC.

Multiple studies have indicated that HNSCC is associated with immune suppression and manipulation, factors that may contribute to the tenacity of this

disease. Patients with acquired or iatrogenic immunodeficiencies have an increased incidence of HNSCC (174, 175). HNSCC patients exhibit increased levels of regulatory T cells (Tregs) (163, 164, 176) and $CD34^+$ progenitor cells (177), both suppressive cell populations. Studies have identified skewing of the Th1/Th2 balance within HNSCC patients to a Th2 dominant phenotype with decreased Th1 activity (28, 176), indicating tumor-induced manipulation of the environment to prevent an effective anti-tumor response. However, while an increase in the $CD34^+$ population is associated with a poor patient outcome (177), Treg infiltration into the tumor was shown to be associated with improved locoregional control (164). In addition, recent studies revealed that peripheral blood mononuclear cell (PBMC) and tumor infiltrating Tregs are elevated even further in patients with no evident disease after oncologic therapy compared to those with active disease (163). Further complicating the matter is the fact that few to none of these studies stratified by HPV status. HPV-positive HNSCC, representing a minority of cases, has a more favorable prognosis compared to classic HPV-negative HNSCC, a phenomenon which appears to be related to the differing immune response to HPV(+) vs. HPV(-) disease (187). In fact, one study showing that $CD3^{high}$ tumors have decreased metastasis rates than $CD3^{low}$ tumors found that their results only held for HPV(+) tumors (188), indicating that conclusions drawn from previous studies may be influenced by HPV status of study participants.

In addition to gaps associated with HNSCC etiology, little to no research thus far has focused on investigating the immune status at the premalignant stage of HNSCC carcinogenesis. One study showed that expression of Fas, a death receptor

that renders a cell susceptible to immune-induced apoptosis through binding with its ligand (FasL), and expression of FasL itself were increased significantly in human oral premalignant tissue compared to normal control. While Fas levels in malignant tissue decreased to below control levels, levels of FasL continued to rise in malignant tissue compared to premalignant tissue, a phenomenon associated with immune escape as tumor-associated FasL can trigger apoptosis of infiltrating lymphocytes (189, 190). Though these results indicate that a significant immune response may take place in dysplastic oral tissue, no further studies into this topic have been carried out. Consequently, research into the immune response during and after the development of HPV-unrelated HNSCC is necessary in order to gain greater insight into possible future immunotherapeutic techniques that may be utilized to prevent or reverse HNSCC progression.

The present study investigates the proportions and total numbers of specific populations of immune cell types within cervical lymph nodes of control mice and mice bearing oral premalignant lesions or HNSCC, using a model of 4-nitroquinoline 1-oxide (4NQO)-induced carcinogenesis (191). While there are multiple models that may be used to study HNSCC, such as the HPV(+) cancer model (192) and the orthotopic SCC VII syngeneic tumor system (193), we chose to use this model of 4NQO-induced carcinogenesis because it allowed the study of HPV(-) disease versus HPV(+) disease, which has a significantly better prognosis (194), and it permitted the analysis of the immune response during the gradual progression to HNSCC rather than the immune response to injection of malignant cells. In addition, 4NQO mimics the carcinogenic effects of tobacco, the highest risk factor for HNSCC (173,

191). As tongue epithelium was determined to contain very low numbers of T cells and immune responses are most often orchestrated in draining lymph nodes, analysis focused on the cervical lymph nodes. Lymph nodes were analyzed to evaluate immune cell makeup of response, and tongue epithelium was lysed to determine presence of various cytokines. Due to trends reported in the literature, it was hypothesized that development of HNSCC would be associated with a gradual increase in immunosuppressive populations such as regulatory T cells and Th2 cells and a decrease in Th1 and Tc1 cell populations. While the current study verifies that HNSCC-bearing mice exhibit an increase in Treg populations, it is also revealed that the presence of HNSCC is associated with an increase in activated conventional $CD4^+$ and $CD8^+$ T cell populations, including an increase in Th1 and Tc1 cells that is not accompanied by an increase in Th2 cells. In addition, it was determined that the premalignant stage is associated with an increase in the percentage of activated and memory conventional T cells, as well as an increase in the percentage of Th1 and Tc1 cells compared to control and an increase in the percentage of Th17 cells compared to control and HNSCC-bearing mice.

Human samples were also tested for cytokine levels as a preliminary indicator of immune status. Normal/adjacent tongue tissue, premalignant tissue, and tumor tissue were obtained from human patients. Peripheral blood was also collected from unaffected individuals, premalignant lesion-bearing individuals, and HNSCC patients. Cytokine bead array analysis of tissue lysate and peripheral blood revealed a similar trend as what was detected in mice: while the expected local increase in IL-10 in HNSCC tissue was observed, there was also a local increase in

IFN-γ in HNSCC tissue compared to normal/adjacent tissue. In addition, analysis showed an impressive increase in the Th1 cytokines IFN-γ and IL-2 and the Th17 cytokine IL-17A in human premalignant tissue compared to both normal/adjacent and HNSCC tissue.

3.2 Materials and Methods

Oral HNSCC carcinogenesis

Five mg/ml 4NQO was administered in propylene glycol stock in the drinking water (diluted to 50 μg/ml) of 2 month old (at start) female C57BL/6 mice (Charles Rivers Laboratory, Wilmington, MA) until development of premalignant oral lesions (6-8 weeks) or HNSCC (12-16 weeks). Control mice received propylene glycol diluent. To monitor development of premalignant oral lesions and HNSCC, oral cavities of 4NQO-treated mice were endoscoped weekly using a Stryker 1.9mm x 30° scope and a Stryker 1088 HD camera. Mice were sedated with inhaled isoflurane (Piramal Healthcare, Mumbai, India) during the procedure.

Cervical lymph node processing

Cervical lymph nodes were harvested from C57BL/6 mice and homogenized via a Stomacher 80 homogenizer (Seward, West Sussex, UK) set on high for 90 sec. Cells were passed through a 40-μm cell strainer (BD Falcon, San Jose, CA) and rinsed with Hank's Buffered Saline Solution (HBSS, Invitrogen, Carlsbad, CA) to remove debris. Cells number was determined by counting cells excluding trypan blue using a hemocytometer.

Human samples

Recruitment of patients into this study was approved by the Institutional Review Board of record. Peripheral blood samples and tissue samples were collected from patients bearing premalignant lesions or HNSCC, and surgically

excised HNSCC and premalignant tissues were cryopreserved at -80° C until used for cytokine analyses. HNSCC tissues from untreated patients were also obtained from the Medical University of South Carolina Tissue Biorepository. Normal, non-carcinogenic oral tissue was procured from the adjacent areas bordering oral cancer tissue, and these tissues were deemed pathologically normal with no microscopic evidence of invasive carcinogenic disease.

Flow cytometric analysis of surface markers and Foxp3 expression

All reagents in this section were from BD Biosciences (San Jose, CA) unless otherwise specified. In order to detect levels of CD8 and CD4 positive cells in relation to Foxp3 and $CD11b^{+}Gr\text{-}1^{+}$ MDSCs, *ex vivo* cervical lymph node cells in single-cell suspension were washed once in Stain Buffer and resuspended at $1x10^{7}$ cells/ml. Nonspecific staining of a total of $1x10^{6}$ cells was blocked with FBS and anti-CD16/32 monoclonal antibody prior to cell surface staining with the following antibodies: PerCP-Cy5.5 CD4, FITC CD8a, PE Gr-1, PE CD25, PE PD-1, APC CD11b, APC CD44, APC CD69, and APC KLRG1 (eBioscience, San Diego, CA). Intracellular staining for PE-Cy7 Foxp3 (eBioscience, San Diego, CA) was performed after fixation with Foxp3 Fixation Buffer and permeabilization with Foxp3 Permeabilization Buffer. Extent and frequency of positively stained cells was visualized using flow cytometry (FACSCanto, San Jose, CA).

Flow cytometric analysis of cytokine expression

All reagents in this section were from BD Biosciences (San Jose, CA) unless otherwise specified. In order to detect intracellular cytokines, single-cell suspensions of cervical lymph node cells were restimulated for 4 hours at 37°C with 50 ng/ml phorbol 12-myristate 12-acetate (PMA), 1 μg/ml ionomycin, and brefelden A solution. Nonspecific staining of total of $1x10^6$ cells was blocked with FBS and anti-CD16/32 monoclonal antibody prior to cell surface staining with the following antibodies: PerCP-Cy5.5 CD4 and PE-Cy7 CD8a. Intracellular staining for PE IL-17A, FITC IFN-γ, APC IL-4, APC IL-10 and Alexa Fluor 488 IL-13 (eBioscience, San Diego, CA) was performed after fixation/permeabilization with Cytofix/Cytoperm. Extent and frequency of positively stained cells was visualized using flow cytometry (FACSCanto, San Jose, CA).

Cytokine bead array

All reagents in this section were from BD Biosciences (San Jose, CA) unless otherwise specified. In order to detect cytokines released into supernatant, single-cell suspensions of cervical lymph node cells were restimulated for 4 hours at 37°C with 50 ng/ml PMA and 1 μg/ml ionomycin without addition of brefeldin A. In order to detect cytokine levels in tongue tissue, mouse tongue epithelium was obtained after incubation of tongue fragments with Dispase II (Roche, Florence, SC). Both mouse and human tissue was lysed through sonication, protein concentration was determined by BCA protein assay (Pierce, Rockford, IL) per manufacturer's instructions, and final cytokine levels in tissue were normalized to pg/100μg of protein. Levels of IFN-γ, IL-2, IL-17A, IL-4, and IL-10 in cell supernatant or tongue

epithelium lysate were determined using a mouse cytometric bead array Th1/Th2/Th17 cytokine kit, while levels of RANTES, MIP-1α, MIP-1β, and IL-13 in cell supernatant or mouse tongue epithelium lysate were determined using cytometric bead array flex sets for the individual cytokines according to the manufacturer's instructions. Relative amounts of each cytokine were analyzed using FCAP Array software (San Jose, CA).

T cell purification and functional assays

$CD4^+CD25^-$ conventional T cells and $CD4^+CD25^+$ regulatory T cells were purified from mouse cervical lymph nodes using Dynabeads FlowComp Mouse $CD4^+CD25^+$ Treg Cell Isolation Kit (Invitrogen, Carlsbad, CA). To measure T cell proliferative response, $CD4^+CD25^-$ Tconv cells from control, premalignant lesion-bearing, and HNSCC-bearing mouse lymph nodes were labeled with CFSE using the CellTrace CFSE Cell Proliferation Kit (Invitrogen, Carlsbad, CA) and cultured for 3 days in the presence of Dynabeads Mouse T-Activatory anti-CD3/anti-CD28 beads (Invitrogen, Carlsbad, CA). To measure T cell suppression, CFSE-labeled control $CD4^+CD25^-$ Tconv cells were incubated for 3 days with control, premalignant lesion-bearing mouse, and HNSCC-bearing mouse $CD4^+CD25^+$ Treg cells at a 1:1 ratio with anti-CD3/anti-CD28 beads. Cell proliferation was measured by percentage of cells with diluted CFSE.

Statistical analysis

Data were reported using the mean as a measure of central tendency ± standard error of the mean. To compare one variable condition between groups, the 2-tailed Student's *t*-test or Mann-Whitney U test was used. Significance was reported in the 95% confidence interval.

3.3 Results

Increase in absolute number of cervical lymph node cells in mice bearing HNSCC compared to control mice and mice bearing premalignant oral lesions

Draining regional lymph nodes may become hyperplastic during the development and progression of malignancy due to reactive lymphadenopathy, metastasis, or both (195). To determine if draining lymph nodes undergo hyperplasia during the premalignant stage or malignant stage of HNSCC development, cervical lymph nodes from control mice, premalignant oral lesion-bearing mice, and HNSCC-bearing mice were processed to single cell suspension, and the number of trypan-blue excluding cells were counted. While mice bearing premalignant oral lesions exhibit equal numbers of cervical lymph node cells as control mice ($4.94\times10^6 \pm 6.25\times10^5$ vs. $5.31\times10^6 \pm 7.29\times10^5$, $p = 0.362$), mice bearing HNSCC exhibit a marked increase in number of cervical lymph node cells to more than double the number found in control mice ($1.17\times10^7 \pm 2.63\times10^6$ vs. $5.31\times10^6 \pm 7.29\times10^5$, $p = 0.006$). This indicates that draining lymph node hyperplasia occurs to a great extent after establishment of HNSCC but not during premalignant stages of HNSCC development.

Increase in total number of conventional and regulatory lymphocytes in cervical lymph nodes of mice bearing HNSCC compared to control mice and mice bearing premalignant oral lesions

Many solid carcinomas, including HNSCC, have classically been associated with immunosuppression involving decreased conventional ($Foxp3^-$) T cells and

increased regulatory ($Foxp3^+$) T cells and myeloid derived suppressor cells (MDSC), contributing to immune escape (163, 164, 176). The upregulation of a conventional T cell response is typically a goal of antitumor immunotherapy. While $CD8^+$ cytotoxic T cells have long been considered to be the most beneficial in actively combatting tumorigenesis through cytotoxic activity, recently $CD4^+$ conventional T cells have recently been shown to exhibit cytolytic activity that is beneficial in eradicating established tumors (196, 197). To determine the relative composition of $CD4^+$ and $CD8^+$ conventional T cells and regulatory T cells, as well as MDSCs, in cervical lymph nodes of control, premalignant lesion-bearing mice, and HNSCC-bearing mice, lymph node cells were stained for surface expression of CD4, CD8, CD11b, and Gr-1 and intracellular expression of the regulatory T cell marker Foxp3. Proportions of populations expressing each marker were determined through flow cytometric analysis of stained cells (Fig 3.1a and b), while total numbers of populations expressing each marker (Fig 3.1c) were determined by multiplying the percent positive by the number of lymph node cells for each individual mouse. While no differences in $CD11b^+Gr\text{-}1^+$ MDSCs were observed among the three groups (data not shown), HNSCC-bearing mouse lymph nodes consist of a greater total number of conventional helper ($CD4^+Foxp3^-$) and cytotoxic ($CD8^+Foxp3^-$) T cells and $CD8^+Foxp3^+$ regulatory T cells as well as a greater proportion and total number of $CD4^+Foxp3^+$ regulatory T cells compared to both control and premalignant lesion-bearing mouse lymph nodes (Fig 3.1b and c). This indicates that establishment of HNSCC is associated not only with increased regulatory T cell levels but also with increased numbers of conventional $CD4^+$ and $CD8^+$ T cells.

Ratios of conventional to regulatory T cells were determined by dividing number of $CD4^+Foxp3^-$ or $CD8^+Foxp3^-$ conventional T cells by number of $CD4^+Foxp3^+$ or $CD8^+Foxp3^+$ regulatory T cells for each individual mouse. As shown in Fig 1d, while there is no significant difference between $CD8^+$ conventional:regulatory T cell ratio between the three groups, both premalignant lesion-bearing mice and HNSCC-bearing mice have a $CD4^+$ conventional:regulatory T cell ratio that is significantly lower than control. This suggests that while lymph node cells from HNSCC-bearing mice exhibit large changes in numbers of both conventional and regulatory $CD4^+$ T cells, the relative degree of these changes results in a relative proportion of these cells which is similar to the proportion resulting from the minor decrease in $CD4^+$ conventional T cells and minor increase in $CD4^+$ regulatory T cells seen in lymph nodes of premalignant lesion-bearing mice.

Decrease in proliferation of conventional T cells from HNSCC-bearing mouse lymph nodes in response to stimulation compared to control.

Due to the unexpected increase in total number of conventional T cells in mice bearing HNSCC compared to control mice, the proliferative capacity of these cells in response to stimulation was evaluated. Conventional $CD4^+CD25^-$ T cells were isolated from control, premalignant lesion-bearing, and HNSCC-bearing mouse lymph nodes by magnetic isolation, stained with CFSE, and cultured for 3 days with or without anti-CD3 and anti-CD28. Conventional CD4 cells from HNSCC bearing mice had a significantly decreased percentage of proliferation compared to control mice (Figure 3.1e). This was supported by cytokine analysis of cell supernatant

which revealed a decrease in IL-2 released into the supernatant of HNSCC-bearing mouse conventional CD4 cells compared to control conventional CD4 cells (Figure 3.1f). The levels of $CD4^{+}CD25^{-}$ conventional T cell proliferation and IL-2 release at the premalignant stage fell in the middle of levels for control cells and HNSCC-bearing mouse cells.

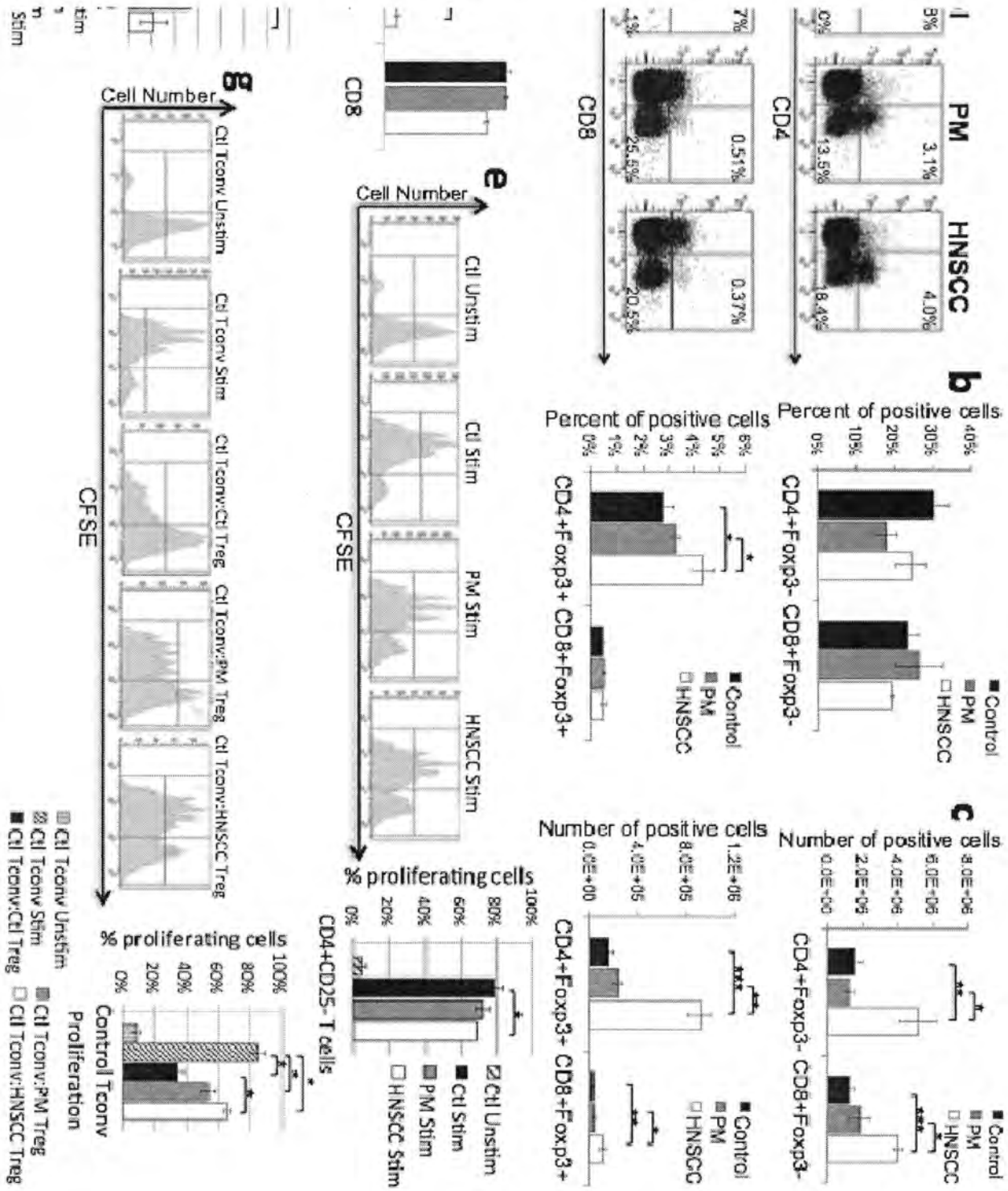
PM
HNSCC
CD4
CD8
3.1%
4.0%
0.51%
0.37%
b
Percent of positive cells
CD4+Foxp3- CD8+Foxp3-
CD4+Foxp3+ CD8+Foxp3+
Control
PM
HNSCC
c
Number of positive cells
CD4+Foxp3- CD8+Foxp3-
CD4+Foxp3+ CD8+Foxp3+
d
CD8
e
Cell Number
CFSE
Ctl Unstim
Ctl Stim
PM Stim
HNSCC Stim
% proliferating cells
CD4+CD25- T cells
g
Ctl Tconv Unstim
Ctl Tconv Stim
Ctl Tconv:Ctl Treg
Ctl Tconv:PM Treg
Ctl Tconv:HNSCC Treg
Control Tconv Proliferation

