

Title: B Cell (CD19+) and Macrophage (CD68+) Spatial Interaction in the Tumor Microenvironment Associated with Higher Survival Probability

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Abstract

Ovarian cancer is the deadliest gynecological malignancy and high-grade serous carcinoma (HGSOC) is the most common histotype. HGSOC is primarily deadly due to low early detection rates and the high prevalence of acquired therapy resistance. Multi-omics techniques have provided a platform for improved predictive modeling of therapy response and patient outcomes. Further, while HGSOC tumors are immunogenic and numerous studies have defined positive correlation to immune cell infiltration, immunotherapies have exhibited low efficacy rates in clinical trials. There is a significant need to better comprehend the role and composition of immune cells in mediating ovarian cancer therapeutic response and progression. We performed multiplex immunohistochemistry with an HGSOC tissue microarray ($n=127$) to characterize the immune cell composition within tumors. We analyzed the composition and spatial context of T cells (CD4/CD8), macrophages (CD68), and B cells (CD19) within the tumor. While T cell and macrophage tumor infiltration did not correlate with overall survival, we found that increased B cell presence did correlate with overall survival. More importantly, we observed that the close proximity between tumor-associated macrophages and B cells correlated with overall survival. The results highlight the anti-tumor role of B cells, and that the spatial interactions between B cell and other cell types (e.g., macrophages) is a novel predictor of therapeutic response and patient outcomes.

Statement of Implication

In high grade serous ovarian carcinoma (HGSOC), there is an urgent need for improved predictive markers of therapeutic response. Cellular composition and spatial profiling of the tumor microenvironment are becoming ubiquitous. This is the first study to examine the spatial relationship of HGSOC tumors with clinical attributes, such as overall survival. We discovered that a “high” interaction between tumor-associated B cells and macrophages significantly correlates to survival. Moreover, the findings provide valuable insight into future predictive models and therapeutic strategies.

Introduction

Ovarian cancer is the most lethal gynecologic malignancy, with over 295,400 new diagnoses and over 184,000 deaths per year, globally [1]. While most patients with high grade serous ovarian cancer

(HGSOC) will respond to first-line treatment, a combination of cytoreductive surgery and platinum/taxane based chemotherapy, over 75% will recur and develop therapy-resistant disease [2]. More recently, advanced stage disease (IV) is treated with neoadjuvant chemotherapy (NACT) prior to cytoreductive surgery, to facilitate optimal tumor resection. The five-year overall survival rate of patients with advanced stage HGSOC is less than 30% [3]. The tumor microenvironment and the ability of the immune system to mount an anti-tumor response directly correlates to patient outcomes [4]. Moreover, current strategies to induce anti-tumor immunity in ovarian cancer (e.g., immune checkpoint blockade) have only had a minimal impact in HGSOC management [5]. Like most tumors, the microenvironment of HGSOC tumors is heterogeneous and complex, and the biological significance of spatial relationships between cell types is not well understood.

Tumor associated macrophages (TAMs) convey an inflammatory microenvironment that can lead to tumor progression and are the most prevalent immune cells in HGSOC tumors [6]. Elevated tumor infiltration of macrophages is a poor prognostic indicator in HGSOC ([7], [8]). TAMs secrete the cytokine IL-6, which binds to the IL-6 receptor to activate The Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway, specifically STAT3. IL-6 expression can lead to both inflammatory and anti-inflammatory responses [9]. Increased IL-6 expression and activation of STAT3 in tumor cells have both been attributed to HGSOC disease progression and therapy resistance ([10], [11]). Currently, there is a significant research gap in understanding tumor promoting properties of TAMs and defining strategies for TAM depletion in the HGSOC tumor microenvironment.

In contrast to TAMs, the role of B cells in HGSOC tumor biology is less understood. As such, there are several research articles with contradictory conclusions about the prognostic value of tumor infiltrating B cells [12]. For example, Yang C and colleagues reported a median 113.3-month overall survival benefit in HGSOC tumors with low B cell (CD19+) infiltration. In contrast, Milne K and colleagues found B cell (CD20+) tumor infiltration promoted an approximately 60-month overall survival benefit [13]. The discrepancies are likely due to different subsets or developmental stages of B cells within the tumor, but additional research is needed. The primary function of B cells is antibody production, but B cells also function to secrete cytokines and initiate paracrine signaling with neighboring immune cells (e.g., macrophages). B cell and macrophage paracrine interactions are proposed to be both tumor promoting

and tumor suppressive. Mechanisms of tumor-promoting B cell-macrophage associations are more established. For instance, secretion of IL-10 by B cells was shown to induce polarize macrophages to an M2-like, pro-tumor phenotype, and subsequently drive tumor progression [14]. In contrast, the tumor suppressive B cell-macrophage associations are not well understood. For example, in a lymphoma model depletion of macrophages blunts the anti-tumor response of an anti-CD20 therapy [15]. With respect to HGSOC, in a mouse model B cell depletion led to a more aggressive progression, highlighting the anti-tumor properties of B cells [16]. Thus, to improve therapeutic response and patient outcome, in HGSOC tumors, there is a significant need to dissect B cell- and TAM-mediated tumor effects.

