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## Assessment of Cytokine mRNA Expression Profiles in Tumor Microenvironment and Peripheral Blood Mononuclear Cells of Patients with High-grade Serous Carcinoma of the Ovary

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### Abstract

**Objective:** Tumor establishment, metastatic spreading and poor survival in ovarian cancer is strongly associated with progressive derangement of the patient's immune system. Accumulating evidence suggests that immune impairment is influenced by the production and presence of cytokines in the tumor microenvironment.

**Methods:** Cytokine mRNA profiles in tumor tissue and peripheral blood mononuclear cells (PBMC) were analyzed in patients with high grade serous carcinoma (HGSC) of the ovary and compared it to patients with benign ovarian conditions and controls with normal ovaries. Cytokine assessment was done by real-time quantitative RT-PCR and specific primers and probes for 12 cytokines-IFN- $\gamma$ , IL-1 $\beta$ , IL-2, IL-4, IL-6, IL-8, IL-10, IL-15, TNF- $\alpha$ , TNF- $\beta$ /LTA, TGF- $\beta$ 1, and GM-CSF chosen to distinguish between cytotoxic Th1, humoral Th2, regulatory Th3/Tr1 and inflammatory responses.

**Results:** The cytokine mRNA response in the HGSC patients was significantly up regulated compared to patients with benign ovarian conditions and normal ovary controls confirming the immunogenicity of HGSC and implying immune recognition and reaction locally in the tumor microenvironment and systemically in the peripheral blood. There was an up-regulation of inflammatory and inhibitory cytokine mRNA promoting tumor progression, T-regulatory cell priming and T-regulatory cell-mediated immune suppression. In contrast, there was an inability to mount the crucially important IFN gamma response needed for upregulation of the cytotoxic anti-tumor response in the local microenvironment. In addition, systemic IL-4-mediated Th2 response prevailed in the peripheral blood deviating the systemic defense towards humoral immunity.

**Conclusions:** Taken together, these results suggest local and systemic cytokine cooperation promoting tumor survival, progression and immune escape. Our study confirms and extends previous investigations and contributes to the evaluation of potential cytokine candidates for diagnostic cytokine mRNA profiles and for future therapeutic interventions based on cytokine inhibition.

**Keywords:** Cytokines; High-grade serous ovarian carcinoma (HGSC); EOC; Tumor microenvironment; Tumor inflammation; Immune suppression

### Introduction

Several reports indicate that tumors develop mechanisms to subvert the immune responses and suppress immune surveillance [1]. Previous studies have shown that these mechanisms down regulate the anti-tumor immunity of the patient. The tumor can directly inhibit the immune cells' function by deviation of their responses, down regulation of cellular receptors or suppression of their mechanisms of action [1-3]. Several of these mechanisms are promoted by immunosuppressive cytokines, chemokines and growth factors that are raised and operate in the tumor microenvironment [3,4]. Ovarian cancer is the most lethal of all gynecological malignancies [5]. It is heterogeneous in nature and classified based on histological type where the epithelial ovarian carcinoma (EOC) is the most common and comprises about 90%. EOC can be subdivided into high-grade serous carcinoma (HGSC), endometrioid, clear-cell, mucinous and low-grade serous carcinoma. The high-grade serous type is the most abundant and malignant [6]. A new classification was presented by Shih and Kurman subdividing the tumors according to tumorigenesis and genetic features into type I and type II [7]. Type I tumors are slow growing and genetically stable. Type II tumors are more aggressive, present themselves at a

late stage and are genetically unstable. Since ovarian cancer has diffuse symptoms and there is no specific diagnostic marker(s), most women are diagnosed with metastasized disease (FIGO stages III and IV) [2]. Cytokines, modulating immune responses, may play a significant role in the establishment and progression of ovarian cancer [8]. Cytokines are cell-signaling proteins/peptides for intercellular communication, secreted by a variety of cells. Different cytokine profiles, designated Th1, Th2, Th3, Tr1 and Th17, are associated with the ability to mediate and regulate immunity and inflammation, promote or halt cell growth and movement or immune responses. Thus, a cytokine profile dominated by IFN- $\gamma$  and interleukin (IL)-15 (Th1) promotes cytotoxicity, a cytokine

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profile dominated by IL-4 (Th2) promotes humoral immunity, while IL-17 (Th17), IL-1 $\beta$ , IL-6, IL-8, tumor necrosis factor (TNF)- $\alpha$  and TNF- $\beta$ /LTA promote inflammation, and TGF- $\beta$  and IL-10 (Th3, Tr1) promote T regulatory cell-induced immunosuppression. These cytokines can be expressed in small amounts in the normal ovaries as well [8]. Previous assessments of protein concentration of cytokines in serum and ascites of EOC patients and in supernatants of EOC cell lines have shown that there is a shift towards immunoinhibitory cytokines [8-13]. Additionally, in earlier reports from the nineties, attempts were made to assess mRNA expression for a limited number of individual cytokines in tumor tissue using Northern blot or semi-quantitative RT-PCR [14-17].

