

Focus on TILs: Prognostic significance of tumor infiltrating lymphocytes in head and neck cancers

Ravindra Uppaluri¹, Gavin P. Dunn² and James S. Lewis Jr.^{1,3}

¹Department of Otolaryngology/Head and Neck Surgery and John Cochran VA Medical Center, Washington University School of Medicine, St. Louis, MO, USA

²Massachusetts General Hospital, Harvard Medical School, Boston, MA, USA

³Department of Pathology/Immunology, Washington University School of Medicine, St. Louis, MO, USA

The expanding and established literature that correlates tumor infiltrating lymphocytes (TILs) with outcomes of patients with solid tumors has contributed greatly to the appreciation of the interaction between the host immune system with neoplastic growth. This analysis has been limited to specific tumors, such as melanoma and ovarian cancer, and our understanding of TILs in relation to many other malignancies has yet to be explored. We review one less well studied malignancy, head and neck squamous cell carcinoma (HNSCC), and the initial attempts to examine the impact of TILs on outcomes of these patients. To provide a context for the discussion of TILs and HNSCC, we first review the epidemiology, relevant head and neck anatomy, immune responses and discuss the historical data regarding the unique immunobiology of these tumors. Finally, with this perspective, we describe our current understanding of tumor infiltrating lymphocyte data for head and neck cancers.

Keywords: human, head and neck cancer, tumor-infiltrating lymphocytes, prognosis, therapy

Head and neck cancer: A clinical perspective

Pathologically, the vast majority of tumors (95%) that arise in the head and neck region are squamous cell carcinomas arising from the upper aerodigestive tract epithelium. The progressive local growth of head and neck squamous cell carcinomas (HNSCCs) impinge on the highly critical functions of speech, swallowing and respiration. Current therapies, whether the modality is surgery alone or combined with radio- or chemoradiotherapy, leave many of these patients with significant functional deficits exacting a unique physical, social and emotional toll. Although significant advances in the areas of reconstructive surgery, minimally invasive surgery, chemotherapy and monoclonal antibody therapy have been achieved in the last two decades, the overall survival rates for patients with these cancers has been minimally affected.

Other tumors of the head and neck region include melanomas where the prognostic significance of tumor infiltrating lymphocytes (TILs) has been clearly established (1, 2). In addition, nasopharyngeal squamous cell carcinomas (NPCs) are a group of head and neck tumors that have a biological behavior quite distinct from conventional HNSCCs. Therefore, as these tumors are strongly associated with Epstein-Barr virus, are geographically localized and as a whole are managed differently than HNSCCs (3-5), they will not be considered in this review.

Epidemiology

HNSCCs are a significant public health entity in that they claim 11,000 lives a year in the United States and represent one of the top ten cancers worldwide (6, 7). The overall 5-year survival rate for patients with HNSCC is approximately 50%. Despite significant advances in the medical and surgical treatment of these cancers, this statistic has remained stable for decades.

The major independent risk factors for development of these tumors are tobacco and alcohol abuse. Many patients have simultaneous addictions to both, which synergize to greatly increase the risk of tumor development. The habits of betel nut and gutkha chewing and reverse smoking, common practices in South Asia, are also contributing carcinogenic insults. The general decrease in smoking in North America has reduced the incidence of these cancers; however, there has been an increased incidence of oral tongue, tonsil and base of tongue carcinomas in patients under the age of 45, an increase that has been attributed to HPV infection in the latter two sites [reviewed in (8, 9)].

Immunoediting in head and neck cancer

Human immunosurveillance of HNSCC

Our expanding concepts about TILs and their relation to patient prognosis have occurred in the context of a better understanding of immunosurveillance. In the last fifteen years, this previously abandoned concept has been resurrected by analysis of multiple tumor models in mice deficient in a variety of immunologically relevant cells and molecules, including T and B lymphocytes, IFN- γ , STAT1 and perforin [reviewed in (10)]. These data revealed that the immune system does indeed protect against the development of primary and chemical carcinogen-induced cancers. This result, however, raised the question of why organisms with intact immune systems develop neoplastic disease. The finding that addressed this issue came from analysis of tumors that arose in wild-type hosts compared to immunodeficient *RAG2*^{-/-} lymphocyte deficient hosts. All tumor cells (whether wild-type or *RAG2*^{-/-} derived) formed progressively growing sarcomas in *RAG2*^{-/-} mice. However, when assessed for their capacity to form tumors upon transplantation into wild-type syngeneic hosts, tumors derived from immunocompetent hosts were significantly more tumorigenic than those from immunodeficient hosts. *Thus, the immune system protects against the development of tumors and yet drives the generation of tumors that have developed mechanisms to evade elimination.* These data have been codified

as the "Cancer Immunoediting" hypothesis which includes as its first phase the older concept of tumor immunosurveillance [reviewed in (10)]. One of the key components in these studies was the ability to experimentally manipulate mice, which allowed the generation of incontrovertible evidence supporting the Cancer Immunoediting hypothesis. However, similar evidence in human tumors can only be gained indirectly and TIL analysis is one of the cornerstones of these studies in humans as exemplified by the studies of Galon, Fridman and Pagès (11-13).

