The Journal of Pathology: Clinical Research
J Path: Clin Res October 2016; **2:** 259–270
Published online 10 August 2016 in Wiley Online Library (wileyonlinelibrary.com). DOI: 10.1002/cjp2.55

Original Article

STAT1-associated intratumoural T_H1 immunity predicts chemotherapy resistance in high-grade serous ovarian cancer

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Abstract

High-grade serous ovarian carcinoma (HGSC) accounts for 70% of all epithelial ovarian cancers but clinical management is challenged by a lack of accurate prognostic and predictive biomarkers of chemotherapy response. This study evaluated the role of Signal Transducer and Activator of Transcription 1 (STAT1) as an independent prognostic and predictive biomarker and its correlation with intratumoural $CD8^+$ T cells in a second independent biomarker validation study. Tumour STAT1 expression and intratumoural $CD8^+$ T cell infiltration were assessed by immunohistochemistry as a multicentre validation study conducted on 734 chemotherapy-naïve HGSCs. NanoString-based profiling was performed to correlate expression of STAT1 target genes *CXCL9, CXCL10* and *CXCL11* with *CD8A* transcript expression in 143 primary tumours. Multiplexed cytokine analysis of pre-treatment plasma from resistant and sensitive patients was performed to assess systemic levels of STAT1-induced cytokines. STAT1 was validated as a prognostic and predictive biomarker in both univariate and multivariate models and its expression correlated significantly with intra-epithelial $CD8^+$ T cell infiltration in HGSC. STAT1 levels increased the prognostic and predictive value of intratumoural $CD8^+$ T cells, confirming their synergistic role as biomarkers in HGSC. In addition, expression of STAT1 target genes (*CXCL9, CXCL10* and *CXCL11*) correlated significantly with levels of, and *CD8A* transcripts from intratumoural $CD8^+$ T cells within the resistant and sensitive tumours. Our findings provide compelling evidence that high levels of STAT1, STAT1-induced chemokines and $CD8^+$ T cells correlate with improved chemotherapy response in HGSC. These results identify STAT1 and its target genes as novel biomarkers of chemosensitivity in HGSC. These findings provide new translational opportunities for patient stratification for immunotherapies based on emerging biomarkers of inflammation in HGSC. An improved understanding of the role of interferon-inducible genes will be foundational for developing immunomodulatory therapies in ovarian cancer.

Keywords: interferon; intratumoural $CD8^+$ T cell; ovarian cancer; chemoresistance; STAT1

Received 5 February 2016; Accepted 6 August 2016

All authors have no potential conflicts of interest to declare.

Background

Epithelial ovarian cancer (EOC) is a leading cause of gynaecological cancer-associated deaths in women around the world [1] with modest improvements in survival rates over the past 30 years [2]. Among all the histotypes of EOC, high-grade serous carcinoma (HGSC) is the most prevalent type [3]. The majority of patients with HGSC are diagnosed at late stages (Stage III and Stage IV) where peritoneal or distant metastasis, respectively, has already occurred [4]. Treatment of HGSC consists of cyto-reductive surgery followed by combination chemotherapy using platinum and taxol-based drugs [1]. Although initial response rates to

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chemotherapy are high [5], 80–90% of HGSC patients unfortunately develop resistance to conventional chemotherapy with increased disease recurrence creating a major hurdle in its management [3,6,7]. The most widely applied classification defines patients exhibiting a progression free survival (PFS) interval of more than 6 months after completion of chemotherapy as chemosensitive whereas those with a PFS interval less than 6 months from initiation or completion of chemotherapy as chemoresistant [6–8].

It is now established that immune cells within the tumour microenvironment contribute significantly towards the survival or killing of tumour cells by chemotherapy [9,10]. The presence of macrophages, dendritic, $CD8^+$ T, $CD4^+$ T, $CD20^+$ B and NK cells [9,11,12] within the tumour microenvironment have been linked to patient prognosis [13,14]. For example, a higher intra-epithelial $CD8^+$ T cell density relative to $CD4^+$ $CD25^+$ $FOXP3^+$ and $FOXP3^-$ T regulatory cells in ovarian tumours is associated with a better clinical outcome [12,15]. The presence of $CD4^+$ T cells and $CD20^+$ B cells has also been associated with a more favourable prognosis in HGSC [16]. In addition to tumour infiltrating lymphocytes (TILs), effector molecules secreted by both tumour and immune cells mediate tumour progression, metastasis and/or response to therapy [10]. Indeed, four molecular subtypes of HGSC were recently defined with the C1 and C2 molecular subtypes of HGSC showing a predominance of immune-related gene signatures [17–20]. Interestingly, the C2 subtype shows characteristic higher levels of *CXCL9*, *CXCL10* and *CXCL11* expression as well as higher intra-epithelial $CD3^+$ T cell infiltration compared to C1 which has a distinct high stromal immune signature [17,21]. Within each subtype, identification of the factors underlying T cell recruitment and effector functions of immune cells in HGSC tumours may allow discovery of prognostic and predictive biomarkers of chemotherapy response.

We previously reported the role of NFκB gene networks associated with intrinsic chemotherapy resistance in HGSC [22]. To further evaluate the role of inflammation in mediating chemoresistance, we focused on profiling of inflammatory genes in HGSCs from sensitive and resistant patients. These studies revealed the prognostic and predictive value of STAT1 expression evaluated in an independent cohort of 183 HGSCs and its association with chemosensitivity at both transcriptional and protein levels [7]. Following activation by Type I or II interferon (IFN), STAT1 induces expression of the angiostatic chemokine CXCL10 by multiple cell types including antigen-presenting dendritic cells, macrophages, T cells, fibroblasts and epithelial cells [23]. Stimulation by IFN-γ also promotes STAT1 activation, which further induces the production of the T helper 1 (T_H1) type chemokines CXCL9, CXCL10 and CXCL11 that bind to the common chemokine receptor CXCR3 to mediate their functions [23]. CXCR3 signalling promotes chemotaxis, tissue recruitment of $CD8^+$ T cells and NK cells, and favours a T_H1 immune response [24]. Several studies in HGSC have demonstrated that levels of CXCL10 are associated with improved clinical outcome [24–26]. Increased levels of CXCL10 and CCL5/RANTES are also associated with enhanced $CD8^+$ T cell infiltration in melanoma, colorectal and gastric cancer but has not been studied in HGSC [27–29].

The current study builds on our previous findings linking STAT1 with a pre-existing tumour inflammatory microenvironment in differential response to chemotherapy in HGSC [7]. Here, we provide evidence that favourable outcome in HGSC patients treated with chemotherapy is associated with high levels of STAT1 and intratumoural $CD8^+$ T cells. This is likely explained by increased expression of STAT1 target genes, including several chemokines (*CXCL9*, *CXCL10* and *CXCL11*) that were highly expressed within chemosensitive HGSC tumours, but were not enriched systemically. This study provides further validation of the STAT1 signalling axis as predictive of chemotherapy response and outcomes in HGSC.

Materials and methods

Ethics statement

Ethical approval for the study was obtained from the Queen's University Institutional Ethics Review Board, the Centre hospitalier de l'Université de Montréal (CHUM) institutional ethics committee (Comité d'éthique de la recherche du CHUM) and the Ottawa Health Research Institute (OHRI) Research Ethics Board. Informed patient consent was obtained prior to sample collection.

Patient samples

HGSCs in the form of fresh frozen tumour tissues and formalin fixed paraffin embedded (FFPE) tissues were obtained from the Terry Fox Research Institute Canadian Ovarian Experimental Unified Resource (TFRI-COEUR), the OHRI and the Ontario Tumour Bank (OTB). Inclusion criteria for the study were: no chemotherapy pre-treatment before ovariectomy and HGSC histopathology (supplementary material, Table S1). All specimens were collected at the time of surgical resection prior to administration of systemic chemotherapy. Eighty percent of tumours in this study were from Stage III or IV disease. Similarly, plasma samples were

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derived from blood collected pre-operatively from patients undergoing cytoreductive surgery at the CHUM. Age matched normal plasma samples were collected from women with benign disease diagnosed after surgery or from women with no known ovarian disease.

