Cancer Research

Chemoradiotherapy-Induced Upregulation of PD-1 Antagonizes Immunity to HPV-Related Oropharyngeal Cancer

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Abstract

While viral antigens in human papillomavirus (HPV)-related oropharyngeal cancer (HPVOPC) are attractive targets for immunotherapy, the effects of existing standard-of-care therapies on immune responses to HPV are poorly understood. We serially sampled blood from patients with stage III–IV oropharyngeal cancer undergoing concomitant chemoradiotherapy with or without induction chemotherapy. Circulating immunocytes including $CD4^+$ and $CD8^+$ T cells, regulatory T cells (Treg), and myeloid-derived suppressor cells (MDSC) were profiled by flow cytometry. Antigen-specific T-cell responses were measured in response to HPV16 E6 and E7 peptide pools. The role of PD-1 signaling in treatment-related immunosuppression was functionally defined by performing HPV-specific T-cell assays in the presence of blocking antibody. While HPV-specific T-cell responses were present in 13 of 18 patients before treatment, 10 of 13 patients lost these responses within 3 months after chemoradiotherapy. Chemoradiotherapy decreased circulating T cells and markedly elevated MDSCs. PD-1 expression on $CD4^+$ T cells increased by nearly 2.5-fold after chemoradiotherapy, and *ex vivo* culture with PD-1–blocking antibody enhanced HPV-specific T-cell responses in 8 of 18 samples tested. Chemoradiotherapy suppresses circulating immune responses in patients with HPVOPC by unfavorably altering effector:suppressor immunocyte ratios and upregulating PD-1 expression on $CD4^+$ T cells. These data strongly support testing of PD-1–blocking agents in combination with standard-of-care chemoradiotherapy for HPVOPC. *Cancer Res; 74(24); 7205–16. ©2014 AACR.*

Introduction

Squamous cell carcinoma is the most frequently occurring malignant tumor of the head and neck and a major cause of morbidity and mortality worldwide. While head and neck squamous cell carcinoma (HNSCC) related to environmental carcinogens (tobacco, alcohol) has declined in the United States, incidence of HNSCC related to the human papillomavirus (HPV), primarily oropharyneal cancer, is rapidly increasing (1). HPV-related oropharyngeal cancer (HPVOPC) is a different disease from classical environmentally related HNSCC, with distinct epidemiology and natural history including a more favorable prognosis (2–4). The carcinogenic mechanism of HPVOPC is also distinct from tobacco/alcohol-associated HNSCC, driven by expression of viral antigens such as the E6 and E7 oncoproteins, which bind and inactivate p53 and RB tumor suppressor genes, respectively, to transform oropharyngeal epithelial cells (5).

The viral antigens in HPVOPC provide compelling targets for immune-based therapy, a strategy both for reducing the roughly 20% recurrence rate seen with existing treatment (5), and for de-escalating chemoradiotherapy, which is associated with significant short- and long-term toxicity (6). Tumor-mediated immunosuppression by upregulation of signaling through negative costimulatory molecules such as CTLA4 and PD-1, release of soluble mediators, and induction of suppressive/regulatory immunocytes including myeloid-derived suppressor cells (MDSC) and regulatory T cells (Treg; refs. 7, 8) is an important mechanism

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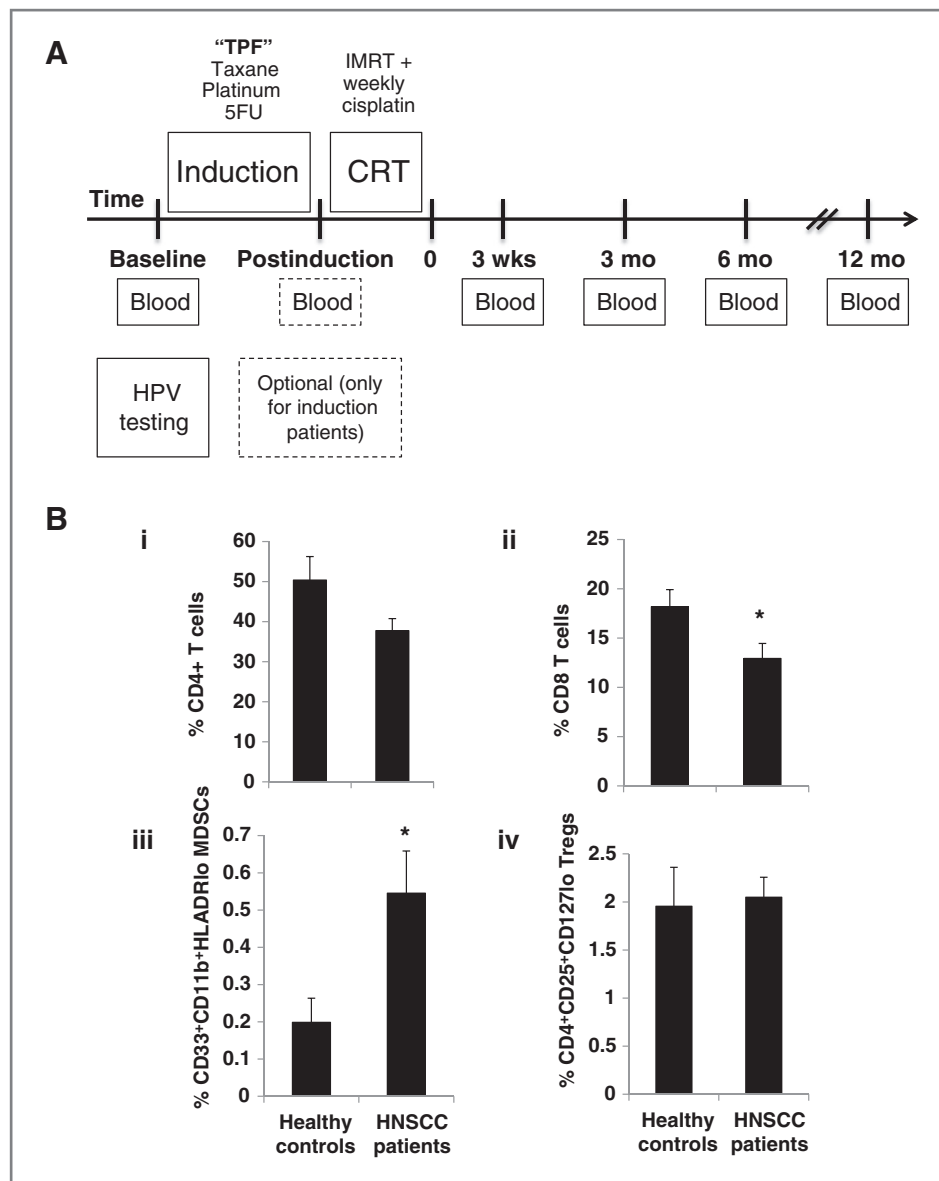
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Figure 1. Comparison of effector and regulatory immunocyte populations in healthy control and HPV+ HNSCC patients at baseline. A, outline of study design. Patients with biopsy-proven HPV+ squamous cell cancer of the oropharynx underwent blood sampling at baseline before treatment and at multiple time points after the completion of induction chemotherapy and/or chemoradiation therapy. Patients treated with induction chemotherapy received three cycles of TPF before concomitant chemoradiation. All patients received a standard course of 7 weeks of concomitant chemoradiation therapy with IMRT and platinum-based chemotherapy. CRT, chemoradiotherapy. B, PBMCs were harvested from healthy donors ($n = 7$) or study patients ($n = 19$), and flow cytometry was performed to determine the relative number of circulating CD4+ T cells (i), CD8+ T cells (ii), CD33+ CD11b+HLADR^{lo} MDSCs (iii), and CD4+CD25+CD127^{lo} Tregs (iv). Patients with cancer had a significant increase in circulating MDSCs and significant decrease in CD8+ T cells compared with healthy controls. *, $P < 0.05$.

driving tumor progression and resistance to host immunity. Tumor-mediated immunosuppression is also understood to be a major barrier to successful cancer immunotherapy. Thus, understanding how conventional chemoradiation interacts with the endogenous immune response to HPV will provide insight into the mechanism of current antitumor therapies in oropharyngeal cancer, and may accelerate development of immune-based prognostic biomarkers and therapeutic approaches. This latter goal is particularly relevant given recent interest in combining immunotherapeutic approaches with standard-of-care chemotherapy and radiation.

