CD20^+ Tumor-Infiltrating Lymphocytes Have an Atypical CD27^- Memory Phenotype and Together with CD8^+ T Cells Promote Favorable Prognosis in Ovarian Cancer

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Abstract

**Purpose:** Tumor-infiltrating lymphocytes (TIL), in particular CD8^+ T cells and CD20^+ B cells, are strongly associated with survival in ovarian cancer and other carcinomas. Although CD8^+ TIL can mediate direct cytolytic activity against tumors, the role of CD20^+ TIL is poorly understood. Here, we investigate the possible contributions of CD20^+ TIL to humoral and cellular tumor immunity.

**Experimental Design:** Tumor and serum specimens were obtained from patients with high-grade serous ovarian cancer. CD8^+ and CD20^+ TIL were analyzed by immunohistochemistry and flow cytometry. Immunoglobulin molecules were evaluated by DNA sequencing. Serum autoantibody responses to the tumor antigens p53 and NY-ESO-1 were measured by ELISA.

**Results:** The vast majority of CD20^+ TIL were antigen experienced, as evidenced by class-switching, somatic hypermutation, and oligoclonality, yet they failed to express the canonical memory marker CD27. CD20^+ TIL showed no correlation with serum autoantibodies to p53 or NY-ESO-1. Instead, they colocalized with activated CD8^+ TIL and expressed markers of antigen presentation, including MHC class I, MHC class II, CD40, CD80, and CD86. The presence of both CD20^+ and CD8^+ TIL correlated with increased patient survival compared with CD8^+ TIL alone.

**Conclusions:** In high-grade serous ovarian tumors, CD20^+ TIL have an antigen–experienced but atypical CD27^- memory B-cell phenotype. They are uncoupled from serum autoantibodies, express markers of antigen-presenting cells, and colocalize with CD8^+ T cells. We propose that the association between CD20^+ TIL and patient survival may reflect a supportive role in cytolytic immune responses.

Introduction

High-grade serous ovarian cancer (hereafter abbreviated HGSC) is the most common and lethal subtype of ovarian cancer, with a 5-year survival rate of only 30% (1). Although most patients are highly responsive to primary surgery and chemotherapy, the majority experience recurrence within 1 to 3 years and ultimately succumb to their disease (2). Despite these unfortunate statistics, a subset of patients experience prolonged disease-free survival. Favorable prognostic indicators for HGSC include early stage and optimal surgical debulking (2). Furthermore, studies over the past 2 decades have revealed strong links between host immunity and survival. In particular, multiple studies have shown that the presence of CD3^+ and CD8^+ tumor-infiltrating lymphocytes (TIL) is associated with markedly prolonged survival (3–8).

In addition to CD8^+ T cells, we have shown that tumor-infiltrating CD20^+ B cells (CD20^+ TIL) are strongly associated with patient survival in HGSC (6). Tumor-infiltrating B cells are also correlated with favorable outcomes in breast, cervical, and non–small cell lung cancer (9–11). In breast cancer and germ cell tumors, tumor-infiltrating B cells have been shown to consist of activated, antigen-experienced, oligoclonal subpopulations (12–18). Beyond this information, the mechanistic role of CD20^+ TIL in tumor immunity remains poorly defined. A priori, tumor-infiltrating B cells could mediate their effects through the production of tumor-specific antibodies. Indeed, more than 40% of HGSC patients show autoantibody responses to tumor antigens such as p53 and NY-ESO-1 (19, 20). In theory, tumor-specific autoantibodies could mediate antitumor responses by direct inhibition of target proteins, activation of...
Tumor Tissue Repository (cohort A) or OvCaRe (cohorts B and C) under protocols approved by the Research Ethics Board of the BC Cancer Agency and the University of British Columbia.

Tumor tissue was obtained at the time of primary cytoreductive surgery before any other treatment. Samples were either preserved in formalin and processed in paraffin for preparation of tissue microarrays, frozen in OCT medium for sectioning and DNA extraction, or processed into single-cell suspensions as previously described (31). Serum was processed using serum separator tubes, aliquoted, and stored at –80°C. Pretreatment serum was collected before primary surgery (except for patient IROC037, which was collected 1 month postsurgery), and subsequent samples were collected approximately 3, 6, 12, and 24 months following surgery. Peripheral blood mononuclear cells (PBMC) from patients and healthy controls were collected in heparinized Vacutainer tubes, isolated by Ficoll density centrifugation and cryopreserved.

