Neoadjuvant Chemotherapy Modulates the Immune Microenvironment in Metastases of Tubo-Ovarian High-Grade Serous Carcinoma

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Abstract

**Purpose:** The purpose of this study was to assess the effect of neoadjuvant chemotherapy (NACT) on immune activation in stage IIIC/IV tubo-ovarian high-grade serous carcinoma (HGSC), and its relationship to treatment response.

**Experimental Design:** We obtained pre- and posttreatment omental biopsies and blood samples from a total of 54 patients undergoing platinum-based NACT and 6 patients undergoing primary debulking surgery. We measured T-cell density and phenotype, immune activation, and markers of cancer-related inflammation using IHC, flow cytometry, electrochemiluminescence assays, and RNA sequencing and related our findings to the histopathologic treatment response.

**Results:** There was evidence of T-cell activation in omental biopsies after NACT. CD4+ T cells showed enhanced IFNγ production and antitumor Th1 gene signatures were increased. T-cell activation was more pronounced with good response to NACT. The CD8+ T-cell and CD45RO+ memory cell density in the tumor microenvironment was unchanged after NACT but biopsies showing a good therapeutic response had significantly fewer FoxP3+ T regulatory (Treg) cells. This finding was supported by a reduction in a Treg cell gene signature in post-versus pre-NACT samples that was more pronounced in good responders. Plasma levels of proinflammatory cytokines decreased in all patients after NACT. However, a high proportion of T cells in biopsies expressed immune checkpoint molecules PD-1 and CTLA4, and PD-L1 levels were significantly increased after NACT.

**Conclusions:** NACT may enhance host immune response but this effect is tempered by high/increased levels of PD-1, CTLA4, and PD-L1. Sequential chemoimmunotherapy may improve disease control in advanced HGSC. Clin Cancer Res; 22(12); 3025–36. ©2016 AACR.

Introduction

The aim of this study was to determine the influence of neoadjuvant chemotherapy (NACT) on the immune microenvironment in peritoneal metastases of high-grade serous ovarian cancer (HGSC). Early peritoneal and pleural spread is a feature of HGSC with a majority of patients presenting with FIGO stage IIIC and stage IV disease (1).

Platinum-based chemotherapy is the only backbone medical first-line treatment approved for HGSC in the past 30 years and although most patients respond initially, resistance eventually develops in a majority of them. Peritoneal metastases are frequently the site of relapse and eventually lead to bowel obstruction that contributes to death in many patients (2, 3).

In patients with stage IIIC or IV disease who are not suitable for primary debulking surgery (PDS), three cycles of platinum-based NACT followed by interval debulking surgery (IDS) and adjuvant chemotherapy is an accepted alternative approach. This is equally effective while potentially associated with lower morbidity (4, 5) although there is a need for international consensus criteria for patient selection in this approach.

There is good evidence that HGSC has the potential to be an immunogenic tumor, and activated T cells have been characterized in the tumor microenvironment and ascitic fluid (6, 7). There is an association between increased tumor-infiltrating leukocyte (TIL) density and longer survival (8, 9) and TIL subpopulations recognize shared tumor antigens, gene products from somatic mutations, as well as amplified or aberrant genes (10, 11). Mutation frequency also correlated with an immune cell cytolytic activity transcriptional signature in ovarian cancer databases (12). However, TILs in HGSC biopsies are often suppressed or functionally exhausted, and immunotherapies, until recently, have
not yet had a major impact on patients with advanced chemoresistant disease (10, 11, 13).

Recent studies in animal cancer models have shown how the immune system plays an important role in the response to some cancer chemotherapies, inducing an ‘immunogenic’ cell death, presentation of neoantigens, and an increase in acute inflammatory and tumor-destructive responses (14–16). Furthermore, recent mouse cancer experiments showed the efficacy of blocking the PD-1/PD-L1 axis following paclitaxel chemotherapy (17). The ability to sample HGSC biopsies at diagnosis and after NACT during surgery gives us an opportunity to ask whether similar responses occur in a clinical setting and if they do, whether this provides a rationale for introducing immunotherapy after NACT rather than in relapsed disease. We therefore studied a cohort of 54 women with stage IIIC and stage IV HGSC receiving NACT, as well as 6 women who underwent surgery before chemotherapy. Using transcriptional and protein analyses of prospectively collected metastatic peritoneal (omental) specimens, as well as plasma samples, we investigated the effects of platinum-based NACT on the immune microenvironment in patients and compared this to their response to chemotherapy as assessed by a recently published prognostic histologic score (18).

