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Mechanisms for Immune Escape in Epithelial Ovarian Cancer

Pernilla Israelsson

Department of Clinical Sciences, Obstetrics and Gynecology
Department of Clinical Microbiology, Infection and Immunology
Department of Medical Biosciences, Pathology

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Till min familj

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ABSTRACT

Tumors develop mechanisms to subvert the immune system, constituting immune escape. Epithelial ovarian cancer (EOC), the deadliest of all gynecological malignancies, uses a variety of mechanisms to undermine immune surveillance, aiding its establishment and metastatic spreading. Despite progress in oncoimmunology, a lot remains unknown about the cancer-immune system interplay. The aim of this thesis was to study tumor-mediated mechanisms for immune escape in EOC patients, focusing on the role of cytokines and EOC-derived exosomes.

Cytokines are key molecules regulating immune effector functions in health and disease. We used real-time RT-qPCR and a set of primers and probes for 12 cytokines, discriminating between different immune responses and compared the cytokine mRNA expression profiles locally in the TME and systemically in peripheral blood immune cells of EOC patients, to women with benign ovarian conditions and women with normal ovaries. The cytokine mRNA expression was in general most prominent in EOC patients, confirming the immunogenicity of EOC. We found significant dominance of inflammatory and immunosuppressive/ regulatory cytokines, known to promote tumor progression by priming and activating T regulatory cell-mediated immune suppression. In contrast, IFN-γ, crucially important for evoking a cytotoxic anti-tumor response, was not upregulated. Instead, a systemic increase of IL-4 prevailed, deviating the immune defense towards humoral immunity. With regard to our cytokine study, we performed comparative analyses of cytokine mRNA versus protein expression in the EOC cell lines OVCAR-3 and SKOV-3. We found that cytokine mRNA signals were universally detected, and in some instances translated into proteins, but the protein expression levels depended on the material analyzed and the method used. Due to the high sensitivity of real-time RT-qPCR, we suggest that cytokine mRNA expression profiles can be used for some instances, such as in studies of mechanistic pathways and in comparisons between patient groups, but cannot replace expression at the protein level.

Exosomes are nanometer-sized vesicles of endosomal origin, released by virtually all cells, participating in normal and pathological processes. Like many tumors, EOC is a great exosome producer. We isolated exosomes from EOC ascitic fluid and supernatant from tumor explant cultures to study their effect on the NK cell receptors NKG2D and DNAM-1, involved in tumor killing. We found that EOC exosomes constitutively expressed NKG2D ligands on their surface while DNAM-1 ligand expression was rare and not associated with the exosomal membrane. Consistently, the major cytotoxic pathway of NKG2D-mediated killing was dysregulated by EOC exosomes while the accessory DNAM-1-mediated pathway remained unchanged. Our results provide a mechanistic explanation to the previously made observation that in EOC patients, tumor killing is only dependent on the accessory DNAM-1 pathway. Following these

results, we studied NKG2D-mediated cytotoxicity *in vivo* in EOC patients before and after surgery. We found that the serum exosomes isolated from EOC patients were able to downregulate the NKG2D receptor and suppress NKG2D-mediated cytotoxicity in NK cells from healthy donors, in a similar way as exosomes from EOC ascites. We also found that surgery of the primary EOC tumor has a beneficial effect on the patients' anti-tumor cytotoxic immune response. One mechanistic explanation could be a decrease in circulating NKG2D ligand-expressing exosomes, thus improving the cytotoxic NK cell function.

In conclusion, our results contribute to the understanding of the mechanisms responsible for tumor immune escape in general, and in EOC patients in particular, and might be useful in developing novel antitumor therapies. Our studies highlight the prevailing immunosuppression in the local TME and the immunosuppressive role of EOC exosomes. Furthermore, they support the notion that cancer surgery is also a way of removing exosome-producing cells and reducing the serum concentration of immunosuppressive exosomes, thus boosting the patients' cytotoxic anti-tumor response.

Keywords: human, ovarian cancer, high-grade serous cancer, EOC, HGSC, HGSOC, tumor microenvironment, immune escape, immune suppression, cytokines, exosomes, NKG2D, MICA/B, ULBP1-3, DNAM-1, surgery

POPULÄRVETENSKAPLIG SAMMANFATTNING

Äggstockscancer är den dödligaste av alla gynekologiska cancersjukdomar. Sjukdomen ger sällan några symtom hos kvinnan förrän den har spridit sig och upptäcks således oftast sent. Därför är det viktigt att genom forskning försöka förstå mekanismerna bakom cancerns spridning och hitta markörer för tidigare diagnostik och nya sätt att behandla sjukdomen. En del av vårt immunförsvar är programmerat för att kunna känna igen och eliminera förändrade eller skadade celler, exempelvis cancerceller. Ibland utvecklar cancerceller en förmåga att nedreglera och undkomma immunförsvaret. När det sker märker inte kroppen att en tumör bildas och sprids. Syftet med denna avhandling var att studera biologiska mekanismer som ger äggstockscancer förmåga att inaktivera immunförsvaret. Äggstockscancer är nämligen en tumörtyp som har utvecklat flera mekanismer för att nedreglera kroppens immunförsvar i syfte att undvika upptäckt. Det är ännu inte helt klarlagt hur detta går till. För att försöka förstå hur äggstockscancer kan undkomma immunförsvaret har vi i detta forskningsprojekt undersökt cytokiner i tumörvävnad och exosomer som tumören utsöndrar. Cytokiner är proteiner som agerar som lösliga signalmolekyler. Exosomer är mycket små ”signalbubblor” som innehåller olika molekyler som också finns i deras modercell. Cytokiner och exosomer utsöndras av såväl kroppens friska som sjuka celler för att cellerna ska kunna kommunicera med varandra utan att vara i direkt kontakt.

I det första delarbetet kartlades nivåerna av cytokin-mRNA, dvs. det genetiska förstadiet till cytokinproteiner, i tumörvävnad och i cirkulerande vita blodkroppar från kvinnor med äggstockscancer, och jämfördes med motsvarande uttryck hos kvinnor med godartade tillstånd i äggstockarna och kvinnor med normala äggstockar. Vi fann förhållandevis högre uttryck av cytokin-mRNA hos cancerpatienterna vilket tyder på att immunförsvaret i större utsträckning är aktiverat hos dem. Vidare dominerade nedreglerande och inflammatoriska cytokin-mRNA hos kvinnor med äggstockscancer. Cytokin-mRNA som är viktiga för vårt eget cancerförsvar och kroppens egna mördarceller visades vara nedreglerade. Mönstret sågs också i cirkulerande vita blodkroppar, vilket visar att tumören har en förmåga att påverka immunförsvaret, inte bara lokalt i tumören, utan i hela kroppen.

