Neoplasia 44 (2023) 100934

Contents lists available at ScienceDirect

Neoplasia

journal homepage: www.elsevier.com/locate/neo

Original Research

Increased expression of IDO1 is associated with improved survival and increased number of TILs in patients with high-grade serous ovarian cancer

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ARTICLE INFO

Keywords:
HGSOC
TILs
IDO1
Ovarian cancer
Prognostic marker

ABSTRACT

Background: The enzyme indoleamine 2,3-dioxygenase 1 (IDO1) plays a crucial role in regulating the immune system's response to tumors, but its exact role in cancer, especially in high-grade serous ovarian cancer (HGSOC), remains controversial. We aimed to investigate the prognostic impact of IDO1 expression and its correlation with tumor-infiltrating lymphocytes (TILs) in HGSOC.

Methods: Immunohistochemical (IHC) staining and bioimage analysis using the QuPath software were employed to assess IDO1 protein expression in a well-characterized cohort of 507 patients with primary HGSOC. Statistical evaluation was performed using SPSS, and *in silico* validation considering *IDO1* mRNA expression in bulk and single-cell gene expression datasets was conducted. Additionally, IDO1 expression in interferon-gamma (IFNG) stimulated HGSOC cell lines was analyzed.

Results: Our findings revealed that IDO1 protein and mRNA expression serve as positive prognostic markers for overall survival (OS) and progression-free survival (PFS) in HGSOC. High IDO1 expression was associated with a significant improvement in OS by 21 months ($p < 0.001$) and PFS by 6 months ($p = 0.016$). Notably, elevated IDO1 expression correlated with an increased number of CD3+ ($p < 0.001$), CD4+ ($p < 0.001$), and CD8+ TILs ($p < 0.001$). Furthermore, high *IDO1* mRNA expression and protein level were found to be associated with enhanced responsiveness to pro-inflammatory cytokines, particularly IFNG.

Conclusions: Our study provides evidence that IDO1 expression serves as a positive prognostic marker in HGSOC and is associated with an increased number of CD3+, CD4+ and CD8+ TILs. Understanding the intricate relationship between IDO1, TILs, and the tumor microenvironment may hold the key to improving outcomes in HGSOC.

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https://doi.org/10.1016/j.neo.2023.100934
Received 22 June 2023; Accepted 31 August 2023

Introduction

With 313,959 new cases and 207,252 new deaths in 2020, ovarian cancer is the 8th most common cancer-related death in females worldwide [1]. High-grade serous ovarian carcinoma (HGSOC) constitutes the most frequent histological subtype and is often diagnosed in advanced stage [2]. With a 5-year survival rate of 43%, the prognosis of this disease is very poor and novel therapeutic strategies are urgently required [3].

Cancer immunotherapy has shown efficacy in various types of cancer, such as melanoma, lung cancer, and bladder cancer. This therapeutic approach mainly comprises immune checkpoint inhibition targeting the programmed cell death protein 1 (PD-1)/programmed death-ligand 1 (PD-L1) pathway. However, inhibition of PD-1/PD-L1 has shown low efficacy in HGSOC [4]. Besides PD-1 and PD-L1, other immune checkpoints might have to be targeted to overcome intrinsic immunotherapy resistance that characterizes HGSOC.

One of these immune checkpoints is indoleamine 2,3-dioxygenase 1 (IDO1), an intracellular enzyme that plays a key role in regulating the immune system's response to invading pathogens and tumors. IDO1 expression can be modulated by a variety of signals, including growth factors and pro-inflammatory cytokines, such as interferon-gamma (IFNG) that is secreted in large quantities during anti-tumor response. In fact, IDO1 was the first gene described to be activated by IFNG [5].

IDO1 catalyzes the initial and rate-limiting step in tryptophan degradation, thereby limiting the availability of this essential amino acid to cells and thus modulating their function and survival. In healthy conditions, IDO1 balances the immune system by suppressing the immune response through inhibiting T cell activation and promoting regulatory T cell recruitment. In numerous types of cancer, IDO1 has been shown to be overexpressed [6]. While some studies demonstrate immunosuppressive effects of IDO1, indicated by reduced number of tumor-infiltrating lymphocytes (TILs) and worse survival [7,8], other studies found IDO1 correlated with higher TILs and better survival [9–11]. In HGSOC, existing studies report a negative correlation between IDO1 expression and survival, but these studies are limited by small cohort sizes [12–14]. IDO1 is considered a potential new target for cancer immunotherapy [15,16], but its role in HGSOC remains unclear. Due to the controversial findings on the role of IDO1 in predicting cancer prognosis and its suitability for therapeutic approaches, we reassessed its impact on HGSOC in a well characterized large cohort with well documented follow-up. We immunohistochemically investigated IDO1 protein expression patterns in a cohort of 507 patients correlating the results with survival data. Additionally, we validated our results using *in silico* analyses of *IDO1* mRNA expression levels from publicly available data sets. Finally, we performed exploratory biological studies using RNA single cell analysis and *in vitro* HGSOC cell data.

Materials and methods

Study population and histopathological examination

The study cohort included tumor tissue samples of 507 patients diagnosed with high-grade serous ovarian carcinoma (HGSOC). All patients underwent cytoreductive surgery at the Department of Gynaecology, Charité - Universitätsmedizin Berlin, Germany between 2000 and 2019. The samples were examined by two board-approved gynecological pathologists (E.T., S.D.-E.) at the Institute of Pathology at Charité - Universitätsmedizin Berlin, Germany and histological subtypes were re-evaluated in accordance with the current WHO criteria [17]. As quality control, all samples were tested for mutational p53 expression by IHC. For the entire cohort, data on overall survival (OS) were available, while data on progression-free survival (PFS) were available for 333 (66.7%) of these patients. The Charité Comprehensive Cancer Center (https://cccc.charite.de) and the Tumor Bank Ovarian Cancer Network (www.toc-network.de) were used to obtain clinical data of the included patients. A detailed overview of the study cohort characteristics can be found in Supplementary Table 1. This study was performed in accordance with the Declaration of Helsinki and the guidelines of the local ethics committee (EA1/110/22).

