CD44<sup>+</sup> Cells in Head and Neck Squamous Cell Carcinoma Suppress T-Cell–Mediated Immunity by Selective Constitutive and Inducible Expression of PD-L1

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Abstract

**Purpose:** Human tumors consist of heterogeneous populations of cells with distinct marker expression and functional properties. In squamous cell carcinoma of the head and neck (SCCHN), CD44 is a well-characterized marker of a resilient subpopulation of cells associated with increased tumorigenesis, radioresistance, and chemoresistance. Evidence indicates that these cells have an immunosuppressive phenotype; however, mechanisms have been elusive.

**Experimental Design:** Using primary human SCCHN tumor samples and patient-derived xenografts, we examined the phenotypes of subsets of tumor cells and investigated mechanisms regulating their immunogenicity.

**Results:** CD44<sup>+</sup> cells in primary human SCCHN were found to have an epithelial-to-mesenchymal (EMT) phenotype and were less immunogenic than CD44<sup>−</sup> cells when cultured with autologous CD8<sup>+</sup> tumor-infiltrating T cells. Selective expression of the programmed death-ligand 1 (PD-L1) was observed on CD44<sup>+</sup> cells compared with CD44<sup>−</sup> cells and was associated with constitutive phosphorylation of STAT3 on CD44<sup>+</sup> cells. Importantly, inhibition of STAT3 decreased expression of PD-L1 on CD44<sup>+</sup> cells. IFNγ treatment preferentially induced further PD-L1 expression on CD44<sup>+</sup> cells and was associated with enhanced IFNγ receptor expression and phosphorylation of STAT1. Finally, the decreased immunogenicity of CD44<sup>+</sup> cells was partially reversed by antibody blockade of the programmed death 1 (PD-1) receptor, indicating that the differences in PD-L1 expression between CD44<sup>+</sup> and CD44<sup>−</sup> cells are biologically and clinically relevant.

**Conclusions:** Our findings provide a mechanism by which long-lived CD44<sup>+</sup> tumor-initiating cells can selectively evade host immune responses and provide rationale for targeting the PD-1 pathway in the adjuvant therapy setting of SCCHN.

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Introduction

Phenotypic and functional intratumor cellular heterogeneity has been described and observed in multiple tumor types (1, 2), including squamous cell carcinoma of the head and neck (SCCHN; ref. 3). This heterogeneity can arise from cell-intrinsic factors, such as DNA mutations or epigenetic modifications, or from cell-extrinsic factors, such as signals from cells in the microenvironment (2). In mouse models of cancer immunoeediting, tumors evolve under constant immune pressure and develop resistance to immune recognition, often reaching a state of equilibrium with the immune system (4). The resulting poorly immunogenic tumor cells may persist for long periods and contribute to tumor dormancy in the context of minimal residual disease (5, 6). It is not clear if this applies to human tumor dormancy; however, within human tumors, subpopulations of cells possessing the capacity for long-term self-renewal and tumor initiation have been isolated, and there is growing evidence for an intrinsic immunosuppressive phenotype associated with these cells (6–10). Mechanisms underlying this, however, are not well understood.

SCCHN is the most common malignancy of the upper aerodigestive tract mucosa, and advanced cases of human papilloma virus (HPV)-negative SCCHN have recurrence rates of 30% to 50%. In this malignancy, CD44, a cell-surface glycoprotein receptor for hyaluronic acid, involved in cell migration and signal transduction (11, 12), is a well-characterized marker of a resilient subpopulation of cells with increased tumorigenesis (13). Increased proportions of CD44<sup>+</sup> cells have been correlated with worse clinical outcome (14). Furthermore, CD44<sup>+</sup> cells in SCCHN can propagate in vivo tumor engraftment and give rise to CD44<sup>+</sup> cells in serial passing of xenografts, supporting the idea that a “cancer stem cell”–like population is contained within the CD44<sup>+</sup> compartment (13).
recent registration of an anti-PD-1 antibody for advanced tumors in a constitutive and IFN-γ-inducible manner. PD-L1 is expressed on many tumor types, including squamous cell carcinoma of the head and neck (SCCHN). In SCCHN, CD44 is a well-characterized marker of a resilient subpopulation of cells with increased tumorigenesis, radioresistance, and chemoresistance. In this study, we observed a greater constitutive and inducible expression of PD-L1 on CD44+ cells, associated with constitutive phosphorylation of STAT3 and enhanced responsiveness to IFNγ. As CD44+ cells have the capacity for long-term self-renewal and tumor initiation, their preferential expression of PD-L1 provides a mechanism by which these cells can selectively evade immune responses and contribute to tumor dormancy in the context of minimal residual disease. Our findings provide an explanation for the durable responses observed with PD-1 blockade in the treatment of advanced disease and provide rationale for use of PD-1 blockade in the adjuvant setting following surgical resection of SCCHN tumors at high-risk for recurrence.

