Biology of Human Tumors

Complement Pathway Is Frequently Altered in Endometriosis and Endometriosis-Associated Ovarian Cancer

Swati Suryawanshi1, Xin Huang1, Esther Elishaev2,3, Raluca A. Budiu1, Lixin Zhang1, SungHwan Kim4, Nicole Donnellan2, Gina Mantia-Smaldone5, Tianzhou Ma4, George Tseng6, Ted Lee7, Suketu Mansuria3, Robert P. Edwards1,3, and Anda M. Vlad1

Abstract

Purpose: Mechanisms of immune dysregulation associated with advanced tumors are relatively well understood. Much less is known about the role of immune effectors against cancer precursor lesions. Endometrioid and clear-cell ovarian tumors partly derive from endometriosis, a commonly diagnosed chronic inflammatory disease. We performed here a comprehensive immune gene expression analysis of pelvic inflammation in endometriosis and endometriosis-associated ovarian cancer (EAOC).

Experimental Design: RNA was extracted from 120 paraffin tissue blocks comprising of normal endometrium (n = 32), benign endometriosis (n = 30), atypical endometriosis (n = 15), and EAOC (n = 43). Serous tumors (n = 15) were included as nonendometriosis-associated controls. The immune microenvironment was profiled using Nanostring and the nCounter GX Human Immunology Kit, comprising probes for a total of 511 immune genes.

Results: One third of the patients with endometriosis revealed a tumor-like inflammation profile, suggesting that cancer-like immune signatures may develop earlier, in patients classified as clinically benign. Gene expression analyses revealed the complement pathway as most prominently involved in both endometriosis and EAOC. Complement proteins are abundantly present in epithelial cells in both benign and malignant lesions. Mechanistic studies in ovarian surface epithelial cells from mice with conditional (Cre-loxP) mutations show intrinsic production of complement in epithelia and demonstrate an early link between Kras- and Pten-driven pathways and complement upregulation. Downregulation of complement in these cells interferes with cell proliferation.

Conclusions: These findings reveal new characteristics of inflammation in precursor lesions and point to previously unknown roles of complement in endometriosis and EAOC. Clin Cancer Res; 20(23); 6163–74. ©2014 AACR.

Introduction

The immunological makeup of the host can protect against cancer development via complex mechanisms com-

1Department of Obstetrics, Gynecology and Reproductive Sciences, University of Pittsburgh School of Medicine and Magee Women’s Research Institute, Pittsburgh, Pennsylvania. 2Department of Pathology, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania. 3Magee Women’s Hospital of Pittsburgh, University of Pittsburgh Medical Center, Pittsburgh Pennsylvania. 4Department of Statistics University of Pittsburgh, Graduate School of Public Health, Pittsburgh Pennsylvania. 5Section of Gynecologic Oncology, Department of Surgical Oncology, Fox Chase Cancer Center, Philadelphia, Pennsylvania.

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S. Suryawanshi and X. Huang contributed equally to this article.

Corresponding Author: Anda M. Vlad, Department of Obstetrics, Gynecology and Reproductive Sciences, University of Pittsburgh, School of Medicine and Magee Women’s Research Institute, Room B403, 204 Craft Avenue, Pittsburgh PA 15213. Phone: 412-641-2985; Fax: 412-641-6156; E-mail: vladam@upmc.edu

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endometriosis by either measuring cellular phenotypes or previously focused on profiling immunity in patients with inflammatory T suppressor cells all of which may favor chronic patients show dysfunctional macrophages, depressed killers, and mediators like TNF. Peritoneal fluid often shows increased levels of proinflammatory cytokines (17). In women with endometriosis, the peritoneal inflammation is a major factor that contributes to disease pathogenesis (17). It is estimated that 60% to 80% of all endometriosis-associated ovarian cancers (EOAC) occur in the presence of atypical endometriosis (AE), often found in direct continuity with the tumor, suggesting AE as the transitioning entity from benign lesions to malignant variants. The intermediate, “atypical” lesions, are defined by several histologic criteria, including large nuclei with moderate to marked pleomorphism, increased nuclear-to-cytoplasmic ratio, cellular crowding, stratification, or tufting (14, 15).

EOACs are believed to be a culmination of a multifaceted complex of pathogenic factors (16). In conjunction with endocrine imbalance and oxidative stress, immune dysregulation is a major factor that contributes to disease pathogenesis (17). In women with endometriosis, the peritoneal fluid often shows increased levels of proinflammatory mediators like TNF-α, IL-1β, and IL-6 (18). In addition, the patients show dysfunctional macrophages, depressed killing capacity of NK cells, increased accumulation of regulatory T suppressor cells all of which may favor chronic inflammation and promote the initiation and progression of EOAC (18, 19). Notably, the vast majority of studies have previously focused on profiling immunity in patients with endometriosis by either measuring cellular phenotypes or soluble mediators in the peritoneal fluid or peripheral blood (20).

