The immune environment in ovarian cancer
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Chapter 4

Treatment regimen, surgical outcome and T cell differentiation influence prognostic benefit of tumor-infiltrating lymphocytes in high-grade serous ovarian cancer


*Authors contributed equally

Abstract

**Purpose** Tumor-infiltrating lymphocytes (TIL) are associated with a better prognosis in high grade serous ovarian cancer (HGSC). However, it is largely unknown how this prognostic benefit of TIL relates to current standard treatment of surgical resection and (neo-)adjuvant chemotherapy. To address this outstanding issue, we compared TIL infiltration in a unique cohort of advanced stage HGSC cancer patients primarily treated with either surgery or neo-adjuvant chemotherapy.

**Experimental Design** Tissue Microarray (TMA) slides containing samples of 171 patients were analyzed for CD8+ TIL by immunohistochemistry. Freshly isolated CD8+ TIL subsets were characterized by flow cytometry based on differentiation, activation and exhaustion markers. Relevant T cell subsets (CD27+) were validated using immunohistochemistry and immunofluorescence.

**Results** A prognostic benefit for patients with high intratumoral CD8+ TIL was observed if primary surgery had resulted in a complete cytoreduction (no residual tissue). By contrast, optimal (< 1 cm of residual tumor) or incomplete cytoreduction fully abrogated the prognostic effect of CD8+ TIL. Subsequent analysis of primary TIL by flow cytometry and immunofluorescence identified CD27 as a key marker for a less-differentiated, yet antigen-experienced and potentially tumor-reactive CD8+ TIL subset. In line with this, CD27+ TIL was associated with an improved prognosis even in incompletely-cytoreduced patients. Neither CD8+ nor CD27+ cell infiltration was of prognostic benefit in patients treated with neo-adjuvant chemotherapy.

**Conclusions** Our findings indicate that treatment regimen, surgical result and the differentiation of TIL should all be taken into account when studying immune factors in HGSC or, by extension, selecting patients for immunotherapy trials.

**Translational Relevance**

The presence of tumor-infiltrating lymphocytes (TIL) is associated with a longer survival in high-grade serous ovarian cancer (HGSC). However, the relationship of this prognostic benefit to current treatment schedules is largely unknown. Here, we demonstrate that the treatment modality and surgical result are important factors to take into account when studying immune responses and prognostic benefit thereof in HGSC. Further, we demonstrate that the differentiation status of infiltrating cytotoxic T cells is an important parameter for studying prognosis. A less-differentiated TIL phenotype, as determined by expression of CD27, was shown to be associated with improved survival after incomplete cytoreductive surgery. Our findings have potential implications for assessment and characterization of TIL infiltrate as a biomarker for disease, relevant for prognosis of individual patients and patient selection in immunotherapeutic trials.
Introduction

Epithelial ovarian cancer (EOC) is the most deadly gynecological malignancy with an overall 5-year survival of 38-40% (1). EOC is a heterogeneous disease with multiple histological subtypes, of which high grade serous carcinoma (HGSC) is the most common (2). The poor prognosis of the disease is largely due to diagnosis at advanced stage and therapy-resistant disease relapses that occur in the majority of patients following initial treatment consisting of cytoreductive surgery and platinum-containing chemotherapy (2). Nevertheless, a subset of HGSC patients appears to remain disease-free for prolonged periods of time. To date, several factors have been identified to define this subset of patients.

Arguably the strongest prognostic factor is the amount of residual tumor tissue after surgery (3). Indeed, patients in which a complete resection, leaving no residual macroscopic lesions, is achieved have an approximately 1.6-fold longer survival than patients with remaining macroscopic disease (4). As such, neo-adjuvant chemotherapy (NACT) is increasingly considered as the treatment option of choice in patients in whom the chances of up-front complete cytoreduction are minimal (e.g. patients with stage IIIC/IV disease with widespread tumor dissemination) or as an effort to reduce morbidity due to aggressive primary cytoreductive surgery (PS). NACT may reduce tumor load before cytoreductive interval surgery, increasing the likelihood of completely resected tumors (5), as well as reduce perioperative morbidity and mortality in this group of patients.

A second major factor in determining the prognosis of HGSC is the presence of tumor-infiltrating lymphocytes (TIL). Infiltration of CD3+ T cells, and particularly CD8+ cytotoxic T cells (CTL) is associated with a better prognosis for HGSC patients (6,7). Moreover, the ratio between CTL and immune-inhibitory cells (FoxP3+ regulatory T cells, CD33+ myeloid-derived suppressor cells (MDSC)), the activation status of T cells (CD45RO), and their cytolytic activity (measured by TIA-1/Granzyme B expression) are all predictive for survival (8–10). Despite these well-established prognostic roles of both surgical outcome and TIL, the relationship between both factors has remained largely unknown. Indeed, most studies on the prognostic value of TIL in ovarian carcinomas used heterogeneous patient populations including various subtypes of EOC, early and advanced stages and different grades and included patients treated with various chemotherapeutic regimens. In addition, many studies use different outcome measures for the result of cytoreductive surgery, further complicating analysis.