One clear indication of the contribution of the immune system in controlling HNSCC is the relative increase in incidence in the context of acquired or iatrogenic immunodeficiency. King *et al.* (14) identified premalignant lip leukoplakia in 13% of renal transplant patients as compared to 0.6% of control age- and sex-matched individuals. Of the renal transplant patients with leukoplakia, a majority demonstrated dysplastic conversion and 10% of these patients (i.e. 2/21 patients with leukoplakia) had squamous cell carcinoma. Many other reports examining databases of transplant recipients have confirmed this increased incidence of lip (15, 16) and other cancers (17). In addition, analysis of patients who underwent bone marrow transplantation for hematologic malignancies also demonstrated a 17.4-fold increased risk for oral cancer, which was second only to the risk of liver cancer development (18). Again, many other studies (which are not mentioned here due to space limitations) have confirmed these general findings [for example see (19)]. An additional contributing risk factor for buccal cancers in patients who have undergone hematopoietic stem cell transplantation is chronic graft versus host disease (GVHD) that may contribute to local inflammation and tumor development (20). Due to the relatively recent appreciation of the contribution of HPV to HNSCC, all of these studies lack documentation of HPV status in lip and oral tumors in the transplant recipients. The increase in infection-related cancers in HIV positive and transplant patients has received recent attention (21). We discuss this topic in the HPV section below.

Anatomy relevant to HNSCC immunosurveillance

The complex anatomy of the upper aerodigestive tract allows for multiple functions including mastication, deglutition, phonation and airway maintenance. This portal also serves as the entry point to both the gastrointestinal and respiratory systems. Thus, cancerous growths in any part of the upper aerodigestive tract impinge on multiple overlapping functions. The mucosal subsites of the head and neck where tumors develop include the nasopharynx, paranasal sinuses, oral cavity, oropharynx, larynx and hypopharynx. Patients with tumors that develop in the larynx or oral cavity, where subjective symptoms manifest earlier, have a clinical behavior quite different than patients with tumors in the hypopharynx. Patients present much earlier because of symptoms such as hoarseness for laryngeal lesions or masses in the oral cavity, whereas lesions in the hypopharynx are tolerated much longer and thus these patients typically present with advanced disease. This varied clinical presentation must also be considered in any TIL analysis of tumor samples.

The associated lymphatic drainage of the upper aerodigestive tract tumors serves as the first echelon of regional metastatic cancer spread. The lymphatic chains within the neck are divided into 6 major levels—level I includes the submental and submandibular nodes, levels II through IV encompass the jugular chain lymph nodes by dividing the nodes into three equal segments, level V nodes are in the "posterior triangle"

which track with the eleventh cranial nerve as it leaves the sternocleidomastoid muscle and heads towards the trapezius muscle and, finally, level VI nodes are the central compartment nodes which surround the trachea near the thyroid gland. More recently, tumor involvement in the retropharyngeal lymph nodes (which include the node of Rouviere) has garnered attention as a site of tumor metastasis that was often missed in older surgical approaches (22, 23). A large body of literature based on retrospective analysis of primary tumors and their associated resected lymph node dissections showed that specific lymphatic groups serve as recipients of regional metastases (24-26). Thus, for example, tumors of the oral cavity (lip and anterior floor of the mouth) drain to level I nodes whereas tonsil tumors drain to the level II nodes. Lymph node involvement with tumor is one of the worst prognostic factors for patients with HNSCC (27)—for example, patients with oral tongue cancer have close to a 50% decrease in survival if cervical lymph nodes are involved with tumor at initial presentation [reviewed in (28)]. In addition, patients with extracapsular spread of tumor out of the lymph node have been shown to be at high risk for recurrent tumor (29-32). The underpinnings of the propensity of certain tumors for lymph node involvement remains under investigation but likely involves a combination of dependent drainage (i.e. certain subsites have easier access to lymphatic pathways than other sites) and specific molecular pathways.

