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High serum levels of interleukin-6 in endometrial carcinoma are associated with uterine serous papillary histology, a highly aggressive and chemotherapy-resistant variant of endometrial cancer

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Abstract

Purpose. To evaluate and compare autocrine expression and production of interleukin-6 (IL-6), a pleiotropic cytokine involved in the resistance to cytotoxic agents and inhibition of anti-tumor immune function in endometrial carcinoma in vitro as well as in vivo.

Patients and methods. IL-6 gene expression levels were evaluated in twenty-four primary endometrial tumors including 14 endometrioid carcinomas (EC) and 10 uterine serous papillary carcinoma (USPC) as well as in normal control endometrial cells (NEC) by real-time PCR. Secretion of IL-6 protein by 6 primary endometrial tumor cultures including USPC and EC was measured using a sensitive enzyme-linked immunosorbent assay (ELISA) in vitro. Finally, IL-6 concentration in 71 serum samples including 20 apparently healthy women, 19 women with benign abdominal diseases, 19 women with primary EC, and 13 USPC patients was studied.

Results. IL-6 gene expression levels were significantly higher in USPC when compared to EC (mean copy number by RT-PCR = 313 ± 55 vs. 53 ± 11, USPC vs. EC, respectively: $P < 0.01$). IL-6 serum concentrations between normal healthy females (range 0.01–21.23 pg/ml; mean 3.1 pg/ml) and benign disease patients (range 0.01–95.77 pg/ml; mean 13.07 pg/ml) were not statistically different. In contrast, significantly higher levels of IL-6 were detected in both patients with EC (range 2.86–82.13 pg/ml; mean 20.43 pg/ml) and patients with UPSC (range 16.3–500.1 pg/ml; mean 125.7 pg/ml) when compared to the healthy females ($P < 0.01$), with a mean serum IL-6 level in USPC patients 6.1-fold higher when compared to EC patients ($P < 0.03$). Accordingly, higher levels of IL-6 secretion were noted in primary USPC cell lines (mean 3121 pg/ml, range between 1099 and 5017 pg/ml/10^5 cells/48 h) when compared to primary EC (mean 88, range between 19 and 112 pg/ml/10^5 cells/48 h) ($P < 0.01$) in vitro.

Conclusions. IL-6 is highly expressed in USPC, and it is released in high concentration in the serum of USPC patients. IL-6 may be a novel biomarker for USPC. Drugs used to inhibit the expression of IL-6 or the IL-6 signal transduction pathway may potentially be highly beneficial in USPC.

Keywords: Uterine serous papillary carcinoma; Interleukin-6; Biomarkers; Endometrial cancer

Introduction

Endometrial carcinoma is the most prevalent gynecologic tumor in women, with an estimated 40,320 cases and 7090 deaths in the United States in 2004 [1]. On the basis of clinical and histopathologic variables, two subtypes of endometrial carcinoma, namely Type I and Type II tumors, have been described [2]. Type I endometrial cancers account

Abbreviations: USPC, uterine serous papillary carcinoma; IL-6, Interlukin-6.

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for the majority (i.e., about 80%) of cases, are usually associated with a history of hyperestrogenism as the main risk factor, are well or moderately differentiated in their histology, and typically have a favorable prognosis with appropriate therapy. In contrast, Type II endometrial cancers are poorly differentiated tumors, often with serous papillary or clear cell histology and are not associated with hyperestrogenic factors. These tumors are biologically highly aggressive and account for about 50% of all relapses which occur in endometrial cancer patients [2,3].