Materials and Methods

Patients and samples

Institutional review board approval was granted for the Barts Gynae Tissue Bank to collect and store biologic material and clinical information. Patients were treated at St. Bartholomew’s Cancer Centre (London, UK) between 2010 and 2015 and gave written informed consent. Clinical parameters were collected using tissue repository databases and chart review.

Omental metastases and plasma samples from 54 FIGO stage IIIC and IV HGSC patients were collected prospectively before and after platinum-based NACT (details of patients and treatment are shown in Supplementary Table S1) and analyzed in immunohistochemical, flow cytometric, and transcriptomic experiments. Surgery was usually performed between 3 and 4 weeks after the last NACT. Samples from an additional 6 patients with FIGO stage IIIC and IV HGSC who underwent PDS were used in the flow cytometry and RNAseq studies described below (Supplementary Table S2). These samples were matched to the NACT cohort in terms of amount of tumor and stroma in the biopsy, pretreatment levels of plasma cytokines, age, and stage. Immunostaining cytokine levels were measured in paired pre- and post-NACT plasma samples of 23 patients and 22 plasma samples were from healthy female volunteers (median age 47.5 years; range 32–64 years).

Supplementary Table S3 lists the numbers of samples that were used in each of the analyses.

The response to chemotherapy was assessed in IDS biopsies using a chemotherapy response score (CRS) that separates the patients into three major subgroups (18). CRS1 samples show minimal response to chemotherapy, CRS2, ‘poor’ responders, have easily identifiable malignant cells in the omentum after NACT and CRS3, ‘good’ responders, usually show extensive regression-associated fibroinflammatory changes with absent or minimal numbers of malignant cells (18).

Formalin-fixed paraffin embedded tumor biopsies

Pretreatment omental or peritoneal biopsies were obtained at diagnostic laparoscopy or diagnostic core biopsy. Sections of 4-μm thickness were mounted on glass slides. Blocks of omentum removed at IDS after NACT, were reviewed by a pathologist according to the ICCR guidelines (19). Blocks representing the area of worst response were selected and used to construct tissue microarrays (TMA) with a 1-mm core size and up to 6 cores per patient sample.

Fresh tumor biopsies

Omental biopsies were collected in the operating theatre from untreated patients undergoing PDS or diagnostic laparoscopy and from patients undergoing IDS after NACT and fresh-frozen samples were histologically matched to FFPE specimens of the same patient by hematoxylin and eosin review.

Blood samples

Sodium heparin blood (BD Vacutainer Systems) was immediately placed on ice within one week before NACT and within 2 weeks before IDS. The minimum time after the last cycle of chemotherapy was 3 weeks. After centrifugation, plasma from patients and controls was snap frozen and stored at −80°C.

Extraction of stroma vascular fraction

Fresh omental biopsies were subjected to mechanical dissection and enzymatic treatment. Briefly, the tissue was dissected with a scalpel, incubated in RPMI medium (Gibco) containing 5% FBS, 1 mg/ml Collagenase D from clostridium histolyticum (Roche) and 25 μg/ml DNase (Roche) at 37°C under agitation for 40 minutes. The extract was then filtered through a 70-μm pore strainer; red blood cells were lysed (eBioscience) and the stroma vascular fraction (SVF) was frozen for later FACS analyses or freshly restimulated in vitro for intracellular cytokine staining.

Flow cytometry analyses

SVFs and peripheral blood mononuclear cells (PBMC) were stained for flow cytometry analyses in PBS containing 2.5% BSA and 2 mmol/L EDTA for 30 minutes at 4°C. The following markers were used: CD45 APC efluor780 (eBioscience), CD3 BV650 (Biolegend), CD3 PE (eBioscience), CD4 PerCP-Cy5.5 (eBioscience), CD8 APC (eBioscience), CD8 FITC (eBioscience), PD-1 BV421 (Biolegend), CTLA4 PE (Biolegend), CD69 APC (eBioscience), and CD25 PECy7 (eBioscience). Viability of the cells was assessed by staining with the fixable viability dye (FVD)
eFluor506 (eBioscience) or DAPI (Sigma). Appropriate Fluorescence Minus One (FMO) controls were used in these experiments.