Translational Relevance

Endometrioid and clear-cell ovarian cancers are partly derived from endometriosis, a chronic inflammatory disease that often shows signs of local invasion, treatment resistance, and recurrence. Majority of studies have previously focused on profiling immunity in endometriosis patients by either measuring cellular phenotypes or soluble mediators in the peritoneal fluid or peripheral blood. We performed here a comprehensive tissue immune gene expression analysis in lesions of endometriosis and endometriosis-associated ovarian cancer (EOAC). Some of the patients with endometriosis revealed a cancer-like inflammation profile, suggesting that cancer-like immune signatures may develop earlier, in patients with lesions that are classified as clinically benign. Gene expression analyses revealed the complement pathway as most prominently involved in both endometriosis and EOAC. Our studies provide a novel insight into immune dysregulation in endometriosis and EOAC and reveal complement as a potential target for prevention and treatment.

Although endometriosis remains largely benign, malignant transformation may occur in up to 1% of cases, most commonly from ovarian lesions (7, 9, 10). Gene profiling studies recently revealed that clear-cell and low-grade endometrioid carcinomas share a similar gene expression pattern, consistent with a common origin. As a prevailing trait, inactivating mutations of ARID1A, a chromatin-remodeling gene, have been found in 49% of clear-cell carcinomas and 30% of endometrioid ovarian cancers (11–13). It is estimated that 60% to 80% of all endometriosis-associated ovarian cancers (EOAC) occur in the presence of atypical endometriosis (AE), often found in direct continuity with the tumor, suggesting AE as the transitioning entity from benign lesions to malignant variants. The intermediate, “atypical” lesions, are defined by several histologic criteria, including large nuclei with moderate to marked pleomorphism, increased nuclear-to-cytoplasmic ratio, cellular crowding, stratification, or tufting (14, 15).

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Here, we focus on the tissue immune microenvironment and provide the first comprehensive immune transcriptome profiling of tissues from 120 cases comprising normal controls, benign endometriosis, AE, and EOAC cases. With a broad collection of 511 immune gene probes, we identified gene expression changes associated with each disease state. One of the immune pathways enriched for differentially expressed genes in all disease categories was the complement pathway. Complement proteins were significantly upregulated in epithelial cells. Using a novel, ovarian surface epithelium (OSE)-derived cell line from genetically engineered mice with conditional mutations that trigger EOAC, we demonstrate that activation of Kras and Pten tumor-driving pathways leads to upregulation of complement in epithelial cells. These results reveal for the first time the link between tumor-initiating events and immune surveillance via complement, and point to this pathway as a potential target for therapy and early prevention in EOAC.

Materials and Methods

Ethics statement

This research study protocol was approved by the Institutional Review Board (IRB) at the University of Pittsburgh, (Pittsburgh, PA).

Patients

We accessed 120 formalin-fixed paraffin-embedded (FFPE) tissue samples, through the Health Science Tissue Databank at the University of Pittsburgh Medical Center (UPMC, Pittsburgh, PA). The FFPE tissue blocks comprised of normal endometrium (n = 32, equally distributed between secretory and proliferative stage), benign endometriosis (n = 30) consisting of ovarian endometriosis (n = 11), and extra ovarian endometriosis cases (n = 19), AE (n = 15), clear-cell tumors (n = 12), and endometrioid ovarian cancer (n = 16). Serous tumors (n = 15) were included as nonendometriosis-associated controls.

RNA extraction and Nanostring

The RNeasy FFPE Kit (Qiagen) was used to isolate total RNA from FFPE tissues. The nCounter GX Human Immunity Kit (Nanostring) was used to profile 511 immune genes. Genes in this kit belong to several pathways such as inflammatory disease, cell to cell signaling, cellular development, cell death, and hematologic system development. Additional details are included in Supplementary Materials and Methods.

Immunohistochemistry and immunocytochemistry

Immunohistochemical studies were performed in FFPE tissue with the following antibodies: anti-human C7, C3, C4b, MASPI1, CFH, CFD, membrane attack complex (MAC). Detailed protocols, clone identification, antibody dilutions, and image acquisition are included in Supplementary Materials and Methods.
Reverse transcriptase quantitative PCR

Two micrograms of purified RNA was used for reverse transcription using the iScript cDNA Synthesis Kit (Bio-Rad). Quantitative reverse transcriptase PCR (qRT-PCR) was carried out with the CFX96 Touch Real-Time PCR Detection System (Bio-Rad) using the SsoAdvanced SSO SYBR Green Supermix (Bio-Rad). Detailed protocols are provided in Supplementary Materials and Methods.

Establishment of the murine MUC1/Cras/Pten (MKP) OSE cell line

MKPOSE cells were derived in Anda Vlad’s laboratory at Magee Women’s Research Institute, from primary OSE cells isolated from healthy MKP mice (21) via gentle trypsinization of six ovaries collected at necropsy from three healthy female mice, according to previously published protocols (22) and as further detailed in Supplementary Materials and Methods. Authentication was performed through genomic PCR of MKPOSE cells for the presence of loxP cassettes at the (murine-specific) Kras and Pten loci before and after AdCre infection, as shown in Supplementary Fig. S2. This verification approach was performed for each experiment described herein.