Fig 3.1 Established HNSCC is associated with an increase in numbers of regulatory and conventional T cells in cervical lymph nodes of affected mice Representative results (a) and graphical representation (b) of flow cytometric staining of cervical lymph node cells from control, premalignant lesion-bearing (PM), and HNSCC-bearing mice (HNSCC) with at least 3 mice per group. *, $p < 0.05$. Total numbers of populations (c) were determined by multiplying the percent positive by the number of cervical lymph node cells for each individual mouse. *, $p < 0.05$. **, $p < 0.01$. ***, $p < 0.001$. The $CD4^+$ conventional T cell to regulatory T cell ratio of both premalignant lesion-bearing and HNSCC-bearing mice is significantly decreased compared to control (d). *, $p < 0.05$. Representative results and graphical representation (e) of flow cytometric analysis of CFSE-stained $CD4^+CD25^-$ conventional T cells from control, premalignant lesion-bearing mouse, and HNSCC-bearing mouse lymph nodes (at least 3 mice per group) after three days culture with no stimulation (unstim) or stimulation with anti-CD3/anti-CD28 beads (stim). Cell proliferation was measured by percentage of cells with diluted CFSE. *, $p < 0.05$. Cytometric bead array analysis (f) of $CD4^+CD25^-$ conventional T cell supernatant. *, $p < 0.05$. Representative results and graphical representation (g) of flow cytometric analysis of CFSE-stained control $CD4^+CD25^-$ conventional T cells after coincubation at a 1:1 ratio for three days with control mouse, premalignant lesion-bearing mouse, or HNSCC-bearing mouse (at least 3 mice per group) $CD4^+CD25^+$ regulatory T cells in the presence of anti-CD3/anti-CD28 beads. *, $p < 0.05$.

Decrease in suppressive capacity of regulatory T cells from HNSCC-bearing mouse lymph nodes compared to control regulatory T cells.

As it was counterintuitive to observe a simultaneous increase in both conventional and regulatory T cells in the HNSCC-bearing mouse lymph nodes, the suppressive function of the regulatory T cells was investigated. Magnetically isolated control, premalignant-bearing, and HNSCC-bearing mouse lymph node $CD4^+CD25^+$ regulatory T cells were co-incubated with CFSE-stained control $CD4^+CD25^-$ conventional T cells at a 1:1 ratio for 3 days in the presence of anti-CD3 and anti-CD28. As seen in Figure 3.1g, HNSCC-bearing mouse regulatory T cells were found to be significantly less suppressive of control conventional T cells compared to control mouse regulatory T cells, and the premalignant lesion-bearing mouse regulatory T cells suppressed control Tconv cells at a level that was

intermediate between control and HNSCC-bearing mouse Tregs. This indicates that while Tregs increase in number during HNSCC development, these cells may also lose their ability to inhibit conventional T cell proliferation.

Increased percentage of lymphocytes expressing markers for activation, memory, and exhaustion in cervical lymph nodes of mice bearing premalignant oral lesions compared to both control mice and mice bearing HNSCC

An optimal antitumor response depends on the activation, expansion and survival of memory T cells. Suboptimal immune responses involve increased numbers of T cells expressing markers of exhaustion (198, 199). To determine the make-up of these phenotypic subsets between cervical lymph node cells from control, premalignant lesion-bearing mice and HNSCC-bearing mice, $CD4^+Foxp3^-$ helper T cells and $CD8^+Foxp3^-$ cytotoxic T cells were analyzed for expression of the activation markers CD25 and CD69, the marker of antigen experience, CD44, and the exhaustion markers PD-1 and KLRG1. As shown in Fig 3.2a and b, lymph node cells from premalignant lesion-bearing mice exhibit a nearly 4-fold increase in percent of $CD25^+$ helper T cells compared to control and a nearly doubled percentage of $CD25^+$ helper T cells compared to HNSCC-bearing mice. HNSCC-bearing mice show a significant but minor increase in percent of $CD25^+$ cytotoxic T cells compared to control. When factoring in total number of positive cells, while premalignant lesion-bearing mice maintain an increased number of $CD25^+$ helper T cells compared to control, HNSCC-bearing mice have an increased number of $CD25^+$ and $CD69^+$ helper

and cytotoxic T cells compared to both control and premalignant lesion-bearing mice due to the increase in overall cellularity (Fig 3.2c).

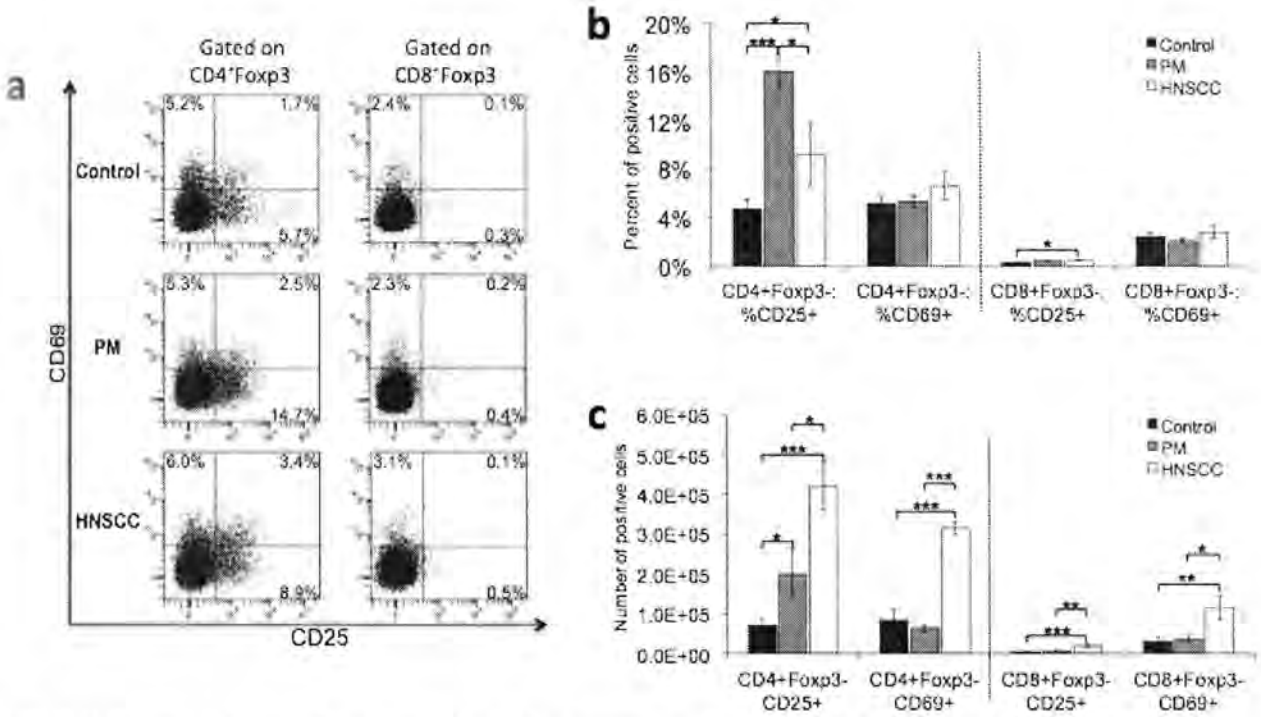

Fig 3.2 Premalignant lesion-bearing mouse and HNSCC-bearing mouse cervical lymph nodes exhibit an increase in activated conventional T cells compared to control mouse cervical lymph nodes
Representative results (a) and graphical representation (b) of flow cytometric analysis of cervical lymph node cells from control, premalignant lesion-bearing (PM), and HNSCC-bearing mice (HNSCC) with at least 3 mice per group. *, $p < 0.05$. ***, $p < 0.001$. Total numbers of populations (c) were determined by multiplying percent positive by number of cervical lymph node cells for each individual mouse. *, $p < 0.05$. **, $p < 0.01$. ***, $p < 0.001$.

Increased expression of the surface marker CD44 is used to distinguish T cells with antigen experience from those that are naïve (200, 201). Analysis of expression of the T cell memory marker CD44 reveals that lymph nodes of mice bearing premalignant oral lesions consist of a greater percentage of $CD44^{high}$ helper T cells compared to control and a greater percentage of $CD44^{high}$ cytotoxic T cells compared to both control and HNSCC-bearing mice (Fig 3.3a and b). However, HNSCC-bearing mouse lymph nodes have a greater total number of $CD44^{high}$ helper

T cells compared to both control and premalignant lesion-bearing mice and a greater number of CD44^{high} cytotoxic T cells compared to controls, again due to an increase in overall lymph node cellularity.

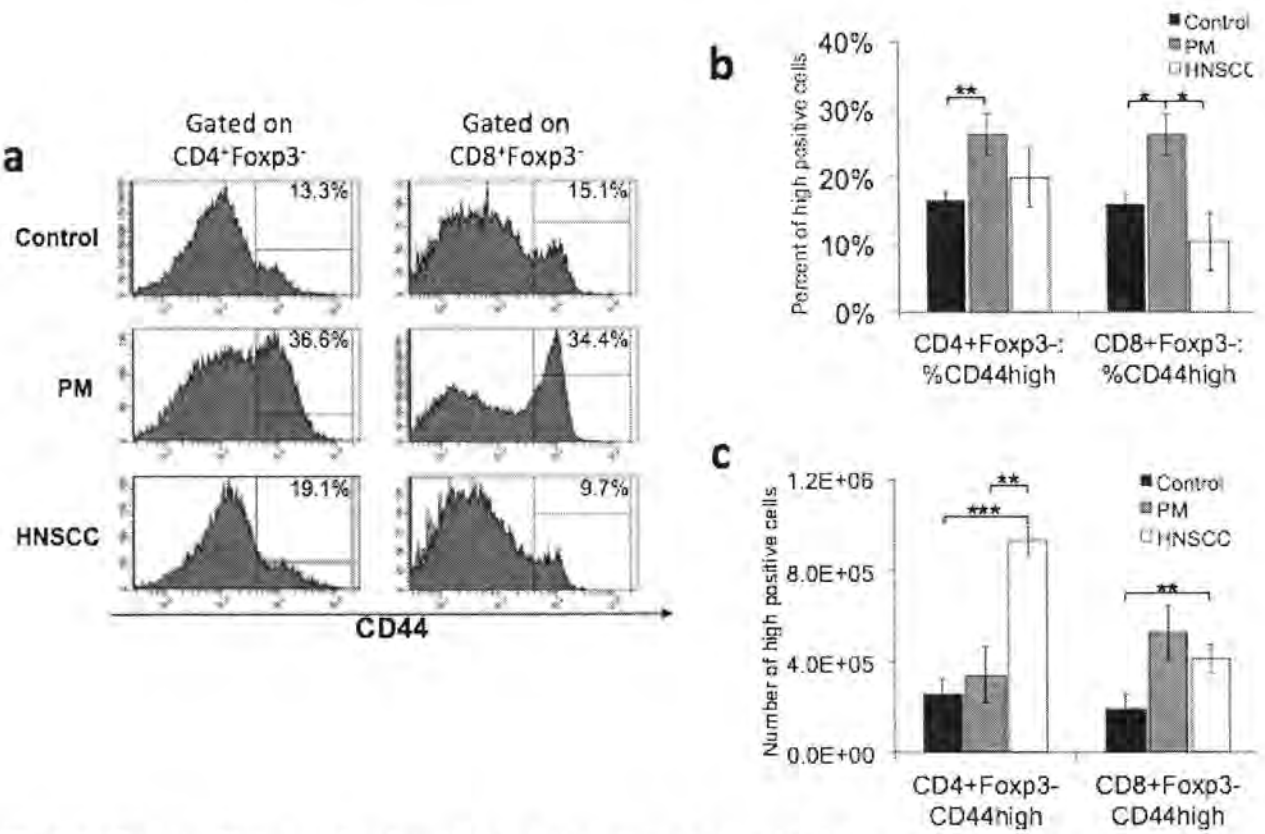

Fig 3.3 Premalignant lesion-bearing mouse cervical lymph nodes consist of a greater percentage of CD44^{high} memory conventional T cells compared to control and HNSCC-bearing mice

Representative results (a) and graphical representation (b) of flow cytometric analysis of cervical lymph node cells from control, premalignant lesion-bearing (PM), and HNSCC-bearing mice (HNSCC) with at least 3 mice per group. *, $p < 0.05$. **, $p < 0.01$. Total numbers of populations (c) were determined by multiplying percent positive by number of cervical lymph node cells for each individual mouse. **, $p < 0.01$. ***, $p < 0.001$.

Expression of the markers PD-1 or KLRG1 indicates that a T cell has been activated yet is at the point in its lifespan where, upon further stimulation, it will undergo programmed cell death rather than perform effector functions (199, 202). As such, these markers are often used to indicate T cell exhaustion. Analysis of

expression of PD-1 and KLRG1 on conventional T cells in the lymph nodes of control, premalignant lesion-bearing and HNSCC-bearing mice revealed that premalignant lesion-bearing mice have a significant but minor increase in percent of KLRG1$^+$ helper T cells compared to both control and HNSCC-bearing mice, while HNSCC-bearing mice have a decreased percentage of KLRG1$^+$ helper T cells compared to control as well (Fig 3.4a and b). When comparing total numbers of positive cells (Fig 3.4c), HNSCC-bearing mouse lymph nodes consist of a greater number of PD-1$^+$ helper and cytotoxic T cells compared to both control and premalignant lesion-bearing mice. Taken together, this data indicates that the premalignant stage of HNSCC development is associated with an increase in activated, memory conventional T cells, some of which become exhausted, compared to control and HNSCC-bearing mice. When it comes to total number of positive cells, however, the prominent increase in cervical lymph node cellularity in mice with established HNSCC makes it appear as though there is an increase in activated, memory and exhausted T cells at this stage compared to the premalignant stage and control.

Cervical lymph nodes of premalignant oral lesion-bearing and HNSCC-bearing mice exhibit an increase in percent and total number of Th1, Tc1 and Th17 cells as well as an increase in secretion of Th1, Tc1 and Th17-related cytokines and chemokines compared to control

To investigate the degree of upregulation or downregulation of different subtypes of cytokine-secreting effector T cells during the progression to HNSCC, cervical lymph node cells from control mice, premalignant lesion-bearing mice, and

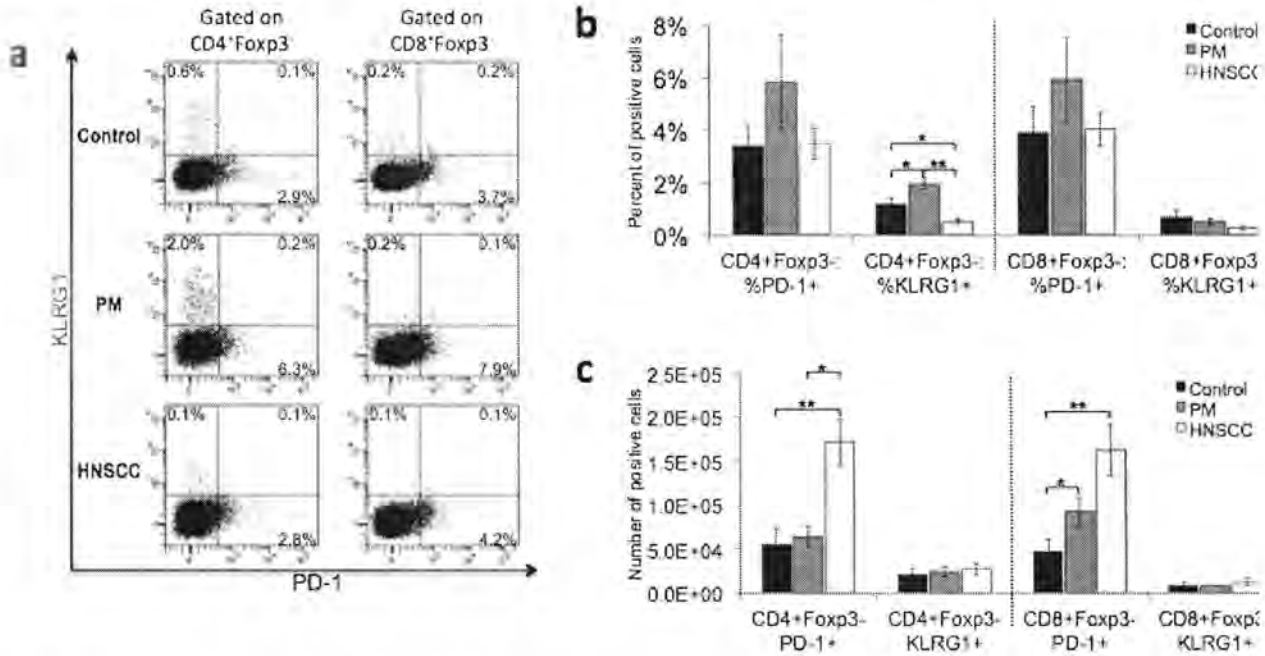

Fig 3.4 Premalignant lesion-bearing mouse cervical lymph nodes contain an increased percentage of KLRG1⁺ helper T cells, while HNSCC-bearing mouse cervical lymph nodes contain a greater total number of PD-1⁺ conventional T cells

Representative results (a) and graphical representation (b) of flow cytometric analysis of cervical lymph node cells from control, premalignant lesion-bearing (PM), and HNSCC-bearing mice (HNSCC) with at least 3 mice per group. *, $p < 0.05$. **, $p < 0.01$. Total numbers of populations (c) were determined by multiplying percent positive by number of cervical lymph node cells for each individual mouse. *, $p < 0.05$. **, $p < 0.01$.