Staining for Foxp3+ T regulatory (Treg) cells was performed using the Human Treg Kit (eBioscience) containing CD45 eFluor 780, CD4 FITC/CD25 APC, FOXP3 PE, and the isotype control for Foxp3. Viability of the cells was assessed with FVD eFluor450 (eBioscience).

To study the production of IL10 and IFNγ by omental T cells from pre- and posttreatment patients, 2.5 × 10⁶ cells from the extracted SVF were restimulated in vitro in RPMI 10% FBS, 50 ng/mL phorbol 12-myristate 13-acetate (PMA; Sigma) and 1 μg/mL ionomycin (Sigma) for 5 hours at 37°C, 5% CO₂. After the first hour of restimulation 10 μg/mL Brefeldin A (Sigma) was added to the culture. After restimulation, cells were stained for CD4 PerCP-Cy5.5 (eBioscience), CD8 FITC (eBioscience), and FVD 450 (eBioscience) in PBS 2.5% BSA + 2 mMol/L EDTA for 20 minutes at 4°C. Cells were then washed, fixed for 20 minutes at room temperature with the Fixation Buffer (eBioscience), permeabilized with the Permeabilization Buffer (eBioscience), and stained with anti-human IL10 PE (eBioscience), peroxidase with the Permeabilization Buffer (eBioscience), and stained with anti-human IL10 PE (eBioscience) and IFNγ PECy7 (eBioscience) for 20 minutes at room temperature. Corresponding isotype controls for IL10 and IFNγ were used to generate FMO controls. Stained samples were analyzed using an LSRFortessa cell analyzer (BD Biosciences) and data were analyzed with FlowJo 9.4.6 (Treestar Inc.).

IHC

FFPE-embedded tissue sections were dewaxed and rehydrated. Antigen retrieval was performed using a pressure cooker and a citrate-based antigen unmasking solution (Vector Laboratories) followed by staining in an autostainer (Dako) using an ultrasensitive HRP Polymer Kit (Biogenex). The following antibodies were used: CD8 clone C8/144B (Dako), FOXP3 clone 236A/E7 (Abcam), CD45RO clone UCLH (Dako), and PD-L1 clone SP142 (Spring Biosciences). Stained samples were analyzed using an LSRFortessa cell analyzer (BD Biosciences) and data were analyzed with FlowJo 9.4.6 (Treestar Inc.).

Immune cell quantification on digitalized slides

Immunohistochemically stained slides were scanned at 20× with a Pannoramic Flash Scanner (3D Histech). Images of 5 areas representing the entire biopsy of paired pre- and post–NACT samples were taken with an area of 0.58 mm² per high-power field (Pannoramic software, 3D Histech). Intraepithelial cells (within tumor islets) and intrastromal cells were counted independently by three investigators, including one histopathologist, blinded for clinical information. Each HPF was scored as 0, 1+, 2+, 3+, or 4+ for tumor (within or direct contact with the epithelial component) and stromal regions. The cut-off was based on the typical density of these cell populations in HGSC omentum and prior joint discussion of the investigators (CD8 and CD45RO: no cells, 1–10, 11–60, 61–150, >150 per HPF, respectively. FOXP3: no cells, 1–10, 11–40, 41–100, >100 per HPF, respectively). The average score of all HPFs of one slide of one investigator was added to the corresponding scores of the other two and then divided by three.

Scoring of PD-L1 expression

PD-L1 was scored by a study pathologist using the criteria described in ref. (20). Briefly, samples were scored for PD-L1 expression on tumor-infiltrating immune cells, which included macrophages, dendritic cells, and lymphocytes. Specimens were given a score of 0–3 if <1%, 1%–<5%, 5%–<10%, and ≥10% of cells were positive for PD-L1.

Plasma cytokine analysis

Cytokines were measured by electrochemiluminescence multiplex assay (V-Plex, Meso Scale Discovery) on a Sector Imager (Meso Scale Discovery) according to the manufacturer’s protocol. The detection range for the assays was as follows: TNFα: 0.076–311 pg/mL; IL8: 0.133–546 pg/mL; IL6: 0.369–749 pg/mL; IFNγ: 0.369–1,510 pg/mL; IL10: 0.076–313 pg/mL; IL17: 1.38–5,670 pg/mL.