Complement-mediated cell lysis

A total of 5 × 10⁵ AdCre infected and uninfected MKPOSE cells were incubated with 90 μL mouse ascites and 5 μL of anti-MUC1 antibody (50 μg/mL) and 15 μL of DMEM medium. Either reagents alone or cells in serum-free medium served as controls. As positive controls for lysed cells, we incubated MKPOSE cells with 0.2% Triton for 15 minutes at 37°C. Number of live and dead cells were measured after staining with either 7-Aminoactinomycin-D (7-AAD) or propidium iodide (BD Biosciences), via flow cytometry. Similarly treated, normal mouse splenocytes served as control for complement-induced cell lysis.

siRNA transfection

Commercially available ON-TARGETplus siRNA consisting of mixture of four mRNA regions directed against mouse C7 or mouse Capdh were used (Thermo Scientific Dharmacon RNAi Technologies). Transfection methods were according to the manufacturer’s instructions and are further detailed in Supplementary Materials and Methods.

nCounter data preprocessing

Experiments were compliant with nCounter mRNA Expression Assay (http://www.genetics.pitt.edu/forms/nCounter_Gene_Expression_Data_Analysis_Guidelines.pdf) and the complete datasets are available in the Gene Expression Omnibus database under accession number GSE57545. To minimize the impact of detection anomalies, we normalized the dataset to the positive control normalization factor outside the recommended range of 0.3 to 3 or with an estimated background >3 SDs from the mean.

Statistical analyses

To identify differentially expressed genes between any two patient categories, we used the generalized linear model with negative binomial distribution family for count response data. Once negative binomial models were fitted and dispersion estimates were obtained, we performed testing procedures for determining differential expression using an exact test with the quantile-adjusted conditional maximum likelihood (qCML) method. For a paired group, we evaluated the average effect of disease type over involving patients. All statistical programming was implemented in R, using the edgeR package (23).

For hierarchical clustering, we first filtered out features with means or SDs under the 50th quantile of all the features. The filtering procedure left 100 expressed (large means) and informative (large SDs) features, based on which hierarchical clustering with complete linkage was then applied.

Results

Immune gene expression profiles can distinguish different disease categories

The clinical demographics of all patients and controls used in this study are summarized in Table 1. Most of the individuals self-identified as Caucasians, regardless of disease category. The majority of patients in control group (18/32, 56%) underwent hysterectomy due to leiomyomas. The remaining of cases were treated due to ovarian cysts (7 cases), adenomyosis, pelvic organ prolapse, and endometrial polyps (2 cases of each). One patient underwent prophylactic procedure due to risk for Lynch syndrome.

Patients with endometriosis were younger than patients with ovarian cancer, as expected (P = 4.635e−11). Gravidity and parity scores were also lower in patients with endometriosis and EAOC as compared with healthy individuals (P < 0.05), in line with findings pointing to hormonal imbalances that accompany these conditions (24).

Although disease staging was not consistently performed for the endometriosis cases in this retrospective study, we classified the subjects according to the type of lesions, using predefined criteria (25–27). Of all endometriosis cases, 9 cases (47%) were classified as “peritoneal” disease and 9 as “deeply infiltrating lesions” (DIE). One case presented with abdominal wall disease. The vast majority (13/15 cases, 87%) of all AE lesions presented in the context of ovarian endometrioma. The remaining two cases had peritoneal lesions.

To profile the immune microenvironment in endometriosis and EAOC, we extracted mRNA from formalin-fixed and paraffin-embedded (FFPE) tissue and performed NanoString measurements of 511 immune gene transcripts, using the nCounter GX Human Immunology Kit. Multidimensional scaling, constructed using 65% cut-off threshold for differentially expressed genes, shows a good level of separation of control individuals, endometriosis, and
EAOC patients (Fig. 1A). Unsupervised clustering of 100 filtered genes (as described in statistical analyses section in Materials and Methods) also distinguished the three disease categories well (Fig. 1C). Twenty-two of 28 (79%, after removal of outliers as described in Materials and Methods) healthy control samples and 22 of 28 (79%) of cancer cases cluster within their disease category and separate from each other (Fig. 1C clusters 1 and 2, respectively). Endometriosis shows a mixed immune gene profile with some (n = 11, 37% of cases) clustering with healthy controls (cluster 1) while others (n = 10, 33%) display profiles more similar to control (cluster 2). The remaining 30% were also more similar to cancer, albeit in the most distant cluster 3. These results suggest that in some patients with endometriosis, endometriosis-associated inflammation carries features typically associated with cancer immune environments. In addition, similar comparisons using the AE cohort show that the vast majority (n = 13, 85%) of cases in this disease category have a cancer-like immune environment as they homogeneously cluster closer to the EAOC cases than to the control group (Fig. 1B and D), providing further support for AE as a tumor precursor lesion.