Immunohistochemical staining

For IHC, tissue microarrays (TMAs) were prepared from formalin-fixed and paraffin-embedded (FFPE) tissues of the HGSOC patients. Briefly, two cores of 1.0 to 1.5mm diameter for each patient were punched out and assembled on 20 TMA blocks. The staining process was performed on the DISCOVERY ULTRA autostainer (Ventana Medical System, Inc., Tucson, Arizona, USA) with previous establishment of the IDO1 antibody staining on normal tissue based on manufacturer's instructions. For immunohistochemical IDO1 staining, tissue sections were incubated with a monoclonal rabbit anti-IDO1 antibody (Ventana, #86630) at 1:100 dilution after a Heat Induced Epitope Retrieval (HIER) in standard CC1 Tris-EDTA buffer. Tissue of palatine tonsils served as positive controls, while liver tissue was used as a negative control.

Image analysis

To perform digital image analysis, the stained TMA slides were digitized using the Pannoramic Slide Scanner (3D Histech, Budapest, Hungary) and subsequently analyzed with the open-source software platform QuPath (version 0.3.0, [18]). A detailed description of the procedure can be found in the Supplementary Materials, detailed QuPath analysis parameters are listed in the Supplementary Tables S2 and S3.

In silico validation of survival analysis

To explore the expression of *IDO1* on mRNA level and its effects on survival, we used the online Kaplan-Meier plotter that enables the genome-wide validation of several biomarkers in different cancer entities [19,20]. The Kaplan-Meier plotter calculates an auto-selected cut-off based on the best possible threshold to divide the cohort into two groups and generates survival curves for them. Since HGSOC could not be selected as a specific entity, we examined all serous carcinomas of grade 2 and 3 together for OS and PFS in all available data sets (GSE14764, GSE15622, GSE18520, GSE19829, GSE23554, GSE26193, GSE26712, GSE27651, GSE30161, GSE3194, GSE51373, GSE63885, GSE65986, GSE9891, TCGA).

In silico bulk gene expression analysis

RSEM-normalized gene expression data of the HGSOC cohort [21,22] of The Cancer Genome Atlas (TCGA) was downloaded from cBioPortal (https://www.cbioportal.org/, accessed on Nov 5, 2022). Gene expression data was available for 300 cases. Spearman correlation coefficients were calculated for all genes. Genes were ranked by correlation with *IDO1* expression. Based on the ranked gene list, gene set enrichment analysis (GSEA) was performed using the "clusterProfiler" R package [23]. The code used for TCGA bulk gene expression analysis is available from: https://github.com/bischofp/HGSOC_IDO1.

In silico single-cell gene expression analysis

Single-cell gene expression data and metadata were downloaded from two public datasets [24,25]. Analyses were performed using the open-source software "R" (version 4.1.1) and package "Seurat" (version 4.1.0; [26]) and the gene signatures Hallmark [27], KEGG [28], as well as two antigen processing and presentation signatures [29,30]. For detailed description of all analysis parameters, see Supplementary Materials.

Cell culture and interferon-gamma stimulation

OAW-42, OVCAR-3, and SKOV-3 cells were cultured in DMEM (Gibco #21885-025) and supplemented with 10% fetal bovine serum, no added antibiotics, at 37°C with 5% CO2 and 95% humidity. Prior to the study, cytogenetic analysis and cell authentication was performed at the DNA-Fingerprinting Facility at Charité Berlin using short tandem repeat DNA. All cell lines were tested for mycoplasma contamination using PCR Mykoplasmen kit (Biontex #M030/050). Cells were plated in 10cm dishes and when a 70% confluence was reached, they were stimulated with human IFNG (Sigma-Aldrich #SRP3058), 1000U/ml. After 24 hours, the cells were harvested for subsequent experiments. As controls, we used unstimulated cell lines undergoing the same plating and incubation protocol. Western blotting was performed subsequently according to established standards at the Institute of Pathology. For a detailed protocol, see Supplementary Materials.

Statistical evaluation

For statistical analysis, optimal cut-offs to group the cohort depending on IDO1 protein expression levels (high vs. low) were determined using the online tool "cut-off Finder" (https://molpathoheidelberg.shinyapps.io/CutoffFinder_v1, [31]). Cut-offs were considered optimal as the point with the most significant split that was calculated by a log-rank test. Survival analyses were performed using IBM SPSS Statistics (Version 27.0.0.0 64-Bit-Version). OS and PFS of the patient cohort were analyzed with the Kaplan-Meier method and the Kaplan-Meier estimate of potential follow-up ("reverse Kaplan-Meier") was used for the calculation of the median follow-up [32]. PFS was assessed as previously defined [33]. Cox proportional hazard models were used to further investigate the influence of IDO1 expression on the cohort's survival in univariate and multivariate testing situations. The additionally considered variables in multiple regressions included the established clinical parameters age ($\leq$ or $>$ 60 years), FIGO stage (FIGO I-II or FIGO III-IV) and the occurrence of residual tumor burden (no residual or residual tumor). Patients were censored at the time of their last follow-up in case of a missing defined event for their survival, or when they were not in follow-up care for PFS at the time of our analyses. Additionally, possible correlations between IDO1 and the expression of TIL markers were investigated using data sets generated in previous studies [33,34]. Due to the exploratory approach of our study, we refrained from adjustment for multiple testing. P-values of <0.05 were considered statistically significant.

Results

IDO1 staining pattern in HGSOC

Immunohistochemical staining revealed mainly cytoplasmic IDO1 protein expression in 500 out of 507 samples (98.62%) with strong and clear staining quality. Representative images of a stained and annotated TMA core are shown in Fig. 1A-B. Using the QuPath software, positive staining was detected at different intensities (weakly, moderately, and strongly positive, as indicated by different colors in Fig. 1C). In all subsequent analyses, all IDO1-expressing cells, regardless of staining intensity, were considered IDO1-positive.