Next to this effect of surgical outcome on the prognostic value of TIL, neo-adjuvant chemotherapeutic treatment might also alter TIL infiltration and function and therefore the overall effectiveness of a (pre-existing) immune response. Indeed, platinum-based NACT in patients with breast cancer can increase TIL infiltrate (11) and levels of TIL before treatment are reportedly predictive for the subsequent response to chemotherapy (12–14) as well as response to Trastuzumab, a monoclo-
nal antibody targeted against HER2 (15). While no association of TIL infiltrate and (neo-)adjuvant chemotherapy has been described in HGSC, a single study found that patients with high TIL were more likely to be completely cytoreduced, potentially due to a better tumor control by the immune system (6).

To address the effects of cytoreductive surgery and neo-adjuvant chemotherapy on the prognostic role of CD8+ TIL, patient cohorts therefore need to be highly standardized in terms of treatment schedule, stage, grade and outcome of cytoreductive surgery. Here, we generated two such cohorts of advanced stage HGSC patients treated with identical chemotherapy, but with primary treatment being either PS or NACT.

We show here that the prognostic benefit of CD8+ TIL is restricted to patients in whom a complete cytoreductive surgery was achieved (no residual tumor) and is not demonstrable in patients in which up-front complete cytoreduction was considered to be unattainable (NACT patients). Interestingly, we also found that patients performed better when the tumor was infiltrated by less-differentiated, CD8+ T cells, despite the presence of a residual macroscopic tumor after cytoreduction. Taken together, our findings indicate that treatment regimen, surgical result and the differentiation of TIL should all be taken into account when studying immune factors in HGSC or, by extension, selecting HGSC patients for immunotherapy trials.
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Methods

Patient selection
     An anonymized database was created containing information on clinico-pathological characteristics and follow-up of patients diagnosed with serous ovarian cancer at the University Medical Center Groningen (Groningen, The Netherlands) between January 2000 and December 2012. Patients were staged according to International Federation of Gynecology and Obstetrics (FIGO) criteria, and graded by a gynecologic pathologist based on World Health Organization (WHO) guidelines. Patients were selected if sufficient formalin-fixed paraffin-embedded (ffpe) tissue was available for TMA construction. Tissue was obtained either from primary cytoreductive surgery (PS cohort) or from interval surgery (NACT cohort). Patients that underwent PS subsequently received six cycles of platinum-based chemotherapeutic regimen often combined with paclitaxel. Patients that were selected for NACT first received three cycles of chemotherapy, followed by interval surgery and three more cycles of chemotherapy. Follow-up was calculated from date of initial treatment (either surgery or neo-adjuvant chemotherapy) and was last updated in April 2014.

Ethical review
     Patient data were retrieved from the institutional database into a new anonymous database, in which patient identity was protected by unique patient codes. According to Dutch law no approval from our institutional review board was needed. Primary patient TIL were isolated from surgical tumor waste for which no approval from our institutional review board was needed according to Dutch law.

Tissue MicroArray (TMA)
     From 265 HGSC patients FFPE tissue was available for the construction of a tissue microarray (TMA). A gynecologic pathologist confirmed the presence of tumor tissue on H&E slides and selected representative locations with tumor tissue. Triplicate cores with a diameter of 1 mm were taken from each paraffin-embedded tissue block and placed in a recipient block by using a tissue microarrayer (Beecher instruments, Silver Spring, USA). An asymmetrical grid was chosen with a 14x9 layout. Both normal and tumor tissue were included as orientation cores and controls. The seventh column from the fourth row onwards, and the fourth row from the seventh column onwards were left empty as a points of reference for grid layout. From each TMA block, 4 µm sections were cut and applied to APES-coated slides (Starfrost, Braunschweig, Germany). Core-loss was on average 9.0% (PS cohort) and 10.6% (NACT cohort). The presence of tumor in the arrayed samples was confirmed by H&E staining.
Immunohistochemistry and multi-color immunofluorescence

TMA slides were stained with mouse anti-human CD8 antibody (DAKO, Heverlee, Belgium; clone: C8/144B, 1:25 in blocking buffer (1% BSA/PBS with 1% human AB serum)) or rabbit anti-human CD27 antibody (Abcam, Cambridge, UK; clone: EPR8569, 1:150 in blocking buffer) by use of immunohistochemistry using standard methods (Supplementary methods). Furthermore, on the basis of the highest infiltration with CD8+ cells, 10 patients were selected from the TMA dataset and full tumor tissue slides were retrieved for analysis of CD8 and CD27 costaining by use of multicolor immunofluorescence. Antibody binding was visualized with goat anti-rabbit Alexa Fluor-488 and goat anti-mouse Alexa Fluor-555 (1:150, Life Technologies, Bleiswijk, The Netherlands). Counterstaining was done by 4’,6-diamidino-2-phenylindole (DAPI).

Isolation of TIL from fresh tumor tissue

Fresh tumor material was obtained for the isolation of TIL from patients undergoing cytoreductive surgery. With a scalpel, tumor pieces of approximately 0.5 cm³ were cut, and subjected to digestion in digestion medium (RPMI supplemented with 1 mg/ml collagenase type IV (Life technologies) and 31 U/ml rhDNase (Pulmozyme, Genentech, California, USA) for 30 minutes at 37°C. Subsequently, the digestion medium containing remaining tumor pieces was filtered over a 70 µm cell strainer (Corning, Amsterdam, The Netherlands) and cells were pelleted, washed, and cryopreserved until further use.

