B Cells Improve Overall Survival in HPV-Associated Squamous Cell Carcinomas and Are Activated by Radiation and PD-1 Blockade

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ABSTRACT

Purpose: To characterize the role of B cells on human papilloma virus (HPV)-associated cancer patient outcomes and determine the effects of radiation and PD-1 blockade on B-cell populations.

Experimental Design: Tumor RNA-sequencing data from over 800 patients with head and neck squamous cell carcinoma (HNSCC) and cervical cancer, including a prospective validation cohort, was analyzed to study the impact of B-cell gene expression on overall survival (OS). A novel murine model of HPV+ HNSCC was used to study the effects of PD-1 blockade and radiotherapy on B-cell activation, differentiation, and clonality including analysis by single-cell RNA-sequencing and B-cell receptor (BCR)-sequencing. Human protein microarray was then used to quantify B-cell–mediated IgG and IgM antibodies to over 16,000 proteins in the serum of patients treated on a clinical trial with PD-1 blockade.

Introduction

The essential role that the immune system plays in tumor control is now at the forefront of oncology, and understanding how innate and adaptive antitumor responses work in concert is vital to improving the efficacy of immunotherapy (1, 2). Many studies evaluating antitumor immune responses have focused on T cells and myeloid cells; however, the role of B cells in oncology has been studied much less frequently. There exist multiple distinct subsets of human B cells including B-cell progenitors, immature B cells, plasma cells, memory B cells, and immunosuppressive or regulatory B cells (B-regs; refs. 3, 4). In addition, formation of germinal centers (GC) is the hallmark of B-cell–mediated adaptive immunity and is required for proper B-cell affinity maturation and antibody diversification (5). While B cells are principally known for humoral immune responses, the function of B cells that migrate and infiltrate primary tumors remains poorly understood.

In melanoma, B-cell depletion was associated with decreased local tumor control, increased lung metastases, and impaired tumor antigenspecific CD8+ T-cell proliferation (6). A recent report has shown that tumor-associated B cells may play a role in maintaining inflammatory responses in melanoma (7). In head and neck squamous cell carcinoma (HNSCC), an early study of 33 patients did not observe an association between tumor-infiltrating immune cells at the primary site and patient outcomes, but did demonstrate improved outcomes with increased peritumoral B cells in lymph node metastases (8). Recent analyses of B-cell phenotypes and responses in HNSCC described significant heterogeneity; however, the effect of B cells on survival or responses to immunotherapy or radiation was not explored (9, 10). Conversely, other studies have described an immunosuppressive or protumorigenic role for B cells including a subset of IL10-producing B-regs (11-13), and B cells have been implicated in contributing to chronic inflammation that can then lead to de novo carcinogenesis in squamous cell carcinomas (14). Recently, two studies have described a major role of B cells in melanoma and soft-tissue sarcomas in mediating responses to immunotherapy; however, the role of B cells in HNSCC, and the effect of specific treatment regimens, including PD-1 blockade and radiotherapy, on modulating B-cell populations remains largely unknown (15, 16).

Anti-PD-1/PD-L1 checkpoint blockade immunotherapy (CBI) is approved for metastatic squamous cell carcinomas including HNSCC, cervical cancer, and lung cancer (17–19). For HNSCC, radiotherapy and radiotherapy combined with concurrent chemotherapy are two of the most effective treatment options and a standard of care for locally advanced disease (20). Importantly, human papilloma virus (HPV)-
Translational Relevance

The human papilloma virus (HPV) is thought to be the causative agent for approximately 5% of all cancers worldwide, resulting in significant morbidity and mortality. Many studies evaluating immune correlates in the tumor microenvironment have focused on T cells or myeloid cells; however, the role of B-cell populations is rarely studied. Here, we discovered a remarkable beneficial role for B cells in overall survival (OS) of patients with squamous cell carcinomas and used cutting edge technologies to comprehensively characterize the effects of PD-1 blockade and radiotherapy on B-cell populations. Specifically, we have identified CD19 and IGJ as single-gene B-cell-specific prognostic biomarkers for 3-year OS. Furthermore, we discovered that B-cell-mediated IgG and IgM antibody responses correlate with response patterns to PD-1 blockade. These data establish a key role for B cells in HPV-associated squamous cell carcinomas and provide strong rationale for development of additional diagnostics and novel therapeutics targeting B cells.

Flow cytometry, cell surface staining, and tumor-infiltrating lymphocytes preparation

Single-cell suspensions were generated from spleens, inguinal lymph nodes, and tumors. To isolate tumor-infiltrating lymphocytes, density gradient centrifugation using an 80%/40% Percoll (GE Healthcare) gradient was done on the single-cell suspensions from excised tumors. Cells were then stained with fluorochrome-conjugated antibodies from BioLegend and BD Biosciences and analyzed using an LSR II Flow Cytometer (BD Biosciences). Antibodies against the following cell surface markers (clone) were used: CD19 (6D5), B220 (RA3-6B2), IgD (11-26c.2a), IgM (RM1-1), IgG (Poly4053), CD80 (16-10A1), MHC II (MS/114.15.2), CD27 (LG.3A10), PD-L2 (TY25), CD1d (1B1), CD5 (53-7.3), CD138 (281-2), and CD45.2 (104). In addition, a live/dead Zombie Yellow stain and fluorochrome-conjugated streptavidin with biotinylated-ovalbumin at approximately 1:10 molar ratio were used. For the apoptosis assay, a Flow Cytometry-Based Kit (BioLegend) utilizing Annexin V, propidium iodide (PI), and select cell surface markers were used following the manufacturer’s protocol. Flow cytometry data were then analyzed using FlowJo v10 (BD Biosciences).