The spatial relationships between tumor cells and tumor associated immune cells are not well understood, but multispectral immunohistochemistry has provided a unique opportunity to assess the complex spatial organization within a single tumor section. In this study, we utilized a tissue microarray of HGSOC tumors and correlated the tumor composition (TAM, B cell, T cell) to clinical attributes. Specifically, in the cohort of HGSOC tumors, while TAM infiltration did not correlate with survival, the distance of TAMs to B cells, did significantly correlate to improved survival. We also observed that B cell tumor infiltration was a positive predictor of survival.

Methods

Regulatory Compliance

The tissue microarray (TMA) was constructed with Institutional Review Board approval from the University of Colorado (COMIRB numbers 17-7788). A board-certified gynecologic pathologist (MDP), selected the tumor sections included on the array. Patient and clinical attributes of the tissues included on the TMA were previously published ([17], [18]).

Data Collection/Attributes

Tissue samples from tumor regions of 128 ovarian cancer patients were selected by a board-certified gynecologic pathologist (MDP). Following immunohistochemical analysis, a patient's tumor was removed due to lack of an exact date for "known alive as of". Primary tumor tissue samples from 127 patients were used for subsequent analyses. For Cox Proportional Analyses, three additional samples were removed

due to lack of PARPi (1) and debulking (2) data. All samples are from patients with high grade serous ovarian cancer (n=118).

Vectra Staining

The TMA containing the high grade serous ovarian cancer tumors was previously analyzed via Vectra Automated Quantitative Pathology Systems (Akoya Biosciences) [17]. Sequential 5-micron slides from the TMA were stained with antibodies specific for CD8 (T cells, C8/144B, Agilent Technologies), CD68 (macrophages, KP1, Agilent Technologies), cytokeratin (CK, tumor cells, AE1/AE3, Agilent Technologies), CD3 (T cells, LN10, Leica), and CD19 (B cells, BT51E, Leica). The entire TMA core was imaged using the 20x objective on the Vectra 3.0 microscope (Akoya Biosciences) and image analysis was performed using inForm software version 2.3 (Akoya Biosciences), including tissue segmentation to define cells within the tumor regions (tumor-associated cells), cell segmentation to define cellular borders, and cell phenotyping.

Statistical Analysis

All statistical analyses were conducted in R Studio (version 1.3.1073) [19]. TMA slides were imaged, and cells classified by their phenotypes using Inform Software from Akoya Biosciences. Cells were classified using the following marker designations: B cell (CD19+), macrophage (CD68+), CD8 T cell (CD3+ CD8+), CD4 T cell (CD3+ CD8-), and tumor cell (CK+). Nearest neighbor distances were calculated using the “Phenoptr” package, which is a software provided by Akoya Biosciences to analyze images from their Vectra Polaris Imaging System [20] downstream of cell segmentation and phenotyping. Kaplan-Meier curves and Cox proportional hazards models were used to obtain p-values and hazard ratios. A p-value of less than 0.05 was considered significant.

An interaction variable was derived for each pair of immune cell types. Using the B-cell to macrophage interaction for illustration, this variable was calculated as follows. Within each image, the total count of B cells that had at least one macrophage within 25 microns was counted towards the B cell-macrophage interaction variable. To avoid noise from B cell or macrophage cell presence alone, count was then divided by the total number of B cells and macrophages within the tumor region for a given

image and multiplied by 100 (Figure 1C for representation of interaction variable between B cells and macrophages). For Kaplan-Meier curves, patients were classified as high interaction samples between two immune cells if they expressed above the median value of the interaction variable.

To construct Kaplan-Meier curves for high and low infiltration of immune cells, groups were split at the median percentage of a given immune cell in tumor regions within the tumor microenvironment (TME). Tumor-associated immune cell percentages were calculated as total immune cells divided by total number of cells within tumor regions for a given sample and given immune cell type. For example, a patient was classified as having a high macrophage (CD68+) presence if their percentage of macrophage cells in tumor regions was above the median for all samples of 2.51% (Table 1A).

Results

Our models examine how spatial interactions among different immune cells in the ovarian cancer TME are associated with overall survival. We analyzed the immune TME of over 100 HGSOC tumors using the Vectra-Polaris multi-spectral immunohistochemistry platform.

Quantification of Immune Infiltrate

Immune cell composition across patients for B cells (CD19+), macrophages (CD68+), CD4+ T cells (CD3+ CD8-), and CD8+ T cells (CD3+ CD8+) is summarized in Table 1A. Average B cell presence across patients was only 0.323% of cells in tumor regions. Macrophages were more prevalent with a mean of 3.9%. Of the T cells, CD4+ T cells and CD8+ T cells made up, on average, 0.846% and 2.88%, respectively. Most of the cells in the tumor regions were CK+ cells with a mean of 81.4%.