In the current study, we hypothesize that platinum-based concomitant chemoradiation, with or without taxane–platinum–5-fluorouracil (5-FU; TPF) induction chemotherapy profoundly alters circulating immunocytes and HPV-specific T-cell responses in patients with HPVOPC. To test this hypothesis, we performed serial blood sampling of 22 patients with oropharyngeal cancer at multiple time points before and after chemoradiotherapy, and analyzed both the profile of effector and suppressor immunocytes by multicolor flow cytometry, and HPV-specific T-cell responses to pooled HPV E6 and E7 peptides. We found that chemoradiotherapy led to globally unfavorable changes in circulating immunity, including increased numbers of circulating MDSC, significant decrease in CD8+ T cells and CD8:MDSC and CD8:Treg ratios, and loss of HPV-specific T-cell responses. We further demonstrated that chemoradiotherapy dramatically upregulates PD-1 expression on circulating CD4+ T cells and that chemoradiation-induced immunosuppression is potentially reversible by PD-1 blockade.

Table 1. Demographics

Characteristics	Summary
Age (average)	58.8
Gender	
Male (%)	19/22 (86.4%)
Female (%)	3/22 (13.6%)
HPV Status	
Positive (%)	20/22 (90.9%)
p16 status	20/20 positive
Negative (%)	2/22 (9.1%)
p16 Status	2/2 negative
Subsite	
Tonsil	7/22 (31.8%)
Tongue base	15/22 (68.2%)
T	
T1	4/22 (18.2%)
T2	12/22 (54.5%)
T3	4/22 (18.2%)
T4	2/22 (9.1%)
N	
N1	4/22 (18.2%)
N2a	2/22 (9.1%)
N2b	8/22 (36.4%)
N2c	5/22 (22.7%)
N3a	3/22 (13.6%)
Stage	
III	4/22 (18.2%)
IV	18/22 (81.8%)
Chemoradiotherapy alone	8/22 (36.4%)
Induction + chemoradiotherapy	14/22 (63.6%)

Patients and Methods

Human subjects

We included 22 patients with biopsy-proven stage III–IV (T1-3, N0-2b, M0) squamous cell cancer of the oropharynx who were scheduled to be treated with standard-of-care concomitant chemoradiotherapy with or without induction chemotherapy. Twenty patients were HPV-positive by PCR testing and 2 HPV⁻. Clinical Laboratory Improvement Amendments-approved PCR-based HPV testing was performed as part of routine management of patients with oropharyngeal cancer at Icahn School of Medicine at Mount Sinai (ISMMS, New York, NY). All patients were treated with 7 weeks of platinum-based (cisplatin or carboplatin) concomitant chemoradiotherapy using intensity-modulated radiotherapy (IMRT). A subset of patients also received induction chemotherapy using the TPF regimen (9), which at ISMMS is offered to patients with advanced (stage III–IV) disease without medical contraindications to therapy. A minority of patients receiving TPF induction (7015, 7016, 7019, 7026, and 7032) was cross-enrolled in another clinical trial and thus received cabazitaxel instead of taxotere as part of the induction regimen. Induction chemotherapy regimens were three cycles of taxane, cisplatin, 5-FU before 7 weeks of chemoradiotherapy. All patients underwent baseline blood sampling before treatment and serial blood sampling after the completion of induction chemotherapy and/or chemoradiation. All patients were enrolled under the ISMMS Institutional Review Board-approved human subjects protocol GCO# 10-1219 (Principal Investigator, A.G. Sikora).

Sample collection and processing

Peripheral blood mononuclear cells (PBMC) were isolated from fresh heparinized blood by Ficoll–Hypaque density gradient centrifugation and cryopreserved.

MDSC and T-cell staining and immunophenotyping

Thawed PBMCs were surface stained for MDSCs (CD33, CD11b, HLADR, CD14) and T cells (CD4, CD8, CD25, CD127, CD45RO, PD-1; BD Pharmingen] for 1 hour at 4°C. The cells were washed with FACS buffer (2% FBS–PBS). The data were acquired with a BD LSR Fortessa flow cytometer and analyzed using TreeStar Flowjo software.

Assessment of HPV-specific T-cell responses by cytokine release

PBMCs were cultured in medium consisting of RPMI1640 (Invitrogen) supplemented with 25 mmol/L HEPES buffer (Invitrogen), 2 mmol/L L-glutamine, 1% nonessential amino acids, 1mmol/L sodium pyruvate, 50 U/mL penicillin, 50 μg/mL streptomycin (all from Sigma Aldrich), and 10% normal human serum AB (GemCell) with or without pooled HPV16 E6 and E7 peptides (20 nmol/L) for 11 days. At day 2, 50 U/mL IL2 (Peprotech) and 50 U/mL IL7 (R&D Systems) were added. On day 8, cells were washed and cultured in medium without serum. On day 11, cells were restimulated with HPV16 E6 and E7 peptide pool (1 μmol/L) or control peptide pool for 48 hours and cytokine levels in the supernatants assessed by the BeadLyte Cytokine Assay Kit (Upstate) as per the manufacturer's protocol. Overlapping 15-mer peptides spanning HPV16 E6 and HPV16 E7 proteins were purchased from Mimotopes. A positive response was defined as >100 pg/mL IFNγ release and >3-fold greater than control peptide.