### Differential expression of immune genes that differentiate between controls, endometriosis, and EAOC reveal complement pathway

Analyses of differentially expressed genes using a false discovery rate (FDR) of 5%, and log2 fold change in expression ≥1 identified 39 immune genes that distinguish normal from endometriosis, 73 immune genes that differentiate between endometriosis and EAOC, and 99 immune genes that are different in EAOC compared with controls (Fig. 2A). Analyses of AE reveal 95 and 75 differentially expressed genes when compared with controls and EAOC, respectively (Fig. 2A). The complete list of all differentially expressed genes and their P values is shown in Supplementary Table S1. Analysis of the differentially expressed gene lists comparing endometriosis with control or EAOC revealed that a total of 74 genes were present in at least two of the three signatures (Fig. 2B). Nine of the 74 genes were present in all three datasets and of these 9, 5 genes belong to the complement pathway: complement factors 7, D, B, H (C7, CFD, CFB, CHF), and mannose-associated serine protease 1 (MASP1; Fig. 2B). Two other complement genes encoding for complement factors 3 and 4a (C3, C4A) were also revealed in other two-way intersections such as normal versus endometriosis, endometriosis versus EAOC and normal versus EAOC (Supplementary Table S1). In addition, C3, C7, and CFH remain deregulated in AE (Supplementary Fig. S1) and when analyzed together, a differentiating pattern of the 7 complement genes (C3, C7, CFH, CFD, CFB, CHF, and MASP1; Fig. 2B). Twenty compound genes encoding for complement factors 3 and 4a (C3, C4A) were also revealed in other two-way intersections such as normal versus endometriosis, endometriosis versus EAOC and normal versus EAOC (Supplementary Table S1). In addition, C3, C7, and CFH remain deregulated in AE (Supplementary Fig. S1) and when analyzed together, a differentiating pattern of the 7 complement genes (C3, C4A, C7, CFH, CFD, CFB, and MASP1) was detected among all four disease categories (control, endometriosis, AE, and EAOC; Fig. 2C). Notably, changes in expression for most of the complement genes displayed a consistent trend, from low levels seen in control endometrium to higher expression in endometriosis, AE, and EAOC (Fig. 2D). In contrast, MASP1, which encodes for mannan-binding lectin serine protease 1, follows the opposite pattern, suggesting that lectin pathway of complement activation may be not involved in endometriosis and EAOC-associated inflammation. In addition, Ingenuity Pathway Analyses confirms that

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**Table 1. Demographic and clinical characteristics of patients**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Normal, n = 32 (26.7%)</th>
<th>Benign endo, n = 30 (25%)</th>
<th>Atypical endo, n = 15 (12.5%)</th>
<th>EAOCb, n = 28 (23.3%)</th>
<th>SOCb, n = 15 (12.5%)</th>
<th>Pab</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at presentation (years), mean (SD)</td>
<td>46.5 (6)</td>
<td>40.1 (10.9)</td>
<td>48.01 (6.5)</td>
<td>54.8 (11.6)</td>
<td>65.4 (12.4)</td>
<td>1.135e-09</td>
</tr>
<tr>
<td>Gravidity, median (IQR)c</td>
<td>2 (1)</td>
<td>1 (2.5)</td>
<td>1 (2)</td>
<td>1.5 (2.25)</td>
<td>2 (2)</td>
<td>0.016</td>
</tr>
<tr>
<td>Parity, median (IQR)c</td>
<td>2 (2)</td>
<td>0.5 (1.25)</td>
<td>1 (1.75)</td>
<td>0.5 (2)</td>
<td>2 (2)</td>
<td>0.002</td>
</tr>
<tr>
<td>Body mass index (kg/m²), mean (SD)</td>
<td>30.8 (9.9)</td>
<td>28 (7.3)</td>
<td>31.5 (12.7)</td>
<td>28.7 (8.1)</td>
<td>28.4 (6.6)</td>
<td>0.8</td>
</tr>
<tr>
<td>Race (white), n (%)</td>
<td>9 (33%)</td>
<td>10 (36%)</td>
<td>3 (21%)</td>
<td>9 (36%)</td>
<td>6 (50%)</td>
<td>0.7</td>
</tr>
<tr>
<td>History of tobacco use, n (%)</td>
<td>9 (33%)</td>
<td>10 (36%)</td>
<td>3 (21%)</td>
<td>9 (36%)</td>
<td>6 (50%)</td>
<td>0.7</td>
</tr>
<tr>
<td>History of alcohol use, n (%)</td>
<td>14 (54%)</td>
<td>13 (46%)</td>
<td>8 (57%)</td>
<td>11 (46%)</td>
<td>4 (33%)</td>
<td>0.7</td>
</tr>
<tr>
<td>Preoperative CA125 level (U/mL)</td>
<td>79.1</td>
<td>18.8</td>
<td>736.3</td>
<td>1,920</td>
<td>1,920</td>
<td>0.005</td>
</tr>
<tr>
<td>History of diabetes, n (%)</td>
<td>10 (35%)</td>
<td>5 (17%)</td>
<td>6 (46%)</td>
<td>12 (50%)</td>
<td>9 (75%)</td>
<td>0.007</td>
</tr>
<tr>
<td>Stage of diseaseb, n (%)</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Early</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>15 (53%)</td>
<td>3 (20%)</td>
<td>0.08</td>
</tr>
<tr>
<td>Late</td>
<td>N/A</td>
<td>N/A</td>
<td>N/A</td>
<td>12 (42%)</td>
<td>11 (73%)</td>
<td>—</td>
</tr>
</tbody>
</table>