Kaplan-Meier curves were constructed to assess the relationship between different immune cell types and overall survival for the 127 patients. Subjects with high B cell infiltration in tumor regions expressed higher survival probability ($p=0.03$), with median survival for the high B cell presence group of 2112 days (95% CI: 1884 – 3401) compared to 1587 days (95% CI: 1424 – 2311) for the low group (Figure 2A). Subjects with high infiltration of macrophages, CD8+ T cells, and CD4+ T cells in tumor regions did not differ in survival probability from low infiltration groups ($p = 0.94, 0.26$, and 0.12 , respectively) (Figure 2B,

2C, and 2D). These findings indicate a positive correlation of B cell tumor infiltration with improved survival.

Immune Cell Spatial Relationships

We examined the distance relationships between the different immune cell types and correlated these spatial parameters with overall patient survival. Spatial relationships between cell types were represented through our derived cell type interaction variables (see Figure 1C for pictorial representation) and assessed overall survival using Kaplan Meier curves and unadjusted Cox proportional hazards models. Kaplan Meier curves show the “high” and “low” interaction groups did not differ in survival probability for the CD8+ to CD68+ cell relationship (Figure 3B, $p = 0.47$), for the CD4+ to CD68+ relationship (Figure 3C, $p = 0.46$), or for the CD4+ to CD8+ relationship (Figure 3D, $p = 0.57$).

Cox proportional hazards models similarly found that most cell type interactions failed to predict overall survival. Specifically, no statistically significant relationships with overall survival were found for the CD8+ to CD4+ T cell interaction ($p = 0.22$), the CD4+ T cell to macrophage interaction (0.06), or the CD8+ T cell to macrophage interaction (0.28) variables. B cell interaction with CD4+ and CD8+ T cells was low across samples, with over 50 percent of samples not containing any B cells within 25 microns of a CD4+ or CD8+ T cell. Cox regression models were still implemented and found to be not statistically significant with p-values of 0.22 and 0.12, respectively.

B Cell and Macrophage Spatial Relationship

Given the discrepant literature on the role of B cells within the HGSOC TME, we hypothesized that B cell interaction with different immune cells, rather than B cell presence alone, is an important indicator of overall survival. Subjects with high B cell-to-macrophage interactions (median survival of 2301 days, 95% CI: 1992-3401) exhibited a higher survival probability, compared to subjects with low B cell-to-macrophage interactions (median survival of 1587 days, 95% CI: 1424-2311)(Figure 3A). The unadjusted hazard ratio for overall survival, comparing the high B cell-to-macrophage interaction group to the low B cell-to-macrophage interaction group is 0.64 (95% CI: 0.40 – 0.99).

Cox proportional hazards models, adjusted for clinical covariates presented in Table 1A, were used to compare B cell presence alone versus B cell-to-macrophage interaction as predictors of overall survival. In both the unadjusted model and the covariate adjusted model, the total B cell percentage variable was not a significant predictor of overall survival ($p=0.27$ and 0.26 , respectively). The B cell-to-macrophage interaction variable was a significant predictor of overall survival in both the unadjusted model and model adjusted for clinical attributes ($p = 0.0428$ and 0.0318 , respectively). The adjusted hazard ratio for the B cell-to-macrophage interaction variable is 0.8937 (95% CI: $0.8066 – 0.9902$), suggesting a decreased hazard of death for increased B cell-to-macrophage interaction. Hazard ratios, confidence intervals, and p-values for all covariates in the adjusted B cell-to-macrophage interaction model are presented in Table 1B.

Discussion

HGSOC tumors are heterogeneous and cellular composition provides critical insight into how the cancer patients may respond to primary chemotherapy. In this data set of 127 HGSOC tumors samples of the TME, each patient received the standard of care: a combination of surgical debulking and chemotherapy (platinum/taxane-based treatment). Thus, the overall survival benefits and positive immune cell correlations observed in our study may be a direct reflection as to how effective the standard-of-care is within the patient cohort. We observed that, within the TME, the interactions between B cells (CD19+) and macrophages (CD68+) were associated with better overall survival outcomes. Higher infiltration of B cells into tumor regions was also associated with better survival outcomes. Using Cox proportional hazards models, the interaction of B cells and macrophages was shown to be a better predictor of survival probability than the presence of B cells alone. Taken together, elevated B cell and macrophage interactions within tumors suggest a more favorable response to chemotherapy.