I delarbete II undersökte vi sambandet mellan cytokinernas mRNA respektive proteinuttryck. Proteinet är aktivt i kroppen medan mRNA är dess genetiska förstadium. Eftersom cytokinproteiner endast uttrycks kortvarigt och lokalt samt påverkas mycket av hur ett prov tas och senare hanteras i laboratoriet, är det svårt att dra slutsatser av en proteinanalys. I en modell med odlade cancerceller jämförde vi skillnaden mellan cytokinernas mRNA- och proteinuttryck. Vi fann att metoden för proteinanalys har ett snävt spann för att kunna upptäcka protein. Om inget protein kan detekteras behöver det inte betyda att det inte finns utan provet kan behöva spädas eller koncentreras. Analys av mRNA är mindre

ömtåligt och har hög träffsäkerhet. Vi fann att äggstockscancerceller producerade cytokin-mRNA som kan leda till mätbara proteinnivåer. Det finns vissa fördelar med mRNA-analysen som gör att den kan användas t ex för att kartlägga en individuell cytokin mRNA-profil som kan användas vid diagnostik eller behandling.

I delarbete III analyserades exosomer utsöndrade av äggstockscancer i form av odlade celler, vävnad, blod och ascites. Ascites är vätska i bukhålan som kvinnor med äggstockscancer kan utveckla. Vi ville undersöka om exosomer utsöndrade av cancerceller påverkar kroppens mördarceller. Vid äggstockscancer förefaller en aktiverande signalväg i mördarcellerna delvis vara utslagen. Vår hypotes var att exosomerna orsakar detta. Signalen går via något som kallas ligand-receptorsystem. En ligand är en molekyl som kan binda till en mottagarmolekyl (receptor) fäst på mördarcellernas yta. När liganden binder till receptorn får mördarcellen en signal om att den ska aktiveras och döda en målcell. Vi fann att äggstockscancer-exosomer uttrycker ligander för receptorn NKG2D på sin yta. Det är den viktigaste signalvägen i avdödandet av cancerceller. På så vis agerar exosomerna ”lockbete” och lurar mördarcellerna att nedreglera sina NKG2D-receptorer. Därmed aktiveras inte mördarcellerna för att döda cancerceller. En mindre effektiv receptor, DNAM-1, verkar istället dominera avdödande av äggstockscancerceller. Vi fann att dess ligander inte uttrycks på exosomernas yta och således lämnas denna bana opåverkad.

Om en tumör kan opereras bort i sin helhet förbättras kvinnans prognos avseende överlevnad. I delarbete IV ville vi studera effekten av kirurgi genom att undersöka blodprover tagna före och efter operationen. Vi undersökte hur en operation påverkar mängden exosomer i blodet, de molekyler som exosomerna uttrycker och om effektiviteten hos kvinnans mördarceller påverkas. Oavsett om hela tumören opererats bort eller inte kunde vi hos samtliga patienter se en förbättrad funktion hos mördarcellerna efter operationen. En möjlig förklaring till detta är den minskade mängden exosomer i blodet som kunde observeras hos en grupp patienter efter kirurgi. Dessa kvinnor hade dessutom ett högre uttryck av NKG2D receptorn hos mördarcellerna efter operationen.

Sammanfattningsvis har vi funnit nya mekanismer för hur äggstockscancer nedreglerar och undkommer immunförsvaret. Förhoppningsvis kan denna nya kunskap utgöra ytterligare en pusselbit i sökandet efter nya diagnostiska verktyg, nya effektiva behandlingar och i förlängningen bidra till en förbättrad överlevnad för kvinnor som drabbas av äggstockscancer.

LIST OF PUBLICATIONS AND MANUSCRIPT

This thesis is based on the following papers, referred to in the text by their Roman numerals:

I. **Assessment of cytokine mRNA expression profiles in tumor microenvironment and peripheral blood mononuclear cells of patients with high-grade serous carcinoma of the ovary**
Israelsson P, Labani-Motlagh A, Nagaev I, Dehlin E, Nagaeva O, Lundin E, Ottander U, Mincheva-Nilsson L.
J Cancer Sci Ther. 2017. 9:422-429. doi: 10.4172/1948-5956.1000453

II. **Cytokine mRNA and protein expression by cell cultures of epithelial ovarian cancer - Methodological considerations on the choice of analytical method for cytokine analyses**
Israelsson P, Dehlin E, Nagaev I, Lundin E, Ottander U, Mincheva-Nilsson L.
Am J Reprod Immunol. 2020;84:e13249. doi: 10.1111/aji.13249

III. **Differential expression of ligands for NKG2D and DNAM-1 receptors by epithelial ovarian cancer-derived exosomes and its influence on NK cell cytotoxicity**
Labani-Motlagh A, Israelsson P, Ottander U, Lundin E, Nagaev I, Nagaeva O, Dehlin E, Baranov V, Mincheva-Nilsson L.
Tumor Biol. 2015. 37:5455-66. doi: 10.1007/s13277-015-4313-2

IV. **The influence of surgery on circulating ovarian cancer exosomes and NKG2D-mediated cytotoxicity**
Israelsson P, Björk E, Nagaev I, Nagaev O, Lundin E, Mincheva-Nilsson L, Ottander U.
(Manuscript)

ABBREVIATIONS

ACT	Adoptive cell therapy
ADCC	Antibody-dependent cell-mediated cytotoxicity
Ag	Antigen
APC	Antigen presenting cell
BRCA	Breast related cancer antigen
CA-125	Cancer antigen 125
CAF	Cancer-associated fibroblast
CAM	Cell adhesion molecule
CCL	Chemokine (C-C motif) ligand
CD	Cluster of differentiation
CTL	Cytotoxic T lymphocyte
CTLA	Cytotoxic T lymphocyte-associated antigen
DAMP	Danger-associated molecular pattern
DC	Dendritic cell
DNAM	DNAX accessory molecule
DNAX	DNA-X frameshifting element
EMT	Epithelial-mesenchymal transition
EOC	Epithelial ovarian cancer
ESCRT	Endosomal sorting complex required for transport protein
EV	Extracellular vesicle
FIGO	International Federation of Gynecology and Obstetrics
HGSC	High-grade serous cancer
IDO	Indoleamine 2,3-dioxygenase
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
ILV	Intraluminal vesicle
LGSC	Low-grade serous carcinoma
lncRNA	Long non-coding RNA
mAb	Monoclonal antibodies

