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A pilot study investigating the effect of pembrolizumab on the tumoral immunoprofile of newly diagnosed mullerian cancers

Stéphanie L. Gaillard [a,b,e,*], Gloria Broadwater [c], Andrew Berchuck [b], William S. Andrews [b], Laura J. Havrilesky [b], Brittany A. Davidson [b], Rebecca A. Previs [b], Mark D. Starr [d], John S. Yi [d], Andrew B. Nixon [e], Jadee L. Neff [f], Jose Ramon Conejo-Garcia [g], Angeles Alvarez Secord [b]

[a] Departments of Oncology and Obstetrics and Gynecology, Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins School of Medicine, Baltimore, MD 21287, United States
[b] Division of Gynecologic Oncology, Duke Cancer Institute, United States
[c] Duke Cancer Institute Biostatistics, Duke Cancer Institute, United States
[d] Department of Surgery, Duke Cancer Institute, United States
[e] Department of Medicine, Duke Cancer Institute, United States
[f] Department of Pathology, United States
[g] Department of Integrative Immunobiology, Duke Cancer Institute, Durham NC 27710, United States

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ABSTRACT

Objective: This pilot window of opportunity study was conducted to assess feasibility, toxicity, and changes in immune parameters in response to one dose of the PD-1 inhibitor, pembrolizumab, in patients newly diagnosed with mullerian epithelial cancers.
Methods: Eligible patients received pembrolizumab 200 mg IV once ≥ 7 days prior to further standard therapy, including adjuvant chemotherapy. Tissue and blood were collected before and ≥ 7 days after pembrolizumab administration. Primary endpoints included change in tumor infiltrating lymphocytes (TIL), feasibility, and toxicity based on frequency and severity of adverse events. Exploratory objectives included tumor assessment of immunohistochemical PD-L1 staining using a quantitative modified proportion score and qualitative assessment of immune presence at the stromal interface. Measurement of cytokine levels and digital spatial profiling were performed from plasma and tissue samples, respectively, before and after pembrolizumab.
Results: Fifteen patients enrolled and received pembrolizumab. TIL levels changed in 4 of 11 paired sets, with 3 decreasing and 1 increasing post-treatment. PD-L1 modified proportion score increased in 7 cases, decreased in 2, and remained unchanged in 2. The stromal interface switched from negative to positive in 3 cases. Collectively, 8 of 10 assessable tumor pairs demonstrated either an increase in PD-L1 modified proportion score or the stromal interface switched from negative to positive. Circulating CXCL10 and TNFα levels increased after pembrolizumab in patients with response, but decreased in the one patient with progression on adjuvant chemotherapy. Digital spatial profiling showed increased IDO1 protein expression in immune and tumor compartments after treatment.
Conclusion: A single dose of pembrolizumab increased PD-L1 modified proportion score and/or stromal interface immune cells suggesting potential for local tumor immunologic recruitment. Additionally, increases in systemic inflammation, measured by cytokine production and differential IDO1 expression, reflect an interferon response. These hypothesis-generating data need to be confirmed and validated in larger subsets.

1. Introduction

Immune checkpoint inhibitors have changed the treatment paradigm for numerous cancers. Inhibitory antibodies directed against the programmed cell death 1 (PD-1) receptor, or its ligand (PD-L1), enhance anti-tumor immune responses through the recovery of T-cell function. In gynecologic cancers, the efficacy of immune checkpoint inhibitors varies based on disease site, setting (treatment-naïve versus previously

* Corresponding author at: Department of Oncology, Johns Hopkins School of Medicine, 201 North Broadway, Viragh 10-260, Baltimore, MD 2128721287, United States.
E-mail address: stephanie.gaillard@jhmi.edu (S.L. Gaillard).

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treated), biomarkers (especially mismatch repair status), and the antibody. Anti-PD-1/-L1 are now standard of care in the treatment of advanced endometrial cancer both in primary therapy and in the second-line setting (Mirza, 2023; Eskander, 2023; Makker, 2022; Mahdi et al., 2023). By contrast in ovarian, peritoneal, and tubal cancers (collectively referred to as ovarian cancers), immune checkpoint inhibitors in multiple settings have yielded disappointing outcomes (Porter and Matulonis, xxxx). However, occasional durable responses stimulate continued interest in understanding factors predicting benefit. Biomarker studies have identified mismatch repair deficiency, MSI-high, and high tumor mutational burden as the most predictive. However, in $\geq$ 50 % of patients with these markers single-agent anti-PD-1/-L1 fails to provide a response. An enhanced understanding regarding the effect of immune checkpoint inhibitors on the tumor microenvironment is needed to identify novel targets.

Despite extensive research, factors influencing immunologic responses and mechanisms of resistance are poorly understood in gynecologic cancers. PD-1 is expressed on the cell surface of activated lymphocytes including peripheral CD4 + and CD8 + T-cells, B-cells, T regs and natural killer cells and down-regulates excessive immune responses, including autoimmune reactions. The PD-1 ligands, PD-L1 and PD-L2, are either constitutively expressed or can be induced in numerous cell types, including non-hematopoietic tissues and cancers. PD-L1 regulates T-cell function in peripheral tissues and may have a critical role in tumor immune evasion by inducing immunosuppression of CD8 + T cell lymphocytes (Hamanishi, 2015). Utility of PD-L1 expression as a potential predictive marker has been inconsistent across ovarian and endometrial studies (Matulonis, 2019; Pignata, 2023; Westin, 2023); thus, PD-L1 expression is not currently an accepted predictive biomarker in these diseases.