SCCHN tumors are highly immunosuppressive in general, and programmed death-ligand 1 (PD-L1, B7-H1, CD274) expression has been proposed to be a potential mechanism for this phenotype (15–17). PD-L1 is expressed on many tumors in a constitutive and IFN-γ-inducible manner. PD-L1 binds to an inhibitory receptor, the programmed death 1 receptor (PD-1, CD279), which is a member of the B7 family of receptors (18), and to the costimulatory molecule CD80 (B7-1; ref. 19). PD-1 is expressed on activated T cells, and binding to PD-L1 results in the inhibition of T-cell receptor-mediated activation (20). Binding of PD-L1 to CD80 prevents activation of CD80 by CD28 and thus, also contributes to the immunosuppressive activity of PD-L1. Durable tumor regression with blockade of the PD-1/PD-L1 checkpoint has been demonstrated in recent clinical experience (21, 22), leading to recent registration of an anti-PD-1 antibody for advanced melanoma and non–small cell lung cancer. In addition, clinical efficacy of an anti-PD-1 monoclonal antibody in SCCHN has been observed in phase I studies (23). In general, response to PD-1 blockade has been commonly correlated with expression of PD-L1 by the treated tumor (22).

In this study, we examined primary human SCCHN tumor samples and compared the immunogenicity of the CD44+ subset of cells with that of the CD44− counterparts. Here, we show that PD-L1 is preferentially expressed on the CD44+ subpopulation, and that suppression of T-cell activity mediated by these cells is conferred, in part, by this elevated expression of PD-L1.

**Materials and Methods**

**Human SCCHN samples and patient-derived xenografts**

Fresh tumor specimens were obtained through the Stanford Tissue Bank from patients undergoing surgical resection of HPV-negative oral cavity SCC at the Stanford Medical Center (Stanford, CA) after informed consent, in accordance with an Institutional Review Board (IRB)-approved protocol. Tumor samples were digested to obtain cell suspensions for assays, or dissected into small tumor chunks (<2 mm) for implantation into Rag2−/− Il2rg−/− immunodeficient mice as xenografts. Briefly, mice were anesthetized with isoflurane-oxygen, and a trocar was used to implant Matrigel (BD Biosciences)-coated tumor chunks subcutaneously into the flanks. Mice were monitored for tumor growth and euthanized when tumors were at least 1 cm in diameter. All experiments were performed using primary tumor samples or xenografts passaged in mice less than four times.

**Mice**

B10.B6-Rag2−/− Il2rg−/− mice (Taconic) were bred and maintained under specific pathogen-free conditions. All animal procedures were performed in accordance with protocols approved by the Administrative Panel on Laboratory Animal Care at Stanford University (Stanford, CA).

**Tumor digestion**

Tumors were minced and digested in 300 U/mL collagenase and 100 U/mL hyaluronidase (StemCell Technologies) in culture media; DMEM/F-12 medium (Mediatech) with 10% FBS, 2 mmol/L L-glutamine, and 1% penicillin-streptomycin-amphotericin B (MP Biomedicals). The tumor digest was pipetted every 15 minutes and incubated at 37°C for 3 hours, until a single-cell suspension was obtained. The dissociated cells were spun down and resuspended in Trypsin-EDTA (StemCell Technologies) for 5 minutes, then further dissociated with 5 U/mL dispase (StemCell Technologies) and 0.1 mg/mL DNase 1 (StemCell Technologies) for 1 minute. Cells were filtered through a 40-μm cell strainer and erythrocytes were lysed with ACK lysing buffer (Lonza).