mRNA	Messenger RNA
MDSC	Myeloid-derived suppressor cell
MHC	Major histocompatibility complex
MIC	MHC class I chain-related protein
miRNA	MicroRNA
MMP	Matrix metalloproteinase
MVB	Multivesicular bodies
NK	Natural killer
NKG2D	Natural-killer group 2, member D
NTA	Nanoparticle tracking analysis
OC	Ovarian cancer
PARP	Poly (ADP-ribose) polymerase
PBMC	Peripheral blood mononuclear cell
PD-1	Programmed cell death protein 1
PD-L	Programmed death-ligand
PGE	Prostaglandin E
PVR	Poliovirus receptor
RT-qPCR	Quantitative reverse transcription polymerase chain reaction
TAM	Tumor-associated macrophage
TCR	T cell receptor
TGF	Transforming growth factor
Th	T helper
TIL	Tumor-infiltrating lymphocyte
TME	Tumor microenvironment
TNF	Tumor necrosis factor
TRAIL	Tumor necrosis factor (TNF)-related apoptosis-inducing ligand
Treg	T regulatory cell
ULBP	UL16 binding protein
US FDA	US food and drug administration
VEGF	Vascular endothelial growth factor
WB	Western blot

x

INTRODUCTION

Epithelial ovarian cancer

Incidence and survival

Ovarian cancer (OC) is the eighth most common cancer among women and the most lethal of all gynecological malignancies (1). Globally it accounts for approximately 295,000 new cases and 185,000 deaths each year (1). The incidence in Northern Europe is 9,2 per 100,000, a higher rate is seen only in Central and Eastern Europe (1). The lifetime risk of developing OC is 1 in 70 (2). Because of diffuse symptoms (2) and lack of screening methods, most women are diagnosed with advanced-stage ovarian cancer, FIGO stages III and IV, with a five-year survival rate in Sweden ranging from 36% to 19% (Table 1) (3). The five-year survival rate for localized-stage disease is however approximately 90%, emphasizing that the most important prognostic factor is stage at diagnosis (3).

Table 1. FIGO ovarian, fallopian and peritoneal cancer staging system summarized (modified from (4)). Stage at diagnosis and five-year survival rates in Sweden (3). Stage at diagnosis is unknown in 6% of cases.

Stage		Stage at diagnosis (%)	Five-year survival rate (%)
I	Tumor confined to ovaries or fallopian tube(s)	32	91
II	Tumor involves one or both ovaries or fallopian tubes with pelvic extension (below pelvic brim) or primary peritoneal cancer	8	90
III	Tumor involves one or both ovaries or fallopian tubes, or primary peritoneal cancer, with cytologically or histologically confirmed spread to the peritoneum outside the pelvis and/or metastasis to the retroperitoneal lymph nodes	38	36
IV	Distant metastasis excluding peritoneal metastases	16	19

Histopathological classification

Ovarian cancer is heterogeneous in nature and current classification is based on histology, where the epithelial ovarian cancer (EOC) is the most common and comprises about 90%. The other much much rarer conditions include malignant germ cell tumors and sex cord-stromal tumors (4). EOC can further be divided according to histopathology and genetic alterations; high-grade serous carcinoma (HGSC), endometroid, clear cell, mucinous and low-grade serous carcinoma (LGSC). These subgroups can be viewed as distinct disease entities based on differences in epidemiological and genetic risk factors, precursors, molecular features, response to chemotherapy and prognosis (5) (Table 2).

Table 2. Characteristics of the different EOC subgroups (modified from (5, 6))

	HGSC	LGSC	Mucinous	Endometroid	Clear cell
Proportion (%)	70	<5	3	10	10
Precursor lesions	Tubal intraepithelial carcinoma?	Serous borderline tumor	Borderline tumor?	Atypical endometriosis	Atypical endometriosis
Molecular abnormalities	BRCA, p53, genetically unstable	BRAF, KRAS	KRAS, HER2	PTEN, ARID1A	HNF1, ARID1A
Response to chemotherapy	High	Intermediate	Low	High	Low
Prognosis	Poor	Intermediate	Favorable	Favorable	Intermediate

A dualistic classification according to similarities in tumorigenesis has been proposed, into type I and type II tumors (7). Type I tumors include: endometroid, clear cell, low-grade serous carcinomas, mucinous carcinomas and malignant Brenner tumors. Type I tumors are thought to develop in a step-wise fashion from well-defined precursor lesions. They are slow-growing, genetically stable with better prognosis. Type II tumors include high-grade serous carcinomas, carcinosarcomas and undifferentiated carcinomas. HGSC is suggested to develop from the precancerous lesion serous tubal intraepithelial carcinoma (STIC) in the fallopian tube, that disseminates as carcinoma in the fallopian tube and to the ovary, peritoneum etc. Type II tumors are fast-growing and more aggressive, with *TP53* mutations and account for 90% of ovarian cancer deaths (7).

Etiology and pathogenesis

The etiology of EOC is unknown. The cell of origin and subsequent pathogenesis has been widely debated and numerous etiological hypotheses have been presented over the years.

The ovarian epithelium is the modified pelvic mesothelium covering the ovary. It consists of a single layer of flat to cuboidal epithelial cells (8). EOC on the other hand is Müllerian in nature with serous columnar epithelium, resembling fallopian tube (serous), endometrium (endometroid), gastrointestinal tract (clear cell) or endocervix (mucinous), and has traditionally been thought to emerge from metaplasia of the ovarian surface epithelium (8, 9). In 1971 Fathalla published the incessant ovulation theory based on the observation that the number of ovulatory events increases the risk of OC, stating that this might be caused by the constant damage and repair of the ovarian epithelium during ovulation, increasing proliferation and subsequently the risk of DNA damage (10). Following this, several proposed causes for genomic instability in the ovary have been highlighted, for example the gonadotropin and estrogen effect (11), the inflammation caused by ovulation (12-14) and the incessant menstruation hypothesis, where the toxic effect of iron is stressed (15). Ovarian, tubal and

peritoneal cancers have thus been viewed as separate entities. This is opposed by the Müllerian hypotheses, on the basis of a higher likelihood that all these tumors are derived from cells where the Müllerian phenotype is already present (6, 9). In 2001, Piek et al. presented the finding of, what would later be known as, serous tubal intraepithelial carcinomas (STIC) in the fallopian tube of BRCA-mutation carriers (16). Subsequent research supports the theory of STIC as a common precursor of HGSC of the fallopian tube, peritoneum and ovary, arising in the distal region of the fallopian tube and then spreading (17-19). The FIGO classification was revised in 2014 following that these tumors seem to arise from Müllerian-derived tissues and share clinical features (4). However, STIC is not always seen in HGSC patients and some HGSC seem to arise without fallopian tube involvement, thus there might be a dualistic origin (20). It can be concluded that the pathogenesis of this tumor type is far from determined (21).