HNSCC-bearing mice were stimulated for four hours with PMA and Ionomycin with or without brefeldin A. Cells incubated with brefeldin A were stained for CD4, CD8, and the cytokines IFN-γ, IL-17A, IL-13, IL-4, and IL-10 and analyzed by flow cytometry. Supernatants of cells incubated without brefeldin A and lysates of tongue epithelium from each mouse group were analyzed by cytometric bead array for presence of the Th1/Tc1-related cytokines IFN-γ and IL-2 and chemokines RANTES, MIP1-α and MIP-1β, the Th17-related cytokine IL-17A, and the Th2-related cytokines IL-13, IL-6, IL-4, and IL-10.

An ideal antitumor immune response is often described as involving an increased in Th1 and Tc1 type immunity, and the upregulation of this population is often considered a target goal of cancer immunotherapy (203). Many solid tumors, including HNSCC, have been linked to a decrease in Th1 immunity in past studies (28, 176). As shown in Fig 3.5a and b, premalignant lesion-bearing mice exhibit an increase in percentage of lymph node cells staining double positive for CD4 and IFN-γ (Th1 cells) and lymph node cells staining double positive for CD8 and IFN-γ (Tc1 cells) compared to controls. HNSCC-bearing mice have an even higher percentage of Th1 cells compared to both control and premalignant-lesion bearing mice and an increased percentage of Tc1 cells compared to control alone. After factoring in total number of lymph node cells (Fig 3.5c), premalignant lesion-bearing mice have a greater number of Tc1 cells compared to control, while HNSCC-bearing mice have a greater number of both Th1 and Tc1 cells compared to control. Levels of secreted cytokines and chemokines help confirm these results, as supernatants of cells from both premalignant lesion-bearing and HNSCC-bearing mice contain increased IFN-γ, IL-2, RANTES, MIP-1α and MIP-1β compared to control mice (Fig 3.5d). While the level of IL-2 in tongue epithelium lysate was lower in HNSCC-bearing mice compared to control, the levels on IFN-γ, RANTES, MIP-1α and MIP-1β were found to be higher in premalignant epithelium lysate and HNSCC epithelium lysate compared to control (Figure 3.5e). This data suggests that Th1 and Tc1 immunity is stimulated early in development of HNSCC, during the premalignant stages, and is sustained even after HNSCC is established, contrary to what some previous studies have indicated (28, 176).

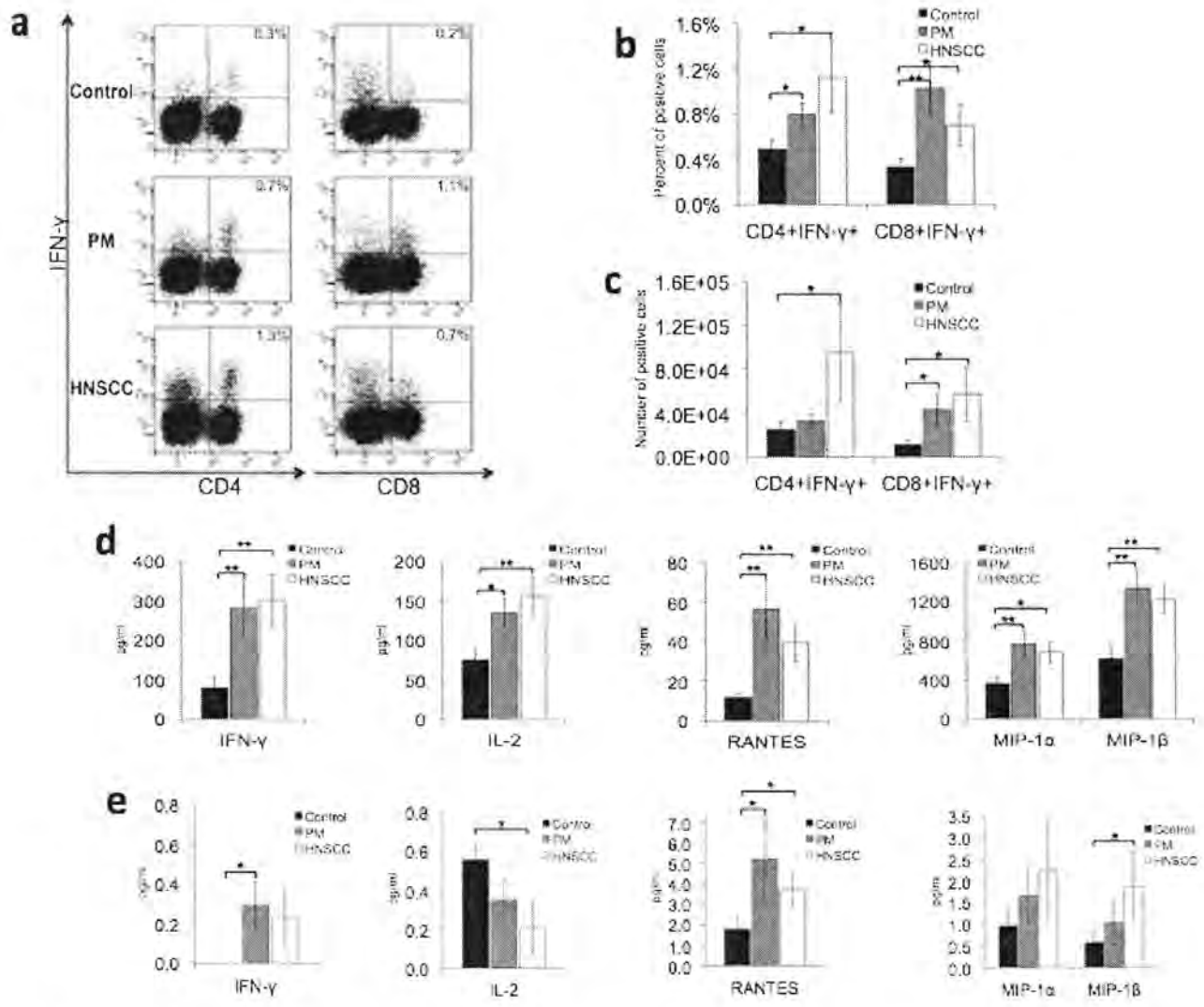

Fig 3.5 Th1/Tc1 cells and related cytokines are increased in cervical lymph nodes of premalignant lesion-bearing and HNSCC-bearing mice
Representative results (a) and graphical representation (b) of flow cytometric analysis of control, premalignant lesion-bearing mouse, and HNSCC-bearing mouse cervical lymph node cells after stimulation for four hours with phorbol 12-myristate 13-acetate (PMA) and ionomycin in the presence of brefeldin A, with at least 9 mice per group. *, $p < 0.05$. **, $p < 0.01$. Total numbers of populations (c) were determined by multiplying percent positive by number of cervical lymph node cells for each individual mouse. *, $p < 0.05$. Cytometric bead array analysis (d) of control, premalignant lesion-bearing mouse, and HNSCC-bearing mouse cervical lymph node cells after stimulation for four hours with PMA and ionomycin, with at least 9 mice per group. *, $p < 0.05$. **, $p < 0.01$. Cytometric bead array analysis (e) of control, premalignant lesion-bearing mouse, and HNSCC-bearing mouse tongue epithelium lysate, with normalization to 100 μg of protein (as determined by BCA protein assay), with at least 10 mice per group. *, $p < 0.05$.

The role of Th17 cells in tumor immunology is less well defined than that of Th1 and Tc1 cells. While populations of Th17 cells have been shown to be expanded in multiple cancer types, including HNSCC, the benefit and/or detriment of these cells remains under debate (204, 205). Both premalignant lesion-bearing and HNSCC-bearing mouse lymph nodes contain a greater percentage (Fig 3.6a and b) and number (Fig 3.6c) of Th17 ($CD4^+IL\text{-}17A^+$) cells compared to control mouse lymph nodes, while premalignant lesion-bearing mouse lymph nodes contain a greater percentage of Th17 cells than HNSCC-bearing mouse cervical lymph nodes (Fig 3.6a and b). This is supported by the cytometric bead array analysis showing a greater amount of IL-17A released into the supernatant of premalignant lesion-bearing mouse lymph node cells compared to control (Fig 3.6d). Levels of IL-17A in tongue epithelium lysate were too low to be detected by cytometric bead analysis. This data indicates that development of premalignant lesions is associated with an inflammatory immune response consisting of Th1, Tc1 and Th17 cells, and that while the Th1 response is sustained throughout HNSCC establishment and progression, the Th17 response wanes.

Among the many immune evasive mechanisms often employed by solid malignancies, the skewing of a Th1-driven cell-mediated response into a less effective Th2-driven humoral response has been described as occurring in the setting of HNSCC (28, 176). The only significant difference among Th2 cytokines identified by flow cytometric analysis of lymph node cells is a minor decrease in percent of $CD4^+IL\text{-}13^+$ cells in lymph nodes of HNSCC-bearing mice compared to premalignant lesion-bearing mice (Fig 3.7a and b). Of the Th2 cytokines measured

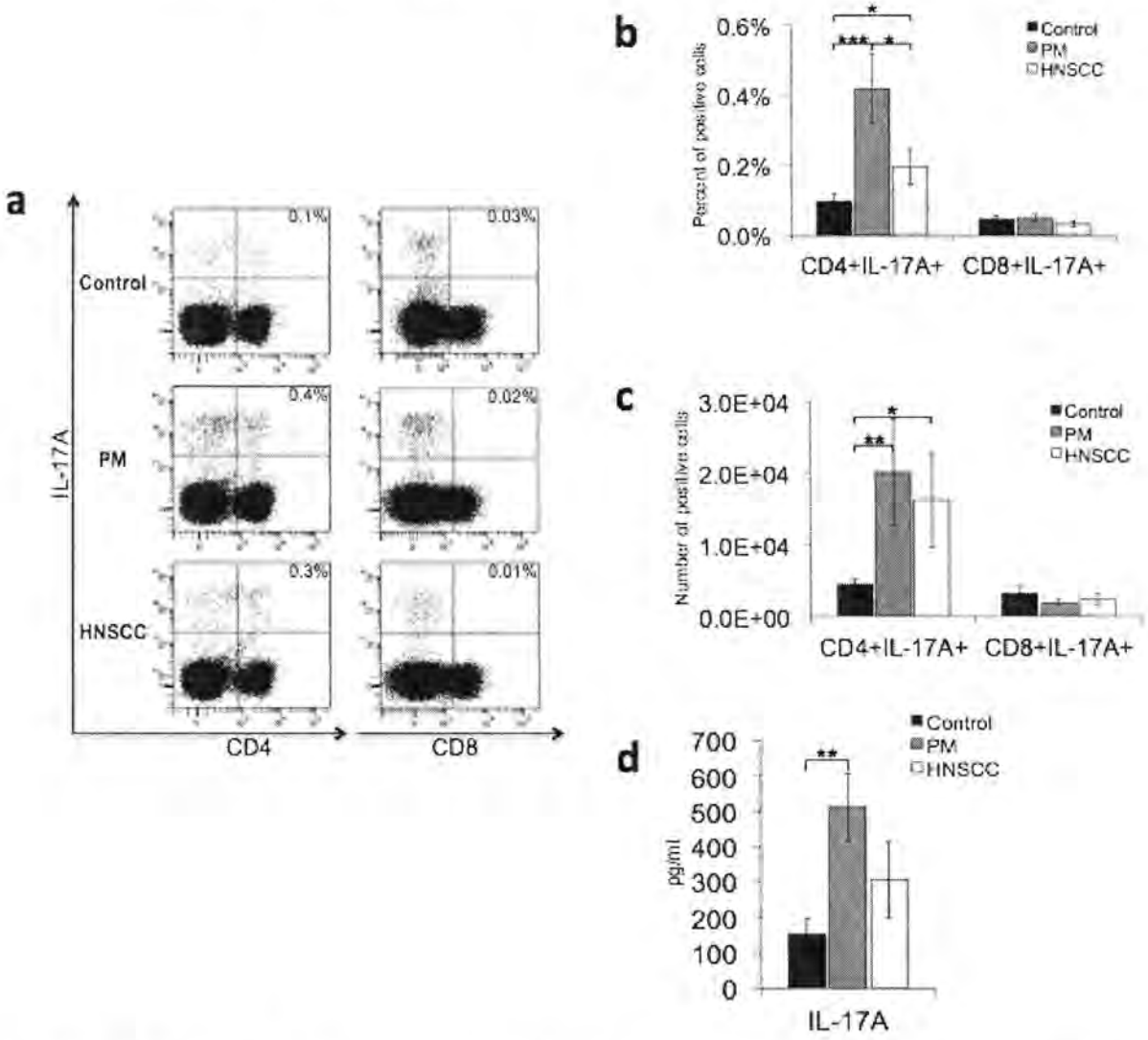

Fig 3.6 Th17 cells are increased in cervical lymph nodes of premalignant lesion-bearing and HNSCC-bearing mice

Representative results (a) and graphical representation (b) of flow cytometric analysis of control, premalignant lesion-bearing mouse, and HNSCC-bearing mouse cervical lymph node cells after stimulation for four hours with phorbol 12-myristate 13-acetate (PMA) and ionomycin in the presence of brefeldin A, with at least 9 mice per group. *, $p < 0.05$. ***, $p < 0.001$. Total numbers of populations (c) were determined by multiplying percent positive by number of cervical lymph node cells for each individual mouse. *, $p < 0.05$. **, $p < 0.01$. Cytometric bead array analysis (d) of control, premalignant lesion-bearing mouse, and HNSCC-bearing mouse cervical lymph node cells after stimulation for four hours with PMA and ionomycin, with at least 9 mice per group. **, $p < 0.01$.

in supernatants, only a small decrease in level of IL-4 released from HNSCC-bearing mouse lymph node cells compared to premalignant lesion-bearing mouse lymph node cells was detected (Fig 3.7d). The measurement of Th2 cytokines in tongue epithelium lysate revealed no significant differences between the three mouse

groups (data not shown). This indicates that HNSCC development is not strongly associated with any change in Th2 cytokines.

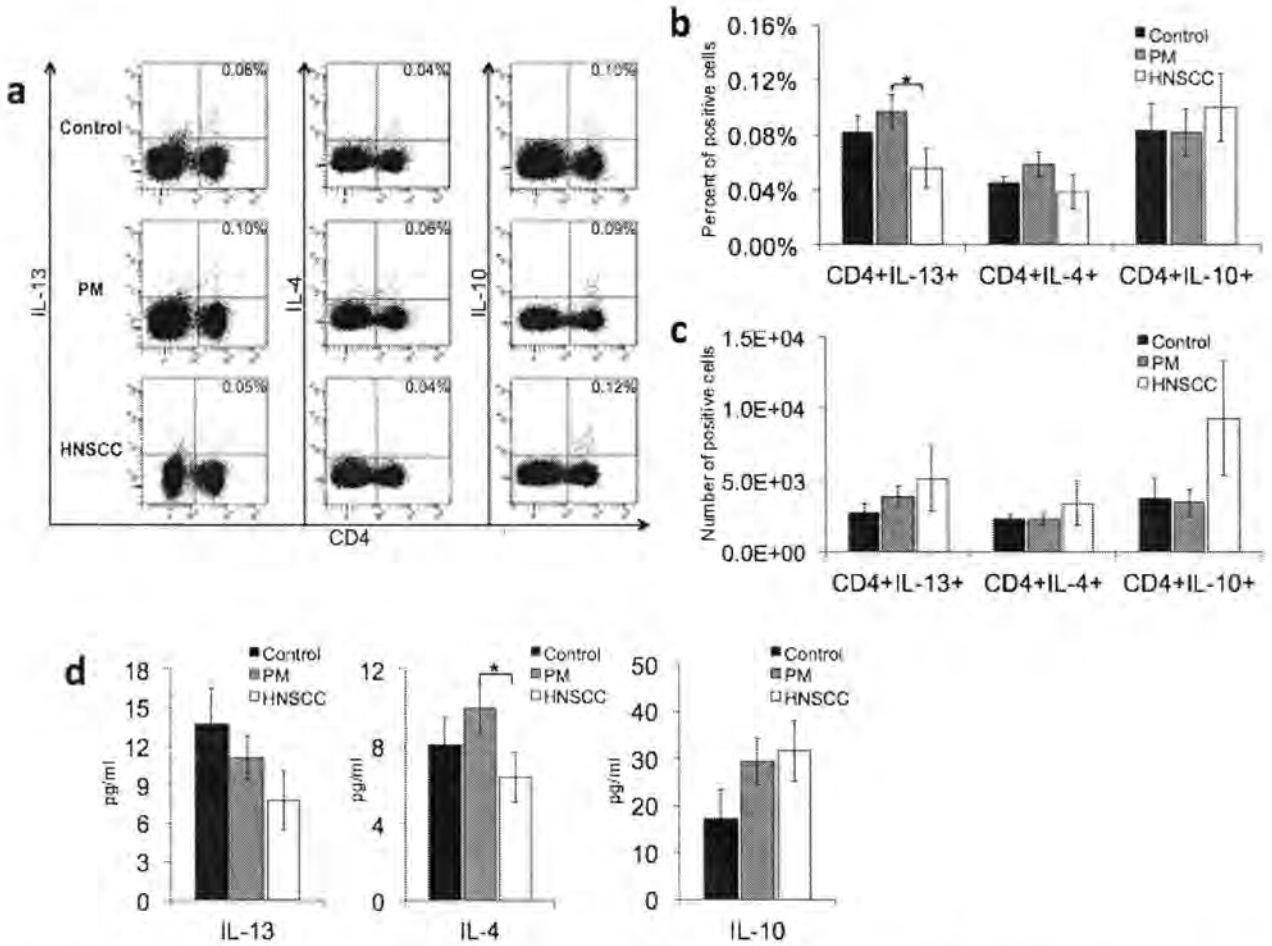

Fig 3.7 No major changes in levels of Th2 cells or cytokines between cervical lymph nodes of control, premalignant lesion-bearing and HNSCC-bearing mice.

Representative results (a) and graphical representation (b) of flow cytometric analysis of control, premalignant lesion-bearing mouse, and HNSCC-bearing mouse cervical lymph node cells after stimulation for four hours with phorbol 12-myristate 13-acetate (PMA) and ionomycin in the presence of brefeldin A, with at least 7 mice per group. *, $p < 0.05$. Total numbers (c) of populations were determined by multiplying percent positive by number of cervical lymph node cells for each individual mouse. Cytometric bead array analysis (d) of control, premalignant lesion-bearing mouse, and HNSCC-bearing mouse cervical lymph node cells after stimulation for four hours with PMA and ionomycin, with at least 9 mice per group. *, $p < 0.05$.