We observed a right-skewed distribution of IDO1-positive cells with a median proportion of 0.8070% of all cells (IQR: 0.2036 - 3.3805%, Fig. 1D) and in 492 out of 507 patients, IDO1 expression was detected in tumor cells. Cut-off determination using a web-based cut-off finder tool resulted in two different cut-offs for our statistical analyses: for IDO1-positive tumor cells and OS (cut-off: 2.304) and for IDO1-positive tumor cells and PFS, respectively (cut-off: 3.2). To eliminate type 2 errors and enhance the overall robustness, we maintained a minimum of 10% for the number of significant tests for the chosen cut-offs, while the range of significant tests varied from 11% to 87.6%, predominantly exceeding 30%. Hereafter, we will refer to the different groups as either "IDO1-low" (percentage of IDO1-positive cells equal to or below the optimal cut-off) or "IDO1-high" (percentage of IDO1-positive cells above the optimal cut-off).

High IDO1 protein expression in tumor cells correlates with better OS and PFS

The median OS in the IDO1-low group ($n = 342$) was 38.9 months (95% CI: 34.0-43.9 months, Supplementary Table S4) and significantly lower than the median OS of 59.7 months (95% CI: 40.4-79.3 months) in the IDO1-high group ($n == 165$, $p < 0.001$, Fig. 2A). Univariate Cox regression revealed that an elevated IDO1 expression alone had a significant positive effect on OS (HR=0.622, 95% CI: 0.481-0.805, $p < 0.001$). Even after the inclusion of other relevant risk factors, such as age ($\leq$ or $>$ 60 years), FIGO stage (FIGO I-II or FIGO III-IV) and residual tumor burden, in a multivariate Cox proportional hazard model, the protein expression level of IDO1 remained an independent significant

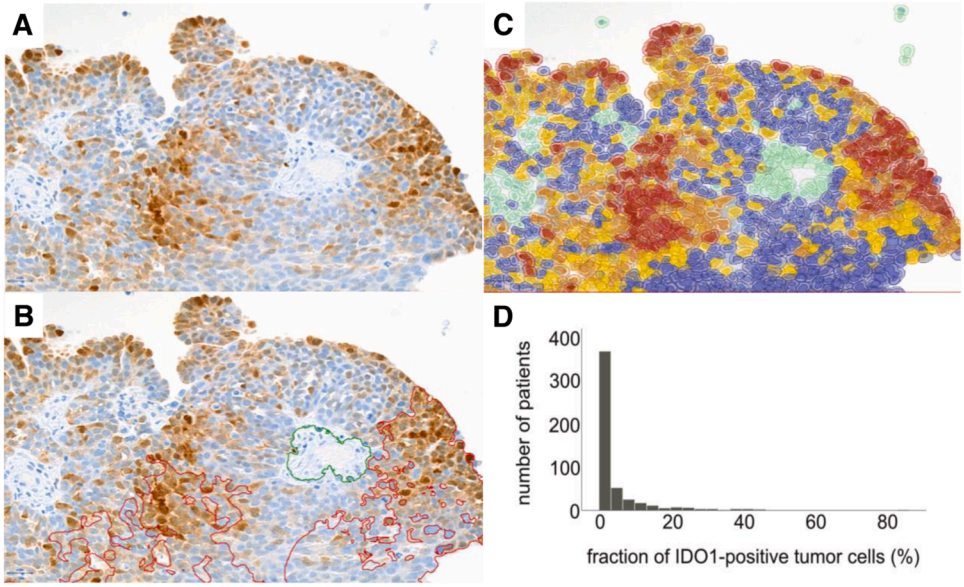

Fig. 1. Images of TMA core in QuPath workflow and percentage of IDO1-positive cells in tumor tissue. (A) Representative close-up image section of an IDO1-stained TMA core without annotations. (B) Image of the same TMA core section including manual annotations of tumor (red) and stroma areas (green). (C) TMA core section after positive cell detection with the color code: green = stroma cells, blue = negative tumor cells, yellow = weakly positive tumor cells, orange = moderately positive tumor cells and red = strongly positive tumor cells. (D) Distribution of the fraction of IDO1-positive tumor cells. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

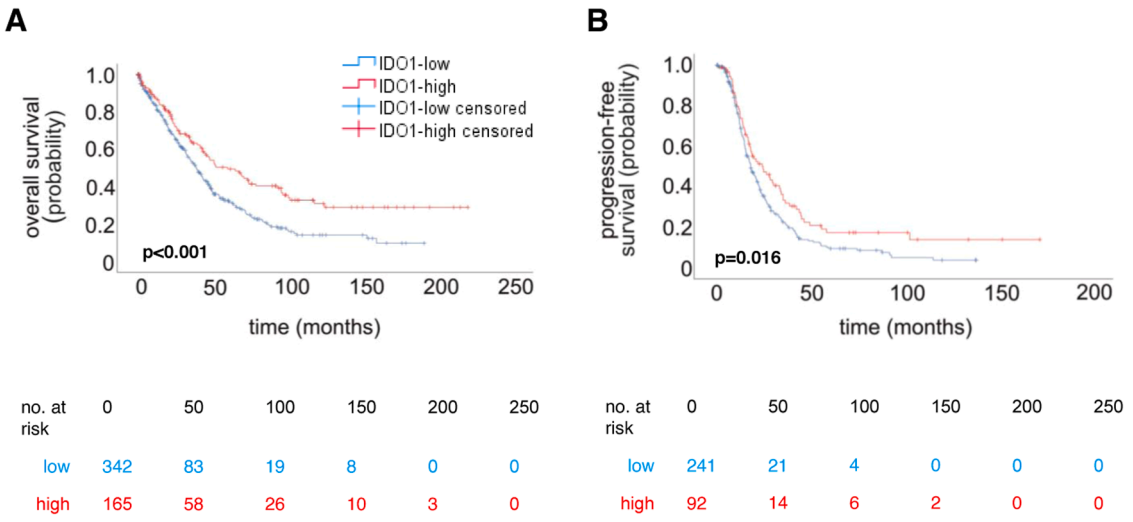

Fig. 2. Kaplan-Meier survival curves of overall and progression-free survival with survival tables for IDO1-low versus IDO1-high. (A) Overall survival, grouped by IDO1 protein expression in tumor cells. (B) Progression-freeIDO1 protein expression in tumor cells and progression-free survival.

prognostic factor (HR=0.696, 95% CI: 0.518-0.935, $p == 0.016$), while among the other covariates, only residual tumor burden also had a significant impact ($p < 0.001$).