**Isolation and culture of tumor-infiltrating lymphocytes**

Tumor-infiltrating lymphocytes (TIL) were cultured using a protocol adapted from Dudley and colleagues (24). Tumor fragments (1–2 mm) were cultured in RPMI-1640 medium (Mediatech) with 10% human AB serum (Cellgro), 2 mmol/L L-glutamine, 1% penicillin-streptomycin-amphotericin B (MP Biomedicals), 55 μL/1 2-mercaptoethanol (Gibco), and 6,000 U/mL recombinant human IL-2 (Roche, provided by the National Cancer Institute). Alternatively, digested tumor cell suspensions containing both tumor and stromal cells were cultured at 1 to 2 × 106 cells/mL. Confluent cultures were sorted to obtain CD8+ CD8+ "CD4−" effector T cells and further expanded in T25 flasks or 96-well plates with 30 ng/mL soluble anti-CD3 (OKT3, BioLegend), 3,000 U/mL IL-2, and irradiated PBMCs pooled from two allogeneic donors in a 200:1 feeder cells-to-TIL ratio in a mixture of 50% culture media and 50% AIM-V serum-free media (Gibco). After 5 days, the media were replaced with fresh media and 3,000U/mL IL-2, and then replaced again every 2 to 3 days thereafter.

**Flow-cytometric analysis and cell sorting**

Cells were stained with a panel of antibodies in PBS containing 2% FBS and 2mmol/L EDTA for analysis and cell sorting. Tumor cells from primary tumor samples were identified by gating out leukocytes, endothelial, and stromal cells expressing human lineage markers: CD2 (RPA-2.10), CD3 (UCHT1), CD18 (6.7), CD31 (WM59, eBioscience), CD45 (HI30), CD64 (10.1), and anti-fibroblasts antibody (TE-7, Millipore). Tumor cells from...
gene concentrations. A standard curve was constructed and assayed using the ELISA MAX Deluxe Kit (BioLegend). A standard curve was fitted using a sigmoidal 4-parameter logistics function.

For TIL profiling, Antibodies to CD3 (OKT3) and PD-1 (eBio105) were obtained from eBioscience. Antibodies to CD4 (RPA-T4), CD8 (RPA-T8), and Tim-3 (F38-2E2) were obtained from BioLegend.

Cells were analyzed on a BD LSFortessa or sorted to >95% purity using a BD FACSAria II. Events collected were analyzed using FlowJo Vers.9.6.4 software (Tree Star). The isotype-subtracted mean fluorescence intensity (MFI) was calculated by subtracting the MFI of the relevant isotype control from the MFI of the antibody-stained cells.

RNA extraction and qRT-PCR
RNA was extracted using the RNeasy Mini Kit (Qiagen) and cDNA was synthesized using the Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Scientific). Validated TaqMan Gene Expression Assays (Applied Biosystems) for HPR1 (Hs02800695_m1), CD274 (Hs01125301_m1), IFNNG (Hs00983034_m1), BMI1 (Hs00180411_m1), TWIST1 (Hs01675818_s1), ZEB1 (Hs00327837_m1), ZEB2 (Hs00207691_m1), SNAI1 (Hs00195591_m1), SNAI2 (Hs00950344_m1), CDH1 (Hs01023894_m1), and CDH2 (Hs00983056_m1) were used with the Applied Biosystems 7900HT system. Gene expression data were normalized to the housekeeping gene HPRT1.

IFNγ ELISA
Tumor cells were sorted into CD44+ and CD44- subsets on the day of the assay. A total of 5 × 10^6 CD8+ T cells and 5 × 10^4 autologous sorted tumor cells were cocultured (1:1) in 200 μL of media for 2 days. Supernatants were collected and assayed for IFNγ levels in at least two replicates using the Human IFN-γ ELISA MAX Deluxe Kit (BioLegend). A standard curve was constructed and fitted using a sigmoidal 4-parameter logistics curve-fitting algorithm to determine the unknown analyze concentrations.

RNA in situ hybridization
Paraffin-embedded tissue sections (5 μm thickness) were processed for RNA in situ detection using the RNAscope 2-plex Detection Kit (Advanced Cell Diagnostics) according to manufacturer’s instructions. CD44 and PD-L1 (CD274) were detected using the Fast Red and the HRP (Green) chromogenic stains, respectively.