Human premalignant oral lesion tissue exhibits a dramatic increase in levels of IFN-γ, IL-2, and IL-17A compared to both normal/adjacent and HNSCC tissue, while human HNSCC tissue exhibits a simultaneous increase in levels of IFN-γ and IL-10 compared to control tissue

To investigate the local levels of Th1, Th17, and Th2 cytokines in humans, normal/adjacent tongue tissue, premalignant tissue, and HNSCC tumor tissue was obtained from patients, mechanically lysed, and analyzed by cytokine bead array for the presence of the Th1 cytokines IFN-γ and IL-2, the Th17 cytokine IL-17A, and the Th2 cytokines IL-4 and IL-10 (Fig 3.8a). Levels of cytokine were normalized to pg/100μg of protein, as determined by BCA protein assay. To investigate the systemic levels of Th1, Th17, and Th2 cytokines in humans, peripheral blood was obtained from normal patients and patients with either premalignant lesions or HNSCC, and plasma was also analyzed by cytokine bead array for presence of IFN-γ, IL-2, IL-17A, IL-4, and IL-10 (Fig 3.8b). While no difference was detected between plasma levels of IFN-γ and IL-2 for all three groups, premalignant lesion tissue was found to contain a great deal more of both of these Th1 cytokines compared to both normal/adjacent and HNSCC tissue (Fig 3.8a). Unexpectedly, HNSCC tissue was found to have a significant increase in IFN-γ compared to normal/adjacent tissue (Fig 3.8a). Analysis of IL-17A revealed a significant decrease in this cytokine in the plasma of HNSCC patients compared to controls and a sizeable significant increase in this cytokine in premalignant tissue compared to both normal/adjacent and HNSCC tissue (Fig 3.8a and b). Finally, while IL-4 levels were found to be undetectable in both plasma and tissue, analysis of the Th2 cytokine IL-10 showed a

trend towards an increase in the level of this cytokine in the plasma of HNSCC patients compared to controls and a significant increase in the level of this cytokine in the HNSCC tissue compared to normal/adjacent tissue (Fig 3.8a and b). This data indicates that, as observed in mice, human premalignant tissue is associated with a large increase in inflammatory Th1 and Th17 cytokines. While human HNSCC tissue is associated with the expected increase in the immunosuppressive cytokine IL-10, it is also unexpectedly associated with an increase in the Th1 cytokine IFN-γ compared to normal/adjacent controls.

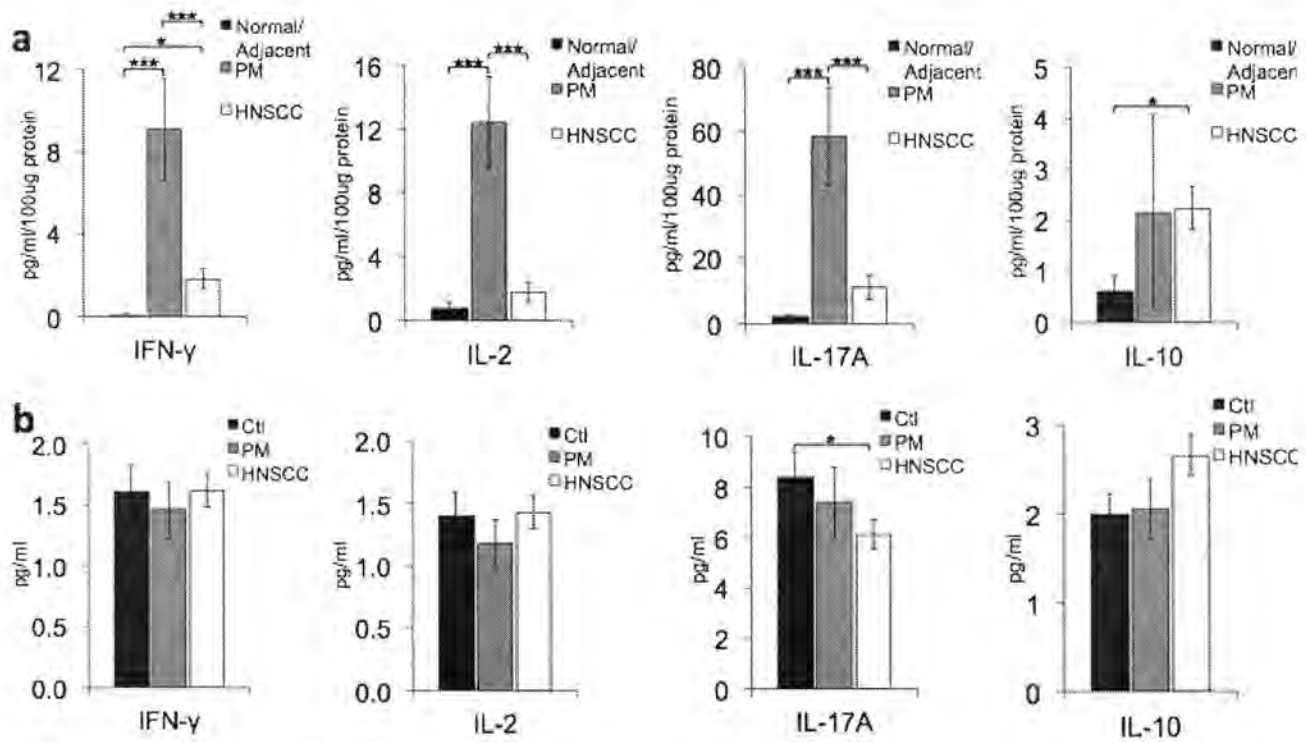

Fig 3.8 Levels of IFN-γ, IL-2, and IL-17A are increased in human premalignant tissue compared to normal/adjacent human tissue and human HNSCC tissue, while levels of IFN-γ and IL-10 in HNSCC tissue are increased compared to normal/adjacent controls.
Cytometric bead array analysis of human normal/adjacent, premalignant, and HNSCC tissue lysate, with normalization to 100 μg of protein (as determined by BCA protein assay), with at least 6 samples per group (a), and cytometric bead array analysis of plasma from normal, premalignant lesion-bearing, and HNSCC-bearing patients, with at least 23 samples per group (b). *, $p < 0.05$. ***, $p < 0.001$.

3.4 Discussion

Depending on a multitude of factors, the immune system is capable of either mounting a beneficial response against dysplastic cells or promoting tumor development and progression. As each individual component of an immune response is not always consistently beneficial or detrimental, it is essential to investigate the changes in immune phenotype during different mechanisms of tumor initiation and development for different types of malignancies. This study examined the alterations in various immune populations during carcinogen-induced development of premalignant oral lesions and HNSCC. The development of oral premalignant lesions is shown to be associated with an increase in percentage of conventional lymphocytes expressing activation and memory markers in cervical lymph nodes of 4NQO-treated mice compared to both control and HNSCC-bearing mice, changes that are not accompanied by a significant increase in regulatory T cells. Specifically, no significant difference was detected between percentages of Tconv cells positive for the early activation marker CD69, a glycoprotein expressed within an hour after stimulation which rapidly declines after 4-6 hours, while levels of Tconv cells positive for CD25, a marker induced within 2-24 hours of stimulation which persists for a few days after stimulus withdrawal, and Tconv cells with upregulated CD44, an adhesion molecule upregulated on T cells after activation with antigen and maintained until memory cell death, were both increased in lymph nodes from premalignant lesion-bearing mice compared to control mice and HNSCC-bearing mice (201, 206, 207). This data indicates that, at the time when lesions become detectable, an immune response has been mounted in the dysplastic

tissue and has been present for some time. In addition, lymph node cells from premalignant lesion-bearing mice have a minor, yet significant, increase in Tconv cells bearing the exhaustion marker KLRG1, a receptor upregulated by T cells upon repetitive antigenic stimulation which induces T cell proliferative dysfunction, and a trend towards an increase in Tconv cells bearing the exhaustion marker PD-1, an inhibitory receptor upregulated during chronic T cell stimulation which induces T cell unresponsiveness, compared to both control and HNSCC-bearing mice (202, 208-211). Also, while the premalignant stage is not associated with a significant decrease in conventional T cells or a significant increase in regulatory T cells, the ratio of $CD4^+$ conventional to regulatory T cells is decreased compared to control to an extent that matches the $CD4^+$ conventional to regulatory T cell ratio of HNSCC-bearing mice. In addition, the proliferative capacity of $CD4^+$ conventional T cells and the suppressive capacity of the $CD4^+$ regulatory T cells appears to progressively decrease throughout the development of HNSCC. This data indicates that, by the time lesions become detectable, the immune reaction might already be starting the transition toward the immune environment seen in the setting of HNSCC.

The classical view of immunoediting typically identifies a beneficial antitumor response as involving an increase in IFN-γ secreting Th1 and Tc1 cells with a concurrent decrease in Th2 cells (203). A shift in the opposite direction is often seen in peripheral blood and tumor-infiltrating cells of cancer patients. The current study reveals that both mice with oral premalignant lesions and, unexpectedly, mice with HNSCC exhibit an increased Th1 and Tc1 response without any concomitant change in Th2 response compared to control. Likewise, human

premalignant tissue and HNSCC tissue was found to have an increase in IFN-γ levels compared to normal/adjacent controls. This data contradicts earlier human studies showing a local and systemic skewing from a Th1 response to a Th2 response in patients with HNSCC (28, 176). This brings up two major questions: #1) is the Th1 and Tc1 response that is so elevated in the setting of HNSCC actually promoting tumor growth, and #2) is the Th1 and Tc1 response at the premalignant stage performing the same role as the Th1 and Tc1 response observed at the HNSCC stage? Interferon-γ expression has previously been shown to be associated with tumor promotion (120, 121, 212, 213), notably in carcinogen-induced tumors of epithelial origin (212) and involving upregulated Th17 expression (121). In addition, the timing of the Th1 and Tc1 response may discern between different purposes of the response. It is interesting to note that the IFN-γ related response at the premalignant stage is characterized by an increase in Tc1 cells to a greater extent than the increase in Th1 cells, while the IFN-γ response at the HNSCC stage shifts to a Th1-dominated reaction. Perhaps the source of the IFN-γ is significant in defining its role in the anti- or pro-tumor immune response. Further investigation into the specific roles of Th1 cells, Tc1 cells, and IFN-γ during tumor development and progression will help elucidate its role in the tumor microenvironment in 4NQO-induced HNSCC carcinogenesis.

The role of the Th17 population remains somewhat ambiguous; some studies have indicated that these cells can work with Th1 cells to help eliminate malignancies while other studies have identified Th17 cells as tumor-promoting, often citing their pro-angiogenic effects (204, 205). This study revealed that the

Th17 response increases in mice and humans bearing premalignant lesions compared to controls, and then falls in mice and humans bearing HNSCC. This could indicate that the Th17 response seen during early development of HNSCC represents an anti-tumor response. As Tregs and Th17 cells have been shown to have an inversely proportional relationship (214), the decrease in Th17 cells seen in HNSCC-bearing mice may be related to the simultaneous increase in the Treg population. It will be important to determine the relationship between these two populations during the progression to HNSCC with future experimentation. In addition, mice bearing premalignant lesions were found to have an increase in a $CD4^-$, IL-17A-expressing population of cells that does not appear to be Tc17 cells (Fig 3.6a). Determining the identity of this cell population through costaining for IL-17A and other cells known to occasionally express IL-17A, such as γδ T cells or mast cells (215, 216), will be important for future investigation into the functional implications of IL-17A-producing populations during HNSCC carcingogenesis.

We have evaluated the multitude of immune reactions in 4NQO-treated mice during the development of HNSCC, and we have begun to evaluate these same reactions in humans during the development of HNSCC. At this point, it is essential to investigate the functional impact of the individual cell populations that are upregulated at the premalignant stage or after HNSCC establishment. This can be accomplished through analysis of tumor progression in 4NQO-treated mice with depleted CD4, CD8, or CD4 and CD8 cells. Investigation into the role of IFN-γ in the progression of HNSCC can be carried out in mice with depleted or neutralized IFN-γ. Future studies will help to elucidate the links between these cell subsets and tumor

development. Investigation into whether the responses described in this study are beneficial in any way, and if they can be further manipulated to combat tumor progression, is highly important to developing new treatment strategies for HNSCC.

Chapter 4

Dendritic Cell Vaccination of Premalignant Lesion-Bearing Mice

CHAPTER 4: DENDRITIC CELL VACCINATION OF PREMALIGNANT LESION-BEARING MICE

Aim 2: ***To use premalignant lesion-pulsed dendritic cells in vivo to skew the immune phenotype within mice bearing premalignant lesions towards immunity against further development of premalignant oral lesions and progression to HNSCC.***

4.1 Introduction

Head and neck squamous cell carcinoma (HNSCC) is an aggressive malignancy that has had a 5-year survival rate of around 50% for over 30 years (173, 217). Developing from oral keratinizing epithelial cells in a progressive fashion, both premalignant oral lesions and HNSCC have a high incidence of recurrence. This malignancy has also been consistently associated with extensive immune manipulation, including upregulation of both immunosuppressive regulatory T cells and inflammatory cell types (163, 164, 176, 177, 218, 219). The high incidence of recurrence and systemic immune manipulation are large contributors to the low survival rate of HNSCC (173, 186, 217).

Dendritic cell vaccination involves the exposure of dendritic cells to tumor-associated antigens (TAAs) through one of a variety of mechanisms. Primed dendritic cells are then matured and injected into the recipient with the intention of stimulating the recipient's own T cells to specifically target tumor tissue (220). In the case of HNSCC, however, the patient's immune system at the point of initiation of immunotherapy may have been compromised to the point that it is not capable of

mounting a sufficient immune response to vaccination. While established HNSCC has been shown to be associated with extensive immune manipulation, during the premalignant stages of progression, levels of activated T cells with a memory phenotype, including inflammatory Th1, Tc1 and Th17 cells increase (218). As such, this time may be optimal for initiation of immunotherapeutic techniques, since the immune system is already stimulated.

The utilization of this technique during the premalignant stage of HNSCC depends on the presence of common antigens that are upregulated at both the premalignant and malignant stages compared to normal tissue. Our lab identified several common TAAs, including EGFR, RAGE, and MUC1, in mice with 4NQO carcinogen-induced premalignant oral lesions and HNSCC, as well as in patients (136, 180). Previous work also revealed that *in vitro* sensitization of human PBML with autologous premalignant lesion lysate resulted in increased IFN-γ release from sensitized PBML upon subsequent challenge with autologous premalignant lesion or HNSCC lysate and increased cytolytic activity of sensitized PBML against challenge with premalignant lesion or HNSCC cells (136). Additional *in vitro* work showed that sensitization of mouse splenocytes with premalignant lesion lysate-pulsed dendritic cells resulted in increased IFN-γ release from sensitized splenocytes upon subsequent challenge with premalignant lesion or HNSCC lysate (180). This provides the rationale for the use of premalignant tissue as the source of antigen to stimulate a protective immune response against the further development of premalignant lesions and HNSCC.

In the current study, mice treated with 4NQO until the development of

premalignant oral lesions were vaccinated with dendritic cells pulsed with either

premalignant lesion lysate or normal tongue epithelium lysate. Lysate was used as

an antigenic source to provide an increased amount of potentially immunogenic

antigens that the dendritic cells could present, rather than limiting presentation to

one specific antigen. As multiple rounds of splenocyte sensitizations was required

for activation in previous *in vitro* studies (180), three separate vaccinations were

administered: the first was given at the onset of premalignant lesions, the second was given 1 week after the first, and the final booster was given 7 weeks after the first (Fig 4.1). To determine immune response in vaccinated mice over time, mice were sacrificed at two timepoints: an early timepoint at 4 weeks after the first vaccination and a late timepoint at 8 weeks after the first vaccination (1 week after the final booster) (Fig 4.1). Lesion progression as well as immune response to vaccination was monitored. Somewhat surprisingly, both mice treated with the premalignant lesion-pulsed dendritic cell vaccine (DCpm) and, to a lesser extent,

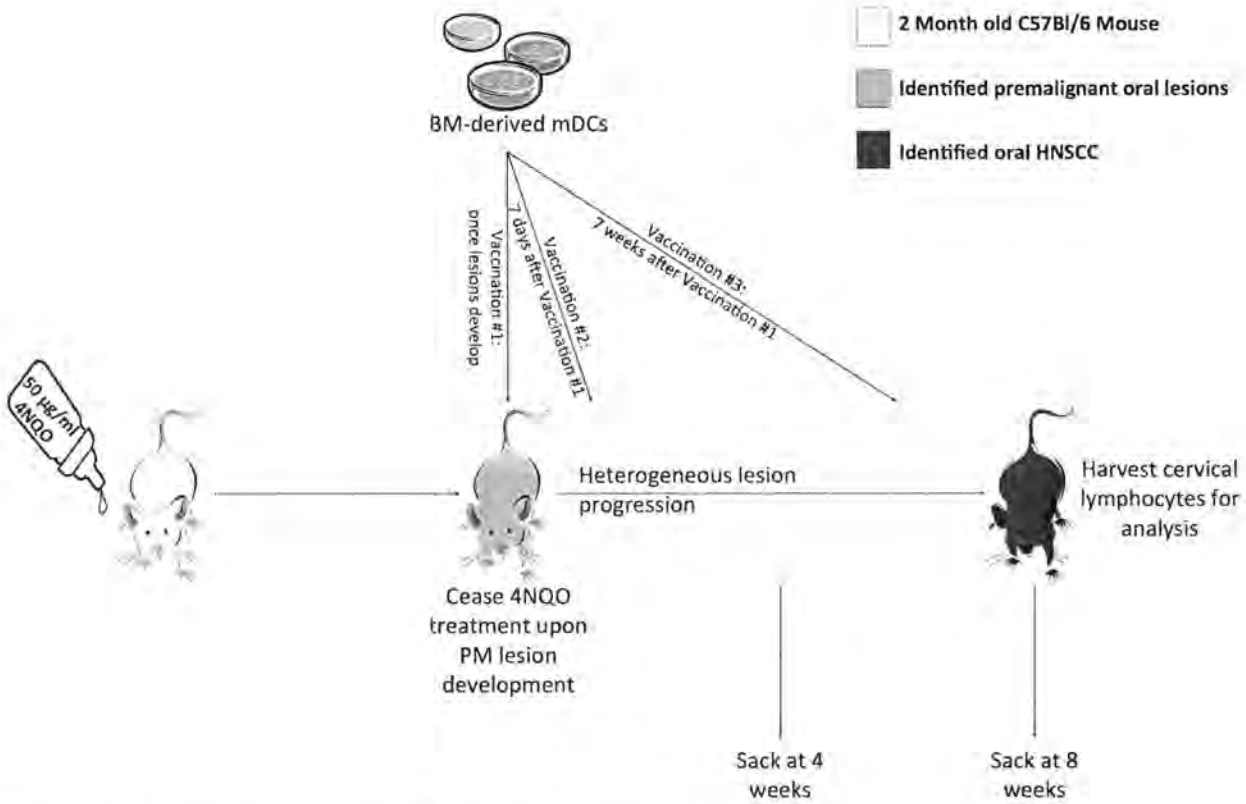

Fig 4.1 Representation of vaccination schedule.

mice treated with the normal tongue epithelium lysate-pulsed dendritic cells (DCnt) had an improved clinical course compared to 4NQO-treated controls. DCnt mice had an early increase in cervical lymph node cellularity and levels of multiple immune effectors, while DCpm treated mice had a delayed increase in these same effectors.

4.2 Materials and Methods

Oral HNSCC carcinogenesis

Five mg/ml 4NQO was administered in the drinking water (diluted to 50 μg/ml) of 2 month old (at start) female C57BL/6 mice (Charles Rivers Laboratory, Wilmington, MA)) until development of premalignant oral lesions (6-8 weeks) or HNSCC (12-16 weeks). Control mice received propylene glycol diluent control. To monitor development of premalignant oral lesions and HNSCC, oral cavities of 4NQO-treated mice were endoscoped weekly using a Stryker 1.9mm x 30° scope and a Stryker 1088 HD camera. Mice were sedated with inhaled isoflurane (Piramal Healthcare, Mumbai, India) during the procedure.

