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Expression profile and in vitro blockade of programmed death-1 in human papillomavirus–negative head and neck squamous cell carcinoma

Ian-James Malm, MD^{1,2}, Tullia C. Bruno, PhD², Juan Fu, PhD¹, Qi Zeng, PhD¹, Janis M. Taube, MD³, William Westra, MD³, Drew Pardoll, MD, PhD², Charles G. Drake, MD, PhD², and Young J. Kim, MD, PhD^{1,2,*}

¹Department of Otolaryngology – Head and Neck Surgery, Johns Hopkins Medical Institutions, Baltimore, Maryland ²Department of Oncology, Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins Medical Institutions, Baltimore, Maryland ³Department of Pathology, Johns Hopkins Medical Institutions, Baltimore, Maryland

Abstract

Background—Treatment with a blocking programmed death-1 (α PD-1) antibody recently showed clinical efficacy for various tumor types. In this study, we characterized the expression profile of PD-1/programmed death-ligand-1 (PD-L1) and the potential of PD-1 blockade in human papillomavirus (HPV)-negative head and neck squamous cell carcinoma (HNSCC).

Methods—Lymphocytes from peripheral blood, draining lymph nodes, and the tumor were phenotyped for PD-1 expression, and their proliferative activity was assessed in the presence of blocking α PD-1 treatment. Primary tumor expression of PD-L1 was also analyzed using immunohistochemistry (IHC).

Results—Lymphocyte PD-1 expression was abundant with highest expression in the tumor, and in vitro mixed lymphocyte reaction demonstrated that PD-1 blockade could induce T cell proliferation. Furthermore, tumor cells were found to have 3 distinct patterns of PD-L1 expression with over 78% of the specimens demonstrating strong PD-L1 positivity.

Conclusion—Our data strongly supports the use of α PD-1 blockade in patients with HPV-negative HNSCC that are refractory to standard treatments.

Keywords

head and neck squamous cell carcinoma (HNSCC); T cell; programmed death-1 (PD-1); anergy; immunotherapy

*Corresponding author: Y. Kim, Department of Otolaryngology – Head and Neck Surgery, Johns Hopkins University School of Medicine, 1650 Orleans Street, CRB1 Building, Suite 4M61, Baltimore, MD 21231. ykim76@jhmi.edu.

INTRODUCTION

Modest improvements in the survival rates of head and neck squamous cell carcinoma (HNSCC) have been reported with the advent of concurrent chemoradiation, surgical ablation advancements, reconstructive methods, and biological treatments, but treatment failures remain common and it is unclear if substantial improvements can be achieved with these standard treatment modalities.¹ In this light, tumor immunotherapy has become an attractive prospect for the treatment of HNSCC. Despite multiple strategies to unleash the adaptive T-cell response against the tumor, cancer immunotherapy had yet to reach its potential until recently.^{2,3}

One obstacle has been the failure to address critical immune checkpoint pathways that can restrain T-cell function in the context of inflammation.⁴ The signaling of these checkpoint molecules in T cells can prevent an anti-tumor response in patients despite the presence or exogenous addition of primed antitumor T cells.^{5,6} Programmed death-1 (PD-1) is one clinically significant checkpoint molecule that has been shown to suppress T-cell function upon the binding of one of its ligands, programmed death ligand-1 (PD-L1), that is expressed on tumor cells in both preclinical models and clinical settings of patients with cancer.^{7,8} In HNSCC, Strome et al⁸ demonstrated that inhibitory co-receptor signals (signal 2) on T cells mediated by PD-1 can impede the antitumor response in a pre-clinical model. Recent clinical trials of blocking α PD-1 (nivolumab; Bristol–Myer Squibb, New York, NY) resulted in significant survival benefit without significant toxicity (grade 1 or 2) in patients with advanced melanoma, lung cancer, and renal carcinoma.^{9,10} What was most striking in these reports was the durable responses with monotherapy on patients whose treatment had failed in previous clinical trials with other modalities. Other studies that combined nivolumab with anti-CTLA-4 antibody ipilimumab (Bristol–Myer Squibb) in advanced melanoma confirmed the demonstrable clinical responses in targeting the immune checkpoint molecules.¹¹