Antigen-multimer generation

OVA purchased from Sigma-Aldrich was biotinylated and isolated using the EZ-Link NHS-PEG4-Biotinylation Kit from Thermo Fisher Scientific per kit protocol. Biotinylation of the OVA protein was successively verified using a 4-hydroxyazobenzene-2-carboxylic acid assay. Biotinylated-OVA was then stored at −80°C until use.

Serum OVA antibody analysis

Mouse whole blood was collected by cardiac puncture at the time of euthanization. Serum was isolated by allowing whole blood to coagulate at room temperature for 90 minutes prior to centrifugation at 1,000 × g for 20 minutes. Serum supernatant was then collected and stored at −80°C. Serum OVA antibodies were analyzed using an in-house generated anti-Ovalbumin total IgG (mouse) ELISA assay. In brief, plates were loaded with 1 μg OVA protein per well and incubated overnight at 4°C. Plates were then washed the next day and blocked with PBS + 1% BSA for 2 hours. Sera were then diluted 1:100 prior to loading onto the ELISA plate for 1 hour. Secondary rabbit anti-mouse antibody conjugated to horseradish peroxidase was then added and incubated for 30 minutes. TMB reagent was then added and then quenched with 2.5 mol/L HCl. Absorbance at 450 nm was then read.
using a TECAN infinite M200 plate reader. Standard murine anti-
OVA IgG antibody (3G2E1D9) was used to generate titration curves.

**Tumor growth experiments, anti-PD-L1 therapy, and radiotherapy**

B16-OVA and AT-84-E7-OVA cells were cultured and injected subcutaneously in the right flank with $1.5 \times 10^5$ cells (B16-OVA) or $5 \times 10^5$ cells (AT-84-E7-OVA) on day 0. The injection sites were monitored until a tumor was palpable. The tumor was then measured using electronic calipers every 3 days and tumor volume was calculated using $(l \times w^2)/2$. Anti-PD-L1 therapy and radiotherapy was started as soon as tumors were palpable. Mice were treated with 200 µg anti-PD-L1 antibody (clone 10F.9G2, BioXcell) via intraperitoneal injections every 3 days for a total of three injections per mouse. Mice were treated with 12–18 Gy of focal radiation to the tumor site using a JL Shepherd Cs-137 Irradiation (JL Shepard and Associates), a dose rate of 2.53 Gy/minute. To direct radiation, customized lead shielding with a jig to immobilize the region receiving radiation were used for each mouse. To deplete B cells, 250 µg of anti-CD20 antibody (clone SA271G2, BioLegend) was given intravenously through the tail vein following the manufacturer’s instructions 5 days prior to tumor injection.

**B-cell sorting, single-cell RNA-sequencing library preparation and sequencing**

B cells from inguinal or tumor-draining lymph nodes (TDLN) were pooled from three different mice to obtain single-cell suspensions. Cells were then stained with fluorophore-conjugated antibodies from BioLegend and sorted on a FACSAria cell sorter. Antibodies against the following cell surface marker (clone) were used: CD19 (1D3/ CD19), CD45.2 (104), CD3e (145-2C22), Gr-1 (RB6-8C5), CD11b (M1/70), and TER-119 (TER-119). Cells were additionally stained with PI. Following sorting, cells were counted using an Invitrogen Countess II FL and loaded into the 10x-Genomics Chromium controller. 10x-Genomics v3 libraries were then prepared as per the manufacturer’s recommendations. Initial quality control was done using a Qubit 2.0 Fluorometer. Libraries were then sequenced aiming for a minimum coverage of 20,000 reads per cell using an Illumina HiSeq 4000 at the Institute for Genomic Medicine at the University of California, San Diego (La Jolla, CA). Single-cell RNA-sequencing data were initially analyzed using Cell Ranger (10x Genomics) and converted into matrix files. The data were then subsequently analyzed using Seurat v3 (30, 31).

**B-cell receptor sequencing**

VDJ recombination sequencing analysis was performed by Adaptive Biotechnologies on genomic DNA isolated from TDLNs and tumors. DNA was extracted using a Qiagen DNeasy Kit per kit protocol. For the sequencing analysis, libraries were prepared by multiplex PCR to cover all possible IgH rearrangements, followed by high-throughput sequencing of CDR3 of the B-cell receptor (BCR). Data were initially visualized and analyzed using the ImmunoSEQ Analyzer.

**HuProt proteome microarray analysis**

Patient sera were collected from consented patients under a University of California, San Diego, Human Research Protections Program Institutional Review Board (IRB)-approved protocol (HRPP# 151570). The study was conducted in accordance with ethical principles from the Declaration of Helsinki. Sera were analyzed using the HuProt Proteome Microarray v3.1 (CDI Laboratories, Inc.). In brief, sera were diluted 1:1,000 before incubating on a slide with >16,000 GST-tagged human proteins. Following sera incubation, a fluorophore-conjugated secondary antibody recognizing either IgG or IgM was added prior to plate reading.

**Patient HNSCC and cervical cancer tumor RNA-sequencing analysis**

Patient HNSCC tumor RNA-sequencing data were obtained from The Cancer Genome Atlas and a prospectively collected, proprietary, curated database of HPV+ HNSCC from JHU (Baltimore, MD). Patient cervical cancer tumor RNA-sequencing data were obtained from TCGA, TCGA RNASeq data was obtained from the NCI Genomic Data Commons (http://portal.gdc.cancer.gov/). Thresholds for determining “high” versus “low” expression were set at both the median and 75th percentile of gene reads.