Dendritic cell generation, pulsing, and maturation

Cells were collected from C57Bl/6 mouse femoral bone marrow and cultured for 5-6 days with 1000 U/ml GM-CSF (R&D Systems, Minneapolis, MN) to stimulate the development of dendritic cells. Dendritic cells were pulsed by 12 hours of incubation with 25 μg/ml lysate of premalignant lesion-bearing tissue from 4NQO-treated mice or normal tongue epithelial tissue from control mice. Tongue epithelium was obtained after incubation of tongue fragments with Dispase II (Roche, Florence, SC) and lysed through sonication, after which protein concentration was determined by BCA protein assay (Pierce, Rockford, IL) per manufacturer's instructions. Dendritic cells were then matured by 48 hrs of culture with 10 ng/ml GM-CSF and 0.1 μg/ml LPS (R&D Systems, Minneapolis, MN).

Vaccine regimen

Vaccination was by injection of $1x10^6$ bone marrow-derived premalignant lesion lysate- or normal tongue epithelium lysate-pulsed dendritic cells in 50 µl serum-free medium into the ventral tongue of 4NQO-treated mice under inhaled isoflurane sedation (Piramal Healthcare, Mumbai, India). 4NQO-treated controls were injected with 50 µl of saline into the ventral tongue of 4NQO-treated mice. The first vaccination was given to mice once all 4NQO-treated mice were determined through oral cavity endoscopy to exhibit premalignant oral lesions. A second vaccination was administered 7 days after the first. Some mouse groups were sacrificed four weeks following the first vaccination. A third vaccination was administered 7 weeks after the first vaccination for the remaining mice, and these mice were sacrificed 7 days later. Tongues were harvested, sectioned, and stained with hematoxylin and eosin for histologic analysis.

Cervical lymph node processing

Cervical lymph nodes were harvested from mice and homogenized via a Stomacher 80 homogenizer (Seward, West Sussex, UK) set on high for 90 sec. Cells were passed through a 40-µm cell strainer (BD Falcon, San Jose, CA) and rinsed with Hank's Buffered Saline Solution (HBSS, Invitrogen, Carlsbad, CA) to remove debris. Cell number was determined by counting cells excluding trypan blue using a hemocytometer.

Flow cytometric analysis of surface markers and Foxp3 expression

All reagents in this section were from BD Bioscience (San Jose, CA) unless otherwise stated. In order to detect levels of CD8 and CD4 positive cells in relation to Foxp3, *ex vivo* cervical lymph node cells in single-cell suspension were washed once in Stain Buffer and resuspended at $1x10^7$ cells/ml. In order to detect expression of MHCII (I-A^b for C57/bl6 mice), CD80, and CD86 on CD11c positive dendritic cells, cultured matured dendritic cells in single-cell suspension were washed once in Stain Buffer and resuspended at $1x10^7$ cells/ml. Nonspecific staining of total $1x10^6$ cells was blocked with FBS and anti-CD16/32 monoclonal antibody prior to cell surface staining with the following antibodies: PerCP-Cy5.5 CD4, FITC CD8a, FITC CD11c, PE CD25, PE I-A^b, APC CD69, APC CD80, and PE-Cy7 CD86. Intracellular staining for PE-Cy7 Foxp3 was performed after fixation with Foxp3 Fixation Buffer and permeabilization with Foxp3 Permeabilization Buffer. Extent and frequency of positively stained cells was visualized using flow cytometry (FACSCanto, San Jose, CA).

Flow cytometric analysis of cytokine expression

All reagents in this section were from BD Bioscience (San Jose, CA) unless otherwise stated. In order to detect intracellular cytokines, single-cell suspensions of cervical lymph node cells were restimulated for 4 hours at 37°C with 50 ng/ml phorbol 12-myristate 12-acetate (PMA), 1 μg/ml ionomycin, and brefelden A

solution. Nonspecific staining of total of $1x10^6$ cells was blocked with FBS and anti-CD16/32 monoclonal antibody prior to cell surface staining with the following antibodies: PerCP-Cy5.5 CD4 and PE-Cy7 CD8a. Intracellular staining for PE IL-17A, FITC IFN-γ, APC IL-4, APC IL-10 and Alexa Fluor 488 IL-13 (eBioscience, San Diego, CA) was performed after fixation/permeabilization with Cytofix/Cytoperm. Extent and frequency of positively stained cells was visualized using flow cytometry (FACSCanto, San Jose, CA).

Cytokine bead array

All reagents in this section were from BD Bioscience (San Jose, CA) unless otherwise stated. In order to detect cytokines released into supernatant, single-cell suspensions of cervical lymph node cells or dendritic cells were restimulated for 4 hours at 37°C with 50 ng/ml PMA and 1 μg/ml ionomycin without addition of brefeldin A or cervical lymph node cells were activated for 3 days in the presence of anti-CD3/anti-CD28 beads. To determine degree of cytokine release in response to challenge, some lymph node cells were incubated for three days with 25μg/ml of normal tongue epithelium lysate, premalignant epithelium lysate or HNSCC epithelium lysate in the presence of anti-CD3/anti-CD28 beads. Presence/level of IFN-γ, IL-17A, IL-4, IL-10, and IL-13 in lymph node cell supernatant and IL-12, IL-6, TNF, IL-10, IL-1α and IL-1β in dendritic cell supernatant was determined using a mouse cytometric bead array Th1/Th2/Th17 cytokine kit and cytometric bead array flex sets for individual cytokines according to the manufacturer's instructions. Relative amount of each cytokine was analyzed using FCAP Array software.

Statistical analysis

Data were reported using the mean as a measure of central tendency ± standard error of the mean. To compare one variable condition between groups, the 2-tailed Student's *t*-test was used. To evaluate significant differences between lesion numbers for each mouse group, the Mann-Whitney *U* Test was used. To evaluate if any significant differences exist between histologic scores, a chi squared test was used. Significance was reported in the 95% confidence interval.

4.3 Results

DCpm vaccination and, to a lesser extent, DCnt vaccination result in an improved clinical response compared to 4NQO-treated controls.

The ultimate goal of any interventional immunotherapeutic approach is an improvement in clinical status. To monitor development of lesions in 4NQO-treated mice, oral cavities were examined through weekly endoscopy and number of visible lesions was counted. Analysis of lesion number revealed that DCpm vaccination resulted in a significant decrease in the number of lesions compared to 4NQO control at 6, 7, and 8 weeks (Fig 4.2a). DCnt vaccination resulted in a significant decrease in number of lesions compared to 4NQO control at 8 weeks only (Fig 4.2a).

Mice were sacrificed at 4 and 8 weeks post-vaccination and tongues were harvested, processed, and random paraffin-embedded sections were analyzed for histologic score (normal, mild dysplasia, moderate-severe dysplasia, invasive carcinoma). Histologic analysis revealed a trend towards an increase in the overall degree of dysplasia at 4 weeks in mice administered the DCnt vaccine compared to 4NQO-treated controls. Similar to the results from gross analysis, histologic analysis also revealed a trend towards a decrease in the overall degree of dysplasia at 8 weeks in mice administered either the DCnt or DCpm vaccine compared to 4NQO-treated controls (Fig 4.2b). This indicates that DCpm vaccination may decrease the clinical lesion burden in mice treated with 4NQO, and, surprisingly, DCnt vaccination also appears to decrease clinical lesion burden, though at a later timepoint.

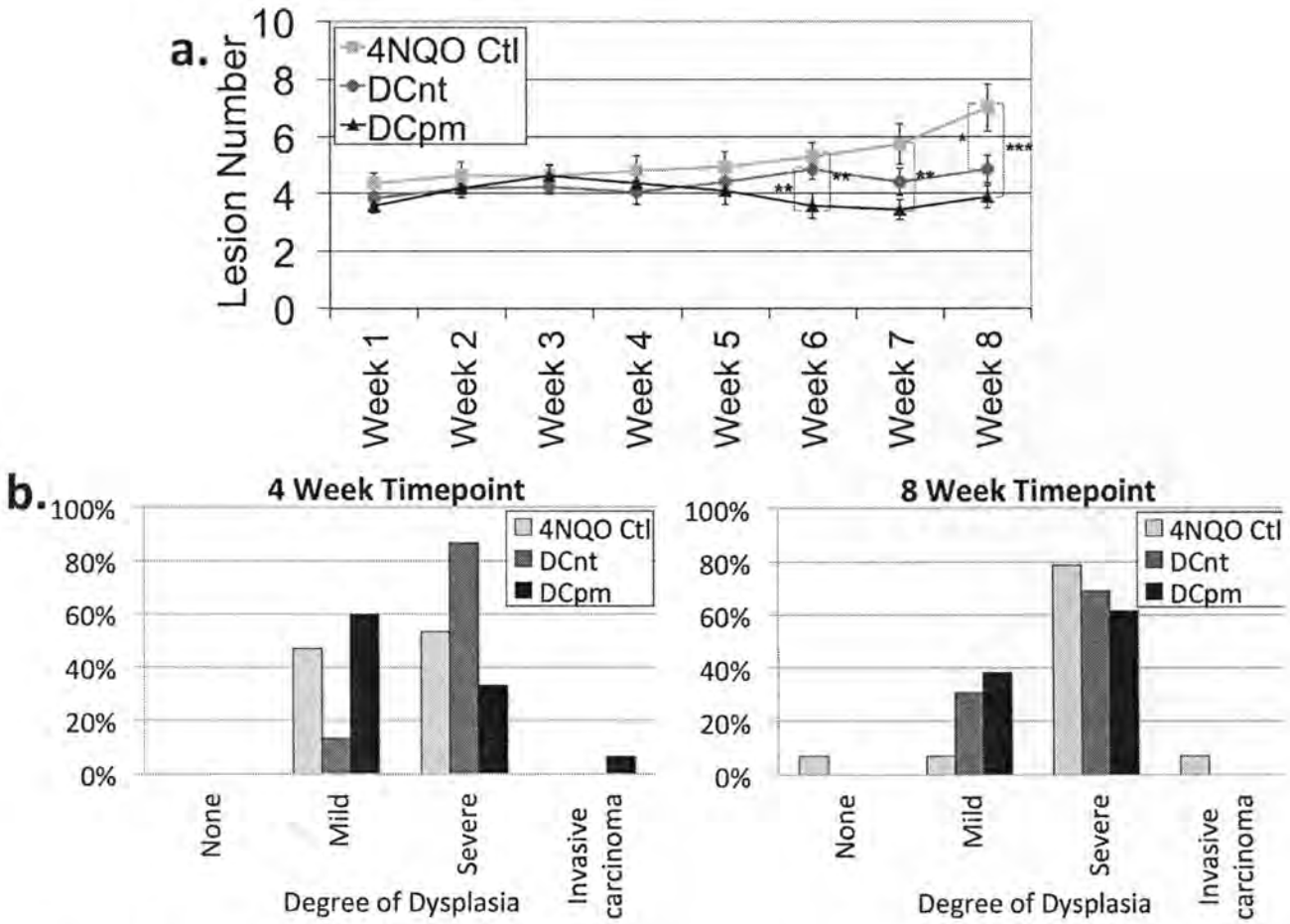

Fig 4.2 DCpm vaccination and DCnt vaccination result in an improved clinical response compared to 4NQO-treated controls.
Number of visible lesions (a) as determined by weekly endoscopy of oral cavities of 4NQO-treated mice administered dendritic cells pulsed with normal tongue epithelium lysate (DCnt), dendritic cells pulsed with premalignant lesion lysate (DCpm), or saline (4NQO Ctl) with at least 15 mice per groups. Data represent mean ± SEM. *, $p < 0.05$. **, $p < 0.01$. ***, $p < 0.001$ (Mann-Whitney *U* test) . At 4 weeks and 8 weeks post-first vaccination, 4NQO Ctl, DCnt and DCpm mice were sacrificed and tongues were harvested, sectioned, and random sections were stained with hematoxylin and eosin for histopathologic analysis (b) with tongues from at least 11 mice per group. Data represent percentage, and statistical significance was determined by chi squared test.

DCnt vaccination results in an early increase in lymph node cell count, while DCpm vaccination results in a delayed increase in lymph node cell count.

The development of malignancies is often associated with hyperplasia of draining lymph nodes, whether due to reactive hyperplasia, metastasis, or both

(195). To determine the degree of lymph node hyperplasia among the various mouse groups, cervical lymph nodes from control mice, 4NQO-treated control mice, 4NQO-treated DCnt mice, and 4NQO-treated DCpm mice were harvested at 4 and 8 weeks post-first vaccination, and the number of trypan blue-excluding cells were counted. At 4 weeks post-first vaccination, DCnt vaccinated mouse lymph nodes contained significantly more cells than all other groups (Fig 4.3a). At 8 weeks post-first vaccination, however, DCpm and DCnt vaccinated mouse lymph nodes contained greater numbers of cells compared to control and 4NQO control mouse lymph nodes (Fig 4.3b). Also at 8 weeks, 4NQO control mouse cervical lymph nodes contained significantly more cells than control mice (Fig 4.3b). This indicates that there is a more rapid response in lymph node cell proliferation after DCnt vaccination compared to DCpm vaccination. However, the proliferative response to the DCpm vaccine occurs to a great extent by the later timepoint.

DCnt vaccination results in an early increase in levels of Tconv cells and CD4 Tregs, while both DCnt and DCpm vaccination result in a decreased percentage of CD4 Tconv cells and increased absolute number of CD8 Tconv cells and Tregs at 8 weeks compared to 4NQO-treated controls.

Many solid carcinomas have been shown to be associated with local and systemic immunosuppression (163, 164, 176). This includes downregulation of stimulatory immune cells, such as $CD4^+$ and $CD8^+$ conventional T cells, and upregulation of suppressive immune cells. One suppressive cell population that is often upregulated as a part of this phenomenon is the regulatory T cell population,

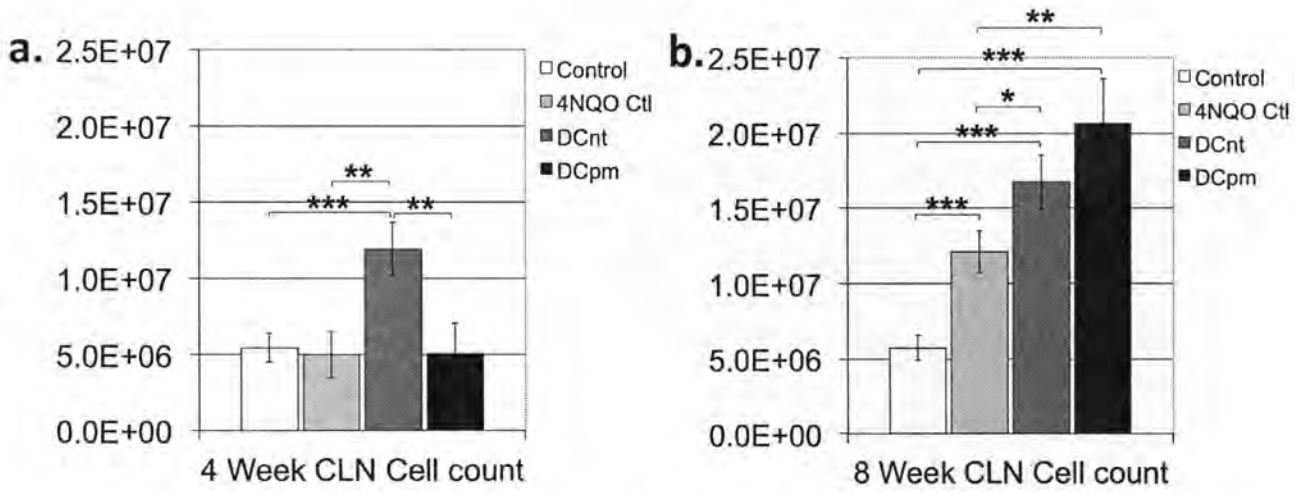

Fig 4.3. DCnt vaccination results in an early increase in lymph node cellularity, while DCpm vaccination results in a delayed increase in lymph node cellularity.
At 4 weeks and 8 weeks post-first vaccination, control mice, 4NQO-treated control mice (4NQO Ctl), DCnt-vaccinated mice (DCnt) and DCpm-vaccinated mice (DCpm) were sacrificed and cervical lymph nodes were harvested and processed to single-cell suspensions, and trypan-blue excluding cells were counted with at least 5 mice per group. Data represent mean $\pm$ SEM of cell number at 4 weeks post-first vaccination (a) and 8 weeks post-first vaccination (b). *, $p < 0.05$. **, $p < 0.01$. ***, $p < 0.001$ (2-tailed Student's *t*-test).

identified by expression of the transcription factor Foxp3 (163, 164, 176). Our lab had previously shown that locoregional regulatory and conventional T cell numbers are both increased in mice with HNSCC (218). To determine the relative composition of conventional and regulatory T cells in 4NQO-treated mice administered DC vaccination, lymph node cells collected at 4 and 8 weeks post-first vaccination were stained for surface expression of CD4 and CD8 and intracellular expression of Foxp3. Proportions of cells expressing each marker were determined through flow cytometric analysis, and numbers of cells expressing each markers were determined by multiplying the percent positive by the number of lymph node cells for each individual mouse. At 4 weeks post-first vaccination, all 4NQO-treated mouse groups had a decreased percentage of $Foxp3^{-}$ $CD4^{+}$ Tconv cells and an

increased percentage of $CD4^+$ Treg cells compared to controls (Fig 4.4a and b). At this timepoint, DCnt mice exhibited an increase in percentage and absolute number of Tconv cells and an increase in absolute number of CD4 Tregs compared to 4NQO-treated controls (Fig 4.4c). At 8 weeks post-first vaccination, both DCnt and DCpm mice exhibited a decrease in percentage of $CD4^+$ Tconv cells compared to 4NQO controls (Fig 4.4a and b). Analysis of cell numbers revealed an increase in the absolute number of CD8 Tconv cells and Treg cells in both DCnt and DCpm mouse lymph nodes compared to 4NQO controls (Fig 4.4c). This data indicates that DCnt vaccination may work early on to stimulate an increase in Tconv and Treg cells. While DCpm mice show no differences from 4NQO controls early on, DCpm vaccination appeared to affect a delayed increase in Tconv and Treg cells at 8 weeks after the initial vaccination.

DCnt vaccinated mice exhibit a decrease in percentage but increase in absolute number of activated CD4 Tconv cells 4 weeks, while both DCnt and DCpm vaccinated mice exhibit an increase in absolute number of activated CD8 Tconv cells at 8 weeks compared to 4NQO-treated controls.

