Serum CD95L Level Correlates with Tumor Immune Infiltration and Is a Positive Prognostic Marker for Advanced High-Grade Serous Ovarian Cancer

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Abstract

Soluble CD95L (s-CD95L) is a chemoattractant for certain lymphocyte subpopulations. We examined whether this ligand is a prognostic marker for high-grade serous ovarian cancer (HGSOC) and whether it is associated with accumulation of immune cells in the tumor. Serum s-CD95L levels in 51 patients with advanced ovarian cancer were tested by ELISA. IHC staining of CD3, CD4, CD8, CD20, CD163, CD31, FoxP3, CCR6, IL-17, Granzyme B, PD-L1, and membrane CD95L was used to assess tumor-infiltrating immune cells. Although the intensity of CD3, CD4, CD8, and CD163 in tumor tissues remained constant regardless of membrane CD95L expression, tumors in patients with HGSOC with s-CD95L levels ≥516 pg/mL showed increased infiltration by CD3+ T cells (P = 0.001), comprising both cytotoxic CD8+ (P = 0.01) and CD4+ (P = 0.0062) cells including FoxP3+ regulatory T cells (P = 0.0044). Also, the number of tumor-infiltrating CD20+ B cells (P = 0.0094) increased in these patients. Multivariate analyses revealed that low s-CD95L concentrations (<516 pg/mL, HR, 3.54; 95% confidence interval (CI), 1.13–11.11), and <1,200 activated CD8+ (Granzyme B+) cells (HR, 2.63; 95% CI, 1.16–5.95) were independent poor prognostic factors for recurrence, whereas >6,000 CD3+ cells (HR, 0.34; 95% CI, 0.15–0.79) was a good prognostic factor. Thus, low levels of s-CD95L (<516 pg/mL) are correlated with lower numbers of tumor-infiltrating lymphocytes (CD3+ and CD8+, and also CD4 and FoxP3 T cells) in advanced HGSOC and are a poor prognostic marker.

Implications: Serum s-CD95L is correlated with a number of tumor-infiltrating immune cells in HGSOC and could be used as a noninvasive marker of tumor immune infiltration to select patients referred for immunotherapy trials that evaluate checkpoint inhibitor treatment.

Introduction

Ovarian carcinoma is the seventh most common cancer in women and the eight most common cause of cancer-related death worldwide (1). At the time of diagnosis, the majority of patients with epithelial ovarian cancer (EOC) present with advanced disease, which is characterized by a high and widespread tumor load in the peritoneal cavity, often accompanied by malignant ascites. Thus, the prognosis of women with ovarian cancer remains poor, with a 5-year overall survival (OS) rate estimated at <45% for all cancer stages, and only 20% to 30% for patients with Federation of Gynecologists and Obstetricians (FIGO) stage III or IV disease (1, 2).

Patients with EOC and other cancers who exhibit a robust immune response show increased survival rates (3). Recent studies show that number of tumor-infiltrating lymphocytes (TILs), which are lymphocytes that extravasate from blood vessels to access the tumor, may be a positive predictive factor for melanoma (4), colorectal cancer (5), esophageal carcinomas (6), breast cancer (7, 8), or endometrial cancer (9). The presence of TILs affects the outcome of ovarian cancer; high numbers of CD8+ T cells in the immune infiltrate are associated with improved OS (10–15), particularly in patients with high grade serous ovarian carcinoma (HGSOC; refs. 16–18). In contrast, clinical outcome of ovarian cancers infiltrated by regulatory FoxP3+ T cells (Tregs) remains unclear; studies suggest either decreased OS (19–21) or improved clinical outcomes (22–24). Furthermore, some works establish that ovarian cancer is often
accompanied by systemic immunosuppression (25, 26), which correlates with a poor prognosis (27, 28). New treatment strategies based on neutralizing antibodies that target checkpoint inhibitors represent a revolution in the fight against cancer; indeed, such treatments have shown survival benefits in patients with melanoma (29) or lung cancer (30). Therapeutic antibodies that block the PD1/PD-L1 checkpoint (such as nivolumab or pembrolizumab) or the CTLA4 (such as ipilimumab) show therapeutic responses linked to tumor immune infiltration (31, 32). Phase II studies of anti-PD1/PD-L1 therapy in ovarian cancer suggest that it triggers antitumor responses (33–35); and, several phases III studies are underway. In such cases, a serum thermanostic biomarker would be useful for selecting patients that are eligible for immunotherapy trials.