Immunohistochemistry

For cohort A, a tissue microarray (TMA) was constructed using a manual tissue arrayer from Beecher Instruments. The TMA consisted of duplicate 1-mm cores taken from representative central regions of nonnecrotic tumor epithelium with some stromal regions, as defined upon review of hematoxylin and eosin–stained sections by a pathologist (PHW). For cohorts B and C, TMA's were constructed in a similar manner but consisted of 0.6-mm duplicate cores and were constructed using a Pathology Devices tissue arrayer. TMAs from cohorts A and B were prepared and

Translational Relevance

High-grade serous ovarian cancer has a 5-year survival rate of only 30%, so there is an urgent need for improved treatments. Recent studies have provided unequivocal evidence that patients who mount strong immune responses against their tumor experience markedly prolonged survival. Although tumor-infiltrating T cells are clearly important for tumor immunity, we have recently shown an equally prominent role for B cells. Here, we investigate the underlying mechanisms. Unexpectedly, we found that tumor-infiltrating B cells were uncoupled from antibody responses to common tumor antigens. Instead, they colocalized with T cells and showed characteristics of antigen-presenting cells. The presence of both B cells and T cells in tumors was associated with better prognosis than T cells alone. Thus, tumor-infiltrating B cells seem to support T-cell responses to cancer. Immunotherapeutic strategies that engage both lymphocyte subsets may have more potent and sustained antitumor effects.
stained as previously described (31) using an anti-CD20 rabbit polyclonal primary antibody (catalog #RB-9013; Lab Vision). The cohort C TMA was prepared and stained as previously described (30).

Scoring of TMAs for cohorts A and B was carried out in a blinded fashion by a pathologist (PHW). Tumor cores were initially scored by assessing at low magnification the proportion of each core that comprised tumor or stroma and then scoring at 20× magnification the number of positively stained TIL within the core area (through direct counting up to 20 or by estimation in 10s when in excess of this number), as well as the proportion of TIL present in tumor epithelium versus stroma. For cohort C, cores were scored either 0 (no cells present), 1 (1–5 cells), 2 (6–19 cells), or 3 (20+ cells) and then binarized to negative versus positive (score 0 vs. scores 1, 2, and 3). This binarization was used to generate the survival data in Fig. 6E. Survival curves were not determined for cohorts A and B due to small sample size and insufficient follow-up time.

Two-color IHC was carried out on FFPE sections following deparaffinization in a decloaking chamber (Biocare Medical) with Diva Decloaker. Samples were blocked with Peroxidased-1 and Background Sniper and then labeled with a cocktail of antibodies to CD8 (mouse monoclonal C8/144B; Cell Marque) and CD20 (rabbit polyclonal catalog # E2560; Spring Bio) for 30 minutes at room temperature. The two-color polymer kit Mach-2 DS2 was used to amplify the primary signal, and Betazoid DAB and Warped Red chromogens were used for detection. Slides were counterstained with hematoxylin. All staining reagents were from Biocare Medical. Images were captured using an Olympus BX53 microscope and Nuance multispectral imaging system (CRI).

Flow cytometry
Cryopreserved cells were labeled with antibodies listed in Supplementary Table S2. Data were acquired using a BD Influx cell sorter (BD Biosciences) and analyzed using FlowJo software (Tree Star). All samples are gated on lymphocytes based on forward and side scatter plots. Antibody specificity was tested using matched isotypes and fluorescence-minus-one controls.

Idiotype sequencing
Six independent PCRs were carried out using primer pairs designed to amplify VH1, VH2, VH3, VH4, VH5, and VH6 sequences. After amplification, PCRs were cloned into the pENTR/D-TOPO plasmid vector (Invitrogen). At the BC Cancer Agency’s Genome Sciences Centre (Vancouver, Canada), plasmids were electrotopered into DH10B T1 phage-resistant cells, plated onto selective growth media, and robotically arrayed in 384-well plates. Plasmid DNAs were then extracted and BigDye Terminator on ABI 3730 384-well plates. Plasmid DNAs were then extracted and BigDye Terminator on ABI 3730 384-well plates. Plasmid DNAs were then extracted and BigDye Terminator on ABI 3730 384-well plates. Plasmid DNAs were then extracted and BigDye Terminator on ABI 3730 384-well plates.