Similar results were found for PFS. Patients in the IDO1-high group ($n == 92$) showed a significantly prolonged PFS ($p == 0.016$, Fig. 2B) with a median survival time of 25.0 months (95% CI: 15.7-34.4 months, Table. 2), compared to those in the IDO1-low group ($n == 241$) that had a median PFS of 18.9 months (95% CI: 16.0-21.8 months). Cox regression analyses revealed a significantly improved survival on a univariate level (HR=0.709, 95% CI: 0.535-0.939, $p == 0.016$), as well as an almost significant impact in combination with the standard prognostic covariates age, FIGO stage and residual tumor burden (HR=0.736, 95% CI: 0.535-1.011, $p == 0.059$).

Higher expression of IDO1 mRNA is linked to a better OS and PFS

To validate our findings in an independent cohort, we analyzed the impact of *IDO1* mRNA expression on survival using the web-based Kaplan-Meier plotter tool. We found that a higher expression of *IDO1* mRNA significantly correlated with improved OS (median survival in months: *IDO1*-low=38.77 and *IDO1*-high=50.00, $p < 0.001$, Fig. 3A), as well as with improved PFS (median survival in months: *IDO1*-low=16.00, *IDO1*-high=19.02, $p == 0.003$, Fig. 3B).

Increased IDO1 expression correlates with an increased number of TILs

Since IDO1 is known to affect T cell immunity [35,36], we investigated possible correlations of IDO1 with different T cell subsets. Data on protein expression of the T cell markers CD3, CD4 and CD8 were available for 119, 105 and 101 patients, respectively [34]. The number of IDO1-positive tumor cells significantly correlated with the number of CD3-positive cells ($n == 119$, Spearman's ρ=0.609, $p < 0.001$; Fig. 4A+D), CD4-positive cells ($n == 105$, Spearman's ρ=0.419, $p < 0.001$, Fig. 4B+D), and CD8-positive cells ($n == 101$, Spearman's ρ=0.582, $p < 0.001$, Fig. 4C+D), respectively.

Additionally, we clustered the cohort based on the respective expression levels and compared the OS and PFS between the different groups. For each survival analysis, four groups were created based on

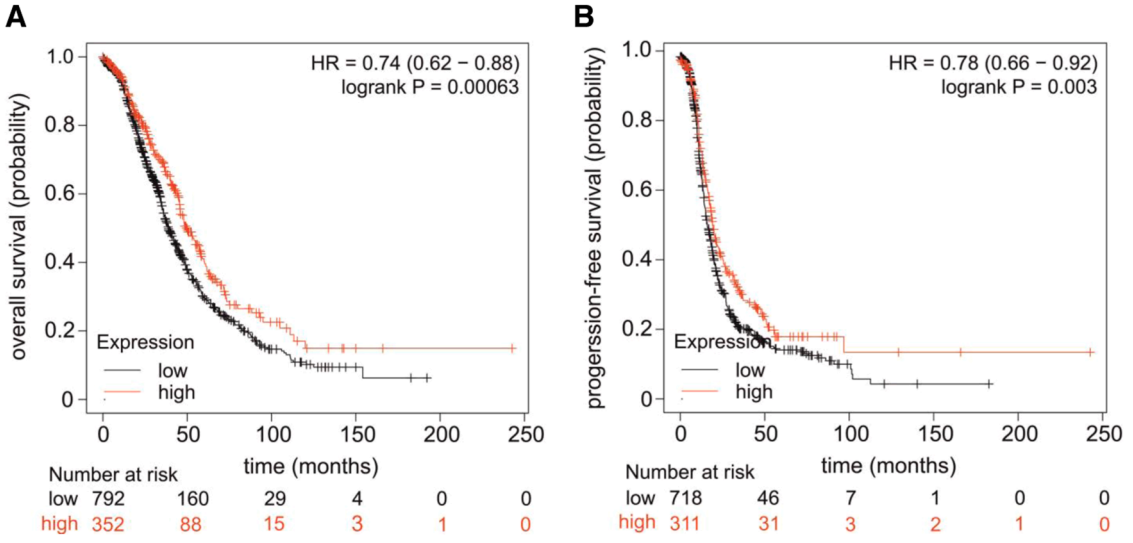

Fig. 3. Kaplan Meier plot of survival rates for IDO1 mRNA expression in publicly available data sets of HGSOC patients. (A) Overall survival: $n = 1144$, $p = 0.000063$, HR=0.74 (0.62-0.88), median survival in months: IDO1-low=38.77 and IDO1-high=50.00. (B) Progression-free survival: $n = 1029$, $p = 0.003$, HR=0.78 (0.66-0.92), median survival in months: IDO1-low=16.00, IDO1-high=19.02. N = number of cases, HR = hazard ratio.