CD95L (also called Fasl), which belongs to the TNF family, binds to the receptor CD95 (also known as Fas). Whereas CD95 is ubiquitously expressed, CD95L shows a more restricted expression pattern; it is expressed mainly on the membrane of lymphocytes, where it plays a pivotal role in eliminating infected and transformed cells (36). Binding of membrane bound CD95L to CD95 recruits the adaptor protein Fas Associated Death Domain (FADD; ref. 37), which in turn aggregates caspase-8 and caspase-10 to induce apoptosis (38). CD95L is also expressed by endothelial cells lining the blood vessels of patients with tumors and chronic inflammatory disorders (39–41). These CD95L-expressing endothelial cells seem to behave as a selective immune barrier, killing CD8 T cells while being permissive for Treg cells (41). CD95L can be cleaved by metalloproteases, thereby releasing soluble CD95L (s-CD95L) into the bloodstream. Binding of s-CD95L to CD95 fails to trigger cell death but rather induces a nonapoptotic signaling pathway that promotes the migration of T cells (42). Here, we wondered whether s-CD95L plays a role in ovarian cancer and examined its impact on the immune landscape in patients with HGSOC.

**Materials and Methods**

**Cell lines and culture conditions**

IGROV-1, OVCAR-3, OVCAR-8, and SKOV-3 cell lines were obtained from ATCC (LGC Standards) and were authenticated by short tandem repeat. Each month, routine testing was conducted on all cultured cells using the sensor-cell approach PlasmoTest - short tandem repeat. Each month, routine testing was conducted using the Biinchonic Acid Method (Pierce). Proteins were separated on an 8% SDS-PAGE gel and transferred to a nitrocellulose membrane (GE Healthcare). The membrane was blocked for 30 minutes with TBST (50 mmol/L Tris, 160 mmol/L NaCl, 0.05% v/v Tween 20, pH 7.8) containing 5% w/v dried skimmed milk or BSA and incubated overnight at 4°C with primary antibodies (mouse anti-E-Cadherin, clone 36, BD Pharmingen; mouse anti-Vimentin, clone V9, DAKO; and mouse anti-α-actin, clone AC-74, Sigma) diluted in the same buffer used for saturation (milk for anti-E-cadherin and anti-α-actin antibodies and BSA for anti-vimentin antibodies). The membrane was washed with TBST, followed by incubation for 1 hour with a peroxidase-conjugated anti-mouse antibody (SouthernBiotech). Proteins were visualized using the enhanced Chemiluminescence Substrate Kit (GE Healthcare).

**Flow cytometry analysis**

Cells were stained for 30 minutes at 4°C with anti-CD24 (clone ML5, BD Pharmingen), CD44 (clone G44-26, BD Pharmingen), and anti-CD95 (clone DX2, BD Pharmingen) mAbs. Isotype-matched murine fluorochrome-conjugated immunoglobulins (PE mouse IgG2a, APC mouse IgG2b, and PE mouse IgG1, respectively; BD Pharmingen) were used as negative controls.

**qRT-PCR**

Total RNA was extracted from the cells using the NucleoSpin RNA Isolation Kit (Macherey-Nagel) and cDNA was generated from 1 μg of total RNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Quality and quantity of total RNA and cDNA was measured using a DS-11 Spectrophotometer (DeNovix). Expression of mRNA was measured using the TaqMan Gene Expression Assay Kit (Applied Biosystems), which contains primers and TaqMan MGB probes specific for the following genes: FAP1 (assay no: Hs00196632_m1), ZEB1 (Hs00611018_m1), ZEB2 (Hs00207691_m1), CDH1 (Hs00170423_m1), SNAI1 (Hs00195591_m1), TWIST1 (Hs01675818_s1), and human GAPDH (Hs99999905_s1; endogenous control). The probes targeting SLUG (ENST00000020945.3|ENSG00000019549.9) were designed using the Universal Probe Library's Assay Design Center Tool (Roche), and the forward (TGGTGTCTCCTACGGCAGCAT) and reverse (GCAAATGCCTCTGTACGCGT) primers were purchased from Eurogentec. Expression of SLUG mRNA was measured using PowerUp SYBR Green Master Mix (Applied Biosystems), with

**Generation of cleaved CD95L**

HEK/293T cells cultured in 1% FCS containing medium were transfected with 3 μg of empty plasmid or a wild-type CD95L-containing vector using the calcium/phosphate precipitation method. Medium containing s-CD95L and exosome-bound full-length CD95L was harvested 5 days after transfection. Dead cells and debris were removed by centrifugation (2 × 4,500 rpm/15 minutes). Exosomes were pelleted by ultracentrifugation at 100,000 × g for 2 hours. Finally, debris- and exosome-free supernatants were concentrated (Centricon; 10 kDa cutoff) and dialyzed against PBS.