DNA sequences were analyzed using the IMGT/V-QUEST tool (32) of IMGT, the international ImMunoGeneTics information system (33). Up to 96 sequences were analyzed from each tumor sample. Sequences were compared with germline sequences to identify somatic hypermutations, and V-D-J junctions were compared to identify clonal sequences.

ELISA to detect serum antibodies to NY-ESO-1 and p53
cDNAs encoding the autoantigens NY-ESO-1 and p53 were amplified by reverse transcriptase PCR (RT-PCR) from the ovarian cancer cell line OVCAR3, cloned into the pDEST17 vector, and used for recombinant protein production, as previously described (NY-ESO-1; ref. 31) or using the primer pair: 5’-CACCATGGAGGAGCCGCAGTCA and 5’-TATAGGAGTCGAGCCCTCCTGCT (p53). Serum was assayed in triplicate for IgG antibodies to recombinant NY-ESO-1 and p53 by ELISA as previously described (31). Triplicate measurements of control sera were used to establish the mean and SD of autoantibody responses to each antigen. For cancer patients, autoantibody responses were scored as positive if they were significantly greater than the mean of the controls. Specifically, the mean of the controls was subtracted from individual patient responses, which were then divided by the SD of all controls. Responses greater than 3.0 SDs from the control mean were considered positive (34). Association between CD20 status and autoantibody responses was assessed using the Fisher exact test with a 2-tailed P value (GraphPad Prism). To assess quantitative changes over time, autoantibody titers were determined using a method similar to Gnjatic and colleagues (19). Each serum sample was subjected to 4-fold serial dilutions from 1:100 to 1:6,553,600. Reciprocal titers were determined based on the highest dilution that gave a positive response against NY-ESO-1. The cutoff for positive responses was based on optical density values greater than twice the value of the average optical density of the 4 lowest dilutions of a pool of 5 healthy donor serum samples.

Results
CD20+ TIL are mature, antigen-experienced B cells
We previously reported that CD20+ TIL are strongly associated with prolonged survival in HGSC (6). To investigate potential immunologic mechanisms underlying this association, we first assessed whether CD20+ TIL exhibit characteristics of antigen-experienced B cells. We selected 9 HGSC tumors containing CD20+ TIL, as determined by IHC (Fig. 1A) and used multiparameter flow cytometry to assess surface immunoglobulin expression on CD20+ B cells from tumor tissue and matched blood samples. In peripheral blood, HGSC patients showed a normal distribution of naive (IgD- IgM+ IgG-) and activated (IgD+ IgM+ IgG+) CD20+ B cells, which was similar to cancer-free controls (Fig. 1B). In contrast, the vast majority of CD20+ TIL showed an activated phenotype (IgD+ IgM+ IgG+), indicating they had undergone Ig class switching in response to antigen exposure (Fig. 1B).

To further investigate whether CD20+ TIL were antigen experienced, we sequenced up to 96 IgG heavy chain variable regions (including V, D, and J gene segments) from
tumor-infiltrating B cells isolated from 3 HGSC patients. We observed a diverse cross-section of sequences from all major VH families (Supplementary Fig. S1). Moreover, tumor-infiltrating B cells exhibited a high level of somatic hypermutation: on average 20 nucleotides were mutated in each approximately 400 nucleotide variable region, resulting in an average of 13 amino acid (aa) substitutions (Fig. 2A). This is consistent with reference values for antigen-experienced B cells (~18 nucleotide and 10 aa substitutions) versus naive B cells (~3 nucleotide and 3 aa substitutions; ref. 18). Moreover, in all 3 tumors, there was evidence of B cells actively undergoing somatic hypermutation, as numerous sequences were identified with identical V-D-J junctions but distinct mutation patterns. An example of a clone with multiple mutated sequences is depicted in Fig. 2B.