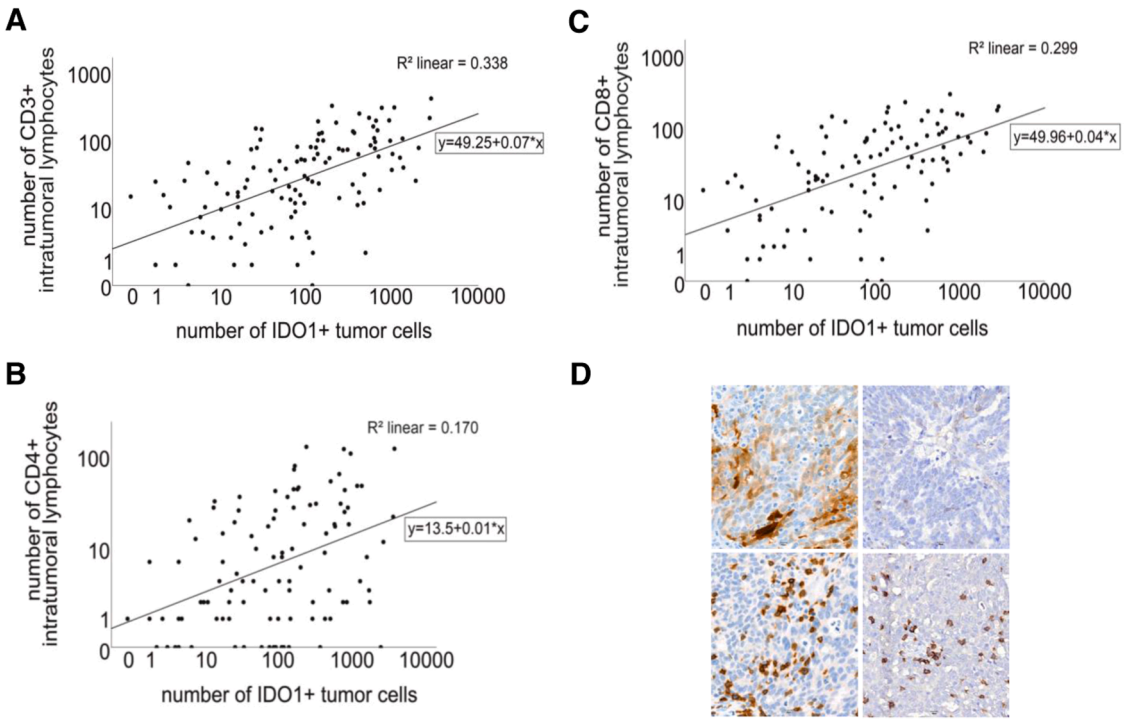

Fig. 4. Association of IDO1 with tumor-infiltrating lymphocytes. (A) Correlation of number of IDO1-positive cells with number CD3-positive cells. (B) Correlation of number of IDO1-positive cells with number of CD4-positive cells. (C) Correlation of number of IDO1-positive cells with number of CD8-positive cells. (D) Representative HGSOC TMA core sections with immunohistochemical stainings IDO1 (top left), CD3 (bottom left), CD4 (top right) and CD8 (bottom right).

their respective cut-offs determined with the Cutoff-Finder: IDO1-high/TILs-high, IDO1-high/TILs-low, IDO1-low/TILs-high and IDO1-low/TILs-low. We found a significantly prolonged OS and PFS in both the groups IDO1-high/CD3-high (OS: $p < 0.001$, PFS: $p < 0.001$, Fig. S1A+D) and IDO1-high/CD8-high (OS: $p < 0.001$, PFS: $p == 0.002$, Fig. S1C + F). For IDO1/CD4+ TILs, the best survival rates were found in the subgroup IDO1-high/CD4-low (Fig. S1B+E).

IDO1 gene expression correlates with enhanced antigen presentation on tumor cells and is linked to a pro-inflammatory tumor microenvironment

To analyze potential mechanisms which are correlated with *IDO1* gene expression and might contribute to differences in patient survival, we performed gene set enrichment analyses in the TCGA ovarian cancer dataset using the Hallmark and KEGG gene sets [27,28]. Within the Hallmark gene sets, the gene signatures "interferon alpha response" and "interferon gamma response" showed the strongest correlation with *IDO1 gene* expression (Fig. 5A+B). Furthermore, we found many other inflammation-related gene signatures positively correlated with *IDO1* gene expression, such as "allograft rejection", "IL6/JAK/STAT3 signaling", "inflammatory response" and "TNFalpha signaling via NFkappaB". Within the KEGG gene sets, correspondingly, *IDO1* gene expression was positively correlated with multiple inflammation-related gene signatures, such as "antigen processing and presentation" (Fig. 5C+D). In contrast, in both the Hallmark and the KEGG gene sets, we observed a negative correlation with "TGFbeta signaling" gene signatures. These results indicate that *IDO1* gene expression might be correlated with a pro-inflammatory tumor microenvironment (TME).

In the TCGA bulk gene expression data, the expression of genes or gene signatures cannot be attributed to distinct cell populations. Therefore, we analyzed two public single-cell gene expression datasets of ovarian cancer to study the expression of *IDO1* and correlation with gene signatures on the single-cell level (Fig. 6A). In both datasets, *IDO1* was expressed mainly in tumor cells and myeloid dendritic cells (Fig. 6B). Interestingly, *IDO1* gene expression in tumor cells was heterogeneous across patients (Fig. 6C). Within the myeloid immune cells, *IDO1* was specifically expressed in LAMP3+/CLEC9A+ dendritic cells (Fig. 6D). *IDO1*-positive tumor cells were characterized by higher expression scores of the Hallmark gene signatures "interferon gamma response" and "interferon alpha response" and lower expression scores of the "TGFbeta signaling" signature (Fig. 6E). Additionally, *IDO1*-positive tumor cells exhibited higher expression scores of the KEGG gene signature "antigen processing and presentation" (Fig. 7E), which was substantiated by other antigen processing and presentation-related gene signatures as well as individual genes, such as *CD74, B2M* and different *HLA* genes (Fig. S2).

Together, our results indicate that high *IDO1* gene expression in tumor cells is linked to tumor cell response to interferon alpha and gamma as well as higher capability of antigen processing and presentation by tumor cells which might sustain a pro-inflammatory TME.

Interferon-gamma increases IDO1 in stimulated HGSOC cell lines

To validate the association between IFNG response and increased IDO1 expression, we selected three ovarian cancer cell lines: two cell lines reported in the literature as possibly HGSOC with TP53 mutations (OVCAR-3 and SKOV-3) and one cell line defined as unlikely HGSOC and without *TP53* mutations ([37,38], Supplementary Fig. S3A). The cells were stimulated with 1000U/ml interferon-gamma for 24 hours and subsequently proteins were harvested for western blotting (Supplementary Fig. S3B). As expected, interferon-gamma increased the protein expression of IDO1 compared to unstimulated controls and the effect was more pronounced in OVCAR-3 (possibly HGSOC, *TP53*_Mut) and SKOV-3 (possibly HGSOC, TP53Mut) compared to OAW-42 an unlikely HGSOC cell line with *TP53*_WT status (Supplementary Fig. S3C+D).