RNA isolation

Total RNA was isolated from frozen whole tissue using the RNeasy Kit (Qiagen) following the manufacturer’s instructions. Briefly, whole tissue was homogenized in RLT lysis buffer, passed through a Qiashredder (Qiagen), and then purified on the mini spin columns, including the on-column DNAse treatment to remove any remaining DNA. The purified total RNA was then analyzed by Bioanalyzer 2100 expert (Agilent) as per manufacturer’s instructions. RIN numbers between 9.9 and 8.0 were obtained.

Transcriptomic analysis

RNA Sequencing was performed by Oxford Gene Technology using the Illumina HiSeq2500 platform and generated approximately 42 million 101 base-pair paired end reads per sample. Sequenced reads were mapped to Human RefSeq genes archived in the Illumina iGenomes resource (http://support.illumina.com/sequencing/sequencing_software/igenome.html) using RSEM (version 1.2.4; ref. 21) in dUTP strand–specific mode. As part of the RSEM pipeline, bowtie (version 0.12.7) (22) was used to perform the mapping stage. Subsequent mapped read counting was performed using RSEM. Differentially expressed genes were identified with the EdgeR package (23) using Biocductor (version 2.7; www.biocductor.org), running on R (version 2.12.1; R-REF). Genes with logCPM > 0 and FDR < 0.05 were judged to be differentially expressed. Immunologic signatures that represent cell states and perturbations within the immune system were extracted from Msigdb (24) and used to perform GSEA with default settings comparing pairwise comparisons. GSEA analysis on pre and post NACT samples, were interrogated using the MsigDB C7 immunologic collection available from the Broad Institute (Cambridge, MA). This consisted of 1,910 signatures, which comprise approximately 200 genes per signature. Signatures were considered significant if the corrected FDR q value was <0.05. Heatmaps shown in Figs. 3 and 4 are truncated to show only the genes identified as contributing to the enrichment score.

Availability of data and materials

FASTQ data files supporting the RNASeq analysis have been uploaded to NCBI GEO database GSE71340.

Statistical analysis

For continuous variables that were approximately normal or normally distributed, mean and SEM are shown and t test was conducted. For ordinal variables, Mann–Whitney test or Wilcoxon matched-pairs signed rank test were applied and Kruskal–Wallis test was used in addition to perform multiple group comparisons. Progression-free survival (PFS) was calculated from the date of first NACT to progression or death (whichever came first) using GCG CA-125 criteria for biochemical progression.
Overall survival (OS) was calculated from the date of first NACT to death from HGSC. Survival functions were estimated using the Kaplan–Meier method and the log-rank test was applied.

Results
Response to chemotherapy in the study cohort
Samples from a total of 54 patients receiving NACT and 6 patients who had surgery prior to chemotherapy were used in the experiments in this article. Supplementary Table S3 summarizes the sample numbers used in each set of experiments. As described above, the response to chemotherapy was assessed in the IDS biopsies using the CRS (18). CRS1 samples show minimal response to chemotherapy, CRS2, "poor" responders, have easily identifiable malignant cells in the omentum after NACT and CRS3, "good" responders, usually show extensive regression-associated fibroinflammatory changes with absent or minimal numbers of malignant cells (18). None of the IDS samples in our experiments, by chance and due to prevalence, scored as CRS1. "Good responder" patients with samples scored as CRS3 had significantly improved progression-free survival (P = 0.002) and OS (P = 0.03) compared with "poor responder" patients whose biopsies scored as CRS2 (Supplementary Fig. S1).

T-cell density pre- and post-NACT
From 25 of the HGSC patients described above, we obtained matched omental biopsies taken at pretreatment and at IDS. Using IHC, the biopsies were stained for CD8⁺ T cells, CD45RO⁺ memory cells, and Foxp3⁺ Treg cells. Figure 1 and Supplementary Fig. S2 show the cell density in malignant cell areas and adjacent stromal areas. Pretreatment, there was no difference in the density of cells positive for the above markers between CRS2 "poor" and CRS3 "good" responders. After

![Figure 1](https://example.com/figure1.png)