Evaluation of STAT1 expression and intra-epithelial $CD8^+$ T cell infiltration in HGSC by immunohistochemistry

Tumour STAT1 expression was evaluated using immunohistochemistry (IHC) on an HGSC tissue microarray (TMA) (TFRI-COEUR) consisting of 0.6 mm diameter FFPE tumour cores, in duplicate, from a cohort of 734 chemotherapy naïve HGSC cases. This large multicentre TFRI-COEUR cohort contained 550 chemotherapy naïve HGSC cases that formed our STAT1 second independent validation cohort in addition to duplicates for the 184 cases (designated as CHUM cohort; first independent validation [7]) from our previous study. The rabbit anti-human polyclonal STAT1 primary antibody (1:2000 dilution, Abcam #ab2415) [7] and mouse anti-human monoclonal CD8 primary antibody (1:25 dilution, Abcam # ab17147) were applied on separate TMA slides using the Ventana automated immunostaining system. In brief, antigen retrieval was carried out with Cell Conditioning 1 (Ventana Medical System Inc.) for 60 min. The slides were incubated with primary STAT1 or CD8 antibody with appropriate negative controls, at 37°C for 60 min. Reactions were carried out using the ultraView DAB detection kit (Ventana Medical System Inc.). Slides were counterstained with haematoxylin and bluing reagent (Ventana Medical System Inc.) for 4 min. The IHC stained TMAs were scanned on Aperio Scanscope and digitally conserved.

Analysis of STAT1 expression by immunohistochemistry

STAT1 protein expression on IHC stained TMAs was scored according to staining intensity in the tumour compartment of each core (value of 0 for absent, 1 for weak, 2 for moderate, 3 for strong) as per our previously published scoring criteria [7]. The staining was relatively homogeneous and the intensity score used represents the vast majority of cells (80%). Each array was independently analysed in a blinded study by two independent observers. Inter-rating correlation was 0.86. The final score for each patient tumour core was obtained by the average score from the independent observers and the average of two cores on the TMA. Based on receiver operating curve analysis, STAT1 expression of ≥ 0.5 was categorised as high/positive whereas < 0.5 was categorised as low/negative STAT1 expression. Spearman correlation coefficient was used to estimate the correlation between clinical data and tumour STAT1 expression.

$CD8^+$ T cell enumeration on HGSC TMAs

For enumeration of $CD8^+$ T cells using Aperio® Digital Pathology, the tumour epithelial compartments were annotated in Aperio® Imagescope (Leica Biosystems) to distinguish them from the stromal compartment. Only $CD8^+$ T cells within the epithelial compartment were enumerated using an automated algorithm created in HALO™ (Indica Lab), in each core that was also evaluated for STAT1 expression.

Statistical analysis of IHC data

The Statistical Package for the Social Science software version 21.0 (SPSS, Inc) was used to perform statistical analysis. Overall survival was defined as the interval between initial diagnosis to death or the date of last follow-up. Progression free survival was defined as the period between the date of surgery to the first recurrence or last follow up. The Kaplan–Meier method was applied for survival analysis and a Log-Rank test was applied to determine the association between STAT1 expression as a dichotomised variable with overall and progression free survival. Logistic multivariate Cox-regression analysis was performed to determine STAT1 association with age, stage and debulking status as variables. A p-value <0.05 was considered significant.

Plasma cytokine and chemokine analysis

Plasma samples from 27 sensitive, 27 resistant and 26-age matched normal controls were subjected to multiplex cytokine analysis using the Luminex™ 100 system (Eve Technologies, Calgary, AB, Canada). A custom multiplex assay consisting primarily of STAT1 induced cytokines (IP-10/CXCL10, VEGF, IL-6, IL-8, IL-10, TNF-α, FGF-2, IL-17 and IFN-γ) was applied on all 80 plasma samples. Comparisons between the resistant and sensitive plasma cytokines with normal controls were derived by a one-way ANOVA test (Graphpad Prism 6.0, Inc.). A p-value <0.05 was considered significant.

Expression analysis of STAT1 induced genes using NanoString gene expression profiling

A 34-gene custom NanoString gene panel (supplementary material Table S3), consisting of target genes primarily induced by STAT1 activation via interferon stimulation and additional immune phenotypic markers, was designed [30]. 143 fresh frozen tumour tissue RNA samples (103 cases from the TFRI-

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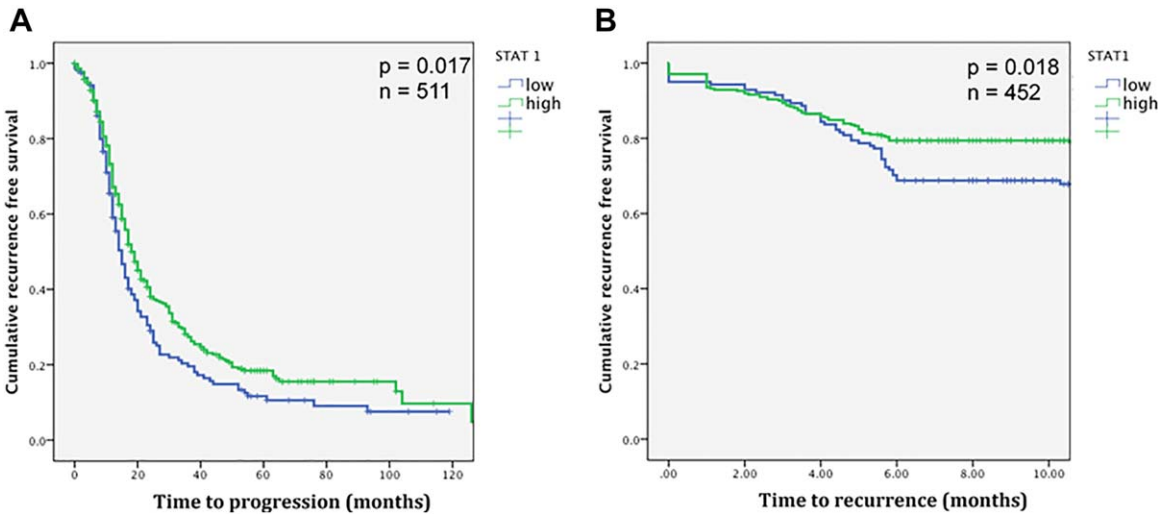

Figure 1. STAT1 expression associates with progression free survival in HGSC. Kaplan–Meier estimates showing significant association between high STAT1 expression and increased progression free survival ($p = 0.017$) (A) and response to chemotherapy ($p = 0.018$) (B) in the Phase II COEUR validation cohort. STAT1 expression in duplicate tumour cores on a HGSC TMA was evaluated using immunohistochemistry using a previously validated antibody [7].

COEUR cohort evaluated for STAT1 expression and $CD8^+$ T cell infiltration and additional tumours from our previous [7] study) were subjected to custom gene expression profiling. Of the total 143, 77 cases were classified as sensitive and 66 as resistant based on our previously described classification criteria [7]. Digital multiplexed NanoString nCounter system (NanoString Technologies, Seattle, WA, USA)-based gene expression profiling was performed on 100 ng total RNA from each sample as per our previous protocols [7].

NanoString gene expression data analysis

The NanoString nSolver platform was used for initial quality control based on fields of view and probe binding density. The raw probe counts were then imported into the R statistical environment and normalised using the Bioconductor package NanoStringNorm [31,32]. We specifically applied the most recommended normalization method in the R NanoStringNorm package '*CodeCount (positive control) geometric mean, Background (negative control) mean + 2 standard deviations and House keeping geometric mean*' as the raw data normalization code [32]. Welch's *t*-test with false discovery rate ($q < 0.05$) correction was applied to derive statistically significant gene expression differences between the sensitive and resistant tumours. Spearman correlation analysis was performed to evaluate within group gene expression correlations. A *p*-value of <0.05 was considered significant.