Assessment of HPV-specific T-cell responses by ELISPOT

PBMCs were presensitized with peptide pools from HPV16 E6 and E7 (20 nmol/L; Sigma Aldrich) and cultured in RPMI1640 medium (Invitrogen) supplemented with glutamax (2 mmol/L, Invitrogen), penicillin (50 U/mL), streptomycin (50 μg/mL), HEPES buffer (10 mmol/L, all from Life Technologies), and 10% normal human serum AB (GemCell) in the presence of anti–PD-1 monoclonal antibody (mAb; J116, 10 μg/mL) or control isotype IgGκ (P3.6.2.8.1, 10 μg/mL; both from Affymetrix). On day 2 and 8, IL2 (Roche) and IL7 (R&D Systems) were added to a final concentration of 10 U/mL and 20 ng/mL, respectively. On day 5, half of the medium was replaced with complete medium containing IL2 (20 U/mL) and IL7 (40 ng/mL), and anti–PD-1 mAb (20 μg/mL) or control isotype IgG κ (20 μg/mL). On day 11, ELISPOT assays were performed to determine Ag-specific effector cells. Flat-bottom, 96-well nitrocellulose plates

(MultiScreen-HA; Millipore) were coated with IFNγ mAb (2 μg/mL, 1-D1K; Mabtech), incubated overnight at 4°C, washed with RPMI1640, and blocked with 10% human AB-type serum for 2 hours at 37°C. Peptide pool-presensitized PBMCs (5×10^4 or 1×10^4 cells) were added to wells and tested for reactivity to the HPV peptide pool (1 μmol/L) or control DMSO, in the presence of anti–PD-1 mAb (10 μg/mL) or of control IgG (10 μg/mL). After 20-hour incubation, plates were washed thoroughly with water containing 0.05% Tween 20 and anti-IFNγ mAb (0.2 μ/mL, 7-B6-1-biotin; Mabtech) was added to each well. After incubation for 2 hours at 37°C, plates were washed and developed with streptavidin–alkaline phosphatase (1 μg/mL; Roche) for 1 hour at room temperature. After washing, substrate (5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium; Sigma-Aldrich) was added and incubated for 7 minutes. Plate membranes displayed dark-violet spots that were scanned and counted using CTL ImmunoSpot Analyzer and software (Cellular Technologies).

PD-L1/PD-L2 staining

PBMCs were irradiated (30 Gy; J.L. Shepherd and Associates Mark I Model 68A Irradiator Cesium-137 source) and checked by flow cytometry for PD-L1 and PD-L2 expression after 4 days in culture in the presence of complete medium (RPMI + 10% SAB), with IL2 (10 U/mL) added at day 2. For each sample, isotype controls were used to determine the autofluorescence of lymphocyte and antigen-presenting cell (APC) populations (selected on the basis of SSC and FSC). PD-L1/PD-L2 expression was calculated as the difference of mean fluorescence intensity (MFI) between PD-L1- and PD-L2–stained samples and isotype control stained samples.

Statistical analysis

For most figures, aggregated data are presented using mean values to represent the central tendency, and SEM to represent variability. Two-tailed paired or unpaired t tests were used to determine significance of differences, except for categorical data, which were analyzed by the Fisher exact test. $P \leq 0.05$ was used as the cutoff for significance.

Results

Chemoradiation has profound and divergent effects on circulating immunocytes

To profile the effect of chemoradiotherapy on effector and suppressor immunocyte populations in oropharyngeal cancer, we collected blood from 20 HPV-positive and two HPV⁻ patients with stage III–IV oropharyngeal cancer undergoing standard-of-care chemoradiation before, during, and after treatment for up to one year (Fig. 1A). The demographics of the patient population (Table 1) are representative of our nonsurgical oropharyngeal cancer population: predominantly middle-aged males with tongue base
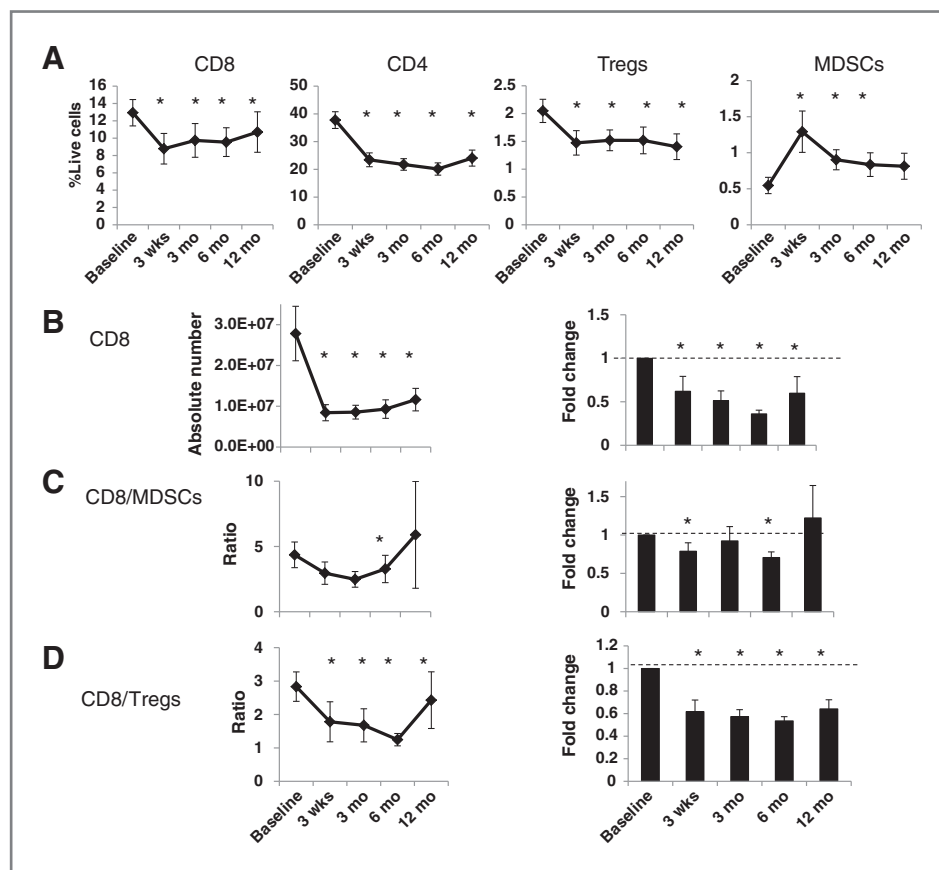
Figure 2. Effect of chemoradiation on circulating immunocytes. A, time course of changes in the relative number (expressed as percentage of all live cells) of effector (CD4⁺ and CD8⁺ T cells) and suppressor (CD11b⁺CD33⁺MHC^{lo} MDSCs and CD4⁺CD127^{lo} Tregs) immunocytes before and after treatment. B–D, treatment-induced changes in the absolute number CD8⁺ T cells (B), and the CD8⁺/MDSCs (C) and CD8⁺/Treg (D) ratios based on absolute numbers (left column) and expressed as the fold change of these ratios with respect to baseline (right column). *, $P < 0.05$ with respect to baseline.

tumors, and a clinical stage distribution biased toward advanced nodal stage (N2-3) disease. The two HPV[−] patients were included in the immunophenotyping results presented in Fig. 2 only; all other data shown are limited to the HPV-positive patient population.

The balance between effector (such as CD8^{+} cytotoxic T cells and CD4^{+} "helper" T cells) and suppressor (such as Tregs and MDSCs) immunocytes is a critical determinant of effective antitumor activity. We sought to determine the effect of chemoradiation on peripheral immunocyte levels, and to compare both pre- and posttreatment levels to those of cancer-free control patients (average age, 44 years; 86% male; neither age nor sex distribution were significantly different from the oropharyngeal cancer patient population). At pretreatment baseline, patients with HPVOPC had a trend toward decreased numbers of CD4^{+} T cells and significantly decreased CD8^{+} T cells as compared with cancer-free controls (Fig. 1B, i and ii; gating scheme shown in Supplementary Fig. S1). Although we did not observe increased numbers of circulating Tregs at baseline, MDSC levels were significantly increased in patients with HPVOPC (Fig. 1B, iii and iv). Overall, these results suggest that the pretreatment peripheral effector/suppressor balance in patients with oropharyngeal cancer is already skewed toward immunosuppression.