Figure 1. T-cell density and location in matched pre- and posttreatment omental biopsies. Amount and localization of CD8⁺ T cells, CD45RO⁺ memory cells, and Foxp3⁺ cells in omental metastasis in 50 matched pre- and postchemotherapy peritoneal biopsies from 25 patients. Analysis of cell amount pre- to post-NACT based on a 5-tier score in tumor and stroma. Intratumoral cells post-NACT in CRS3 good responders are "not applicable" as no large tumor islets are left in the omentum after NACT in these patients. Note that all CRS2 biopsies post-NACT were positive for intratumoral and intrastral CD8⁺ and CD45RO⁺ cells, in many samples increased compared with prechemotherapy. Foxp3⁺ Treg cells do not increase in tumor and stroma of CRS2 biopsies and decrease significantly in stroma of CRS3 biopsies (*, P < 0.05).
NACT, there were still marked infiltrates of CD8\(^+\) T cells and CD45RO\(^+\) memory cells in the stroma and again no difference between CRS2 or CRS3 biopsies. CD8\(^+\) T cells and CD45RO\(^+\) memory cells in the malignant cell areas of CRS2 biopsies remained at high levels; in fact, CD8\(^+\) cell density increased in approximately 50\% of patients and the tumor:stroma ratio of CD8\(^+\) and CD45RO\(^+\) cells in individual patients was essentially unchanged pre- and postchemotherapy in the CRS2 biopsies (Supplementary Fig. S3).

In contrast, there was a significant decline in the density of Foxp3\(^+\) cells in the stromal areas of the CRS3 “good” responder biopsies after NACT \((P = 0.02)\) but there was no significant change in the CRS2 biopsies (Fig. 1). As Foxp3 is a marker of immunosuppressive Treg cells, we next asked whether NACT increased T-cell activation.

**Evidence for T-cell activation after NACT**

We assessed the phenotype of the T cells in the HGSC omental metastases by flow cytometry analysis of TILs from 22 of the HGSC biopsies. As it was not possible to obtain sufficient material for flow cytometry from diagnostic biopsies for pretreatment samples in these experiments, to assess pretreatment T-cell phenotypes, we used six samples obtained at primary debulking and two laparoscopic biopsies obtained before NACT. In agreement with the results in Fig. 1, the percentage of CD3\(^+\) cells in the CD45\(^-\) leukocyte population did not change pre- or post-NACT, irrespective of response to chemotherapy (Fig. 2A). Likewise, there were no significant changes in CD69\(^+\), CD4\(^+\) T cells, or CD4/CD8 ratio (Fig. 2B–D). A subpopulation of the CD4\(^+\) T cells was CD25\(^+\)Foxp3\(^+\) suggesting they were T regulatory cells. When comparing CRS2 and CRS3 patients, we observed a decrease in the percentage of CD4\(^+\) T cells that were CD25\(^+\)Foxp3\(^+\) in the CRS3 biopsies \((P = 0.015,\) Mann–Whitney test; Fig. 2E), again supporting the results in Fig. 1. The ratio of CD4\(^+\)CD25\(^-\)Foxp3\(^+\) cells to CD4\(^+\)CD25\(^+\)Foxp3\(^-\) cells also decreased in post-NACT CRS3 and CRS2 biopsies compared with pretreatment, and the difference between CRS2 and CRS3 was significant \((P = 0.03,\) Mann–Whitney test; Fig. 2F). When applying a correction for multiple group comparison, the differences were not deemed significant \((P = 0.06)\) (Fig. 2E) and \(P = 0.09\) (Fig. 2F). Kruskal–Wallis test

We next studied the functional ability of the T cells in the biopsies to produce IFN\(\gamma\) as a marker of T-cell activation and antitumor response, and IL10 as a marker of immunosuppression. Cells extracted from eleven HGSC omental samples, as well as PBMCs from healthy control women \((n = 3–5)\), were stimulated in vitro and then stained for intracellular IFN\(\gamma\) and IL10 (Fig. 2G and H). A significantly higher proportion of omental CD4\(^+\) and CD8\(^+\) T cells were able to produce IFN\(\gamma\) compared with peripheral T cells from healthy controls. When compared with pretreatment biopsies, post-NACT CRS3 patients had a significantly higher proportion of IFN\(\gamma\)–CD4\(^+\) T cells \((P = 0.002,\) Fig. 2G). No significant difference in the proportion of IL10–producing CD4\(^+\) and CD8\(^+\) T cells was observed between pre- and post-NACT samples (Fig. 2G and H).