Results

Validation of STAT1 as an independent prognostic biomarker in HGSC

For second independent validation of STAT1 as a prognostic biomarker in HGSC, a total of 550 HGSCs were evaluated for STAT1 expression by IHC on a TMA derived from the TFRI-COEUR tumour repository. The median follow up in the TFRI-COEUR cohort was 34 months with a mean age of 65 at the end of follow up in this cohort. All patients were chemotherapy naïve and confirmed to have HGSC by a pathologist. The Intra Class Correlation (ICC) of STAT1 expression as single and average measures were 0.75 and 0.85, respectively. Kaplan–Meier analysis and Log-Rank (Mantel-Cox) test showed high STAT1 expression level as a predictor of increased progression-free survival ($p = 0.017$; Figure 1a) in our second independent biomarker validation study. Spearman correlation analysis revealed significant positive association between both stage of disease and debulking status and STAT1 expression, analyzed as either a dichotomised ($p = 0.002$) or continuous variable ($p = 0.005$; supplementary material Table S2A). Multivariate analysis using COX proportional hazard regression analysis ($n = 340$) revealed a significant association with stage ($p < 0.001$, HR, 1.42; 95% CI, 1.13–1.77) and debulking status ($p < 0.001$, HR, 1.22; 95% CI, 1.10–1.36) as variables.

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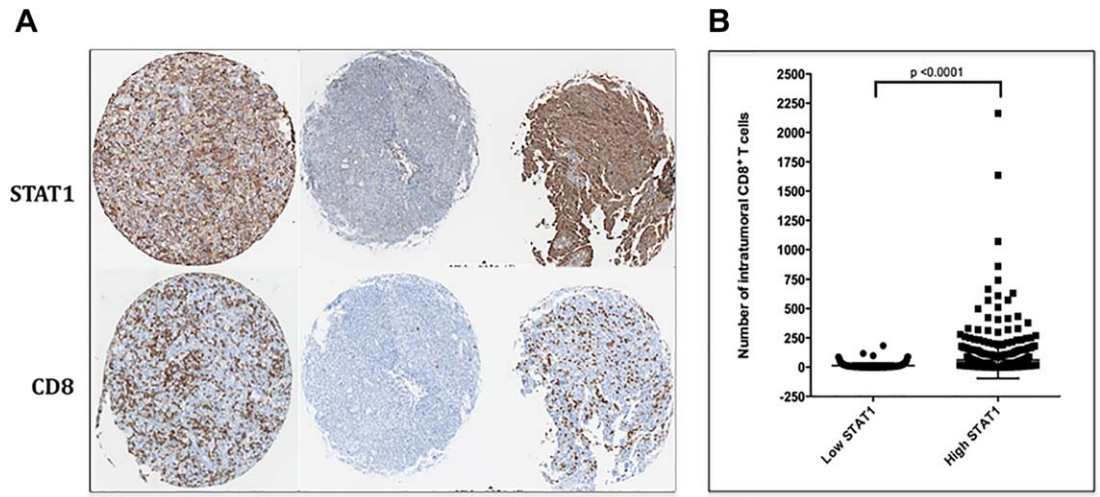

Figure 2. STAT1 expression correlates with intratumoural $CD8^+$ T cell density. (A) Immunohistochemical analysis of STAT1 expression and $CD8^+$ T cells in HGSCs (B) Spearman correlation analysis showing significant association between STAT1 expression and intratumoural $CD8^+$ T cell numbers.

Validation of STAT1 as a predictive biomarker of response to chemotherapy in HGSC

Kaplan–Meier based analysis of STAT1 expression and response to platinum based chemotherapy ($n = 452$) also identified STAT1 as a response predictive biomarker ($p = 0.018$; Figure 1b). When analyzed as a continuous variable (supplementary material, Table S2B) STAT1 expression showed significant association with debulking status ($p < 0.001$; HR, 2.77; 95% CI, 1.58–4.85) but not with patient age and stage.

An important finding from our study is the reproducibility of our previous findings on STAT1 as both prognostic and predictive biomarker between the replicates from CHUM cohort in our two independently conducted validation studies [7]. The Spearman correlation coefficient between these two validation studies of the CHUM cohort was 0.47 ($p < 0.000$, $n = 175$ repeated in second independent validation of STAT1). The inter-class correlation between both results was 0.54. With the observed significant positive correlation our CHUM cohort thus served as an internal control to our TFRI-COEUR cohort ($n = 550$) for evaluation of biomarker assay reproducibility (supplementary material, Figure S1A, B).

STAT1 expression correlates with intra-epithelial $CD8^+$ T cell infiltration in chemotherapy naïve HGSC tumours

With the established role of STAT1 in immune cell recruitment [33] we hypothesised that STAT1 activation is key to recruitment of intratumoural $CD8^+$ T cells in the chemotherapy naïve HGSCs. We evaluated the numbers of $CD8^+$ T cells in the epithelial compartment of all HGSCs evaluated for STAT1 expression by IHC (Figure 2a, b). The intra-epithelial $CD8^+$ T cell number ranged between 0 to 2164 with a median of 13. Spearman correlation analysis ($n = 550$) revealed significant positive correlation between STAT1 expression and intra-epithelial $CD8^+$ T cells ($p = 0.001$) dichotomized as high (>5 per core) and low (<5 per core) based on previously established cut offs [34,35] (Correlation coefficient = 0.460). We further analysed associations between STAT1 expression and $CD8^+$ T cell infiltration relative to stage of disease and significant associations between STAT1 expression and $CD8^+$ T cell infiltration were observed: Stage 2 ($n = 48$; $r = 0.545$; $p = 0.000$), Stage 3 ($n = 392$; $r = 0.465$; $p = 0.000$) and Stage 4 ($n = 50$, $r = 0.435$; $p = 0.002$). In Cox univariate and multivariate analysis as a prognostic and predictive biomarker, CD8 associated significantly with debulking and stage, respectively (supplementary material, Table S4, S5A, B). These data provide strong evidence that STAT1 expression within HGSCs correlates with density of intra-epithelial $CD8^+$ T cells in HGSCs regardless of stage and predicts response to chemotherapy.

$CD8^+$ T cells and association with survival in HGSC

We analysed the association between intra-epithelial $CD8^+$ T cell numbers dichotomised as high and low, based on previously established thresholds for HGSC [34,35], in both cohorts. In the CHUM cohort intra-epithelial $CD8^+$ T cell numbers were significantly