Multi-parameter flow cytometry

From the digested tumor samples TIL were phenotyped by multiparameter flow cytometry. The Zombie Aqua Fixable Viability Kit was used for live/dead stain according to manufacturer’s instructions (BioLegend, Uithoorn, The Netherlands). Antibodies used were CD3-PerCP-Cy5.5 (OKT3), CD8-APC-eFluor780 (RPA-T8), CD45RO-PE-Cy7 (UCHL1), CD137-PE (4B4-1), and PD1-APC (MIH4) (all eBioscience, Vienna, Austria), CCR7-BV421 (150503) (BD Biosciences, Etten-Leur, The Netherlands), and CD27-FITC (9F4) (Sanquin, Amsterdam, The Netherlands). All flow cytometry was performed on a FACSVerse (BD Biosciences) and samples were analyzed with Cytobank software (cytobank.org).

Image acquisition and analysis

Scoring of TMA samples was performed if cores had at least 20% tumor epithelium present, and if at least two cores per patient were analyzable. All CD8+ or CD27+ stained cells localized in tumor epithelium in each core were counted manually by two individuals that were blinded for patient characteristics. The two individual scores were compared and differences in counts of >10% were reanalyzed until consensus was reached. Cell counts were represented as total number of cells per mm² of tumor epithelium. H&E slides were used for comparison in cases
tumor/stroma regions were not clearly definable.

Immunofluorescent slides were scanned using a TissueFaxs imaging system (TissueGnostics, Vienna, Austria). Processed channels were merged using Adobe Photoshop. On each slide an area of 1 mm² of tumor epithelium was selected based on DAPI staining, and cells were counted manually.

**Statistical analysis**

All statistical analyses were performed using IBM SPSS version 22 (SPSS Inc., Chicago, USA) or Graphpad Prism. Disease-specific survival (DSS) was defined as the time period from date of surgery or first chemotherapeutic treatment until death due to ovarian cancer or last follow-up and was analyzed by using Kaplan-Meier method, with Log Rank test to determine differences between groups. Variables that were significantly associated with DSS in univariate analyses were entered into a multivariate analysis using the Cox proportional hazards model. Differences in cell infiltration between 43 matched ovarian and omental primary tumor tissues were assessed by Wilcoxon signed ranks test, no differences were found and therefore primary ovarian and omental tissues were used in the analyses. To determine differences in cell populations between clinicopathological variables or between different TIL subsets, the Mann Whitney U or one-way ANOVA test were used. P-values <0.05 were considered significant, and all tests were performed two-sided.
Results

Primary surgery and neo-adjuvant chemotherapy cohort

From a total of 265 patients, tissue in FFPE blocks obtained at primary or interval surgery was available to construct the TMA. Two cohorts were created on the basis of treatment strategy, a primary surgery (PS) (n=134) and a neo-adjuvant chemotherapy (NACT) (n=121) cohort. From 15 patients tissue from a recurrence and from 5 patients tissue from both primary and interval surgery were included on the TMA. Patients diagnosed with high grade (grade III and undifferentiated), advanced stage (FIGO ≥ IIB) serous ovarian carcinoma were selected for analysis in the current study (n=171), recurrences were excluded (Table 1). The five patients of which both primary and interval tissue was available, were analyzed in the cohort of their primary treatment, which was for all 5 NACT.

For the PS cohort 87 patients were included, who received 6 cycles of platinum-based chemotherapeutic treatment after surgery. Five patients did not receive chemotherapy, either because they received palliative treatment and refused additional chemotherapy, or died due to surgical complications. Eight patients progressed on chemotherapy (recurrence within 6 months of treatment). Surgical cytoreduction resulted in 39 (44.8%) patients with no residual macroscopic lesions. The mean age was 64.1 years (standard deviation (SD)=11.3) and median duration of follow-up was 31.0 months (interquartile range (IQR): 40). Analysis of the disease-specific survival (DSS) of these patients showed a better prognosis for patients who had no residual macroscopic lesions as compared to optimal (≤1 cm residual tumor tissue; p=0.036) and suboptimal (>1 cm remaining tumor nodules; p<0.001) cytoreduced tumors (Supplementary Fig. S1A), confirming the value of complete cytoreduction. Age was also a prognostic parameter in this cohort, older patients (cut-off 59 years of age) had a worse prognosis (p=0.002, Supplementary Fig. S1B).

In the NACT cohort, all 84 included patients received 3 cycles of platinum-based chemotherapy before and 3 cycles following surgical cytoreduction. Complete cytoreduction was achieved at interval surgery in 27 of the 84 patients (32.1%). Three patients had a recurrence within 6 months of treatment. The mean age of patients in this cohort was 64.4 years (SD: 8.6) and median duration of follow-up was 22.0 months (IQR: 24.5). DSS was significantly shorter in patients in this cohort in comparison to the PS cohort (median DSS 24.0 vs. 34.0 months, p=0.042, Table 1). For DSS analysis concerning residual tumor tissue after surgery, the same trend could be observed as in the PS cohort, though the difference in DSS between non-macrosopic disease and optimal cytoreduction was not significantly different (p=0.073), while the difference between no residual tumor and >1 cm tumors was (p<0.001, Supplementary Fig. S1C). Age was not a significant prognostic marker in this cohort (Supplementary Fig. S1D).
CD8+ TIL are predictive for DSS only in completely cytoreduced patients

In these two cohorts of advanced stage HGSC patients treated with identical chemotherapeutics, we first validated the previously published observation that CD8+ TIL are associated with prognosis in HGSC. TMA slides were analyzed for the infiltration of CD8+ CTL in the tumor epithelium. In the PS cohort, 87.9% of the samples had infiltrating CD8+ cells with a median infiltration of 21.31 cells/mm² tumor (IQR: 69.78) (Fig. 1A; 1B). Prognostic characteristics were analyzed for differences in CD8+ cell count in order to determine whether these factors influence intratumoral CTL infiltration. Age (p=0.215), FIGO stage (p=0.172), or surgical cytoreductive outcome (p=0.194) did not show differences in total CD8+ count.