Window studies offer an opportunity to investigate molecular mechanisms of response and resistance to immune checkpoint inhibitors and may provide insight into other targets or rationally-directed immunotherapy combinations. We hypothesized that tumor immune infiltrates would increase after administration of pembrolizumab and that pembrolizumab would be well tolerated when given prior to standard chemotherapy +/- surgery and as maintenance after completion of chemotherapy in patients with gynecologic cancers of müllerian origin. The current trial was developed to assess changes in immune parameters from paired tissue biopsies and longitudinal blood samples after treatment with pembrolizumab in patients with a new diagnosis of mullerian cancer. The primary objectives were to (1) describe changes in tumor immune infiltrates after administration of pembrolizumab, and (2) to determine feasibility and toxicity of pembrolizumab when given prior to standard of care therapy. Exploratory objectives included changes in PD-L1 expression, qualitative assessment of PD-L1 staining reflecting immune presence at the stromal interface, plasma cytokine levels, and digital spatial profiling of immune and tumor signaling proteins after pembrolizumab treatment.

2. Methods

This open-label, pilot, window study (NCT02728830) was approved by the Duke Health System Institutional Review Board (Pro00068544). The study required a two-part enrollment, with written informed consent obtained for each part. Initial consent was obtained for biopsy of tumor tissue or tumor cells from paracentesis or thoracentesis, if appropriate. Patients were eligible to be consented for treatment if the diagnosis of a new gynecologic tumor of mullerian origin, specifically epithelial ovarian, fallopian tube, primary peritoneal, or epithelial endometrial cancer was confirmed and disease was amenable to surgical resection or biopsy. Additional eligibility requirements included adequate organ function; an Eastern Cooperative Oncology Group performance status $\leq$ 1; and age $\geq$ 18 years. Key exclusion criteria included prior treatment for the current malignancy; prior diagnosis of immunodeficiency; chronic systemic steroid or immunosuppressive therapy within 7 days of pembrolizumab administration; prior checkpoint inhibitor therapy; active infection or conditions that would interfere with study procedures.

The primary efficacy objective was to assess the change in tumor immune infiltrates after administration of one dose of pembrolizumab compared to baseline. The primary safety objective was to assess the feasibility and toxicity, based on the frequency and severity of adverse events, of pembrolizumab in patients with newly diagnosed mullerian cancer prior to standard therapy. The exploratory objectives were to assess changes in 1) PD-L1 expression scored using a quantitative modified proportion score, 2) immune presence at the stromal interface based on qualitative assessment of PD-L1 staining, 3) circulating cytokine levels, and 4) change in immune protein expression by digital spatial profiling.

Participants received pembrolizumab 200 mg intravenously once on Day 1, at least 7 days prior to post-treatment surgical resection or biopsy. Participants then underwent standard cytoreductive surgery or research biopsy followed by standard chemotherapy for their cancer as deemed appropriate by their treating physician. Based on patient and physician feedback, the protocol was amended to allow pembrolizumab maintenance after standard of care therapy to increase acceptability. For participants enrolled prior to this addition, maintenance was optional. After the amendment, participants received pembrolizumab 200 mg intravenously every 3 weeks until unacceptable toxicity, progression, or for up to 12 months. Participation was continued until disease progression, intolerable toxicity, or withdrawal of consent.

Fresh tissue (or archival if permitted by investigator) was collected at baseline. Tumor tissue was obtained by surgery or biopsy at least 7 days after Day 1. Safety follow-up evaluations occurred 30 days +/- 7 days after dosing. Patients receiving pembrolizumab maintenance therapy were followed for adverse effects throughout therapy. Toxicity was graded according to the National Cancer Institute Common Toxicity Criteria version 4.0.

Changes in tumor immune infiltrates in formalin-fixed, paraffin-embedded tumor specimens were assessed by QualTek Molecular Laboratories (now Discovery Life Sciences, Newtown, PA). Tumor sections were mounted onto unstained microscope slides and examined by an independent pathologist blinded to clinical data. Samples were deemed not evaluable if there was a significant problem with fixation or processing, or if fewer than 50 evaluable tumor cells were present and fewer than five were PD-L1 positive.

Tumor infiltrating lymphocyte abundance was denoted with a categorical score (0–3) with 0 being the absence of and 3 indicating high profusion of tumor infiltrating lymphocytes. Assessment of tumor infiltrating lymphocyte abundance was validated for exploratory use and was performed as previously described and in accordance with International TIL Working Group guidelines (Tolaney, 2020; Salgado, 2015). PD-L1 staining was performed using a validated immunohistochemistry assay for PD-L1 (CD274, B7-H1) and the mouse monoclonal antibody clone 22C3 (Merck, Palo Alto, CA) with appropriate controls (Dolled-Filhart, 2016). Representative staining is shown in Supplemental Fig. 1.