Finally, the clonality of CD20⁺ TIL was estimated by calculating the prevalence of each variable gene sequence in the dataset. Sequences were defined as clonal if at least 2 independent sequences contained identical V-D-J junctions but differing mutational patterns. Using these criteria, we found evidence for at least 11 to 14 B cell clones in each tumor, some of which were highly represented. For example, in IROC015, 16% of sequences were derived from a single B cell clone, an additional 17% of sequences were derived from 2 other predominant clones, a further 33% of sequences were derived from 8 minor clones, and the remaining 34% of sequences were unrelated (Fig. 2C). These trends were very similar across all 3 patients (Fig. 2C), indicating that CD20⁺ TIL represent oligoclonal populations. Note that these are conservative estimates of clonal abundance, as we disregarded any identical sequences (i.e., sequences that had the same mutational profiles), reasoning that these could result from RT-PCR amplification of mRNA molecules derived from the same B cell. When the analysis was repeated with the inclusion of duplicate sequences, the predominant B-cell clones in each patient represented 22% to 24% of all sequences (compared with 8%–16% of sequences in the initial analysis). We saw no evidence of shared clonotypes between patients (Supplementary Fig. S1). Collectively, these results indicated that CD20⁺ TIL have undergone activation, Ig class switching, somatic hypermutation, and clonal expansion, all of which are hallmarks of antigen exposure.

CD20⁺ TIL have an atypical CD27-negative memory phenotype

Given the above results, we further characterized the phenotype of antigen-experienced CD20⁺ TIL by flow cytometry to determine whether they exhibit features of germinal center or memory B cells. Triple staining with antibodies against CD20, CD38, and IgD revealed that CD20⁺ B cells from HGSC patients and healthy controls consisted of 2 populations: 50% to 65% of CD20⁺ B cells were IgD⁻CD38⁻IgD⁻/low, indicative of naive B cells, whereas 35% to 50% were IgD⁺CD38⁻/low, indicative of memory B cells (Fig. 3A, top panels). The results for CD20⁺ TIL were strikingly different. Naïve CD20⁺ B cells were not seen. In 3 of 9 TIL samples, a small population of IgD⁻CD38⁻ B cells was observed, consistent with germinal center B cells (Fig. 3A, rightmost CD20⁺ TIL panel). Most notably, in all 9 patients the vast majority of CD20⁺ TIL were IgD⁻CD38⁻/low, indicative of a memory B-cell phenotype (Fig. 3A).

CD27 is a cell-surface protein that is expressed under normal physiologic conditions (35–37). As expected, the vast majority of peripheral blood IgG⁺ B cells from HGSC patients and healthy controls expressed CD27 (Fig. 3B), consistent with a memory phenotype, whereas IgG⁻ (naïve) B cells lacked CD27 expression (data not shown). In sharp contrast, in 8 of 9 tumor samples, virtually all IgG⁺CD20⁺ TIL failed to express CD27 (Fig. 3B), suggesting that they represent atypical CD27⁻ memory B cells, as described in other physiologic settings (38–41).

In addition to CD20⁺ B cells, we analyzed tumor and blood samples for the presence of plasma cells and plasmablasts. There was a complete absence of CD20⁺CD38⁺CD138⁺ plasma cells in all analyzed tumors, whereas the majority of analyzed tumors (80%) contained small numbers of cells with a plasmablast phenotype (CD20⁺CD38⁻CD138⁻CD3⁻CD56⁻; Fig. 3C). In summary, B-cell lineage TIL in HGSC are composed predominantly of CD27⁻ memory B cells, with small numbers of germinal center B cells and plasmablasts.

CD20⁺ TIL are uncoupled from tumor-specific serum autoantibody responses

The low abundance of plasmablasts and plasma cells in tumors suggested that CD20⁺ TIL, though antigen-
experienced, might not contribute significantly to systemic humoral immunity. To further explore this possibility, we evaluated whether CD20^+ TIL were correlated with tumor-specific serum autoantibody responses. We and others have shown that at least 40% of HGSC patients have serum autoantibody responses to one or more tumor antigens (19, 20). Of the target antigens identified to date, NY-ESO-1 and p53 are among the most frequently recognized in ovarian cancer (19, 20), so these 2 antigens were selected for further analysis. By ELISA, 8 of 40 (20%) HGSC patients in cohort A showed a serum autoantibody response to NY-ESO-1 or p53 (Fig. 4A and B). Notably, these responses were equally prevalent in patients with or without CD20^+ TIL (19% vs. 20%, respectively; P = 1.00; Fig. 4A and B). Similar results were seen in an independent cohort of HGSC patients (n = 30; cohort B), in which autoantibody responses were equally prevalent in patients with or without CD20^+ TIL (30% vs. 29%, respectively; P = 1.00; data not shown).