**Western blot analysis**

Cells were lysed for 30 minutes at 4°C in lysis buffer (25 mmol/L HEPES pH 7.4, 1% v/v Triton X-100, 150 mmol/L NaCl, 2 mmol/L EGTA supplemented with a mix of protease inhibitors; Sigma-Aldrich). Protein concentration was determined using the Biinchonic Acid Method (Pierce). Proteins were separated on an 8% SDS-PAGE gel and transferred to a nitrocellulose membrane (GE Healthcare). The membrane was blocked for 30 minutes with TBST (50 mmol/L Tris, 160 mmol/L NaCl, 0.05% v/v Tween 20, pH 7.8) containing 5% w/v dried skimmed milk or BSA and incubated overnight at 4°C with primary antibodies (mouse anti-E-Cadherin, clone 36, BD Pharmingen; mouse anti-Vimentin, clone V9, DAKO; and mouse anti-α-actin, clone AC-74, Sigma) diluted in the same buffer used for saturation (milk for anti-E-cadherin and anti-α-actin antibodies and BSA for anti-vimentin antibodies). The membrane was washed with TBST, followed by incubation for 1 hour with a peroxidase-conjugated anti-mouse antibody (SouthernBiotech). Proteins were visualized using the enhanced Chemiluminescence Substrate Kit (GE Healthcare).

**Spheroid formation assays**

Cells (1,000/well) were seeded in 96-well ultra-low attachment plates (Corning) and cultured for 7 days in serum-free culture medium (MammoCult Human Medium Kit, Stemcell Technologies) supplemented with heparin (Stemcell Technologies) and cultured (39, 40). After resuscitation of frozen aliquots, spheres per well was counted manually by taking five large field pictures of each well using a 4 × objective lens.

**Transcription Kit (Applied Biosystems). Quality and quantity of total RNA and cDNA was measured using a DS-11 Spectrophotometer (DeNovix). Expression of mRNA was measured using the TaqMan Gene Expression Assay Kit (Applied Biosystems).**

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**Flow cytometry analysis**

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human GAPDH used as an endogenous control. cDNA (50 ng) was used for each qPCR reaction. All qPCR experiments were performed in a QuantStudio5 Machine (Applied Biosystems) and the data were analyzed using Thermo Fisher Connect Software (Thermo Fisher Scientific). Relative expression of each gene was calculated using the 2–ΔΔCt method and expressed as a fold change.

Cell death assay
Cell viability was measured in an MTT assay. Briefly, 4 × 10⁴ cells were cultured for 24 hours in flat-bottom, 96-well plates along with the indicated concentrations of apoptosis inducer (final volume, 100 µL). Next, 15 µL MTT (5 mg/mL in PBS) solution was added and incubated at 37°C for 4 hours. Absorbance was measured using the Infinite F200 Pro (Tecan) at a wavelength of 570 nm.

Cell migration assay
To determine whether s-CD95L contributed to T-cell migration, T cells were exposed to s-CD95L or control medium in Boyden chambers. This migration assay was described previously (43). Briefly, to measure trans-endothelial migration of activated T cells, a 3-µm sized porous membrane of a Boyden chamber was used. Human umbilical vein endothelial cells (HUVEC) were plated to form a monolayer mimicking endothelial barrier. Membranes were first hydrated in sterile PBS, then, CD3/CD28-activated peripheral blood lymphocytes (PBL) isolated from healthy donors (3 × 10⁵ cells/300 µL) were added to the top chamber covered with a monolayer of HUVEC in a low serum (1%)-containing RPMI. Bottom chamber contained 500 µL of RPMI 1% FBS in presence or absence of s-CD95L (100 ng/mL). Cells were cultured in a CO₂ incubator at the same conditions as adherent cells for 24 hours. Transmigrated cells were then counted in the lower reservoir.

Patients
All clinical investigations were conducted in accordance with the principles outlined in the Declaration of Helsinki. Blood samples were collected from patients diagnosed with ovarian cancer after written informed consent was obtained. The study was approved by the local institutional review board (CEROG 2016-GYN-1003). Samples collected prospectively from patients diagnosed with ovarian cancer between January 2010 and December 2013 were reviewed retrospectively. All samples were obtained at the time of diagnosis and before chemotherapy treatment. A total of 51 patients with advanced stage ovarian cancer were analyzed: 37 with HGSOC, 6 with endometrioid subtype, 4 with mucinous subtype, 2 with clear cell subtype, and 2 with low grade serous ovarian cancer. Thirty-six patients received neoadjuvant chemotherapy after tumor sample plus interval debulking surgery, and 15 underwent primary debulking surgery followed by adjuvant chemotherapy. The surgical specimens were evaluated histologically at the Department of Pathology. All patients were staged according to the FIGO staging system (44).

After surgery, all patients received standard chemotherapy comprising carboplatin plus paclitaxel. For this study, the main inclusion criterion was ovarian cancer with FIGO stage IIC and IV (i.e., carcinomatosis stage or higher) and a serum sample in which the s-CD95L level before chemotherapy could be measured. Progression-free survival (PFS) was defined as the time from diagnosis of ovarian cancer to the time of recurrence or death. OS was defined as the time from diagnosis of ovarian cancer to the time of death. Observation time was defined as the interval between diagnosis and time of last contact (death or last follow-up). Data were censored at death or at the last follow-up for patients without recurrence.

Measurement of s-CD95L by ELISA
S-CD95L concentrations in the serum of patients with HGSOC and healthy donors were measured by ELISA (Diaclone). All blood samples were harvested at the time of diagnosis.