After chemoradiotherapy, we observed that both CD4^{+} and CD8^{+} T-cell levels decreased sharply as, to a lesser degree, did CD4^{+}CD127^{lo} Treg levels (Fig. 2A). In contrast to the ablative effect of chemoradiation on lymphoid populations, CD11b^{+}CD33^{+}HLADR^{lo} MDSC levels strikingly increased (nearly 3-fold) immediately after completion of chemoradiotherapy (Fig. 2A). After chemoradiotherapy, we also observed a sharp reduction in the absolute number of CD8^{+} T cells and significantly decreased CD8^{+}:MDSC and CD8^{+}:Treg effector/suppressor ratios, which remained suppressed even at 6 months posttreatment (Fig. 2B–D and Supplementary Fig. S2). Taken together, our peripheral immunophenotyping data strongly support an overall immunosuppressive effect of chemoradiation on the systemic immunocyte milieu in patients with oropharyngeal cancer.

When we examined the subset of patients who received TPF induction chemotherapy, we again found evidence of posttreatment immunosuppression after completion of chemoradiotherapy (Fig. 3). However, several interesting trends consistent with immune activation were observed in the interim postinduction blood samples. There were trends toward increased numbers of CD8^{+} T cells (Fig. 3A) and increased CD8/MDSC ratio (Fig. 3B), as well as a trend toward decreasing MDSC numbers, which, although not statistically significant in aggregate, was driven by a sharp decrease in
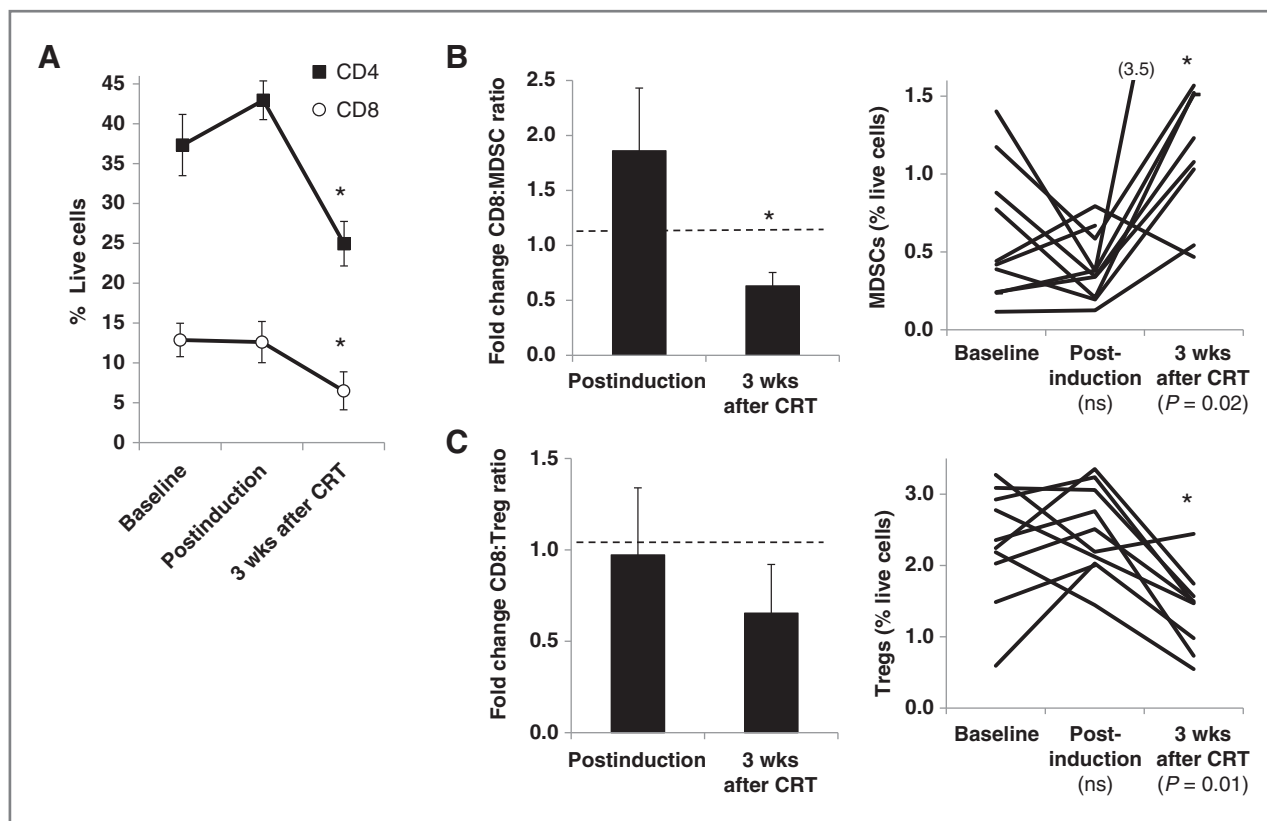
Figure 3. Comparison of circulating immunocytes in patients treated with induction chemotherapy. Changes in the circulating immunocytes after induction and completion of chemoradiotherapy (CRT). A, relative number (expressed as percentage of live cells) of CD4^{+} and CD8^{+} T cells. B, fold changes in CD8^{+}/MDSC ratio (left) and relative MDSC number (right). C, fold changes in the CD8^{+}/Treg (left) and relative Treg number (right).

MDSC in 5 of 10 patient specimens (Fig. 3B). The Treg/MDSC ratio was not significantly altered after induction. While these favorable trends in CD8^{+} T-cell and MDSC number missed statistical significance (likely because of the smaller sample size in the induction group), they are consistent with a potentially immunostimulatory effect of chemotherapy, as has been previously described for 5-FU (10) and the combination of taxol plus platinum chemotherapy (11).

Chemoradiation suppresses preexisting circulating HPV-specific T-cell responses

To determine the effect of chemoradiation on HPV-specific responses in HPVOPC, we analyzed HPV E6/E7-specific T-cell responses before and after treatment by determining the level of IFNγ production after *ex vivo* stimulation of PBMCs with pooled HPV16 E6/E7 peptides. We found that 13 of 18 tested patients had measurable HPV-specific T-cell responses (IFNγ release in response to pooled HPV peptides) at pretreatment baseline (Fig. 4A); an additional two patients who lacked baseline responses acquired them transiently after induction chemotherapy (7004 and 7012). Of 13 patients with preexisting responses, 10 of 13 (77%) lost these responses by 3 months after completion of chemoradiotherapy (loss defined as <100 pg/mL IFNγ at 3 weeks and/or 3 months posttreatment). The fraction of chemoradiotherapy-only and induction plus chemoradiotherapy patients losing preexisting responses was roughly similar (5/6 and 5/7, respectively). Interestingly, 4 of 10

patients who lost responses regained these responses at some point during follow-up (#7008, #7003, #7013, and #7034) although in at least two cases (#7008, #7013) this was transient. When data were analyzed in aggregate, a significant decrease in average IFNγ level with respect to baseline was seen at 3 and 6 months after chemoradiotherapy, and a borderline ($P = 0.05$) decrease at 3 weeks after chemoradiotherapy (Fig. 4B). While the relatively low numbers of HPV-specific cells obtained from cryopreserved PBMCs were rarely enough to perform additional studies in defined T-cell subsets, in the small number of patients samples where we could analyze intracellular IFNγ production we saw concordance with the levels measured in supernatants from unfractionated PBMCs, particularly for CD4^{+} T cells (Supplementary Fig. S3).