The major lymphoid tissues in *direct* contact with the epithelium giving rise to HNSCC are encompassed in the Waldeyer's ring, which includes the laterally located palatine tonsils, the inferiorly positioned base of the tongue with lingual tonsillar tissue, and finally the superiorly based nasopharyngeal adenoid pad. How these structures contribute to immune responses in the oral cavity is still undefined (see below). Importantly, HNSCC originating within these regions encompass a significant number of diagnosed cases. Tumors arising in the oropharyngeal and nasopharyngeal subsites may have a constitutive heavy lymphocyte content complicating immunohistochemical analysis of lymphocytes within tumors. A second consideration for these subsites is that specific viral associations with nasopharyngeal carcinoma (Epstein-Barr virus or EBV) and oropharyngeal carcinoma (human papilloma virus or HPV) may reflect a viral antigen-specific immune response to these tumors rather than to host-derived tumor-specific antigens (see the separate section on HPV-related tumors).

Finally, the specific lymphatic drainage of the subsites is variable, thus, early regional lymphatic metastasis from supraglottic HNSCC is typical whereas glottic cancers manifest a delayed lymphatic involvement. In addition, the supraglottis is considered to be a midline structure and thus lymph flow is directed to bilateral regional lymphatic structures. It is unclear whether this type of increased drainage to regional lymphatics by one subsite results in an augmented histologic immune response. Clinically, patients with supraglottic tumors do not fare as well as similar stage glottic tumors. Thus, a hypothetical increase in exposure to the regional lymphatics does not translate to better outcomes.

Immunosurveillance of HPV-associated tumors

A significant amount of scientific literature supports an oncogenic role for HPV as a causative agent in oropharyngeal cancer leading some authors to suggest that we will, or already have, reached epidemic proportions (9). As many other excellent reviews detail the biology, clinical presentation, behavior and

Table 1
HNSCC tumor antigens.

Antigen	HLA Restriction (if known) or Incidence Information	Specific Technique	Reference
From HNSCC-specific CTLs:			
CASP-8 (mutated)	HLA-B*3503	CTL screening of cDNA	39
Cyclin B1	HLA-A2.1	CTL screening of cDNA	40
Aldehyde dehydrogenase 1 A1	HLA-A*0201 (HLA-A2) binds ALDH1A188-96 peptide	Specific CTL line used to screen tumor lysate fractions	44
From SEREX:			
AU-HN-15 (KIAA0530) most tumor specific	Total of 37 genes identified: 31 known, 6 novel	SEREX	41
KIAA0530	Total of 17 products identified including KIAA0530; similar to ref. 41	SEREX	43
From candidate approach (CT antigens or know antigens in other tumors):			
MAGEA3	MAGEA3 (72%),	RT-PCR for 23 CT antigens, no IHC or other confirmation at the level of protein	42
SSX1	SSX1 (45%),		
MAGEC2	MAGEC2 (33%),		
MAGEC1	MAGEC1 (28%),		
BAGE	BAGE (17%),		
SSX2	SSX2 (16%),		
SCPI	SCPI (12%),		
NY-ESO-1	NY-ESO-1 (6%),		
HOM-TES-85	HOM-TES-85 (4%)		
MAGE-1	RT-PCR 18/45, IHC 6/45	RT-PCR and IHC of 45 tumors	47
MAGE-3	RT-PCR 20/45, IHC 12/45		
NY-ESO-1	RT-PCR 3/45, IHC 1/45		
Wild type p53 peptides	HLA-A2 or HLA-A24	Candidate approach	45
Mutant p53 (Y220C) peptide	HLA-A*0201(HLA-A2)	Candidate approach	46

treatment of these tumors (9, 33, 34), we limit our discussion to the potential immune responses manifest as TILs.

The successful development of an HPV vaccine that targets the key oncogenic subtypes 16 and 18 has garnered a significant amount of lay press attention, focused the public's awareness on cervical cancer etiology and generated a fierce debate about this intervention in prepubescent females [for example see (35)]. However, the current strategy to vaccinate young girls does not take into consideration the vast potential benefit of also including young men in this public health campaign. For unclear reasons, men disproportionately (at a 2:1 ratio) develop HPV-associated oropharyngeal tumors. Although the vaccine is not approved for use in males, a benefit in oropharyngeal and genital tumors would be gained with immunization. Increased public awareness of HPV associations with oropharyngeal cancer [for example see (36)] will likely lead to further calls for including all youth prior to sexual maturity.