Here, we quantified the mRNA transcription of a broad number of cytokines in paired samples of tumor tissue and peripheral blood mononuclear cells (PBMC) from HGSC, benign ovarian conditions and normal controls by real-time RT-qPCR. The cytokine primers and probes used distinguish between the main cytokine mRNA patterns, i.e. Th1-, Th2-, Th3/Tr1- and inflammatory pattern, known to be the key cytokines that control cytotoxic-, humoral-, regulatory- and inflammatory responses respectively. Our goal was to elucidate the local cytokine mRNA production in the tumor tissue and to compare it to the systemic cytokine profile measured in PBMC of the same patients as well as to compare it to benign ovarian conditions and normal ovaries aiming to reveal differences in the systemic and local modulation of the patient's immune response to HGSC.

## Materials and Methods

### Ethical statement

This investigation was approved by the Human Ethics Committee of the Medical Faculty, Umeå University (dnr 09-108M). All patients and controls donated samples after informed consent.

### Patient samples

Tissue samples from patients with HGSC/EOC type II of the ovary, benign ovarian conditions, normal ovaries and corresponding peripheral blood samples were collected between 2005 and 2015 at surgery from 22 women with HGSC, 19 women with benign ovarian

conditions and 8 with normal ovaries. Information about the patients and the histopathologic diagnosis according to the World Health Organization Classification [18], was extracted from their medical records.

### Preparation of tissue and blood samples

Tissue samples, snap-frozen in liquid nitrogen and stored at -150°C, and corresponding Buffy coats from the patients with HGSC, benign ovarian conditions and normal ovaries were retrieved from the Ovarian Cancer Biobank at Norrland's University Hospital. PBMC were isolated from Buffy coats by Lymphoprep (Nycomed) gradient centrifugation as previously described [19,20]. The collected interphase containing lymphocytes and macrophages was washed, counted, frozen and kept at -80°C until use.

### Total RNA extraction and real-time quantitative reverse transcription-polymerase chain reaction (RT-qPCR)

Cytokine gene expression analysis was performed by real time RT-qPCR following the MIQE requirements [21]. A summary of the positive samples from the total number of samples for the individual cytokines analyses are given in Table 1.

**RNA extraction:** About 0.5 mg of frozen ovarian tissue/sample was sliced into 25  $\mu$ m slices in a cryostatic microtome, dissolved in 350  $\mu$ l of lysis buffer and total RNA was extracted using RNeasy Mini Kit (Qiagen, Hilden, Germany). PBMC from Buffy coats were thawed by layering 200  $\mu$ l RNA later stabilization solution (AM7020, ThermoFisher Scientific inc., USA) mixed by pipetting and transferred to a tube with TRIzol Reagent (ThermoFisher Scientific). Total RNA was isolated and cleaned up with RNeasy Mini kit. RNA yield (on average 3961 ng in total volume of 50  $\mu$ l) and purity (average  $A_{260}/A_{280}=1.7$ ) were assessed by spectrophotometry (NanoDrop, ThermoScientific).

**Reverse transcription:** For each sample 400 ng total RNA in a reaction volume of 20  $\mu$ l was transcribed into cDNA by random hexamers, MuLV reverse transcriptase and dNTP mix using high-capacity cDNA reverse Transcription Kit (ThermoFisher) according to the manufacturer's description. Thereafter, 60  $\mu$ l sterile milliQ water was added to each sample to adjust the cDNA concentration to 5 ng/ $\mu$ l total RNA.

Group	EOC		Benign		Normal		Assay controls		Assay ID (ThermoFisher Scientific)
	ovary	blood	ovary	blood	ovary	blood	sPBMC	18S rRNA	
<b>Total number</b>	22	14	18	18	8	7	1	88	
<b>Gene</b>	<b>Number of positive samples</b>						<b>Ct value*</b>		
IL1 $\beta$	22	14	15	13	8	4	21.7	16.0 $\pm$ 2.03	Hs01555410_m1
IL2	14	9	10	11	4	4	26.7	14.4 $\pm$ 1.81	Hs00174114_m1
IL4	8	12	7	10	3	3	35.5	15.3 $\pm$ 1.91	Hs00174122_m1
IL6	22	13	16	8	8	4	26.4	15.1 $\pm$ 1.81	Hs00985639_m1
IL8	22	12	14	18	8	7	20.7	15.9 $\pm$ 2.10	Hs00174103_m1
IL10	21	10	15	11	7	4	28.5	14.2 $\pm$ 1.91	Hs00961622_m1
IL15	22	14	15	13	7	6	32.3	14.7 $\pm$ 1.76	Hs01003716_m1
TNF $\alpha$	22	14	13	14	8	5	25.6	16.3 $\pm$ 2.08	Hs00174128_m1
TNF $\beta$ /LTA	20	12	13	13	6	4	26.6	15.8 $\pm$ 1.97	Hs04188773_g1
TGF $\beta$ 1	21	14	13	16	7	5	24.9	15.4 $\pm$ 1.94	Hs99999918_m1
IFN $\gamma$	21	11	12	11	6	4	25.2	15.7 $\pm$ 1.92	Hs00989291_m1
GM-CSF	17	7	5	10	5	5	28.2	15.2 $\pm$ 1.88	Hs00929873_m1