**Gene set enrichment analysis of immune gene signatures pre- and post-NACT**

We extracted total RNA from 20 of the HGSC omental metastasis biopsies for which we had sufficient material and analyzed gene expression by RNAseq. Nine samples were from CRS3 good responders post-NACT and 7 from CRS2 poor responders. For pretreatment samples, we used two biopsies from stage IIC or IV HGSC patients undergoing PDS and two biopsies obtained from patients before NACT as described in Fig. 2. Gene set enrichment analysis (GSEA) was conducted. Changes to immune cell phenotypes from both the adaptive and innate pathways were seen when comparing CRS3 to CRS2 samples, and individually (CRS2 or CRS3) to pre-NACT samples. In particular, changes associated with T-cell phenotype were found to have the highest significance (lowest FDR value). In agreement with the results in Figs. 1 and 2, we found that the FOXP3-associated Treg cell gene signatures were differentially regulated in pre- versus post-NACT patients. FOXP3-regulated genes whose expression is decreased following differentiation to a Treg phenotype \((25)\) were enriched in pretreatment versus both CRS2 and CRS3 post-NACT biopsies (Fig. 3A and B). We also observed an enrichment of this gene signature in CRS2 poor responder biopsies compared with CRS3 biopsies (Fig. 3C).

Further GSEA analysis revealed an increase in expression of T helper 1 (Th1)-associated genes after NACT in both poor (CRS2) and good (CRS3) responders (Fig. 4A and B). The gene set used contained genes that were experimentally upregulated when naïve T cells were activated to a Th1 phenotype in vitro \((26)\). We also noted that the Th1 phenotype was enriched in CRS3 patients compared with CRS2 (Fig. 4C). Meta-analysis of TCGA data has identified GZMA (encoding granzyme A) and PRF1 (encoding perforin 1) as specifically coexpressed by cytotoxic T cells \((12)\). We compared gene expression levels for both markers within our dataset (Fig. 4D–F). There was a tendency for both genes to be upregulated in the post-NACT versus pretreatment samples and the difference with PRF1 was significant \((P = 0.03)\) (Fig. 4D and E). The geometric mean of both genes (Fig. 4F) again showed a tendency towards upregulation post-NACT. These experiments suggest that NACT reduced Treg cell activity in HGSC metastases while increasing T-cell activation and Th1 responses and these effects were most profound in the CRS3 good responder patients. We next asked whether the potential for efficient host antitumor immune responses was influenced by immune checkpoint molecules.

**The effect of NACT on immune checkpoint molecules**

More than 60\% of CD4\(^+\) and CD8\(^+\) T cells in omental metastases from 6 pre- and 16 post-NACT patients expressed the immune checkpoint molecule programmed cell death-1 (PD-1) as assessed by flow cytometry (Fig. 5A and B). Between 15\% and 30\% of the CD4\(^+\) cells and 5\% to 10\% of the CD8\(^+\) cells expressed CTL-associated protein 4, CTLA4, in 22 biopsies (Fig. 5C and D) and most of the CTLA4\(^+\) T cells coexpressed PD-1 (Fig. 5E and F).

Immunohistochemical staining showed that the PD-1 ligand PD-L1 was frequently associated with the immune cell compartment of the HGSC tumor microenvironment. Using an antibody developed for anti-PD-L1 clinical trials (clone SP142) and a scoring method on immune cells \((20)\) we found that PD-L1 protein was significantly increased in post-NACT compared with pre-NACT biopsies irrespective of response to treatment in paired pre- and post-NACT biopsies of 26 patients \((P = 0.03,\) Fig. S5 and S4). Hence, potential beneficial effects of chemotherapy in stimulating T-cell activation


might be impaired by the high expression of immune checkpoint molecules.