These data indicate that most patients had brisk anti-HPV T-cell responses at baseline, but lost these responses after concomitant chemoradiotherapy. Our finding that two patients (7004 and 7012) acquired new HPV-specific responses, and that one patient (7016) with modest baseline response had a nearly 10-fold increase in response following induction therapy, is consistent with the favorable trends in immunocyte populations seen in the postinduction samples (shown in Fig. 3). Together with the immunophenotyping data, these data suggest a predominantly suppressive effect of chemoradiotherapy and a potentially favorable effect of TPF chemotherapy alone on immune function.
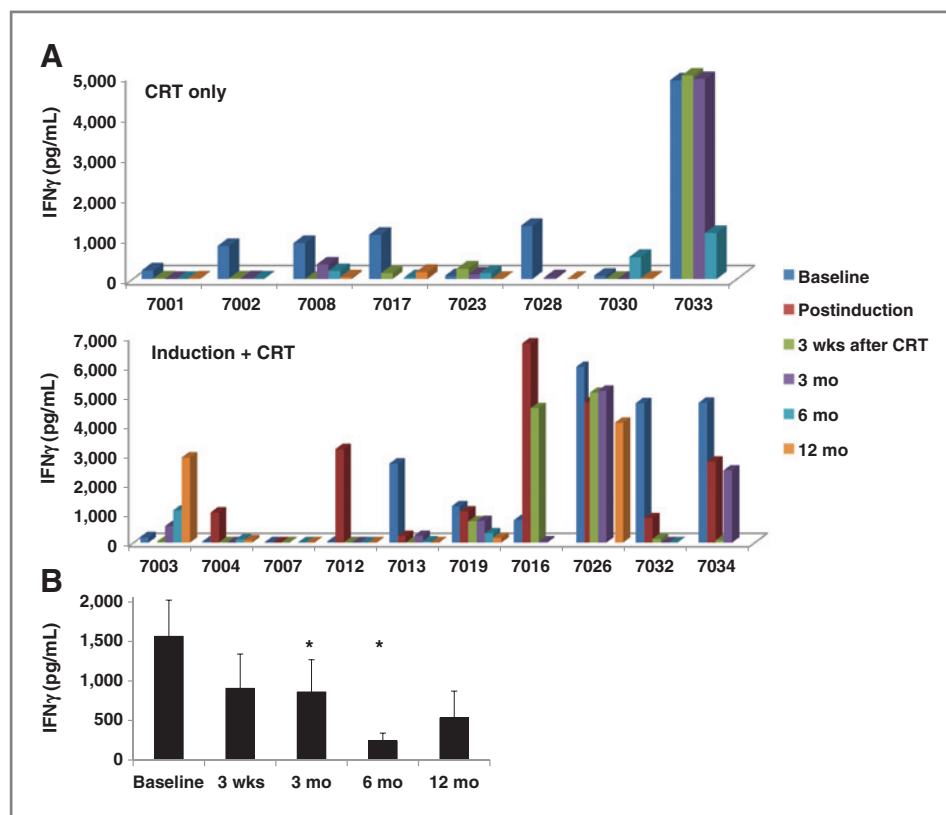
Figure 4. Effect of chemoradiation on circulating HPV-specific T-cell responses. A, batched analysis of cryopreserved PBMCs was performed to determine the level of IFNγ production after *in vitro* stimulation with pooled HPV16 E6/E7 peptides at baseline and posttreatment time points. A, values are shown for patients who received chemoradiation only (top) and from patients who received induction chemotherapy followed by chemoradiation (bottom). In these graphs, very low or zero values are represented by a flat colored square, whereas missing time points are represented by a blank. All values reflect IFNγ production from HPV peptide-stimulated PBMCs minus the value of PBMC stimulated with irrelevant control peptide. B, aggregated IFNγ release data representing averaged data from all patients. *, $P < 0.05$ with respect to baseline.

PD-1 expression on CD4+ T cells increases after chemoradiotherapy and *in vitro* radiation upregulates PD-L2 expression on leukocytes

Many patients lost HPV-specific T-cell responses after chemoradiation, suggesting the induction of specific immunosuppressive mechanisms. PD-1 is a costimulatory/checkpoint molecule, which has been shown to negatively regulate T-cell responses, and both PD-1 and its ligand PD-L1 have been demonstrated to play a role in limiting the immune response to HPV+ and HPV− HNSCC (12). The PD-1 ligand PD-L2 has also been shown to be expressed in cervical cancer (13), but has not previously been examined in HPVOPC. Both PD-L1 and PD-L2 can be expressed by tumor cells or by infiltrating and circulating leukocytes, and can potentially play a role in tumor-mediated and radiation-induced immune dysfunction.

PD-1 expression at baseline was observed on a minority (2%–6%) of circulating CD4+ T cells (Fig. 5), primarily on the CD45RO memory CD4+ population (Fig. 5A). There was a trend toward elevated PD-1 expression on CD4+ T cells from patients with HPVOPC compared with normal controls (median 2.3% and 1.0% of CD4+ cells, respectively; Fig. 5B). We observed that PD-1 expression levels on CD4+ T cells were increased nearly 2.5-fold at 3 weeks after completion of chemoradiation, decreasing somewhat by 3 months after completion of therapy,

but remaining significantly elevated for up to 1 year (Fig. 5C). When analysis was limited to CD45RO+ CD4+ T cells, we also found PD-1 expression to be increased at 3 weeks after therapy (data not shown). While there was also a weaker and nonsignificant trend toward increased PD-1 on circulating CD8+ T cells (Supplementary Fig. S4), PD-1 expression on Treg cells was relatively low and not modulated by therapy (data not shown). Interestingly, we found that baseline PD-1 expression on CD4+ T cells is correlated with expression at 3 months and 6 months posttreatment (Supplementary Fig. S5), suggesting that PD-1 expression at baseline can predict posttreatment levels and potentially be used to select patients who could benefit from combining PD-1/ligand blocking therapy with chemoradiotherapy.