To further address this issue, we assessed whether removal of CD20^+ TIL, which occurs during primary surgery and chemotherapy, abrogated tumor-specific serum
autoantibody responses. We quantified autoantibody titers to NY-ESO-1 in 6 HGSC patients using serum samples collected before and 2 to 6 months after primary surgery. As expected, CD20⁺ PBMC from healthy controls and HGSC patients consist primarily of naive (IgD⁻CD38⁺) and memory (IgD⁻CD38⁻) B cells (top). In contrast, the vast majority of CD20⁺ TIL show a memory phenotype (IgD⁻CD38⁻; bottom). Data are representative of 9 HGSC patients. In 3 of 9 patients (as shown for IROC015), a small population of germinal center CD20⁺ B cells (IgD⁻CD38⁻) is also seen. B, CD27 expression: Flow cytometric analysis of CD27 expression by PBMC and TIL from a representative healthy donor and HGSC patient. Samples are gated on CD20⁺ IgG⁺ lymphocytes. As expected, the majority of CD20⁺ IgG⁺ B cells in PBMC express CD27. In contrast, the vast majority of CD20⁺ IgG⁺ TIL fail to express CD27. Data are representative of 9 HGSC patients. C, Plasmablasts and plasma cells: Flow cytometric analysis of CD38 and CD138 expression by PBMC and TIL from a representative healthy donor and a HGSC patient positive for CD20⁺ TIL. Samples were gated on CD45⁺CD20⁻ lymphocytes. Left, PBMC and TIL samples generally lack CD20⁻CD38⁺CD138⁺ plasma cells (expected to be in top right quadrant). Right, further gating of CD38⁺ cells from the left panels reveals the presence of low numbers of cells with a plasmablast phenotype (CD20⁻CD38⁻CD3⁺CD56⁻; bottom left quadrants) in PBMC and TIL samples. Data are representative of 4 of 5 tumor samples.

decreased after treatment, although one patient (IROC065) showed a reproducible increase (Fig. 4C). Irrespective of these changes, all patients maintained high-titer NY-ESO-1–specific autoantibody responses (range: 1:3,200 to 1:204,800; Supplementary Table S3) after cytoreductive treatment. This indicated that the majority of anti–NY-ESO-1 autoantibody production occurs outside of tumor tissue. Thus, by multiple lines of evidence, CD20⁺ TIL are uncoupled from humoral immune responses in HGSC.
antibodies. B cells can facilitate T-cell responses by serving as APC and by recruiting T cells to affected tissues (42). We therefore assessed whether CD20⁺ TIL might serve as APC to T cells, as described in other settings (23, 26, 42, 43). Unfortunately, the low absolute numbers of CD20⁺ TIL in human specimens precluded in vitro functional analyses. However, sufficient material was available to carry out multiparameter flow cytometry to determine whether CD20⁺ TIL express cell-surface molecules involved in antigen presentation. CD20⁺ TIL expressed MHC class I (pan-HLA-A, B, and C) and class II (HLA-DR) molecules, as well as CD40 (Fig. 5A). Furthermore, CD20⁺ TIL expressed the costimulatory molecules B7-1 (CD80) and B7-2 (CD86), albeit at moderate levels (Fig. 5A). Accordingly, the majority of tumor-infiltrating CD4⁺ and CD8⁺ T cells displayed an activated effector phenotype (HLA-DR⁺CD45R0⁺CD45RA⁻CD62L⁻; Fig. 5B). Collectively, our results are consistent with the possibility that CD20⁺ TIL serve as APC to T cells in the tumor environment.