Interestingly, Taube et al¹² found that tumor expression of PD-L1 was tightly correlated with clinical responsiveness to nivolumab in the early clinical trials, supporting the adaptive immune resistance hypothesis. Using human melanoma samples, they showed that tumor PD-L1 expression, driven by interferon-gamma (IFN- γ), inversely correlated with tumor infiltrating lymphocytes in melanoma. For HNSCC, similar expression patterns of PD-L1 and IFN- γ in human papillomavirus (HPV)-associated HNSCC further corroborated the theory of adaptive immune resistance in the context of virus-driven oncogenesis.¹³ In conjunction with these clinical correlates, our laboratory demonstrated the mechanistic features of this adaptive immune resistance in murine models (in press). Cumulatively, there are now both mechanistic and clinical findings to justify the targeting of the adaptive immune resistance mechanism in malignancies that are refractory to standard treatments.

As for clinical translation in HNSCC, patients with HPV-associated tumors are known to have a significantly improved prognosis in comparison to HPV-negative oropharyngeal HNSCC, and because of this, we focused our attention on the expression profile of PD-1 and PD-L1 in HPV-negative tumors.¹⁴ HPV-associated oropharyngeal tumors are more responsive to both chemoradiation and surgical treatments, and there are currently multiple

deintensification trials for HPV-associated tumors.^{14,15} Because of these reasons, accrual rates for enrolling patients with HPV-associated oropharyngeal HNSCC who have undergone unsuccessful standard treatment modalities in a clinical trial may be problematic. We recently examined our series on HPV-associated patients with HNSCC who either had recurrence locoregionally or distally in a busy tertiary academic center, and we found that there were less than 5 patients per year that would meet the aforementioned criteria (submitted).

Furthermore, in order to directly characterize the PD-1+ T cells from patients with HNSCC, we examined the functional consequences of PD-1 blockade in harvested CD4 and CD8 T cells from patients with HNSCC. This is scientifically significant because previous reports suggested that PD-1+ T cells can display an “exhausted” anergic phenotype that may affect the efficacy of PD-1 blockade in patients.^{16,17}

MATERIALS AND METHODS

Patient population and clinical samples

All specimens were acquired under a Johns Hopkins Medicine Institutional Review Board approved protocol and written informed consent was obtained from all patients. None of the patients were previously treated with immunosuppressive therapy. Patient-matched peripheral blood samples were collected on the day of surgical resection and refrigerated until a Ficoll gradient (GE Healthcare, Little Chalfont, Buckinghamshire, UK) was used to separate lymphocytes. Tumor tissue and draining lymph nodes with no tumor involvement were collected for processing on the day of surgery. Lymphocytes were collected from the draining lymph nodes by gentle homogenization. Tumor was treated with the enzyme Liberase (Roche Applied Science, Penzburg, Upper Bavaria, Germany) at 35 µg/mL for 1 hour at 37°C in Roswell Park Memorial Institute medium to release tumor infiltrating lymphocytes (TILs). Total lymphocytes from all 3 compartments were then washed in phosphate-buffered saline (PBS) and resuspended in 1 mL of Dynal Buffer (PBS supplemented with 25% bovine serum albumin). If required, CD4 and CD8 T cells were positively isolated using the Dynal CD4 or CD8 Positive Isolation Kit (Invitrogen, Carlsbad, CA). Positively isolated CD4 and CD8 T cells were then washed once with Roswell Park Memorial Institute media supplemented with 1% L-glutamate, 1% nonessential amino acids, 1% sodium pyruvate, 1% Pen-Strep, and 10% fetal calf serum (complete media). Monoclonal antibodies against hCD4 or hCD8 (APC, BD Biosciences, San Jose, CA) were used to stain 5000 cells to confirm the purity of positively isolated CD4 and CD8 T cells.

Antibody staining and flow cytometry

CD4 and CD8 T cells from the peripheral blood lymphocytes (PBLs), draining lymph nodes, and TILs were stained for PD-1 (APC conjugate; BD Biosciences) and lymphocyte-activation gene 3 (LAG-3; FITC; Lifespan Biosciences, Seattle, WA) expression. The cells were also co-stained with CD4 or CD8 (PE or PE-Texas Red, PerCP-Cy5.5, respectively, BD Biosciences) for these analyses. For sorting, peripheral blood mononuclear cells were stained with CD3 (AF-700; BD Biosciences) and PD-1 (APC; BD Biosciences), and CD3+ cells were sorted based on PD-1 expression using the FACSaria (BD Biosciences). Sorted