As little is known about the potential effects of chemotherapy and radiation on PD-L1 and PD-L2 expression, we explored the possibility that chemoradiotherapy could also upregulate PD-1 ligand expression on tumor and/or leukocytes. While we found strong PD-L1 but nearly no PD-L2 expression on patient PBMC at baseline, we observed modest upregulation of PD-L2 on APCs after chemoradiotherapy in 5 of 5 patients examined (data not shown), leading us to hypothesize that there may be direct effects of chemoradiotherapy on PD-1 ligand expression. To further
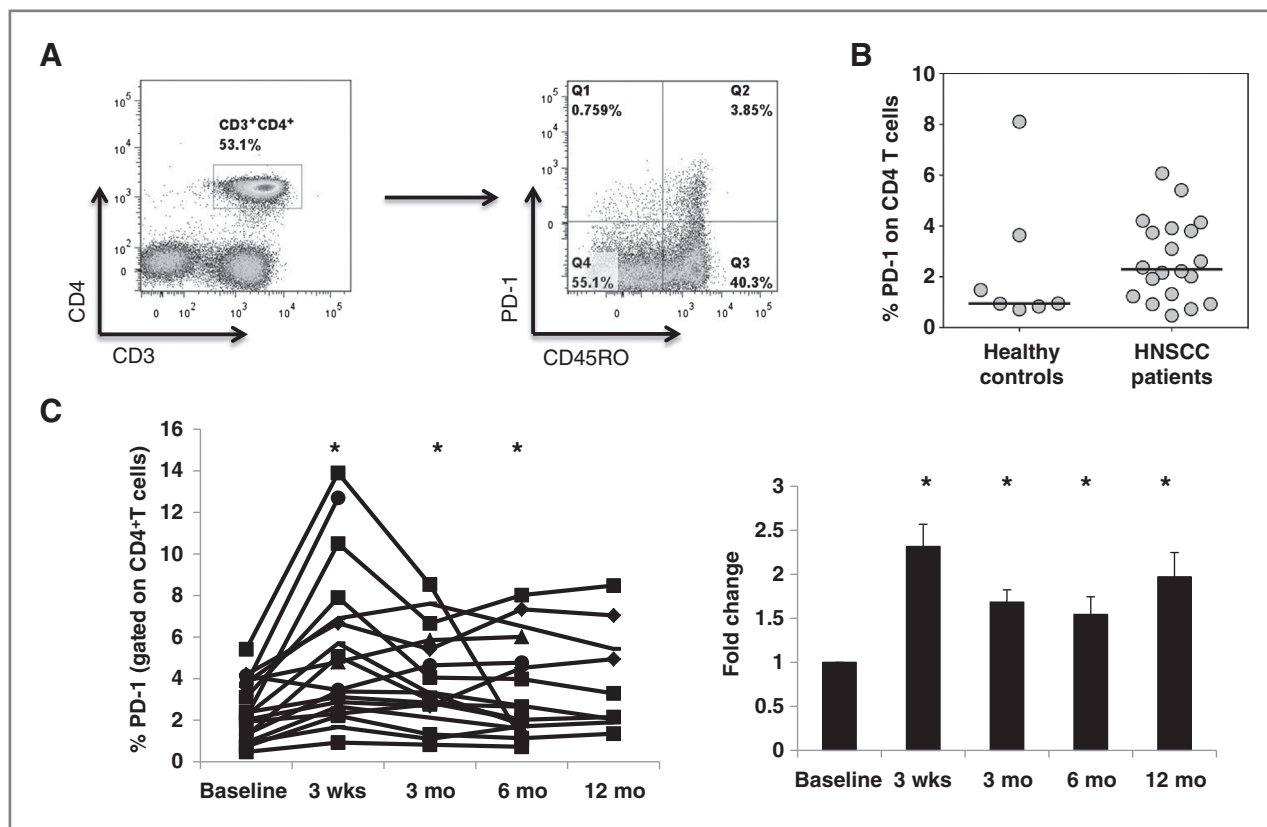
Figure 5. Effect of chemoradiotherapy on PD-1 expression on CD4+ T cells. A, representative FACS plots showing gating scheme of PD-1 and CD45RO expression on CD4+ T cells, gated on all live cells. B, expression of PD-1 on CD4+ T cells from healthy control donors (*n* = 7) and HPVOPC patients (*n* = 19). C, percentage of PD-1+ expressing CD4+ T cells for individual patients (left) and aggregated fold change with respect to baseline (right). *, *P* < 0.05 with respect to baseline.
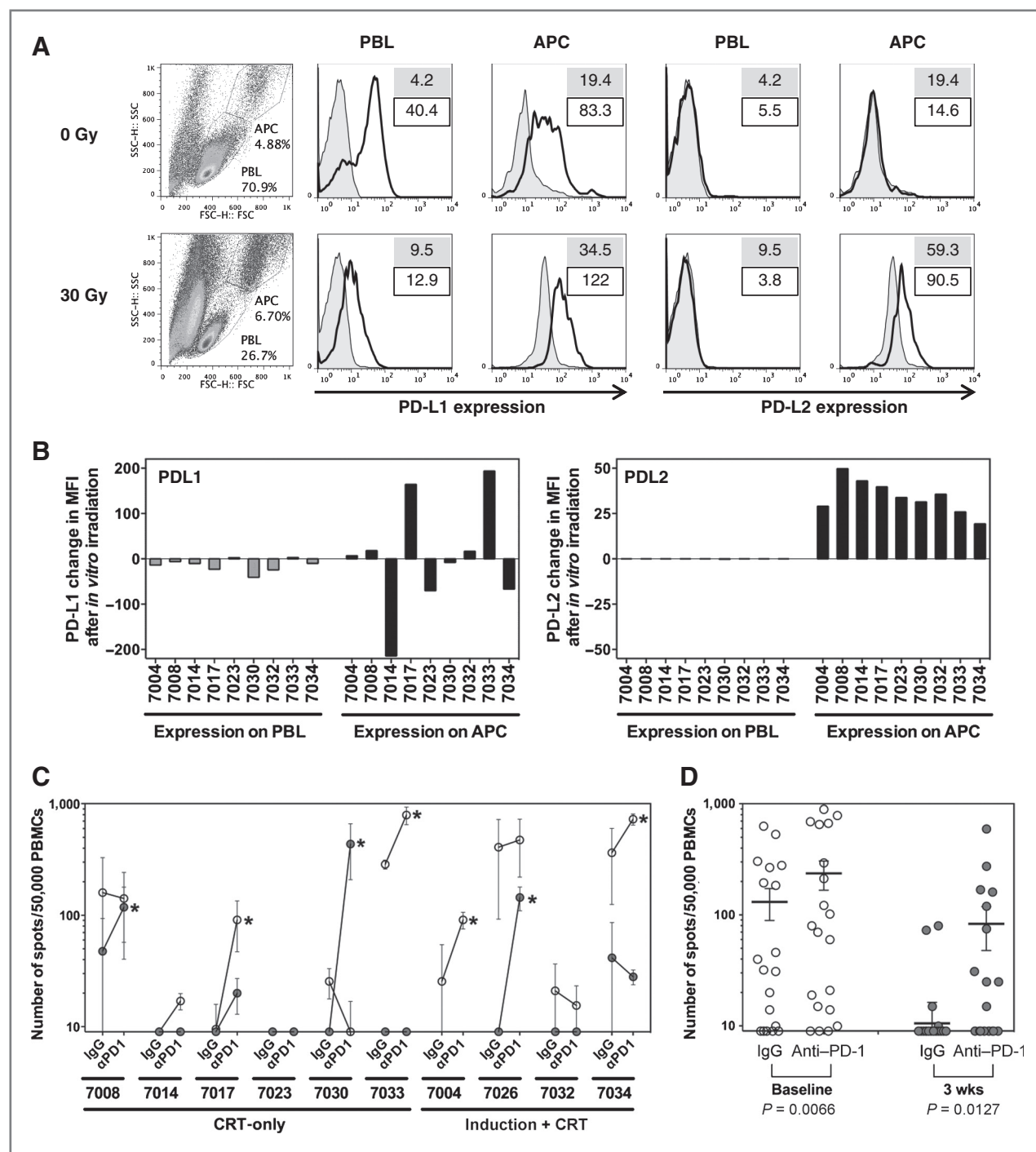
Figure 6. Modulation of PD-1 ligand expression and effect of PD-1 blockade. A and B, effect of *ex vivo* irradiation on PD-L1 and PD-L2 levels on patient PBMC. A, representative flow cytometry of PBMC for PD-L1 and PD-L2. Cells were gated independently for lymphocytes and APC based on forward and side scatter. Numbers on histogram plots indicate MFI of isotype control (filled) and PD-L1/PD-L2 mAb (line), respectively. B, changes in PD-L1 and PD-L2 expression on lymphocytes (PBL) and APCs from PBMCs of patients following *in vitro* irradiation. The changes in MFI were calculated on the basis of MFI of PD-L1 and PD-L2 by flow cytometry after subtraction of MFI from respective isotype control, and results were expressed as the differences of these control-adjusted MFI levels before and after *in vitro* irradiation. Gating of lymphocytes and monocytic/granulocytic populations as shown above. While radiation increased the overall number of dead cells, viability of PBMC in the APC and PBL gates was >90%. Decrease in PD-L1 on PBL and increase in PD-L2 on APC after *in vitro* irradiation were found significant by the paired Wilcoxon *t* test ($P = 0.0185$ and <0.0001, respectively). C, effect of PD-1 blockade on HPV-specific T-cell responses. HPV-specific T-cell responses measured by ELISPOT in ten patients at baseline and 3 weeks after treatment, in the presence or absence of PD-1 blockade. (*Continued on the following page*.)