**NOTE:** The values in bold are statistically significant.

**Abbreviations:** Endo, endometriosis; SOc, serous ovarian cancer; N/A, not applicable.

bAEOC group comprises endometrioid (n = 16, 57%) and clear-cell (n = 12, 43%) tumors.

ANOVA or Kruskal—Wallis test for continuous variables; χ² or Cochran—Mantel–Haenszel χ² test for categorical variables.

iIQR, interquartile range (25th percentile, 75th percentile).

Tumor stage was available in 27 (96%) and 14 (93%) of EAOC and SOC cases, respectively.

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Complement is the most significantly dysregulated immune pathway in endometriosis and EAOC (Supplementary Table S2). Importantly, complement was not among the top pathways in serous ovarian cancer, further demonstrating heterogeneity among ovarian cancer histotypes and suggesting that complement involvement may be specific to EAOC.

Humoral immunity is also included in the top networks in endometriosis and EAOC (Supplementary Table S3A and S3B), but not in serous ovarian cancer (Supplementary Table S3D) although both EAOC and serous cancer seems to trigger changes predominantly in cellular immunity.
### A

<table>
<thead>
<tr>
<th>DE genes = 39</th>
<th>DE genes = 73</th>
<th>DE genes = 99</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls E</td>
<td>E</td>
<td>EAOC</td>
</tr>
<tr>
<td>Benign E</td>
<td>Controls EAOCC</td>
<td>EAOC</td>
</tr>
</tbody>
</table>

### B

<table>
<thead>
<tr>
<th>ID</th>
<th>Ctrl. Vs. E</th>
<th>Ctrl. vs. EOAC</th>
<th>E. vs. EAOC</th>
</tr>
</thead>
<tbody>
<tr>
<td>C7</td>
<td>23.7 (3.39E–61)</td>
<td>7.84 (2.56E–26)</td>
<td>0.33 (9.30E–05)</td>
</tr>
<tr>
<td>CFB</td>
<td>2.8 (3.36E–08)</td>
<td>7.38 (1.66E–26)</td>
<td>2.62 (1.88E–09)</td>
</tr>
<tr>
<td>CFD</td>
<td>2.59 (2.73E–08)</td>
<td>0.39 (0.000178)</td>
<td>0.15 (2.57E–21)</td>
</tr>
<tr>
<td>CFH</td>
<td>7.17 (2.49E–28)</td>
<td>3.19 (2.56E–11)</td>
<td>0.44 (0.001844)</td>
</tr>
<tr>
<td>MASP1</td>
<td>0.37 (6.84E–11)</td>
<td>0.036 (6.66E–41)</td>
<td>0.096 (5.48E–16)</td>
</tr>
<tr>
<td>GNLY</td>
<td>0.35 (2.89E–10)</td>
<td>0.036 (3.79E–5)</td>
<td>0.1 (7.67E–23)</td>
</tr>
<tr>
<td>IL2RB</td>
<td>0.39 (2.37E–09)</td>
<td>0.17 (4.27E–19)</td>
<td>0.44 (0.000221)</td>
</tr>
<tr>
<td>LTF</td>
<td>9.9 (5.61E–16)</td>
<td>31.46 (4.32E–37)</td>
<td>7.19 (3.65E–08)</td>
</tr>
<tr>
<td>PIGR</td>
<td>0.32 (2.12E–13)</td>
<td>2.34 (2.30E–06)</td>
<td>7.22 (8.49E–22)</td>
</tr>
</tbody>
</table>

### C

<table>
<thead>
<tr>
<th>Controls</th>
<th>E</th>
<th>AE</th>
<th>EAOC</th>
</tr>
</thead>
<tbody>
<tr>
<td>C4A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CFH</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CFB</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CFD</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MAS1</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### D

- **C7**: Controls [6.84E–11], Benign E [6.66E–41], EAOC [5.48E–16]
- **CFB**: Controls [2.89E–10], Benign E [3.79E–5], EAOC [7.67E–23]
- **CFD**: Controls [2.37E–09], Benign E [4.27E–19], EAOC [0.000221]
- **LTF**: Controls [5.61E–16], Benign E [4.32E–37], EAOC [3.65E–08]
- **PIGR**: Controls [2.12E–13], Benign E [2.30E–06], EAOC [8.49E–22]
Taken together, these results suggest that gene expression of several complement components is high and that complement activation may be a common biologic process involved in immune surveillance in endometriosis and EAOC (Supplementary Table S3C and S3D). Furthermore, presence of humoral immunity points towards complement as a possible mediator of epithelium–immune stoma interactions, potentially via antibody-dependent, complement-mediated immunity.