Blocking antibodies
Cells were pre-coated with 10 μg/mL blocking antibodies before coculture. Antibodies used were anti-PD-L1 (6E11.1.9, Genentech), anti-PD-1 (EH12.2H7, BioLegend), anti-Tim-3 (F38-2E2, BioLegend), mouse IgG2a isotype (BioLegend), and mouse IgG1 isotype (BioLegend).

Western blot analysis
Tumor cells were sorted from SCCHN patient-derived xenografts and lysed. Proteins from total cell lysates were resolved on a 4% to 12% Tris-Glycine gel (Life Technologies) and transferred to a polyvinylidene fluoride (PVDF) membrane. Membranes were blocked in TBS containing 0.05% Tween 20 and 5% skim milk powder, and incubated overnight with specific primary antibodies. Secondary donkey-anti-rabbit antibodies conjugated to HRP was used for chemiluminescence detection with Pierce ECL Western blotting substrate (Thermo Scientific). Antibodies to p-STAT1 (pY705; D3A7) and STAT3 (79D7) were obtained from Cell Signaling Technology.

p-STAT1 detection
Cells were treated with 50 U/mL recombinant human IFNγ (eBioscience) at 37°C for 15 minutes and immediately fixed with 4% PFA at 37°C for 10 minutes. Cells were then stained with antibodies against surface markers at 4°C for 30 minutes, permeabilized with Perm Buffer III (BD Phosflow) at 4°C for 30 minutes, and finally stained with antibodies against phosphoproteins at 4°C for 1 hour.

Statistical analysis
The two-tailed Student t test (paired and unpaired, as indicated) was used for statistical comparison between groups. Paired t-tests were used for experiments with matched pairs of cell subsets obtained from the same tumor sample. P < 0.05 was considered significant. Statistical analyses were performed using GraphPad Prism version 6.01 for Windows.

Results
CD44+ cells in human SCCHN tumors have an EMT phenotype and are less immunogenic
To examine the phenotypes of tumor cells from human SCCHN, fresh tumor specimens were implanted into immunodeficient Rag2−/−Il2rg−/− mice as patient-derived xenografts (PDX) and also cultured in vitro separately to obtain autologous TILs (Fig. 1A). The tumor samples and PDXs were subsequently dissociated and analyzed by flow cytometry after excluding leukocytes, endothelial, stromal, and non-viable cells from our analysis gates (Supplementary Fig. S1). Tumor cells, sorted into CD44+ and CD44- subsets (Fig. 1B), were analyzed for their expression of EMT-associated genes by qRT-PCR (Fig. 1C). We observed higher mRNA levels of EMT regulators TWIST1 and BMI1 in CD44+ cells compared with CD44- cells, as previously reported (13). Higher levels of the EMT-inducing genes ZEB1, ZEB2, SNAI1, and SNAI2 were also observed in CD44+ cells (Fig. 1C and Supplementary Fig. S2D).

To investigate the immunogenicity of primary SCCHN cells, autologous TILs were expanded in vitro for use as effector cells. Freshly isolated CD8+ TILs were observed to express PD-1 in approximately 35% of the total cells with low levels of T-cell immunoglobulin and mucin domain 3 (Tim-3; Fig. 1D). Of note, following expansion, Tim-3 expression on the CD8+ TILs was significantly elevated (Fig. 1E and F). PD-L1 was not expressed on the CD8+ TILs after expansion (Fig. 1E and F). CD44- and CD44+...
SCCHN cells were separately cocultured with expanded autologous CD8\(^+\) TILs (Fig. 1A) and analyzed for their ability to stimulate IFN\(\gamma\) production by the TILs. Lower levels of IFN\(\gamma\) were detected in the cultures with CD44\(^+\) cells (Fig. 1G), indicating that CD44\(^+\) cells were less immunogenic, or more immunosuppressive, compared with CD44\(^-\) cells. IFN\(\gamma\) secretion was not detected in wells with tumor cells alone (data not shown).

As ZEB1 has been shown to regulate PD-L1 expression via miR-200 (26) and because the more immunosuppressive CD44\(^+\) cells had higher ZEB1 expression (Fig. 1C), we hypothesized that the observed decreased immunogenicity of CD44\(^+\) cells was due to expression of PD-L1, a ligand for the immune checkpoint inhibitor PD-1, on the tumor cells.