Uterine serous papillary carcinoma (USPC) represents the most aggressive variant of Type II endometrial cancer and may constitute up to 10% of endometrial tumors [4–11]. The microscopic criteria for diagnosis of USPC were first outlined by Hendrickson in 1982 [11]. Typically, the neoplastic epithelium is characterized by serous differentiation with psammoma bodies present and with predominantly papillary architecture [11]. Pleomorphism, grade III nuclear atypia with prominent nucleoli and a vesicular chromatin pattern, as well as a high mitotic activity are commonly detected in this tumor. Clinically, USPC has a propensity for early intra-abdominal and lymphatic spread even at presentation and is characterized by a highly aggressive biologic behavior [4–11]. Unlike the histologically similar high grade serous ovarian carcinomas, USPC is a chemoresistant disease from onset, with responses to combined cisplatinum-based chemotherapy in the order of 20% and of short duration [7–9]. The survival rate is dismal, even when USPC is only a minor component of the histologically more common endometrioid adenocarcinoma, and widespread metastasis and high mortality may occur even in those cases in which tumor is confined to the endometrium or to an endometrial polyp [4–11]. The overall 5-year survival is about 30% for all stages and the recurrence rate after surgery is extremely high (50% to 80%). The discovery of novel diagnostic and therapeutic markers against this aggressive subset of endometrial cancers remains a high priority.

With the goal of identifying genes highly and differentially expressed in USPC and to use this knowledge for the development of novel diagnostic and therapeutic markers against this disease, our group has recently used high-throughput technologies, such as high-density oligonucleotide microarrays to analyze USPC genetic fingerprints [12]. Among the several candidate target genes identified, the gene encoding for human interleukin-6 (IL-6) was consistently found as one of the most highly up-regulated genes in USPC. In this regard, IL-6 is a pleiotropic cytokine endowed with a variety of effects on hematopoiesis, the immune system, and acute-phase responses [13]. IL-6 has been previously shown to regulate cell growth of a variety of human cancers, and high serum levels of this cytokine have been correlated with shorter survival in patients harboring renal carcinoma, prostate cancer, and ovarian carcinoma [14–17]. Recently, autocrine secretion of IL-6 in cholangiocarcinoma and breast human tumors has been correlated with an altered expression of apoptosis regulatory proteins causing high resistance of these tumors to chemotherapy [18,19]. Because of these intriguing observations and the fact that no studies have investigated autocrine production of IL-6 in USPC, a highly aggressive and chemotherapy-resistant variant of endometrial cancer, in this study, we have carefully investigated IL-6 gene expression and protein secretion in Type I (i.e., endometrioid) and Type II (i.e., USPC) endometrial cancer in vitro as well as in vivo.

Patients and methods

Primary tumors

Tumor samples were derived from primary specimens staged according to the F.I.G.O. operative staging system. Fresh tumor biopsies from twenty-four endometrial tumors including 14 EC and 10 USPC were derived from patients harboring invasive tumors at the time of surgery through the Gynecologic Oncology Division and the Pathology Department, UAMS, under approval of the Institutional Review Board. Patient characteristics from which tumor biopsies were obtained are described in Table 1. Total abdominal hysterectomy and bilateral lymph node dissection were performed in all endometrial cancer patients. Three normal endometrial control cell samples (NEC) were obtained from biopsies of benign hysterectomy specimens from similar age women. Primary tumor samples and NEC were established as short-term cultures following previously reported standard tissue culture techniques [12,20]. Briefly, normal tissue obtained from healthy endometria and tumor tissues obtained from cancer patients were mechanically minced and enzymatically dissociated with 0.14% collagenase Type I (Sigma, St. Louis, MO) in RPMI 1640 medium (Invitrogen, Grand Island, NY) as described previously by Bongso et al., with minor modifications [20], and Santin et al. [12,21]. After 1–2 h incubation with enzyme on a magnetic stirring apparatus at 37°C in an atmosphere of 5% CO_2, the resulting suspension

Table 1
Characteristics of the patients from which primary tumor biopsies were obtained

	NEC $n = 3$	EC $n = 14$	USPC $n = 10$
Age (mean ± SD)	49 ± 3	58 ± 11	63 ± 10
Stage			
I	–	11	1
II	–	2	1
III	–	1	5
IV	–	–	3
Grading			
G1	–	8	–
G2	–	4	–
G3	–	2	10