Tissue specimens and IHC staining
Surgical specimens were fixed in 4% formalin, embedded in paraffin, and stained with hematoxylin–eosin–saffron (HES). Sections from each histologic specimen were reviewed by two experienced pathologists (University Hospital, Rennes, France) to confirm the diagnosis and grade according to the method of Silverberg or Malpica (35). For each patient, a representative HES slide and the corresponding paraffin block were selected. The selected slide had to contain both tumor and adjacent stroma. The formalin-fixed, paraffin-embedded blocks were cut into 5-µm slices and mounted on SuperFrost Plus Microscope Slides (Menzel-Glaser). Expression of CD3 (clone SP7; Thermo Fisher Scientific), CD4 (clone SP35; Cell Marque), CD8 (clone C8/144B; Dako) CD20 (clone L26; Dako), CD163 (clone 10D6; Leica), CD31 (clone JC70A; Dako), Podoplanin (clone D2-40; Invitrogen), FoxP3 (clone SP97; Eurobio Scientific), Granzyme B (clone GbR-7; Millipore), IL17 (clone bx-2140R; Bios antibodies), and PD-L1 (clone E1L3N; Cell Signaling Technology) was assessed by IHC (Ventana Discovery XT automaton, Ventana Roche). CD95L (clone G247-4, BD Pharmingen) was immunostained manually. After deparaffinization with toluene and rehydration with ethanol, sections were incubated at 95°C and bathed in Tris-EDTA at pH 8 prior to staining for CD4, CD8, FoxP3, CD20, CD3, CD31, CD163, CCR6, and Granzyme B. For CD95L sections were deparaffinized and rehydrated, incubated at 95°C, and immersed in EDTA (pH 9). For all preparations, endogenous peroxidase activity was blocked by incubation in 0.3% hydrogen peroxide. The reactivity of all antibodies, except CD95L, was revealed with a horseradish peroxidase (HRP)-labeled polymer-conjugated secondary antibody followed by diaminobenzidine (DAB; OmniMap DAB Roche). CD95L was revealed with an anti-mouse HRP-labeled polymer-conjugated secondary antibody followed by DAB (DAB Dako).

TIL count
According to the breast cancer guidelines (45) TILs can be subdivided according to their location within the tumor: (i) stromal TILs when located in the peritumoral space and (ii) intraepithelial TILs, when they have penetrated the tumor islets. These recommendations are for breast cancer, and yet, there is no standardized approach to evaluate TILs in EOC. Thus, to avoid a biased evaluation, we decided to perform a representative HES slide, which had to contain both tumor and adjacent stroma, to evaluate overall immune cells whatever their localization in tumor. Each immunostained slide was scanned with a NanoZoomer (Hamamatsu). For each patient, five large field pictures were taken using a 5 × objective lens and Hamamatsu’s Software (NDPview). All fields were analyzed using NIS-Elements Software (Nikon) and positively stained cells were counted; for analysis, a specific threshold was applied for each antibody.
Statistical analysis

Statistical analysis was performed using SAS, v.9.4 (SAS Institute) and R logical Version 3.4.1 software programs. Quantitative results were expressed as the statistical mean ± SD and qualitative results as percentages (%). The Mann–Whitney–Wilcoxon test was used to compare the distribution of quantitative variables between two groups (abnormal statistical distributions). The χ^2 or Fisher exact test was used to compare the distribution of qualitative variables between two groups with theoretical head-counts <5. The method of Contal and O’Quigley was used to determine cut-off values for the continuous variables used to examine prognosis (46). The correlation between TILs and levels of cleaved CD95L was assessed using Spearman correlation. The Kaplan–Meier method was used to compare survival curves between groups. All tests were two-sided and a P < 0.05 was deemed significant.

Ethical statement

This study was agreed to by local institutional review board and French laws. All patients consented to participate.

Results

Serum CD95L is a prognostic marker for HGSOC

The clinical data and outcomes of our current HGSOC cohort match those of previously described cohorts (47, 48), indicating that, although the number of patients was relatively small the cohort is likely to be representative of larger HGSOC cohorts (Supplementary Table S1). In agreement with known clinical cohort is likely to be representative of larger HGSOC cohorts (Supplementary Table S1). In agreement with known clinical 

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m-CD95L and the immune landscape in HGSOC

Next, because tumor-infiltrating immune cells (10, 11, 18) are associated with a good clinical outcome in HGSOC, we examined tissues for the presence of tumor-infiltrating T cells (CD3/CD4/CD8/Th17), B cells (CD20), and macrophages (CD163). The cytolytic activity of CD8⁺ T cells was monitored by staining for Granzyme B. Checkpoint inhibitors are promising therapeutic regimens for patients with HGSOC; therefore, we also stained tissues for PD-L1. Although the intensity of CD3, CD8, CD4, CD20, and CD163 markers did not change regarding the staining of membrane CD95L in tumor tissues, transmembrane CD95L expression was significantly associated with the number of tumor-infiltrating FoxP3 T cells (Table 2). This finding is in agreement with results published by Coukos and colleagues (41), who suggested that m-CD95L is a selective immune barrier; it killed CD8⁺ cells but spared FoxP3-T cells. Nonetheless, we found no significant correlation between the intensity of m-CD95L staining and PFS or OS, indicating that unlike metalloprotease-cleaved s-CD95L, its membrane bound counterpart is not a prognostic marker for patients with HGSOC and that a yet unidentified metalloprotease plays a pivotal role in the disease progression.