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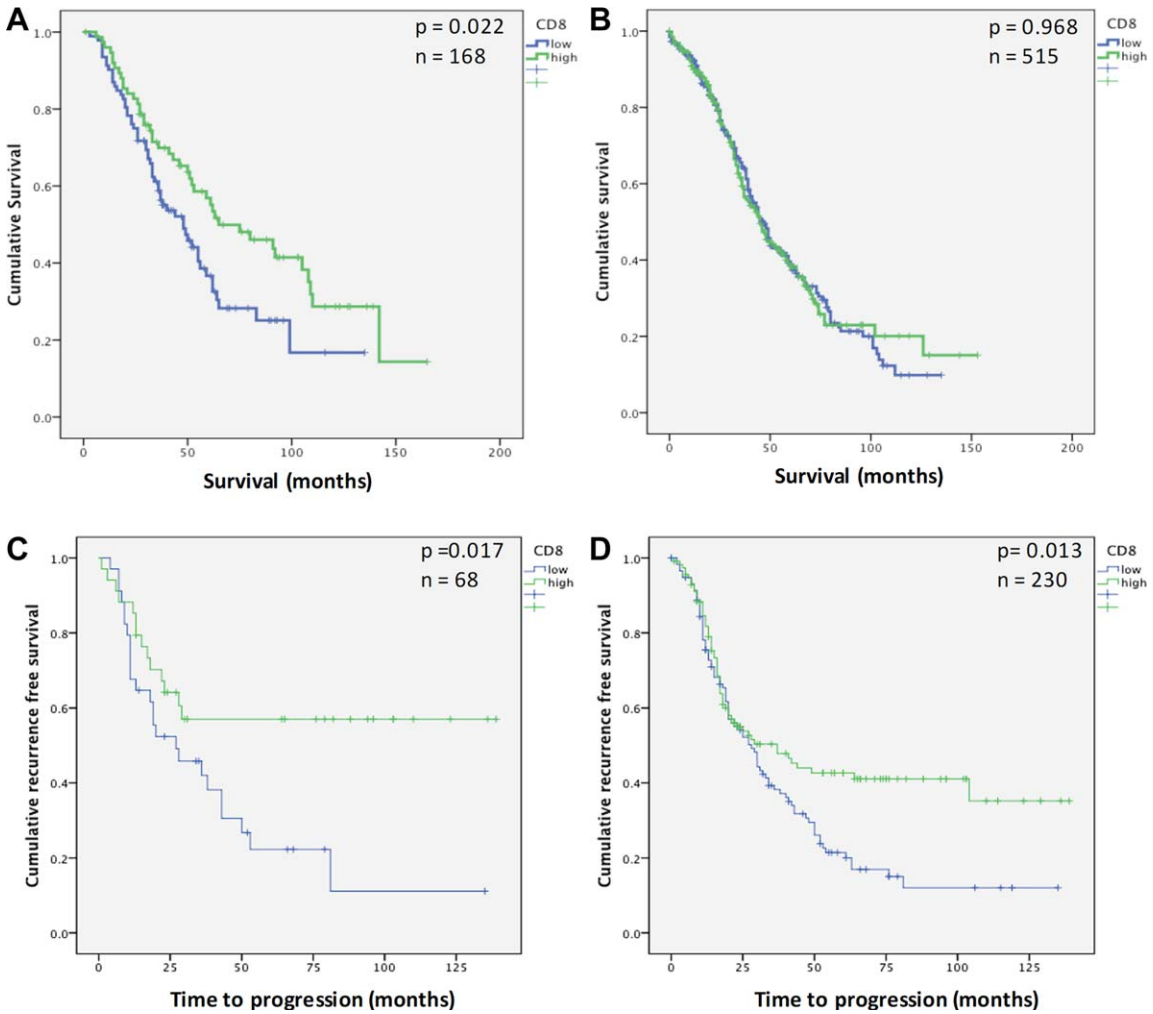

Figure 3. Prognostic relevance of intratumoural CD8$^+$ T cell density in HGSC. Kaplan–Meier analysis was performed using intratumoural CD8$^+$ T cell density (using average of two cores) dichotomised with the threshold of ≤ 5 vs >5 as established for ovarian cancer. Tumour samples were derived from two HGSC retrospective cohorts; (A) CHUM cohort, ($n=$ 184) and (B) TFRI-COEUR cohort, ($n = 515$). Analysis performed on optimally debulked cases on two independent retrospective HGSC patient cohorts (C, CHUM and D, TFRI-COEUR) showed significant prognostic relevance of intratumoural CD8$^+$ T cells. Log-rank test was applied to determine statistical significance ($p < 0.05$).

associated with overall survival (Figure 3a; $p = 0.02$), but not with response to chemotherapy (supplementary material, Figure S2; dichotomised as high vs low). In the TFRI- COEUR cohort, intra-epithelial CD8$^+$ T cell numbers did not associate with overall survival (Figure 3b; $p = 0.96$), progression-free survival or response to chemotherapy (supplementary material, Figure S3A, B). However, intra-epithelial CD8$^+$ T cell numbers, dichotomised as high and low, did show significant association with progression-free survival in patients who were optimally debulked in both CHUM (Figure 3c; $p = 0.017$) and TFRI-COEUR cohorts (Figure 3d; $p = 0.013$). These findings suggest that prognostic relevance of intratumoural CD8$^+$ T cell numbers is affected by debulking status in HSGC.

We next analysed the value of combining high or low CD8$^+$ T cell infiltration with high or low STAT1 expression as prognostic and predictive markers. Interestingly, with STAT1 expression as a categorised variable, intratumoural CD8$^+$ T cell infiltration displayed significant associations with both PFS and response to chemotherapy in both the CHUM (Figure 4a, b), and TFRI-COEUR cohorts (Figure 4c, d). These findings provide evidence for enhanced prognostic relevance of intratumoural CD8$^+$ T cells in combination with STAT1 expression.

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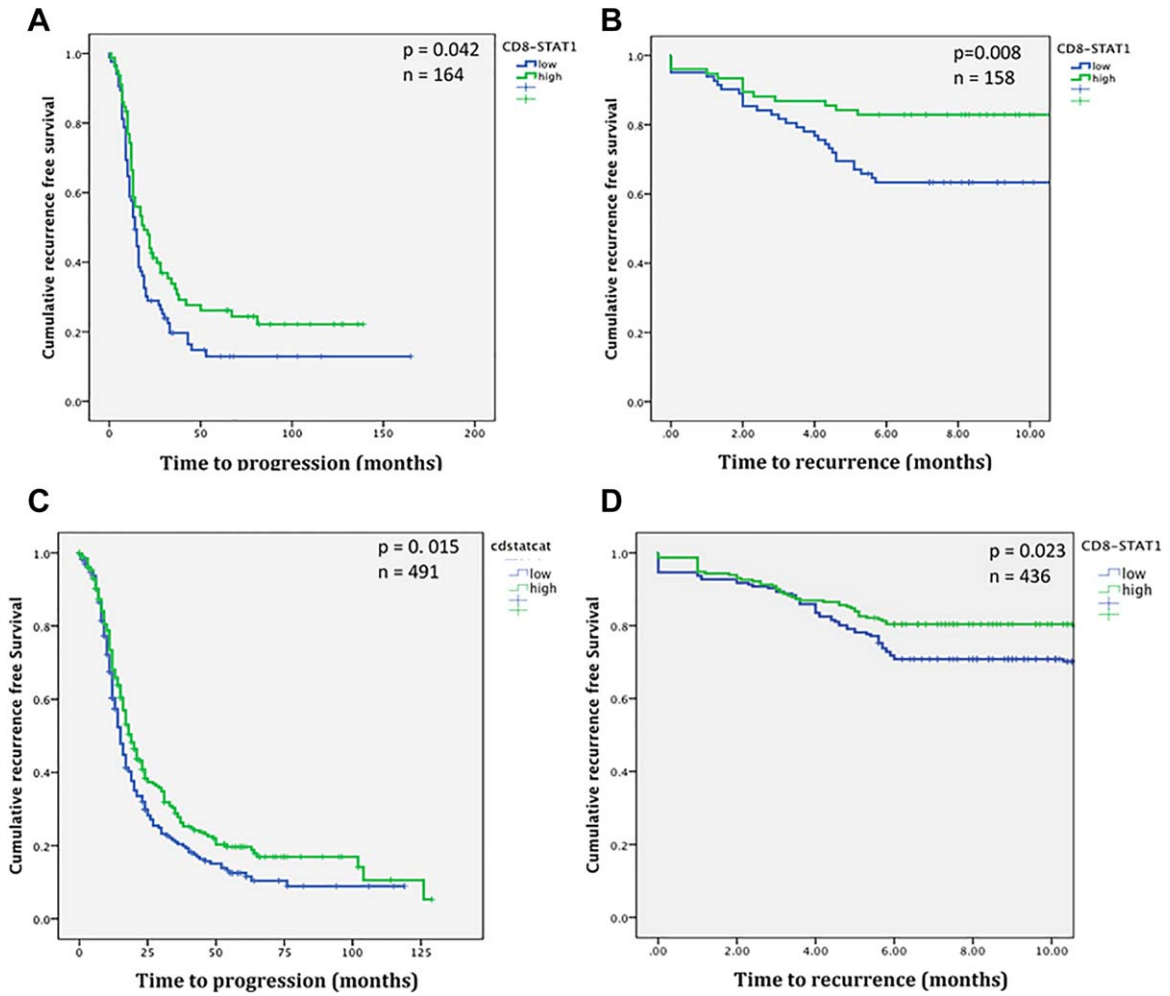

Figure 4. STAT1 expression enhances the prognostic and predictive value of intratumoural $CD8^+$ T cells in HGSC. Average intratumoural $CD8^+$ T cell numbers and STAT1 expression levels in corresponding tumour cores from CHUM (A & B) and TFRI-COEUR (C & D) cohorts were included in the Kaplain Meier survival analysis. Log-rank test was applied to derive statistical significance ($p < 0.05$).