cells were stimulated in a 96-well plate for 3 hours at 37°C with PMA (20 ng/mL) and ionomycin (1 µg/mL) in the presence of Golgi-stop (BD Biosciences). Cells were then stained for CD4 (PE-Texas Red; BD Biosciences), CD8 (PerCP-Cy5.5; BD Biosciences), and intracellular IFN-γ (FITC; BD Biosciences) using the BD Cytotfix/Cytoperm kit, in accord with the manufacturer's recommended protocol. Flow cytometry was conducted using a FACSCalibur (BD Biosciences) or LSR II (BD Biosciences) and data were analyzed using FlowJo software (Tree Star, San Carlos, CA).

Mixed lymphocyte reaction

Patient CD4 or CD8 T cells were cultured in complete media with dendritic cells generated from a normal donor at a ratio of 10 T cells to 1 dendritic cell in a 96-well round-bottom plate in duplicate or triplicate. Because of the limited number of T cells from TIL, 30,000 T cells per replicate from PBL and draining lymph node was standard, whereas only 5000 T cells per replicate from the TIL were used. The αPD-1 or isotype control (which was a generous gift from A. Korman) was added to the culture at a final concentration of 50 µg/mL. If cultures were given interleukin (IL)-2, the final concentration was 200 U/mL (R&D Systems, Minneapolis, MN). The mixed lymphocyte reaction (MLR) reaction was cultured for 5 days. On day 5, supernatants from the culture were collected, tridium (H^3) was added at a concentration of 1:200, and 18 to 24 hours later the cultures were harvested to analyze proliferation via thymidine incorporation.

Dendritic cell generation

CD14+ monocytes were isolated from peripheral blood mononuclear cells of a normal donor post Ficoll (GE Healthcare) separation of lymphocytes using the Miltenyi positive isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). Counted cells were resuspended in a 24-well plate at 0.5×10^6 /mL in complete media supplemented with IL-4 (100 ng/mL; Peprotech, Rocky Hill, NJ) and granulocyte-macrophage colony-stimulating factor (50 ng/mL, R&D systems). Between days 4 and 6 of cell culture, cells were matured with lipopolysaccharide (100 ng/mL; Sigma-Aldrich, St. Louis, MO). Maturation of the dendritic cells was assessed by staining for major histocompatibility complex class II (FITC; BD Biosciences), B7-2 (CD86 PE; BD Biosciences), and PD-L1 (PE; BD Biosciences).

Enzyme-linked immunosorbent assay

Supernatants from the mixed lymphocyte reactions were analyzed for IFN-γ production via a human enzyme-linked immunosorbent assay kit from R&D systems, in accord with the manufacturer's recommended protocol.

Immunohistochemistry

Harvested HNSCC tumor specimens were fixed in PBS with 10% formalin overnight at room temperature. Tissue was embedded in paraffin, cut into 5-µm sections, and mounted on glass slides. Immunohistochemistry (IHC) for human PD-L1 and isotype control was performed as previously described.¹² IHC for select cell lineage markers, including CD3, was performed on adjacent 5-µm sections according to standard automated protocols.

Statistical analyses

Differences between data groups were compared using a one-sided *t* test in the PRISM software (Graphpad Software, San Diego, CA).

RESULTS

Programmed death-1 is expressed on CD4 and CD8 T cells from patients with head and neck squamous cell carcinoma in peripheral blood lymphocytes, draining lymph nodes, and tumor infiltrating lymphocytes

We first analyzed PD-1 expression on patients' with HNSCC CD4 and CD8 T cells from the PBLs, draining lymph nodes, and TILs to determine the distribution of the immune checkpoint molecule on the cell surface. Overall, we found abundant PD-1 expression on both the CD4 and CD8 T cells at all 3 sites. In comparison to LAG-3, another immune checkpoint molecule expressed on T cells, we found abundant PD-1 expression and its relative expression level was significantly higher than LAG-3 expression on both the CD4 and CD8 T cells at all 3 sites (Figure 1A). PD-1 expression was comparable on CD4 and CD8 T cells from the PBL and draining lymph node in our HNSCC population. PD-1 expression in healthy peripheral blood donors is typically under 15% (data not shown); however, over 30% of the lymphocytes from our study population were PD-1 positive in all 3 sites that were surveyed (Figure 1B). In comparing CD4 and CD8 TILs for PD-1 expression, they both had a significantly higher expression of the checkpoint molecule compared to the PBL ($p < .0001$ and $p = .003$, respectively). At the site of the tumor, over 50% of both CD4 and CD8 T cells expressed PD-1. Over 20 patients were analyzed and, cumulatively, these phenotypic data indicated that CD4 and CD8 T cells from patients with HNSCC have abundant PD-1 expression, which has been described as a marker of T-cell exhaustion in the context of chronic infection.^{17–19}