Clearly, in TIL analysis of HNSCC, virus-associated tumors must be specifically identified as the infiltrate in these tumors may be a reflection of a host T cell response to viral gene products. No studies have specifically addressed this issue but several investigators have examined the peripheral blood of patients with HNSCC for the presence of virus-specific T cells using tetramer technology. Both Albers *et al.* (37) and Hoffmann *et al.* (38) identified a 2-3 fold elevated level of HLA-A*201 restricted viral E7-specific T cells in the peripheral blood of patients with HPV+ HNSCC versus patients with HPV-HNSCC or normal controls. Interestingly, Ferris and colleagues (37) found that E7-specific cytotoxic T lymphocytes (CTLs) expanded from peripheral blood did not directly recognize an HLA-A*201 expressing HPV+ tumor cell line. They found a significant decrease of antigen processing machinery components in this cell line and in primary HPV+ tumors and determined that interferon-gamma (IFN- γ) pre-treatment of the cell line greatly enhanced CTL recognition. Thus, these investigators postulated that although virus-specific CTLs exist in HPV+ HNSCC patients, immune escape occurred due to the

inability of these CTLs to recognize the tumor. As we gain a better understanding of HPV antigens and HNSCC immune evasion mechanisms, these viral antigens may emerge as a means to target this subset of tumors.

Tumor antigens and recognition of HNSCC

Some of the most significant data supporting immune recognition of tumors derives from the identification of specific tumor antigens. The HPV-derived antigens described above obviously develop in the context of host recognition of viral machinery. However, similar to other tumors, efforts to identify novel tumor-derived products using serological identification or tumor-specific cytotoxic T lymphocytes (CTLs) have resulted in the discovery of both novel HNSCC-specific and common cancer/testis (CT) antigens (39-47). These studies are summarized in Table 1. Immunotherapy using these antigens for vaccination is at an early stage in HNSCC (48).

Development of oral tolerance in anti-HNSCC immune responses

Just as a grasp of the gross anatomy of the head and neck is a prerequisite for understanding the biology of HNSCC, understanding the local immunologic "anatomy" and immune response is important in the context of examining TIL responses. Similar to the gastrointestinal tract and brain, there are important differences in the local response that are a consideration in how an individual develops a reaction to a growing tumor, and this may play a role in the development of any immunotherapeutic strategy. However, as opposed to the gastrointestinal tract, fewer studies are available, and the vast majority is derived from the dental literature which is focused on chronic adult periodontitis in the oral cavity, or studies where application of allergens to the nasal mucosa is used to examine immune responses. We review some of these studies due to their relevance in host responses to cancer development, especially in the context of tolerogenic mechanisms driven by oral antigen exposure.

The upper aerodigestive tract environment is bathed in approximately 1 liter of saliva per day which helps in digestion, via the α -amylase and lingual lipase enzymes, and in deglutition (49). Saliva also serves an immunologic function in that it is rich in anti-microbial peptides and IgA. The mucosa of the oral cavity and oropharynx also acts as a barrier where one finds keratinization over areas that encounter shear forces (such as the tongue and hard palate) with the remainder having a nonkeratinized epithelium [reviewed in (50)]. This barrier function is distinct from skin in that there is significant permeability and vascularization, which presumably allows sampling of antigens in the oral cavity. Similar to the bacterial load within the gastrointestinal (GI) tract, the oral cavity has over 500 distinct bacterial species that exist in a commensal relationship (51). The development of tolerance to these bacteria and the lack of inflammatory reactions in the oral cavity clearly suggests that a mechanism to induce tolerance exists that likely parallels that seen in the GI tract.

Here a distinction must be drawn between oral tolerance driven by gut exposure to antigens and oral *mucosal* tolerance where the structures in the oral cavity drive immunosuppression. The former, which has been extensively reviewed, is a well-studied mechanism whereby GI tract antigens are sampled by specific cells [including CX3CR1+ dendritic cells (52)], transported to mucosa-associated lymphoid tissues (MALT - including Peyer's patches and mesenteric lymph nodes) and subsequently induce tolerogenic responses via specific cell populations (i.e. IL-10 producing regulatory T cells) and cytokines such as TGF- β . These mediators in turn suppress inflammatory responses driven by Th1 and Th17 cells, which allows tolerance to commensal organisms and food/environmental antigens. In contrast, oral mucosal tolerance is a less well-defined mechanism where similar sampling and transport to undefined inductive sites also induces a tolerogenic state. This is well illustrated in the example of adolescents who have nickel containing dental appliances who then demonstrate reduced T cell mediated responses (53). More recently, sublingual immunotherapy (SLIT) has been gaining popularity as a means to induce tolerance to environmental allergens [reviewed in (54, 55)].