Differential expression of interferon inducible STAT1 target genes in chemosensitive and chemoresistant HGSC tumours

Gene expression analysis of STAT1 target genes revealed their significant differential expression between the chemoresistant and sensitive HGSC tumours (Figure 5). The primary STAT1 target genes, *CXCL9, CXCL10* and *CXCL11*, showed a fold change difference of >1.5 ($p < 0.05$; FDR < 0.05%) between the two groups. Spearman correlation analysis revealed significant correlations between *STAT1*, *CXCL9, CXCL10* and *CXCL11*, with *CD8A* (T cell surface marker) expression within the two cohorts (Figure 6a–c, supplementary material, Figure S5). A significant positive correlation between *STAT1* expression with *IRF1, MX1 and IFITM1* genes was also observed (supplementary material, Figure S6). Collectively, increased expression of *STAT1, CXCL9, CXCL10*, *CXCL11* and *CD8A* correlated with chemosensitivity. Our gene expression analysis-based findings thus support the STAT1-induced CXC chemokine mediated recruitment of prognostically relevant $CD8^+$ T cells in HGSCs. Spearman correlation analysis conducted between gene expression and IHC data showed a positive correlation between STAT1 levels evaluated by these two assays (supplementary material, Figure S7).

Multiplex cytokine analysis of major STAT1 induced cytokines showed increased levels of only IL-6, CXCL-10 and VEGF (p <0.05) in pre-treatment plasma from resistant patients compared to the age matched normal women (supplementary material, Figure S4) suggesting a tumour microenvironment restricted effect of STAT1 induced cytokine alterations.

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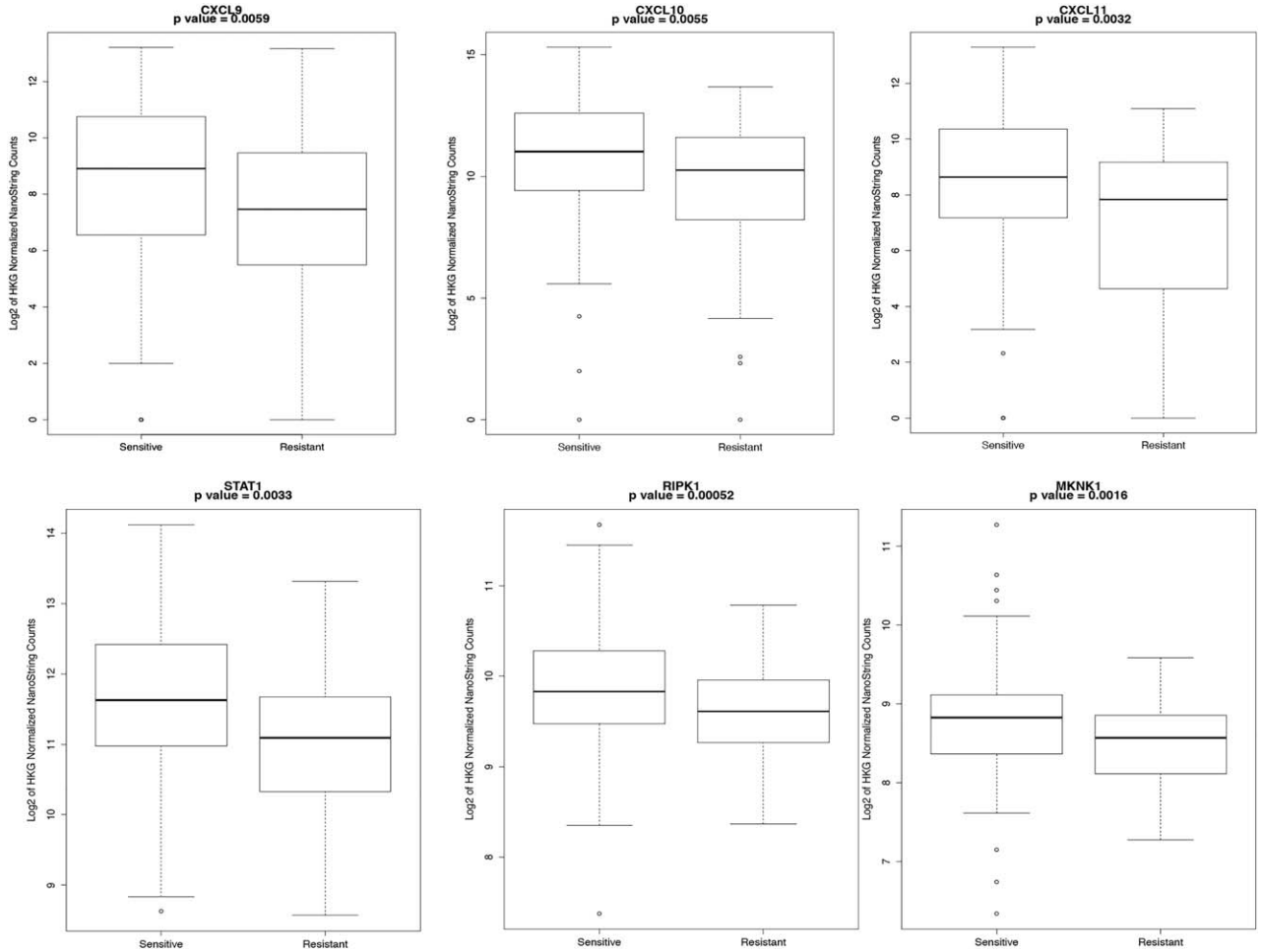

Figure 5. Differential expression of STAT1 target genes in chemoresistant and sensitive HGSC. NanoString based expression profiling of a customised gene panel of 34 genes including those induced by STAT1 as well as immune phenotypic markers in 77 sensitive and 66 resistant HGSCs. NanoString Data analysis was performed using nSolver software from NanoString and R Bioconductor NanoStringnorm package. Statistical significance ($p < 0.05$) between the two groups was determined using Welch's *t*-test.