Risk factors and risk reducing factors

The most prominent risk factor of OC is family history of the disease. About 18% of OC cases are hereditary (22). The majority of these families have mutations in the breast cancer (BRCA) 1 and 2 genes (23). The cumulative life-time risk for OC is 36-53% in BRCA1 mutation carriers and 11-25% in BRCA2 mutation carriers (24). Several acquired somatic mutations are seen in sporadic cases (25). Other risk factors include height ≥170 cm (26), nulliparity (27), menopausal hormone use (28) and for mucinous cancer smoking (29). The risk of developing EOC is higher in women with a history of pelvic inflammatory disease (30) or endometriosis (specifically clear cell and endometroid EOC), the latter associated with a state of chronic inflammation (31). Risk reducing factors are parity (32), use of oral contraceptives (33), breastfeeding (34), tubal ligation (35) and sterilization, as well as surgical hysterectomy and salpingectomy, alone or in combinations including oophorectomy (36). The value of opportunistic salpingectomy is still under evaluation (37, 38).

Diagnosis and treatment

The symptoms of OC are diffuse and easily mistaken for other abdominal problems (2). In suspicion, the first step in the investigation will be a gynecological examination, including transvaginal ultrasonography. CA-125 is the most widely used serum marker. This is a glycoprotein expressed by a variety of epithelial cell surfaces, benign and malignant (39). In OC, elevated levels (≥35 U/ml) can be seen in 50-60% of early stages and 90% of late stages (40). The ultrasonographic picture is interpreted according to IOTA's (International Ovarian Tumor Analysis group) simple rules, offering a standardized examination technique (41). An ultrasound score (U) can also be combined with the CA-125 value and menopausal status (M), calculating a risk of malignancy index (RMI = CA-125 × U × M) (42). With a cut-off value of 200, RMI reaches a sensitivity of 85% and a specificity of 97% (42). Other biomarkers have been

suggested, including cytokines, acute phase reactants, growth factors, proteases, hormones, coagulation factors, microRNAs (miRNAs), circulating tumor DNA, mRNA etc. (43, 44). Only Human Epididymis protein 4 (HE4) is US FDA approved, used with CA-125 in an algorithm (risk of ovarian malignancy algorithm, ROMA) (45).

Recommended treatment for advanced EOC is surgery followed by chemotherapy. In selected cases there is evidence in support of offering neoadjuvant chemotherapy to decrease tumor burden and optimize conditions before attempted radical surgery. Surgery is performed for diagnostic purposes, tumor staging and to remove all visible and palpable tumor (4). The importance of maximal debulking surgery for the overall survival of advanced EOC patients is extensively validated (46, 47). Standard chemotherapy treatment for this patient group is a combination of carboplatin and paclitaxel, given every three weeks for six cycles (48). Bevacizumab, a monoclonal antibody against vascular endothelial growth factor (VEGF) has shown to improve survival in HGSC patients with stage III, not optimally debulked, and stage IV patients, given in combination with traditional chemotherapy and thereafter in maintenance regime (49, 50). DNA repair inhibitors, such as inhibitors of poly (ADP-ribose) polymerase (PARP), have shown greatest results in patients with BRCA positive tumors and in patients with other homologous recombination deficiencies, seen in about 30% of HGSC patients, but also in other patients (51, 52).

Immunotherapy

Immunotherapy is a type of treatment that helps the patient's own immune defense fight cancer. Immunotherapeutic approaches include monoclonal antibodies, cancer vaccines, adoptive T cell therapy and checkpoint inhibitors. None are approved for treatment of OC. Vaccines against different tumor antigens are the most studied immunotherapeutic approaches in OC. The intention is to increase the tumor antigen presentation by antigen presenting cells (APCs), generating tumor-specific T cells. Advantages are the relatively low toxicity and theoretically, in relation to conventional therapy, the ability to establish immunologic memory. Several different trials have been conducted with modest results (53). Problems include the molecular heterogeneity of OC, the absence of distinguished tumor-specific antigens, the immunomodulatory tumor microenvironment (TME) and the risk of tumor immunoediting with antigen loss (54). Different strategies are investigated to circumvent these difficulties. Novel techniques make it possible to map the genome of each individual patient's tumor, finding mutations and neoantigens, to be used in the development of personalized vaccines (55). Early phase studies, mainly of the effectiveness of vaccines made from autologous dendritic cells (DCs) pulsed with autologous whole-tumor cell lysate have shown promising results and several studies are undertaken (56).

A successful example of immunotherapy is immune checkpoint blockades. Monoclonal antibodies against CTLA-4 (cytotoxic T lymphocyte-associated antigen) showed remarkable results in metastatic melanoma patients and in 2011, US FDA approved the drug ipilimumab for treatment of advanced melanoma (57). Since then, antibodies against checkpoint molecules have been approved for numerous cancer types (58). In OC, results from early phase trials for single treatment with checkpoint inhibitors have been modest (median response rates 10-15%) (summarized in (59)). This might reflect the heterogeneity of OC, that OC exhibits lower intrinsic immunogenicity and mutational burden (lack of targetable antigens), that the number of tumor infiltrating lymphocytes (TILs) varies between tumors, and these TILs express several regulatory T cell (Treg) associated inhibitory receptors such as programmed cell death protein 1 (PD-1), lymphocyte-activation gene 3 (LAG-3) and CTLA-4 (60). In a murine model it was shown that blocking PD-1, CTLA-4 or LAG-3 alone in ovarian tumors led to an upregulation of the other inhibitory checkpoints and tumor growth, whereas a combinatorial blockade resulted in improved outcome and increased anti-tumor immunity (61). Moreover, there are redundant immunosuppressive mechanisms in the TME of OC: indoleamine 2,3-dioxygenase (IDO, an enzyme involved in catabolizing tryptophan), transforming growth factor (TGF) β, interleukin (IL) 10, non-tumor cells with a pro-tumorigenic phenotype and others (60). Although the same modest overall results were seen in the keynote-100 trial of the PD-1 mAb pembrolizumab, there was a higher overall response rate and survival with increased programmed death-ligand (PD-L) 1 expression in the TME (62).