CD44\(^+\) SCCHN cells express higher levels of PD-L1

To determine the expression of PD-L1, we examined 21 human HPV-negative oral SCCHN tumors by flow cytometry. Tumor cells were identified by excluding stromal cells from the analysis gates (Supplementary Fig. S1) and by their expression of EGFR (Supplementary Fig. S2A). Among the tumors analyzed, 71% were classified as PD-L1\(^+\) (Fig. 2A), consistent with PD-L1 positivity rates reported in other studies (15–17, 23). Interestingly, among the PD-L1\(^+\) tumors, we observed higher levels of PD-L1 expression on the CD44\(^+\) subset of cells compared with matched CD44\(^-\) subsets at both the protein level, measured by flow cytometry (Fig. 2A and B), and at the transcript level, measured by qRT-PCR (Fig. 2C and D). Similarly, RNA in situ hybridization also indicated greater PD-L1 mRNA expression on the CD44\(^+\) cells (Fig. 2E). Given the recent identification of CD271 as another marker of tumor-initiating cells within the CD44\(^+\) compartment in SCCHN (27), we assessed for differences in PD-L1 expression between the CD44\(^+\)CD271\(^-\) and CD44\(^+\)CD271\(^+\) cells and found comparable elevated levels of PD-L1 expression between these two subsets (Supplementary Fig. S3). Hence, among the PD-L1\(^+\) SCCHN tumors, the CD44\(^+\) subpopulation had selectively higher constitutive expression of PD-L1 both at the transcriptional and translational levels.

CD44\(^+\) PD-L1\(^+\) and CD44\(^+\) PD-L1\(^-\) SCCHN cells have similar tumor-initiating capacity in vivo

Since the CD44\(^+\) compartment in SCCHN tumors has been associated with increased tumorigenesis and has been shown to...
Selective PD-L1 Expression on CD44+ SCCHN Cells

Figure 2.

CD44+ cells in human SCCHN tumors express higher levels of PD-L1. A, representative plots of CD44 and PD-L1 expression in PD-L1+ (n = 15, 71%) and PD-L1− (n = 6, 29%) SCCHN. B, PD-L1 MFI of matched pairs of CD44− (○) and CD44+ (▲) gated tumor cells from PD-L1− tumors (n = 15, *P < 0.05, paired t-tests). C, PD-L1 mRNA transcript levels of sorted CD44− (○) and CD44+ (▲) tumor cells from PD-L1− tumors (n = 6, *P < 0.05, paired t-tests). D, CD44+/PD-L1+ fold change of PD-L1 MFI (n = 15) and mRNA (n = 6) in PD-L1+ tumors, mean ± SEM. E, H&E-stained SCCHN tumor with the corresponding RNA in situ hybridization of CD44 (red) and PD-L1 (green).

to possess cancer stem cell-like properties (13), we next sought to determine whether CD44+ cells that differed in their expression of PD-L1 had different tumorigenic potential in vivo. SCCHN tumors were sorted into CD44−, CD44+PD-L1−, and CD44+PD-L1+ subsets (Fig. 3A) and transplanted subcutaneously into Rag2−/−H2rg−/− immunodeficient mice. We observed that both CD44+PD-L1− and CD44+PD-L1+ cells formed xenograft tumors similarly over a range of cell numbers injected, while CD44− cells either did not form tumors (Fig. 3C) or had a substantially lower tumorigenic capacity despite higher cell numbers (Fig. 3D and E). Analysis of the xenograft tumors from CD44+PD-L1− and CD44+PD-L1+ transplants also showed that the tumors arising from the sorted transplants recapitulated the parental tumor expression of CD44 and PD-L1 (Fig. 3A and B). Thus, although CD44+ cells possess greater tumor-initiating capacity compared with the CD44− cells, PD-L1 expression does not distinguish intrinsic tumorigenic capacity within the CD44− subset.

PD-1/PD-L1 blockade restores IFNγ secretion by TILs against CD44+ SCCHN cells

To understand the biologic relevance of the differential expression of PD-L1, we assessed the ability of CD44+ and CD44− cells to stimulate autologous CD8+ TILs by in vitro coculture experiments. Consistent with the greater expression of PD-L1 on the CD44+ cells, this subset induced lower amounts of IFNγ secretion by the cocultured CD8+ TILs (Figs. 1G and 4A) compared with the CD44− subset. Importantly, the addition of blocking antibodies to PD-1/PD-L1 resulted in a significant increase in IFNγ levels produced in the CD44+ cocultures. (Fig. 4A and B); however, no increase in IFNγ levels was seen in the CD44− cocultures. An increase in IFNγ levels in the CD44+ cocultures was also observed with anti-PD-1 blockade alone (Supplementary Fig. S4). These data indicate that the greater PD-L1 expression on the CD44+ cells was biologically relevant in specifically contributing to T-cell inhibition.