An optimal immune response to antigenic stimulation involves the activation of conventional T cells (198). To determine the proportions of conventional T cells bearing markers for activation, $CD4^+Foxp3^-$ and $CD8^+Foxp3^-$ T lymph node cells were analyzed for expression of CD25 and CD44. While often used as a marker for regulatory T cells, CD25 expression on $foxp3^-$ conventional T cells is expressed within 2-24 hours after stimulation and persists for a few days after withdrawal of

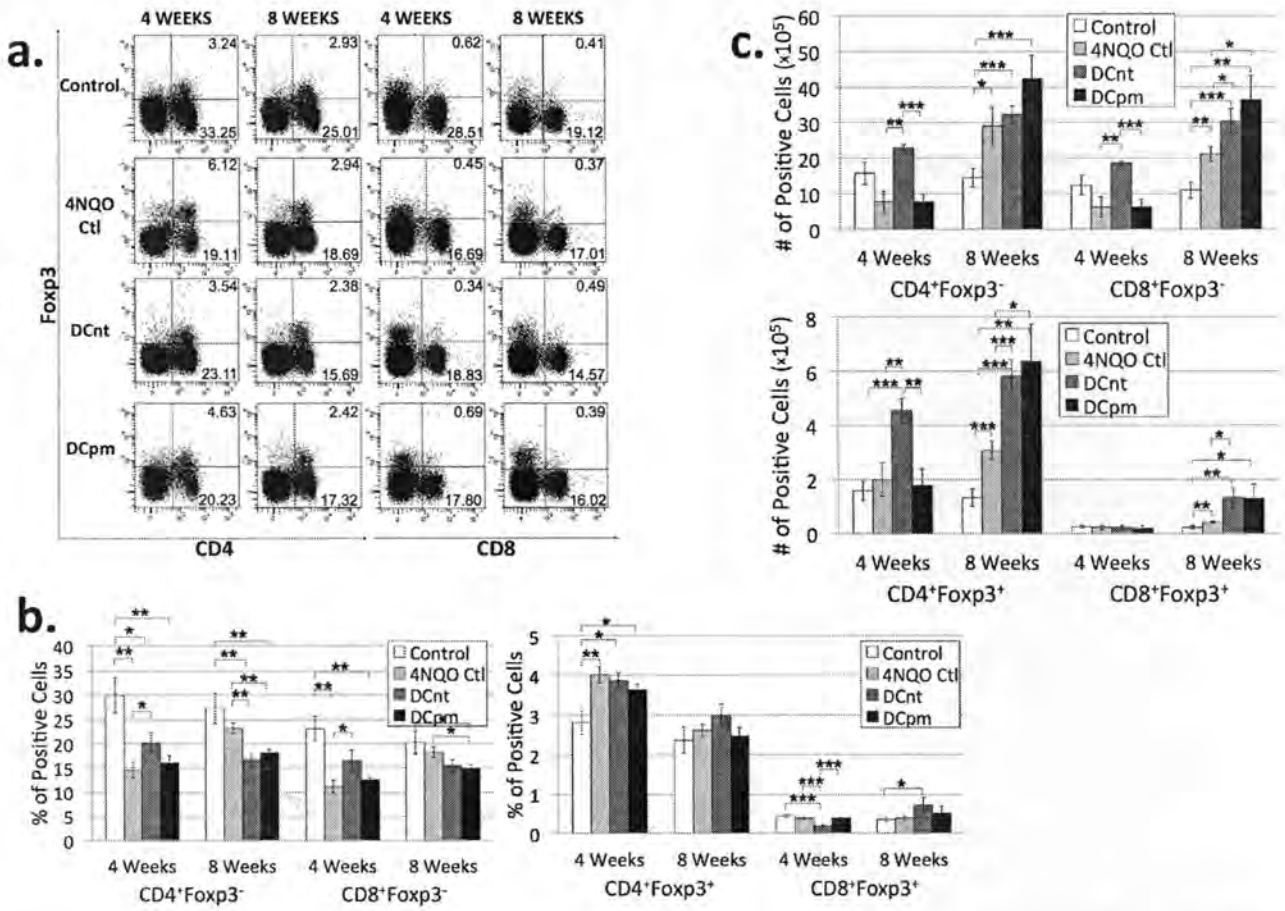

Fig 4.4 DCnt vaccination results in an increase in levels of Tconv cells and CD4 Tregs at 4 weeks, while both DCnt and DCpm vaccination result in a decreased percentage of CD4 Tconv cells and increased absolute number of CD8 Tconv cells and Tregs at 8 weeks compared to 4NQO-treated controls.
Representative results (a) and graphical representation (b) of flow cytometric staining of cervical lymph node cells from control, 4NQO-treated control (4NQO Ctl), DCnt-vaccinated (DCnt) and DCpm-vaccinated (DCpm) mice at 4 and 8 weeks post-first vaccination with at least 5 mice per group. Data represent mean ± SEM. *, $p < 0.05$. **, $p < 0.01$ (2-tailed Student's *t*-test). Total numbers of populations (c) were determined by multiplying the percent positive by the number of cervical lymph node cells for each individual mouse. Data represent mean ± SEM. *, $p < 0.05$. **, $p < 0.01$. ***, $p < 0.001$ (2-tailed Student's *t*-test).

the stimulus. CD44 is a glycoprotein involved in cell adhesion and migration, and its increased expression is indicative of T cell antigen experience (200, 201). Percentages of cells bearing CD25 and CD44 were determined by flow cytometric analysis, and total numbers of cells bearing these markers were determined by

multiplying percent positive by the number of lymph node cells for each individual mouse. At 4 weeks post-first vaccination, percentages of $CD25^+$ and $CD44^{hi}$ CD4 and CD8 Tconv cells are decreased in DCnt mouse lymph nodes compared to 4NQO controls (Fig 4.5a and b). After factoring in cell numbers, DCnt vaccinated mouse lymph nodes were found to have an increased absolute number of $CD25^+$ and $CD44^{hi}$ CD4 Tconv cells compared to 4NQO controls (Fig 4.5c). At this timepoint, the only significant difference between the DCpm and 4NQO control group is a decrease in percentage of $CD44^{hi}$ CD8 Tconv cells in DCpm-vaccinated mice (Fig 4.5a and b). This could possibly be due to antigen-specific cells dying upon TCR restimulation *in vivo*. At 8 weeks post-first vaccination, both vaccinated groups see a significant increase in number of $CD25^+$ and $CD44^{hi}$ CD8 Tconv cells compared to 4NQO controls, and DCpm-vaccinated mice also exhibit an increase in $CD44^{hi}$ CD4 Tconv cells compared to 4NQO controls (Fig 4.5c). This data indicates that both DCnt and DCpm vaccination tend to result in an increase in activated T cells compared to 4NQO treatment alone by 8 weeks post-first vaccination.

DCnt vaccination results in an early increase in Th1 and Tc1 levels, while both DCnt and DCpm vaccination result in an increase in Tc1 levels at 8 weeks compared to 4NQO-treated controls.

T cells can be further subdivided based on their expression of certain cytokines that dictate differing effector functions. Th1 cells (and a smaller subset of $CD8^+$ cells known as Tc1 cells) secrete IFN-γ and are generally considered as inflammatory. They are traditionally thought of as beneficial for anti-tumor

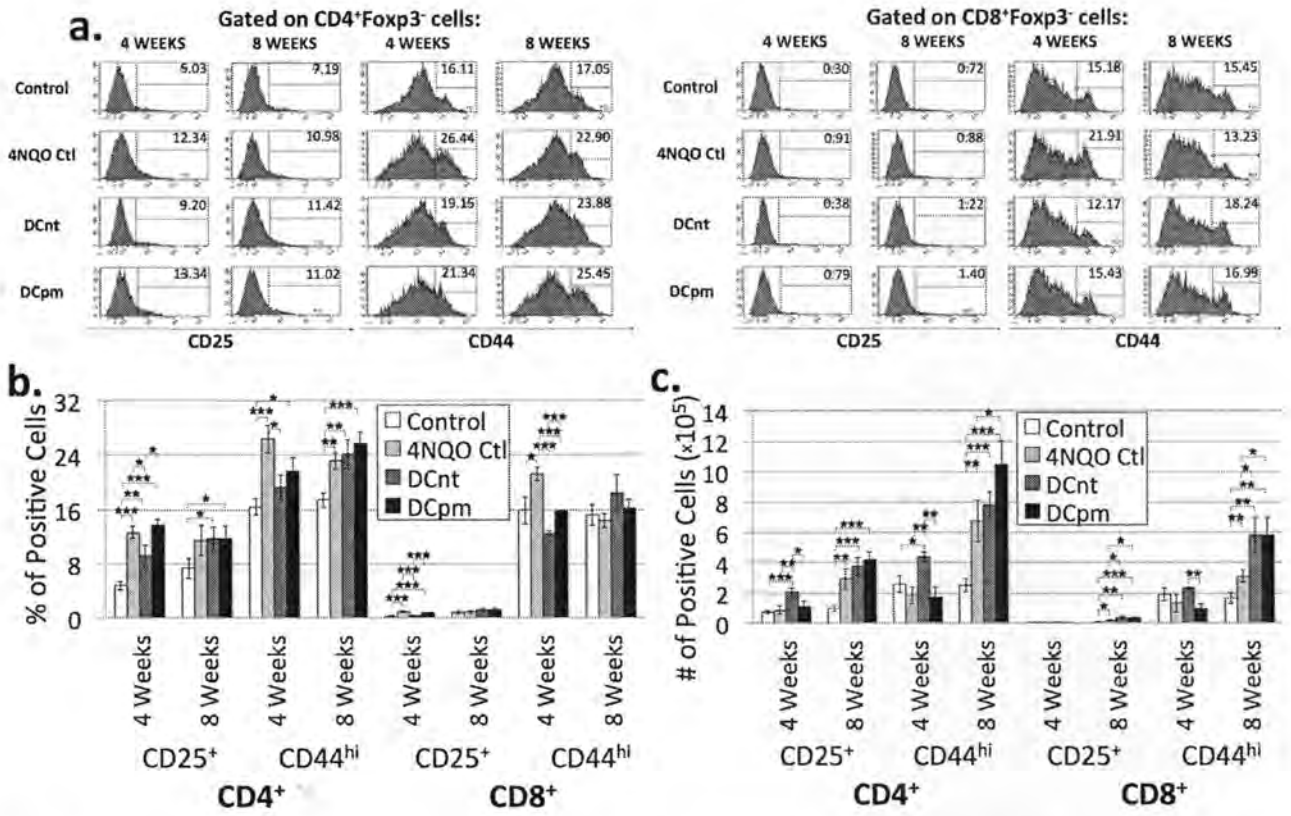

Fig 4.5 DCnt vaccinated mice exhibit a decrease in percentage but increase in absolute number of activated CD4 Tconv cells 4 weeks, while both DCnt and DCpm vaccinated mice exhibit an increase in absolute number of activated CD8 Tconv cells at 8 weeks compared to 4NQO-treated controls.
Representative results (a) and graphical representation (b) of flow cytometric staining of cervical lymph node cells from control, 4NQO-treated control (4NQO Ctl), DCnt-vaccinated (DCnt) and DCpm-vaccinated (DCpm) mice at 4 and 8 weeks post-first vaccination with at least 5 mice per group. Data represent mean ± SEM. *, $p < 0.05$. **, $p < 0.01$. ***, $p < 0.001$ (2-tailed Student's *t*-test). Total numbers of populations (c) were determined by multiplying the percent positive by the number of cervical lymph node cells for each individual mouse. Data represent mean ± SEM. *, $p < 0.05$. **, $p < 0.01$. ***, $p < 0.001$ (2-tailed Student's *t*-test).

immunity (203). However, recent research in our laboratory has shown that Th1 and Tc1 cells are upregulated in both the premalignant and malignant stages of HNSCC (218). To evaluate the populations of Th1 and Tc1 cells in draining lymph nodes of control, 4NQO control, DCnt, and DCpm mice, lymph node cells were stained for surface expression of CD4 and CD8 and intracellular expression of the Th1 and Tc1 cytokine IFN-γ after 4 hours incubation with PMA, Ionomycin and

brefeldin A. In addition, supernatants of lymph node cells incubated for 4 hours in PMA and Ionomycin were analyzed by cytometric bead array for levels of IFN-γ. At 4 weeks post-first vaccination, lymph nodes from DCnt vaccinated mice contain a greater percentage and number of Th1 cells and a greater absolute number of Tc1 cells compared to all other groups (Fig 4.6a, b, c). This is supported by CBA data showing an increase in IFN-γ released into the supernatant of DCnt mouse lymph node cells compared to all other groups (Fig 4.6d).

At 8 weeks post-first vaccination, DCnt mouse lymph nodes have a decreased percentage of Th1 cells and an increased percentage of Tc1 cells compared to 4NQO controls (Fig 4.6a and b). After factoring in cell number, both DCnt and DCpm mouse lymph nodes were found to contain a greater absolute number of Tc1 cells compared to 4NQO controls (Fig 4.6c). Analysis of cytokines secreted in response to 4 hour stimulation with PMA and Ionomycin revealed no significant changes in the levels of IFN-γ in the supernatant of ex vivo lymph node cells from vaccinated mice compared to 4NQO controls (Fig 4.6d). To determine cytokine release in response to more extended stimulation, lymph node cells from 8 week post-first vaccination mice were cultured for 3 days in the presence of anti-CD3/anti-CD28. After the three day stimulation, cytometric bead array was used to measure levels of IFN-γ in the supernatant. Cytokine analysis revealed a huge increase in IFN-γ levels in the supernatant of DCpm and DCnt mouse lymph node cells compared to control and 4NQO control (Fig 4.6e). This data indicates that DCnt vaccination induces an early increase in Th1 and Tc1 cells, but by the later timepoint, both DCnt and DCpm vaccination induce a Tc1-type reaction.

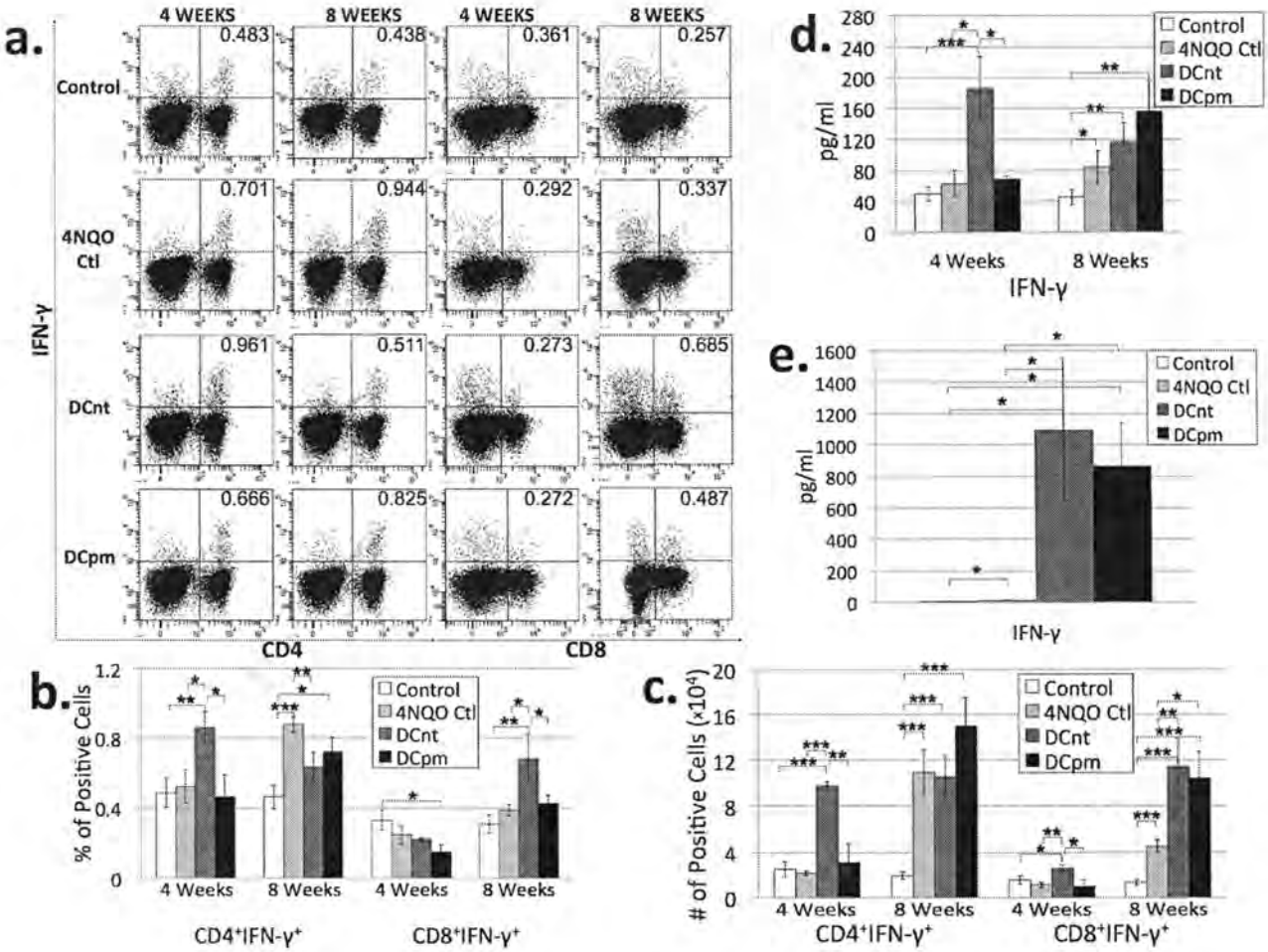

Fig 4.6 DCnt vaccination results in an increase in Th1 and Tc1 levels at 4 weeks, while both DCnt and DCpm vaccination result in an increase in Tc1 levels at 8 weeks compared to 4NQO-treated controls.
Representative results (a) and graphical representation (b) of flow cytometric staining of cervical lymph node cells from control, 4NQO-treated control (4NQO Ctl), DCnt-vaccinated (DCnt) and DCpm-vaccinated (DCpm) mice at 4 and 8 weeks post-first vaccination with at least 5 mice per group. Data represent mean ± SEM. *, $p < 0.05$. **, $p < 0.01$. ***, $p < 0.001$ (2-tailed Student's *t*-test). Total numbers of populations (c) were determined by multiplying the percent positive by the number of cervical lymph node cells for each individual mouse. Data represent mean ± SEM. *, $p < 0.05$. **, $p < 0.01$. ***, $p < 0.001$ (2-tailed Student's *t*-test). Cytometric bead array analysis (d) of supernatant of 4 week and 8 week control, 4NQO Ctl, DCnt, and DCpm mouse cervical lymph node cells after stimulation for four hours with PMA and ionomycin, with at least 5 mice per group. Data represent mean ± SEM. *, $p < 0.05$. **, $p < 0.01$. ***, $p < 0.001$ (2-tailed Student's *t*-test). Cytometric bead array analysis (e) of supernatant of 8 week control, 4NQO Ctl, DCnt, and DCpm mouse cervical lymph node cells after 3 day stimulation with anti-CD3/anti-CD28 beads with at least 4 mice per group. Data represent mean ± SEM. *, $p < 0.05$ (2-tailed Student's *t*-test).

DCnt vaccination results in an early increase in absolute number of Th17 and Tc17 cells, while both DCnt and DCpm vaccination result in an increase in absolute number of Th17 and Tc17 cells at 8 weeks compared to 4NQO-treated controls.

Th17 cells (and a smaller subset of $CD8^+$ cells known as Tc17 cells) secrete IL-17A, among other cytokines, and induce a greater degree of inflammation compared to Th1 cells. Their role in antitumor immunity is less clear. They are highly proinflammatory and proangiogenic, qualities that generally promote tumorigenesis. They are also upregulated in many different tumor types; however, in some tumor types, like lung cancer and ovarian cancer, an increased presence of IL-17A and Th17 cells is actually associated with improved prognosis (123, 125, 204, 205). Recent data from our lab showed that Th17 cells are highly upregulated in mice bearing premalignant oral lesions, only to decrease in the draining lymph nodes of HNSCC-bearing mice (218). To evaluate the populations of Th17 and Tc17 cells in draining lymph nodes of control, 4NQO control, DCnt, and DCpm mice, lymph node cells were stained for surface expression of CD4 and CD8 and intracellular expression of the Th17 and Tc17 cytokine IL-17a after 4 hours incubation with PMA, Ionomycin and brefeldin A. In addition, supernatants of lymph node cells incubated for 4 hours in PMA and Ionomycin were analyzed by cytometric bead array for levels of IL-17A. At 4 weeks post-first vaccination, DCnt mouse lymph nodes have an increased absolute number of Th17 and Tc17 cells compared to 4NQO controls, corresponding to an increased secretion of IL-17A from DCnt lymph node cells compared to 4NQO lymph node cells in response to 4 hour stimulation (Fig 4.7c and d).