determine whether radiation may directly modulate PD-L1 and PD-L2 expression, we exposed baseline patient PBMC samples to *ex vivo* radiation and measured PD-L1 and PD-L2 levels after 4 days *in vitro* culture. While no clear trends were seen for PD-L1 expression, PD-L2 was modestly but consistently upregulated on APC (Fig. 6A and B), suggesting that radiation could directly modulate PD-L2 expression in patients with HPVOPC. Taken together, our data suggest that loss of HPV-specific immune response after chemoradiotherapy in our patient population could potentially be driven by a combination of increased expression levels of PD-1 on $CD4^+$ T cells and PD-L2 on APC.

Ex vivo PD-1 blockade enhances HPV-specific T-cell responses and reverses chemoradiation-induced hyporesponsiveness

Dysregulated PD-1/ligand interaction is a mechanism of immune dysfunction amenable to targeting by clinically available anti–PD-1 or anti–PD-L1–blocking antibodies. We sought to determine whether blocking PD-1 signaling could enhance HPV-specific T-cell responses in baseline patient PBMC samples and/or restore responsiveness in postchemoradiotherapy samples where preexisting baseline responses had been lost. HPV16 E6- and E7-stimulated PBMCs were cultured in the presence of anti–PD-1–blocking mAb for 11 days before peptide restimulation and ELISPOT analysis of HPV-reactive T cells (Fig. 6C and D). Consistent with our observation that PD-1 and PD-L1 are expressed on PBMC in baseline samples and that PD-1 and PD-L2 are elevated following chemoradiotherapy, we observed a >2-fold increase in the number of HPV-reactive T cells in presence of anti–PD-1 in 4 of 9 (44%) pretreatment samples and 4 of 9 (44%) posttreatment samples (8 of 18 total samples). This confirms that dysregulated PD-1 signaling contributes to both tumor-induced (baseline) and chemotherapy-induced (posttreatment) immune dysfunction in patients with HPVOPC, and provides proof-of-principle that therapeutic PD-1/ligand blocking antibodies could restore T-cell responsiveness to HPV antigens and reverse chemoradiotherapy-induced immune suppression.

Discussion

The impact of chemoradiation on immune function is widely agreed to be significant, but there is little agreement as to whether this impact skews primarily in the direction of immunosuppression or enhanced antitumor immunity. This is an important question, because there is increasing interest in combining radiotherapy and chemoradiotherapy with checkpoint inhibitors and other immune-based therapeutic approaches. This is particularly true for HPVOPC, where standard-of-care chemoradiotherapy is relatively effective and thus likely to be combined with, rather than replaced by immunotherapy. In the current study, we find evidence that systemic immunosuppression predominates in patients with HPVOPC treated with concomitant platinum-based chemoradiotherapy, and that unfavorable skewing of effector:suppressor immunocyte ratios and dysregulated PD-1/ligand signaling are significant mechanisms of chemoradiotherapy-induced immune dysfunction.

The potentially suppressive effects of radiation to the head and neck on systemic immunity, and the impact of global immune function on prognosis in HNSCC, have long been described (14–19), and recently described again with reference to HPV status (20). Conversely, radiation and chemoradiation have been suggested to have an enhancing effect on the immune response to both HPV$^-$ (21) and HPV$^+$ (22) head and neck cancer. Intact host immunity has also been proposed to be critical for efficacy of conventional radiation and chemoradiation therapy for HPVOPC (23, 24), suggesting immune-dependent mechanisms of action. Such a beneficial role for (chemo)radiation in host immune response to HPVOPC would be consistent with recent clinical literature describing immunostimulating effects of chemotherapy and radiation in melanoma (25, 26) and esophageal squamous cell carcinoma (27). However, the results of clinical studies in HPV$^+$ cervical and oropharyngeal cancers have been mixed, with some studies showing evidence of posttreatment immune activation (28, 29) and others immune suppression (30–32). It is possible that different treatment regimens may have differing effects on antitumor immunity, as has been shown in at least one study that found an immune-enhancing effect in the draining nodes after low-dose radiation (XRT) but an immunosuppressive effect of high-dose XRT (50 Gy, consistent with standard-of-care clinical practice; ref. 32). However, an important difference between *in vivo* mouse models of radiation and XRT for patients with head and neck cancer is that most of the existing mouse models employ relatively few fractions of radiation, whereas patients with head and neck cancer are treated in many small fractions over the course of weeks. Thus, it is important to analyze potential immune effects of chemoradiation by directly studying the effect of specific treatment regimens on host immune function in cancer patients undergoing standard-of-care treatment.

We sought to perform such a study of patients with HPVOPC treated at a single institution with concomitant platinum-based chemoradiotherapy, with and without induction chemotherapy. At pretreatment, baseline HPVOPC patients had paradoxical evidence of peripheral immune

(*Continued.*) PBMCs from individual time points were sensitized with a pool of overlapping long peptides from HPV E6 and E7, cultured in the presence of anti–PD-1 mAb or control IgG for 11 days, and assayed for reactivity against the same HPV peptide pool by ELISPOT. Spot numbers indicate HPV-specific IFN(γ-secreting T cells out of 50,000 cells tested, following removal of nonspecific responses (DMSO control, typically <20 spots). Open and gray symbols indicate responses from samples at baseline and 3 weeks after treatment, respectively. Asterisks indicate samples with a greater than two times increase in response following PD-1 blockade. CRT, chemoradiotherapy. D, dot plots showing mean (bar) and SE of individual replicates from samples of all patients cultured with IgG versus anti–PD-1. There were significantly more spots following PD-1 blockade both at baseline and 3 weeks after treatment, as shown by the paired Wilcoxon t test ($P = 0.0066$ and 0.0127, respectively).

activation (measurable HPV-specific T-cell responses, which in many cases were quite strong) and immunosuppression [decreased CD8$^+$ T cells and elevated levels of circulating MDSC as compared with normal controls (Fig. 1B), and elevated PD-1 expression on CD$^+$ T cells (Fig. 5B)]. This is consistent with the widely accepted model of immunity in HPV-related cancers, in which a vigorous antigen-specific peripheral immune response is thwarted by immunosuppressive mechanisms upregulated in the tumor immune microenvironment. Such mechanisms found in the microenvironment of HPVOPC include induction of immunoregulatory immunocytes (MDSC, Treg, alternatively activated macrophages) and negative costimulatory molecules (PD-1 and CTLA4), as well as other immunosuppressive mechanisms common to many solid tumors (33). Thus, host immunity to HPVOPC is characterized by a balance between immune activation and suppression that results in a vigorous but ineffective immune response incapable of controlling tumor growth. We next sought to determine whether chemoradiotherapy tends to drive this balance in the direction of enhanced immunity or toward worsening immunosuppression.