Adoptive cell therapy (ACT) uses autologous or allogeneic lymphocytes to induce cancer regression. Tumor-reactive lymphocytes are isolated from peripheral blood or the tumor and expanded *in vitro* before reinfusion. Attempts have also been made using tumor-draining lymph-node-derived T cells (63). ACT was first proven effective in malignant melanoma (64). Although, tumor-reactive TILs in OC seem to be low in numbers in general, it has been shown that TILs from OC patients can be successfully expanded and show anti-tumor activity *ex vivo* (65). There are ongoing studies on how to increase the tumor infiltration of T cells. In an OC mouse model, it was shown that DNA methylation and histone modification repress the production of T cell attracting chemokines and that the effector T cell tumor infiltration could be increased by treatment with epigenetic modulators (66).

In recent years, the opportunity to genetically modify T cells has emerged, resulting in a highly potent, *ex vivo* expanded T cell directed against specific antigens through a chimeric antigen receptor (CAR) or engineered T cell receptor (TCR). These have been successful in several cancers like ALL and lymphoma (67, 68). There are also trials for CAR and TCR engineered T cells targeting different antigens, like MUC-16, mesothelin, NY-ESO-1 and Folate receptor-α, in OC (69).

In conclusion, due to the nature of ovarian tumors, a multidimensional approach with a combination of old and new drugs involving a variety of strategic attacks on the tumor, such as chemotherapy, PARP-inhibition, checkpoint blockades, vaccines, adoptive T cell therapy, cytokine therapy, anti-angiogenesis etc., will hopefully increase the survival rates.

A brief overview of the immune system

The immune system has three main functions: 1) to protect against infection and invading microbes - like viruses, bacteria, fungi and parasites, 2) to defend against damaged or transformed self, constituting immune surveillance and 3) to uphold homeostasis at mucosal sights in the body, such as lung, intestine and urogenital mucosa.

Traditionally, the immune system is divided into two branches, innate and adaptive immunity. Many of the components of the innate immunity are constitutively present in the body, offering a non-specific initial competent defense, a rapid "first line of defense". It comprises our physical barriers, soluble proteins like cytokines, chemokines, complement factors and acute-phase proteins as well as phagocytic cells (granulocytes, macrophages), DCs, natural killer (NK) cells, NKT cells and γδ T cells. Many of the components have the ability of pattern recognition, expressing pattern recognition receptors (PRR). PRR recognizes two types of molecules, pathogen-associated molecular patterns and damage-associated molecular patterns (DAMPs). DAMPs are released by the cells during damage or death and include heat-shock proteins, hyaluronan fragments, DNA and RNA outside the nucleus etc. PRR signaling leads to the expression of genes encoding important proteins in innate immunity, such as chemokines and cytokines (70-72).

Key features of the adaptive branch include specificity, memory and self-nonself discrimination. The immune effector responses consist of a cellular (T helper and T cytotoxic cells) and a humoral (B- and plasma cells, antibodies and cytokines) response. As this system is antigen-specific, its cells have to meet an antigen and proliferate in order to mount a sufficient response, which usually takes up to seven days. T helper cells and cytotoxic T cells are MHC (major histocompatibility complex) restricted, meaning that they have to recognize both an MHC self-component and the antigen during antigen presentation to evoke an immune response. MHC molecules are cell-surface glycoproteins which comprise of two classes: 1) MHC class I that is expressed on all nucleated cells and 2) MHC class

II that is expressed on immune cells, such as antigen presenting cells (APCs) and B cells. MHC class I molecules are expressed on the cell surface together with β2-microglobulin and present intracellular peptides. MHC class II molecules are loaded with internalized foreign- or self-peptides intracellularly before they are expressed at the cell surface. Besides antigen presentation, B cells can mount an immune response, both T cell-dependent and T cell-independent, the latter without isotype switch and B cell memory. The innate and adaptive systems are complementary and work closely together (70-72).

Immune surveillance and the immunoediting concept

Cancer is a sort of altered self. Immune surveillance constitutes the immune system's ability to recognize and eliminate transformed precancerous cells that have escaped intrinsic tumor-suppressor mechanisms, that should trigger senescence or apoptosis in case of uncontrolled growth (73). Both the innate and adaptive immune system contribute to this process. As malignancies still develop, it is clear that immune surveillance sometimes fails. The fact that some tumor cells evade the immune system and that this system also seems to select for tumor variants resistant to immune surveillance, led to the development of the immunoediting concept, presenting the three phases elimination, equilibrium and escape (74). The elimination phase is initialized by the innate immune system recognizing the tumor. This may be caused by stromal remodeling and local tissue disruption, leading to the release of proinflammatory molecules and chemokines, recruiting cells of the innate branch. They may react to stress inducible ligands or DAMPs expressed by tumor cells, producing cytokines such as IFN-γ and IL-12, and to some extent also kill tumor cells. Damaged tumor cells will release tumor associated antigens and eventually cells of the adaptive branch will be activated (75). Below is a brief overview of the major cytotoxic effector cells and activating receptor-ligand interactions involved in immune surveillance.

Cells with cytotoxic function

Cells with cytotoxic function belong to the innate or adaptive immunity and have the ability to kill infected and transformed cells. They protect us from infections and tumors and preserve homeostasis of the mucosal surfaces and organs of the body.

Natural killer cells (NK cells)

In humans, NK cells are defined as large granular lymphocytes that lack CD3 and express the neuron cell adhesion molecule CD56. NK cells constitute about 15% of all lymphocytes in peripheral blood and represent innate cytotoxic effector cells with ability to mount a direct cytotoxic response against virally infected and tumor cells (76). Two major subsets can be distinguished depending on the level of CD56 expression and they also differ in the expression of homing molecules: $CD56^{dim}$ and $CD56^{bright}$ (77). In peripheral blood, the majority (~90%) of NK cells are $CD56^{dim}$ and express high levels of the Fcγ receptor III/CD16 and are primarily involved in cytotoxicity, including ADCC (76, 78). The $CD56^{bright}$ (~10%) NK cells constitute the majority in lymph nodes and tonsils, they express none or lower amounts of CD16 but secrete larger amounts of cytokines (76, 79). One role of this subset may be to prime cells of the innate immune response at an early stage of infection or cell transformation, by providing IFN-γ and other cytokines (76).