**Complement proteins are abundantly present in epithelial cells endometriosis and EAOC**

Using immunohistochemistry (IHC), we confirmed protein expression for all five complement factors (C7, CFD, CFB, CFH, MASP1) differentially expressed in endometriosis, control, and EAOC (Fig. 2B). Complement factor 7 (C7) is highly expressed in endometriosis and ovarian cancer while normal endometrium shows little to no mRNA expression (Fig. 2D). Accordingly, expression of C7 protein, a member of the membrane attack complex (MAC), is minimal in eutopic (control) endometrial glands and stroma, whereas epithelial cells in endometriosis, AE, and EAOC show a clearly increased protein production (Fig. 3A). Scoring of C7 protein shows that at least for this complement component, its expression levels are in line with the C7 gene expression data (Fig. 2D).

Expression of other complement proteins such as CFB, CFD, CFH, and MASP1 was confirmed by IHC (Fig. 3C). Similar to C7, most of the complement proteins were present in epithelial cells lining endometriotic glands or endometrioma and in epithelial tumor cells (in EAOC). Staining pattern reveals complement proteins are distributed both intracellularly and on the cell surface, suggesting an endogenous production and consumption in epithelial lesions of endometriosis and cancer.

**Complement genes in a murine model for EAOC that mirrors expression seen in human tumors**

On the basis of the above ex vivo findings that complement proteins are abundant in epithelial cells in endometriosis, premalignant, and malignant lesions, we proposed next to investigate in vitro the link between the complement pathway and early carcinogenic events in ovarian epithelial cells. To accomplish this, we employed ovarian surface epithelial cells derived from triple transgenic mice that progress to human mucin 1 (MUC1)-expressing endometrioid ovarian cancer that closely mirrors the human disease (21). The mice heterozygously express human MUC1 as a transgene, and simultaneously carry the conditional KrasG12D oncoallele and the floxed PtenKrasPten gene (28). In this Cre-LoxP in vivo system, injection of Cre recombinase encoding adeno virus (AdCre) under the ovarian bursa of female MKP mice triggers progression to endometrioid ovarian tumors in about 7 to 8 weeks (21). The mouse tumors show similarly increased epithelial cell expression of complement proteins (Fig. 4A), further demonstrating that the MUC1KrasPten (MKP) mouse model, which replicates with high fidelity the histopathology of human EAOC (21, 28), may also provide an useful preclinical tool for exploring the complement biology in the ovarian tumor microenvironment.

**Conditional activation of tumor driving pathways leads to complement gene upregulation**

Using conditional mice with a Kras-activating mutation and Pten deletion, we studied next how engagement of these classical oncogenic and tumor suppressor pathway, respectively, affects complement activation in primary epithelial cells. To accomplish this, we generated a novel cell line (MKPOSE) from primary OSE cells of healthy MUC1KrasPten mice (21), using previously established protocols (22). At baseline, Kras and Pten/Pi3K pathways are unaffected in these mice (21, 28). However, as with our in vivo experiment, in vitro exposure of the MKPOSE cells to AdCre flexes out loxp sites and triggers Pten deletion and oncogenic Kras activation (Supplementary Fig. S2A), similarly to levels seen in vivo in tumors (21).

We postulated that MKPOSE cells exposed to AdCre create a surrogate in vitro system for early transformation and tumor formation and offer an experimental setting in which we can monitor early changes in complement gene expression before and after Cre-induced genetic events that effectively trigger in vivo tumorigenesis. Focusing on C7 as the prototype member of the MAC, the end-product of complement activation, we show that in MKPOSE cells, C7 is present at detectable levels at baseline and is further upregulated (alongside other complement genes) following exposure to AdCre (Fig. 4B–D). In addition, treatment of cells with small molecule inhibitors that inhibit the AdCre-induced Kras and Pi3k pathways (AZD6422 or BEZ235, respectively) reverses C7 upregulation (Supplementary Fig. S2B). These drugs are in preclinical development as cancer therapeutics, AZD6244 as a highly selective inhibitor of Erk1 and Erk2 (29) and BEZ235 as a pan-Pi3K-mTOR inhibitor (30).