Dendritic cells (DCs) are at the front line of the immunologic infrastructure and have initial contact with antigen. Studies in the sublingual region of BALB/c mice identified a rich network of CD11b+ CD11c+ cells that were also MHC class II+, expressed the CD40, CD80 and CD86 costimulatory molecules, and the CCR6 chemokine receptor (56)—cellular features which are similar to Langerhan's cells found in skin. Sublingual administration of ovalbumin induced antibody responses, specific cytokine expression and T cell proliferation but required a cholera toxin adjuvant to achieve significant levels. Studies in humans have also identified similar DC populations [reviewed in (57)], again suggesting that these cells serve as the sentinels for foreign invaders in the oral mucosa.

Once these DCs sample oral antigens, the inductive site(s) where the immune response is initiated is most likely in the cervical lymph nodes. Inductive sites for tolerance in the gut were identified using mice deficient in various components of the lymphotoxin family and revealed that mesenteric lymph nodes are critical for inducing tolerance (58) whereas the role of Peyer's patches was felt to be dispensable. The parallel MALT in the head and neck in humans is represented by the lymphoid tissue in Waldeyer's ring. Although not providing definitive proof (as not all oropharyngeal lymphoid tissue is removed), surgical removal of adenoids and tonsils in humans has gone on

for decades with no evidence of increased infections, inflammation or autoimmunity. Clearer evidence has been described in mice where tolerance to a nasal challenge with ovalbumin was absolutely dependent on the presence of cervical lymph nodes (59). Interestingly, transplanting functioning peripheral axillary lymph nodes into the cervical region of mice that had all neck lymph nodes removed did not allow for development of tolerance. However, control cervical lymph node transplants into similar mice did show induction of tolerance suggesting that regional differences in specific lymph node architecture can dictate this immune response. Studies to define the inductive sites in mice using the oral cavity as the initiating site have not been performed to date. Thus, although many of the specifics of oral mucosal tolerance have not been defined, significant parallels and differences are emerging with respect to the better defined mechanisms in the gut.

With respect to developing cancers in the upper aerodigestive tract, how does the propensity of this site for tolerance induction to commensal organisms and environmental antigens relate to the host response to nascently transformed cells? Although speculative, we suggest that this local immune response may reduce the host response to developing tumors, which obviously would be reflected in any analysis of tumor infiltrating lymphocytes. This concept has been proposed for the gut where intra-cecal injection of a BALB/c syngeneic colon carcinoma showed an increased growth rate compared to the subcutaneous flank site (60). These investigators hypothesized that tumor exposure through the MALT would result in systemic immunosuppressive effects and indeed this was borne out as they found an increased concentration of immunosuppressive TGF- β in the serum of mice 14 days after subserosal cecal implantation of tumor cells relative to the subcutaneous site. O'Sullivan and colleagues (61, 62) have proposed the same idea in relation to cancers of the foregut.

Escape: How HNSCC evades the immune response

Tumor cells from all sites have evolved multiple pathways for both active and passive immune evasion [reviewed in (63)] and HNSCC echoes many of these themes [reviewed in (64)]. Many early studies examining global immunosuppression induced by cancers primarily involved patients with HNSCC (65-69). These studies utilized reactivity to 2,4-dinitrochlorobenzene (DNCB) as an indicator of cell-mediated immune response, and the general sense at that time was that patients with HNSCC were more immunocompromised than patients with other cancers and that worsening reactivity to DNCB correlated with a poorer prognosis for patients (66, 67).

A better understanding of the molecular and cellular basis of immune responses and regulation has led to a more detailed analysis of the underpinnings of these early studies. Several pathways utilized by HNSCC have been delineated to account for tumor immune evasion [reviewed in (64)]. Some of these mechanisms include immunosuppressive myeloid-derived suppressor cells (MDSCs) (64, 70), decreased HLA class I expression by tumor cells (71-74), tumor-induced T cell apoptosis (75), regulatory CD4+ CD25+ T cells (76, 77), galectin-1 expression by tumor cells (78, 79) and tumor-induced senescent T cells with suppressor function (80). The interrelationships between these putative mechanisms and how they may influence TILs has not been explored in detail.

Table 2
Studies on TILs in HNSCC.

