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Characterization of the evolution of immune phenotype during the development and progression of squamous cell carcinoma of the head and neck

A. Anna-Maria De Costa,

Research Service (151), Ralph H. Johnson Veterans Affairs Medical Center, Charleston, SC 29401, USA. Department of Otolaryngology, Head and Neck Surgery, Medical University of South Carolina, Charleston, SC 29401, USA. Department of Medicine, Medical University of South Carolina, Charleston, SC 29401, USA

Corinne A. Schuyler,

Research Service (151), Ralph H. Johnson Veterans Affairs Medical Center, Charleston, SC 29401, USA

David D. Walker, and

Department of Otolaryngology, Head and Neck Surgery, Medical University of South Carolina, Charleston, SC 29401, USA

M. Rita I. Young

Research Service (151), Ralph H. Johnson Veterans Affairs Medical Center, Charleston, SC 29401, USA. Department of Otolaryngology, Head and Neck Surgery, Medical University of South Carolina, Charleston, SC 29401, USA. Department of Medicine, Medical University of South Carolina, Charleston, SC 29401, USA

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. The present study utilized a mouse model of 4-nitroquinoline 1-oxide-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 cervical lymph nodes of control and premalignant lesion-bearing mice, though the Tconv cells appear to be less proliferative and the Treg cells appear to be less suppressive at the HNSCC stage. 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. Also, lymph nodes' cells from both premalignant lesion-bearing and HNSCC-bearing mice include increased levels of Th1, Tc1, and Th17 cells, with no differences in levels of Th2 cells, compared to control mice. The data show that while there is the expected increase in immunosuppressive Tregs in lymph nodes when HNSCC is present, there is also an unexpected increase in immune populations usually associated

with a beneficial antitumor response, including Tconv cells and Th1 and Tc1 cells. In addition, the results demonstrate that the premalignant stage of HNSCC development is associated with a robust immune response involving an increase in inflammatory Th1, Tc1, and Th17 cells.

Keywords

HNSCC; Premalignant; Immunoediting; Th1/Tc1 cells; Th17 cells

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 5-year survival rate remains around 50% [1], with a 5-year survival rate of 28% for patients with advanced HNSCC [2]. 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 [1, 2]. 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 [1]. Therefore, our focus is shifting toward identifying this disease in its premalignant stages and ultimately developing treatment strategies to halt progression toward 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 [3, 4]. HNSCC patients exhibit increased levels of regulatory T cells (Tregs) [5–7] and CD34⁺ progenitor cells [8], 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 [5, 9], indicating tumor-induced manipulation of the environment to prevent an effective antitumor response. However, while an increase in the CD34⁺ population is associated with a poor patient outcome [8], Treg infiltration into the tumor was shown to be associated with improved locoregional control [7]. 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 [6]. 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(+) versus HPV(–) disease [10]. 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 [11], 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 [12, 13]. 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 [14]. While there are multiple models that may be used to study HNSCC, such as the HPV(+) cancer model [15] and the orthotopic SCC VII syngeneic tumor system [16], 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 [17], 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 [1, 14]. As tongue epithelium was determined to contain very low numbers of T cells, draining cervical lymph nodes were analyzed to evaluate immune cell makeup of response and tongue epithelium was lysed to determine the 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.

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)

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 1.9 mm $\times$ 30° scope and a Stryker 1088 HD camera. Mice were sedated with inhaled isoflurane (Piramal Healthcare) during the procedure.

Cervical lymph node processing

Cervical lymph nodes were harvested from C57BL/6 mice and homogenized via a Stomacher 80 homogenizer (Seward) set on high for 90 s. Cells were passed through a 40- μ m cell strainer (BD Falcon, San Jose, CA) and rinsed with Hank's Buffered Saline Solution (HBSS, Invitrogen) to remove debris. Cells' 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 Biosciences unless otherwise specified. In order to detect levels of CD8- and CD4-positive cells in relation to Foxp3 and CD11b⁺Gr-1⁺ MDSCs, ex vivo cervical lymph node cells in single-cell suspension were washed once in Stain Buffer and resuspended at 1×10^7 cells/ml. Nonspecific staining of a total of 1×10^6 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). Intracellular staining for PE-Cy7 Foxp3 (eBioscience) 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).