Previous studies have defined how chemotherapy remodels the HGSOC TME. Notably, chemotherapy often induces a robust IL-6 mediated inflammatory response that may directly remodel the TME ([17], [21]). In HGSOC, IL-6 induction is mainly described as tumor promoting; thus, dampening inflammatory or IL-6-driven responses may ultimately be beneficial. While the mechanisms driving the improved survival benefit from B cell/macrophage interactions are not well understood, we speculate that

B cell-dependent paracrine signaling via immunomodulatory cytokines, such as IL-10, serves to attenuate inflammation. Moreover, macrophages can present antigens to B cells, potentially enriching for tumor-specific antigen antibody production, leading to a more robust anti-tumor immune response following chemotherapy. Dissecting these B cell/macrophage anti-tumor mechanisms will be the subject of future investigations.

Small populations of immune cells, as well as intratumoral heterogeneity, can obfuscate investigations into the TME by making it difficult to ascertain whether results will replicate to other similar data sets. To better understand the relationships between different immune cells within the TME, further research will need to be conducted with larger TME samples and with stronger phenotyping of immune cells (for example, double positive or double negative T cells were not a focus of this study). Regardless, based on previous studies ([16], [17], [21]), there is reason to believe that immune cell interaction within the TME leads to better overall survival outcomes, as demonstrated by the “interaction” of B cells (CD19+) and macrophages (CD68+) in this data set . To further the study of the TME, data and code will be provided in a public GitHub repository to assist in reproducibility. Understanding which immune cell interactions are most important in the TME may lead to more accurate diagnosis and development of novel therapy techniques.