TIL Correlation with Survival	Study Type	TIL Data and Outcome	Number and Type of Tumors	Reference
Positive	IHC for CD4 ⁺ or CD8 ⁺ T cells/HPF	Parenchymal CD4 ⁺ but not CD8 ⁺ T cells associated with survival benefit	40; diverse stages/subsites	82
	FACS based	Improved prognosis for patients with CD4/CD8 ratios less than 1	16; diverse stages/subsites	84
	H&E, Multivariate analysis of pathologic grading of lymphocytic infiltrate	Weak/limited response is associated with increased recurrence, decreased survival	208; oral cavity	81
	IHC for class I components-graded. CD8 ⁺ T cells counted in 0.25 mm ² of tumor	CD8 ⁺ infiltration assoc. with better CSS but not DFS. Decreased class I components assoc. with poor prognosis	63; laryngeal	73
	IHC for CD3 ⁺ T cells, infiltrate was graded. Also, analyzed galectin-1 staining	Inverse correlation between CD3 ⁺ T cells and galectin-1, weak CD3 ⁺ correlated with poor overall survival.	101; diverse stages/subsites	78
	Double/triple IF for CD4 ⁺ , CD8 ⁺ , CD69 or FoxP3, 5 HPFs per tumor	No correlation with CD8s, but improved overall survival with CD4 ⁺ CD69 ⁺ cells and better locoregional control with Tregs	84; 5 subsites, 47/84 T3 or T4, 44/84 with +nodes	86
Negative	IHC for CD3 ⁺ T cells in tissue microarray, graded the infiltrate in the 0.6 mm of tumor	Overall, no statistical difference	48; oropharyngeal/diverse stages. 32/48 patients with HPV-16 DNA	85
	IHC for CD4 ⁺ or CD8 ⁺ T cells/HPF	No statistical difference with increased T cells	26; diverse stages/subsites	83

Current status of TIL analysis in HNSCC

The available studies on TILs in HNSCC are not definitive (summarized in Table 2). The major limitation in existing reports is the low number of patient samples available for analysis and the heterogeneity in tumor stages, which eventually impacts the interpretation of histologic data. Given this backdrop, specific studies have arrived at conclusions either supporting or dismissing the prognostic value of lymphocyte infiltration into HNSCC.

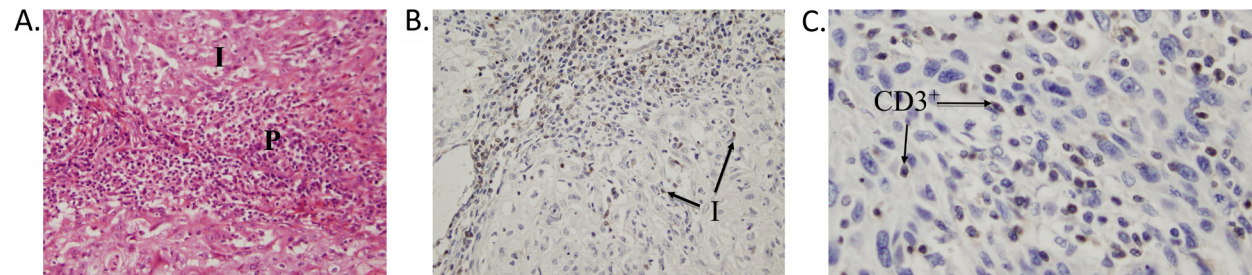
As for many other tumor types, initial prognostic studies on HNSCC were limited to subjective observation of lymphocyte infiltration on H&E (hematoxylin and eosin) stained tumor specimens. One detailed study that illustrates this type of analysis assessed lymphocyte infiltration into over 200 oral squamous cell carcinomas (81). Using multivariate analysis, these investigators identified that a weak or limited lymphocyte response at the tumor and stromal margin was associated with increased locoregional recurrence and decreased overall survival. Many other pathologic criteria were also examined but the lack of definition of what actually constitutes the lymphocytic infiltrate demonstrates the limitation of this approach.