**Statistical analysis**

Statistical analysis was performed using Prism 7 (GraphPad) and R. One-way ANOVA with a Tukey post hoc test for multiple comparisons was conducted for flow cytometry and BCR-sequencing analysis. Cox proportional hazard models were used to characterize the difference between gene expression levels in patient survival analysis. The effect of clinical, demographic, and gene expression levels on 3-year OS was assessed using multivariate logistic regression. All data are shown as mean ± SEM. Results were considered significant if $P < 0.05; **P < 0.01; ***P < 0.001.$

**Study approval**

Patients in the JHU cohort were recruited under protocol NA_00-36235, which was approved by the JHU IRB. Patients in the University of California, San Diego (UCSD) cohort were recruited under IRB-approved protocol UCSD HRPP 151570 (ClinicalTrials.gov identifier: NCT02843165). Animal experiments were performed at the University of California, San Diego, Moores Cancer Center (La Jolla, CA) under protocols approved by the Institutional Animal Care and Use Committee Office.

**Results**

RNA-sequencing analysis demonstrates clear survival advantage with high expression of B-cell–specific markers in squamous cell carcinomas

To study the importance of B cells in survival of patients with HPV+ squamous cell carcinomas, we analyzed B-cell–specific genes in tumor samples from TCGA RNA-sequencing database. CD19 is a coreceptor for the BCR and is a canonical B-cell marker (32). On the basis of CD19 expression, we divided all TCGA patients with HNSCC into either high-expressing or low-expressing relative to median expression, we divided all TCGA patients with HNSCC into either high-expressing or low-expressing relative to median expression (Supplementary Table S1). We did observe baseline differences between these groups with an increased percentage of HPV+ oropharyngeal subsite patients in the CD19-high cohort. When stratifying all patients by CD19 status, we observed a dramatic increase in 3-year OS in patients with high expression of CD19 (Fig. 1A). There was a median survival of 5.65 years in CD19-high patients versus 2.70 years in CD19-low patients [3-year OS HR of 0.345; 95% confidence interval (CI), 0.41–0.72; $P < 0.001.$ CD19-high patients had 3-year survival rates of 68.9% (95% CI, 0.63–0.76) compared with 47.4% (95% CI 0.41–0.55) in CD19-low patients. When stratifying by HPV status, we found HPV+ CD19-low patients also had worse survival, and that HPV+ CD19-low patients had an unexpected dramatically worse survival (Fig. 1B; Supplementary Fig. S1A). These findings demonstrate the
impact of CD19 B-cell expression on outcomes regardless of HPV status.