NEC = normal endometrial cells.
EC = endometrial cancer.
USPC = uterine serous papillary carcinoma.

was collected by centrifugation at 100 × *g* for 5–10 min and washed twice with RPMI 1640 medium containing 10% fetal bovine serum (FBS, Invitrogen, Grand Island, NY). The final pellet was then placed in RPMI 1640 containing 10% FBS, 200 u/ml penicillin, and 200 μg/ml streptomycin in tissue culture flasks or Petri dishes (Invitrogen). The epithelial explants and tumor cells were then allowed to attach and proliferate. Explants were trypsinized and subcultured for 1 to 2 passages before being collected for RNA extraction while tumor cells were collected for RNA extraction at a confluence of 50% to 80% after a minimum of two to a maximum of ten passages in vitro. The epithelial nature and the purity of tissue cultures were verified by immunohistochemical staining and flow cytometric analysis with antibodies against cytokeratin and vimentin as previously described [12,20,21]. Only primary cultures which had at least 90% viability and contained >99% epithelial cells were used for IL-6 quantification by real-time PCR and analyzed for IL-6 secretion in vitro by ELISA as described below.

RNA isolation

RNA isolation from all primary samples including twenty-four primary endometrial cancers (i.e., 10 USPC and 14 EC) as well as 3 normal endometrial cell controls was performed using TRIzol Reagent (Invitrogen) according to the manufacturer's instructions. To verify integrity, 4 μg of RNA from each sample was run in 1% agarose gel using 18S+28S ribosomal RNA (Sigma) as a positive control. RNA extracted from CaOV3 serous papillary ovarian cancer cell line, previously reported to express IL-6 [22], was used as a positive control.

Quantitative real-time PCR

q-RT-PCR was performed with an ABI Prism 7000 Sequence Analyzer using the manufacturer's recommended protocol (Applied Biosystems, Foster City, CA) to evaluate expression of IL-6 gene in samples from all primary cell lines. Each reaction was run in triplicate. The comparative threshold cycle (C_T) method (PE Applied Biosystems) was used to determine gene expression in each sample relative to the value observed in the nonmalignant endometrial epithelial cells, using GAPDH (Assay-on-Demand Hs99999905_m1) RNA as an internal control. Briefly, 5 μg of total RNA from each sample was reverse-transcribed using SuperScript III first strand cDNA synthesis (Invitrogen, Carlsbad, CA). Ten microliters of reverse-transcribed RNA samples (from 500 μl of total volume) were amplified by using the TaqMan Universal PCR Master Mix (Applied Biosystems) to produce PCR products specific for IL-6. IL-6 primers were obtained from Applied Biosystems as assay on demand products (Assay ID: Hs00174131_m1). Differences among NEC, USPC, and EC in the q-RT-PCR expression data were tested using a Student's *t* test.

Analysis of IL-6 secretion

An important issue is whether differences in IL-6 gene expression level in tumor tissues result in meaningful differences in protein expression. To validate IL-6 data obtained by RT-PCR on primary USPC and EC cell lines at the protein level, supernatants obtained from 6 primary endometrial specimens including 3 USPC and 3 EC were evaluated by ELISA. Briefly, tumor supernatants tested for IL-6 secretion were collected from primary cell lines seeded at a density of 1×10^5 cells/ml in tissue culture Petri dishes (Gibco) in RPMI 1640, supplemented with 10% FBS (i.e., USPC and EC). After a 48 h incubation at 37°C, supernatants were aspirated, rendered cell-free by centrifugation at 1500 rpm for 10 min, and stored at −80°C before being analyzed for IL-6 by ELISA (RςD Systems Inc. Minneapolis, MN).