Blockade of programmed death-1 enhances T-cell function in vitro

After phenotyping the T cells from patients with HNSCC for PD-1 expression, we queried whether this immune checkpoint molecule has functional significance in patients. We used the MLR assay with cultured dendritic cells from normal subjects as antigen presenting cells, and assayed T cells from PBLs and lymph nodes from cancer patients with or without blocking antibodies. For the purpose of MLR, there were insufficient TILs for this assay, so we examined only T cells from PBLs and draining lymph nodes. Figure 2 is representative of MLR from draining lymph nodes in the presence of a blocking α PD-1 antibody. MLRs for both CD4 and CD8 T cells from the PBLs were comparable to that from the draining lymph nodes (data not shown). In both draining lymph nodes and PBLs, we observed a consistent enhancement of T cell function with PD-1 blockade. Blocking α PD-1 antibody enhanced CD4 and CD8 T cell proliferation significantly ($p < .0001$ and $p = .0004$, respectively). This was correlated with significantly greater IFN- γ production with PD-1 blockade in both CD4 ($p = .0179$) and CD8 ($p = .0427$) populations. These MLRs demonstrated that PD-1 blockade can potentially reverse the immunosuppressive phenotype in patients with HNSCC, but they also questioned the notion that PD-1+ cells are irreversibly exhausted T cells in patients with HNSCC.

Interleukin-2 treatment alone enhances CD4 and CD8 T cell function

To corroborate MLR assays, we determined if draining lymph node CD4 and CD8 T cell function could be rescued with the addition of IL-2, a physiologic stimulator of both CD4 and CD8 T cells, alone or in combination with PD-1 blockade (see Figure 3). We found that the addition of IL-2 increased CD4 and CD8 T cell proliferation significantly ($p = .0001$ and $p < .0001$, respectively), and, like the α PD-1 blockade, IL-2 significantly increased IFN- γ production in both the CD4 and CD8 T cells ($p < .0001$ and $p = .0002$, respectively). Interestingly, combining IL-2 and α PD-1 blockade only had a statistically significant effect on T-cell proliferation in CD4 T cells compared to just IL-2 alone ($p = .0038$).

Programmed death-1+ T cells can be stimulated to express a potent Th1 cytokine, interferon-gamma

The MLR functional assays noted above stimulated both PD-1 positive and PD-1 negative T cells, so to directly test the effect of PD-1 blockade on PD-1+ cells from HNSCC, we sorted PD-1 high and negative CD3+ T cells from PBLs from patients with HNSCC. Upon stimulation with PMA/ionomycin, PD-1^{high} CD8 T cells produced IFN- γ (see Figure 4). When we compared CD4 versus CD8 PD-1^{high} cells, we noted that CD8 cells were more amenable for activation (data not shown).

Human papillomavirus-negative head and neck squamous cell carcinoma tumors express programmed death ligand-1

Based on the abundance of PD-1+ T cells in HNSCC and the potential for blockade in the HNSCC population with commercially available anti-PD-1 antibody, we stained for its ligand, PD-L1, on HPV-negative tumor tissue. PD-L1 expression has been intimately associated with efficacy of anti-PD-1 blockade in multiple clinical trials of other solid malignancies.¹² Using IHC, we found abundant expression of PD-L1 on HPV-negative tumor specimens from the oropharynx and larynx. As previously described in melanoma, we found 3 patterns of PD-L1 expression, as seen in Figure 5: absence of PD-L1, regional expression of PD-L1 colocalized with invading CD3+ lymphocytes, and global PD-L1 expression that is out of proportion to invading CD3+ lymphocytes. Of the 9 tumor specimens we analyzed, 2 were PD-L1 negative (11%), 5 had regional expression with colocalized CD3+ lymphocytes (56%), and 4 had diffuse PD-L1 with few infiltrating lymphocytes (44%).