As HPV⁺ oropharyngeal subsite patients are known to have improved outcomes compared with HPV⁻ patients, we performed multivariate logistic analysis of survival based on CD19 expression controlling for clinical–pathologic features including HPV status, smoking status, disease subsite, tumor–node–metastasis stage, and CD8 or CD45 expression status among others (Supplementary Tables
S2 and S3). CD8 and CD45 expression status were separately included to control for an inflamed tumor microenvironment. On multivariate analysis, CD19 expression remained highly significant, and above-median expression was protective, with a 3-year OS HR of 0.58 (P = 0.001) and 0.55 (P = 0.003) when accounting for CD8 and CD45 expression, respectively. Univariate regression analyses for individual variables used in the multivariate analysis are shown in Supplementary Table S4. Intriguingly, CD19 was shown to be a stronger predictor for OS than either CD8 or CD45 expression status, supporting the independent prognostic capacity of CD19 expression as a biomarker for survival in HNSCC. Moreover, there was a significant interaction between CD19 expression and HPV status such that CD19 expression level has an increased effect size in stratifying survival in HPV+ patients compared with HPV− patients (Fig. 1; Supplementary Table S5). Thus, pretreatment CD19 expression is highly prognostic of OS independent of anatomic subsite, HPV status, and clinical stage, and may reflect the underlying biology of HNSCC tumors. While the expression of CD19 indicates the presence of B cells, we wanted to confirm these findings with additional B-cell–specific genes. The IGJ gene (also referred to as JCHAIN) encodes the J-chain, an adaptor protein for IgM and IgA antibodies, and is one of the most specific B-cell markers (33). In all HNSCC cases, there was a median survival of 7.40 years for IGJ-high patients versus 3.91 years for IGJ-low patients (3-year OS HR of 0.49; 95% CI, 0.34–0.70; P < 0.001; Fig. 1E). IGJ-high patients had a 3-year survival rates of 74.8% (95% CI, 0.67–0.84) compared with 52% (95% CI 0.47–0.58) for IGJ-low patients. Analysis of survival by HPV status and IGJ expression are shown in Figs. 1F and Supplementary Fig. S1B. Multivariate analysis was repeated and demonstrated a significant correlation with 3-year OS with a HR of 0.70 (P = 0.001) and 0.57 (P = 0.002) when accounting for CD8 and CD45 expression, respectively; complete results are shown in Supplementary Tables S6 and S7. In addition, we analyzed the effect of multiple B-cell genes involved in development, differentiation, and memory formation on OS using both median and 75th percentile gene expression cutoffs to define high versus low expression (10). We found that high expression of multiple B-cell genes correlated with improved survival, and these results calculating 3-year OS HR, using both thresholds of median and 75th percentile expression levels, are summarized in Supplementary Table S8. To cross-validate these findings in a different HPV-related malignancy, we performed a similar analysis of TCGA data from patients with cervical cancer. We observed no baseline differences in various clinical–pathologic variables in patients classified as being CD19 high versus CD19 low (Supplementary Table S9). Similar to HNSCC, high expression of either CD19 or IGJ was associated with improved OS in patients with cervical cancer compared with those who had low expression of CD19 or IGJ, respectively (Fig. 1C and G). Prospective analysis confirms survival advantage with CD19 and IGJ B-cell markers in independent HPV+ validation cohort To further validate these findings, we analyzed an independent dataset of prospectively collected tumor RNA-sequencing data from 35 patients with HPV+ HNSCC (Supplementary Table S10). In this validation dataset, we observed concordant increases in survival in patients with high expression of CD19 (HR, 0.23; 95% CI, 0.06–0.95; P = 0.04; Fig. 1D) or IGJ (HR, 0.25; 95% CI, 0.06–0.997; P = 0.058; Fig. 1H). Taken together, these findings identify CD19 and IGJ as novel B-cell biomarkers that are highly prognostic of 3-year OS in patients with squamous cell carcinomas. Importantly, while HPV+ patients are known to have a better overall prognosis compared with HPV− patients, CD19 or IGJ expression was able to stratify HPV+ patients biologically into CD19+HPV+ patients with an excellent prognosis, and CD19−/HPV+ patients with a dramatic and unexpected worse prognosis. B-cell depletion promotes tumor growth To analyze the functionality of B cells in HNSCC and effects of treatment we used the AT-84-E7 murine model (34). AT-84-E7 is an HPV oncogene expressing syngeneic HNSCC model derived from oral squamous mucosa of the C3H mouse (34), which was also engineered to express OVA (AT-84-E7-OVA). We found that treatment with anti-PD-L1 CBI alone did not significantly decrease tumor growth, while radiotherapy led to reduced tumor growth, and the addition of anti-PD-L1 CBI to radiotherapy (referred to as “combined therapy”) further improved tumor control (Fig. 2A). To evaluate the effect of B-cell function on local tumor control, mice were treated with anti-CD20 antibody intravenously to deplete peripheral B cells (35). Mice were treated 5 days prior to tumor injection to ensure that B cells were depleted prior to tumorigenesis to mitigate the early effects of B-cell immunity derived from injection. We found that B-cell–depleted mice developed larger tumors with a faster tumor growth velocity than matched control mice (Fig. 2C). In addition, B-cell–depleted mice trended to having larger tumor size after treatment with radiotherapy, anti-PD-L1 CBI, or combined therapy (Supplementary Fig. S2). Anti-PD-L1 immunotherapy and radiotherapy enhance B-cell maturation and activation Next, we used multiparametric flow cytometry to further analyze the impact of CBI and radiotherapy on B-cell phenotypes. We analyzed surface expression of MHC II on mature (IgM−, IgA−), transition type 1 (T1; IgD−IgM+), and transitional type 2 (T2; IgD−IgMhigh) B cells in the TDLN of AT-84-E7-OVA tumor-bearing mice by looking at geometric mean fluorescence index (Fig. 2D). A schematic of the full gating strategy used is provided in Supplementary Fig. S3. MHC II expression on B cells is important for its antigen-presenting capabilities to CD4 T cells and successive differentiation and effector functions (36, 37). In the TDLN, combined therapy led to the greatest increase in surface MHC II in mature and T2 B cells, while in T1 B cells, anti-PD-L1 CBI was associated with the highest MHC II expression (Fig. 2E). In the spleen, a similar analysis found that radiotherapy paradoxically decreased MHC II expression in T1 and T2 B cells, and this was rescued with combined therapy (Supplementary Fig. S4A and S4B). To examine these findings in a different model system and mouse background, we repeated these experiments using a B16-OVA melanoma model in C57BL/6 mice. Combined therapy led to the greatest local tumor control as previously shown (Fig. 2B; ref. 38). In the TDLN, we identified mature, T1, and T2 B cells (Fig. 2F), and there were concordant findings with combination therapy leading to the greatest increase in MHC II expression across all B cells (Fig. 2G). On splenic B cells in this system, we found that combined therapy led to the highest upregulation of MHC II across all B cells (Supplementary Fig. S4C and S4D). Overall, these data demonstrate radiotherapy can prime mature B cells and skew them toward a more activated state, and anti-PD-L1 CBI can augment this effect. PD-1 blockade can reverse radiation-induced increases in B-regs We next examined immunosuppressive B-regs in the TDLN. As described by Yanaba and colleagues, B-regs were described by Yanaba and colleagues, B-regs were identified as...
Figure 2.
B-cell depletion promotes tumor growth, and anti-PD-L1 immunotherapy and radiotherapy modulate B-cell activation. A, Tumor volumes of mice (n = 6–7 per group) inoculated with 5 × 10^5 cells of AT84-E7-OVA tumor cells. Treatment with radiation (XRT, 12 Gy × 1) and/or anti-PD-L1 CBI (200 μg every 3 days for three total doses) beginning on day 6. This experiment was conducted three times with similar results. B, Tumor volumes of mice (n = 6–7 per group) inoculated with 1 × 10^5 cells of B16-OVA tumor cells. C, Tumor volumes of mice (n = 5 per group) inoculated with 5 × 10^5 cells of AT-84-E7-OVA tumor cells. Mice were treated with anti-CD20 antibody to deplete B cells. D and F, Representative contour plots for IgD and IgM expression to define mature, T1, and T2 B cells in the TDLN. E and G, MHC II expression within each subpopulation of B cells in the TDLN. H, Representative contour plot and gating schematic for B-regs in the TDLN. I, Percent B-regs (CD1dHigh, CD5−) in the TDLN. Statistical analysis was evaluated by one-way ANOVA with multiple comparisons (*, P ≤ 0.05; **, P ≤ 0.01; ***, P ≤ 0.001; ****, P ≤ 0.0001).
Radiotherapy enhances development of antigen-specific B cells, promotes plasma cell and memory B-cell differentiation, and stimulates antibody production.