In the NACT cohort, tumors obtained at interval surgery were infiltrated with CD8+ cells in 96.2% of patients with a median infiltration of 35.00 cells/mm² (IQR: 81.76) (Fig. 1B). There were no differences in number of cells within patient groups regarding age (p=0.856), FIGO stage (p=0.564), or surgical result (p=0.535). Comparing the two cohorts revealed that the infiltration of CD8+ TIL did not seem to be affected by the received neo-adjuvant chemotherapeutic treatment (Fig. 1B; p=0.084).

For subsequent survival data analysis, patients were dichotomized based on the group of patients with the highest infiltration of cells (highest tertile) vs. the group with low or no infiltration. DSS analysis based on infiltration of CD8+ cells revealed a non-significant improvement of survival (p=0.052) in the PS cohort for the group with high infiltration compared to patients with low or no infiltration (Fig. 1C). In the NACT cohort, no differences in survival were detected between the groups with either a high or a low infiltration of CD8+ (Fig. 1D, p=0.992).
Since the result of cytoreductive surgery is a major predictor for prognosis and determines the course of disease, we next analyzed the prognostic benefit of CD8+ TIL in relation to primary surgical outcome. Patients were categorized based on patients that had no residual macroscopic lesions after surgery and those with residual macroscopic disease. Within the group without residual tissue, a clear survival benefit was observed for patients who had a high infiltration of CD8+ T cells (Fig. 1E, p=0.028), suggesting a role of CD8+ in tumor immunosurveillance. By contrast, no survival benefit of CD8+ T cell infiltration was detected in the group of patients that received an incomplete cytoreduction. No difference was found in total number of infiltrating CD8+ TIL in completely resected tumors as compared to tumors of patients with remaining tumor tissue (Fig. 1F), suggesting that the number of TIL present does not affect surgical outcome in these patients. Concluding, a survival benefit of infiltration with CD8+ TIL is present only in patients that had a complete tumor resection at time of primary surgery.

Figure 1. CD8+ tumor-infiltrating T cells associate with improved prognosis in completely cytoreduced patients only. **A)** Representative images of a 1 mm tissue core with high infiltration of CD8+ cells (top) and without CD8+ cells (bottom). Insets indicate magnification at 4x and 16x. **B)** Total CD8+ cell counts within 1 mm2 tumor epithelium per patient in tissue from the primary surgery cohort (PS) and from the neo-adjuvant chemotherapy cohort (NACT). **C)** Disease-specific survival (DSS) of patients in the primary surgery cohort with a high or low infiltration of CD8+ cells in the tumor epithelium (p=0.052). **D)** DSS of patients in the neo-adjuvant chemotherapy cohort with a high or low infiltration of CD8+ cells in the tumor epithelium (p=0.992). **E)** DSS of patients in which cytoreductive surgery was complete (no residual tumor tissue) or incomplete (residual tumor tissue) and that displayed either as having a high or low infiltration of CD8+ cells in the tumor epithelium (complete: p=0.028; incomplete: p=0.897). **F)** Numbers of infiltrating CD8+ cells in patients in which cytoreductive surgery was complete or incomplete.
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Figure 1

A 4x 4x
1 mm

1 mm

B

CD8+ cells/mm² tumor

n=70
n=53

0
100
101
102
103
104
105

PS NACT

Disease-specific survival

Follow-up in years

1 2 3 4 5 6 7 8 9 10

Disease-specific survival

Follow-up in years

1 2 3 4 5 6 7 8 9 10

Disease-specific survival

Follow-up in years

1 2 3 4 5 6 7 8 9 10

Disease-specific survival

Follow-up in years

1 2 3 4 5 6 7 8 9 10

Disease-specific survival

Follow-up in years

1 2 3 4 5 6 7 8 9 10

E 250 225 200 175 150 125 100 75 50 25 0

CD8+ cells/mm² tumor

PS cohort

Disease-specific survival

high CD8+ cells

low CD8+ cells

no residual tumor

residual tumor

n=13
n=19
n=10
n=28

p=0.052

p=0.992

p=0.283

p=0.123
CD27 is expressed on CD8+ TIL of HGSC in situ

Interestingly, in adoptive cell transfer (ACT) studies, undifferentiated T cells provide greater control over established large tumor masses, with terminally differentiated T cells providing only poor tumor control (16). Therefore, we wondered whether less-differentiated T cells were equally associated with better tumor control in HGSC patients in which complete cytoreduction could not be achieved. Here-to, we analyzed expression of the differentiation marker CD27, known to be a key marker associated with improved outcome in ACT. First, to assess co-expression of CD27 on CD8+ TIL, whole slides from 10 patients with a high infiltration of CD8+ cells (95.9-2555.0 cells/mm²) were selected from the TMA dataset and stained for CD27 and CD8 using immunofluorescence. First, we determined if tumor regions could be differentiated from stromal regions by using DAPI nuclear staining. Tumor slides were stained with anti-EpCAM and anti-fibronectin antibodies, followed by DAPI staining of the nuclei. By means of DAPI, tumor islands were selected and checked for expression of EpCAM and fibronectin, showing that indeed the differentiation between tumor islands (EpCAM⁺) and stromal regions (fibronectin⁺) could be made (data not shown).