PD-L1 expression was assessed by determining the percentage of tumor, including tumor infiltrating mononuclear inflammatory cells, demonstrating membrane staining and the presence of a distinctive PD-L1 staining pattern at the tumor/stroma interface. Full or partial PD-L1 plasma membrane staining was scored while cytoplasmic staining was not scored. The percent of tumor with membrane-specific staining was directly estimated at four intensity levels [negative (<1), low (1 +), moderate (2 +), and high (3 +)] and reported as any one of the following: 0, 1, 2, 5, 10, 15, 20, 30, 40, 50, 60, 70, 80, 85, 90, 93, 95, 99, or 100 %. To calculate modified proportion score, the percent tumor staining at low, moderate, and high levels were summed. To calculate modified H-score, the percent staining was multiplied by an intensity factor (1 for low stain, 2 for moderate, 3 for high) and then the three products were summed. Dichotomous assessment (present or absent) for a distinctive lichenoid pattern PD-L1 membrane staining at the stromal

interface was performed at low-power evaluation.

EDTA plasma samples were processed within an hour, frozen, and stored at −80 °C. Plasma levels of multiple cytokine markers (Supplemental Table 1) were assessed using the Meso Scale Discovery imaging system (Meso Scale Discovery, Inc., Rockville, MD). Assays were performed in duplicate per manufacturer's instructions and assessed in a single batch to minimize variability. Results are expressed in pg/ml. Coefficients of variation were ≤ 10 %. Lab personnel were blinded to demographic and clinical data.

Statistical analyses were primarily descriptive given the small sample size. Median and range summarize tumor infiltrating lymphocyte and PD-L1 expression. Stromal interface was categorized as positive or negative. Adverse events were summarized using frequencies and percentages. To test cytokine changes in response to treatment, log transformed ratios (Lratios) were calculated for each biomarker using the formula: Log_2(post-treatment level / baseline level). Waterfall plots graphically illustrate the change in cytokine levels from baseline after treatment with pembrolizumab. Pairwise associations between biomarkers were conducted using Spearman correlation coefficients. Wilcoxon signed-rank test was used to estimate the p-value on the difference in post-treatment to pre-treatment values. P-values ≤ 0.05 were considered statistically significant. Statistical analyses were conducted using SAS software version 9.4 (SAS Institute, Inc. Cary, NC) and graphs were created using S-Plus software (TIBCO Software Inc., Palo Alto, CA).

For digital spatial profiling, patient samples mounted on slides were stained for immune (CD45) and tumor/epithelial (PANCK) tissue using fluorescently conjugated antibodies ("morphology probes") and barcoded antibodies against the protein antigens of interest ("protein probes"). Guided by the morphology probes, regions of interest within each tissue type and slide were selected by a pathologist and barcodes from the protein probes were collected from each region of interest using the NanoString GeoMx digital spatial profiling instrument. Protein expression counts for 46 antigens (plus positive hybrid and negative IgG controls) were measured by quantifying the barcodes collected from each region of interest using the NanoString nCounter instrument. The following protein probe sets were used (individual proteins listed in Supplemental Table 2): the Protein Core, IO Drug Target, Immune Activation Status and Immune Cell Typing panels. Additional methodology included in the Supplement.

In accordance with the journal's guidelines, we will provide our data for independent analysis by a selected team by the Editorial Team for the purposes of additional data analysis or for the reproducibility of this study in other centers if such is requested.

3. Results

This study required a two-part enrollment; the initial consent allowed screening biopsy and if eligible, patients were consented for treatment. Between May 2017 and December 2018, 39 patients were screened with 24 screen failures (Supplemental Fig. 2). Reasons included opting for neoadjuvant chemotherapy (n = 8) and non-eligible pathology (n = 7). Fifteen patients received at least one dose of pembrolizumab (Table 1). Thirteen (87 %) patients had ovarian or fallopian tube cancer, while 2 (13 %) had endometrial cancer. The median age was 65.7 years (range 43–76); 12 (80 %) had serous adenocarcinoma, 13 (87 %) had high-grade disease and 12 (80 %) had stage III or IV disease. There were no delays in planned standard of care treatment post-pembrolizumab. The median number of days between pembrolizumab administration and post-treatment surgery or biopsy was 8 (range 7–15). Extent of cytoreductive surgery was optimal in 12 patients (n = 12, R0 in 7, R1 in 5), suboptimal in 2, and not applicable in 1 due to early-stage disease.

Lymphocyte and PD-L1 staining assessments are summarized in Supplemental Table 3. Eleven patients had adequate matched pre- and post-treatment tissue samples for quantifying a tumor infiltrating lymphocytes score and PD-L1 expression by PD-L1 modified proportion score (Table 2). Four patients did not have matched samples; 3 had insufficient tissue on biopsy and 1 was missed. Pre- and post-treatment tumor infiltrating lymphocytes ranged from 0 to 3 with medians of 2 and 1, respectively. Tumor infiltrating lymphocyte levels changed in 4 of 11 paired sets, with 3 decreasing and 1 increasing post-treatment. Pre- and post-treatment PD-L1 modified proportion score values ranged from 0 to 95 (median 1.5) and 0–85 (median 6), respectively. The stromal interface was negative at baseline in 7 of 9 assessable cases (Table 2). After exposure to pembrolizumab, PD-L1 modified proportion score increased in 7 cases, and the stromal interface switched from negative to positive in 3 cases. When evaluating changes in either factor, 1 sample had no change in modified proportion score and insufficient sample to assess change in stromal interface. Eight of the other 10 assessable tumor specimens demonstrated either an increase in PD-L1 modified proportion score or a switch from a negative to a positive stromal interface. The only patient who developed progressive disease during standard of care

Table 1
Patient demographics and tumor characteristics.