**A** and **C**, Representative contour plot of antigen-specific B cells in AT-84-E7-OVA and B16-OVA models. **B** and **D**, Percent IgG⁺OVA-Multimer⁺ B cells in the TDLN. Percent plasma cells (CD138⁺, IgG⁺) in the TDLN (E) and spleen (F). **G**, Antibody concentrations against OVA in B16-OVA-bearing mice. **H**, Representative contour plot for SP and DP memory populations, gated on IgG⁺ cells. Percent of each memory population in AT-84-E7-OVA-bearing mice (I) and B16-OVA-bearing mice (J). **K–M**, Percent of cells in each group that were live (PI⁻/AnnV⁻), in early apoptosis (PI⁻/AnnV⁺), and in late apoptosis (PI⁺/AnnV⁺). Groups contained 6–8 mice per group. ns, not significant; UN, untreated; XRT, treatment with radiotherapy. Statistical analysis was evaluated by one-way ANOVA with multiple comparisons (*, P ≤ 0.05; **, P ≤ 0.01; ***, P ≤ 0.001; ****, P ≤ 0.0001).
CD1dHighCD5−, and secrete IL10 to inhibit T-cell–dependent responses; there was also a distinct population of CD1dLowCD5− whose relevance is not clearly defined (11). We applied this gating strategy to CD19+ B220− lymphocytes (Fig. 2H). In the TDLN in both the AT-84-E7-OVA and B16-OVA models, radiotherapy increased CD1dHighCD5− Bregs; however, this increase was abrogated with combined therapy (Fig. 2I). As radiotherapy was associated with increased B-regs in the TDLN, there may be a negative effect of radiotherapy alone in inducing immunosuppressive cells, which can be partially reversed with the addition of PD-1 blockade. This ability for PD-1 blockade to mitigate the increase in B-regs due to radiotherapy alone is analogous to what we observed for CD4+CD25+Foxp3+ regulatory T cells in the tumor (27). The ability for radiotherapy to induce immunosuppressive T regulatory cells has been reported by multiple groups; however, this is the first report to our knowledge demonstrating that radiotherapy alone can induce B-reg populations (27, 39). Radiation increases tumor antigen–specific B cells and plasma cell differentiation

To analyze antigen–defined B cells using our AT-84-E7-OVA model we generated an “OVA-Multimer” reagent by biotinylating OVA molecules and staining with a fluorophore-conjugated streptavidin molecule to identify B cells possessing BCRs that recognize OVA. We observed that B cells possessing BCRs that bind OVA could be divided into IgG+ and IgG− (Fig. 3A). When analyzing IgG+ cells in the TDLN, there were dramatic changes to antigen–specific B cells as radiotherapy alone significantly increased the proportion of IgG+ OVA-Multimer+ B cells (Fig. 3B). Interestingly, the addition of anti-PD-L1 CBI appeared to blunt this increase, perhaps due to relative changes in other B-cell populations, which can downregulate BCR upon activation. Interestingly, we did not observe any increase in OVA-Multimer+ B cells in the spleen, which may be related to differences in antigen processing and capture in the TDLN versus spleen (Supplementary Fig. S5A). When tested in a B16-OVA model, similar effects to the populations of IgG+ OVA-Multimer+ B cells in the TDLN and spleen were observed (Fig. 3C and D; Supplementary Fig. 5B).

To study the effect of CBI and radiotherapy on B-cell plasma cell differentiation from CD19+IgD− lymphocytes, we analyzed CD138+IgG+ and CD138+IgG− subsets. CD138 is a canonical marker for plasma cells and is a member of the heparan sulfate proteoglycan family involved in many cellular pathways, including cellular adhesion (40). In both AT-84-E7-OVA and B16-OVA models, mice that were treated with combined therapy exhibited a significant increase in CD138+IgG+ plasma cells in the TDLN (Fig. 3E). In the spleen, combined therapy similarly significantly increased populations of CD138+IgG+ plasma cells in both AT-84-E7-OVA and B16-OVA models (Fig. 3F). To account quantify absolute counts of plasma cells, we irradiated spleens with 8 Gy or 20 Gy and confirmed an increase in absolute count of plasma cells in the spleen, which supports our findings of increased frequency of plasma cells induced with radiotherapy (Supplementary Fig. S5C). To quantify changes in B-cell–mediated humoral antibody responses, we measured concentrations of antibodies against OVA and confirmed that mice bearing B16-OVA tumors treated with combined therapy had the highest anti-OVA antibody concentrations (Supplementary Fig. S3G). Taken together, these data indicate that anti-PD-L1 CBI and radiotherapy can increase B-cell differentiation into plasma cells and enhance B-cell–mediated antigen–specific antibody responses.

PD-1 blockade and radiation modulate development of B-cell memory cells in the TDLN

Memory B-cell subsets are rare populations, which can dramatically expand upon exposure to cognate antigen. To analyze memory B cells, we use a combination of CD80, PD-L2, and surface expression of IgG and IgM to identify four distinct B-cell memory subsets: IgG+CD80−PD-L2+ [IgG+ single positive (SP)], IgG+CD80−PD-L2+ [IgG+ double positive (DP)], IgG−IgM+CD80−PD-L2+ (IgM−SP), and IgG−IgM+CD80+PD-L2− (IgM+ DP) as defined by Zuccarino-Catania and colleagues (Fig. 3H; ref. 41). In the TDLN of the AT-84-E7-OVA model, treatment with radiotherapy led to a dramatic increase in the populations of IgG+ SP and DP memory B cells, and this was augmented in mice that received combined therapy (Fig. 3I). In the spleen, combined therapy led to decreased IgG− and IgM− SP memory B cells (Supplementary Fig. S6A).