Figure 2A shows a representative image for the double staining of CD8 and CD27, with the background staining of DAPI. Within the tumor islands, both CD8 and CD27 positive cells could be detected which were either single or double-positive (Fig. 2B, 2C). While CD8+CD27+ cells were the dominant subtype, clear differences were detected in the percentages of CD8+CD27- and CD8+CD27+ cells/mm² tumor epithelium within this group of patients (Fig. 2D). Thus CD27 is expressed on CD8+ TIL in HGSC, with variability in infiltration between patients.
Figure 2. CD27 is expressed on CD8+ T cells in situ. 

A) Image of a highly infiltrated tumor (full slide) with CD8 in magenta, CD27 in green and DAPI nuclear staining in blue. Magnification 1.5x and 2.5x. 

B) Single stains for CD8, CD27 and DAPI, as well as double staining for CD8 and CD27. Inset represents the indicated area of panel A. 

C) CD8 and CD27 double positive cells pseudocolored in yellow, with DAPI nuclear staining in blue. 

D) Bar graph representing the total percentage of CD8 and CD27 single positive and CD8CD27 double positive cells of all counted cells within 1 mm² of tumor epithelium. Each bar represents 1 patient, from left to right in order of total CD8 count.
CD27+ TIL represent a less-differentiated antigen-experienced subset of CD8+ T cells

To further analyze the phenotype of CD8+ and CD27+ infiltrating cells, TIL were isolated from fresh tumor tissue (N=9, Table 1) and analyzed by flow cytometry. The gating strategy for live CD8+CD27+ cells is depicted in Figure 3A. First, we compared TIL populations isolated from fresh tumor tissue to fluorescent staining results of full slides. The percentages of CD27+ cells within the CD8+ subpopulation found were comparable for samples analyzed by either of the two techniques, confirming the reproducibility of the used methods of analysis (Fig. 3B; p=0.440). Within the population of CD8+ TIL, we then compared the CD27+ with the CD27- cells for differentiation markers. Here, no differences were found in expression of CD45RO (Fig. 3C; p=0.421), but CCR7 was predominantly expressed in the CD27+ group (Fig. 3D; p=0.042). Indeed, most of the CD27+CD8+ cells were double positive for CD45RO and CCR7 (Fig. 3E), confirming the less-differentiated, yet antigen-experienced phenotype of these TIL. CD8+CD27- cells had relatively comparable levels of CD45RO+ cells positive or negative for CCR7, suggesting this cell subset represents a further differentiated phenotype compared to the CD8+CD27+ population (Fig. 3F).

PD1 was expressed on most of the CD8+ TIL (Fig. 3G) with no difference within the subsets with or without CD27 expression (p=0.095). On the other hand, the marker for recent T cell activation CD137, was found to be expressed on a significantly higher percentage of CD27+CD8+ cells in comparison to the CD8+CD27- population (Fig. 3H; p=0.008). Conversely, within the total CD8+CD137+ population, most cells were CD27+PD1+, which was a significantly higher percentage compared to the CD8+CD137- subpopulation (Fig 3I, p=0.016). Comparing the subsets with or without expression of CCR7 within the CD8+CD27+ population, showed that there was no difference in the percentage of CD137+PD1+ cells (Fig. 3J; p=0.440). Taken together, CD27+ TIL represent a less-differentiated, potentially activated, antigen-experienced subset of CD8+ T cells.
Figure 3. CD27 is predominantly expressed on antigen-experienced, recently activated CD8+ T cells. Ovarian tumor tissue was subjected to enzymatic digestion and analyzed by flow cytometry. A) Gating strategy for live CD8+CD27+ TIL. B) Comparison of the total percentage of CD27+ cells within the CD8+ population in tumor digest vs tissue slides (immunofluorescence; Fig. 2). C) Percentage of CD45RO+ cells on the total CD8+ population or the indicated CD8+CD27+ and CD8+CD27- populations. D) CCR7 expression on the total CD8+ population or the indicated CD8+CD27+ and CD8+CD27- populations. E) CD45RO and CCR7 expression on CD27+CD8+ TIL. F) CD45RO and CCR7 expression on CD27+CD8+ TIL. G) PD1 expression on the total CD8+ population or the indicated CD8+CD27+ and CD8+CD27- populations. H) CD137 expression on the total CD8+ population or the indicated CD8+CD27+ and CD8+CD27- populations. I) Expression of CD27 and PD1 on the indicated CD8/CD137+ subpopulations. J) Expression of CD137+/PD1+ on the indicated CCR7+/ CD8+CD27+ subpopulations.
CD27 infiltration in HGSC is not different between the PS and NACT cohort

Based on the flow cytometry results, we hypothesized the CD27 subset of TIL to resemble more naive-like TIL which are therefore better capable of immune control of the tumor, presumably due to a greater expansion potential (17). In order to further analyze this, expression of this marker was determined in both the PS and the NACT cohort. In the PS cohort 85.5% out of a total of 76 patients demonstrated CD27+ cell infiltration. Median cell count was 13.94 cells per mm$^2$ of tumor epithelium (IQR: 30.92), revealing lower total cell counts compared to CD8+ (Fig. 4A; 4B vs. 1B). Infiltration was not influenced by any of the prognostic factors age (p=0.832), stage (p=0.161), or surgery status (p=0.749).