**CD8⁺ and CD20⁺ TIL colocalize in HGSC**

If B cells serve as APC in the tumor environment, they would be expected to be located in close proximity to T cells. To assess this, we carried out 2-color IHC with antibodies to CD8 and CD20. As previously reported, CD8⁺ and CD20⁺ TIL were found in both tumor stroma and epithelium (Fig. 6A and B). Intriguingly, CD8⁺ and CD20⁺ lymphocytes were frequently seen in close proximity, often in loosely structured aggregates (Fig. 6A). Such aggregates were even found in tumors with low densities of TIL, suggesting the colocalization of CD20⁺ and CD8⁺ TIL as an active process. There were numerous examples in which CD8⁺ and CD20⁺ TIL were directly juxtaposed, consistent with cell–cell interactions (Fig. 6C and D). In summary, these imaging studies indicated that CD8⁺ and CD20⁺ TIL are frequently colocalized in HGSC, further supporting a possible role for CD20⁺ TIL as APC.

**The combination of CD8⁺ and CD20⁺ TIL is associated with prolonged survival in HGSC**

Finally, we used data from a large previously published cohort (6) to assess the relationship between CD20⁺ and CD8⁺ TIL and patient survival. CD20⁺ and CD8⁺ TIL were found in 41% and 84% of cases, respectively. Of the cases that were positive for CD20⁺ TIL, almost all were also positive for CD8⁺ TIL. Conversely, of the cases that were positive for CD8⁺ TIL, approximately half also contained CD20⁺ TIL. By Kaplan–Meier analysis, cases that were positive for both CD8⁺ and CD20⁺ TIL showed markedly greater disease-specific survival compared with those positive for CD8⁺ TIL alone (Fig. 6E). Thus, it seems that CD20⁺ TIL can potently enhance the antitumor effect of CD8⁺ TIL in ovarian cancer.

**Discussion**

We have investigated potential mechanisms by which CD20⁺ TIL contribute to tumor immunity in HGSC,
expressed surface markers characteristic of APC, including recently described subset of CD27 failed to express CD27, suggesting that they belong to a

tumor tissue, often in loose aggregates. CD20

thereby promoting prolonged patient survival. CD20 TIL showed clear evidence of antigen exposure, including expression of activation markers, class switching to IgG, somatic hypermutation, and oligoclonality. The majority failed to express CD27, suggesting that they belong to a recently described subset of CD27 memory B cells (39–41, 44). Despite expressing IgG, CD20 TIL showed no correlation with tumor-specific serum autoantibodies and hence seemed to be uncoupled from humoral immunity. Instead, CD20 TIL correlated strongly with CD8 TIL. The 2 lymphocyte subsets were found in close proximity in tumor tissue, often in loose aggregates. CD20 TIL expressed surface markers characteristic of APC, including MHC class I, MHC class II, CD80, and CD86. Furthermore, tumors positive for both CD8 and CD20 TIL were associated with prolonged survival compared with those positive for CD8 TIL alone. Collectively, our results support the novel concept that CD20 TIL enhance tumor immunity through functional interactions with CD8 TIL.

Sequencing of IgG molecules from CD20 TIL yielded clear evidence of oligoclonality and somatic hypermutation, which are hallmarks of antigen exposure. The degree of B-cell clonality we measured in HGSC is similar to prior studies in breast cancer and germ cell tumors. Specifically, we found evidence for 11 to 14 B-cell clones in HGSC, compared with 6 to 13 clones in germ cell tumors (18) and 3 to 6 clones in breast cancer (12, 17). Similarly, 58% to 66% of sequences were clonally derived in HGSC, compared with 18% to 79% in germ cell tumors and 30% to 69% in breast cancer. Notably, these are minimal estimates, as it is

Figure 5. CD20 TIL have a surface phenotype characteristic of APC, whereas CD4 and CD8 TIL have a surface phenotype characteristic of activated T cells. A, representative flow cytometry data showing expression of MHC class I (pan-HLA-A, B, C), MHC class II (pan-HLA-DR), CD40, CD80, and CD86 in CD20 B cells from healthy control PBMC (top), HGSC patient PBMC (middle), and TIL (bottom). Black lines: specific staining with the indicated antibody. Gray lines: negative controls lacking the indicated antibody. Samples were gated on CD45 CD20 lymphocytes (n = 9). B, representative flow cytometry data showing an activated (HLA-DR CD45RA CD45RO CD62L) surface phenotype on the majority of CD4 TIL and CD8 TIL. Samples are gated on CD3 lymphocytes (n = 9).
possible that additional less abundant clones were present in all of these studies. In addition to clonality, the degree of somatic hypermutation we observed in HGSC is similar to that reported for breast cancer and germ cell tumors.