The increase in IFNγ secretion by the T cells in the CD44+ cocultures with PD-1/PD-L1 blockade was significant but did not reach the levels produced in response to CD44+ cells, suggesting that additional mechanisms of immunosuppression were involved. As noted above, there was a significant increase in Tim-3, a negative regulator on CD8+ T cells (28, 29), on the CD8+ TILs post-expansion (Fig. 1D and E). We, therefore, assessed if Tim-3 blockade could further restore T-cell activity against CD44+ cells. Indeed, we observed a significant increase in CD8+ T-cell responses to CD44+ cells; however, this augmented response was also seen with CD44− cells and was not specific to CD44+ cells (Fig. 4A and D). Furthermore, the purported ligand for Tim-3, galectin-9 (30), was not detected in either the CD44+ or CD44− population (Supplementary Fig. S2C). The combination of PD-1 and Tim-3 blockade resulted in the greatest increase in IFNγ secretion against CD44+ cells, indicating a potential for significant combined activity in the clinical setting. Higher expression of CD47, a ligand that inhibits phagocytosis by macrophages, was also observed on CD44− cells (Supplementary Fig. S2B), consistent with previous studies where increased CD47 levels was observed on leukemia stem cells in human acute myeloid leukemia (AML) compared with bulk AML cells (31).
Thus, combination of PD-1 and CD47 blockade may also have additive or synergistic anti-tumor activity clinically.

**STAT3 regulates constitutive PD-L1 expression on CD44**

As STAT3 has been shown to bind to the PD-L1 promoter and regulate PD-L1 expression (32,33), we hypothesized that STAT3 activation may be a potential mechanism contributing to the constitutive PD-L1 expression on the CD44\(^+\) cells. Consistent with this possibility, we observed constitutive phosphorylation of STAT3 on CD44\(^+\) cells but not on CD44\(^-\) cells isolated from SCCHN xenografts (Fig. 5A and Supplementary Fig. S5), consistent with previous observations on SCCHN tumors (34). Correspondingly, tumor cells treated with Stattic, a selective STAT3 inhibitor (35), showed a specific decrease in PD-L1 expression on CD44\(^+\) cells (Fig. 5B and C), indicating that STAT3 activation was necessary for PD-L1 expression on CD44\(^+\) cells. Hence, the specific constitutive phosphorylation of STAT3 on CD44\(^+\) cells, in...
Selective PD-L1 Expression on CD44⁺ SCCHN Cells

Figure 4. Blockade of the PD-1/PD-L1 pathway partially restores IFNγ secretion by TILs against CD44⁺ SCCHN cells. A, CD44⁺ and CD44⁻ SCCHN cells were cocultured with autologous CD8⁺ TILs with 10 μg/mL of control mAb, 10 μg/mL of anti-PD-1, or 10 μg/mL anti-Tim-3. Graph shows the IFNγ concentrations from a single representative coculture experiment (n = 4, *P < 0.05, unpaired t tests), mean ± SD. B, IFNγ secretion fold-change with anti-PD-1/PD-L1 versus control mAb (n = 4), C, anti-PD-1/PD-L1/Tim-3 versus anti-Tim-3 (n = 6), and D) anti-Tim-3 versus control mAb (n = 4), mean ± SEM (*, P < 0.05, paired t tests).

and robust phosphorylation of STAT1, the transcription factor downstream of the IFNγ receptor signaling pathway, in CD44⁺ cells (Fig. 6G and H). Because IFNγ signaling also regulates MHC class I levels, we examined the expression of HLA-ABC following exposure to IFNγ, but found no significant increase; however, the levels of HLA-ABC were very high in both CD44⁺ and CD44⁻ cells and were likely at the inducible limit (Supplementary Fig. S6B and S6C). Thus, the greater responsiveness of the CD44⁺ subpopulation to IFNγ at the signaling level, compared with the CD44⁻ subpopulation, may contribute to the selective inducible expression of PD-L1 on the CD44⁺ cells.