Similarly, in the B16-OVA model, combination therapy resulted in the greatest increased IgG+ SP memory B cells (Fig. 3J, left). Radiotherapy itself increased IgM+ SP memory B cells, and this was not significantly altered with combined therapy (Fig. 3J, right). In the spleen of these mice, combined therapy led to the highest increases in the populations of IgG+ SP and IgM+ SP B cells (Supplementary Fig. S6). The modulation of only a subpopulation of memory B-cell populations demonstrates the differential effect of radiotherapy and anti-PD-L1 CBI on B cells and warrants further investigation into the molecular mechanisms modulating memory development.

Differential radiosensitivity of B-cell subsets

Next, we investigated whether specific B-cell subsets had variable radiosensitivity by irradiating spleens of wild-type mice and analyzing apoptosis ex vivo of CD19+ B220+ cells using a combination of PI and Annexin V (AnnV). We quantified the proportion of cells that were live (PI−AnnV−), undergoing early apoptosis (PI−AnnV+), and undergoing late apoptosis (PI+AnnV+). We observed that CD19+ B220+ B cells in general are susceptible to undergoing apoptosis by 20 Gy (Fig. 3K). Nonclass-switched memory B cells showed an increased susceptibility to apoptosis at 8 Gy and 20 Gy, while plasma cells have a nonsignificant increase in apoptotic cells (Fig. 3L and M). This suggests that B cells are susceptible to radiation-induced apoptosis, and specific subsets possess differential radiosensitivity.

BCR-sequencing demonstrates changes to B-cell repertoire and clonality due to radiation and anti-PD-L1 CBI

To characterize changes in the antigen repertoire of B cells, we performed BCR-sequencing on matched TDLN and tumor in mice bearing AT-84-E7-OVA tumors treated with CBI and/or radiotherapy. Within all TDLN samples, there were multiple shared B cell clones across treatment groups (Fig. 4A). Productive templates, defined as sequences that are in-frame and do not contain a stop codon, can be assayed using BCR-sequencing. Mice treated with radiotherapy had a lower number of total productive templates, but higher fraction of productive templates in the TDLN (Supplementary Fig. S7A and S7B). Importantly, we observed an increase in productive clonality, which measures the clonality of productive templates, and maximum productive frequency in mice treated with radiotherapy alone. The addition of radiotherapy to anti-PD-L1 CBI significantly increased productive clonality but not maximum productive frequency (Fig. 4B and C).

We next looked at somatic hypermutation (SHM), which is a critical process for affinity maturation of GC B cells. Anti-PD-L1 CBI or radiotherapy alone did not significantly alter the mean number of
SHM in each productive BCR sequence; however, mice treated with combined therapy had more SHM per productive sequence (Fig. 4D).

BCR-sequencing of tumor samples demonstrated less drastic changes to B-cell phenotype, likely due to the lower frequency of tumor-infiltrating B cells. There was a trend toward increased total productive templates and productive clonality in mice treated with radiotherapy alone (Supplementary Fig. S7D and S7E). Radiotherapy alone significantly increased maximum productivity frequency, whereas radiotherapy and anti-PD-L1 CBI alone but not in combination increased mean number of SHM (Supplementary Fig. S7F and S7G).

Radiotherapy drives changes in BCR CDR3 loop length

On average, mature B cells that have undergone affinity maturation and antigen selection have BCRs with shorter CDR3 regions than immature B cells (42). We observed that in the TDLN, radiotherapy alone decreased the mean CDR3 loop length (Supplementary Fig. S7C). Mice treated with combined therapy had a mean CDR3 loop length that was similar to mice treated with radiotherapy alone, but statistically lower than mice treated with anti-PD-L1 CBI alone. When calculating the fraction of BCR sequences with CDR3 loops less than 12 amino acids, mice treated with radiotherapy alone had the highest proportion of short CDR3 loops (Fig. 4E). In the tumor, we did not observe significant changes in CDR3 loop length based on treatment (Supplementary Fig. S7H).

Single-cell RNA-sequencing reveals distinct subpopulations of B cells

To analyze changes in B-cell subpopulations in further detail, we performed single-cell RNA-sequencing (scRNA-seq) on FACS B cells. CD3 CD11b Gr-1 Ter-199 CD19 B cells from naïve nontumor-bearing mice, and TDLN from AT-84-E7-OVA flank tumor-bearing mice treated with anti-PD-L1 CBI, radiotherapy, radiotherapy combined with anti-PD-L1 CBI, and controls, were subjected to scRNA-seq. The B-cell sorting procedure and quality control measures for sequencing are shown in Supplementary Fig. S8. We first compared all tumor-bearing mice, both as individual datasets and as a single, integrated dataset (Supplementary Fig. S9A). Uniform manifold approximation and projection (UMAP) and unsupervised clustering of the integrated data revealed two clearly distinct subsets of B cells divided into six clusters (Fig. 5A). These two subsets were found to represent either GC or follicular B cells based on differential gene expression analysis of genes previously characterized to be upregulated in GC B cells, such as Bcl6, Mki67, and Aicda (Fig. 5B; ref. 43). We identified four unique clusters, named F1–F4, representing follicular B-cells (Fig. 5C). Cluster F1 represented naïve follicular B cells, with increased expression of genes such as Jun, CD55, and Vpreb3 (Fig. 5B). Cluster F2 had particularly high expression of Mif, a proinflammatory cytokine with chemokine functionality, and Il4i1; cluster F3 had increased expression of genes such as Plack, Fert5, and Apoe, and
Figure 5.