Characteristic	Median (range)
Age, years	65.7 (43–76)
Days between pembrolizumab and post-treatment surgery/ biopsy	8 (7–15)
Race	**Number (%)**
Caucasian	13 (88.7)
African American	1 (6.7)
Unknown	1 (6.7)
Ethnicity	
Non-Hispanic	14 (93.3)
Unknown	1 (6.7)
Primary Tumor Site	
Ovary	9 (60.0)
Fallopian Tube	3 (20.0)
Uterus	2 (13.3)
Other[a]	1 (6.7)
Histologic Subtype	
Serous	12 (80.0)
Clear cell	3 (20.0)
Tumor Grade at Diagnosis	
High Grade	13 (86.7)
Low Grade	2 (13.3)
Stage	
I	1 (6.7)
II	1 (6.7)
III	10 (66.7)
IV	2 (13.3)
Unstaged	1 (6.7)
ECOG Performance Status	
0	5 (33.3)
1	10 (66.7)
Extent of Cytoreductive Surgery[b]	
Optimal R0	7 (46.7)
Optimal R1	5 (33.3)
Suboptimal	2 (13.3)
Not Applicable[c]	1 (6.7)

[a] Primary gynecologic cancer of mullerian epithelial origin (consistent with ovarian, tubal, peritoneal), unable to determine site of origin further.
[b] Four patients had a research biopsy followed by neoadjuvant chemotherapy prior to undergoing cytoreductive surgery.
[c] One patient had early stage endometrial cancer and cytoreduction status is not applicable.

Table 2
Intrapatient evaluation of tumor infiltrating lymphocytes (TILS), modified proportion score, and stromal interface pre- and post-pembrolizumab.

	TILS			modified proportion score			stromal interface		
	Pre	Post	Change	Pre	Post	Change	Pre	Post	Change
01	0	0	–	0	6	+6	No	Yes	+
02	2	1	↓	0	6	+6	No	No	–
03	3	3	–	15	50	+35	Yes	Yes	–
04	1	1	–	0	20	+20	Yes	Yes	–
05	2	2	–	1	10	+9	No	Yes	+
06	3	3	–	95	85	−10	No	Yes	+
07	3	1	↓	0	0	0	NA	No	NAA
08	1	2	↑	5	4	−1	No	No	–
09	1	1	–	2	4	+2	NA	Yes	NAA
10	1	1	–	0	0	0	No	No	–
11	2	1	↓	10	20	+10	No	No	–

NA, Not available; ***NAA***, Not able to assess; ***TIL***, Tumor infiltrating lymphocyte.

chemotherapy had an ovarian cancer exhibiting the following: decreased tumor infiltrating lymphocyte score from 3 pre-treatment to 1 post-treatment, PD-L1 modified proportion score of 0 both pre- and post-treatment, and a negative stromal interface on post-treatment sample.

All patients who received pembrolizumab were evaluable for toxicity. During the window phase, 3 (20 %) patients experienced treatment-related elevation in alkaline phosphatase and 2 (13 %) experienced diarrhea (Table 3). Over the course of the study, 10 (67 %) patients had a treatment-related adverse event of any grade (Supplemental Table 4). The most common treatment-related adverse events were elevation in alkaline phosphatase (n = 4, 27 %), hypothyroidism (n = 3, 20 %), and cough (n = 3, 20 %). No Grade 4 or 5 events occurred.

Key immune-response related cytokines were assessed using multiplex ELISA at baseline and after pembrolizumab (Table 4). Strong pairwise correlations between several of the biomarkers at baseline (Supplemental Table 5) and after pembrolizumab (Supplemental Table 6) were noted. There were no significant pairwise correlations between pre-treatment and change in PD-L1 expression and any of the blood-based biomarkers. Following pembrolizumab treatment, median levels for CXCL10, TNFα, IL10, and IL2ra were found to be statistically different in post-treatment versus pre-treatment samples (Table 4). CXCL10 and TNFα levels increased in all responding patients (patients who had a complete or partial response after standard of care therapy) but decreased in the one patient who progressed during adjuvant chemotherapy (Fig. 1). Additionally, the patient with progression exhibited the largest increases in IL-6 and largest decreases in IL-1β levels after treatment.

Five patients had both pre- and post-treatment samples suitable for digital spatial profiling while one patient had only post-treatment samples. Immune tissue regions of interest per patient ranged from six to nine for a total of 47 immune tissue regions of interest. Tumor tissue regions of interest per patient ranged from six to 10 for a total of 49 tumor tissue regions of interest. Following quality control and normalization, nine regions of interest appeared to be outliers under principal component analysis, but were retained in the differential expression analysis due to the already small sample size. Differential expression analysis comparing post- versus pre-treatment timepoints was performed using preprocessed protein expression data from all pre- and post-treatment regions of interest from the six patients. Three antigens produced singular fit warnings in different tissue subsets due to estimated random effect variances of zero (SMA in the immune tissue subset; HLA-DR and CD20 in the tumor tissue subset). The estimated coefficients for these antigens were similar with and without the inclusion of the random effect intercept for patient, so the mixed effect results were reported.