What antigens might be recognized by CD20⁺ TIL? Although this issue has yet to be addressed in HGSC, prior studies in breast cancer identified 2 antigens recognized by recombinant antibodies derived from tumor-infiltrating B cells: ganglioside D3 and a cleaved form of β-actin that was shown to become exposed on the surface of apoptotic cells (13, 45). These results suggest that CD20⁺ TIL responses reflect a breakdown of tolerance to self-proteins, possibly due to apoptosis of tumor cells. Identification of additional antigens recognized by CD20⁺ TIL may yield further insights into the mechanisms by which B-cell responses are triggered and contribute to tumor immunity.

Even though CD20⁺ TIL express surface immunoglobulin, we found that they are not associated with serum autoantibodies to the common tumor antigens p53 and NY-ESO-1. This lack of correlation was seen across 2 independent cohorts of patients, as well as within individual patients undergoing cytoreductive surgery and chemotherapy. Although only 2 antigens were assessed, these are the most frequently recognized tumor antigens in HGSC identified to date (19, 20). This finding is consistent with the fact that CD20⁺ TIL are associated with prolonged survival in HGSC, whereas serum autoantibodies show little or no positive prognostic signal in most studies (31, 46–48).

Figure 6. CD20⁺ and CD8⁺ TIL colocalize and are associated with prolonged survival in HGSC. A, IHC of HGSC tumor (IROC006) showing CD20⁺ TIL (red) and CD8⁺ TIL (brown) primarily in stromal regions. B, IHC of a densely infiltrated HGSC tumor (IROC042) showing CD20⁺ TIL (red) and CD8⁺ TIL (brown) in epithelial and stromal regions. C, high power IHC image of a HGSC tumor (IROC010) showing CD20⁺ TIL (red) and CD8⁺ TIL (brown) in close proximity. D, the same image as shown in (C), but with false coloring to further contrast the CD20⁺ (red) and CD8⁺ (green) signals. All samples were counterstained with hematoxylin (blue/purple). Original images were taken with a 20× (A and B) or 40× objective (C and D). Scale bars: 100 μm (A and B) or 50 μm (C and D). Representative of 5 tumor samples. E, Kaplan–Meier curves showing that the presence of both CD8⁺ and CD20⁺ TIL is associated with prolonged disease-specific survival compared with CD8⁺ TIL alone or lack of CD8⁺ TIL. Primary data is from cohort C (6). Log-rank tests were used to derive P values for comparisons between each group and between all 3 groups.
Collectively, our data supports the conclusion that serum autoantibodies are produced by cells outside the tumor. Prime candidates are the plasma cells in bone marrow, which are the primary source of serum antibodies in healthy individuals. It remains possible that CD20+ TIL, and the small populations of germinal center–like B cells and CD38+ plasmablasts we identified in tumors, are precursors to plasma cells that in turn produce serum antibodies in some patients.

Although CD20+ TIL exhibit the hallmarks of mature, antigen-experienced B cells, the majority failed to express the conventional memory B-cell marker CD27. CD27− memory B cells are present at low numbers in healthy donors (39–41, 44). In patients with systemic lupus erythematosus, the number of CD27− memory B cells is elevated in proportion to disease severity (44). To our knowledge, this study is the first to identify CD27− memory B cells in cancer. Although the functional differences between CD27+ and CD27− memory B cells have yet to be elucidated, several possibilities can be imagined. Activated helper T cells express CD70, which can ligate CD27 on B cells to induce Ig secretion (35). Perhaps the lack of CD27 expression on CD20+ TIL allows them to forego antibody production and instead contribute to cellular immunity. Alternatively, the lack of CD27 expression could indicate recent activation of B cells, as exposure of B cells to CD70− T cells leads to rapid CD27 downregulation (35). Perhaps the presence of CD27− memory B cells reflects a response to CD70 signals by CD4+ and/or CD8+ TIL in HGSC.