ScRNA-seq demonstrates that radiation combined with anti-PD-L1 immunotherapy significantly enhances development of GC B cells. 

A, UMAP plot of integrated analysis of sorted B cells from the TDLN of tumor-bearing mice that were untreated, or treated with radiotherapy, anti-PD-L1 CBI, or combination therapy divided with clear clusters corresponding to follicular and GC B cells. 

B, Ridge plots demonstrating expression of select genes between the GC and follicular B-cell clusters. 

C, Heatmap demonstrating the expression profile of the top 10 most differentially expressed genes in each subcluster of follicular B cells (clusters F1–F4). 

D, Ridge plots demonstrating expression of select genes between two subclusters of GC B cells corresponding to light zone (GC 1) and dark zone (GC 2). 

E, UMAP plots of GC B cells from individual treatment groups. 

F, Proportion of total B cells from each sample belonging to combined GC clusters. XRT, treatment with radiotherapy. Statistical analysis was evaluated by one-way ANOVA with multiple comparisons (*, P ≤ 0.05; **, P ≤ 0.01; ***, P ≤ 0.001; ****, P ≤ 0.0001).
B Cells Improve Survival in HPV-associated Cancers

Patients responding to CBI demonstrate increased antibody production after treatment

To characterize the effects of PD-1 blockade on B-cell–mediated antibody responses in humans, we used the HuProt proteome microarray to quantify individual IgM and IgG serum antibody responses to over 16,000 proteins in 6 patients with HPV-associated cancers enrolled on a phase II randomized clinical trial comparing the use of CBI ± stereotactic body radiotherapy (SBRT) in patients with advanced metastatic cancer (UCSD HRPP 151570; ClinicalTrials.gov identifier: NCT02843165). Clinical and pathologic details of all patients analyzed are shown in Supplementary Table S11. We compared baseline pretreatment sera to sera collected 2 months after treatment and determined fold changes in both IgG and IgM antibody responses against each protein in the array. Importantly during an initial humoral immune response for thymus-dependent antigens, multimeric IgM antibodies predominate prior to class switching, affinity maturation, and development of a robust IgG response. We defined two distinct regions: region 1 represents antigens that elicited an increased IgG to IgM relative fold change after treatment, which we term “secondary antibody response antigens” and region 2 represents antigens that elicited an increased IgM to IgG relative fold change after treatment, which we term “primary antibody response antigens.” To isolate targets that had significantly increased fold change in antibodies, a fold change of 8 was used as a threshold. Representative antibody profiles are shown in Fig. 6.

We observed that patients with objective responses to therapy (Fig. 6A and B) had dramatically increased IgG and IgM responses posttreatment (region 1 and region 2) compared with patients with progressive disease (Fig. 6C and D). The absolute number of antigens recognized in each region for each patient is also included in Supplementary Table S11. The fluorescence value changes for the top 25 or fewer targets in each region for each representative patient is shown for IgG, region 1 (Fig. 6E) and IgM, region 2 (Supplementary Fig. S11A). Finally, we compared the number of highly elevated IgG and IgM responses (>8-fold change) in patients with a clinical benefit to therapy versus those with progressive disease. Clinical benefit was defined as a complete response, objective partial response, or stable disease lasting greater than 6 months. We found that patients with a clinical benefit from PD-1 blockade had remarkably increased absolute number of IgG and IgM responses with >8-fold change compared with patients with progressive disease (Fig. 6F).

Discussion

B cells are a critical arm of the adaptive immune system and play dual roles as both professional antigen-presenting cells and specialized antibody-producing cells. Although they are not known to have direct cytotoxic capability, understanding how B cells contribute to a multifaceted immune response targeting tumor-associated antigens or tumor-associated viruses is vital. Our data support an antitumorigenic role of B cells in HPV-related squamous cell carcinomas and a remarkably beneficial impact on patient outcomes. Fortunately, patients with HPV+ HNSCC are known to have improved outcomes compared with HPV− patients, although the reasons behind this are not fully understood and additional biomarkers are needed to help stratify these populations. Currently, the only biological prognostic marker in widespread clinical use is p16, which is a surrogate marker of HPV positivity in oropharyngeal HNSCC. Importantly, the CD19 and IGI biomarkers reported here remained significant for OS on multivariate analysis, demonstrating that analysis of the underlying biology within HNSCC tumors adds significant prognostic value beyond HPV or p16 status. Interestingly, we observed an increased effect size and a statistical interaction between CD19/IGI and HPV-positivity when compared with patients with HPV-negative HNSCC. This finding suggests a biological interaction between B cells and the HPV and perhaps reflects the importance of B cells reacting against HPV-associated antigens.

Among HPV+ patients there is known to be significant heterogeneity and HPV+ subpopulations have been identified with disparate clinical outcomes (45, 46). Zhang and colleagues identified two subtypes of HPV-related HNSCC—one characterized by expressing genes involved in immune responses and mesenchymal differentiation that trended toward a better prognosis, and the other expressing genes involved in keratinocyte differentiation and cell adhesion (46). However, as opposed to hierarchical clustering based upon a large number of various genes, we were able to stratify our HPV+ patients based...
Figure 6. B-cell-mediated IgG and IgM antibody responses correlate with objective responses to CBI. Representative plot of IgG and IgM antibody responses for all matched targets in patients treated with CBI ± SBRT in patients with objective responses (A and B) and progressive disease (C and D). E, Fluorescence values for top 25 of fewer IgG antibodies in region 1 for each patient are shown. F, Number of antibody responses with an IgG or IgM posttreatment fold change greater than 8 in patients with clinical benefit versus progressive disease. Statistical analysis was evaluated by Mann-Whitney test (*, P ≤ 0.05).
analyses were performed on pretreatment biopsies, these B-cell–specific markers have the potential to guide biologically driven management protocols in the future, for example, to escalate therapy in high-risk patients or deescalate therapy in biologically defined low-risk patients. Moreover, in addition to HNSCC and cervical cancer, these B-cell markers may play important roles in other HPV-associated malignancies including anal, penile, or vulvar cancer. Further investigation into the predictive capacity of these B-cell markers in prospective clinical trials is certainly deserved.