\*Average Ct-VIC of 18S rRNA  $\pm$  standard deviation calculated for the entire 88-sample test

**Table 1:** Number of positive samples, average Ct-values for assay- and house-keeping gene controls and gene assay ID.

**Real-time quantitative PCR amplification:** Multiplexed qPCR tests were performed detecting the target gene and 18S rRNA as endogenous control on an ABI PRISM 7900HT Sequence Detection Instrument (Applied Biosystems). One  $\mu$ l cDNA/well in 20  $\mu$ l reaction volume was used in all tests and run for 40 cycles with factory default settings for TaqMan Gene Expression Master Mix (#4369016) and TaqMan<sup>®</sup> FAM/MGB probe Assays (all purchased from Thermo Fisher). The following cytokines were assessed: IFN- $\gamma$ , IL-1 $\beta$ , IL-2, IL-4, IL-6, IL-8, IL-10, IL-15, TNF- $\alpha$ , TNF- $\beta$ /LTA, TGF- $\beta$ 1, and GM-CSF. The catalogue numbers are given in Table 1. The constancy of Ct values for the endogenous, house-keeping gene 18S rRNA is shown in Table 1. The Ct values were similar between samples (standard deviation on average was 1.5) with a normal variation between different gene assays but very stable during the whole study. Cytokine mRNA amplifications of PMA-ionomycin stimulated PBMCs from healthy donors were used as positive assay controls and template omission as a negative control. The obtained cytokine mRNA profiles of tissue and blood were compared between patients with HGSC, patients with benign ovarian conditions and controls with normal ovaries. Data was analyzed with AB 7900 Sequence Detection Software version 2.4.

### Statistical Analysis

Comparative Ct ( $\Delta\Delta$ Ct) method was applied for computing relative quantities (RQ) and an average RQ (aRQ) was calculated for each study group. Every individual fold difference value is a result of division of a test group aRQ by a reference group aRQ, thus standard error and standard deviation is not applicable for calculation and presentation of the results. Statistical tests were used to estimate the variance for each group. We used Student's T test to evaluate statistical significance. P-values  $\leq 0.05$  were considered significant.

## Results

### Patients

Twenty-two patients with HGSC/serous EOC type II diagnosis, aged from 45 to 77 years, participated in the study. The characteristics of the patients are presented in Tables 2,3 and 4. The differentiation grade was low, except for 3 patients where it was low to moderate and 1 where it was undifferentiated (Table 2). Six of the patients had received neoadjuvant preoperative chemotherapy. Standard cytostatic treatment according to guidelines [22] was given for 2-4 cycles, 3 weeks apart, surgery following within 6 weeks of the last cycle. The cytokine mRNA profiles of the patients treated with cytostatic drugs (n=6) are presented separately. Of the remaining 16 patients with HGSC, 14 had both tissue and blood samples. Twenty patients with benign ovarian conditions were included in the benign group, 18 of those had corresponding ovarian tissue- and peripheral blood samples. Their individual diagnoses are presented in Table 3. The normal control group, presented in Table 4, consisted of 8 women with normal healthy ovaries. All patients were matched referring to age and body mass index (BMI). The peripheral blood samples were taken on the day of operation. There were blood samples available from 14 HGSC/EOC patients, 18 patients with benign ovarian conditions and from 7 with normal ovarian tissue, annotated in the tables as paired tissue- and peripheral blood samples.

### The cytokine mRNA profiles in paired HGSC tissue and PBMC samples revealed a statistically significant mobilization of inflammatory and T-regulatory responses combined with an enhanced systemic humoral response and a failure to mount a local cytotoxic response

The cytokine mRNA profiles in tissue- and blood samples from

Patient (n=22)	Stage (FIGO)	Grade of differentiation	Age	BMI <sup>a</sup>	Paired samples tissue and blood <sup>b</sup>	Preop chemo <sup>c</sup>
1	II	low	70	27	-	-
2	IV	low	56	20,9	-	-
3	IV	low	52	23,4	-	-
4	II	low	45	22,3	-	-
5	IV	low	54	32,3	+	+
6	III	low	55	21,3	+	-
7	III	low	63	28,6	+	-
8	III	low	56	30,6	+	+
9	III	low	63	29,3	+	+
10	III	low	76	34,1	+	-
11	I	low	77	24,5	+	-
12	III	low	54	29,4	+	-
13	III	low	56	32,5	+	-
14	IV	low	56	21,1	-	-
15	III	low	67	22,5	+	-
16	III	low	68	21,6	+	-
17	IV	low	70	23,5	+	-
18	III	low	51	22,5	-	-
19	IV	low/moderate	51	21,9	-	+
20	I	low/moderate	75	29,6	+	-
21	III	low/moderate	49	28,4	+	+
22	III	undifferentiated	54	23,2	-	-
Mean $\pm$ SD			60,2 $\pm$ 9	26,4 $\pm$ 5		
body mass index						
<sup>b</sup> + paired samples of blood and tissue available						
- no blood sample						
<sup>c</sup> + received preoperative chemotherapy						
- no preoperative chemotherapy						