DISCUSSION

Based on the recent publication of successful clinical studies using blocking α PD-1 antibodies in several types of advanced malignancies, we analyzed the expression of PD-1 on both CD4 and CD8 T cells from HPV-negative patients with HNSCC undergoing surgical resections.^{10,20} Although the PD-1/PD-L1 expression pattern was recently described in HPV-associated oropharyngeal HNSCC,¹³ those tumors are more responsive to both surgical and nonsurgical treatments,^{14,15} and current efforts are focused on the de-escalation of treatment for these tumors. Our clinical series found only 47 locoregional recurrences and distant metastasis for HPV-positive tumors in nearly 10 years at an active tertiary academic center, consistent with the excellent prognosis seen in HPV-positive

tumors from other head and neck cancer centers (submitted). We therefore focused on HPV-negative patients with HNSCC, as these patients are more likely to have recurrences after standard treatment modalities and to be candidates for immune checkpoint blockade clinical trials.

Currently, there are several “targetable” immune checkpoint blockade molecules.⁴ Of these, the 2 molecules that have been shown to be clinically effective are CTLA-4 and PD-1. Significant clinical responses from ipilimumab (anti-CTLA-4) and nivolumab (anti-PD-1) in multiple metastatic cancers^{20,21} have prompted combination therapy with ipilimumab and nivolumab, which demonstrated an objective response rate of 40%.¹¹ Of these two antibodies, nivolumab has a better profile in terms of toxicity. Most of nivolumab’s toxicity is grade 1 or 2, whereas ipilimumab’s trial in melanoma showed a higher percentage of grade 3 toxicities.^{20,21} With the goal of initiating clinical trials with PD-1 blockade, we focused on the expression profile of PD-1 in patients with HNSCC as well as looking at PD-L1 expression on the tumor specimens.

In all of our samples tested, PD-1 was expressed at some level on both CD4 and CD8 T cells. The PD-1 expression profile was comparable on T cells from the peripheral blood and draining lymph nodes, but was significantly higher on T cells from the tumor microenvironment. Although about 20% to 30% of CD4 and CD8 T cells from the peripheral blood and draining lymph nodes were positive for PD-1, over 50% of the CD4 TILs were PD-1+ and over 60% of the CD8 TILs were PD-1+. Blockade of PD-1 significantly increased the proliferative index and IFN- γ production of the T cells under stimulating conditions using an MLR. Sorted PD-1 positive cells showed that they can be induced to express IFN- γ . Interestingly, we observed a more robust stimulation of PD-1+ CD8 cells in the peripheral blood when compared to the PD-1 negative population. In animal models of chronic infection, PD-1 is seen as a marker of anergy,¹⁶ but results from our laboratory and our colleagues (unpublished) suggest that, although PD-1+ cells in the TIL compartment may be anergic, PD-1+ cells in the peripheral blood have the capacity to be activated. Further work is needed to determine the full functional capacity of circulating and tumor infiltrating PD-1+ T cells. Nonetheless, both of these functional experiments strongly suggest that PD-1 positive cells from patients with HNSCC do not demonstrate the exhausted “anergic” cells as described in murine studies.¹⁶

In the nivolumab clinical trial, expression of PD-L1 on the tumor correlated with clinical response to the blocking PD-1 antibody.²⁰ Strome et al⁸ has previously demonstrated PD-L1 expression in HNSCC samples; however, they did not comment on PD-L1 expression patterns nor did they correlate their findings with the presence of TILs. Because of this, we wanted to further characterize PD-L1 expression and lymphocyte infiltration in non-HPV HNSCC. In this study, we report patterns of tumor PD-L1 expression and lymphocyte infiltration similar to those previously described in melanoma.¹² As shown in Figure 5, over 80% of the tumor samples tested showed some form of PD-L1 expression on immunohistochemistry. Interestingly, the patterns we observed may be driven by 2 distinct mechanisms. As Taube et al¹² described in their theory of adaptive resistance, IFN- γ expression by TIL leads to local PD-L1 expression by the tumor, allowing it to “escape” immune surveillance. This adaptive change in PD-L1 expression would be expected to

correlate with local TIL, as seen in Figure 5B. On the other hand, diffuse expression of PD-L1 with little or no lymphocytic infiltration, as seen in Figure 5C, may be a result of intrinsic expression by the tumor, unrelated to infiltrating lymphocytes. Intrinsic tumor expression of PD-L1 has been described in association with phosphatase and tensin homolog deficiency in gliomas and in some HPV-negative HNSCC cell lines.^{13,22} Distinct tumor biology may thus account for the differential expression of PD-L1 and further research is needed to determine whether this distinction is clinically relevant.