Discussion

In this retrospective study, we demonstrate that IDO1 protein and mRNA expression serve as a positive prognostic marker for both OS and

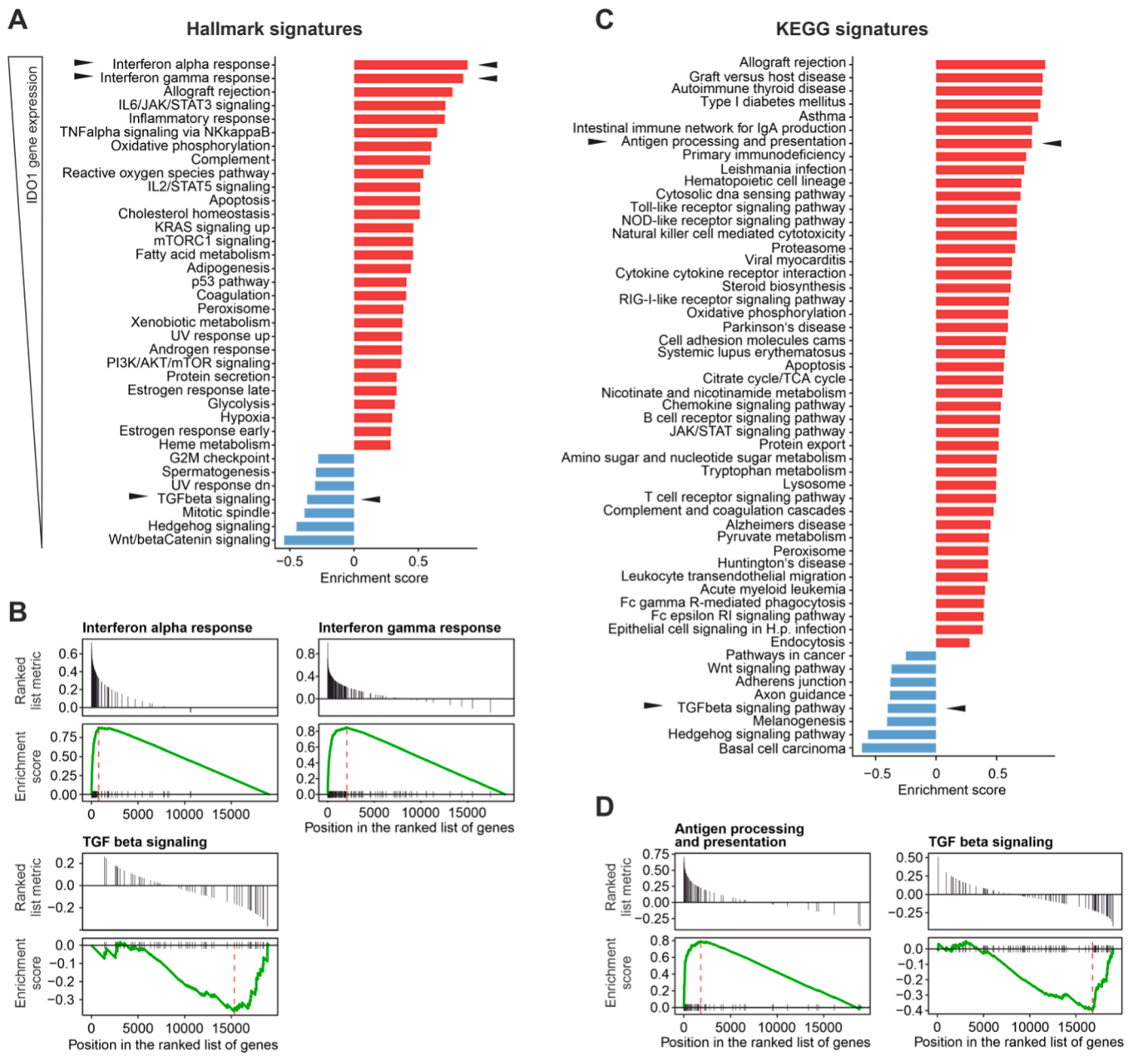

Fig. 5. Correlation of gene signatures with IDO1 expression in the TCGA HGSOC dataset. (A) Gene set analysis (GSEA) enrichment scores of Hallmark gene sets, genes ranked by Spearman correlation coefficient with IDO1 expression, only significant enrichment scores shown (Bonferroni-adjusted $p < 0.05$). (B) GSEA plot for Hallmark gene sets indicated in (A). (C) GSEA enrichment scores of KEGG gene sets, genes ranked by Spearman correlation coefficient with IDO1 expression, only significant enrichment scores shown (Bonferroni-adjusted $p < 0.05$). (D) GSEA plot for KEGG gene sets indicated in (C).

PFS in a large independent HGSOC cohorts. Furthermore, we show that high IDO1 protein expression correlates with increased numbers of CD3+, CD4+ and CD8+ TILs, and high *IDO1* mRNA expression is associated with an enhanced response to pro-inflammatory cytokines, especially IFNG, providing a potential mechanistic link to improved patient survival.