There was no evidence of differential expression of PD-1 in the immune tissue ($p = 0.86$; FDR = 0.959; log2FC = −0.141) or tumor tissue ($p = 0.0559$; FDR = 0.184; log2FC = 0.165). For the exploratory differential expression analysis, there were a total of five proteins identified as differentially expressed with unadjusted p-values < 0.01 within immune tissue (Supplemental Table 7). Two of these proteins were up-regulated (log2 fold change > 1; GAPDH and IDO1) and two were down-regulated (log2 fold change < -1; CD66b and Fibronectin) in post-treatment relative to pre-treatment regions of interest (Fig. 2). Within tumor tissue, five proteins were identified as differentially expressed with unadjusted p-values < 0.01 (Supplemental Table 8). Two of these proteins, GAPDH and IDO1, were up-regulated in post-treatment relative to pre-treatment regions of interest (Fig. 2).

Table 3
Window Phase Treatment-Related Adverse Events of Any Grade in ≥ 10 % of Patients or Grade ≥ 3 in Any Patient.

	Maximum Grade						Total (any grade)	
	1		2		3			
Adverse Event	**n**	**%**	**n**	**%**	**n**	**%**	**n**	**%**
ALK Phos increased	3	20	0		0		3	20
Diarrhea	1	7	1	7	0		2	13

ALK Phos, alkaline phosphatase.
No Grade 4 or 5 events reported.

4. Discussion

This study assessed changes in response to pembrolizumab in the tumor-immune microenvironment and circulating cytokines in patients newly diagnosed with mullerian cancers. Pembrolizumab enhanced immune cell recruitment to the tumor microenvironment in the majority of patients based on increases in PD-L1 expression by modified proportion score and PD-L1 staining at the stromal interface. There were more dynamic changes in these parameters compared to tumor infiltrating lymphocytes. A single dose of pembrolizumab resulted in PD-L1 staining patterns reflective of immune cell recruitment. Clinically single-dose pembrolizumab did not delay standard of care treatment with either surgery or chemotherapy, was well tolerated without significant toxicity, and no new safety signals were identified.

Despite rapid development and treatment indications for many cancer types, many questions persist regarding integrating immune checkpoint inhibitors into standard therapy to induce an optimal anti-cancer immune response. These include questions about patient selection and predictive biomarkers, efficacy in combination with chemotherapy and targeted therapies, and timing/sequencing of therapy in regards to surgery and chemotherapy. Patient selection based on mismatch repair deficient/MSI-H tumor status is associated with increased response rates to immune checkpoint inhibitor therapy across tumor types, but for mismatch repair proficient/microsatellite stable tumors the utility of predictive biomarkers is less clear. While PD-L1 expression is associated with improved responses in some cancer types

Table 4
Blood-based biomarker levels at baseline (pre-pembrolizumab) and post-pembrolizumab treatment.

	Pre-treatment (pg/ml)		Post-treatment (pg/ml)			
Biomarker	**Median**	**Range**	**Median**	**Range**	**FC**	**p***
CXCL10	139.0	45.3–283.0	250.5	69.4–630.5	1.5	0<.001
TNFα	10.5	6.8–16.2	13.2	7.9–31.1	1.1	0.006
IL-10	3.2	1.7–6.7	4.2	1.8–9.1	1.2	0.01
IL-2ra	1864.5	1,054.0–3,943.5	2,599.5	1,474.0–6,341.0	1.2	0.01
IFNγ	0.5	0.1–3.5	0.8	0.3–3.0	1.6	0.057
IL-6	6.3	1.5–12.4	5.2	1.4–22.4	1.1	0.31
IL-1β	0.2	0.0–0.7	0.3	0.0–0.6	1.9	0.37
IL-12p70	0.3	0.1–1.2	0.3	0–1.4	1.1	0.52

CXCL10, C-X-C motif chemokine 10; ***FC***, fold change; ***IFNγ***, interferon gamma; ***IL-10***, interleukin-10; ***IL-12p70***, interleukin-12p70; ***IL-1β***, interleukin-1β; ***IL-2rα***, interleukin-2ra; ***IL-6***, interleukin-6; ***TNFα***, tumor necrosis factor alpha.
* Wilcoxon sign test.

including cervical cancer, PD-L1 expression alone does not appear to be a strong predictive biomarker for response in ovarian and endometrial cancers (Gaillard and Coleman, 2019; Smith, 2022). Little information exists regarding the association of tumor infiltrating lymphocytes with tumor response to immune checkpoint inhibitors in gynecologic cancers. In ovarian cancers, tumor infiltrating lymphocytes are associated with enhanced progression-free and overall survival suggesting that host immune response may lead to better survival outcomes (Zhang, 2003). In endometrial cancer, tumor infiltrating lymphocytes are associated with mismatch repair deficient/MSI-H status but not necessarily survival and have not been independently associated with improved response to immunotherapy (Bounous, 2022; Asaka et al., 2019; Konstantinopoulos, 2022).

Given the dynamic nature of PD-L1 expression and tumor immune infiltration, sequential evaluation of adaptive immune responses during early treatment has been proposed as an improved method of determining likelihood of benefit (Chen, 2016; Vilain, 2017). In melanoma, increased tumoral PD-L1 expression and immune cell infiltration in on-treatment biopsies (median time to biopsy, 11 days) was more predictive of response to immune checkpoint inhibition than pre-treatment PD-L1 expression (Vilain, 2017). Our study establishes that dynamic changes in PD-L1 expression and immune presence at the stromal interface are identifiable in early on-treatment biopsies (median time, 8 days) and typically increase as was seen in melanoma (Vilain, 2017; Dummer, 2020; Clouthier, 2019; Cottrell, 2018). However given the small size in our study the clinical relevance in ovarian/endometrial cancers is currently unknown.