In the NACT cohort the same trend could be observed. In comparison to CD8, a lower percentage of patients had CD27 infiltration (86.4%) and the total number of cells in these tumors was lower (median: 10.83, IQR: 30.25) (Fig. 4B vs. 1B). There were no differences in amount of cells within patient groups regarding age (p=0.965), FIGO stage (p=0.773), or surgical result (p=0.902). In order to determine whether the chemotherapy had influenced the infiltration of CD27+ TIL, we compared cell numbers between the two datasets and found no differences between the two cohorts (Fig. 4B, p=0.891). Thus, in the majority of patients in both cohorts infiltration of CD27+ cells was detected, with higher numbers of CD8 as compared to CD27, which was not influenced by any clinicopathological factors or neo-adjuvant chemotherapy.

CD27+ TIL are strongly associated with survival in the PS cohort

To determine the prognostic value of CD27+ TIL infiltration, patients were subdivided based on cut-off for the highest tertile of CD27 cell counts. In the PS cohort, DSS analysis based on CD27 expression showed a clear survival benefit for the highly infiltrated group (p=0.001, Fig. 4C). In the NACT cohort, no differences in survival could be detected within the groups with a high or low infiltration of CD27+ (cut-off highest tertile) (Fig. 4D).

If indeed CD27+ TIL show better tumor control, it is to be expected that these cells show prognostic value in completely resected tumors as well as in patients who had remaining tumor tissue following surgery. To determine whether CD27+ cells can compensate for incomplete removal of the tumor, we again analyzed groups based on surgical outcome in the PS cohort. Indeed, a clear survival benefit could not only be observed in patients without residual disease (p=0.011), but also in the group of patients which were incompletely cytoreduced (p=0.017; Fig. 4E). The survival benefit in the incomplete group can be attributed completely to the optimally cytoreduced patients (≤1 cm; p=0.021), while no benefit was observed in the patients that had >1 cm of residual tumor after surgery (p=0.369) (Supplementary Fig. 2).
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Figure 4

A. Disease-specific survival

Follow-up in years

Disease-specific survival

n=25
n=51
p=0.001

B. CD27+ cells/mm² tumor

n=76
n=59

PS NACT

C. PS cohort

n=12
n=13
n=21
n=30

D. NACT cohort

n=40
n=19
p=0.201

E. PS cohort

n=326; 407

F. PS cohort

CD27+ cells/mm² tumor

no residual tissue residual tissue

320, 407

↑
To confirm the value of CD27 over CD8 as a marker for prognosis in HGSC, we performed a multivariate Cox regression analysis (Table 2), including all variables shown to be associated with survival in univariate analyses (FIGO stage, surgery result and age). In this model, only surgical result (hazard ratio (HR): 1.50, 95% CI: 1.24-1.80) and CD27+ cell infiltration (HR: 0.23, 95% CI: 0.10-0.56) proved to be of prognostic value.

Taken together, the CD27+ TIL subset is more strongly associated with a favorable prognosis compared to the CD8+ TIL in HGSC patients, due to survival benefit in both patients with residual macroscopic disease and patients with no residual macroscopic disease. Whereas CD8+ TIL provide survival benefit only in patients where no macroscopic disease is present after cytoreductive surgery.

<table>
<thead>
<tr>
<th>Variable</th>
<th>HR</th>
<th>P value</th>
<th>95% CI</th>
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<td>FIGO stage</td>
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<td>0.24</td>
<td>0.80-2.42</td>
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<tr>
<td>Surgical result (residual)</td>
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<td>&lt;0.001</td>
<td>1.24-1.80</td>
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<td>Age (&gt;59 years)</td>
<td>1.92</td>
<td>0.123</td>
<td>0.84-4.41</td>
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<td>CD8 (highest tertile)</td>
<td>1.44</td>
<td>0.385</td>
<td>0.63-3.30</td>
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<td>CD27 (highest tertile)</td>
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Figure 4. CD27+ TIL are strongly associated with survival in both completely and incompletely-cytoreduced HGSC patients. A) Representative images of a 1 mm tissue core of a highly infiltrated tissue (top) and one with low infiltration (bottom) of CD27+ cells. Insets indicate magnification at 4x and 16x. B) Total CD27+ cell counts per mm2 tumor tissue for patients in the primary surgery (PS) and neo-adjuvant chemotherapy (NACT) cohort. C) Disease-specific survival (DSS) of patients in the PS cohort with a high or low infiltration of CD27+ cells in the tumor epithelium (p=0.001). D) DSS of patients in the NACT cohort with a high or low infiltration of CD27+ cells in the tumor epithelium (p=0.201). E) DSS of patients in which cytoreductive surgery was complete (no residual tumor tissue) or incomplete (residual tumor tissue) and that displayed as either a high or low infiltration of CD27+ cells in the tumor epithelium (complete: p=0.011, incomplete: p=0.017). F) Numbers of CD27+ infiltrating cells in patients in which cytoreductive surgery was complete or incomplete.
Discussion

In the present study we demonstrate that the prognostic value of TIL in advanced stage HGSC is variable for patients primarily treated with surgery or neo-adjuvant chemotherapy. Furthermore, the differentiation status of infiltrating CD8+ T cells, as determined by CD27 expression, proved to be associated with a survival benefit superior to that observed for the overall CD8+ TIL population. This differential impact of various T cell subsets is related to surgical result of primary surgery. The less-differentiated CD27+ TIL were found to have a prognostic benefit even in patients with residual tumor tissue after surgery.