Using two-color IHC, we showed that CD20+ TIL are often found in close proximity to CD8+ TIL (Fig. 6A–D). Moreover, the presence of both CD20+ and CD8+ TIL was associated with markedly prolonged patient survival compared with CD8+ TIL alone (Fig. 6E). These findings suggest that CD20+ TIL may promote cytolytic antitumor responses. In theory, this could occur through multiple mechanisms. First, CD20+ TIL could help recruit and retain T cells at the tumor site, facilitating the formation of the lymphoid aggregates we observed (Fig. 6A–D). Indeed, in autoimmunity, allograft rejection, and chronic infection, B cells are central to the formation of so-called tertiary lymphoid structures in affected tissues (28, 49). For example, in a xenograft model of rheumatoid arthritis, depletion of B cells with anti-CD20 antibody led to decreased T-cell activation and infiltration (27). Conversely, depletion of T cells led to disruption of tertiary lymphoid structures and loss of immunoglobulin production by infiltrating B cells (50). Thus, during prolonged immune responses, B cells and T cells can engage in cooperative interactions to maintain a strong presence at the affected site.

Second, CD20+ TIL could serve as APC in the tumor environment. It is well documented that B cells can present antigens to T cells, including cross-presentation to CD8+ T cells (51–53). B cells have been shown to serve as APC under a wide variety of physiologic conditions, including autoimmunity and allograft rejection. For example, B cells serve as APC to induce T-cell responses in nonobese diabetic mice (26). Similarly, B cells from multiple sclerosis patients have been shown to present myelin basic protein to CD4+ T cells in vitro (43). Thus, B cells have the capacity to function as APC in multiple autoimmune disorders and may function similarly in antitumor responses. In support of this notion, we found that CD20+ TIL express MHC class I and II, as well as the costimulatory molecules CD80 and CD86. Although speculative, the presence of professional APC in the tumor environment may allow for sustained T-cell responses, for example, by providing supportive niches for memory T-cell proliferation. Notably, CD20+ TIL are far more numerous than immature or mature dendritic cells (as defined by CD1a or CD208 expression, respectively) in immune infiltrates in HGSC (6). Moreover, compared with dendritic cells, B cells have the advantage that B-cell receptor–mediated endocytosis facilitates concentration of small quantities of specific antigens, which may allow the amplification of responses to tumor antigens expressed at low levels. To definitively show that CD20+ TIL serve as APC in the tumor environment, it will be necessary to identify their cognate antigens and assess recognition by CD4+ and CD8+ TIL.

Our findings have implications for the immunotherapy of cancer. Most cancer vaccines are modeled on acute viral infections, which are characterized by large yet transient T-cell responses. By contrast, in autoimmunity or allograft rejection, T-cell responses are sustained over many months or years and involve cooperative interactions with colocalized B cells. Thus, it may be possible to generate more potent, sustained T-cell responses in the tumor environment by promoting the infiltration of tumor-reactive B cells. In support of this idea, adoptive transfer of activated B cells has been shown to induce tumor-specific T-cell immunity in murine models (54). Thus, improved understanding of the functional phenotype of CD20+ TIL, their target antigens, and their mechanism of recruitment to target tissues may facilitate the design of more effective immunotherapies for the treatment of cancer.

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

Authors’ Contributions
Conception and design: J.S. Nielsen, N.J. Nesslinger, B.H. Nelson Development of methodology: J.S. Nielsen, K. Milne, N.J. Nesslinger Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J.S. Nielsen, R.A. Sahota, K. Milne, S.E. Kost, N.J. Nesslinger, P.H. Watson Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J.S. Nielsen, R.A. Sahota, K. Milne, P.H. Watson, B.H. Nelson Writing, review, and/or revision of the manuscript: J.S. Nielsen, R.A. Sahota, K. Milne, S.E. Kost, N.J. Nesslinger, P.H. Watson, B.H. Nelson Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J.S. Nielsen, R.A. Sahota Study supervision: J.S. Nielsen, B.H. Nelson

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