Figure 1.
Serum cleaved CD95L is increased in patients with HGSOC with better prognosis. A, Serum s-CD95L level in all ovarian cancer cohort compared with healthy donor. B, Kaplan–Meier analysis of patients with recurrence ovarian cancer with s-CD95L higher (thick line) or lower (dotted line) than 516 pg/mL. C, Kaplan–Meier analysis of recurrence in patients with HGSOC with cleaved CD95L higher (thick line) or lower (dotted line) than 516 pg/mL. D, Serum cl-CD95L level according to DFS (< or ≥12 months) in patients with HGSOC; *P < 0.05. E, Kaplan–Meier analysis of OS in patients with HGSOC with cleaved CD95L higher (thick line) or lower (dotted line) than 516 pg/mL.
De La Motte Rouge et al.

Table 1. Clinical characteristics of patients with HGSOC according to a serum s-CD95L threshold of 516 pg/mL

<table>
<thead>
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<th>Variable(s)</th>
<th>s-CD95L ≥516 pg/mL</th>
<th>s-CD95L &lt;516 pg/mL</th>
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</thead>
<tbody>
<tr>
<td>n = 7</td>
<td>n = 30</td>
<td>P</td>
</tr>
<tr>
<td>Age (years; mean ±SD)</td>
<td>65.1 ± 16</td>
<td>63.3 ± 11.0</td>
</tr>
<tr>
<td>Mammography</td>
<td>No</td>
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<tr>
<td></td>
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<td>6 (89.7%)</td>
</tr>
<tr>
<td>Missing data</td>
<td>0 (0%)</td>
<td>4 (7.7%)</td>
</tr>
<tr>
<td>CA125 level (UI/mL; mean ± SD)</td>
<td>1,032 ± 1,036</td>
<td>4,155 ± 9,370</td>
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<tr>
<td>FIGO stage</td>
<td>IIC</td>
<td>7 (100.0%)</td>
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<tr>
<td></td>
<td>IV</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Nodes Involved</td>
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<tr>
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<td>2 (28.5%)</td>
</tr>
<tr>
<td></td>
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<td>1 (14.2%)</td>
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<tr>
<td>Neoadjuvant chemotherapy</td>
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<td>1 (14.2%)</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>6 (85.7%)</td>
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<tr>
<td>Residual disease after surgery</td>
<td>No</td>
<td>7 (100.0%)</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>0 (0.8%)</td>
</tr>
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</table>

NOTE: *P < 0.05 in bold.
Abbreviation: BRCA, breast cancer gene.

lupus (ref. 52; Supplementary Fig. S4C) and expression of matrix-metalloproteinase-7 (MMP7) is higher in these patients (53). MMP7 might be a good candidate for the metalloprotease involved in CD95L cleavage in HGSOC. Although the number of tumor-infiltrating CD8+ T cells did not correlate with the expression of m-CD95L, we found that the number of activated (GZB+) CD8+ T cells was higher in tumor tissues with greater expression of m-CD95L (Supplementary Fig. S3C). Because m-CD95L levels were not associated with a good prognosis, but activated (GZB+) CD8+ T cells were, this observation suggests immunosuppressive activity even in tumors showing high endothelial expression of m-CD95L.

S-CD95L and the immune landscape in HGSOC

Because high levels of serum s-CD95L are associated with a good prognosis in patients with HGSOC, we next investigated whether the concentration of s-CD95L was associated with the number of TILs. We observed that the concentration of s-CD95L was correlated with the number of tumor-infiltrating CD3+ and CD4-expressing T cells (CD3: r = 0.4373, P = 0.0068; CD4: r = 0.3284, P = 0.0472; Supplementary Fig. S4A). Counterintuitively, the number of tumor-infiltrating Treg cells (FoxP3+) in patients with HGSOC also correlated with s-CD95L expression (r = 0.4584, P = 0.0043); in contrast, the number of infiltrating IL17-producing Th17 cells did not (Fig. 3B; Supplementary Fig. S4A). The number of B cells was associated significantly with the concentration of s-CD95L (Fig. 3B; Supplementary Fig. S4A). Finally, we found no correlation between s-CD95L levels and the number of tumor-infiltrating macrophages (CD163+ cells; Fig. 3B; Supplementary Fig. S4A). Also, there was no correlation between s-CD95L and Granzyme B (a marker of CD8+ T-cell activation) or PD-L1 (Fig. 3B; Supplementary Fig. S4A), suggesting that s-CD95L is not involved in tumor recruitment/activation of cytolytic CD8+ T cells (GZB) or expression of PD-L1, which contributes to exhaustion of CD8+ T cells.