**Plasma levels of cytokines**

Cancer-related inflammatory pathways can also contribute to the immunosuppressive tumor microenvironment (27) and ovarian cancers have complex tumor-promoting inflammatory cytokine networks (28) that can be reflected by increases in systemic cytokine levels (29). We measured key cytokines implicated in tumor-promoting inflammation, as well as T-cell activation and immune suppression, in 46 matched pre- and post-NACT plasma samples from 23 patients. Levels of three
major inflammatory cytokines with potential tumor-promoting activity, TNF, IL8, and IL6, significantly decreased after NACT ($P = 0.0008$, $P = 0.001$, and $P = 0.0006$, respectively, Fig. 6A–C). All plasma levels were elevated pretreatment but post-NACT levels were not statistically different from values obtained from 22 healthy females. There were no differences between patients whose samples were scored CRS2 and CRS3. In contrast, plasma IFNγ was increased in posttreatment patients compared with healthy controls ($P = 0.005$) with a trend towards an increase posttreatment versus pretreatment (Fig. 6D). Pretreatment patient plasma IL10 levels were significantly higher compared with controls before NACT ($P = 0.04$) and decreased after NACT ($P < 0.001$; Fig. 6E). IL17 levels were significantly elevated in HGSC patients compared with controls ($P = 0.006$) and remained elevated after NACT ($P = 0.02$, Fig. 6F).

**Discussion**

As well as in malignant melanoma, there have been some encouraging responses to immune checkpoint inhibitor therapy in patients with difficult-to-treat metastatic solid cancers such as non–small cell lung cancer (30), bladder cancer (20), and ovarian cancer (13). However many patients show no benefit, not all cancer types respond and most trials are conducted after multiple treatments and with drug-resistant
disease. The hypothesis of our study was that immunotherapies such as immune checkpoint blockade may result in enhanced clinical benefit if given after or during first-line chemotherapy. The NACT protocols used for many patients with stage IIIC and stage IV HGSC gave us an opportunity to investigate this in a clinical setting.

Figure 4.
Gene expression analysis of T-cell responses to NACT. A and B, GSEA Th1 signature generated from naive T cells that have been activated to a Th1 phenotype in vitro (26) is enriched after NACT in CRS2 poor responders (FDR $= 0.02$; A) and CRS3 good responders (FDR $= 0.004$; B) compared with pretreatment and the same signature compared between good and poor responders to NACT (FDR $= 0.014$; C). Red squares indicate genes of the core enrichment, shown in the corresponding heatmap. D and E, transcripts per million (TPM) of PRF1 and GZMA expression between pre- and post-NACT samples, and the geometric mean of PRF1 and GZMA expression (F) compared between pre- and post-NACT samples (* $P < 0.05$).
We believe that this is the first in-depth analysis of the effects of first-line chemotherapy on a human metastatic tumor microenvironment. We demonstrate that NACT induces activation of CD4⁺ T cells and that CD8⁺ T cells and CD45RO⁺ memory cells are present in omental metastases after NACT. By incorporating a recently developed prognostically significant histologic score (18), we show that omental metastases biopsies from patients with good response (CRS3) to NACT have more pronounced T-cell activation and reduced Treg cell infiltration compared with poor responders (CRS2). Importantly, even those patients who had a poor response to three cycles of platinum-based chemotherapy had high densities of CD8⁺ T cells and CD45RO⁺

Figure 5.
Expression of checkpoint molecules in omental metastasis before treatment and according to response to chemotherapy. A–F, expression of PD-1 and CTLA4 on CD4⁺ and CD8⁺ T cells assessed on 22 samples separated into pretreatment and post-NACT according to CRS scores. G, examples of positive staining for PD-L1 before and after NACT (IHC, antibody clone SP142). Analysis of IHC staining for PD-L1-positive immune cells (H) in 52 paired samples before and after NACT (*, P < 0.05).
memory cells and their ability to produce IFNγ was preserved. Moreover, increased levels of transcripts coding for cytotoxic markers post-NACT suggested chemotherapy might enhance the cytotoxicity of immune effector cells, such as CD8^+ T cells and NK cells towards the tumor. These local changes were accompanied by a decline, usually back to healthy control values, in systemic levels of cytokines implicated in cancer-related inflammation in all patients. The potential enhancement of host antitumor immune response and reduction in mediators of cancer-related inflammation was tempered by the fact that levels of the immune-checkpoint molecules PD-1 and CTLA4 on CD4^+ and CD8^+ T cells remained high after NACT. Levels of PD-L1 ligand on tumor-infiltrating immune cells were significantly increased. The fact that the immune checkpoint targets are present or increased after NACT, in the presence of significant densities of CD8^+ T cells and memory cells provides a rationale for cancer immunotherapy, especially immune checkpoint blockade, in the HGSC first-line setting at this early time point. The presence or absence of T cells in solid tumors is a key limiting factor for cancer immunotherapy (31) and the presence of CD8^+ T cells at the invasive margin was predictive of response to anti PD-1 treatment in malignant melanoma (32).