**Table 2:** Characteristics of patients with HGSC of the ovary.

Patient (n=20)	Diagnosis	Age	BMI <sup>a</sup>	Paired samples tissue and blood <sup>b</sup>
1	Serous cysts <sup>c</sup>	71	22	+
2	Serous cysts	66	26,4	+
3	Serous cyst	18	20,6	+
4	Serous cyst	65	25,6	+
5	Serous cystadenoma	74	25,3	+
6	Serous cystadenoma	59	24,8	+
7	Serous cystadenoma	74	17,4	+
8	Serous cystadenoma	68	30,2	+
9	Serous cystadenoma and torsion	59	22,4	+
10	Serous cystadenofibroma	57	26,3	+
11	Serous cystadenofibroma	24	46,1	+
12	Serous cystadenofibroma	53	25,7	+
13	Mucinous cystadenoma	45	21,8	-
14	Mucinous cystadenoma	66	34,4	-
15	Mucinous cystadenoma	72	31,1	+
16	Mucinous cystadenoma	68	24,4	+
17	Cyst <sup>c</sup> , unspecific	71	22,7	+
18	Cysts <sup>c</sup> , unspecific	68	25,6	+
19	Inclusion cysts <sup>c</sup>	52	33,2	+
20	Torsion	48	24,5	+
Mean ± SD		58,9±15,6	26,5±6,2	

<sup>a</sup> body mass index  
<sup>b</sup> + paired samples of blood and tissue available  
- no blood sample  
<sup>c</sup> ≥ 8 mm in size

**Table 3:** Characteristics of patients with benign conditions.

Patient (n=8)	Diagnosis	Age	BMI <sup>a</sup>	Paired samples tissue and blood <sup>b</sup>
1	Normal tissue	41	21.6	+
2	Normal tissue	75	21.8	+
3	Normal tissue	57	27.1	-
4	Serous inclusion cysts <sup>b</sup>	63	19.4	+
5	Serous inclusion cysts	54	17	+
6	Serous inclusion cysts	73	19.9	+
7	Inclusion cyst <sup>c</sup>	67	33.7	+
8	Inclusion cysts <sup>c</sup>	71	32.9	+
Mean ±SD		63.9 ± 11	24.2 ± 6.3	

<sup>a</sup> body mass index; <sup>b</sup> + paired samples of blood and tissue available; - no blood sample;  
<sup>c</sup> < 8 mm in size

**Table 4:** Characteristics of patients with normal ovarian tissue.

cytostatic drug-treated and untreated HGSC patients are presented in Figure 1A and 1B. As can be seen there was an enhanced cytokine mRNA expression in the untreated EOC patients (Figure 1A) compared to normal controls. Most prominent and significant mRNA upregulation in both tumor tissue and the corresponding peripheral blood was found for IL-1 $\beta$ , IL-6, IL-8, TNF $\alpha$  and TNF $\beta$ /LTA i.e cytokines mediating inflammatory response. The upregulation was more prominent in the tumor tissue compared to peripheral blood (Figure 1A). One of the cytokines, IL-8 was highly upregulated in the tumor tissue while in the peripheral blood the expression was somewhat lower or unchanged (Figure 1A). Both TNF $\alpha$  and TNF $\beta$ /LTA, the latter an inflammatory cytokine secreted only by immune cells, were significantly upregulated (Figure 1A). The second important observation was that, in the tumor tissue, there was a highly significant upregulation of the T regulatory responses measured by TGF- $\beta$ 1 mRNA (innate- and/or Th3 adaptive T-regulatory cells) and IL-10 mRNA (adaptive Tr1 regulatory cells). The mRNA upregulation in the peripheral blood reached higher significance compared to tumor tissue in the HGSC patients compared to normal controls (Figure 1A). In contrast, IFN $\gamma$  was unchanged or

downregulated without reaching a statistical significance. Messenger RNA for IL-15, another Th1-oriented cytokine, remained also statistically unchanged (Figure 1A). IL-4, the index cytokine of humoral immune responses was differentially expressed in tumor tissue and peripheral blood. Although not reaching a statistical significance, IL-4 was downregulated in the tumor tissue but significantly upregulated in the peripheral blood (Figure 1A). Interleukin-2 mRNA was enhanced in the tumor microenvironment compared to PBMC (Figure 1A). In Figure 1B the cytokine mRNA profile of the 6 patients that received preoperative chemotherapy is presented. As can be seen the overall cytokine mRNA expression was highly depressed compared to the normal control group although statistical significance could not be achieved due to the small number of patients.