TIL have long been known to be associated with a favorable prognosis in EOC, with ratios between subtypes of cells, their activation status and cytolytic activity as determining factors (8,9). We analyzed two patient cohorts of advanced stage HGSC treated with either surgery or neo-adjuvant chemotherapy as the primary treatment modality. Patient selection for the latter treatment is based on tumor dissemination, comorbidity and performance status. Therefore, not surprisingly, the patients in the NACT cohort showed a worse prognosis in general. Also, when studying parameters that were of clear prognostic value in the PS cohort, namely residual tissue after surgery and age, the effect on prognosis was less clear or not present at all in the NACT group. This may in part explain why the effect of infiltrating CD8+ or CD27+ cells was also not associated with a survival benefit in this group. Whether this is due to the response of T cells to chemotherapy cannot be excluded, although total cell counts of these cell populations do not indicate a difference in infiltration. This is in line with the observation in mice that high dose carboplatin or paclitaxel treatment does not affect the amount of circulating T cells, nor the number of ovalbumin-specific T cells after vaccination (18). Further studies will have to reveal whether functional differences are present in the TIL of tumors treated with these agents. Also, it is of interest whether response to chemotherapy can be predicted by TIL infiltrate as is the case in breast cancer (12–14).

In both cohorts, the most pronounced indicator of survival is the result of cytoreductive surgery, with a more distinct effect in the primary surgery cohort, consistent with observations by Rosen et al. (19). In line with the immune tumor control hypothesis, one would expect that the influence of TIL infiltration on prognosis is affected by the amount of residual tumor tissue after surgery. Indeed, our data indicate that CD8+ TIL are of prognostic benefit only when maximum tumor cytoreduction can be achieved. Less-differentiated CD27+ TIL were able to compensate for incomplete surgical resection of the tumor, but only to a limited extent (residual tumor mass <1 cm). These data clearly indicate that the level of cytoreduction should be taken into account when studying immunomodulating effects. Of note, surgical outcome may not be a predictor of survival itself, but rather a reflection of the underlying tumor biology. Indeed, it was recently shown that a specific gene expression signature correlates with surgical outcome in ovarian can-
cer (20). In our cohort, this may have led to a selection bias for the NACT cohort, since patients were selected for NACT if chance of up-front complete cytoreduction in primary surgery was minimal, thus these patients possibly differ in expression of key genes. Therefore, the lack of prognostic effect of infiltrating CD8 and CD27 cells in the NACT cohort could potentially be due to a difference in tumor biology. It will be of interest to determine whether patients with the two profiles show contrasting prognostic effects of immune infiltrates, and if so, whether a combined immune and cytoreduction gene signature can be identified for the stratification of patients or as a selection criterion for patients likely to respond to immunotherapy.

Our first in-human analysis of intraepithelial CD27+ TIL suggests these cells to represent a more potent tumor-controlling subset of CD8+ TIL in HGSC. These observations are in line with recent adoptive cell transfer (ACT) studies in melanoma. In particular, CD27+ TIL were found to persist longer after ACT and had a higher reactivity upon re-administration (21), presumably due to a greater expansion potential in response to antigen exposure (17). Not surprisingly, the proportion of CD8+CD27+ cells within the transferred TIL was therefore strongly associated with tumor regression in patients (22). Furthermore, introduction of a CD27 intracellular signaling domain to chimeric antigen receptor (CAR) T cells led to longer persistence after infusion and improved efficacy in an ovarian cancer xenograft mouse model (23). This matches our results showing longer survival in patients that have infiltrating CD27+ cells in their tumors. Interestingly, while CD27 is constitutively expressed on naive T cells, further upregulation is induced upon activation via TCR signaling, while loss of CD27 occurs in more terminal stages of differentiation. Therefore, the CD8+CD27+ subpopulation found within tumors might represent a more activated, tumor-reactive subset of cells. Indeed, when compared to the CD27- CTL, CD27+ CTL demonstrated a significantly higher percentage of cells expressing CD137, the recently-described marker for tumor-reactive T cells in HGSC (24). Conversely, CD137+ CTL were predominantly (>80%) double-positive for CD27 and PD-1. In addition to this increased capacity for tumor recognition, CD27+ CTL also expressed CD45RO and CCR7, indicating that these cells are indeed antigen-experienced, but not yet terminally differentiated (25).

Our results suggest CD27 as an interesting target for immunotherapy in HGSC, in which stimulation of T cells via CD27 signaling might evoke anti-tumor responses in patients. A first humanized anti-CD27 antibody (1F5) was well-tolerated in non-human primates and was explored for the treatment of hematological malignancies overexpressing CD27 (26). Treatment with the 1F5 antibody further inhibited growth of CD27+ human lymphoma cells in SCID mice and, in a human CD27-transgenic mouse model, led to an increase in antigen-specific CTL with concomitant anti-tumor activity (27). The 1F5 antibody is currently in phase I trial for the treatment of hematological malignancies and solid tumors. Based on the co-expression of CD27 and PD1 on TIL, combining agonistic CD27 antibody with a checkpoint blockade targeting the PD1/PD-L1 axis might be of further interest,
since preclinical studies have shown a synergistic effect of combinatorial antibody treatment (28).