There is already some evidence that prior chemotherapy enhances the effect of immunotherapy. Successful responses to adoptive therapy with CAR T cells, TCR T cells, and TILs are regularly seen in cancer patients who have become
chemotherapy-resistant. In terms of ovarian cancer, there were responses to IL2 immunotherapy in patients who had become resistant to platinum therapy (33), while in a first-line study, chemotherapy plus IFNγ 1-b immunotherapy showed a decreased OS compared with chemotherapy alone (34). This highlights the need for careful monitoring of patients during combination chemotherapy.

The relative contribution of platinum versus taxane to the immunostimulatory effects seen in our study is an open question because almost all patients received the combination therapy. Experimental studies suggest that cisplatin does not induce immunogenic cell death (ICD; ref 35) but carboplatin and docetaxel produce partial features of ICD (36). Chemotherapy can also activate the host immune system by several other mechanisms (37) such as increased presentation of neoantigens. Chemotherapy before immunotherapy is of particular interest since efficacy of PD-1 blockade has recently been shown to correlate with neoantigen burden in non–small cell lung cancer (38).

In ovarian cancer, there are conflicting results relating to the prognostic impact of Treg cells (39, 40). Our data obtained at transcriptional, protein, and cellular level show a reduced Treg cell signature and density after NACT is most apparent in biopsies of tumors classified as CRS3 good responders, providing evidence for a link between chemotherapy efficiency and Treg density. In addition to reduced Tregs after NACT in good responders, we observed by flow cytometry a shift of the Foxp3+ population towards increased CD25 expression, again most pronounced in good responders. This population has been shown to have an “exhausted” phenotype that could effectively been reversed using an anti-PD-1 mAb (41). We suggest that blocking the PD-1/PD-L1 axis is a way to inhibit Treg cell function and enhance the antitumor effectiveness of the CD4+CD25+ T cells. Future work in larger cohorts may reveal insights into functionally different subsets of Treg cells with potentially different prognostic and predictive value (42, 43).

There is much evidence that cancer-related inflammation is also tumor promoting (44) although clinical trials that target inflammatory mediators or cells are not as advanced as some other immunotherapy approaches (27, 34). As the adaptive immune response is heavily dependent on innate immunity, inhibiting some of the tumor-promoting immunosuppressive actions of the innate immune system might enhance the potential of immunotherapies that activate a nascent antitumor response. It is therefore encouraging that NACT had a significant impact on systemic levels of three key inflammatory cytokines in our study. TNF and IL6 are major players in the complex cytokine networks that drive tumor progression in ovarian cancer (28, 29, 45). Elevated IL6 produced by malignant cells was a cause of paraneoplastic thrombocytosis in HGSC patients (29) and plasma IL8 correlated with tumor burden and treatment response across a range of human and experimental cancers (45). The observation that all three cytokines decrease to normal plasma levels after NACT further suggests that this is a favorable time to introduce immunotherapy, taken with the finding of an increase in the Th1 cytokine IFNγ in the patient plasma.

In summary, we have been able to study the human tumor microenvironment at the transcriptomic, proteomic and cellular level at a key site of cancer dissemination and under conditions that reflect current practice of patient care—the disease at diagnosis and after NACT. Our results suggest that NACT opens a window of opportunity for immunotherapies such as immune checkpoint blockade for patients with different levels of response to chemotherapy. We conclude that incorporation of immunotherapies into postchemotherapy treatment options could be of benefit for prolonged disease control in patients with advanced HGSC and, if confirmed, in patients with other cancer types.

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

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Acknowledgments
The authors thank the patients for donating samples to the Barts Gynaecological Cancer Centre for their support. The authors thank Dr. Dha Al Okati, Queens Hospital, Romford and Dr. Konstantinos Giaslakiotis, Whipps Cross University Hospital (London, UK) for providing biopsy samples for the study. The authors also wish to thank Dr. Ian Said, Barts Health NHS Trust and George Elia, Andrew Owen, and Dr. Linda Hammond, Barts Cancer Institute, for technical support.

Grant Support
This research was funded by Swiss Cancer League (Bill KIS-2883-02.2012), the European Research Council (ERC225266), Cancer Research UK (A16354), and Barts and The London Charity (467/1307).

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Received November 5, 2015; revised January 29, 2016; accepted February 1, 2016, published online June 15, 2016.

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