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Visual Overview

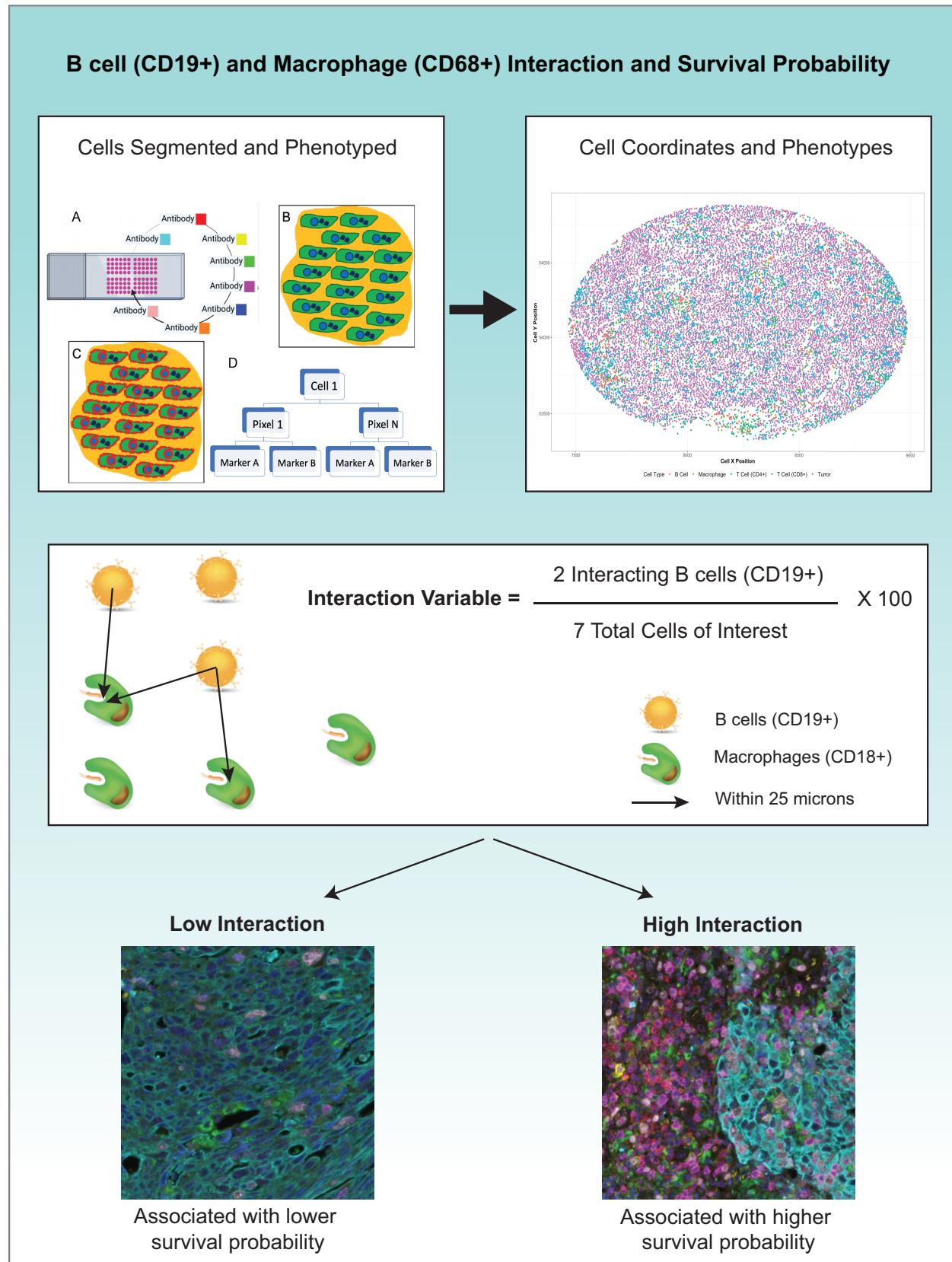


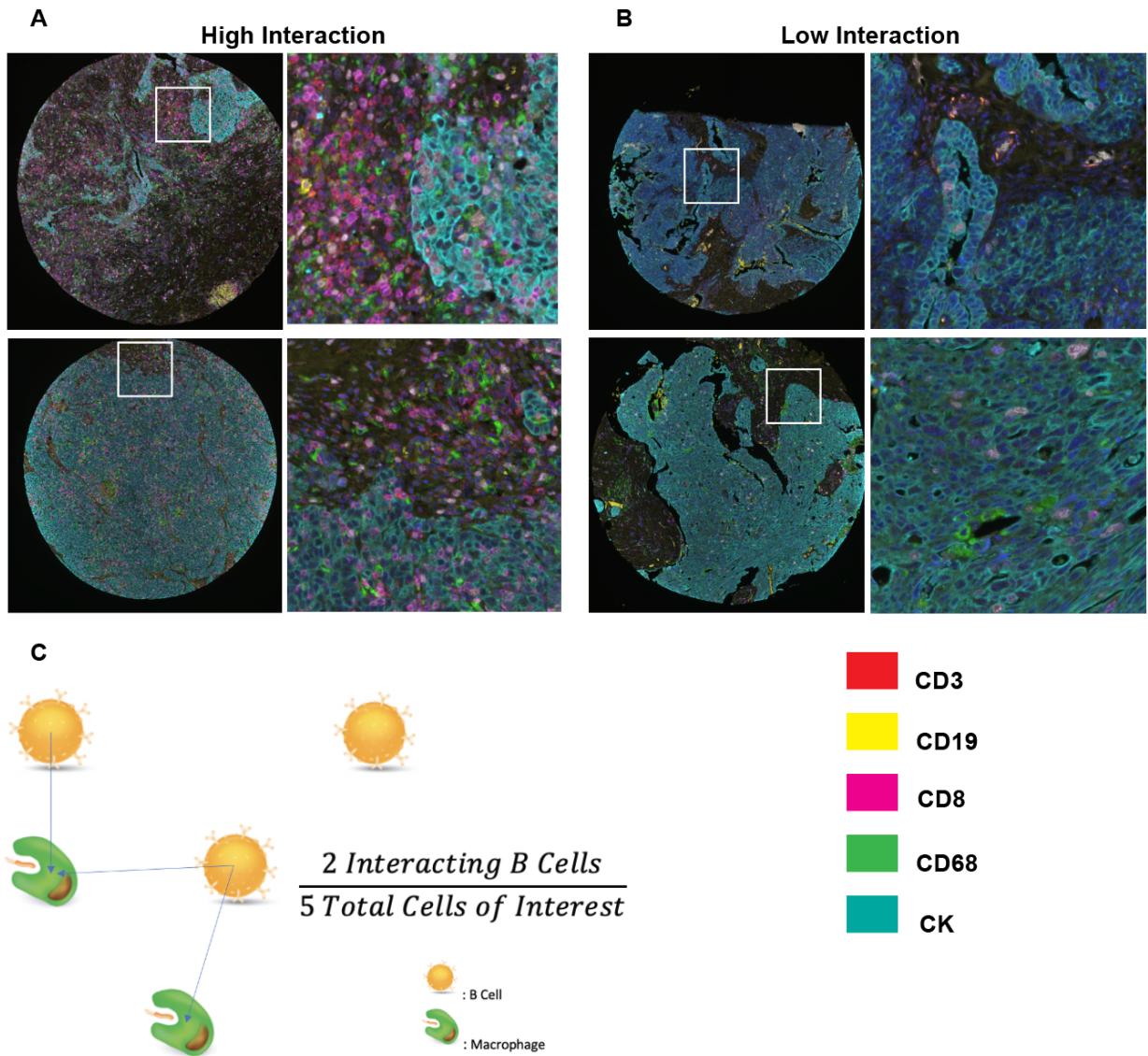
Figure 1:

Figure 1. Tissue microarray (TMA) slides of ovarian tumor samples stained with antibodies specific for CD4 (T cells, 4B12, Leica Biosystems), CD8 (T cells, C8/144B, Agilent Technologies), CD68 (macrophages, KP1, Agilent Technologies), and cytokeratin (tumor cells, AE1/AE3, Agilent Technologies). **A**, TMA slides exhibiting high values for the interaction variable for B cells and macrophages (classified as high if greater than the median for all samples). **B**, TMA slide exhibiting low values for the interaction variable for B cells and macrophages (classified as low if less than the median for all samples). **C**, Explanatory diagram of how the B cell (CD19+) and macrophage (CD68+) interaction variable was calculated. The interaction variable was calculated by taking the total number of B cells that had a macrophage within 25 microns of it, dividing by the total number of B cells and macrophages for that sample, and multiplying by 100.