Discussion

A major hurdle in HGSC management is the development of resistance to chemotherapy via both intrinsic and acquired mechanisms [36–38]. In the current study, we investigated elements within the IFN pathway that potentially mediate the STAT1 associated pre-existing tumour immune state and determine eventual response to chemotherapy via recruiting effector cytotoxic $CD8^+$ T cells in the chemotherapy naïve HGSC tumours. An important determinant of successful immune based therapies is a guided and stratified approach such that maximum survival benefit is achieved in addition to reducing therapy associated side effects. The role of Type I IFN has been previously correlated with therapeutic outcome in cancer [39]. Further, its role in establishing anti-tumour immune response as danger signals initiating specific T cell responses has also been suggested [33]. We initially extended our first independent validation findings [7,40] on STAT1, as a biomarker of prognosis and response to chemotherapy, to a larger multicenter independent HGSC cohort derived from the TFRI-COEUR repository, as a second independent biomarker validation study. In a cohort of 550 HGSC patient tumours, STAT1 expression was confirmed as prognostic and predictive biomarker in both univariate and multivariate cox-regression models. Similar prognostic relevance of STAT1 has been reported in other cancers such as pancreatic, lung, colorectal and breast [41–43]. As per our previous report these findings are suggestive of tumour evolutionary pathways affected by the co-evolving tumour microenvironment, in these cancers [7]. These common associations across cancers suggest that tumour inflammatory pathways follow and

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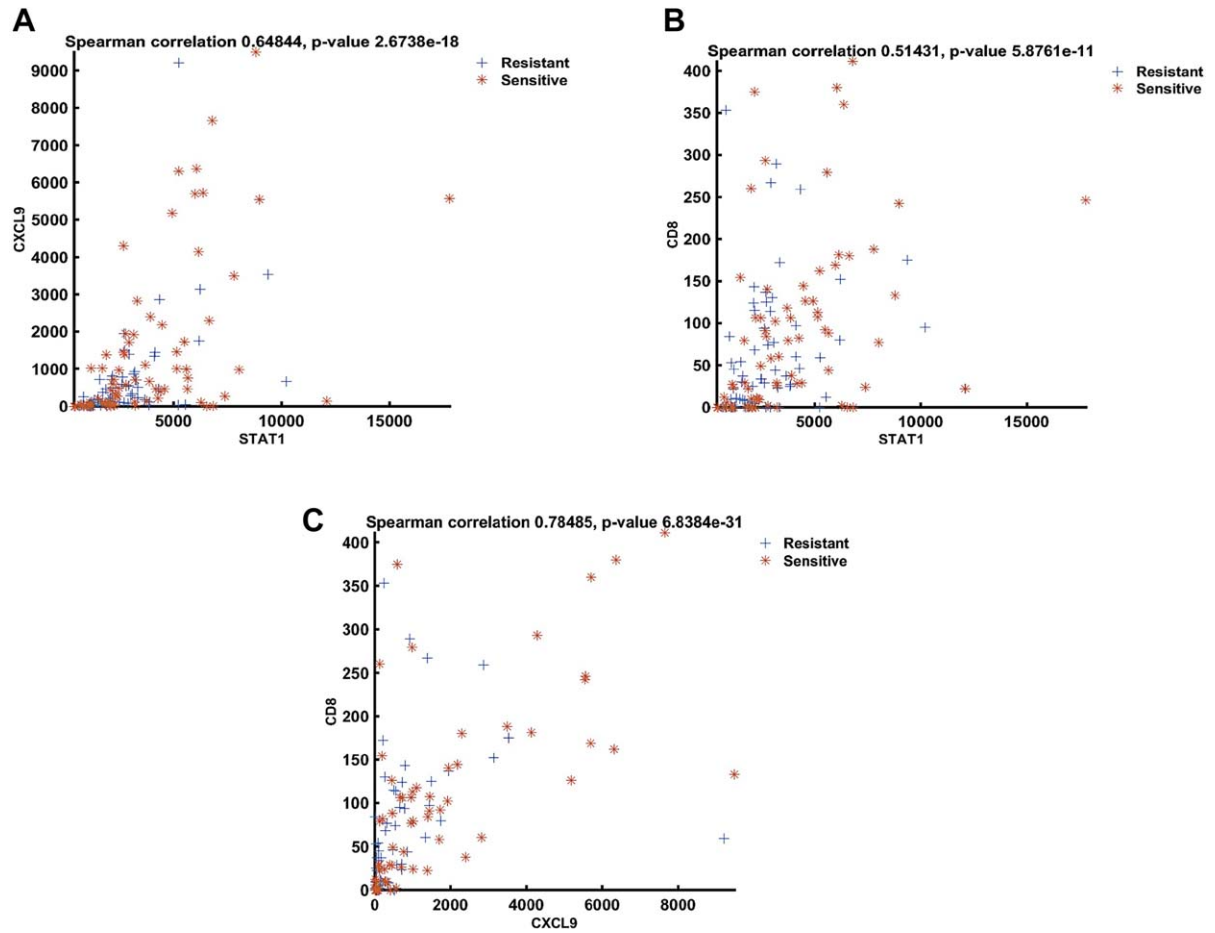

Figure 6. STAT1 target chemokine genes exhibit significant correlation with *CD8A* expression. Spearman correlation analysis was performed between *STAT1*, *CD8A*, *CXCL9*, *CXCL10* and *CXCL11* gene expression in the sensitive and resistant tumours. Significant positive correlation was noted between *STAT1* and *CXCL9* (A), *STAT1* and *CD8A* (B) as well as *CXCL9* and *CD8A* (C) gene expression (measured as house keeping gene normalised NanoString counts) within the two groups.

culminate into a specific axis (IFN in this case) across these cancer types that evolves into a pre-existing tumour immune state and eventually corresponds to disease prognosis. Intra-epithelial $CD8^+$ T cells in ovarian cancer have been reported to be of prognostic significance [12,44]. IFN-induced STAT1 activates chemokines that recruit $CD8^+$ T cells at the site of induction. In the current study, we therefore evaluated the intra-epithelial $CD8^+$ T cells in the same tumours that were assessed for STAT1 expression. The correlation between tumour STAT1 expression and intra-epithelial $CD8^+$ T cells within the sensitive and resistant cohorts confirmed the significant and possible key role of tumour STAT1 in immune cell recruitment within the HGSC tumours. Intratumoural $CD8^+$ T cells exhibited a significant association with overall survival in the CHUM cohort, however, did not associate with overall survival in the TFRI-COEUR cohort. These findings could reflect heterogeneity in the tumour microenvironment and presence of different molecular subtypes of HGSC within these cohorts. Lack of significant association between intratumoural $CD8^+$ T cells with progression free survival and response to chemotherapy in both cohorts is also indicative of their activation state as a determinant in disease prognosis. Similar findings were recently reported where additional activation markers enhance the prognostic relevance of intratumoural $CD8^+$ T cell density in HGSC [34,45]. An important finding in our study is that STAT1 expression enhanced the prognostic value of intratumoural $CD8^+$ T cells. It can thus be speculated that STAT1 activation induced key chemokines that recruit tumour reactive $CD8^+$ T cells to the tumour microenvironment. However, these associations need functional evaluation to confirm the effects of STAT1 in immune cell recruitment in HGSC tumours. Furthermore, in our study we confirmed the prognostic relevance of $CD8^+$ TILs with PFS in only optimally debulked cases in both cohorts. Webb and colleagues

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[34] recently reported that $CD103^+$ (marker for T cell activation) expression enhanced the prognostic relevance of intratumoural $CD8^+$ T cells [34]. A recent report from Wouters and colleagues also suggests that the prognostic relevance of $CD8^+$ TILs in HGSC is affected by their differentiation status, where $CD27^+$ $CD8^+$ TILs associated with patient survival [46]. This study also attributed the variability in TIL association and restricted survival benefit to tumour resection and residual volume. Since STAT1 activation also leads to clonal expansion of memory T cells that express $CD27^+$/$CD103^+$ [47,48] it is possible to some extent that increased levels of STAT1 expression is also represented by activated $CD8^+$ T cells. However, in our cohorts we observed STAT1 expression in tumour compartment with and without $CD8^+$ T cells. Further studies based on co-localization of these markers could possibly address this question. Further evaluation of these companion markers in the context of STAT1 is essential to confirm if the variability in immune cell type infiltration is affected with regards to their prognostic relevance. Overall, these findings provide compelling evidence towards the significance of surrogate markers in application of $CD8^+$ T cell infiltration as a marker of disease prognosis. We thus provide the first evidence for an association between STAT1 induced CXC chemokine recruited cytotoxic T cells with chemotherapy resistance in HGSC and their putative role in mediating immune mediated chemosensitivity. Nevertheless, our findings based on these large independent HSGC cohorts confirm STAT1 as an independent prognostic and predictive biomarker in HGSC and thus the immune independent effect of STAT1 in mediating chemosensitivity cannot be undermined. Gene expression analysis further confirmed significant differential expression of *STAT1* and its downstream effector genes including *IRF1, MX1, IFITM1, CXCL9, CXCL10* and *CXCL11* that correlated significantly with *CD8A* expression. Plasma cytokine levels did not reflect the changes induced by tumour STAT1 expression in the systemic circulation further emphasising their tumour restricted functions. However, significant differences between IL-6 and VEGF in the resistant compared to age matched control plasma are in concordance with previous findings on these cytokines and their association with poor outcomes [49,50]. It is necessary to determine the activation state of intratumoural $CD8^+$ T cells in HGSC that correlate strongly with STAT1 expression levels which will aid in proper design of immunotherapies.