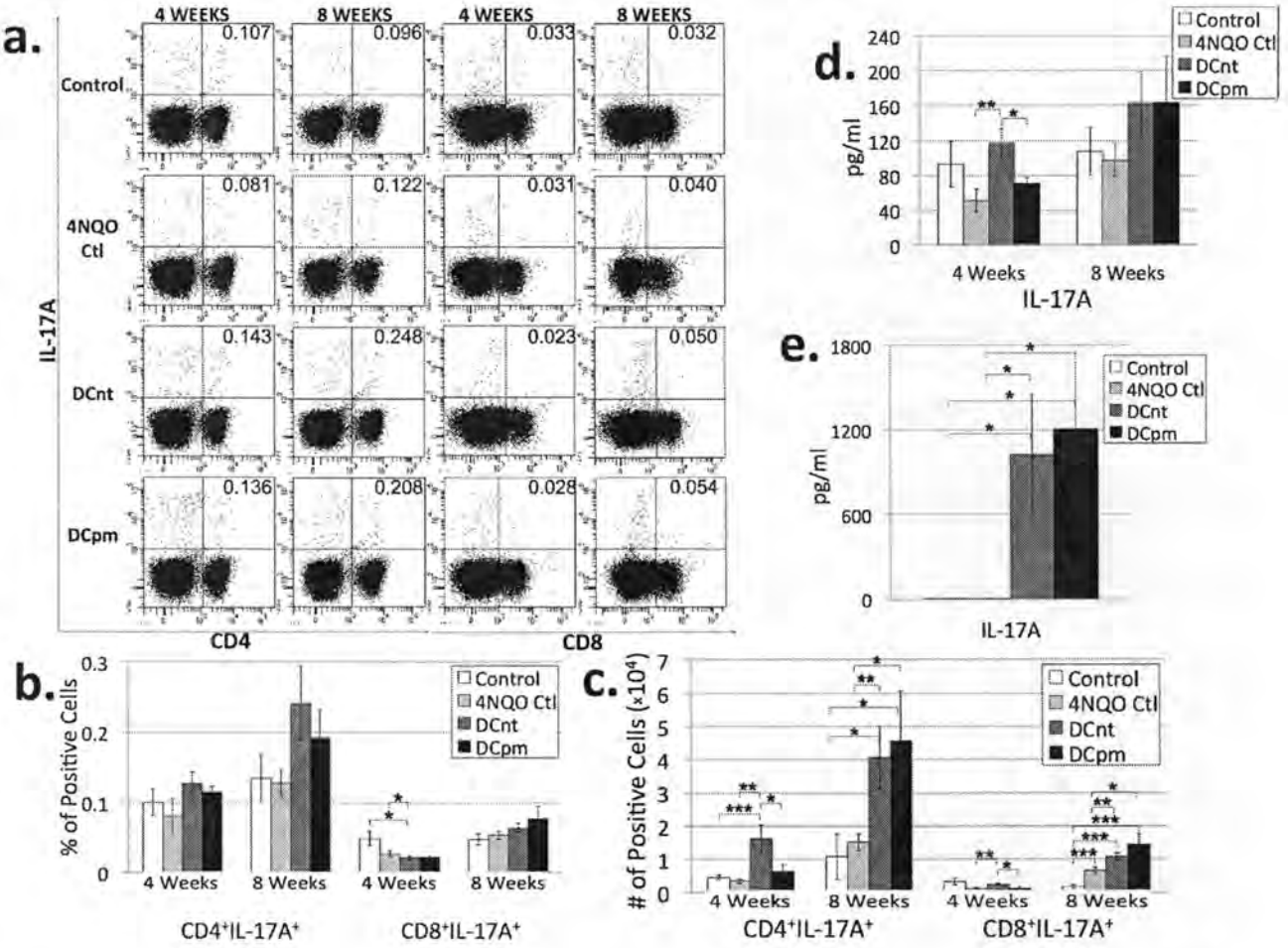

Fig 4.7 DCnt vaccination results in an increase in absolute number of Th17 and Tc17 cells at 4 weeks, while both DCnt and DCpm vaccination result in an increase in absolute number of Th17 and Tc17 cells at 8 weeks compared to 4NQO-treated controls.

Representative results (a) and graphical representation (b) of flow cytometric staining of cervical lymph node cells from control, 4NQO-treated control (4NQO Ctl), DCnt-vaccinated (DCnt) and DCpm-vaccinated (DCpm) mice at 4 and 8 weeks post-first vaccination with at least 5 mice per group. Data represent mean ± SEM. No significant differences were found in percentages of $CD4^+IL\text{-}17A^+$ or $CD8^+IL\text{-}17A^+$ cells at 4 or 8 weeks (2-tailed Student's *t*-test). Total numbers of populations (c) were determined by multiplying the percent positive by the number of cervical lymph node cells for each individual mouse. Data represent mean ± SEM. *, $p < 0.05$. **, $p < 0.01$. ***, $p < 0.001$ (2-tailed Student's *t*-test). Cytometric bead array analysis (d) of supernatant of 4 week and 8 week control, 4NQO Ctl, DCnt, and DCpm mouse cervical lymph node cells after stimulation for four hours with PMA and ionomycin, with at least 5 mice per group. Data represent mean ± SEM. *, $p < 0.05$. **, $p < 0.01$. ***, $p < 0.001$ (2-tailed Student's *t*-test). Cytometric bead array analysis (e) of supernatant of 8 week control, 4NQO Ctl, DCnt, and DCpm mouse cervical lymph node cells after 3 day stimulation with anti-CD3/anti-CD28 beads with at least 4 mice per group. Data represent mean ± SEM. *, $p < 0.05$ (2-tailed Student's *t*-test).

At 8 weeks post-first vaccination, both DCnt and DCpm mice have an increased absolute number of Th17 and Tc17 cells compared to control and 4NQO control (Fig 4.7c). While the supernatant of ex vivo lymph node cells shows no significant change in IL-17A levels in vaccinated mice compared to controls (Fig 4.7d), supernatant of DCnt and DCpm mouse lymph node cells stimulated for 3 days with anti-CD3/anti-CD28 contained much higher levels of IL-17A compared to controls (Fig 4.7e). This indicates that vaccination with DCnt results in an increase in Th17 and Tc17 cells early on, while vaccination with either DCnt or DCpm results in an increase in the Th17 and Tc17 response at 8 weeks post-vaccination.

DCpm vaccination results in increased levels of IL-10 at 4 weeks, while both DCnt and DCpm vaccination results in increased levels of IL-13 at 8 weeks compared to 4NQO-treated controls.

Th2 cells can have inflammatory or anti-inflammatory effects, and they can release multiple different cytokines, including IL-4, IL-10, and IL-13. These cells have been traditionally associated with a pro-tumorigenic phenotype. They are often upregulated in cancers, antagonizing a beneficial Th1 response (221-224). However, our lab has shown that levels of Th2 cytokines remain largely unchanged in the draining lymph nodes of premalignant lesion-bearing and HNSCC-bearing mice compared to controls (218). To evaluate the populations of Th2 cells in draining lymph nodes of control, 4NQO control, DCnt, and DCpm mice, lymph node cells were stained for surface expression of CD4 and intracellular expression of the Th2 cytokines IL-4, IL-10, and IL-13 after 4 hours incubation with PMA, Ionomycin and brefeldin A. In addition, supernatants of lymph node cells incubated for 4 hours

in PMA and Ionomycin were analyzed by cytometric bead array for levels of IL-4, IL-10, and IL-13. At 4 weeks post-first vaccination, DCpm mice have an increased percentage of IL-10 expressing CD4 T cells compared to all other groups (Fig 4.8a and b). This is supported by data showing an increase in IL-10 released into the supernatant of ex vivo DCpm mouse lymph node cells incubated for 4 hours with PMA and Ionomycin compared to all other groups (Fig 4.8d). Also at this timepoint, DCnt mouse lymph nodes were found to have an increased absolute number of IL-10 expressing CD4 T cells as well as an increased level of IL-13 in supernatant of ex vivo lymph node cells compared to 4NQO control (Fig 4.8c and d).

At 8 weeks post-first vaccination, DCpm mouse lymph nodes were found to contain a decreased percentage of IL-10-expressing CD4 T cells compared to 4NQO controls and an increased number of IL-4-expressing CD4 T cells compared to 4NQO controls (Fig 4.8a, b, c). DCnt mouse lymph nodes have an increased percentage and both DCnt and DCpm mouse lymph nodes have an increased absolute number of IL-13-expressing CD4 T cells compared to 4NQO control (Fig 4.8c). Analysis of supernatant of ex vivo cells stimulated for 4 hours with PMA and Ionomycin revealed a significant increase in IL-13 in both vaccinated groups compared to 4NQO control and control (Fig 4.8d). Analysis of supernatant of cells stimulated for 3 days with anti-CD3/anti-CD28 revealed a large increase in IL-10, IL-13 and IL-4 in supernatant from DCnt and DCpm mouse lymph node cells compared to control and 4NQO control (Fig 4.8e). This data indicates that, while DCpm vaccination results in an early increase in the immunosuppressive cytokine IL-10, both DCnt and DCpm

vaccination result in the delayed increase in multiple Th2 cytokines, especially IL-13.

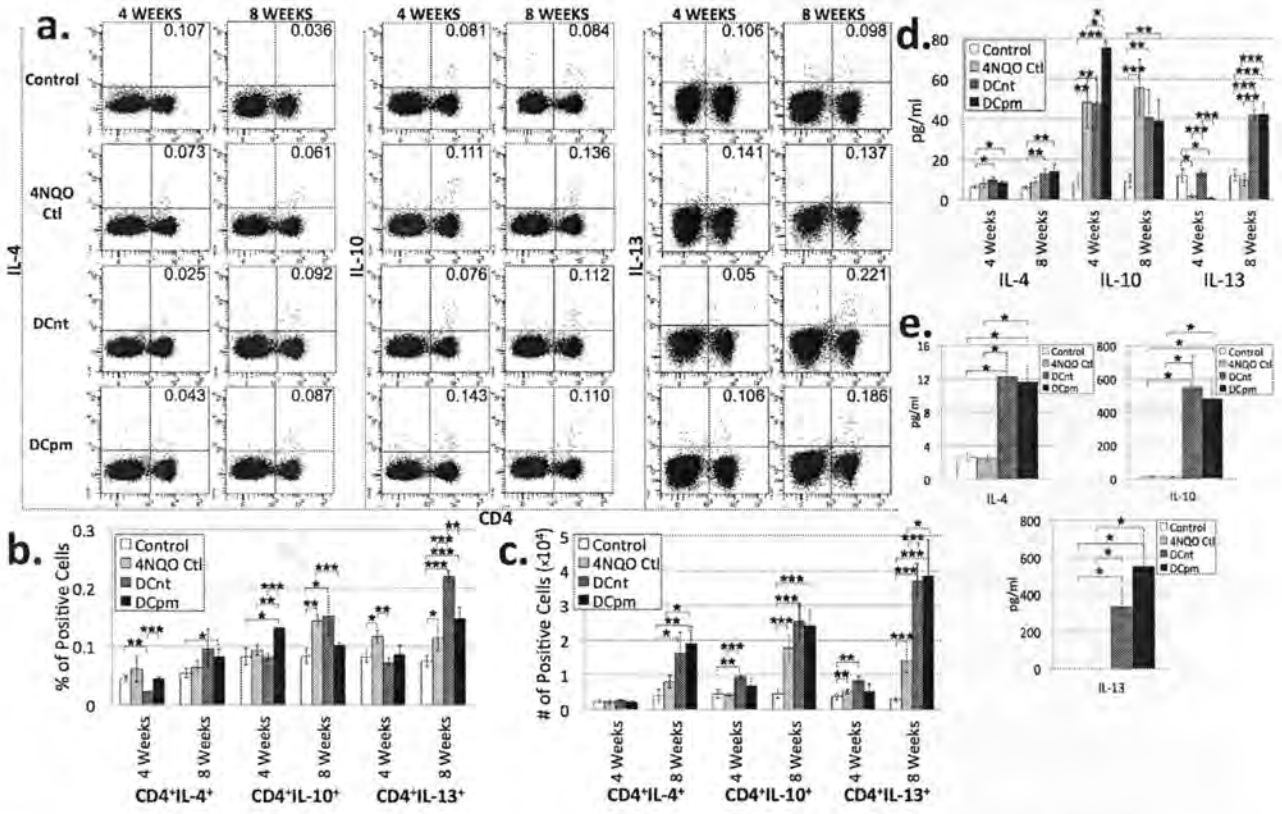

Fig 4.8 DCpm vaccination results in increased levels of IL-10 at 4 weeks, while both DCnt and DCpm vaccination results in increased levels of IL-13 at 8 weeks compared to 4NQO-treated controls.
Representative results (a) and graphical representation (b) of flow cytometric staining of cervical lymph node cells from control, 4NQO-treated control (4NQO Ctl), DCnt-vaccinated (DCnt) and DCpm-vaccinated (DCpm) mice at 4 and 8 weeks post-first vaccination with at least 5 mice per group. Data represent mean ± SEM. *, $p < 0.05$. **, $p < 0.01$. ***, $p < 0.001$ (2-tailed Student's *t*-test). Total numbers of populations (c) were determined by multiplying the percent positive by the number of cervical lymph node cells for each individual mouse. Data represent mean ± SEM. *, $p < 0.05$. **, $p < 0.01$. ***, $p < 0.001$ (2-tailed Student's *t*-test). Cytometric bead array analysis (d) of supernatant of 4 week and 8 week control, 4NQO Ctl, DCnt, and DCpm mouse cervical lymph node cells after stimulation for four hours with PMA and ionomycin, with at least 5 mice per group. Data represent mean ± SEM. *, $p < 0.05$. **, $p < 0.01$. ***, $p < 0.001$ (2-tailed Student's *t*-test). Cytometric bead array analysis (e) of supernatant of 8 week control, 4NQO Ctl, DCnt, and DCpm mouse cervical lymph node cells after 3 day stimulation with anti-CD3/anti-CD28 beads with at least 4 mice per group. Data represent mean ± SEM. *, $p < 0.05$ (2-tailed Student's *t*-test).

4.4 Discussion

The immune reaction to tumor development is a complicated and multifaceted phenomenon. While the immune system is capable of recognizing and attacking most dysplastic tissues, some dysplastic tissue is bound to avoid elimination and, given time and the selective pressure of the immune system, variants arise which have the capacity to escape recognition and/or manipulate the immune system in ways which further the progression of the malignancy (225). This is the case with HNSCC, a cancer that is known to upregulate immunosuppressive cell populations such as Treg cells and promote smoldering inflammation through chronic upregulation of inflammatory cell types (163, 164, 176, 177, 218, 219). In this study, we sought to halt the progression of HNSCC at the premalignant stage through dendritic cell vaccination, with the rationale that premalignant and HNSCC tissue share common TAAs and the immune system at the premalignant stage has not yet been compromised by the advanced immune-manipulative tactics of an established tumor (136, 180, 218). Unexpectedly, both DCpm vaccination and DCnt vaccination, though to a lesser extent, resulted in a decrease in lesion burden compared to 4NQO treatment alone. Also, evaluation of lymph node cells from DCpm vaccinated mice for response to a 3 day challenge with premalignant or HNSCC lysate showed that a specific response was not being generated (Fig 4.9). Analysis of the ex vivo immune response in vaccinated mice, as summarized in Table 4.1, revealed an early increase in draining lymph node cell number and levels of stimulatory immune effectors, including activated CD4 Tconv cells, CD8 Tconv cells, Th1, Tc1, Th17, and Tc17 cells, for DCnt mice, while the

immune response in DCpm mice at 4 weeks mirrored that of 4NQO control mice. At the later timepoint, when both vaccinated groups appear to provide beneficial clinical effects, both DCnt and DCpm mouse lymph nodes have a greater number of cells, consisting of immunostimulatory cells such as activated CTLs, Tc1, Th17 and Tc17 cells, and, to a lesser extent, immunosuppressive Tregs and Th2 cells. These data indicate that DC vaccination, regardless of whether the DCs were pulsed with normal tongue or premalignant antigens, provides a non-specific but substantial immune stimulatory response and may be clinically beneficial.

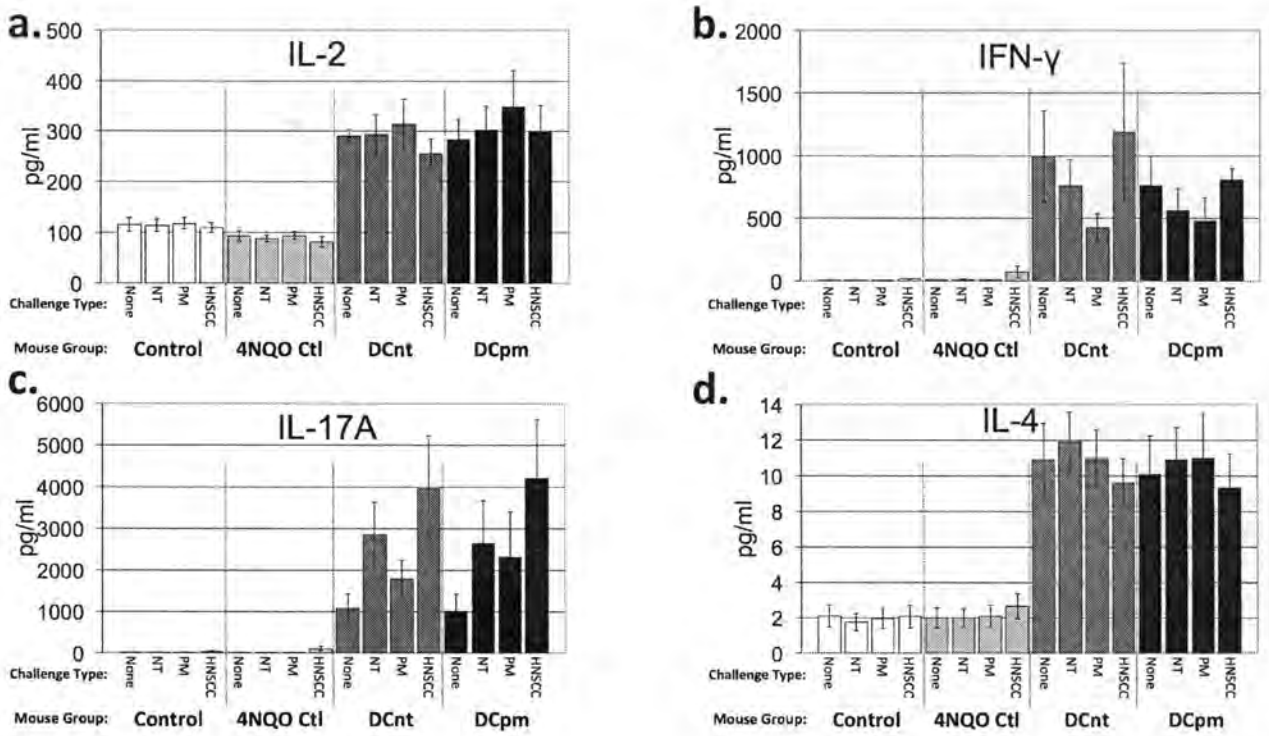

Fig 4.9 Cervical lymph node cells from vaccinated mice do not exhibit specificity of reaction to premalignant lysate or HNSCC lysate challenge. Cytometric bead array analysis of supernatant of 4 week control, 4NQO-treated control (4NQO Ctl), DCnt-vaccinated (DCnt) and DCpm-vaccinated (DCpm) mouse cervical lymph node cells after 3 day challenge with 25 μg/ml of normal tongue epithelium lysate (NT), premalignant epithelium lysate (PM), or HNSCC lysate (HNSCC) in the presence of anti-CD3/anti-CD28 beads with at least 4 mice per group. No significant differences in IL-2 release (a), IFN-γ release (b), IL-17A release (c), or IL-4 release (d) were found for the different challenges within each mouse group (2-tailed Student's *t*-test).