One of the consequences for complement upregulation is mediation of antibody-induced, complement-mediated cell death. Given that humoral immunity was among the

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Figure 2. Differentially expressed genes across disease categories. A, heatmaps of differentially expressed (DE) genes in five different two-group comparisons: control versus endometriosis (E; n = 39), endometriosis versus EAOC (n = 73), normal versus EAOC (n = 99), control versus AE (n = 95), AE versus EAOC (n = 75). B, Venn diagram showing number differentially expressed genes that are differentially expressed in controls, E, and EAOC. Accompanying table lists the 9 common differentially expressed genes (common intersection, arrow), fold change and P value. Complement genes are in bold. C, heatmap of 7 complement genes (C4A, CFH, CFB, C7, CFD, MASP1) shows a differentiating pattern among the four different disease categories (controls, E, AE and EAOC). D, Individual gene expression profile of five common complement differentially expressed genes (C7, CFB, CFD, CFH, MASP1) across the four cohorts (controls, E, AE, and EAOC). All genes are significantly different (FDR < 0.05 and absolute log FC greater than 1) for any of the two group comparisons (normal, endometriosis, and EAOC).
top five networks in both endometriosis and EAOC (Supplementary Table S3A and S3B), we explored next whether complement upregulation triggered by pathways downstream of Kras and Pten/Pi3k modifies antibody-induced, complement-mediated cell death of MKPOSE cells. AdCre-treated MKPOSE cells were exposed to ovarian cancer ascites, collected from mice challenged intraperitoneally (IP) with syngeneic, human MUC1-expressing ovarian tumors. These mice produce anti-MUC1 antibodies at high levels in both serum and ascites (Supplementary Fig. S3A). The MUC1-specific antibodies in ascites have the ability to bind to MUC1 molecules present on tumor cell surface (Supplementary Fig. S3B). We postulated that if complement engagement would be effective, AdCre MKPOSE cells exposed to antibody-containing ascites would undergo antibody-induced, complement-mediated cell death. However,

Figure 3. Tissue validation of complement proteins via IHC. A, tissue deposition of complement C7 protein. B, intensity staining for C7 was scored as follows: 0, no staining; 1, weak staining; 2, moderate staining; and 3, strong staining. y-axis: average score plus SD. Each of the three groups (controls, endometriosis, and EAOC) contained five representative samples, one-way ANOVA; P = 0.001. C, tissue expression of CFB, CFD, CFH, and MASP1 proteins in endometriosis and EAOC FFPE tissues. At least 5 cases in each disease categories were stained for each marker. Representative images for each marker are shown.
we observed either no effect or slight decrease rather than increase in cell death (compared to EV control cells, Fig. 4E). In contrast, control cells (consisting of similarly treated splenocytes exposed to same ascites, as well as mouse serum as control) are successfully lysed (Supplementary Fig. S3C). This suggests that despite upregulation in transformed OSE, complement engagement in the predicted lytic pathways is rather ineffective.

**Complement C7 knockdown inhibits ovarian cell proliferation**

Complement inhibition has been recently reported as having a beneficial effect against tumor growth (31). To investigate roles of C7 expression on proliferation of ovarian epithelial cells in vitro, we knocked down C7 expression in MKPOSE AdCre-infected and control cells, using a mixture of siRNAs targeting four regions of mouse C7 mRNA (Fig. 5A). Mouse Gapdh was used as positive control for knockdown efficacy (Supplementary Fig. S4A), whereas nontarget siRNA, which does not affect C7 expression was used to control for target specificity (Supplementary Fig. S4B). Our results show that inhibition of C7 gene expression in MKPOSE exposed to AdCre inhibits growth curve (Fig. 5B; \(P = 0.03\)). Nontarget siRNA at increasing concentrations does not interfere with cell growth (Supplementary Fig. S4C). We further tested complement inhibition in combination with AZD 6422 (80 nmol/L) or BEZ235 (25 nmol/L). As expected, when cells were treated with C7 siRNA and either of the inhibitor drugs, growth rate of cells was significantly inhibited (\(P < 0.05\)). Combination of complement knockdown and either drug showed additional inhibitory effect (Fig. 5C; \(P < 0.005\)).

Overall, these in vitro results link Kras and Pten/P13K pathways to increased complement gene expression and suggest that complement inhibition reduces tumor cell proliferation an effect further increased, albeit moderately, by specific inhibitors of the above tumor pathways.

**Discussion**

Several inflammatory phenotypes have been associated with endometriosis and efforts are under way to incorporate
these into noninvasive diagnostic tests (32). However, most of the studies to date have focused primarily on secreted chemokine profiles (20) or immune cells resident in the peritoneal cavity (macrophages, NK cells) or isolated from circulation (T lymphocytes, NK cells; refs. 33, 34). We performed here the first comprehensive immune gene expression analysis of pelvic inflammation to date, using a collection of 511 Nanostring probes and RNA extracted from affected tissues. Our results revealed immune gene profiles that can differentiate endometriosis from both healthy and cancer cases. Surprisingly, however, unsupervised clustering shows that several of the patients with endometriosis may have an inflammation profile similar to those with EAOC (Fig. 1). This intriguing finding further confirms heterogeneity of inflammatory milieu in endometriosis and suggests that cancer-like immune signatures may develop earlier, in patients with lesions that are classified as clinically benign. Given the sensitivity and specificity of immune effectors that can sense early molecular changes of cells undergoing transformation, it is conceivable that profiling the immune response in tissue may provide molecular clues for patients with EAOC risk. This hypothesis stimulates further studies, with significantly larger sample sizes and additional controls, to validate and test the efficacy of cancer-like immune signatures as potential predictors of progression to EAOC. We also acknowledge that all the cases used here as controls consisted of a separate cohort of eutopic endometrium/ectopic glands from same patients.