Finally, our findings add to the current knowledge on the immunoreactive C2 molecular subtype of HGSC [17,51] that exhibits high tumour IFN pathway gene expression. Immune mediated chemosensitivity to platinum and taxol based therapy in HGSC could present a classifier for patient stratification in addition to providing novel insights into their role in active or underactive tumour microenvironment. Re-educating the chemotherapy naïve tumour microenvironment in selected patients via incorporation of accurate predictive biomarkers is key to informing benefit with conventional chemotherapy as well as novel immunotherapies.

Acknowledgements

The authors would like to thank Lee Boudreau and Shakeel Virk at the QLMP, Queen's University, for help with IHC staining and scanning of stained TMAs on the AperioScanscope. We thank Dr. Robert J. Gooding for providing guidance in NanoString data analysis. We thank Brooke Snetsinger for help with NanoString experiments. This study uses resources supported by the Terry Fox Research Institute and managed and supervised by the Centre hospitalier de l'Université de Montréal (CRCHUM). The TFRI-COEUR cohort acknowledges the contributions from Institutions across Canada (for a full list see http://www.tfri.ca/en/research/translational-research/coeur/coeur_biobanks.aspx). Biological materials were also provided by the OTB that is funded by the Ontario Institute for Cancer Research. Serum banking was supported by the Banque de tissus et données of the Réseau de recherche sur le cancer of the Fond de recherche du Québec – Santé (FRQS), associated with the Canadian Tumor Repository Network (CTRNet).

Katrina Au is a recipient of a studentship from the TFRI training program in Transdisciplinary Cancer Research in partnership with the Canadian Health Research Institutes. Funding support for the current study was provided by a joint initiative of the Terry Fox Research Institute and Ovarian Cancer Canada as well as support from the Cancer Research Society grants to Dr. M. Koti.

Author contributions

MK and AMM provided conceptual design and experimental planning to this study. KA, LM, IC, and MK performed scoring and evaluation of IHC stained TMAs. CLP performed the statistical analysis for the STAT1 and CD8 IHC data. RR and KT performed NanoString data analysis. JK and NP contributed to the cytokine profiling from patient plasma. TC helped with histopathological evaluation of TMAs. JF, CG, AC and JS provided critical input to the interpretation of the findings and review of the manuscript.

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References

1. Zsiros E, Tanyi J, Balint K, *et al*. Immunotherapy for ovarian cancer: recent advances and perspectives. *Curr Opin Oncol* 2014; **26:** 492–500.
2. Jayson GC, Kohn EC, Kitchener HC, *et al*. Ovarian cancer. *Lancet* 2014; **384:** 1376–1388.
3. Bowtell DD, Böhm S, Ahmed AA, *et al*. Rethinking ovarian cancer II: reducing mortality from high-grade serous ovarian cancer. *Nat Rev Cancer* 2015; **15:** 668–679.
4. Zhang L, Conejo-Garcia JR, Katsaros D, *et al*. Intratumoral T cells, recurrence, and survival in epithelial ovarian cancer. *N Engl J Med* 2003; **348:** 203–213.
5. Oza AM, Cibula D, Benzaquen AO, *et al*. Olaparib combined with chemotherapy for recurrent platinum-sensitive ovarian cancer: a randomised phase 2 trial. *Lancet Oncol* 2015; **16:** 87–97.
6. Marcus CS, Maxwell GL, Darcy KM, *et al*. Current approaches and challenges in managing and monitoring treatment response in ovarian cancer. *J Cancer* 2014; **5:** 25–30.
7. Koti M, Siu A, Clément I, *et al*. A distinct pre-existing inflammatory tumour microenvironment is associated with chemotherapy resistance in high-grade serous epithelial ovarian cancer. *Br J Cancer* 2015; **112:** 1215–1222.
8. Rutherford T, Orr J, Grendys E, *et al*. A prospective study evaluating the clinical relevance of a chemoresponse assay for treatment of patients with persistent or recurrent ovarian cancer. *Gynecol Oncol* 2013; **131:** 362–367.
9. Mittal D, Gubin MM, Schreiber RD, *et al*. New insights into cancer immunoediting and its three component phases–elimination, equilibrium and escape. *Curr Opin Immunol* 2014; **27:** 16–25.
10. Schreiber RD, Old LJ, Smyth MJ. Cancer immunoediting: integrating immunity's roles in cancer suppression and promotion. *Science* 2011; **331:** 1565–1570.
11. Nelson BH. CD20+ B cells: the other tumor-infiltrating lymphocytes. *J Immunol* 2010; **185:** 4977–4982.
12. Milne K, Kobel M, Kalloger SE, *et al*. Systematic analysis of immune infiltrates in high-grade serous ovarian cancer reveals CD20, FoxP3 and TIA-1 as positive prognostic factors. *PLoS One* 2009; **4:** e6412.
13. Preston CC, Maurer MJ, Oberg AL, *et al*. The ratios of CD8+ T cells to CD4+CD25+ FOXP3+ and FOXP3- T cells correlate with poor clinical outcome in human serous ovarian cancer. *PLoS One* 2013; **8:** 1–10.
14. Ali HR, Provenzano E, Dawson SJ, *et al*. Association between CD8+ T-cell infiltration and breast cancer survival in 12 439 patients. *Ann Oncol* 2014; **25:** 1536–1543.
15. Preston CC, Maurer MJ, Oberg AL, *et al*. The ratios of CD8(+) T Cells to CD4(+)CD25(+) FOXP3(+) and FOXP3(-) T cells correlate with poor clinical outcome in human serous ovarian cancer. *PLoS One* 2013; **8:** e80063.
16. Nelson BH. New insights into tumor immunity revealed by the unique genetic and genomic aspects of ovarian cancer. *Curr Opin Immunol* 2015; **33:** 93–100.
17. Tothill RW, Tinker AV, George J, *et al*. Novel molecular subtypes of serous and endometrioid ovarian cancer linked to clinical outcome. *Clin Cancer Res* 2008; **14:** 5198–5208.
18. Bosquet JG, Marchion DC, Chon H, *et al*. Analysis of chemotherapeutic response in ovarian cancers using publicly available high-throughput data. *Cancer Res* 2014; **74:** 3902–3912.
19. The Cancer Genome Atlas Network. Integrated genomic analyses of ovarian carcinoma. *Nature* 2011; **474:** 609–615.
20. Verhaak R, Tamayo P, Yang JY, *et al*. Prognostically relevant gene signatures of high-grade serous ovarian carcinoma. *J Clin Invest* 2013; **123:** 517–525.
21. Nielsen JS, Sahota RA, Milne K, *et al*. CD20+ tumor-infiltrating lymphocytes have an atypical CD27- memory phenotype and together with CD8+ T cells promote favorable prognosis in ovarian cancer. *Clin Cancer Res* 2012; **18:** 3281–3292.
22. Koti M, Gooding RJ, Nuin P, *et al*. Identification of the IGF1/PI3K/NFkB/ERK gene signalling networks associated with chemotherapy resistance and treatment response in high-grade serous epithelial ovarian cancer. *BMC Cancer* 2013; **13:** 549.
23. Groom JR, Luster AD. CXCR3 in T cell function. *Exp Cell Res* 2011; **317:** 620–631.
24. Rainczuk A, Rao J, Gathercole J, *et al*. The emerging role of CXC chemokines in epithelial ovarian cancer. *Reproduction* 2012; **144:** 303–317.
25. Rainczuk A, Rao JR, Gathercole JL, *et al*. Evidence for the antagonistic form of CXC-motif chemokine CXCL10 in serous epithelial ovarian tumours. *Int J Cancer* 2014; **134:** 530–541.
26. Li G, Tian L, Hou JM, *et al*. Improved therapeutic effectiveness by combining recombinant CXC chemokine ligand 10 with Cisplatin in solid tumors. *Clin Cancer Res* 2005; **11:** 4217–4224.
27. Kunz M, Toksoy A, Goebeler M, *et al*. Strong expression of the lymphoattractant C-X-C chemokine Mig is associated with heavy infiltration of T cells in human malignant melanoma. *J Pathol* 1999; **189:** 552–558.
28. Muthuswamy R, Berk E, Junecko BF, *et al*. NF-kappaB hyperactivation in tumor tissues allows tumor-selective reprogramming of the chemokine microenvironment to enhance the recruitment of cytolytic T effector cells. *Cancer Res* 2012; **72:** 3735–3743.
29. Ohtani H, Jin Z, Takegawa S, *et al*. Abundant expression of CXCL9 (MIG) by stromal cells that include dendritic cells and accumulation of CXCR3+ T cells in lymphocyte-rich gastric carcinoma. *J Pathol* 2009; **217:** 21–31.
30. Khodarev NN, Roizman B, Weichselbaum RR. Molecular pathways: interferon/stat1 pathway: role in the tumor resistance to genotoxic stress and aggressive growth. *Clin Cancer Res* 2012; **18:** 3015–3021.
31. Waggott D, Chu K, Yin S, *et al*. NanoStringNorm: an extensible R package for the pre-processing of nanostring mRNA and miRNA data. *Bioinformatics* 2012; **28:** 1546–1548.
32. Waggott D. Introduction to analyzing NanoString nCounter data using the NanoStringNorm package 2015; 1–24. [Accessed 5 October 2016]. Available from: https://cran.r-project.org/web/packages/NanoStringNorm/vignettes/NanoStringNorm_Introduction.pdf.
33. Pilar Gil M, Salomon R, Louten J, *et al*. Modulation of STAT1 protein levels: a mechanism shaping CD8 T-cell responses in vivo. *Blood* 2006; **107:** 987–993.
34. Webb JR, Milne K, Watson P, *et al*. Tumor-infiltrating lymphocytes expressing the tissue resident memory marker CD103 are associated with increased survival in high-grade serous ovarian cancer. *Clin Cancer Res* 2014; **20:** 434–444.
35. Hwang W-T, Adams SF, Tahirovic E, *et al*. Prognostic significance of tumor-infiltrating T cells in ovarian cancer: a meta-analysis. *Gynecol Oncol* 2012; **124:** 192–198.