IL-6 concentration was quantified in the serum of 20 apparently healthy women (ages 26 to 72 years; mean 43 years), 19 women with benign diseases (ages 21 to 76 years; mean 46 years), 19 women with histologically proven primary endometrioid adenocarcinoma (ages 49 to 79 years; mean 63 years), and 13 women with histologically proven primary USPC (ages 53 to 84 years; mean 68 years) by ELISA. This assay has a detection limit of 3.12 pg/ml and a dynamic range up to 300 pg/ml. Of the benign lesions, 12 were classified as endometriosis, 2 as mucinous cystadenomas, 2 as ovarian dermoid cysts, 1 as ovarian benign teratomas, 1 as corpus luteum, and 1 as serous cystadenomas. The characteristics of the patients from which serum samples were obtained are described in Table 2. Serum samples from all patients were collected before surgery and stored at −80°C until analysis.

Statistical analysis

The analyses of differences between IL-6 serum concentrations among the different groups of patients (i.e.,

Table 2
Characteristics of the patients from which serum samples were obtained

Variable	Healthy female *n* = 20	Benign diseases *n* = 19	EC *n* = 19	USPC *n* = 13
Age:				
(mean ± SD)	42.6 ± 10.9	46.2 ± 15.6	62.9 ± 11.3	68.2 ± 9.5
Stage				
I	–	–	14	1
II	–	–	3	1
III	–	–	2	7
IV	–	–	–	4
Grading				
G1	–	–	11	–
G2	–	–	6	–
G3	–	–	2	13

EC = endometrial cancer.
USPC = uterine serous papillary carcinoma.

healthy controls, benign gynecologic diseases, EC, and USPC) as well as among USPC, EC, and NEC specimens in the q-RT-PCR expression data were performed using the Student's *t* test at alpha = 0.05.

Results

IL-6 expression in endometrial cancer by quantitative real-time PCR

Tumor tissue flash frozen biopsies are known to contain significant numbers of contaminant stromal cells as well as a variety of host derived immune cells (e.g., monocytes, dendritic cells, lymphocytes). In addition, USPC represents rare tumors which may present in either pure forms or admixed with endometrioid or clear cell tumor cells (i.e., mixed USPC) [4–11]. To minimize the risk of contamination of USPC RNA with that of normal cells or tumor cells with different histology, we extracted RNA to be evaluated for IL-6 expression by RT-PCR from twenty-four primary endometrial cancers with single type differentiation (i.e., 10 USPC and 14 EC). Normal endometrial cell (NEC) IL-6 levels from 3 samples were used as controls. Short term USPC, EC, and NEC cell cultures, minimizing the risk of a selection bias inherent in any long-term in vitro growth, may provide an opportunity to study differential gene expression between highly enriched populations of normal and tumor-derived epithelial cells. A comparison of the q-RT-PCR data for IL-6 in USPC and EC using NEC as controls is shown in Fig. 1. Significant expression differences between USPC and EC were readily apparent (Figs. 1A and B). All USPC samples (10 out of 10 = 100%, mean copy number ± SEM = 313 ± 55 range from 56 to 553) and the majority of EC samples (11 out of 14 = 79%, mean copy number ± SEM = 53 ± 11 range from 4 to 119) were found positive for IL-6 expression by RT-PCR (Fig. 1B). However, only 2 out of 14 of the EC (14%) had an mRNA copy number above 105 (Fig. 1B). In contrast, 8 out of 10 USPC (80%) were found to highly express the IL-6 gene (Fig. 1B, USPC vs. EC: $P < 0.01$). Low levels of IL-6 gene expression were found in the NEC control cultures tested (mean copy number ± SEM = 5 ± 2 range 1–9) (Fig. 1).