The first studies examining specific lymphocytes were performed in the 1980s using newly available monoclonal antibodies for specific T cells (82). Using a selected cutoff number of cells per high power field (HPF), Wolf and colleagues identified a survival benefit for patients who had intra-tumoral CD4⁺ T cell, but not CD8⁺ T cell, infiltration. However, the significant limitations in this study included a 9.5 month average follow-up (range 2-25 months), limited tumor numbers and heterogeneous tumor sites. The largest subset of tumors in their 40-patient study was a group of 10 patients with oral cavity tumors. In contrast, Guo and colleagues (83) examined 26 patients, again from diverse subsites, and found a trend towards improved survival with increased numbers of all T lymphocyte subsets but statistical significance was not achieved. Finally, Snyderman *et al.* (84) performed a fluorescence activated cell sorter (FACS) based assay on TIL preparations from 16 patients with various stages of HNSCC. These investigators identified a

better prognosis in patients who had CD4/CD8 ratios less than 1 and therefore suggested that the lack of CD8 cells in the tumor may have led to poorer outcomes. Thus, these early attempts at analyzing TILs in HNSCC yielded some provocative but mixed results, and ultimately were inconclusive due to the limitations described.

Following these initial studies, the attention of immunologists studying HNSCC turned away from potential prognostic information provided by TILs towards the incapacitating effects of the tumor on host immune cells. However, with the renewed interest in TIL studies in the current decade, more specific evaluation approached the definition of the composition of the cellular infiltrate, either directly or indirectly, in the context of studies on immunosuppressive molecules. Sewell and colleagues (85) generated a tissue microarray containing 48 different oropharyngeal squamous cell cancers and analyzed CD3⁺ T cells within these tissue cores (by immunohistochemistry). This study concluded that overall tumors that were classified as CD3^{high} exhibited decreased rates of metastasis compared to those that were CD3^{low}. Interestingly, the largest tumors overall (grade T4) had the lowest CD3 levels. However, when the tumors were divided between those that were HPV positive and those that were HPV negative, this finding only held for the virus positive tumors. Notably, 32/48 tumor specimens had detectable HPV-16 DNA. Analysis of overall survival in relation to CD3 content did not show statistical significance. The limitations of this study include the small number of patients and the technique for counting the CD3⁺ cells. The cellular infiltrate was graded, not counted, and was limited to the tissue core which represented 0.6 mm of the tumor.

Other investigators have examined specific lymphocytic infiltration in the context of studies on immunoevasion by HNSCC. Le *et al.* (78) identified the hypoxia inducible production of galectin-1 in the supernatants of squamous cell carcinoma cell lines. As galectin-1 has been shown to have apoptotic effects on T cells, these investigators examined CD3⁺ T cell infiltration in 101 HNSCCs and found that there was a significant inverse association between galectin-1 expression and CD3⁺ T cell infiltration. In multivariate analysis, either

Figure 1

Representative histology and IHC for CD3+ T cells in a supraglottic squamous cell carcinoma. (A) H&E stained section showing lymphocytic infiltrate, particularly prominent at the periphery of large tumor nests (200x). (B) Low power (200x) view of CD3+ T cell infiltration in peritumoral stroma and into the periphery of large tumor nests. (C) High power (400x) view of CD3+ T cell IHC. Abbreviations: P, peritumoral; I, intratumoral.

strong galectin-1 staining or weak CD3+ T cell staining predicted for a poor patient outcome.

CD8+ T cell infiltration and patient outcomes were examined in two studies which came to opposing conclusions. Ferrone and colleagues (73) examined antigen processing machinery (APM) deficiencies in HNSCC as a mechanism of immunoevasion and also quantified CD8+ T cell infiltration. Using specific monoclonal antibodies (mAbs) to various APM components, they analyzed 63 tumor specimens from glottic, supraglottic and subglottic sites and found LMP2 and HLA class I down regulation, in addition to decreased CD8+ T cell infiltration, was correlated with poorer patient outcomes. Decreased class I molecule expression by tumor cells correlated with decreased CD8+ T cell infiltration ($P < 0.001$). CD8+ T cell infiltration was analyzed by dichotomizing patients into those who had more than 20 cells/HPF and those that had fewer than 20 cells/HPF within the tumor. A significant association for cause specific survival (CSS), but not disease free survival (DFS), was observed. How CD8+ T cells do not contribute to recurrence but do contribute to improved cancer-specific survival is unclear. In addition, as opposed to other TIL studies, this study determined CD8+ T cell infiltration by counting cells in a 0.25 mm^2 area of tumor (as opposed to the more typical 10-20 HPFs) and the survival curve analysis did not examine CD8+ T cells as a continuous variable. Notwithstanding these comments, the significant correlation between decreased APM components in tumors and decreased CD8+ T cell infiltration does suggest that tumor antigens being presented in the context of HLA class I are being recognized by specific immune components which impact on survival.