To date, IDO1 expression has been widely studied in various cancer types. While some studies report a negative correlation between IDO1 expression and prognosis, such as in endometrial cancer [39], hormone receptor-positive breast cancer [40], non-small cell lung cancer [41], colorectal cancer [42], prostate cancer [43], and glioblastoma [44], there are also studies showing a positive correlation between IDO1 expression and prognosis, such as in hepatocellular carcinoma [9,45], basal-like breast carcinoma [46], and rectal cancer [47] . To our knowledge, three studies by Okamoto et al. [12], Takao et al. [13], and Inaba et al. [14] specifically investigated IDO1 protein expression in serous ovarian cancer and found an association with poor patient survival. However, these studies are notably limited by small cohort sizes of 24, 33 and 60 patients, while in our cohort data on OS and PFS was available for 507 and 333 patients, respectively. Moreover, all three previous studies applied a semi-quantitative assessment of IDO1 IHC, whereas we applied an automated quantitative image analysis, allowing a more precise cut-off determination and reducing observer bias. In another study, Feng et al. [11] analyzed gene expression data from TCGA in different gynecologic and breast cancers, including ovarian serous cystadenocarcinoma, and found that high *IDO1* mRNA expression correlates with improved survival, which is in line with the findings of our study.

IDO1 is a well-known target of IFNG signaling [5]. Consistent with our study, it has previously been shown that high *IDO1* mRNA

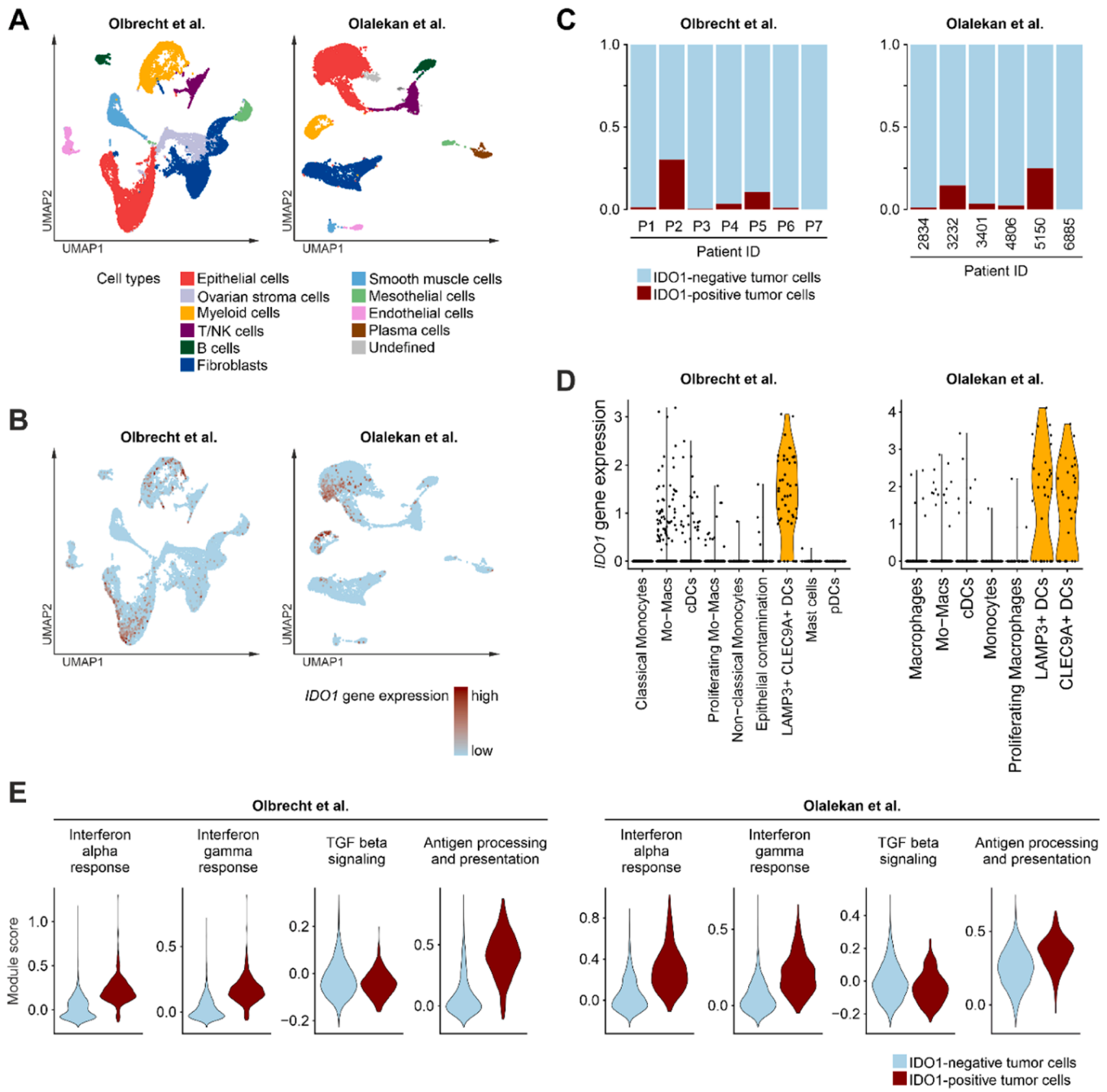

Fig. 6. Gene expression of IDO1 on single-cell level in two ovarian cancer datasets. (A) Visualization of two ovarian cancer single-cell datasets by uniform manifold approximation and projection (UMAP), color-coded by cell type. (B) Gene expression of IDO1 in UMAP plots as shown in (A). (C) Proportion of IDO1-positive and IDO1-negative tumor epithelial cells across different patient samples. (D) Gene expression of IDO1 in different immune cell subtypes. (E) Module scores of indicated Hallmark and KEGG gene sets in IDO1-positive and IDO1-negative tumor epithelial cells.

expression correlates with immune-related hallmarks, including IFNG and IFN-alpha (IFNA) response, in several gynecologic cancers, including ovarian serous cystadenocarcinoma [11]. Since interferons act via auto- and paracrine signaling in the TME, different cell types can respond with increased IDO1 expression. Just as in our study, IDO1 protein expression was associated with improved survival in renal cell carcinoma [48]. Here, IDO1 was mainly expressed by endothelial cells and the authors concluded that tumor growth might be restricted, and survival improved by limiting the influx of tryptophan from the blood to the tumor cells. Ishio et al. [45] identified IDO1 as a necessary factor for the antitumor immune response of tumor-infiltrating cells and found it to be expressed only in TILs, but not tumor cells, leading to the hypothesis that IDO1 expression by TILs might lead to a TME that is depleted of tryptophan, suppressing tumor proliferation. They also correlated *IDO1* mRNA expression in tumorous tissues with the expression of *IFNG* and *TNF-alpha* mRNA, raising the possibility that *IDO1* is expressed due to the presence of these cytokines, which might be produced by activated TILs. In our HGSOC cohort, we found IDO1 to be mainly expressed in tumor cells. This was underlined by single-cell gene expression data of two independent HGSOC cohorts showing *IDO1* mRNA expression mainly in tumor cells and a subset of dendritic cells, but not in TILs. In cell culture experiments, we found that IDO1 protein expression was inducible by IFNG stimulation in two possibly HGSOC cell lines, but not in the unlikely HGSOC cell line, supporting the

hypothesis that IDO1 expression in tumor cells might be induced by IFNG signaling in HGSOC.