**The cytokine mRNA expression in paired tissue and PBMC samples of benign ovarian conditions is generally much lower than in HGSC and comparable to the mRNA expression in normal ovarian tissue and PBMC**

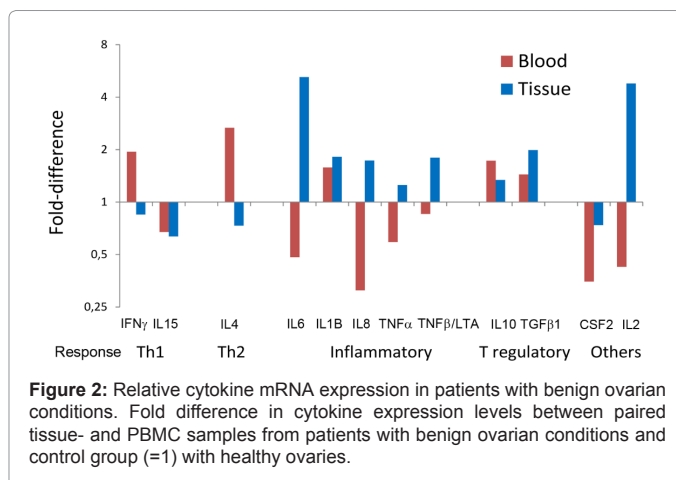
In Figure 2 the relative cytokine mRNA expression in benign



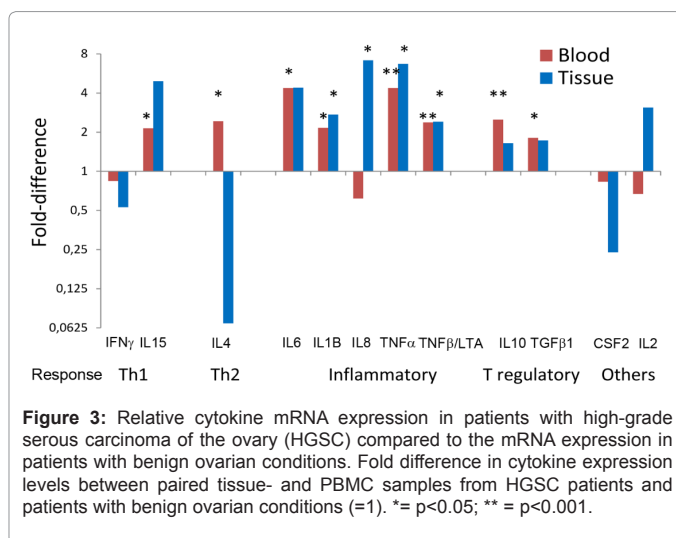
ovarian conditions is compared to the normal control group. The first observation is that generally, the cytokine mRNA expression in benign conditions is much lower compared to HGSC/EOC. As seen in Figure 1B and Figure 2 the fold change in mRNA expression was up to 16 to 32 times higher and statistically significant in HGSC/EOC patients, while the fold change in patients with benign ovarian conditions was at the most 2 to 4 times higher and did not reach statistically significant levels. Furthermore, mRNA expression for several inflammatory cytokines was downregulated in the peripheral blood suggesting that the benign ovarian conditions in this patient group did not evoke a systemic cytokine response.

**Cytokine mRNA profiles in HGSC compared to benign ovarian conditions reveal a statistically significant enhancement of inflammatory and T-regulatory responses, a failure to mobilize a local Th1-primed cytotoxic anti-tumor response and a systemic Th2-primed humoral response**