Our exploratory analyses of circulating immune mediators identified potential biomarkers of interest. The combined secretion pattern of proinflammatory (IL-6, TNFα) and anti-inflammatory mediators (IL-4, IL-8, and IL-10) influences immunologic response. Several of the biomarkers appear to be co-regulated at baseline or during treatment (e.g. IL-6 with CXCL10, IFNγ, IL-10, and IL-2ra and IL-10 with IL-2ra and IL-6). Pembrolizumab significantly increased CXCL10, IL-10, IL-2ra, and TNFα. CXCL10 guides macrophage anti-tumor immune responses and as part of IFNγ immune-response signatures correlate with response and survival in patients treated with immune checkpoint inhibitors, suggesting that patients with a pre-existing interferon-mediated adaptive immune response may respond better to these agents (House, 2020; Ayers, 2017; de Klerk, 2021). CXCL10 and IL-2ra (CD25) are known to increase in response to anti-PD-L1 therapy and have been associated with response to immunotherapy in melanoma (Reschke, 2021; Kasanen, 2020). While other cytokines, such as TNFα and IL10, are known to increase after anti-PD-L1 therapy but have shown contradictory associations with response (Hill et al., 2020; Carlini, 2023). Interestingly, the highest change in IL-6 concentration and largest decrease in IL-1β, IL-2ra, TNFα, and CXCL10 was noted in the patient who developed progressive disease during adjuvant chemotherapy treatment. IL-6 is a poor prognostic marker associated with worse survival in multiple cancers. Interestingly, IL-6 may predict bevacizumab efficacy in ovarian cancer, where women with higher IL-6 were observed to have the most benefit from bevacizumab (Alvarez Secord, 2020). In contrast, IL-1β showed the greatest decrease in the patient who progressed rapidly on adjuvant chemotherapy. IL-1 β has been demonstrated to promote monocyte differentiation into M1 macrophages that express CD68, and the loss of IL-1 β in this patient could be attributed to the PD-L1 modified proportion score of 0 during this study (Bent et al., 2018; Schenk, 2014). While limited due to small numbers, these exploratory findings are hypothesis-generating and identify potential biomarkers for integrative analyses in future studies to determine association with immune checkpoint inhibitor efficacy.

Using digital spatial profiling, we compared the effect of pembrolizumab on proteins involved in immune and tumor signaling. Few proteins were differentially expressed and notably, GAPDH, a putative housekeeping gene, was observed to be differentially increased in both the immune and tumor regions of interest after treatment. The significance of this is unclear, but the observed differential effects may be due to changes in cell type composition rather than treatment-induced changes in abundance of specific antigens. IDO1 expression was also increased in both immune and tumor compartments post treatment. High IDO expression associates with immune tolerance and cancer progression in both ovarian and endometrial cancer (Ino, 2008; Inaba, 2009). However, IDO expression is also associated with response to immunotherapy suggesting that the effect of IDO expression on immune activity may be context dependent (Zhai, 2017; Hamid, 2011).

Taken together, the increase in PD-L1 modified proportion score, increase in circulating cytokines especially CXCL10, and increase in IDO expression in tumor and immune cells suggests inflammatory activation and increased interferon signaling after exposure to pembrolizumab. Functional analyses are necessary to determine whether increases in infiltrating immune cells leads to improved anti-tumor reactivity. The window of opportunity approach allowed for the isolation and broad analysis of effects of pembrolizumab on the tumor microenvironment and circulating cytokines. While most studies have assessed only archival tissue, our study showed the feasibility of on–treatment dynamic assessment of multiple immune parameters. Major limitations of our study include the small sample size, heterogeneity of cancer type, and lack of functional assays. These limit the interpretation of clinical applicability. Moreover, definitions for PD-L1 positivity vary. We used the modified proportion score since it was the favored scoring system at the time of study design. Other scoring systems (tumor proportion score and combined proportion score) were not assessed. Other factors not evaluated include disease site differences in PD-L1 expression (primary vs metastatic), optimal threshold of PD-L1 positivity (1 % vs 10 %) and the relevance of small changes in PD-L1 expression (du Rusquec et al., 2019). While a small dataset, the data presented here suggest that priming with pembrolizumab allowed for greater immune recruitment in most cases. Nevertheless, we have identified potential candidate tumor- and blood-based immune biomarkers for further evaluation.