IL-6 secretion by primary Type I and Type II endometrial cancer in vitro

Cell free supernatants from 6 freshly isolated endometrial specimens including 3 USPC and 3 EC were collected and analyzed for levels of IL-6 expression by ELISA. Because prolonged passages in vitro are known to alter the physiology and phenotype of primary tumor cells, we performed all of our experiments with highly purified fresh tumor cells grown for less than 10 passages in vitro. Growth control medium was always analyzed at the same time. In this regard, RPMI 1640 containing 10% fetal bovine serum had no detectable endogenous levels of IL-6 activity (data not shown). As shown in Fig. 2, primary USPC tumor cell lines tested secreted large amounts of IL-6 (range of secretion from 1099 to 5017 pg/ml/10^5

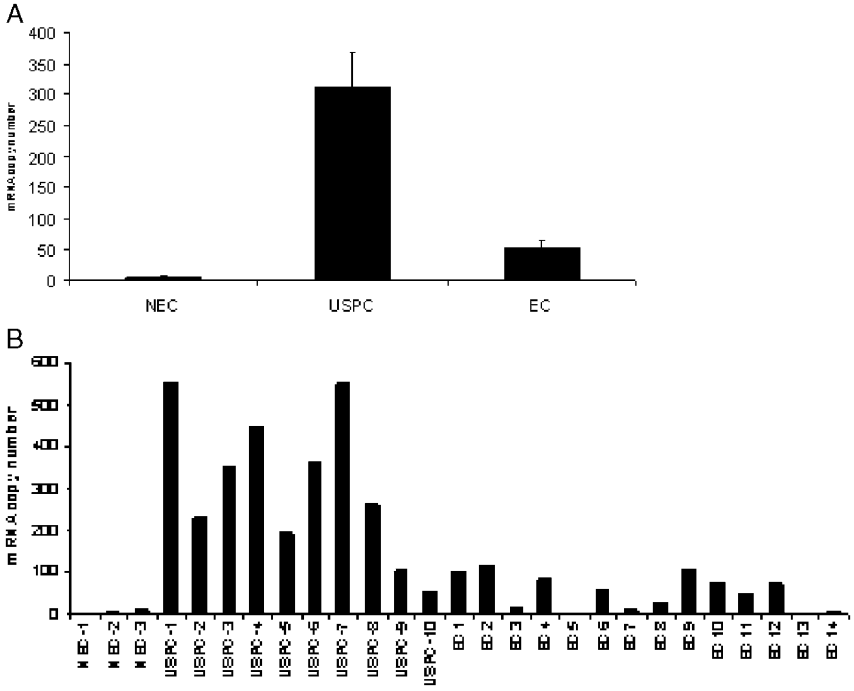

Fig. 1. IL-6 mRNA copy number by quantitative RT-PCR in 24 Type I and Type II endometrial cancers. (A) IL-6 mRNA mean copy number ± SD in 3 normal endometrial control cell samples (NEC), 14 endometrioid carcinoma (EC), and 10 uterine serous papillary carcinoma (USPC). (B) IL-6 mRNA copy number in individual NEC controls and Type I and Type II endometrial cancers.

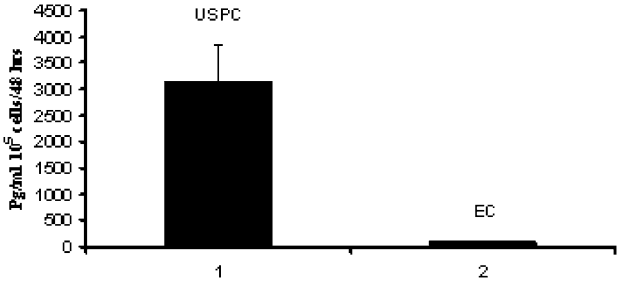

Fig. 2. IL-6 levels by ELISA in the supernatants from 6 primary short-term endometrial tumor cultures including 3 USPC and 3 EC.

cells/48 h, mean 3121 pg/ml). USPC IL-6 secretion levels were significantly higher when compared to EC cell lines (range of secretion from 19 to 112 pg/ml/10^5 cells/48 h, mean 88 pg/ml) ($P < 0.01$).