A recent article by Lyford–Pike et al¹³ characterized the expression profile of PD-1 and PD-L1 in HPV-associated HNSCC. They likewise reported a high percentage of PD-1+ T cells in the tumor compartment and also found a higher percentage of PD-1+ lymphocytes within the CD8+ population. They reported that 70% of their HPV-associated HNSCC tumor samples expressed some level of PD-L1, which is similar to the rate we found in our cohort. However, they only noted a 29% PD-L1 positivity rate among a small cohort of HPV-negative HNSCC. This may be due to the fact that both the HPV-associated and HPV-negative samples in their cohort were from lingual or palatine tonsils. Our cohort of HPV-negative samples, however, is derived from all head and neck subsets, including the oral cavity, oropharynx, and larynx. Furthermore, our data is novel in that we provide functional evidence of PD-1 blockade in these patients. Cumulatively, our findings provide clear clinical evidence to introduce PD-1 blocking antibody in patients with HNSCC whose standard modality treatments have failed.

CONCLUSION

Cumulatively, these studies demonstrate the need to continue the investigation of the PD-1 immune checkpoint blockade in HNSCC. In this study, we show not only the expression of PD-1 on the T cells from HPV-negative patients with HNSCC and the abundance of its ligand in tumor tissue, but we also demonstrate that α PD-1 blockade can increase T-cell proliferation and IFN- γ production in vitro via an MLR assay. Given the relative safety of nivolumab compared to the ipilimumab, PD-1 blockade becomes a strong candidate for future clinical trials in the search of new treatments for HNSCC.

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FIGURE 1.

Programmed death-1 (PD-1) and lymphocyte-activation gene 3 (LAG-3) expression on T cells from patients with head and neck squamous cell carcinoma (HNSCC). (A) CD4 and CD8 T cells isolated from peripheral blood, draining lymph node, or tumor were isolated and stained for PD-1 and LAG-3 expression. Cells were gated on CD4 and CD8 T cells before analysis of checkpoint molecule expression. (B) Synopsis of PD-1 and LAG-3 expression on T cells in patients with HNSCC ($n = 4 - 11$, respectively).

FIGURE 2.

In vitro programmed death-1 (PD-1) blockade enhances draining lymph node CD4 and CD8 T cell function in patients with head and neck squamous cell carcinoma (HNSCC). (A) Synopsis of proliferation in CD4 and CD8 T cells in a mixed lymphocyte reaction ($n = 4$). (B) Synopsis of interferon-gamma (IFN- γ) production from CD4 and CD8 T cells in a mixed lymphocyte reaction ($n = 4$).

FIGURE 3.

Draining lymph node CD4 and CD8 T-cell function is augmented by the addition of interleukin (IL)-2 alone in patients with head and neck squamous cell carcinoma (HNSCC). (A) Synopsis of proliferation in CD4 and CD8 T cells in a mixed lymphocyte reaction ($n = 4$). (B) Synopsis of interferon-gamma (IFN- γ) production from CD4 and CD8 T cells in a mixed lymphocyte reaction ($n = 4$).

FIGURE 4.

Programmed death-1 (PD-1)^{high} CD8 cells can be stimulated to express interferon-gamma (IFN- γ). Synopsis of PMA/ionomycin stimulation of CD8 T cells ($n = 2$). CD3⁺ cells were sorted based on PD-1 expression, stimulated, and then stained for CD8 and IFN- γ .

FIGURE 5.

Human papillomavirus (HPV)-negative head and neck squamous cell carcinoma (HNSCC) tumors express programmed death ligand-1 (PD-L1). Tumor samples from the oropharynx and larynx were sectioned and stained for PD-L1 and CD3. Three patterns of staining were observed: (A) absence of PD-L1 staining with few lymphocytes, (B) regional expression of PD-L1 (membranous “chicken-wire” staining, arrow) colocalized with invading CD3+ lymphocytes (diamond-arrow), and (C) out of proportion PD-L1 staining with rare/sporadic lymphocytes (original magnification $\times 10$). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]