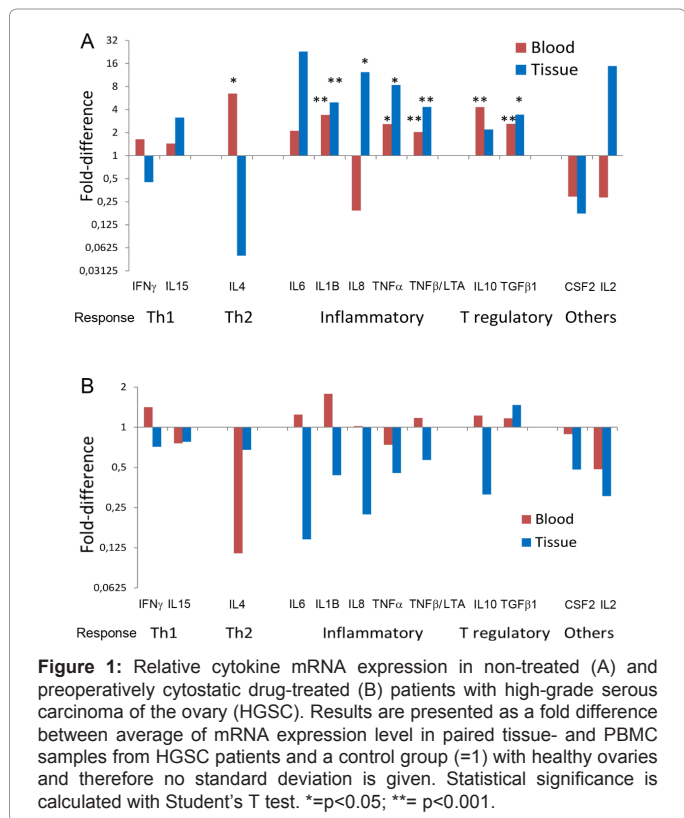
In Figure 3 a comparison between the cytokine mRNA profile in tumor tissue and PBMC in HGSC patients and that of tissue and PBMC in patients with benign ovarian conditions is presented. The relative mRNA expression in HGSC is shown as a fold change compared to the relative mRNA expression in tissue and PBMC of benign ovarian conditions shown in the Figure as =1. In general, there was a statistically significant increase in the cytokine mRNA expression in ovarian cancer tissue and peripheral blood compared to the ovarian tissue and blood from patients with benign conditions. Two significantly enhanced cytokine responses, an inflammatory and a regulatory response, prevailed both in the tumor microenvironment and the peripheral blood Figure 3. In contrast, there was a failure in mounting a Th1 response measured as IFN $\gamma$  mRNA response which



**Figure 2:** Relative cytokine mRNA expression in patients with benign ovarian conditions. Fold difference in cytokine expression levels between paired tissue- and PBMC samples from patients with benign ovarian conditions and control group (=1) with healthy ovaries.



**Figure 3:** Relative cytokine mRNA expression in patients with high-grade serous carcinoma of the ovary (HGSC) compared to the mRNA expression in patients with benign ovarian conditions. Fold difference in cytokine expression levels between paired tissue- and PBMC samples from HGSC patients and patients with benign ovarian conditions (=1). \* = p<0.05; \*\* = p<0.001.



**Figure 1:** Relative cytokine mRNA expression in non-treated (A) and preoperatively cytostatic drug-treated (B) patients with high-grade serous carcinoma of the ovary (HGSC). Results are presented as a fold difference between average of mRNA expression level in paired tissue- and PBMC samples from HGSC patients and a control group (=1) with healthy ovaries and therefore no standard deviation is given. Statistical significance is calculated with Student's T test. \* = p<0.05; \*\* = p<0.001.

was slightly downregulated/unchanged in tissue and blood of the HGSC patients. There was a significantly enhanced IL-15 mRNA response in the peripheral blood of the HGSC patients suggesting a systemic Th1 response. Interleukin-4 was downregulated in the tumor tissue but significantly enhanced in PBMC.

**Discussion**

In this investigation, we assessed the mRNA profile for 12 cytokines in ovarian tumor tissue samples and corresponding PBMC from HGSC patients using real-time RT-qPCR and compared it to corresponding analyses in benign ovarian conditions and normal ovaries. Our results can be summarized as follows: 1) The cytokine mRNA expression in tumor tissue and corresponding PBMC of HGSC patients was significantly upregulated compared to patients with benign ovarian conditions and normal ovaries confirming the immunogenicity of EOC and implying an immune recognition and reaction locally in the tumor microenvironment and systemically in peripheral blood. 2) The significantly enhanced cytokine mRNA expression in HGSC patients was oriented towards inflammation and innate and adaptive T regulatory cells, i.e. responses suppressing anti-tumor immunity and promoting tumor growth and metastasis. 3) In contrast, the IFN $\gamma$  response that would promote cytotoxicity needed for tumor cell elimination was unchanged or downregulated. 4) The absence of Th1

oriented response was combined with a deviation towards systemic humoral Th2 response. 5) Additionally, mRNA for IL-2, a cytokine promoting clonal expansion of immune cells and GM-CSF, a cytokine shown to be associated with EOC prognosis, were differentially expressed in HGSC patients suggesting an unsuccessful attempt to activate anti-tumor immunity and promoting T regulatory cell responses instead. 6) And finally, the cytokine mRNA expression in the 6 HGSC patients that received pre-operative cytostatic treatment was generally and highly depressed showing that preoperative cytostatic therapy knocks down the immune system preventing cytokine mRNA expression thus abrogating activation of any immune response.