At the level of immunophenotyping of peripheral immune cells, nearly all changes observed after chemoradiotherapy were in the direction of immune suppression: decreased CD8$^+$ and CD4$^+$ T cells, and increased MDSCs (Fig. 2A). Even the modest decline in Treg cells observed was offset by a much greater decline in absolute CD8$^+$ T-cell number, leading to unfavorable ratios of CD8$^+$ T cells with Tregs as well as MDSCs. The final effects of treatment on peripheral immune populations were very similar for patients treated with chemoradiotherapy alone and with induction chemotherapy + chemoradiotherapy. These results provide more granular detail about the significant postradiation leukopenia previously reported in patients with head and neck cancer (14, 20) and suggest that interventions that enhance the radioresistance of CD8$^+$ T cells (34) or that deplete Treg and myeloid suppressor populations could potentially restore immune homeostasis.

When we examined HPV-specific T-cell responses, the overwhelming trend observed was loss of preexisting (baseline) responses by 3 months, and often as early as 3 weeks, following completion of therapy. These immunosuppressing effects of chemoradiotherapy seemed to be intrinsic to the treatment itself, as there was no significant association with clinical stage, tumor subsite, initial strength of HPV response, or taxane (taxotere vs. cabazitaxel). The rapid loss of responses in most patients suggests active immunosuppression, rather than extinction of the response due to antigen loss associated with decreased tumor mass after effective treatment; however, we will further explore this hypothesis in a parallel study of the immune response in surgically treated patients to compare chemoradiotherapy with the effects of surgical ablation. We also hypothesize, based on the favorable trends in immunocyte populations following induction chemotherapy, and the markedly increased postinduction HPV-specific responses seen in several patients, that the immunosuppressive effects of chemoradiotherapy are primarily due to radiation, rather than chemotherapy. In fact, our data are suggestive of potentially beneficial immune effects of TPF chemotherapy alone, which we intend to explore in follow-up studies.

A functional role for PD-1 signaling in chemoradiotherapy-induced immunosuppression is supported by the strong upregulation of PD-1 on CD4$^+$ T cells at 3 weeks after completion of therapy (Fig. 5), which remained elevated as long as 12 months following chemoradiotherapy, and the ability of anti–PD-1-blocking mAb to restore HPV-specific T-cell responses in a subset of patients with HPVOPC (Fig. 6). The PD-1 ligand PD-L1 is overexpressed in HPVOPC tumors (12, 35) and tumor-infiltrating APCs (12) and PD-1 is expressed on HPVOPC-infiltrating CD8$^+$ (12) and CD4$^+$ (36) T cells. Thus, our findings are consistent with ample prior evidence suggesting that PD-1 is an important mechanism of HPVOPC immune escape. In fact, expression of PD-1 and other immune checkpoint molecules has been shown to be higher on tumor-infiltrating than circulating T cells (37), and cryopreservation has been shown to decrease PD-1 and PD-L1 expression on PBMC, so it is likely that our findings, derived from observations of cryopreserved circulating immunocyte populations, understate the impact of chemoradiotherapy as a driver of PD-1 expression and concomitant immune suppression.

Further support for this model comes from the observation that ex vivo radiation alone is sufficient to upregulate PD-L2 expression on patient APC (Fig. 6B), suggesting that PD-1 ligand expression may also be modulated by chemoradiotherapy. While the role of PD-L2 in cancer is not as well defined as that of PD-L1, available evidence suggests it can play a role in tumor-associated immunosuppression (38). PD-L2 expression on APC has been shown to limit CD8$^+$ T-cell expansion and cytokine proliferation as well as Th1 cytokine production and tumor killing by human CTL (39). PD-L2 blockade has been shown to have antitumor activity in a mouse model of pancreatic cancer (40), and PDL2 expression predicts poor prognosis in lung (41) and esophageal (42) cancer, suggesting that it may contribute to immune escape. With regard to HPV-related neoplasia, PD-L2 is expressed by benign respiratory papillomas (43) and advanced stage cervical cancer (13). We also observed a small but consistent increase in PD-L2 levels in posttreatment APC populations, which may be indicative of chemoradiotherapy-induced upregulation. Prior evidence of transient direct radiation effects on PD-1 and ligand upregulation has been observed in mouse models of breast cancer and melanoma in which radiation upregulated CD8 T-cell PD-1 expression and anti–PD-1 mAb synergized with radiotherapy in treatment of established tumors (44). In another set of studies, radiation upregulated tumor PD-L1 expression and anti–PD-L1 following radiotherapy led to improved tumor control (45, 46). An additional study in a mouse glioblastoma model found that anti–PD-1 mAb enhanced antitumor efficacy of stereotactic radiotherapy (47). Thus, there is prior biologic plausibility for modulation of PD-1 and receptor expression by (chemo)radiotherapy, and the potentially beneficial effects of combining radiotherapy with clinically available antibodies targeting PD-1 and its ligands. The current data in patient samples add to this preclinical evidence and

provide further support for targeting PD-1 signaling in HPVOPC, and testing PD-1/PD-L1 blockade in combination with chemoradiotherapy.

Our study has several limitations. The most obvious limitation is that our analyses are limited to circulating/systemic immunity, rather than direct interrogation of the tumor microenvironment itself. While many immune processes are anticipated to be regulated similarly in the tumor and circulating compartments, and while systemic immunity has obvious relevance to long-term immune surveillance and risk of relapse, we anticipate that many of our findings would be even more striking were we to sample the tumor immune microenvironment. While our focus on chemoradiotherapy patients, the vast majority of which have a complete initial response to treatment, prohibits a classical pre/posttreatment window-of-opportunity trial approach, we anticipate that follow-up studies will incorporate a mid-treatment tumor biopsy to interrogate this immunologically important compartment. A further limitation is the relatively small clinical series studied ($N = 22$ patients), which makes it difficult to study potentially interesting but statistically nonsignificant differences between, for example, patients treated with induction chemotherapy versus chemoradiotherapy alone, or patients who maintained and those who lost circulating HPV-specific immune responses. Nevertheless, even with this relatively modest sample size, there was sufficient consistency of results to find statistically significant and reproducible treatment-induced changes in immunocyte populations, PD-1 expression, and HPV-specific responses.

Our study also has considerable strengths that add significantly to our understanding of the effects of chemoradiotherapy on immune responses to HPVOPC and other HPV-related cancers. We provide detailed analyses of treatment-induced changes in what is to date the largest immune-focused series of HPV* oropharyngeal cancer patients treated with a single modality (chemoradiation), and identify specific mechanisms of treatment-induced immunosuppresion (effector:Treg and effector:MDSC imbalance, and PD-1 upregulation) potentially amenable to therapeutic intervention. In particular, the current data provide further evidence of the emerging role of PD-1 signaling as a major mechanism of HPVOPC immune escape, and strongly support the development of clinical trials testing the integration of PD-1/PD-L1–blocking antibodies with standard-of-care chemoradiation in HPVOPC.

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Chemoradiotherapy-Induced Upregulation of PD-1 Antagonizes Immunity to HPV-Related Oropharyngeal Cancer

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