NK cells are not MHC restricted or antigen-specific like the adaptive T and B cells. Instead, NK cell recognition is mediated through a number of activating and inhibitory receptors. These receptors interact with the target cell, cytokines and other immune cells, while circulating through blood, tissues and lymphatic organs. The sum of the signals decides if the NK cell activates its' effector functions (and kills the target) or not (80). Under normal, healthy conditions, the NK cell is suppressed by inhibitory receptors recognizing MHC class I molecules, expressed by all nucleated cells. However, under cellular stress, such as malignant transformation, the MHC I molecule may be downregulated ("missing self") and stress ligands upregulated ("induced self"), resulting in a net activating signal (81). Upon activation, effector functions will immediately be carried out through cytotoxicity and/or cytokine secretion (82). Thus, the NK cell is not only responsible for eliminating infected or transformed cells, but also for the activation of cells of both the innate and adaptive immune system. Activating receptors include natural cytotoxicity receptors (NCR) (NKp44, NKp30, NKp46, NKp80), CD16 and the for immune surveillance crucially important Natural-killer group 2, member D (NKG2D) (81). The predominant inhibitory receptors are killer cell immunoglobulin-like receptors (KIRs) and CD94-NKG2A, both recognizing MHC I molecules (self-recognition) (83).

Cytotoxic T lymphocytes (CTLs)

One of the major differences between CTLs and NK cells is that the former are MHC-restricted. T lymphocyte precursors migrate from the bone marrow to the thymus where the development of T cytotoxic and T helper cells takes place. In the secondary lymphoid organs, APCs will present antigens to the naïve $CD8^+$ T cells. $CD4^+$ helper cells will simultaneously be activated and give cytokine help which, together with co-stimulatory signals, lead to proliferation and differentiation into $CD8^+$ effector CTLs and memory cells. CTLs recognize their

targets through the diverse, antigen specific TCR, consisting of variable α and β chains. CTLs are MHC restricted and each TCR recognizes a specific MHC class I-antigen complex presented by an APC or target cell. They are specialized in targeting intracellular pathogens and also cells transformed in other ways. The TCR is associated with the co-receptor CD3, through which the signal is transduced upon activation. The activation of the effector functions of a mature CTL starts with the interaction of the TCR with an MHC I-antigen complex on a target cell, leading to a CTL-target cell conjugate (70, 72, 84).

The mechanism of cytotoxic killing

Cytotoxicity is the effector mechanism that protects the human body by killing of infected or transformed cells. Cytotoxicity may be carried out in two ways: granule-mediated or death ligand-mediated, both causing the target cell to undergo apoptosis. The process is initialized in a similar manner regardless of the cytotoxic cell at play and mode of action – with binding to the target cell, forming an immunological synapse (72). In the granule-mediated initiation of cell death, the activation of the effector cell will lead to the release of lytic granules containing granzymes and perforin, amongst other components (85). Following exocytosis, the pore forming perforin enables the delivery of granzymes to the cytosol of the target cell, where it initiates a cascade leading to apoptosis (86). Death ligand-mediated apoptosis is carried out by three receptor/ligand systems: Fas receptor/Fas ligand, TNF receptor/TNF or TRAIL receptor/TRAIL, all leading to apoptosis through caspase activation (87). Cytotoxic cells may also indirectly induce cytotoxicity through the release of cytokines such as IFN-γ, leading to death through induction of Fas and MHC I complex expression (88).

The NKG2D receptor-ligand system

The NKG2D receptor

NKG2D is a potent activating receptor, the most important in tumor cell recognition, expressed on most NK cells, $CD8^+$ T cells, NKT cells, γδ T cells and a subset of $CD4^+$ T cells (89, 90). It is a type II transmembrane protein that belongs to the C-type lectin-like family. In NK cells, NKG2D acts as a primary activating receptor and in CTLs mainly as a co-stimulatory molecule together with TCR, amplifying the cytotoxic function (91). In humans, it is associated with the DAP10 adaptor protein, through which the signals are transmitted into the cell (89), leading to recruitment and activation of phosphatidylinositol-3 kinase and other activating cascades (81). The expression of NKG2D can be modulated by cytokines; IL-2, IL-7, IL-12, and IL-15 upregulate and TGF-β, interferon-β1 and IL-21 downregulate the receptor (89).

The NKG2D ligands

The NKG2D ligands are cell surface glycoproteins, in structure related to MHC class I (80). They can be divided into two families: the MHC class I chain related antigens (MICA and B) and the UL16-binding proteins (ULBP1-6) (92). An important factor is that the ligand expression on healthy cells is low or absent, but can be induced in, for example, tumor cells (induced self). A number of different factors associated with cell stress have been reported to induce expression, like inflammation, infection, tumorigenesis and cell toxic agents (93).

The DNAM-1 receptor-ligand system

The DNAM-1 molecule and its activation

The DNAX accessory molecule (DNAM-1/CD226) is an adhesion molecule, aiding in conjugate formation by interactions with its ligands on the target cell. It has also been identified as an activating receptor, triggering cytotoxicity and has emerged as an important part of tumor cell recognition (94, 95). In NK cells, DNAM-1 seems to act more co-stimulatory when NKG2D is present, but in its absence, as in ovarian tumor cell recognition, DNAM-1 signaling has been proven dominant (95, 96). DNAM-1 is expressed by NK cells, monocytes and T cells, like $CD8^+$, $CD4^+$ and γδ (97). It is a member of the immunoglobulin (Ig) superfamily. DNAM-1 associates with lymphocyte function-associated antigen 1 (LFA1) and interacts with adhesion molecules (ICAMs) on the target cell and recruits Src kinase FYN to phosphorylate tyrosine (Tyr322), resulting in a downstream phosphorylation leading to activation of cytotoxic effector mechanisms, degranulation and IFN-γ release (98).

The DNAM-1 ligands

The DNAM-1 ligands are nectin and nectin-like proteins: nectin-2 (CD112) and polio virus receptor (PVR/CD155) (99). These are also cell adhesion molecules, involved in the formation of tight junctions and thus in cell movement, homing, proliferation and differentiation. (100). The nectin family consists of four members, nectin 1-4. Nectin-2 is expressed by a variety of cells like fibroblasts, neurons, epithelial cells, B cells, monocytes and spermatids (100). PVR is expressed at low levels on epithelial cells and peripheral blood monocytes (99). Several tumors have been shown to overexpress both these ligands, indicating that they are used by the tumor for spreading and/or metastasizing (94, 99).