Serum IL-6 concentration in endometrial cancer and noncancer patients

To investigate whether IL-6 is detectable in the serum of patients harboring Type I and Type II endometrial cancer, serum samples from 19 EC patients and 13 USPC patients were evaluated by ELISA. In addition, serum samples obtained from 20 healthy female controls and 19 patients diagnosed with benign gynecologic diseases were analyzed at the same time. IL-6 serum levels from 20 healthy female controls (range 0.01–21.23 pg/ml; mean 3.05 pg/ml) and 19 patients with benign gynecologic diseases (range 0.01–95.77 pg/ml; mean 13.07 pg/ml) were not statistically significantly different (Table 3). In contrast, serum IL-6 values in patients with EC (range 2.86–82.13 pg/ml; mean 20.43 pg/ml) and USPC patients (range 16.3–500.1 pg/ml; mean 125.7 pg/ml) were significantly higher than those in the healthy group ($P < 0.01$). When IL-6 levels in the serum of USPC patients were compared to the levels found in EC patients, a significant difference was found with a mean serum IL-6 level in USPC patients 6.1-fold higher when compared to EC patients ($P < 0.03$).

Discussion

This report represents the first evaluation of IL-6, a pleiotropic cytokine previously associated with increased resistance to chemotherapy, apoptosis, and inhibition of anti-tumor immune function in a variety of human tumors [13–19], as a novel biomarker in USPC patients. In this study, we have quantified IL-6 expression by RT-PCR in 24 primary endometrial carcinomas including 14 EC and 10 USPC. In addition, we have studied IL-6 protein secretion in 6 primary endometrial tumor specimens including EC and USPC. In our study, we have confirmed the purity of the tumor cells in fresh tumor specimens by differential counts of Giemsa-stained cytospin slides as well as by cytokeratin expression using immunohistochemical techniques (data not shown). Our fresh tumor samples contained over 99% tumor cells. Finally, we have studied IL-6 levels in 71 serum samples derived from healthy donors, patients harboring benign gynecologic tumors, EC, and USPC.

We report a high level of expression of the IL-6 gene in USPC, a highly aggressive variant of uterine cancer. In addition, we show that IL-6 gene expression is significantly higher in USPC when compared to EC and NEC by RT-PCR. In this regard, the mean copy number of IL-6 gene in USPC was found to be 5.9 times higher when compared to EC cells. Consistent with the gene expression results, primary USPC cultures were found to secrete significantly higher levels of IL-6 by ELISA when compared to primary endometrioid carcinomas. These data highlight for the first time a major difference between EC and USPC in the expression and secretion of IL-6.

Importantly, recent reports have shown autocrine production of IL-6 by human tumor cells to alter the expression of apoptosis regulatory proteins [18] and cause resistance of the tumors to chemotherapy [19]. These data, demonstrating a novel mechanism of IL-6 to promote tumor cell survival, have highlighted the potential of IL-6 as a prognostic indicator for the identification of cancers highly resistant to chemotherapy. In agreement with these data, elevated serum levels of IL-6 in a subset of ovarian cancer patients harboring serous papillary ovarian tumors, a variant of ovarian cancer histologically indistinguishable from USPC, have been previously shown to identify cancer patients who do not respond to chemotherapy [16]. Our results showing high levels of IL-6 secretion by USPC, a variant of uterine carcinoma notorious for its high resistance to chemotherapy and its aggressive biologic behavior, are consistent with these findings. This hypothesis is further supported by the recent demonstration that IL-6 promotes the resistance of tumor cells to chemotherapeutic agents through the activation of the p38 mitogen-activated protein kinase (MAPK) signaling pathway [19,23]. Of interest, the induction of MAPK signaling pathway by IL-6 has been shown to require HER2/neu expression [24], a growth factor receptor recently identified to be highly up-regulated in the majority of USPC [25] and correlated with poor prognosis in USPC [26]. Taken together, these data suggest that high levels of IL-6 gene expression and protein secretion in USPC may