Discussion

In this study, we addressed the intratumoral cellular heterogeneity observed in SCCHN and identified differential expression of PD-L1 between subsets of tumor cells as a major mechanism of heterogeneous immunogenicity. More precisely, analysis of primary human SCCHN tumors and PDXs showed a striking correlation between the constitutive expression of CD44 and PD-L1 at the transcriptional and translational level. This was in agreement with published reports that previously noted PD-L1 expression to be quite heterogeneous within SCCHN tumors (15, 16). Furthermore, our data from coculture experiments between autologous CD8⁺ T cells and purified CD44⁺ tumor cells demonstrated that this preferential expression of PD-L1 is biologically relevant.

Although blockade of the PD-1/PD-L1 axis significantly increased IFNγ production by CD8⁺ T cells cocultured with the CD44⁺ tumor cells, there was only partial restoration of IFNγ secretion to levels produced by CD8⁺ T cells cocultured with CD44⁻ tumor cells. This indicated the presence of additional mechanisms of immunosuppression and/or dysfunction. Our coculture experimental system required the in vitro expansion of sufficient numbers of CD8⁺ T cells from small tumor biopsies,
and as such, the IL-2 stimulation in the culture system led to upregulation of Tim-3 on the expanded T cells, indicating some degree of exhaustion that was unavoidable. The blockade of Tim-3 with the addition of Tim-3 antibodies to the cocultures further augmented the production of IFN-\(\gamma\) by the T cells; however, this was not specific to the cocultures with either the CD44\(^+\) or CD44\(^+/C0\) cells. The ligand responsible for the Tim-3–mediated inhibition remains unknown as Galectin-9 was not detected on the SCCHN cells. Although studies have shown galectin-9 to be the cognate ligand for Tim-3 (30), contradicting studies have also indicated lack of binding between galectin-9 and Tim-3 (39). Other Tim-3 ligands, such as HMGB1 (40) and Ceacam1 (41), have been identified and could potentially regulate Tim-3–mediated inhibition in SCCHN. Preliminary analysis of oral SCCHN cells indicates that Ceacam1 is expressed (data not shown); thus, trans-interaction between Ceacam1 on the tumor cells and Tim-3 on the CD8\(^+\) T cells may result in inhibition (41). The exact mechanism underlying Tim-3–mediated immunosuppression in SCCHN will be interesting in future studies. It is not surprising that immunosuppression in SCCHN is more complex than inhibition by PD-1 alone, and in the tumor microenvironment, there are likely multiple mechanisms of immunosuppression. In fact, clinical studies in melanoma have demonstrated that targeting combinations of checkpoint molecules, such as PD-1 and CTLA-4, have greater efficacy than targeting PD-1 alone (42). The additive effect of blocking Tim-3, in addition to PD-1, on T-cell function in our studies suggests a rationale for this combination therapy in the clinical setting of SCCHN.

PD-L1 expression on tumor cells has been described to be both constitutive and inducible (37). In our study, we observed constitutive phosphorylation of STAT3 on CD44\(^+\) cells, and a specific reduction of PD-L1 expression on CD44\(^+\) cells after addition of a STAT3 inhibitor. As previous studies have shown STAT3 to directly regulate PD-L1 expression (32,33), we propose constitutive STAT3 signaling to be a mechanism by which CD44\(^+\) cells express PD-L1 constitutively, resulting in innate immune resistance of these cells. Hence, the clinical use of STAT3 inhibitors, which has been reported to inhibit malignant cell growth and induce apoptosis in tumor cells (43,44), may also inhibit PD-L1 expression in tumor cells and restore T-cell activity. Currently, phase 0 trials with a STAT3 decoy oligonucleotide in head and neck tumors (45), and phase I trials with AZD9150, an antisense oligonucleotide inhibitor of STAT3, in hepatocellular carcinoma (46) have been completed, whereas phase I/II trials with AZD9150 in lymphomas and metastatic head and neck tumors are currently underway. It will be interesting to examine PD-L1 expression on tumor cells from patients enrolled in these clinical studies targeting STAT3.