Flow cytometric analysis of cytokine expression

All reagents in this section were from BD Biosciences unless otherwise specified. In order to detect intracellular cytokines, single-cell suspensions of cervical lymph node cells were restimulated for 4 h at 37°C with 50 ng/ml phorbol 12-myristate 12-acetate (PMA), 1 μ g/ml ionomycin, and brefeldin A solution. Nonspecific staining of total of 1×10^6 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) was performed after fixation/permeabilization with Cytofix/Cytoperm. Extent and frequency of positively stained cells was visualized using flow cytometry (FACSCanto).

Cytokine bead array

All reagents in this section were from BD Biosciences unless otherwise specified. In order to detect cytokines released into supernatant, single-cell suspensions of cervical lymph node cells were restimulated for 4 h at 37°C with 50 ng/ml PMA and 1 μ g/ml ionomycin without addition of brefeldin A. In order to detect cytokine levels in tongue tissue, tongue epithelium obtained after incubation of tongue fragments with Dispase II (Roche) was lysed through sonication, and protein concentration was determined by BCA protein assay (Pierce) as per manufacturer's instructions. Levels of IFN- γ , IL-2, IL-17A, IL-4, IL-6, 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-1 α , MIP-1 β , and IL-13 in cell supernatant or 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.

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). 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) and cultured for 3 days in the presence of Dynabeads Mouse T-Activatory anti-CD3/anti-CD28 beads (Invitrogen). 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 $\pm$ standard error of the mean. To compare one variable condition between groups, the 2-tailed Student's *t* test was used. Significance was reported in the 95% confidence interval.

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 [18]. To determine whether draining lymph nodes undergo hyper-plasia 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 was counted. While mice bearing premalignant oral lesions exhibit equal numbers of cervical lymph node cells as control mice ($4.94 \times 10^6 \pm 6.25 \times 10^5$ vs. $5.31 \times 10^6 \pm 7.29 \times 10^5$, $P = 0.362$), mice bearing HNSCC exhibit a marked increase in number of cervical lymph node cells to more than double the number found in control mice ($1.17 \times 10^7 \pm 2.63 \times 10^6$ vs. $5.31 \times 10^6 \pm 7.29 \times 10^5$, $P = 0.006$). 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) [5–7]. To determine the relative composition of conventional T cells, regulatory T cells, and 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. 1a, b), while total numbers of populations expressing each marker (Fig. 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-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. 1b, 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 (Fig. 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 (Fig. 1f). The levels of CD4⁺CD25[−] conventional T-cell proliferation or IL-2 release at the premalignant stage fell in the middle of the Tconv proliferation and IL-2 release for control cells and HNSCC-bearing mouse cells.

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 coincubated 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 Fig. 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 become increasingly dysfunctional.

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 [19, 20]. To determine the makeup 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 memory marker CD44, and the exhaustion markers PD-1 and KLRG1. As shown in Fig. 2a, 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. 2c).

Increased expression of the surface marker CD44 is used to distinguish T cells with a memory phenotype from those that are naïve [21, 22]. 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. 3a, 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.

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 [20, 23]. 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. 4a, b). When comparing total numbers of positive cells (Fig. 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, these data indicate 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 HNSCC-bearing mice were stimulated for 4 h 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, MIP-1 α , and MIP-1 β , 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 [24]. Many solid tumors, including HNSCC, have been linked to a decrease in Th1 immunity in past studies [5, 9]. As shown in Fig. 5a, 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. 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-1 α , and MIP-1 β compared to control mice (Fig. 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-1 α , and MIP-1 β were found to be higher in premalignant epithelium lysate and HNSCC epithelium lysate compared to control (Fig. 5e). These data suggest that Th1 and Tc1 immunity is stimulated early in the development of HNSCC, during the premalignant stages, and is sustained even after HNSCC is established, contrary to what some previous studies have indicated [5, 9].