Table 3
Serum IL-6 in noncancer (healthy), benign disease, EC, and USPC patients

Variable	mean ± SEM	Range (pg/ml)
Noncancer ($n = 20$)	3.1 ± 1.1	0.01–21.23
Benign disease ($n = 19$)	13.07 ± 4.9	0.01–95.77
EC ($n = 19$)	20.43 ± 6.1	2.86–82.13
USPC ($n = 13$)	125.7 ± 44.2*	16.3–500.1

* Noncancer vs. benign = P not significant; noncancer cells vs. EC = $P > 0.01$; noncancer vs. USPC = $P > 0.01$. Benign vs. EC = P not significant; benign vs. USPC = $P > 0.02$. EC vs. USPC = $P > 0.03$.

be associated with the HER2/neu signaling cascade and may promote the resistance of USPC cells to cytotoxic agents.

When IL-6 levels were quantified in the serum of endometrial cancer patients, we found significantly higher concentrations of IL-6 in both USPC and EC patients, when compared to the levels found in healthy control women or patients harboring benign gynecologic disease. However, USPC patients had on average 6.1-fold higher levels of IL-6 when compared to EC. These data are consistent with our in vitro results on IL-6 secretion in primary USPC, which were found to secrete significantly higher levels of IL-6 when compared to primary EC. It is worth noting that, in our series of USPC patients, all of whom were surgically staged by a gynecologic oncologist, the striking majority were found to harbor advanced disease. Thus, although most of our patients were considered clinical stage I and up-graded only at the time of the comprehensive surgical staging laparotomy, it is possible that the elevated levels of IL-6 found in this work may reflect a bias related to the high number of patients with advanced disease. Only two patients had surgically confirmed early stage disease. One of them, however, was found to have an elevated level of IL-6. Larger studies including more patients harboring surgically confirmed early stage USPC disease will be necessary to exclude this hypothesis. It is important to point out, however, that because of the propensity of USPC to rapidly manifest extrauterine disease (i.e., positive lymph node metastases or spreading to the abdominal cavity), the USPC series reported here seems most representative of the advanced stage disease commonly detected in comprehensively surgically staged USPC patients [5,27–29].

Tumor-antigen pulsed dendritic cells (DC) [i.e., the most potent professional antigen presenting cells (pAPC) known in humans for triggering the induction of an antigen-specific immune response] (for review see [30]) have recently been reported by our group to induce the generation of tumor-specific cytotoxic T cells in patients harboring advanced USPC in vitro [21]. These data, combined with the promising preliminary clinical data reported in other human malignancies when DC-based vaccinations have been used to induce anti-tumor immunity [31–36], have generated widespread interest in the use of these cells as a novel, potentially effective, immunotherapeutic approach for the treatment of residual/resistant USPC after standard surgical and cytotoxic treatment. Of interest, tumor-derived IL-6 has been reported to dramatically impair DC differentiation and maturation and convert DC into suppressive antigen-presenting cells capable of inducing tumor-specific T cell tolerance [37, 38]. These data combined with our present findings suggest that high IL-6 secretion by USPC may create a highly immunosuppressive microenvironment in vivo and may represent an important mechanism exploited by USPC for modulating host anti-tumor immune responses.

In conclusion, we showed the first evidence that IL-6 is highly expressed in USPC and that high concentrations of IL-6 are present in the serum of USPC patients. Taken together, our results support the premise that IL-6 may play a major role in the induction and progression of this biologically aggressive subtype of endometrial cancer and may represent one of the main mechanisms exploited by USPC for modulating host anti-tumor immune responses and causing resistance to cytotoxic agents. Finally, these findings suggest that drugs used to inhibit IL-6 activity by receptor antagonists, neutralizing antibodies, the expression of IL-6, or the IL-6 signal transduction pathway may potentially be highly beneficial in USPC patients.

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