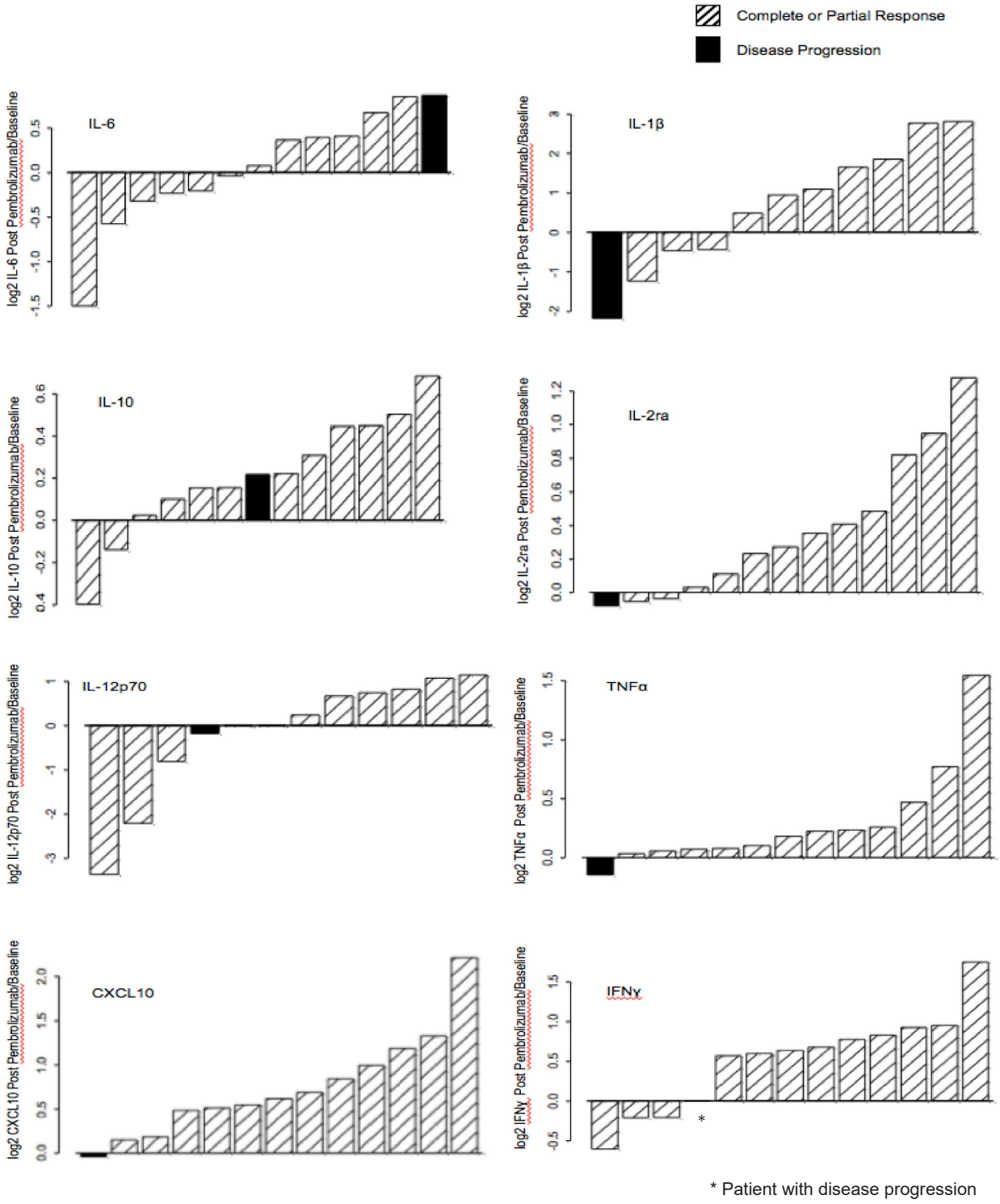

Fig. 1. Waterfall plots showing the post-pembrolizumab: baseline ratio for blood-based biomarkers. The black bar represents the patient with rapidly progressive disease on standard of care adjuvant chemotherapy after pembrolizumab. The hashed bars represent patients who experienced complete or partial response to standard therapy post-pembrolizumab.

5. Conclusions

In conclusion, a single dose of pembrolizumab led to dynamic changes in the tumor microenvironment reflected in increased PD-L1 modified proportion score and/or stromal interface immune cells suggesting potential for local tumor immunologic recruitment and increases in systemic inflammation. Further evaluation in longitudinal sampling studies, post-hoc ancillary studies and ultimately biomarker-specified clinical trials are warranted.

CRediT authorship contribution statement

Stéphanie L. Gaillard: Writing – review & editing, Writing – original draft, Supervision, Data curation, Conceptualization. **Gloria**