HGSC/EOC is known to harbor tumor infiltrating lymphocytes (TILs) in various amounts that could be correlated to the overall patient survival (reviewed in 1). Depending on the subtypes of TILs, they may play opposite roles in the disease outcome. A high frequency of cytotoxic CD8<sup>+</sup> T-cells, NK cells and V $\gamma$ 9V $\delta$ 2  $\gamma$  $\delta$ T cells in the TIL cell population was correlated with better survival rates [23-25] whereas dominance of Tregs among TILs created an immunosuppressive microenvironment helping the tumor escape immune attack [26]. We found differential cytokine mRNA expression in the tumor microenvironment and PBMC of HGSC patients, patients with benign ovarian conditions and the normal control group. Firstly, the HGSC patients could not mount an adequate mRNA expression of the Th1 index cytokine IFN $\gamma$  which promotes T-cell mediated cytotoxicity crucially important in anti-cancer defense. The downregulation of IFN $\gamma$  expression in the tumor microenvironment was accompanied by enhancement of IL-15 mRNA signals. IL-15 has been previously shown to activate binding of STAT1-5 to regulatory sites of the IFN $\gamma$  gene thus participating in IFN $\gamma$  up-regulation [27]. So the observed increase in IL-15 mRNA might be a compensatory but unsuccessful attempt to up-regulate the IFN $\gamma$  expression. Secondly, in line with the dysregulated Th1 response there was an up-regulated IL-4 mRNA response in PBMC suggesting a Th2 deviation of the systemic immune response that further disrupts Th1-mediated cytotoxic function thus promoting immune escape of the tumor. Thirdly, both in the tumor microenvironment and in the peripheral blood prevailed a constitutive and statistically significant mRNA up-regulation of the immunosuppressive cytokines TGF $\beta$ , IL-10, and IL-2, all three being cytokines that are instrumental for the priming, clonal expansion and immunosuppressive function of both innate and adaptive T regulatory cells [28]. The up-regulation of these cytokines is consistent with the assumption that TILs of T regulatory phenotype are present in the analyzed tumor tissue samples. Accumulation of Treg in most tumors, including epithelial ovarian tumors, is a well-documented fact. T regulatory cells of both innate and adaptive type have emerged as "cancer's best friends" in that they act as major immune suppressors of the immune response and thus largely contribute to the compromised status of the immune system of cancer patients [29]. A specific recruitment of CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> Treg cells, tolerogenic dendritic cells, tumor-associated macrophages and myeloid-derived suppressor cells takes place in the tumor microenvironment of HGSC/EOC [30,31], all of these cells being involved in the immune escape of EOC and coinciding with rapid tumor progression and poor survival [32]. In addition to recruitment, there is local Treg expansion and de novo conversion of FoxP3<sup>-</sup> naïve T cells into adaptive Tregs in the tumor microenvironment under the influence of TGF $\beta$ , IL-10 and IL-2 [29,33,34]. Thus, TGF $\beta$ , IL-10 and IL-2 mRNA profile in our analyses suggests Tregs in the TIL population. In addition to the negative influence of Treg cells in tumors, the role of the newly discovered innate lymphoid cells (ILC) should be of interest to study. ILC in cancer have been little studied so far, however,

recent reports suggest a role for ILC subclasses both in the promotion and maintenance but also in the elimination of tumors at various anatomical sites (reviewed in 35). Recent observations indicated that epigenetic mechanisms might be involved in the regulation of ILC and T cells via the enzyme histone lysine methyltransferase G9a. This enzyme regulates ILC2 development and function and, in addition, the T-cell differentiation during inflammation as shown in a mouse model [35-38]. In a G9a double knock out murine model the T cell response was deviated towards inflammation and T regulatory-cell priming, i.e. tumor promoting events [38]. Thus, aberrant G9a expression might affect the immune cell homeostasis in malignant human disorders. These findings, and the participation of G2a and its partner enzyme GLP in maintenance of DNA methylation [39,40] suggest that G9a might serve as an immunological check point and also as an epigenetic regulator and therefore be a good candidate for intervention in future anti-cancer therapy.