The interaction between IDO1 and TILs is complex. IDO1 expression within tumors can lead to the depletion of tryptophan and the accumulation of kynurenine in the TME. This metabolic shift can create an immunosuppressive environment that inhibits the activity of TILs and promotes tumor immune evasion. On the other hand, TILs can produce IFNG, which can induce the expression of IDO1 in tumor cells and other immune cells. This creates a feedback loop where IDO1 expression by the tumor cells further suppresses TILs, leading to a dampened immune response against the cancer. In line with that, several studies have found a correlation between an increased expression of IDO1 and a reduced number of CD8+ cells in ovarian cancer [14,49], endometrial cancer [7] and esophageal squamous cell carcinoma [8]. Other studies detected a correlation with an additional reduction of CD4+ cells [50] or a reduced number of CD3+ cells [42]. While a few studies could not detect any significant coherences between IDO1 and TILs [40], our analyses showed a significant association of elevated IDO1 levels with an increased number of such, corresponding to the findings of Li et al. [9] in hepatocellular carcinoma, Toda et al. [10] in osteosarcoma and Feng et al. [11] in several gynecologic and breast cancers. The controversial findings regarding correlation of IDO1 and TILs in different tumor types point to potentially context-dependent mechanisms regulating IDO1 expression and TIL infiltration.

Our study's findings suggest that the role of IDO1 in ovarian cancer is more complex than originally anticipated. Specifically, IDO1 appears to be beneficial for patients by mediating the suppression of tumor growth. Induced by IFNG, which is produced by natural killer cells, natural killer T cells, CD4, Th1, and CD8 cytotoxic T lymphocyte effector T cells as part of the innate and antigen-specific immunity, it is conceivable that the positive effect of IDO1 is caused by a pro-inflammatory TME, leading to higher IFNG levels and, thus, increased IDO1 expression. Feng et al. (2020) provided additional explanations for the positive effect of IDO1, suggesting that the interaction between tumor cells and TILs expressing IDO1 influences and changes the TME, leading to different outcomes in different cancers. Furthermore, IDO1 can deprive tumor cells of tryptophan, leading to decreased proliferation. Taken together, it remains elusive if IDO1 itself mechanistically contributes to improved survival or serves as a surrogate biomarker for a high anti-tumor immune response mediated by IFNG signaling and TIL infiltration.

At first glance, it may seem debatable whether the very low and specific cut-offs we determined for IDO1 expression are feasible in a clinical setting. However, this alleged flaw can be easily overcome by employing digital methods for the analysis of immunohistochemical staining, which can provide more accurate and reproducible results. Regarding the TILs subgroups and single-cell analyses we performed, it should be noted that the available datasets included only a relatively small number of patients (TILs maximum $n == 119$, single-cell: $n == 19$), allowing for explorative analyses only. Nevertheless, our findings provide valuable insights into the expression patterns of IDO1 in individual cells and suggest potential mechanisms underlying the observed associations between IDO1 expression and immune cell infiltration. Further studies with larger patient cohorts are needed to confirm and extend our findings.

Our study clearly demonstrates that IDO1 is a positive prognostic marker in HGSOC. We identified a correlation between IDO1 expression and the immune response, specifically a positive correlation with increased numbers of CD3+, CD4+ and CD8+ TILs. We propose that further studies are needed to clarify the exact role of IDO1 in HGSOC, especially in the light of modern aspects of immune modulating therapy concepts.

CRediT authorship contribution statement

Inga Hoffmann: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. **Mihnea P. Dragomir:** Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Writing – original draft, Writing – review & editing. **Nanna Monjé:** Data curation, Formal analysis, Writing – review & editing. **Carlotta Keunecke:** Writing – review & editing. **Catarina Alisa Kunze:** Writing – review & editing. **Simon Schallenberg:** Writing – review & editing. **Sofya Marchenko:** Writing – review & editing. **Wolfgang D. Schmitt:** Writing – review & editing. **Hagen Kulbe:** Writing – review & editing. **Jalid Sehouli:** Writing – review & editing. **Ioana Elena Braicu:** Writing – review & editing. **Paul Jank:** Writing – review & editing. **Carsten Denkert:** Writing – review & editing. **Silvia Darb-Esfahani:** Writing – review & editing. **David Horst:** Writing – review & editing. **Bruno V. Sinn:** Writing – review & editing. **Christine Sers:** Supervision, Writing – review & editing. **Philip Bischoff:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Writing – original draft, Writing – review & editing. **Eliane T. Taube:** Conceptualization, Formal analysis, Investigation, Methodology, Supervision, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We would like to thank Ines Koch and Sylwia Handzik for their outstanding technical assistance.

Funding

M.P.D. is a participant in the BIH-Charité Junior Clinical Scientist Program funded by the Charité–Universitätsmedizin Berlin and the Berlin Institute of Health. M.P.D.'s work is funded by a DKTK Berlin Young Investigator Grant 2022 and Berliner Krebsgesellschaft (DRFF202204). PB is participant in the BIH-Charité Clinician Scientist Program funded by the Charité-Universitätsmedizin Berlin and the Berlin Institute of Health. PB's work is funded by a DKTK Berlin Young Investor Grant 2022.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.neo.2023.100934.

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