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 [25, 26]. Both premalignant lesion-bearing and HNSCC-bearing mouse lymph nodes contain a greater percentage (Fig. 6a, b) and number (Fig. 6c) of Th17 (CD4⁺IL-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. 6a, 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. 6d). Levels of IL-17A in tongue epithelium lysate were too low to be detected by cytometric bead analysis. These data indicate 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 [5, 9]. This Th2-type response can be pro-inflammatory, involving cytokines such as IL-13 or IL-6, or anti-inflammatory, involving cytokines such as IL-4 and IL-10, a cytokine also associated with a regulatory T-cell response. The only significant difference identified by flow cytometric analysis of lymph node cells is a minor decrease in percent of CD4⁺IL-13⁺ cells in lymph nodes of HNSCC-bearing mice compared to premalignant lesion-bearing mice (data not shown). Of the Th2 cytokines measured in supernatant, 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 (data not shown). 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, whether inflammatory or anti-inflammatory.

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 h, while levels of Tconv cells positive for CD25, a marker induced within 2–24 h 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 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 [22, 27, 28]. These data indicate 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 toward 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 [23, 29–32]. 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 appear to progressively decrease throughout the development of HNSCC. These data indicate 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 [24]. 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. These data contradict earlier human studies showing a local and systemic skewing from a Th1 response to a Th2 response in patients with HNSCC [5, 9]. 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 before [33–36], notably in carcinogen-induced tumors of epithelial origin [34] and involving upregulated Th17 expression [36]. 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 [25, 26]. This study revealed that the Th17 response increases in mice bearing premalignant lesions compared to control mice and then falls in mice bearing HNSCC. This could indicate that the Th17 response seen during early development of HNSCC represents an antitumor response. As Tregs and Th17 cells have been shown to have an inversely proportional relationship [37], 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.

We have evaluated the multitude of immune reactions in 4NQO-treated mice 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.

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Fig. 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. HNSCC-bearing mouse cervical lymph nodes exhibit an increased percentage of CD4⁺FcγR3⁺ cells compared to both other groups. * $P < 0.05$. Total numbers of populations (c) were determined by multiplying the percent positive by the number of cervical lymph node cells for each individual mouse. HNSCC-bearing mice have greatly increased numbers of both conventional and regulatory CD4⁺ and CD8⁺ lymphocytes compared to control and premalignant lesion-bearing mice. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. 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. Stimulated conventional T cells from HNSCC-bearing mice proliferated significantly less than stimulated control mouse conventional T cells. * $P < 0.05$. Cytometric bead array analysis (f) of CD4⁺CD25⁻conventional T-cell supernatant revealed a decrease in IL-2 release from stimulated HNSCC-bearing mouse conventional T cells compared to stimulated control mouse conventional T cells. * $P < 0.05$. 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. HNSCC-bearing mouse regulatory T cells suppressed control conventional T-cell proliferation significantly less than control mouse regulatory T cells. * $P < 0.05$

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Fig. 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. Premalignant lesion-bearing mice have an increased percentage of CD25⁺ helper T cells (CD4⁺Foxp3⁻) compared to control and HNSCC-bearing mice, while HNSCC-bearing mice have an increased percentage of CD25⁺ helper T cells and cytotoxic T cells (CD8⁺Foxp3⁻) compared to control. * $P < 0.05$; *** $P < 0.001$. Total numbers of populations (c) were determined by multiplying percent positive by number of cervical lymph node cells for each individual mouse. HNSCC-bearing mice have an increased total number of CD25⁺ and CD69⁺ helper T cells and cytotoxic T cells compared to control and premalignant lesion-bearing mice, while premalignant lesion-bearing mice have an increased number of CD25⁺ helper T cells compared to control. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$

Fig. 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. Premalignant lesion-bearing mice have an increased percentage of CD44^{high} helper T cells (CD4⁺Foxp3⁻) compared to control and an increased percentage of CD44^{high} cytotoxic T cells (CD8⁺Foxp3⁻) compared to control and HNSCC-bearing mice. * $P < 0.05$; ** $P < 0.01$. Total numbers of populations (c) were determined by multiplying percent positive by number of cervical lymph node cells for each individual mouse. HNSCC-bearing mice have an increased total number of CD44^{high} helper T cells compared to control and premalignant lesion-bearing mice and an increased number of CD44^{high} cytotoxic T cells compared to control mice. ** $P < 0.01$; *** $P < 0.001$.