Overall, these findings suggested that the chemoattractant s-CD95L increases recruitment of CD8+ T cells and Treg cells, among the CD4+ T cells, to HGSOC tumors, leading to an improved clinical outcome. Of note, we did not find any correlation between serum s-CD95L level and CD3+ T-cell count in blood sample of patients with HGSOC (Supplementary Fig. S4B).

To confirm that s-CD95L was able to promote cell motility of activated PBls, we incubated CD3+CD28-activated PBls in the presence or absence of s-CD95L and evaluated cell migration using Boyden chambers. As shown in Supplementary Fig. S4C, s-CD95L enhanced the migration of activated T cells.

High levels of serum CD95L (s-CD95L) is correlated with the number of tumor-infiltrating immune cells in high-grade serous ovarian cancer (HGSOC). These findings suggest that s-CD95L might be used as a noninvasive marker of tumor immune infiltration. This would avoid tumor biopsy, which is difficult when a patient has relapsed. Immune checkpoint inhibitors such as PD-1/PD-L1 or CTLA4 antibodies are more effective in tumors with immune infiltration. In future, a personalized approach could be envisaged: after initial treatment, patients with HGSOC are tailored according to immune factors. Thus, s-CD95L as a surrogate of tumor immune infiltration could be used to select patients with HGSOC referred for immunotherapy trials. Patients with low s-CD95L levels may be more appropriately directed to clinical trials using molecular therapies.

Discussion

Here, we show that patients with HGSOC with high levels of s-CD95L have a better prognosis than those with low levels. In addition, high s-CD95L levels correlate with increased numbers of tumor-infiltrating immune cells, including T cells (CD8+ lymphocytes and Tregs) and B lymphocytes. These findings suggest that s-CD95L plays a role in regulating tumor immune responses in women with HGSOC and could therefore be a noninvasive marker of tumor immune infiltration. Such a marker would avoid the need for tumor biopsy, which is difficult when a patient has relapsed. Immune checkpoint modulators such as PD-1/PD-L1 or CTLA4 antibodies do not recruit lymphocytes, but break immunosuppression, which lead to restore cytotoxic T-cell activity. Indeed, PD-1/PD-L1 blockade is more effective in tumors with immune infiltration. PD-1/PD-L1 antibodies trigger objective tumor responses in only 20%–30% of patients with recurrent ovarian cancer (34, 35). So far, no biomarkers are associated strongly with high response rates, although PD-L1 expression on both tumor and immune cells can be used to select patients that are more likely to respond to PD1/PD-L1 treatment (34). However, a pathology sample is needed to assess these biomarkers. Moreover, due to the heterogeneous nature of cancer, such a sample may not reflect the disease. In future, a personalized approach could be envisaged: after initial treatment, patients with HGSOC are tailored according to expression of immune factors. Thus, s-CD95L, as a surrogate marker of tumor immune infiltration, could be proposed to select patients with HGSOC for immunotherapy trials: indeed, patients with high s-CD95L expression could be offered check point inhibitor treatment as a maintenance therapy, and patients with low s-CD95L expression may be more appropriately referred to clinical trials of chemotherapies. Nevertheless, a stronger efficiency of PD1/PD-
L1 checkpoint inhibitor has still to be demonstrated for patients with ovarian cancer with high infiltrate of immune cells as compared with women showing a low immune response. By now, the results of immunotherapy trials in ovarian cancer remain disappointing. Mesnage and colleagues showed that neoadjuvant chemotherapy increases tumor immune infiltration in some patients with HGSOC (49); thus s-CD95L as a marker of tumor immune infiltration may allow us to monitor immune responses during neoadjuvant chemotherapy, and to select patients for immunotherapy when a strong tumor immune infiltrate is observed after neoadjuvant chemotherapy.

Although our data required an external validation with an independent cohort, one strength of this study is that we selected only patients with HGSOC and FIGO III and IV stage (i.e., carcinomatosis stage or tumor peritoneal spread), which avoids confusion with other subtypes of ovarian cancer harboring different molecular mutations (e.g., mucinous, endometrioid, or clear cell ovarian cancer) and showing different immune...
responses (54, 55). Indeed, we observed no correlation between survival and s-CD95L levels in patients with HGSOC, mucinous, or endometrioid cancer. Furthermore, IHC experiments identified several types of immune cell in tumor tissue from these highly selected patients: Tregs (FoxP3+ T cells), CD8+ T cells, B cells (CD20 cells), and macrophages (CD163 cells). Zhang and colleagues showed a correlation between TIL numbers and OS and PFS (11), as did Tomsova and colleagues (13), Sato and colleagues (10), and others (56, 57). Nevertheless, these studies suffer from lack of immune cell markers and heterogeneity. A counter-intuitive result is the better prognosis in patients with higher rate of tumor-infiltrating Tregs (FoxP3+ T cells). The magnitude of the immune reaction including tumor-infiltrating CD4+ and CD8+ T cells and FoxP3+ T cells is associated with a better prognosis. Of note, in multivariate analysis, CD3+ and CD8+ T cells remain independent parameters associated with a better survival, while FoxP3 T cells are not (Tables 4 and 5). It is also noteworthy that our IHC analysis fails to discriminate the different subsets of Tregs. Indeed, recent data showed that Tregs are heterogeneous, with five major structurally and genetically distinct cell subsets, each representing a stage of maturation with distinct functional capacities, which could be proinflammation or tolerance (58).