The immunosuppressive ovarian tumor microenvironment

The hallmarks of cancer comprise six capacities acquired by cells during tumor development (101). In 2004, an additional hallmark was proposed – immune escape, the last step of the immunoediting process (75). Highly immunogenic tumor cells will be eliminated, selecting for the growth of weakly immunogenic tumor cells and later for them to actively suppress the immune system (102). For a tumor to develop, transformed cells have to interact with the surrounding host tissue stroma; immune and endothelial cells, fibroblasts, adipocytes and components of the extracellular matrix - constituting the TME (103). Immune escape is thus facilitated both by intrinsic tumor cell changes and by the creation of an immunosuppressive TME (102). The importance of the TME is now widely acknowledged. By looking into the ovarian TME (Fig. 1), its cellular components and their mediators, such as cytokines, exosomes, enzymes and membrane-bound ligands, several mechanisms behind immune escape can be highlighted.

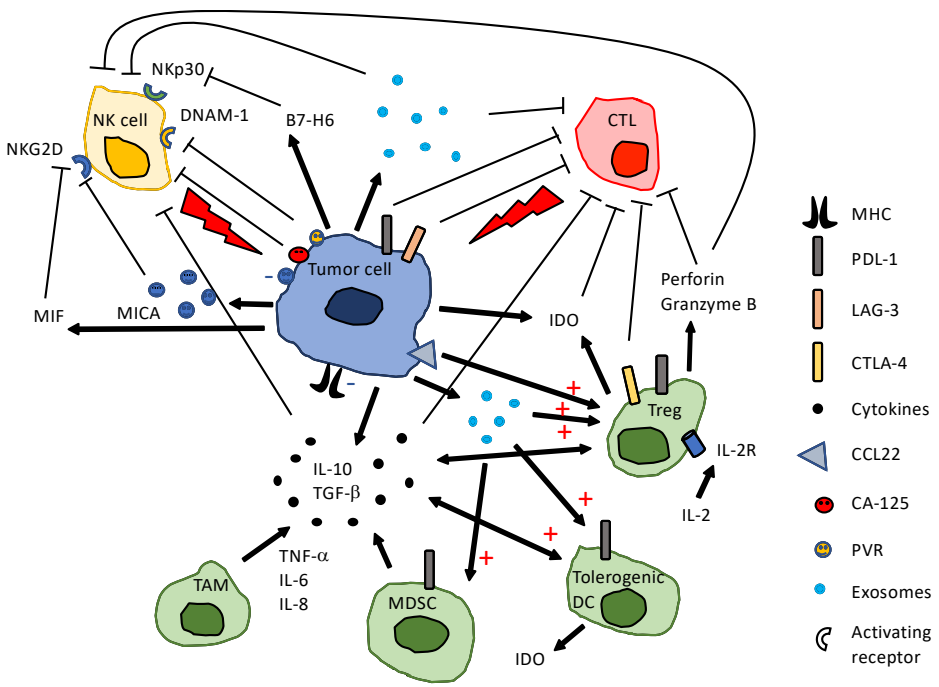

Figure 1. Schematic presentation of the immunosuppressive ovarian tumor microenvironment. CTL, cytotoxic T lymphocyte; Treg, T regulatory cell; DC, dendritic cell; MDSC, myeloid-derived suppressor cell; TAM, tumor-associated macrophage; MIF, macrophage inhibitory factor; IDO, Indoleamine 2,3-dioxygenase.

Ovarian cancer cells

In ovarian cancer cells, mechanisms for immune escape can be carried out through downregulation of immunostimulatory molecules or through surface expression or secretion of immunoinhibiting molecules. It shoud be borne in mind that there is a vast heterogeneity also among OC cells constituting the same tumor, as illustrated by the findings of Alvero et al. (104). They identified at least two EOC cell subtypes, with diverging cytokine profiles, type I (cancer stem cell-like) cells secreting proinflammatory cytokines and type II (characteristics of terminally differentiated) cells secreting immunoinhibitory cytokines, both tumor-promoting through their effect on immune cells.

OC has the ability to downregulate immunostimulatory molecules involved in antigen presentation, such as β2-microglobulin (105). OC and others cells in the TME can express ligands for immune checkpoint molecules, impairing effector functions of CTLs (106, 107). OC overexpress molecules inhibiting cytotoxic cells, like CA-125 that binds to a KIR, an inhibitory NK cell receptor (108). Several mechanisms for evading immune surveillance are carried out through direct or indirect impairment of cytotoxicity by downregulation of activating cytotoxic receptors and/or their ligands, such as the major cytotoxic receptor NKG2D and its ligands (109), and the activating receptor DNAM-1, the latter caused by chronic expression of its ligand PVR (110). High levels of the ligand B7-H6, soluble or expressed by tumor cells, downregulate another activating receptor on NK cells, NKp30 (111). NKG2D ligands shed from the cell through cleavage by matrix metalloproteases (MMPs) are believed to not only diminish the number of ligands expressed by the tumor cell, but also to downregulate the NKG2D receptor due to excessive stimulation, and to block the NKG2D-binding sites for surface expressed ligands (112-115). It was recently shown that the survival of OC patients was inversely correlated to the amount of soluble NKG2D ligands in ascites (116). OC and other TME cells secrete macrophage inhibitory factor (MIF), a proinflammatory cytokine downregulating the transcription of the NKG2D receptor in NK cells (117). Cancer cells and other TME cells express IDO, depleting T cells of tryptophan, rendering them inactive and inducing Treg formation. A high IDO expression is associated with a reduced number of CTLs and poorer prognosis (118). PGE2 is another molecule secreted in the TME, inhibiting NK cell and γδ T cell cytotoxicity and inducing the formation of Tregs (119). Other important secreted factors involved in immune escape are cytokines and exosomes.

Immune cells and other cells

Immune cells with a tumor promoting phenotype are major players in OC immune escape (reviewed in (119-123)). These include Tregs, tolerogenic DCs, myeloid-derived suppressor cells (MDSCs) and tumor-associated macrophages (TAMs). Other stromal cells can also adopt a tumor promoting phenotype, for example fibroblasts.