Fig. 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. Cervical lymphocytes from premalignant lesion-bearing mice consist of a greater percentage of KLRG1⁺ helper T cells (CD4⁺Foxp3⁻) compared to both control and HNSCC, while cervical lymphocytes from HNSCC-bearing mice consist of a lower percentage of KLRG1⁺ helper T cells than control. * $P < 0.05$; ** $P < 0.01$. Total numbers of populations (c) were determined by multiplying percent positive by number of cervical lymph node cells for each individual mouse. HNSCC-bearing mice have an increased total number of PD-1⁺ helper T cells compared to control and premalignant lesion-bearing mice. Both HNSCC-bearing mice and premalignant lesion-bearing mice have an increased number of PD-1⁺ cytotoxic T cells (CD8⁺Foxp3⁻) compared to control. * $P < 0.05$; ** $P < 0.01$

Fig. 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 4 h with phorbol 12-myristate 13-acetate (PMA) and ionomycin in the presence of brefeldin A, with at least 9 mice per group. Both premalignant lesion-bearing mice and HNSCC-bearing mice have a greater percentage of Th1 (CD4⁺IFN- γ ⁺) and Tc1 (CD8⁺IFN- γ ⁺) cells compared to controls. * $P < 0.05$; ** $P < 0.01$. Total numbers of populations (c) were determined by multiplying percent positive by number of cervical lymph node cells for each individual mouse. HNSCC-bearing mouse cervical lymph nodes consist of a greater total number of Th1 and Tc1 cells compared to controls. Premalignant lesion-bearing mouse cervical lymph nodes consist of a greater number of Tc1 cells compared to controls. * $P < 0.05$. Cytometric bead array analysis (d) of control, premalignant lesion-bearing mouse, and HNSCC-bearing mouse cervical lymph node cells after stimulation for 4 h with PMA and ionomycin, with at least 9 mice per group. Cervical lymph node cells from both premalignant lesion-bearing mice and HNSCC-bearing mice release an increased amount of Th1/Tc1-related cytokines (IFN- γ and IL-2) and chemokines (RANTES, MIP-1 α and β) compared to control. * $P < 0.05$; ** $P < 0.01$. Cytometric bead array analysis (e) of control, premalignant lesion-bearing mouse, and HNSCC-bearing mouse tongue epithelium lysate, with normalization to 100 μ g of protein (as determined by BCA protein assay), with at least 10 mice per group. While HNSCC mouse tongue epithelium lysate contained a decreased level of IL-2 compared to control, HNSCC mouse tongue epithelium lysate contained a significantly increased level of

RANTES and MIP-1 β and a trend toward an increased level of IFN- γ and MIP-1 α compared to control tongue epithelium lysate. While premalignant tongue epithelium lysate had a trend toward a decreased level of IL-2 compared to control, premalignant mouse tongue epithelium lysate contained a significantly increased level of IFN- γ and RANTES and a trend toward an increased level of MIP-1 α and β compared to control tongue epithelium lysate. * $P < 0.05$

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Fig. 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 4 h with phorbol 12-myristate 13-acetate (PMA) and ionomycin in the presence of brefeldin A, with at least 9 mice per group. Both premalignant lesion-bearing and HNSCC-bearing mice have a greater percentage of Th17 (CD4⁺IL-17A⁺) cells than controls, and premalignant lesion-bearing mice have a greater percentage of Th17 cells compared to HNSCC-bearing mice as well. * $P < 0.05$; *** $P < 0.001$. Total numbers of populations (c) were determined by multiplying percent positive by number of cervical lymph node cells for each individual mouse. Premalignant lesion-bearing mouse and HNSCC-bearing mouse cervical lymph nodes consist of a greater total number of Th17 cells compared to controls. * $P < 0.05$; ** $P < 0.01$. Cytometric bead array analysis (d) of control, premalignant lesion-bearing mouse, and HNSCC-bearing mouse cervical lymph node cells after stimulation for 4 h with PMA and ionomycin, with at least 9 mice per group. Cervical lymph node cells from both premalignant lesion-bearing mice release an increased amount of IL-17A compared to control. ** $P < 0.01$