In other tumor models such as breast cancer, TILs can be subdivided according to their location within the tumor: stromal
TIls when located in the peritumoral space and intraepithelial TIls, when they have penetrated the tumor islets. This classification was widely used in breast cancer and is now recommended in clinical routine (45). Although these recommendations exist in breast cancer, there is no standardized approach to evaluate TIls in ovarian cancer. In this study, we decided to perform a representative HES slide, which had to contain both tumor and adjacent stroma, to evaluate overall immune cells whatever their localization in tumor. Nonetheless, it would be interesting to perform, in the future, additional studies to address whether a correlation exists between TIL distribution and m-CD95L expression or s-CD95L serum level. Because evaluation of tumor immune infiltration is difficult, the International Immuno-Oncology Biomarkers Working Group advocates evaluating intra-tumor lymphocytes in ovarian carcinoma using standard staining methods such as HES. They suggest a semiquantitative evaluation of the area occupied by inflammatory mononuclear cells (lymphocytes and plasma cells, excluding polymorphonuclear cells; ref. 45). These recent recommendations suggest the need for a more reliable method of evaluating tumor immune infiltration and accordingly, we examined TIls in the recommended way using IHC. The serum concentration of s-CD95L may be a reproducible and simple tool for evaluating tumor immune responses in patients with HGSOC. Furthermore, we found that s-CD95L is an independent prognostic factor of PFS in HGSOC (P = 0.0063). Patients with HGSOC with high level of s-CD95L show a good prognosis, which is opposite to that found for those with TNBC (59). This discrepancy may be due to differences in tumor load, disease history (metastasis for TNBC and carcinomatosis for HGSOC), and different roles played by s-CD95L. In TNBC, s-CD95L triggers a promotive signal in tumor cells, whereas in HGSOC it seems to contribute to the immune landscape through molecular mechanisms that remain to be elucidated.

Although we observed a correlation between expression of m-CD95L (assessed by IHC) in the tumor and serum s-CD95L levels in patients with HGSOC, the 8 patients lacking m-CD95L in the tumor still express s-CD95L in serum. This discrepancy could be due to intratumor heterogeneity; further studies are required to assess m-CD95L expression in a cohort from whom multiple biopsies are obtained from anatomically distinct sites. Another hypothesis could be that the membrane-bound ligand would already have been stripped from the endothelial cell surface.

Miotz and colleagues showed that m-CD95L on endothelial cells in ovarian cancer selectively killed cytotoxic CD8 T

<table>
<thead>
<tr>
<th>Variable(s)</th>
<th>Endothelial membrane CD95L expression (n = 20)</th>
<th>No membrane CD95L expression (n = 8)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>s-CD95L serum level</td>
<td>358.20 ± 350.12</td>
<td>226.04 ± 205.28</td>
<td>0.4485</td>
</tr>
<tr>
<td>CD3</td>
<td>9.0227 ± 7.832.4</td>
<td>9.945.1 ± 13.658</td>
<td>0.4277</td>
</tr>
<tr>
<td>CD4</td>
<td>3.8410 ± 4.7601</td>
<td>1.613 ± 935.65</td>
<td>0.1555</td>
</tr>
<tr>
<td>CD8</td>
<td>4.2623 ± 5.9555</td>
<td>4.879 ± 6.7590</td>
<td>0.4791</td>
</tr>
<tr>
<td>CD20</td>
<td>2.766.7 ± 3.9333</td>
<td>1.411.6 ± 2.6108</td>
<td>0.1006</td>
</tr>
<tr>
<td>FoxP3</td>
<td>717.62 ± 750.59</td>
<td>276.25 ± 135.65</td>
<td>0.0528</td>
</tr>
<tr>
<td>CD163</td>
<td>8.8098 ± 5.4300</td>
<td>9.270.6 ± 796.83</td>
<td>0.5927</td>
</tr>
<tr>
<td>IL7</td>
<td>789.03 ± 937.34</td>
<td>545.25 ± 669.34</td>
<td>0.3660</td>
</tr>
<tr>
<td>PD-L1</td>
<td>904.41 ± 1360.1</td>
<td>527.63 ± 563.54</td>
<td>0.5927</td>
</tr>
<tr>
<td><strong>GRANZYM E</strong></td>
<td><strong>1,722.6 ± 1,332.2</strong></td>
<td><strong>762.88 ± 794.99</strong></td>
<td><strong>0.0173</strong></td>
</tr>
</tbody>
</table>