A constitutive finding in our analyses was a high and statistically significant up-regulation of the inflammatory response in HGSC locally in the tumor microenvironment and systemically in the PBMC as measured by cytokine mRNA expression of IL-1 $\beta$ , IL-6, IL-8, TNF $\alpha$  and TNF $\beta$ /LTA. In contrast, benign ovarian conditions expressed lower amount of inflammatory cytokine mRNA comparable to normal ovarian tissue and PBMC of the normal control patients. The local inflammatory mRNA up-regulation in HGSC tumor tissue was more prominent than that in the corresponding PBMC of paired samples suggesting an enrichment of inflammatory cytokine mRNA signals in the local tumor micromillieu. Tumor-associated chronic inflammation is not specific for ovarian cancer but is a constitutive feature in cancer per se [41,42]. Compelling evidence imply that the inflammatory cytokine response in ovarian cancer promotes tumorigenesis and influences the clinical disease status, progress and prognosis [43,44]. Most of the inflammatory cytokines included in our analysis, such as TNF- $\alpha$ , IL-1 $\beta$ , IL-6, and IL-8 can be produced both by the ovarian tumor itself, supporting stromal cells and/or by activated tumor-infiltrating immune cells and all of these cell types participate in the molding of inflammation in the local tumor microenvironment [42-44]. We have analyzed the general cytokine milieu in the tumor microenvironment without dividing the cytokine response into tumor cell-, stromal cell- or immune-cell associated. The reason for that is that we wanted to see the overall type of cytokine mRNA response that was mobilized and prevailed in the tumor microenvironment as such, regardless of the cell types contributing to the establishment of the response. Our finding of significant upregulation of TNF $\beta$ /LTA, a tumor necrosis factor specifically produced and secreted by activated T lymphocytes, reveals activated immune cells as one of the contributing cells. The up-regulation of the inflammatory cytokine trio of IL-1, TNF- $\alpha$  and particularly IL-6, plays a central role in the evolution of EOC. The mechanisms, by which these cytokines influence the disease progression and outcome, are complex and multifactorial [43]. Importantly, it was shown that IL-1, TNF- $\alpha$  and IL-6 are responsible for development of symptoms, characteristic for cancer like anorexia, anemia, altered energy metabolism, weight loss, depression and fatigue and non-responsiveness to chemotherapy [43,45-47]. Elevated IL-6 levels, present in the EOC serum and malignant ascites, were associated to residual disease after debulking and to disease recurrence [48,49]. Furthermore, elevated IL-6 levels were found to impair membranal IL-2 receptor expression and immune cell responses [50] and play a central role in cancer-related anemia [51,52]. TNF- $\alpha$  was shown to have direct effect on tumor cell spreading and neovascularization [53]. Thus, mounting an inflammatory response promotes several

unwanted effects in HGSC such as stimulation of cancer-cell growth and angiogenesis, disease progression and poor disease outcome. Recently, promising attempts to counteract the negative influence of the inflammatory microenvironment in epithelial ovarian cancer by targeting TNF- $\alpha$  and IL-6 were undertaken with promising results. Inhibition of inflammatory cytokines seemed to restore sensitivity to chemotherapy, counteract angiogenesis, reverse systemic cancer symptoms and improve survival prognosis [54-56].

In this study, we chose to measure cytokine mRNA expression levels using RT-qPCR in lieu of measuring biologically active cytokines at the protein level. The primary reason for that was that there was a time lag from collection to processing and freezing serum/plasma samples and the cytokines are sensitive to degradation in rt. Thus, we would not know the true concentration of cytokines analyzed at the protein level. The high specificity, sensitivity and stability of RT-qPCR as a method and our own long term experience and access to a well-established RT-qPCR assay for simultaneous measurement of mRNA expression for a large set of cytokines was another reason to choose mRNA expression analysis [19,20]. Most important, our choice is supported by a previous study that showed an excellent correlation between the levels of cytokine mRNA measured by this method and measurements of corresponding cytokines at the protein level [57]. Further, RT-qPCR of tumor samples was chosen over immunohistochemical protein staining of tumor tissue since the latter is less sensitive and the semi-quantification can be easily biased. Cytokines are dynamically expressed molecules and the composition of cytokines can vary in the different ovarian cancer subtypes, between patients with the same cancer subtype, stage and grade and also in time. To minimize these expected variations between samples we have concentrated on the most malignant type of ovarian cancer, HGSC/serous EOC type II of similar grade and from the quality of the obtained results and the acceptable standard deviations we feel confident to discuss the cytokine expression in term of cytokine profiles. Furthermore, the comparability of the analyses between the samples is highlighted by the uniform and stable expression of the endogenous control that also ensured reliable quantification.

A major strength of this investigation is that in addition to comparison of HGSC patients with normal controls, patients suffering of benign ovarian conditions were included. Furthermore, simultaneous analyses were done with paired tissue- and corresponding PBMC samples thus opening a possibility to compare the cytokine mRNA expression at the local and systemic level. Another strength is the broad panel of cytokines investigated chosen to cover Th cytokine profiles of major interest. The real-time RT-qPCR analyses of mRNA for all 12 chosen cytokines was done at the same occasion to avoid experimental bias. The study groups were small with heterogeneous diagnoses for the group of benign ovarian conditions. The limited number of participants was due to difficulties to find paired blood and tissue samples from benign ovarian conditions and samples from normal ovaries. For the ovarian cancer group we selected HGSC to achieve a more homogeneous group regarding cancer subtype, grade and stage. The number of patients in the HGSC group was also reduced due to the need to exclude patients that received preoperative treatment with cytostatic drugs.

In summary, four major co-operating cytokine mRNA profiles responsible for tumour immune escape at the local and systemic level were found in HGSC patients, i.e. a profound inflammatory- and Treg immunosuppressive cytokine responses, a dysregulated local Th1 response and a Th2-deviated humoral systemic response. Future studies using larger cohorts are needed to elucidate in detail the role of cytokines in the establishment and disease progression of HGSC.

Our study contributes to evaluation of potential cytokine candidates for diagnostic cytokine mRNA profiles and therapeutic intervention by cytokine inhibition.

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