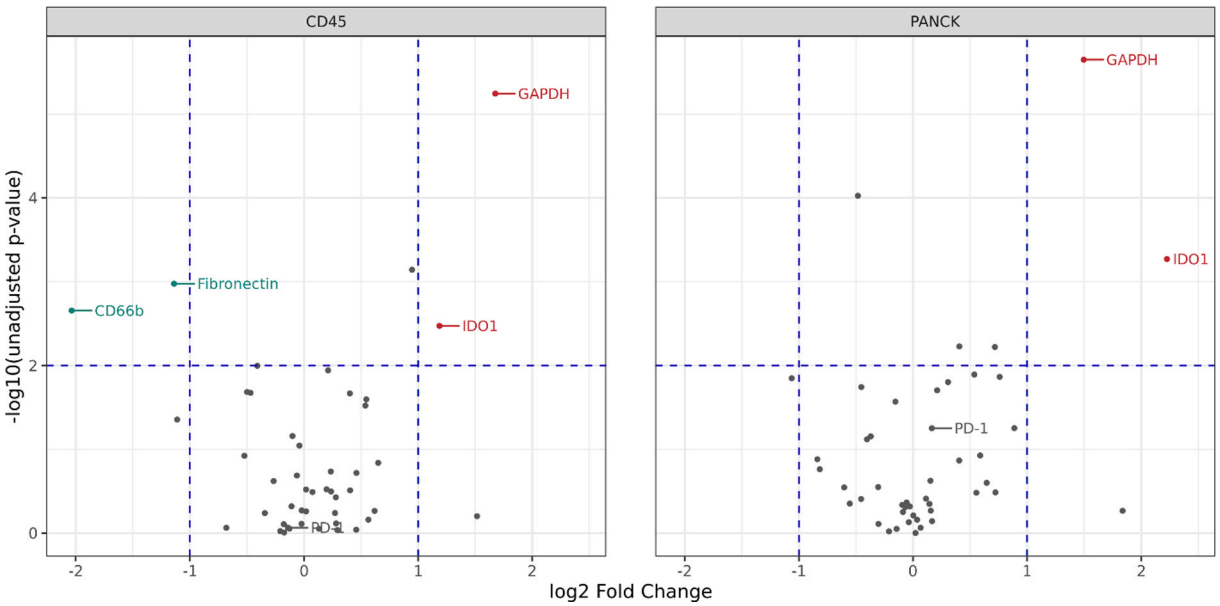

Fig. 2. Volcano plots of differentially expressed proteins in immune (CD45) and tumor (PANCK) tissues. The horizontal dashed lines are plotted at the 0.01 unadjusted p-value threshold, and the vertical dashed lines are plotted at ± 1 log2 fold change. Each point represents a protein. Red points correspond to markedly up-regulated (log2 fold change > 1) proteins post-treatment regions of interest relative to pre-treatment regions of interest. Teal points correspond to markedly down-regulated (log2 fold change < -1) proteins in post-treatment regions of interest relative to pre-treatment regions of interest. PD-1 is labeled in both plots for reference, regardless of p-value or up-/down-regulation.

Broadwater: Writing – review & editing, Writing – original draft, Formal analysis. **Andrew Berchuck:** Writing – review & editing, Data curation. **William S. Andrews:** Writing – review & editing, Data curation. **Laura J. Havrilesky:** Writing – review & editing, Writing – original draft, Data curation. **Brittany A. Davidson:** Writing – review & editing, Writing – original draft, Data curation. **Rebecca A. Previs:** Writing – review & editing, Data curation. **Mark D. Starr:** Writing – review & editing, Data curation. **John S. Yi:** Writing – review & editing, Methodology, Conceptualization. **Andrew B. Nixon:** Writing – review & editing, Writing – original draft, Methodology, Data curation, Conceptualization. **Jadee L. Neff:** Writing – review & editing, Formal analysis, Data curation. **Jose Ramon Conejo-Garcia:** Writing – review & editing, Writing – original draft, Data curation. **Angeles Alvarez Secord:** Writing – review & editing, Writing – original draft, Supervision, Data curation, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: [This investigator-initiated study was supported by Merck and philanthropic anonymous funding for ovarian cancer research. Dr. Gaillard reports grants and personal fees from Merck, during the conduct of the study; grants and personal fees from AstraZeneca, grants from Beigene, grants from Blueprint, grants and personal fees from Compugen, grants from Genentech/Roche, grants from Clovis/Pharma&, grants and personal fees from Immunogen, personal fees from Lumanity, grants from MDAnderson, grants from Tempest, grants from Tesaro/GSK, grants and personal fees from Verastem, grants from Volastra, outside the submitted work; In addition, Dr. Gaillard has a patent US 16/341,033 licensed, and a patent PCT/US2019/026669 licensed. Ms. Broadwater reports grants from NIH during the conduct of the study. Dr. Berchuck has nothing to disclose. Mr. Andrews has nothing to disclose. Dr. Havrilesky reports grants from Astra Zeneca, grants from Tesaro, outside the submitted work. Dr. Davidson reports other from Tesaro, outside the submitted work. Dr. Previs reports grants and other from Myriad, outside the submitted work. Mr. Starr has nothing to disclose. Dr. Yi has nothing to disclose. Dr. Nixon reports personal fees from Eli Lilly, personal fees from Kanghong Pharma, personal fees from Promega Corporation, grants from Genentech, grants from MedImmune/AstraZeneca, grants from Medpacto, grants from Seattle Genetics, grants from HGT Molecular Diagnostics, grants from Tracon Pharmaceuticals, grants from Eureka Therapeutics, grants from Leadiant Biosciences, grants from Acceleron Pharma, grants from OncoMed Pharmaceuticals, outside the submitted work. Dr. Neff reports personal fees from Recordati Rare Diseases, Inc, outside the submitted work. Dr. Conejo-Garcia reports stock options in Compass Therapeutics, Anixa Biosciences and Alloy Therapeutics; receives consulting fees from Alloy Therapeutics; has intellectual property with Compass Therapeutics and Anixa Biosciences; receives licensing fees from Anixa Biosciences; and is co-founder of Cellepus Therapeutics. Dr. Alvarez Secord reports grants and personal fees from Merck, during the conduct of the study; grants from Abbvie, grants from Amgen, grants from Astellas Pharma Inc., grants from Astex Pharmaceuticals Inc, grants and personal fees from AstraZeneca, from Boehringer Ingelheim, grants from Bristol Myers Squibb, grants and personal fees from Clovis, grants and personal fees from Eisai, grants from Endocyte, grants from Exelixis, grants from Immutep Ltd, grants from Incyte, grants from PharmaMar, grants, personal fees and other from Roche/Genentech, grants from Seattle Genetics, Inc., grants and personal fees from Tesaro/GSK, grants and other from VBL Therapeutics, grants from National Cancer Trial Network, personal fees from Aravive, personal fees from Cordgenics, personal fees from Janssen/Johnson & Johnson, personal fees from Mersana, from Oncoquest, outside the submitted work; and GOG Foundation Board member].

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gore.2025.101770.

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