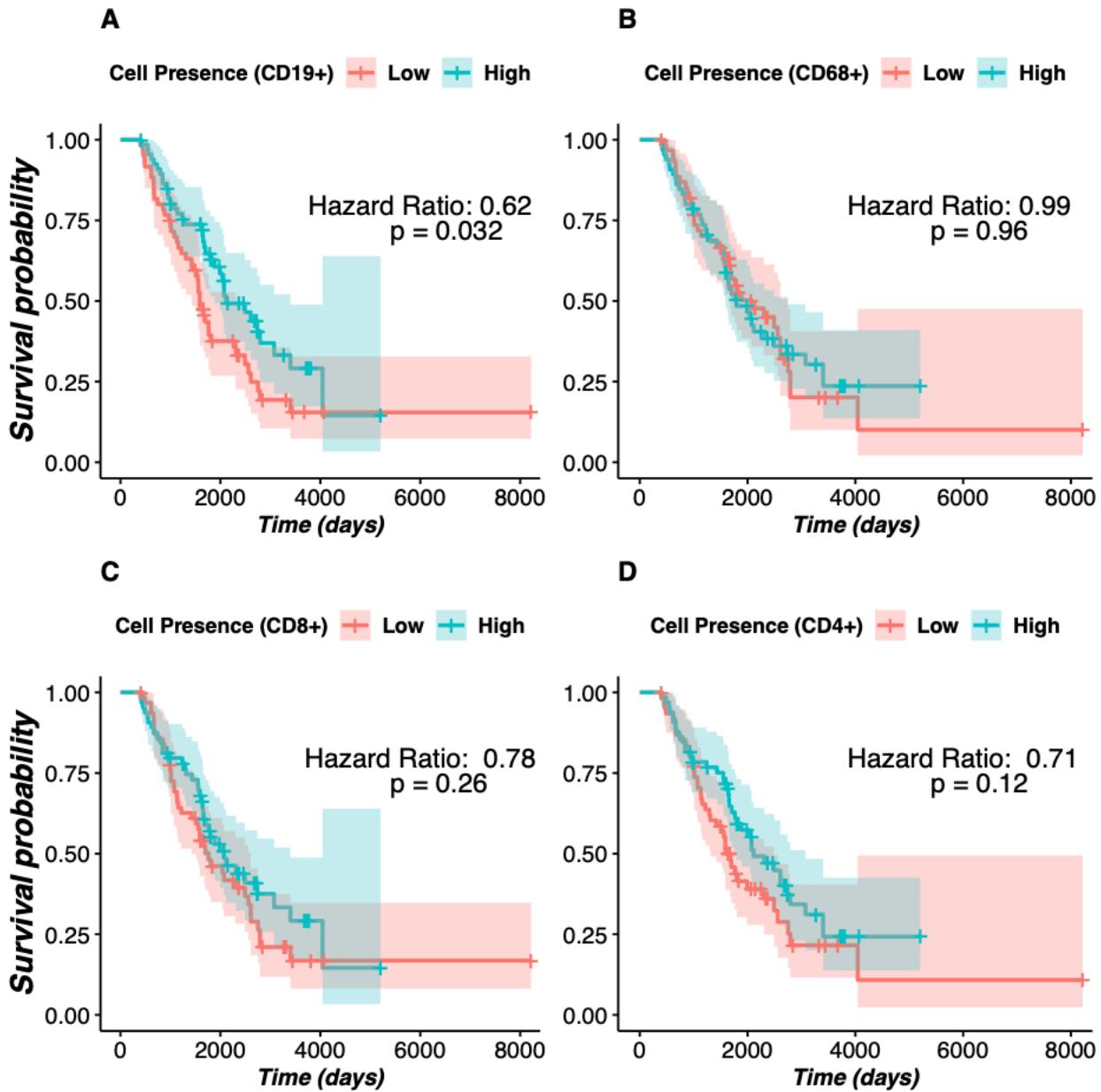
Figure 2:

Figure 2. Kaplan-Meier curves for immune cell infiltration in tumor regions. **A**, Samples with a high percentage of B cell infiltration into tumor regions had higher overall survival probability compared to the low infiltration group ($p=0.032$). The Hazard ratio of 0.62 suggests that the samples with a high presence of B cells in tumor regions had 0.62 times the risk of hazard (death) compared to the low infiltration group (95% CI: 0.4 - 0.96). **B**, Samples with a high percentage of macrophage infiltration into tumor regions did not differ significantly in survival probability from the low infiltration group ($p=0.96$). **C**, Samples with a high percentage of T cell (CD3+ CD8+) infiltration into tumor regions did not differ significantly in survival probability from the low infiltration group ($p=0.26$). **D**, Samples with a high percentage of CD4 T cell (CD3+ CD8-) infiltration into tumor regions did not differ significantly in survival probability from the low infiltration group ($p=0.12$).