PD-L1 expression on tumor cells has also been described to be inducible in response to IFN\(\gamma\) secreted by immune cells in the tumor microenvironment during the endogenous antitumor response (37,47). Interestingly, this IFN\(\gamma\)-inducible expression

**Figure 5.**

CD44\(^+\) SCCHN cells express p-STAT3 constitutively and show reduced PD-L1 expression after STAT3 inhibition. A, Western blot analyses of p-STAT3 and STAT3 protein levels in pairs of sorted CD44\(^+\) cells and CD44\(^+/C0\) cells. B, representative plots of CD44 and PD-L1 expression on two SCCHN tumors with DMSO vehicle control or 10 \(\mu\)mol/L Stattic (Sigma, S7947), a STAT3 inhibitor, after 10 hours. C, timecourse of CD44\(^+/C0\)/CD44\(^+\) fold-change of PD-L1 MFI on two SCCHN tumors with DMSO vehicle control or 10 \(\mu\)mol/L Stattic.
of PD-L1 was restricted to the CD44^+ cells in our experiments. Furthermore, there was greater expression of the IFNGR1 on the CD44^+ cells and more robust phosphorylation of STAT1 after IFNγ exposure, indicating that the IFNγ pathway is more primed for response in this subpopulation. In our studies, the enhanced response to IFNγ by the CD44^+ cells resulted in even greater expression of PD-L1 (above baseline expression) in response to IFNγ. In the tumor microenvironment, this response to IFNγ likely results in a feed forward type of immunosuppression by these cells to TILs. Future studies of mediators enhancing IFNγ production, such as OX-40 and CD137 agonists, may benefit from the addition of PD-1 checkpoint inhibitors.

CD44^+ cells in SCCHN tumors have been proposed to be tumor-initiating cells or cancer stem cells due to their ability to propagate serial in vivo passage and recapitulate the morphology and phenotype of the original parental tumors after engraftment, as compared to CD44^- cells (13). The intrinsic stem cell-like properties of these cells potentially allow them to persist and self-renew following therapy. Our results indicate CD44^+ cells to have higher levels of ZEB1 and STAT3 phosphorylation, both of which have been shown to regulate PD-L1 expression (26,32,33) and contribute to cancer stemness and tumorigenesis (48–51), lending credence to our hypothesis that cancer stemness could potentially be associated with PD-L1-mediated immunosuppression.
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Notably, in human lung squamous cell carcinoma, expression of PD-L1 was reported to be highly correlated with tumor-propagating cells in that disease (52). In addition, higher expression of CD44v3 and v6 variant isoforms has been observed in metastatic lymph nodes compared to primary SCCHN tumors (53), which may result in increased immunosuppression at these sites due to the higher percentage of CD44⁺ tumor cells. Future studies examining SCCHN metastases for PD-L1 expression relative to primary tumors will be interesting. Our data would suggest that there is greater PD-L1 expression on metastatic tumors cells and provide potential rationale for the use of anti-PD-1 therapy in the adjuvant treatment setting for high-risk primary tumors, even in the absence of clinically manifested metastases. Finally, because the cancer stem cell—like phenotype of cells within the CD44⁺ compartment has been demonstrated to be associated with enhanced Wnt signaling (54–56), it is interesting to speculate that the CD44⁺ cells may use additional mechanisms of immunosuppression. In particular, it has been recently reported that T-cell exclusion from the tumor microenvironment is controlled by the Wnt pathway (57), and therefore, patients with tumors having greater CD44⁺ cell proportions may tend to be non-responders to PD-1 blockade. It will be interesting to examine CD44 and other markers of cancer stem cells as potential biomarkers of PD-1 response in ongoing clinical trials.

In summary, our data demonstrate that a major mechanism underlying the immunosuppressive nature of CD44⁺ cells in SCCHN is the significantly greater constitutive and inducible expression of PD-L1 on this population. Because CD44⁺ cells have the capacity for long-term self-renewal and tumor initiation, the immunosuppression conferred by PD-L1 may augment the resilience of these cells and allow them to selectively suppress and evade immune responses and contribute to tumor dormancy in the context of minimal residual disease. Our findings provide an explanation for the durable responses observed with PD-1 blockade in the treatment of advanced disease and provide rationale for use of PD-1 blockade in the adjuvant setting following surgical resection of SCCHN tumors at high-risk for recurrence.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Y. Lee
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