Currently, also other treatment strategies for HGSC are being explored including strategies targeting angiogenesis (e.g. Bevacizumab) and poly(ADP-ribose) polymerase (PARP) inhibitors in tumors with alterations in the homologous recombination repair pathway. Whether supplementation of these strategies to current PS or NACT treatment regimens may affect the surgical outcome and overall prognosis of patients remains to be determined. Furthermore, effect it may have on the immune infiltrate and potential synergism with immunotherapy is of interest (29). Exploration of these ideas can help to stratify patients with respect to different treatment modalities to predict the patients who might benefit most from immunotherapy.

In conclusion, the methodology of interpreting TIL infiltrates in tissue of HGSC and the cut-off values for positive samples need to be standardized before it can be considered as a prognostic marker or serve as a selective marker for treatment strategies. Here we showed that the treatment regimen, surgical outcome, and the differentiation status of TIL should also be taken into account.
References
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Supplementary data

Supplementary Methods

Immunohistochemistry

TMA slides were stained with anti-CD8 and anti-CD27 antibodies. After deparaffinization and rehydration, slides were subjected to heat-induced epitope retrieval (HIER) by microwaving the slides in a citrate buffer (10 mM citrate, pH 6.0) for 15 minutes. After cooling down, endogenous peroxidase was blocked 30 minutes in a 0.3% H2O2 solution. Slides were then incubated with either mouse anti-human CD8 antibody (DAKO, Heverlee, Belgium; clone: C8/144B, 1:25 in blocking buffer (1% BSA/PBS with 1% human AB serum)) or rabbit anti-human CD27 antibody (Abcam, Cambridge, UK; clone: EPR8569, 1:150 in blocking buffer) overnight at 4°C. The next day slides were incubated with either anti-mouse or anti-rabbit Envision secondary antibodies (K4007, K4011; DAKO). Antibody binding was visualized with 3,3’-diaminobenzidine (DAB) and slides were counterstained with hematoxylin.

Multi-color immunofluorescence

On the basis of the highest infiltration with CD8+ cells, 10 patients were selected from the TMA dataset and full tumor tissue slides were retrieved for further analysis. Slides were deparaffinized, rehydrated, and HIER was performed in a citrate buffer (10 mM citrate, pH 6.0). After cooling, endogenous peroxidase was blocked 30 minutes in a 0.3% H2O2 solution. Slides were then incubated overnight with mouse anti-human CD8 (DAKO, clone C8/144B, 1:25 ) and rabbit anti-human CD27 (Abcam, clone EPR8569, 1:150). These were visualized with goat anti-rabbit Alexa Fluor-488 and goat anti-mouse Alexa Fluor-555 (1:150, Life Technologies, Bleiswijk, The Netherlands). Counterstaining was done by 4',6-diamidino-2-phenylindole (DAPI). Slides were mounted in Prolong Gold (Life Technologies) and stored in the dark at RT. In order to differentiate tumor from stromal regions, slides were stained as described above, with a rabbit polyclonal antibody to Fibronectin (1:50, Abcam) and mouse anti-human EPCAM antibody (clone: AUA1, 1:50, Abcam).

Multi-parameter flow cytometry

From the digested tumor samples TIL were phenotyped by multiparameter flow cytometry. The Zombie Aqua Fixable Viability Kit was used for live/dead stain according to manufacturer’s instructions (BioLegend, Uithoorn, The Netherlands). Antibodies used were CD3-PerCP-Cy5.5 (OKT3), CD8-APC-eFluor780 (RPA-T8), CD45RO-PE-Cy7 (UCHL1), CD137-PE (4B4-1), and PD1-APC (MIH4) (all eBioscience, Vienna, Austria), CCR7-BV421 (150503) (BD Biosciences, Etten-Leur, The Netherlands), and CD27-FITC (9F4) (Sanquin, Amsterdam, The Netherlands). All flow cytometry was performed on a FACSVerse (BD Biosciences) and samples were analyzed with Cytobank software (cytobank.org).
Figure S1: Disease-specific survival (DSS) analyses by Kaplan Meier method of cohort treated with primary surgery (PS) and cohort treated with neo-adjuvant chemotherapy (NACT). A) DSS depicted for patients in PS cohort split on surgical outcome. B) Age is a predictor for survival in the PS cohort. C) Prognostic effect of surgical outcome in the NACT cohort. D) Age is not a predictor of survival in the NACT cohort.

Figure S2: Disease-specific survival (DSS) analyses by Kaplan Meier method of the primary surgery (PS) cohort for high and low infiltration of CD27+ cells. CD27 infiltration in the tumor epithelium is a prognostic factor in patients in which the surgical cytoreduction resulted in residual tissue of maximum 1 cm. Groups were created on basis of surgical outcome; red line shows patients that had no residual tumor after surgery, the violet lines depict patients that had residual tumors of ≤1 cm, blue line represents patients with >1cm of residual tumor.
The immune environment in ovarian cancer