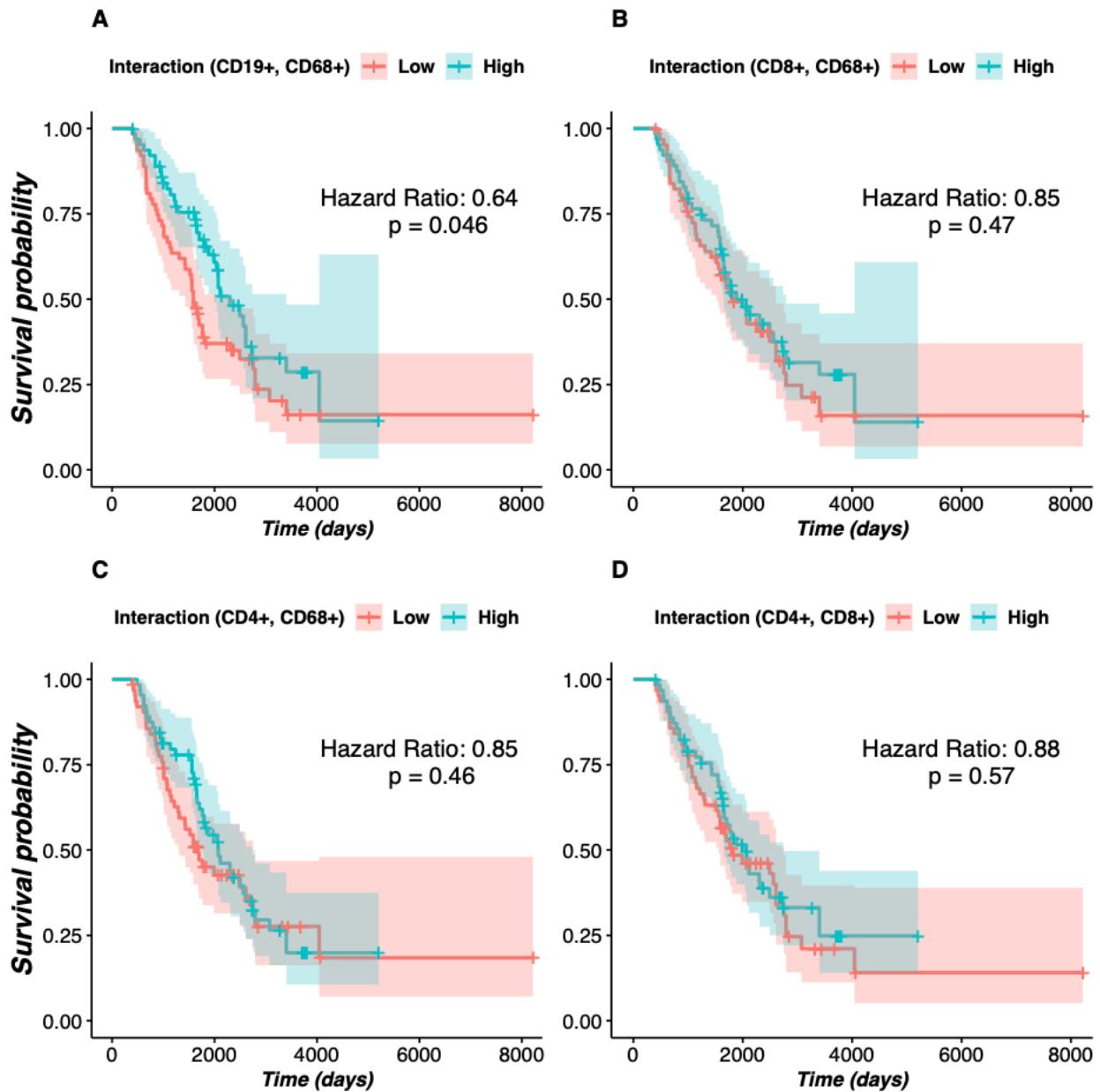
Figure 3:

Figure 3. Kaplan-Meier curves for different combinations of cell distance interactions in tumor regions. **A**, Samples with a high percentage of B cells (CD19+) near macrophages (CD68+) had higher overall survival probability compared to the low interaction group ($p=0.046$). The Hazard ratio of 0.64 suggests the samples with high B cell-macrophage interaction had 0.64 times the risk of hazard (death) compared to the low interaction samples (95% CI: 0.41 - 0.99). **B**, Samples with high CD8 T cell-to-macrophage interaction did not differ significantly in survival probability from the low interaction samples ($p=0.47$). **C**, Samples with high CD4+ T cell-to-macrophage interaction did not differ significantly in survival probability from the low interaction samples ($p=0.46$). **D**, Samples with high CD4+ T cell-to-CD8+ T cell interaction did not differ significantly in survival probability from the low interaction samples ($p=0.57$).