This study presents the evaluation of immune response in the draining cervical lymph nodes of vaccinated and unvaccinated 4NQO-treated mice. This method of evaluation was used because the immune response to tumor is likely to be orchestrated in draining lymph nodes and because tongue epithelium was determined to contain very low numbers of T cells. However, immune response to treatment and to tumor development involves not only loco-regional responses, but also a myriad of changes in cellular localization of immune populations following activation. As such, it will be important to investigate immune populations present in tongue epithelium and in the periphery to fully determine the response to vaccination and tumor development.

Evaluation of dendritic cell maturation markers and cytokines revealed no differences between the dendritic cells pulsed with premalignant lesion lysate and the dendritic cells pulsed with normal tongue epithelium lysate (Fig 4.10). Investigation into response to premalignant lysate or HNSCC lysate challenge showed no indications of specificity of reaction of DCpm mouse lymph node cells or DCnt mouse lymph node cells (Fig 4.9). However, DCnt vaccination clearly elicited a more immediate response compared to DCpm vaccination, though this did not correlate with a stronger clinical response compared to DCpm mice. DCpm vaccination, on the other hand, resulted in very little in the way of an immediate reaction. At the later timepoint, however, both DCnt and DCpm vaccination resulted in increased immune stimulation and improved clinical response. Additional research into what delineates the different responses to these two vaccinations is

necessary to determine why the DCpm vaccination promotes a delayed response compared to the immediate response to the DCnt vaccine.

Part of the rationale for using premalignant lesion lysate as the source of antigen to stimulate a specific *in vivo* response to premalignant and malignant tissue involved the over-expression of multiple common TAAs in both premalignant and HNSCC tissue (136, 180). However, as determined in the current study, pulsing DCs with premalignant antigen did not result in the development of a specific response of lymph node cells from vaccinated mice to premalignant or HNSCC antigens, and both DCnt vaccination and DCpm vaccination resulted in a similar clinical response. To evaluate if the clinical response seen with DCnt and DCpm vaccination is a result of administering activated dendritic cells regardless of pulsing with lysate, another control group was vaccinated with unpulsed, activated DCs. Like both the DCnt and DCpm-vaccinated groups, there was no specific response of DC-vaccinated mouse lymph node cells to either premalignant or HNSCC challenge (data not shown). Also, while DC-vaccinated mice did have a clinically significant decrease in lesion burden, there was no difference in lesion burden between the DC-vaccinated and DCnt- or DCpm-vaccinated groups (data not shown). Therefore, it is likely that the clinical benefit seen is solely due to an increase in numbers of activated DCs. These activated DCs may pick up antigen after injection into the tongue, resulting in cross-presentation to cytotoxic T cells. It is also possible that in using tissue lysate as an antigenic source, the extraneous components of the lysate prevented sufficient stimulation of cells with the intended premalignant and HNSCC tissue-associated TAAs. Future experimentation into vaccination of mice with specific TAA-pulsed

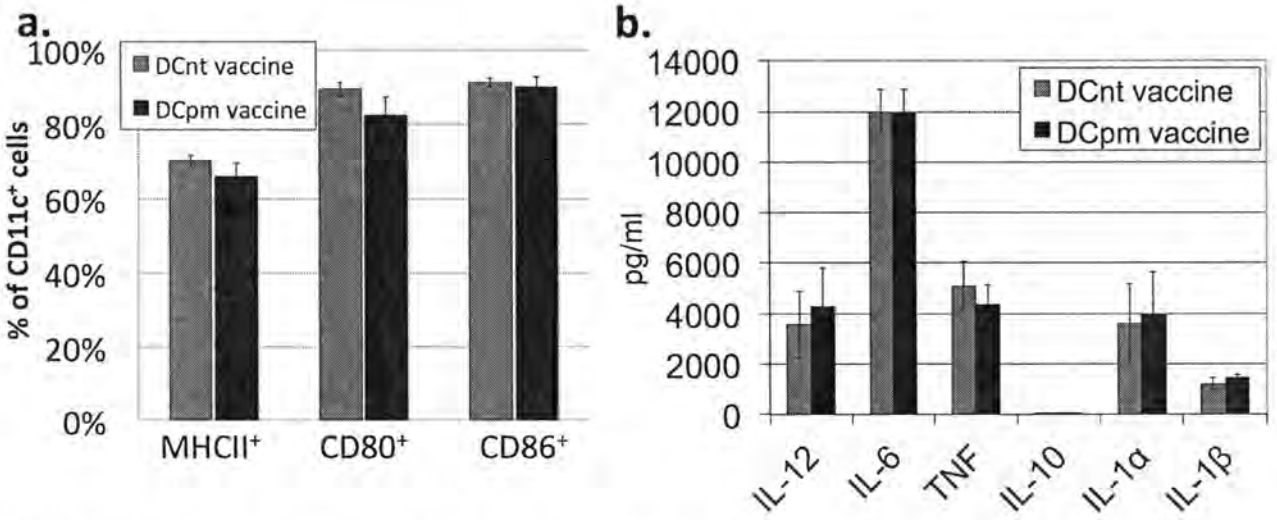

Fig 4.10 Dendritic cells have the same expression of costimulatory markers and cytokines whether pulsed with normal tongue epithelium lysate or premalignant epithelium lysate.
Immature bone marrow-derived dendritic cells were pulsed with lysates of premalignant epithelium (DCpm vaccine) or normal tongue epithelium (DCnt vaccine), then matured with LPS. Phenotyping of dendritic cells by flow cytometric staining (a) and cytometric bead array (b) of at least 4 separate experiments reveal no significant differences between dendritic cell maturation markers or cytokines between the two groups (2-tailed Student's *t*-test).

DCs may lead to the development of an immunotherapy providing a specific immune response with improved clinical benefit.

HNSCC is an aggressive malignancy that has a complicated relationship with the immune system. The development of immunotherapeutic strategies to combat this cancer is highly important to increasing the poor survival rate of patients with HNSCC. While the dendritic cell vaccination described in this study reduced lesion burden by only a small amount, the immune system interactions described help to advance our understanding of the functions of various immune cell subtypes during the development of HNSCC. Further investigation into the intricacies of the tumor-immune system relationship will allow for more efficient development of treatments that have a better chance of being effective in the future.

4 Weeks										
Absolute # (compared to 4NQO Control)	Tconv		Treg	Activated Tconv		Th1/Tc1		Th17/Tc17		Th2
	$CD4^+$ $Foxp3^-$	$CD8^+$ $Foxp3^-$	$CD4^+$ $Foxp3^+$	$CD25^+$ CD4 Tconv	$CD44^{hi}$ CD4 Tconv	$CD4^+$ $IFN\text{-}\gamma^+$	$CD8^+$ $IFN\text{-}\gamma^+$	$CD4^+$ $IL\text{-}17A^+$	$CD8^+$ $IL\text{-}17A^+$	$CD4^+$ $IL\text{-}10^+$
DCnt	↑	↑	↑	↑	↑	↑	↑	↑	↑	↑
DCpm										

8 Weeks											
Absolute # (compared to 4NQO Control)	Tconv	Treg		Activated Tconv			Tc1	Th17/Tc17		Th2	
	$CD8^+$ $Foxp3^-$	$CD4^+$ $Foxp3^+$	$CD8^+$ $Foxp3^+$	$CD25^+$ CD8 Tconv	$CD44^{hi}$ CD4 Tconv	$CD44^{hi}$ CD8 Tconv	$CD8^+$ $IFN\text{-}\gamma^+$	$CD4^+$ $IL\text{-}17A^+$	$CD8^+$ $IL\text{-}17A^+$	$CD4^+$ $IL\text{-}4^+$	$CD4^+$ $IL\text{-}13^+$
DCnt	↑	↑	↑	↑		↑	↑	↑	↑		↑
DCpm	↑	↑		↑	↑	↑	↑	↑	↑	↑	↑

Table 4.1 Summary of significant differences in absolute numbers of cell populations between the 4NQO control group and the DCnt and DCpm-vaccinated groups.

Chapter 5

General Discussion

CHAPTER 5: GENERAL DISCUSSION

5.1 Implications of Immune Response During HNSCC Development

In the theory of immunoediting, there are three stages of immune response during tumor development: elimination, equilibrium, and escape. Elimination involves an anti-tumor response in which Th1 and Tc1 cells direct immune effectors, including CTLs and macrophages, to clear dysplastic tissue (1, 2). Equilibrium involves the co-evolution of tumor variants with the immune system, often resulting in the emergence of non-immunogenic tumor variants that have gained the ability to subvert the immune system through several mechanisms, a stage referred to as escape (2, 17-19). However, the immune system can also contribute to tumor development through the effects of unresolved chronic inflammation (105-111). The mediators that promote tumor growth often overlap with the mediators that combat tumor growth, complicating the classification of certain immune responses as "anti-tumor" or "pro-tumor." Careful investigation into the intricacies of immune responses to the development of specific cancers is essential to determining the true roles of the immune cells in these cancers.

HNSCC is a cancer that has been shown to have a complicated relationship with the immune system. As such, this study aimed to further elucidate the implications of the immune alterations observed in HNSCC by examining the immune responses during HNSCC development. HNSCC is somewhat unique in that, with vigilant surveillance, it may be identified in its premalignant stages as leukoplakia or erythroplakia of the oral mucosa (173). Analysis of these premalignant lesions in humans and mice revealed an increase in inflammatory

cytokine levels, such as IFN-γ, compared to control tissue. Analysis of the draining cervical lymph nodes in premalignant lesion-bearing mice also revealed an increase in stimulatory immune effectors from Tconv cells bearing markers of activation and memory to Th1, Tc1, and Th17 cells compared to control lymph nodes. However, no significant differences were found in systemic levels of any evaluated cytokines between control plasma and plasma from patients bearing premalignant lesions. This data indicates that, at the stage at which premalignant lesions become detectable, a strong local inflammatory immune response, with no accompanying immunosuppression, has been mounted.

While the data clearly indicates that the premalignant stage of HNSCC development is associated with a strong inflammatory immune response, the role of the response is still unclear. Classical thought would indicate that the observed response is part of an anti-tumor immune reaction that, in the case of 4NQO-treated mice, is unable to eliminate the dysplastic effects of ongoing carcinogen treatment. However, it is also possible that the observed inflammatory response is partially responsible for continued progression towards cancer. Further investigation into the roles of these cells through models involving oncogenesis in IFN-γ, IL-17A, CD4, or CD8 knockout mice will serve to uncover the beneficial or detrimental effects of each of the immune cell types during tumor development.

5.2 Complexity of HNSCC-Associated Immune Response

The classic description of immune escape involves downregulation of immunogenic properties, the skewing of a type I response to a type II response, and

an upregulation of multiple immune inhibitory factors, such as regulatory T cells (2). Past research indicates that HNSCC follows this model in several ways, including a downregulation of MHC class I molecules and B7 molecules, an upregulation of Th2 cytokines, and a large increase in peripheral and tumor-infiltrating Tregs (28, 143-145, 163, 164). The present study sought to further investigate the locoregional immune alterations in established HNSCC using both a mouse model of 4NQO-induced carcinogenesis and human samples. While we did observe the expected increase in Treg cells in cervical lymph nodes of HNSCC-bearing mice compared to control, there was also an increase in levels of conventional T cells found in lymph nodes of HNSCC-bearing mice compared to control. In addition, there was an unexpected increase in Th1 cells in cervical lymph nodes of HNSCC-bearing mice compared to control mice. Human tumor samples had a similar increase in levels of IFN-γ compared to normal/adjacent control tissue. This data indicates that, contrary to past research, an inflammatory reaction involving Th1 cells continues in the setting of established HNSCC.

The Th1 reaction observed in mice with established HNSCC could have several implications. It is conceivable that this response may represent an ongoing attempt at an anti-tumor response whose effects are simply overwhelmed by the multitude of other immune manipulative factors induced by the tumor. Conversely, it is possible that this response, and the Th1 and Tc1 response observed at the premalignant stage, is tumor promoting. Though generally associated with an anti-tumor response, IFN-γ expression and Th1 cells have been linked to tumor promotion in several studies, most notably in carcinogen-induced tumors of

epithelial origin similar to 4NQO-induced or tobacco smoking-induced HNSCC development (120, 121). Thirdly, the Th1 and Tc1 response observed at the premalignant stage may be representative of a beneficial immune response, while the Th1 and Tc1 response at the HNSCC stage may be representative of a detrimental immune response. In cervical lymph nodes of premalignant lesion-bearing mice, there is a higher increase in the percentage of Tc1 cells than Th1 cells, while HNSCC-bearing mice have a higher increase in the percentage of Th1 cells than Tc1 cells. In addition, premalignant lesion-bearing mice have a much greater percentage of Th17 cells compared to HNSCC-bearing mice. It is possible that the Tc1 and Th17 cells at the premalignant stage are working together in a concerted effort to combat oncogenesis, while the shift towards Th1 cells and decrease in Th17 cells with a simultaneous increase in Tregs represents a completely separate, pro-oncogenic immune reaction at the HNSCC stage.

Established HNSCC was found to be associated with an increase in both Tconv cells and Tregs. Further analysis revealed that Tconv cells from HNSCC-bearing mice have a reduced proliferative capacity compared to Tconv cells from control mice, providing somewhat of an explanation for how these stimulatory cells could co-exist with what was expected to be an immunosuppressive microenvironment. In addition, analysis of the Treg cells showed that, unexpectedly, Tregs from HNSCC-bearing mice suppress Tconv cells to a lesser degree than Tregs from control mice. It will be important in the future to determine any other role these Tregs may be playing in the HNSCC microenvironment, as they appear to lose their primary function of T cell suppression as the tumor progresses.

5.3 Immunotherapy at the Premalignant Stage

The rationale for initiation of immunotherapy at the premalignant stage of HNSCC development included the fact that oral lesions are identifiable at the premalignant stage, premalignant lesions and HNSCC share overexpression of common tumor antigens, and the hypothesis that the premalignant stage of HNSCC development would not be associated with the systemic immunosuppression that past studies identified in patients with HNSCC (136, 173, 180). Our data showing that both mice and humans with premalignant lesions experience a local increase in inflammatory immune mediators without any immunosuppression supported our reasoning behind initiation of immunotherapy at this point. In this study, premalignant lysate-pulsed dendritic cells were administered to mice bearing premalignant lesions in an attempt to halt or even reverse the progression of these lesions to HNSCC. Unexpectedly, lymphocytes from DCpm mice did not show any specificity of reaction to premalignant or HNSCC lysate challenge. However, both DCpm vaccination and vaccination with the control vaccine were associated with a decrease in lesion burden compared to unvaccinated, 4NQO-treated controls. Analysis of draining cervical lymph node cells from vaccinated mice revealed that DCnt vaccination resulted in an early increase in levels of stimulatory immune effectors compared to DCpm and 4NQO control mice, while DCpm vaccination resulted in a delayed increase in levels of stimulatory immune effectors compared to 4NQO control mice. This data indicated that activated DCs were capable of generating a non-specific immune stimulatory response associated with improved

clinical prognosis regardless of the whether the DCs were pulsed with normal tongue or premalignant antigens. With this large increase in the non-specific immune stimulatory response in mind, it is important to evaluate levels of additional possible contributory cells, such as NK cells, NKT cells, and γδ T cells, to the anti-tumor response in future studies.

Evaluation of dendritic cell maturation markers and cytokines revealed no significant differences between dendritic cells pulsed with normal tongue epithelium lysate or premalignant epithelium lysate, and evaluation of lymphocytes from DCnt and DCpm mice revealed no specificity of response to normal tongue epithelium lysate, premalignant epithelium lysate, or HNSCC lysate challenge. However, DCpm vaccination was found to reduce lesion burden two weeks earlier than DCnt vaccination, and DCnt vaccination was found to increase multiple stimulatory immune effectors at the 4 week point, while DCpm vaccination was not associated with an increase in these effectors until the 8 week point. Additional research into the differing consequences of pulsing dendritic cells with normal tongue epithelium lysate versus premalignant lesion lysate is necessary to determine what is responsible for the differing responses to the two vaccines.

Tumor lysate is often used as a source of antigen for dendritic cell vaccination because it allows sensitization to multiple potentially immunogenic TAAs simultaneously, a method that is especially helpful when the most immunogenic TAAs expressed by the tumor tissue have not yet been identified (226-228). Premalignant lesion lysate was used for the antigenic source for pulsing dendritic cells for vaccination because previous studies showed that both

premalignant lesions and HNSCC overexpress several of the same tumor antigens, though the immunogenicity of each individual antigen has not yet been determined (136, 180). The current study showed that, contrary to expectations, vaccination with premalignant lesion-lysate pulsed dendritic cells did not result in the development of a specific immune response to premalignant or HNSCC epithelium. It is possible that components of the lysate, aside from TAAs, may block or even suppress sensitization of DCs to the intended TAAs (229, 230). As such, future experimentation using specific antigens, rather than premalignant tissue lysate, to pulse dendritic cells prior to vaccine administration may result in the successful development of a specific response with increased clinical benefit.

5.4 Closing Remarks

The current study presented the evaluation of immune responses to the development of HNSCC in carcinogen-treated mice and humans. Experimentation revealed that the premalignant stage is associated with a rise in inflammatory immune effectors without immunosuppression, while established HNSCC is associated with a seemingly contradictory rise in both immunosuppressive and immunostimulatory populations. The genuine roles of the evaluated immune effectors have yet to be established; future experimentation involving carcinogen-treatment of mice lacking expression of these individual cell populations is necessary to determine the anti-tumor versus pro-tumorigenic functions of these populations. The elucidation of these links will provide much-needed information

for the tailoring of future immune therapies aimed at combatting or preventing the development of HNSCC.

The current study also evaluated the feasibility of initiating immunotherapy at the premalignant stage of HNSCC development, thus avoiding the obstacle of systemic immunosuppression seen in established HNSCC, specifically through the use of premalignant antigens in a dendritic cell vaccine. Both the experimental DCpm vaccine and the control DCnt vaccine resulted in clinical benefit to carcinogen-treated mice, even without the development of a specific response to premalignant or HNSCC antigens, a result that is both unexpected and promising. Future investigation involving optimization of dendritic cell preparation, perhaps using specific TAAs as an antigen source, will allow for more efficient development of treatments that have an increased opportunity of being clinically effective and may eventually be used in the clinic to combat this notoriously highly aggressive and tenacious malignancy.

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