In light of the association between B cells and survival, the ability of PD-1 blockade or radiotherapy to modulate B-cell development and function is of paramount importance and likely plays a role in the clinical activity of these therapies. We present a comprehensive approach to characterizing B cells in both a murine model and human patients, including scRNA-seq, flow cytometry, BCR-sequencing, and antibody profiling. Using scRNA-seq on sorted B cells we identified enhanced GC formation due to PD-1 blockade and radiotherapy. Thus, this is direct evidence that PD-1 blockade and radiation can enhance development antigen-specific B cells. We confirmed this by developing a novel "OVA-multimer" reagent to track antigen-specific B cells and demonstrated that combined therapy increased plasma cell differentiation and resultant anti-OVA IgG antibody levels. Analogous to tetramers for T cells, this novel and unique OVA-multimer reagent could be used generally to quantify antigen-specific B-cell populations in other model systems.

Complementing flow cytometry, BCR-sequencing is a powerful new technology that allows for exquisite characterization B-cell clonal diversity. B-cell clones that recognize a specific set of antigens proliferate in the lymph node and GC, altering clonality, although analysis of clonality in B cells has the additional complexity of somatic hypermutation. In support of this, we also observed a significant decrease in CDR3 length after radiotherapy, which is known to correlate with antigen-specific responses (42). Interestingly, we did not observe a significant increase in clonality with CBI alone. The reasons behind this finding remain unclear; although, the PD-1 receptor is known to have important regulatory functions on B cells (47) and PD-L1–high B cells are key regulators of humoral immune responses (48). One possibility is that PD-1 blockade alone does not significantly enhance antigen presentation or that the timing of the effects of PD-1 blockade on tumor cell death and B-cell–mediated antigen processing is delayed compared with radiotherapy. These BCR-sequencing findings certainly deserve further investigation in patients especially in light of the important prognostic information gained from human T-cell receptor–sequencing (49).

Ultimately, the major effector function of B cells is antibody production and the ability for CBI to modulate changes in B-cell-mediated antibody levels is understudied. The two novel regions that we defined have important implications. Region 1 which we term "secondary antibody response antigens" which have developed pre-dominant IgM responses after class switching; and region 2 which we term "primary antibody response antigens" in which IgM responses dominate prior to class switching (or due to antigens which do not elicit class switching) and represent de novo immune responses induced during treatment. For example, patient 3, who had HNSCC that completely responded to combined CBI and SBRT, and demonstrated increased antibodies against ANP32A, a target in HNSCC that is associated with increased mortality in patients with N0 and N1 disease (50). Taken together, these findings demonstrate that CBI can induce antitumor antibodies and patients with objective responses to therapy demonstrate a profound humoral response pattern, which may play a key role in tumor control. As we analyzed a limited number of patients, a larger cohort is needed to comprehensively characterize changes in antibody profiles based upon responses. Nevertheless, the regions defined here provide a novel framework for characterizing enhancements in preexisting humoral immunity (region 1) versus development of de novo antibodies (region 2) after initiation of CBI. Further analyses of these distinct regions in relation to response patterns, as well as immune-related adverse events and novel tumor antigens are deserved.

In summary, we report that CD19 and IGI are B-cell–specific prognostic biomarkers for survival in HNSCC and cervical squamous cell carcinomas. The ability of these single-gene markers to further stratify patient outcomes beyond HPV status is powerful and has implications for personalized management. The observed inferior tumor control in mice whose peripheral B cells have been depleted demonstrates a key role for B cells in HNSCC tumor control. Furthermore, the ability of CBI and radiotherapy to modulate B-cell activation and GC formation provides evidence of systemic B-cell changes that shape adaptive immune responses and may play a role in clinical activity of these therapies. Finally, the identification of distinct regions of IgG versus IgM endogenous antibody responses which develop after CBI provides a novel framework for understanding how B-cell antibodies contribute to patient outcomes and correlate with responses to PD-1 blockade. Cumulatively, these data reveal a remarkably underappreciated role for B cells in squamous cell carcinomas and provide strong rationale for using B-cell markers to stratify patients and developing novel therapeutics targeting B-cell populations.

Disclosure of Potential Conflicts of Interest
L. Mell is a paid consultant for Nanobiotix and Pfizer, and reports receiving commercial research grants from Merck and AstraZeneca. J.S. Gutkind is a paid advisory board member for Domain Therapeutics and Oncoceutics, and is a paid consultant for Vividion Therapeutics. E.E.W. Cohen is a paid consultant for MSD, Bristol-Myers Squibb, Merck, and Regeneron. A.B. Sharabi is a paid consultant for Joune Terapeutics; reports receiving commercial research grants from Varian Medical Systems and Pfizer; holds ownership interest (including patents) in Toragen, and is a paid consultant/advisory board member for AstraZeneca and Merck. No potential conflicts of interest were disclosed by the other authors.

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