Table 1A

	Alive (N=48)	Death (N=79)	Overall (N=127)
B Cell Macrophage Interaction Variable			
Mean (SD)	2.89 (4.51)	1.13 (3.21)	1.79 (3.83)
Median [Min, Max]	1.12 [0, 19.4]	0 [0, 21.4]	0.137 [0, 21.4]
Missing	1 (2.1%)	0 (0%)	1 (0.8%)
CD19+ Percent			
Mean (SD)	0.510 (1.23)	0.209 (0.833)	0.323 (1.01)
Median [Min, Max]	0.0700 [0, 7.01]	0.0200 [0, 6.62]	0.0300 [0, 7.01]
CD68+ Percent			
Mean (SD)	4.32 (4.89)	3.64 (3.51)	3.90 (4.08)
Median [Min, Max]	2.30 [0.0100, 21.9]	2.86 [0.0400, 14.6]	2.51 [0.0100, 21.9]
CD3+ CD8+ Percent			
Mean (SD)	3.12 (4.24)	2.74 (4.35)	2.88 (4.30)
Median [Min, Max]	1.55 [0.0100, 19.9]	1.13 [0, 21.8]	1.25 [0, 21.8]
CD3+ CD8- Percent			
Mean (SD)	1.26 (2.78)	0.596 (1.16)	0.846 (1.95)
Median [Min, Max]	0.155 [0, 16.7]	0.130 [0, 7.15]	0.150 [0, 16.7]
CK+ Percent			
Mean (SD)	81.1 (14.8)	81.6 (15.4)	81.4 (15.1)
Median [Min, Max]	85.3 [29.5, 97.0]	86.7 [30.0, 97.2]	86.4 [29.5, 97.2]
Age at Diagnosis			
Mean (SD)	56.9 (10.8)	61.5 (9.40)	59.8 (10.1)
Median [Min, Max]	54.5 [38.0, 78.0]	63.0 [39.0, 80.0]	60.0 [38.0, 80.0]
Grade			
3	43 (89.6%)	74 (93.7%)	117 (92.1%)
1	1 (2.1%)	0 (0%)	1 (0.8%)
2	4 (8.3%)	5 (6.3%)	9 (7.1%)
Primary			
1	36 (75.0%)	66 (83.5%)	102 (80.3%)
0	12 (25.0%)	13 (16.5%)	25 (19.7%)
Treatment Effect			
0	41 (85.4%)	67 (84.8%)	108 (85.0%)
1	7 (14.6%)	12 (15.2%)	19 (15.0%)
Debulking			
Optimal	39 (81.2%)	49 (62.0%)	88 (69.3%)
Interval	8 (16.7%)	15 (19.0%)	23 (18.1%)
Suboptimal	1 (2.1%)	14 (17.7%)	15 (11.8%)
Missing	0 (0%)	1 (1.3%)	1 (0.8%)
PARPi			
0	27 (56.2%)	55 (69.6%)	82 (64.6%)
1	20 (41.7%)	24 (30.4%)	44 (34.6%)
Missing	1 (2.1%)	0 (0%)	1 (0.8%)

Table 1B

	Hazard Ratio	SE(Hazard Ratio)	Z	Pr(> z)
B Cell Macrophage Interaction	0.899	0.052	-2.055	0.040
Age at Diagnosis	1.028	0.012	2.289	0.022
Primary - 0	0.684	0.337	-1.129	0.259
Treatment Effect - 1	0.621	0.678	-0.704	0.482
Debulking - Interval	2.771	0.631	1.616	0.106
Debulking - Suboptimal	1.539	0.318	1.356	0.175
PARPi - 1	1.064	0.268	0.232	0.817

^a formula = (Survival Time, Death) ~ B Cell Macrophage Interaction Variable + Age at Diagnosis + Primary + Treatment Effect + Debulking + PARPi

^b n = 124, number of events = 78

^c Corresponding p-value for model from Likelihood Ratio Test: p = 0.008

Table 1. A, Descriptive table of variables included in analyses split by whether a patient was still alive or dead at the last follow up.

Cell percentages are calculated as total cells of a specified type divided by total cells in tumor regions for a given sample. Medians of cell percentages were used to split up samples into high or low infiltration groups referenced in Figure 2. **B,** Results from the cox proportional hazards model where coefficients have been exponentiated and interpreted as hazard ratios. The '*B Cell Macrophage Interaction*' variable and age were statistically significant (p <0.05).

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