Analyses of differentially expressed gene sets revealed that complement is the top pathway in endometriosis and EAOC cases. Although this is the first tissue profiling study on complement, changes in peritoneal fluid levels of complement have been recently reported in endometriosis. Using qPCR gene profiling of 84 genes in a cohort of 20 endometriosis cases, Aslan and colleagues recently reported that most significant upregulation was in C5 gene expression, although no other complement genes were detected, possibly due to the limited breadth of the profiler employed (35). We utilized here a comprehensive immune gene set and focused not only on endometriosis but also on EAOC. Our finding that 5 of 9 genes (56%) differentially expressed...
in both endometriosis and EAOC are complement genes strongly supports the importance of complement cascade in these diseases. In addition, the lower prevalence of complement genes in serous ovarian cancer suggests a potential specificity for complement in EAOC.

Primarily classified as a powerful innate immune effector, the complement system and its engagement in both acute and chronic inflammation are well defined (36). However, emerging literature during the past decade has revealed that complement activation may support tumor growth, thus creating a paradigm shift in complement cascade in cancer (37). It is now postulated that the complement pathway may act via several mechanisms that coexist in the tumor environment: directly, by playing an active role in stimulating proliferation of tumor cells or indirectly, via immune suppression (38, 39) and neovascularization (40). Nevertheless, the initiating steps that link epithelial cell biology and complement cascade are not well defined.

Using our recently reported triple transgenic MUC1K-rasPten mice with conditional mutations in Kras and Pten genes (21), we generated a novel primary OSE-derived murine ovarian cancer line (MKPOSE) and a versatile in vitro system that served as a surrogate for early transformation in ovarian epithelial cells. By turning on Kras and deleting Pten in MKPOSE cells, we showed for the first time that activation through Kras and Pten/P13K leads to complement upregulation, an effect reversed when small drug inhibitors acting downstream of Kras and/or Pten are added. Inhibitors of tumor-driving pathways (including AZD6244 and BEZ325 used here) are currently tested in clinical trials for several types of tumors (41, 42). Although complement genes are likely controlled via several pathways, molecular profiling of tumor responses in these trials may provide further molecular evidence on modulation of complement pathways by targeted therapies.

Finally, we report here that one other major biologic pathway active in endometriosis is humoral immunity. In addition to changes in immune cells and cytokines, mostly detected via peripheral blood or peritoneal fluid measurements, women with endometriosis often display increased B-cell activation (43–45). Systemic antibody production and deposition of IgG and complement in tissue has been previously reported in patients with endometriosis, showing humoral responses to various autoantigens (43–46).

Antibody-induced, complement-mediated cell death is very effective in clearing dead cells, mostly via the classical pathway, activated by the Fc portion of immunoglobulins bound to various antigens on either infectious agents or apoptotic cells. The alternative pathway can be triggered via continuous, low level cleavage of C3, and can be activated by bacteria, viruses, or fungi, as well as neoplastic cells. The third complement activation pathway, called the MBL pathway is activated by pathogens, via pathogen-associated molecular patterns (36).

The expression levels for the differentially expressed complement genes detected here suggest that MBL pathway was not involved in these cases (hence ruling out potential infectious causes for pelvic inflammation) and that classical pathway was likely most prominently used. In summary, our findings reveal that chronic inflammation in endometriosis is dominated by complement, which remains active in EAOC but not tumors with serous histology, further demonstrating heterogeneity in the inflammatory milieu within this category commonly referred to as ovarian cancer. Pharmacologic inhibition of complement is currently tested in clinical trials (36), and results from these studies will provide much needed clinical evidence to support (or refute) the recent paradigm shift on protumor roles of complement in cancer. Profiling studies like the one presented here might aid in patient selection for a personalized approach.

Disclosure of Potential Conflicts of Interest
T. Lee is a consultant/advisory board member for Eishcon. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions
Conception and design: X. Huang, E. Elishaev, R. Edwards, A.M. Vlad
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S.M. Suryawanshi, E. Elishaev, L. Zhang, S. Kim, N. Donellan, G. Mantia-Smaldone, S. Manzuria, R. Edwards, A.M. Vlad
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S.M. Suryawanshi, R. Budiu, E. Elishaev, L. Zhang, S. Kim, T. Ma, G. Tseng, R. Edwards, A.M. Vlad
Writing, review, and/or revision of the manuscript: S.M. Suryawanshi, R. Budiu, E. Elishaev, L. Zhang, S. Kim, N. Donellan, G. Mantia-Smaldone, G. Tseng, R. Edwards, A.M. Vlad
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S.M. Suryawanshi, E. Elishaev, T. Lee, R. Edwards, A.M. Vlad
Study supervision: E. Elishaev, A.M. Vlad
Other (provided the surgical specimen for the study): T. Lee

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Swati Suryawanshi, Xin Huang, Esther Elishaev, et al.


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