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36. Cooke SL, Brenton JD. Evolution of platinum resistance in high-grade serous ovarian cancer. *Lancet Oncol* 2011; **12:** 1169–1174.
37. Armstrong SR, Narendrula R, Guo B, *et al*. Distinct genetic alterations occur in ovarian tumor cells selected for combined resistance to carboplatin and docetaxel. *J Ovarian Res* 2012; **5:** 40.
38. Davis A, Tinker AV, Friedlander M. "Platinum resistant" ovarian cancer: what is it, who to treat and how to measure benefit? *Gynecol Oncol* 2014; **133:** 624–631.
39. Fuertes MB, Kacha AK, Kline J, *et al*. Host type I IFN signals are required for antitumor CD8+ T cell responses through CD8 + dendritic cells. *J Exp Med* 2011; **208:** 2005–2016.
40. Le Page C, Ouellet V, Quinn MCJ, Tonin PN, Provencher DM, Mes-Masson AM. BTF4/BTNA3.2 and GCS as candidate mRNA prognostic markers in epithelial ovarian cancer. *Cancer Epidemiol Biomarkers Prev* 2008; **17:** 913–920.
41. Koromilas AE, Sexl V. The tumor suppressor function of STAT1 in breast cancer. *Jakstat* 2013; **2:** e23353.
42. Simpson JA, Al-Attar A, Watson NF, *et al*. Intratumoral T cell infiltration, MHC class I and STAT1 as biomarkers of good prognosis in colorectal cancer. *Gut* 2010; **59:** 926–933.
43. Sun Y, Yang S, Sun N, *et al*. Differential expression of STAT1 and p21 proteins predicts pancreatic cancer progression and prognosis. *Pancreas* 2014; **43:** 619–623.
44. Clarke B, Tinker AV, Lee CH, *et al*. Intraepithelial T cells and prognosis in ovarian carcinoma: novel associations with stage, tumor type, and BRCA1 loss. *Mod Pathol* 2009; **22:** 393–402.
45. Turcotte M, Spring K, Pommey S, *et al*. CD73 is associated with poor prognosis in high-grade serous ovarian cancer. *Cancer Res* 2015; **75:** 4494–4503.
46. Wouters MC, Komdeur FL, Workel HH, *et al*. Treatment regimen, surgical outcome and T cell differentiation influence prognostic benefit of tumor-infiltrating lymphocytes in high grade serous ovarian cancer. *Clin Cancer Res* 2016; **22:** 714–724.
47. Quigley M, Huang X, Yang Y, Alerts E. STAT1 signaling in CD8 T cells is required for their clonal expansion and memory formation following viral infection in vivo. *J Immunol* 2008; **180:** 2158–2164.
48. Farber DL, Yudanin NA, Restifo NP. Human memory T cells: generation, compartmentalization and homeostasis. *Nat Rev Immunol* 2014; **14:** 24–35.
49. Gopinathan G, Milagre C, Pearce OMT, *et al*. Interleukin-6 stimulates defective angiogenesis. *Cancer Res* 2015; **75:** 3098–3107.
50. Gorelik E, Landsittel DP, Marrangoni AM, *et al*. Multiplexed immunobead-based cytokine profiling for early detection of ovarian cancer. *Cancer Epidemiol Biomarkers Prev* 2005; **14:** 981–987.
51. Patch A-M, Christie EL, Etemadmoghadam D, *et al*. Whole–genome characterization of chemoresistant ovarian cancer. *Nature* 2015; **521:** 489–494.

SUPPLEMENTARY MATERIAL ONLINE

Supplementary figure legends

Figure S1. High STAT1 expression associates with increased progression free survival and response to chemotherapy in HGSC

Figure S2. Intratumoural $CD8^+$ T cells associate with overall survival in optimally debulked patients in CHUM cohort

Figure S3. Intratumoural $CD8^+$ T cells do not associate with progression free survival and response to chemotherapy in HGSC TFRI- COEUR cohort

Figure S4. Differential expression of plasma cytokines in chemosensitive and resistant HGSC patients

Figure S5. STAT1 target chemokine genes correlate with *CD8A* expression in chemosensitive and resistant HGSCs

Figure S6. *STAT1* expression correlates with *MX1*, *IRF1* and *IFITM1* expression in chemosensitive and resistant HGSCs

Figure S7. *STAT1* gene expression evaluated by NanoString assay correlates significantly with STAT1 protein expression evaluated by IHC

Table S1. Clinicopathological characteristics of CHUM (n = 184) and Phase TFRI-COEUR (n = 550) high-grade serous ovarian cancer cohorts

Table S2. Univariate and multivariate analysis of STAT1 expression in TFRI-COEUR HGSC cohort

Table S3. Custom gene codeset used for NanoString gene expression analysis

Table S4. Association between STAT1 expression and clinicopathological parameters

Table S5. Univariate and multivariate analysis of CD8 in the TFRI-HGSC cohort

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