**NOTE:** P < 0.05 in bold.

<table>
<thead>
<tr>
<th>Data</th>
<th>Risk of recurrence HR (95% CI)</th>
<th>P</th>
<th>Risk of death HR (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age &gt; 65 years</td>
<td>1.03 (0.52-2.06)</td>
<td>0.9332</td>
<td>1.09 (0.52-2.06)</td>
<td>0.8419</td>
</tr>
<tr>
<td>Serum Cat25 level (UI/mL) &lt;1,200</td>
<td>0.89 (0.42-1.86)</td>
<td>0.7519</td>
<td>0.85 (0.35-2.56)</td>
<td>0.7321</td>
</tr>
<tr>
<td>Stage FIGO IV (vs. IIIc)</td>
<td>1.50 (0.63-3.56)</td>
<td>0.3583</td>
<td>1.97 (0.77-5.05)</td>
<td>0.2645</td>
</tr>
<tr>
<td>Involved nodes</td>
<td>No</td>
<td>1</td>
<td>0.0209</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Yes</td>
<td>2.08 (0.90-4.82)</td>
<td></td>
<td>2.85 (0.90-9.00)</td>
</tr>
<tr>
<td></td>
<td>Not removed</td>
<td>3.80 (1.48-9.75)</td>
<td></td>
<td>7.03 (2.05-24.12)</td>
</tr>
<tr>
<td>Residual disease after surgery</td>
<td>5.74 (2.40-13.74)</td>
<td>&lt;0.0001</td>
<td>6.31 (2.40-13.74)</td>
<td>0.0002</td>
</tr>
<tr>
<td>Neoadjuvant chemotherapy</td>
<td>1.29 (0.60-3.74)</td>
<td></td>
<td>2.69 (0.90-8.04)</td>
<td></td>
</tr>
<tr>
<td>s-CD95L &lt; 5 lg/g/mL</td>
<td>3.44 (1.29-9.15)</td>
<td>0.0135</td>
<td>4.45 (1.03-19.10)</td>
<td>0.0445</td>
</tr>
<tr>
<td>CD3 &gt; 6,000</td>
<td>0.32 (0.15-1.76)</td>
<td>0.0040</td>
<td>0.28 (0.11-0.70)</td>
<td>0.0616</td>
</tr>
<tr>
<td>CD8 &gt; 750</td>
<td>0.45 (0.20-1.01)</td>
<td>0.0532</td>
<td>0.35 (0.37-1.68)</td>
<td>0.0242</td>
</tr>
<tr>
<td>Granzyme B &gt;1,200</td>
<td>1.62 (0.81-3.24)</td>
<td>0.1733</td>
<td>2.52 (1.08-5.92)</td>
<td>0.0335</td>
</tr>
<tr>
<td>CD4 &gt; 803</td>
<td>0.51 (0.25-1.05)</td>
<td>0.0678</td>
<td>0.42 (0.18-0.99)</td>
<td>0.0468</td>
</tr>
<tr>
<td>IL7 &gt; 740</td>
<td>1.58 (0.75-3.33)</td>
<td>0.2318</td>
<td>1.14 (0.47-2.81)</td>
<td>0.7687</td>
</tr>
<tr>
<td>FoxP3 &gt; 700</td>
<td>0.46 (0.21-1.05)</td>
<td>0.0639</td>
<td>0.76 (0.31-1.86)</td>
<td>0.5506</td>
</tr>
<tr>
<td>CD163 &gt; 5,500</td>
<td>0.56 (0.27-1.13)</td>
<td>0.3048</td>
<td>0.62 (0.27-1.45)</td>
<td>0.2577</td>
</tr>
<tr>
<td>PD-L1 &gt; 1,000</td>
<td>0.36 (0.12-1.02)</td>
<td>0.0550</td>
<td>0.37 (0.09-1.60)</td>
<td>0.1858</td>
</tr>
<tr>
<td>CD20 &gt; 1,060</td>
<td>0.39 (0.19-0.82)</td>
<td>0.0134</td>
<td>0.33 (0.13-0.82)</td>
<td>0.0174</td>
</tr>
<tr>
<td>Membrane CD95L expression</td>
<td>0.64 (0.31-1.89)</td>
<td>0.2842</td>
<td>0.56 (0.23-1.38)</td>
<td>0.2001</td>
</tr>
</tbody>
</table>

**NOTE:** P < 0.05 in bold.
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Serum CD95L Level Correlates with Tumor Immune Infiltration and Is a Positive Prognostic Marker for Advanced High-Grade Serous Ovarian Cancer

Thibault De La Motte Rouge, Julien Corné, Aurélie Cauchois, et al.

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