In contrast to the study of Ferrone and colleagues (73), Badoual *et al.* (86) reported that CD4+ T cell, but not CD8+ T cell, infiltration was significantly correlated with patient survival. They examined tissue from 84 patients with HNSCC arising from 5 different subsites with the majority of tumors (47/84; 56%) being stage T3 or T4 and the majority of patients (44/84; 52%) having regional lymph node metastasis. Surprisingly, analysis of intratumoral and stromal regions did not reveal an association between CD8+ T cell infiltration and locoregional control or overall survival. As expected, better locoregional control and overall survival was observed with lower T stage in multivariate analysis. Interestingly, better locoregional control was observed with increased numbers of CD4+ FoxP3+ regulatory T cells, which the authors postulate decrease local inflammation thus impeding tumor growth. This finding is in contrast to studies in ovarian cancer, where the presence of regulatory T cells bode poorly for patients (87). For overall survival, only the presence of increased numbers of CD4+

CD69+ activated phenotype T cells correlated with improved patient outcomes. Thus, this study is the first to correlate CD4+ CD69+ T cells in TILs as a positive prognostic factor for overall survival.

Finally, our own analysis (JSL and RU, unpublished) of over 35 supraglottic squamous cell carcinomas did not identify any correlation between specific CD3+, CD4+ or CD8+ T cell infiltration and survival. We counted 10 HPFs per tumor and performed statistical analysis using these counts as a continuous variable. A representative section of immunohistochemistry for CD3+ T cells infiltrating a supraglottic squamous cell tumor is shown in Figure 1.

Additional immune cell subsets within HNSCC

Although the focus on TILs implies antigen-specific infiltrating cells, other adaptive and innate hematopoietic cells contribute to the HNSCC tumor microenvironment and several reports have addressed their possible contribution to tumor behavior (64, 76, 88, 89). These other cellular subsets include myeloid-derived suppressor cells (MDSCs), FoxP3+ regulatory CD4+ T cells, tumor-associated macrophages (TAMs) and plasmacytoid dendritic cells (pDCs). MDSCs in mice are a bone marrow-derived Gr-1+ CD11b+ immature myeloid population that has been shown to inhibit T cell responses by several mechanisms, including expressing arginase I which deprives T cells of the critical amino acid arginine [reviewed in (63)]. HNSCC was one of the first human cancers where similar cells were identified as CD34+ progenitor cells that inhibited T cell responses (70, 90) and in a small analysis were shown to correlate with poor patient outcomes (91). Further, analysis of these cells in HNSCC has been limited. A second major immunosuppressive subset of cells are regulatory CD4+ T cells that express the forkhead/winged-helix transcription factor FoxP3 and at least in part mediate their actions via IL-10 and TGF- β . Whiteside and colleagues (76) have identified these cells in the peripheral blood and TILs of patients with HNSCC. Interestingly, these cells persist in the peripheral blood after complete tumor eradication in patients with no evidence of disease. As detailed above and in contrast to the studies of Curiel and colleagues (87), Badoual *et al.* (86) found that Treg infiltration into tumors was correlated with better locoregional control.

Macrophage infiltration into tumors including breast, prostate and bladder has been shown to correlate with poor patient prognosis [reviewed in (92)]. Similarly, Teknos and colleagues (89) examined 102 oral cavity HNSCCs and identified that TAMs were correlated with an increased propensity for tumors

to exhibit regional metastatic capacity and extracapsular extension, which are known risk factors for worse patient outcomes. Interestingly, this connection also held true for smaller grade T1 or T2 tumors where regional metastasis is seen less frequently than in advanced tumors. Further mechanistic exploration of how macrophages may promote regional metastatic activity has not been explored in HNSCC.

Concluding remarks

As we analyzed the literature in preparing this review, it became apparent that no definitive studies exist either supporting or dismissing the prognostic relevance of TIL analysis in HNSCC. As with many studies on human cancer, a limited number of samples and diverse stages and subsites clouds the interpretation of the available data. In addition, the very recent appreciation of HPV-associated HNSCC as a relatively distinct clinical entity necessitates a reanalysis of TIL studies with viral infection as a dichotomizing variable. The significant implications of virus-associated HNSCC and other points highlighted in this review indicate arenas for future work on the important topic of TILs and HNSCC solid tumor biology.

Abbreviations

HNSCC, head and neck squamous cell carcinoma

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Contact

Address correspondence to:

Dr. Ravindra Uppaluri
Washington University School of Medicine
Department of Otolaryngology/Head and Neck Surgery
Box 8115, 660 South Euclid Avenue
St. Louis, Missouri 63110
USA
Tel.: + 1 314 362-6599
E-mail: uppalurr@wustl.edu