Regulatory T cells (Tregs)

Regulatory T cells are crucial for immune tolerance and homeostasis. The main and only action of Tregs is suppression of the functions of immune cells and molecules. The immunosuppressive abilities of Tregs are many and mainly carried out through cell contact with dendritic or effector cells, or through secretion of immunoinhibitory cytokines (IL-10 and TGF-β) (124). In OC, Tregs have been shown to express immune checkpoint molecules PD-L1 and CTLA-4, the latter also inducing IDO and IFN-γ release in DCs and other immune cells (106, 125, 126). Tregs also have the ability to downregulate CD80 and CD86 on DCs, rendering them less capable as activators of T cells (127). The majority of Tregs express CD25 (IL-2R) and it has been suggested that Tregs have the ability to "starve" cells in its vicinity by consuming IL-2 (128). Thymic-derived Tregs have been shown to express both granzyme B and perforin, causing cytolysis of the target cell (129) and apoptosis through the TRAIL-DR5 pathway (130). Their accumulation in OC is well documented and associated with poorer prognosis (126, 131, 132). OC have the ability to recruit Tregs by CCL22 expression (126) or induce $CD4^+CD25^+$ Tregs from $CD4^+CD25^-$ T cells by secreting TGF-β (133) or Il-10 (134). The importance of optimal surgery in OC patients is supported by the finding that this leads to a Treg decrease (135), whereas sub-optimal debulking seems to enhance the Treg proportion in peripheral blood (136).

Tolerogenic dendritic cells

Dendritic cells have a crucial role in antitumor adaptive immunity as APCs priming naïve T cells. These are cells with high plasticity and depending on the ovarian TME they can switch towards an immunoinhibitory phenotype, for example by encountering IL-10, VEGF, IL-6, TGF-β or surface molecules like IDO, PD-L1 or PD-1 (137). Regulatory/tolerogenic DCs act immunosuppressive either by direct cell-cell contact with effector T cells or through inducing or "boosting" other immunosuppressive cells in the TME. These actions are mediated by the release/expression of IL-10 and TGF-β (138), arginase (139), IDO (140) and PD-L1 (141).

Myeloid-derived suppressor cells (MDSCs)

Myeloid-derived suppressor cells represent a mixture of immature myeloid cells of immunosuppressive phenotype, accumulating in tumors in response to various factors (142). Their immunosuppressive functions are carried out through various mechanisms: 1) depletion of nutrients like L-arginine and L-cysteine which causes downregulation of the ζ-chain in the TCR-CD3 complex, inhibiting T cell proliferation (142); 2) production of reactive oxygen and nitrogen species, causing immunosuppressive oxidative stress (143); 3) interfering with lymphocyte trafficking and viability by decreasing cell adhesion molecules and chemokine expression (144, 145) or affecting other immune cells through cell-cell contact by expressing immunosuppressive molecules like TGF-β1 (146), PD-L1 (147) and IL-1β (148) and; 4) activation and expansion of the Treg population (149). In OC, a higher level of MDSC tumor infiltration is associated with significantly lower disease-free interval and shorter overall survival (150).

Tumor-associated macrophages (TAMs)

Tumor-associated macrophages are important mediators of tumor progression. Macrophages are plastic and will respond to the TME and adopt a phenotype ranging somewhere from M1 to M2 (151). M2 represents a pro-tumorigenic, immunosuppressive phenotype (152). The cytokine environment favoring polarization towards an M2 phenotype include colony stimulating factor-1 (CSF-1), IL-4, IL-10 and TGF-β (153). OC cells can induce such polarization (154). TAMs have the ability to: 1) create a proinflammatory environment through TNF-α and IL-6; 2) promote angiogenesis through VEGF and IL-8; 3) enhance tumor cell migration and invasion through EGF and MMPs and 4) favor immune escape by production of IL-10, CCL22, PGE2, TGF-β (155). TAMs either reside in the tumor tissue or are recruited from precursors in the bone marrow, the circulation or the spleen by for example CCL2 (156), a chemokine overexpressed by OC cells (157). High amounts of M2 tumor infiltration is associated with a poorer prognosis for OC patients (158).

Cancer-associated fibroblasts (CAFs)

Cancer-associated fibroblasts may originate from normal fibroblast or from mesenchymal stem cells, reprogrammed under the influence of factors, such as TGF-β, PDGF and proinflammatory cytokines, secreted by tumor cells and other cells in its vicinity. This is also seen in OC. Fibroblasts are normally “inactive” but can be activated, as in inflammation, to produce connective tissue ECM, proteases and TGF-β to stimulate epithelial-mesenchymal transition (EMT), allowing the epithelial cells to move as a part of wound healing. These are characteristics also seen in CAFs and used by the tumor, aiding in growth, matrix remodeling and metastasis, angiogenesis and influencing immune cells by cytokine production (159).

B cells

B cell subsets - naïve, memory, plasma cells and B regulatory cells are found in OC. Their effect on overall survival for OC patients are diverging, illustrating their dualistic role in the TME (160). In immune surveillance, they have an anti-tumor role in their capacity as APCs, expressing co-stimulatory molecules and enabling ADCC. B cells have the potential to affect all cells with a Fc receptor, such as DCs, NK cells, granulocytes and MDSCs. Antibodies produced by B cells may facilitate in immune escape since their binding to the tumor may block CTL access to tumor antigens. B regulatory cells also produce immune suppressive cytokines like IL-10 and TGF-β (161).

Cytokines

Cytokines are small peptides/proteins for intercellular communication, produced and secreted in a highly regulated way mainly by immune cells. They participate in both normal and pathological processes. They are characterized by pluripotency, i.e. one cytokine can have many different functions depending on the microenvironment in which they act, and redundancy, i.e. different cytokines can act in a similar way. They can act synergistically, potentiating each other's effects, or antagonistically, opposing each other (162). It has been shown that different cytokine profiles, designated Th1, Th2, Th3/Tr1, Th17 and other proinflammatory cytokines, are associated with the ability to mediate and regulate immunity and inflammation, promote or halt growth, movement or immune responses. Thus, a cytokine profile dominated by IFN-γ, IL-12 and IL-15 (Th1) promotes cytotoxicity, a profile dominated by IL-4 and IL-13 (Th2) promotes humoral immunity, IL-17 (Th17) promotes inflammation, most prominent in autoimmunity and chronic inflammation, IL-1β, IL-6, IL-8, TNF-α and LTA promotes inflammation and TGF-β and IL-10 (Th3/Tr1) promotes immunosuppression and priming of T regulatory cells (72). Cytokines play a complex role in tumor pathogenesis (163). As soluble factors in the TME, they carry out diverse functions and at different stages, like the TME in general, act in a tumor suppressive or promoting way. Cytokines are highly pleiotropic and their output of response is context-dependent. A summary of some of the characteristics and functions of individual cytokines are presented in Table 3.

Table 3. Cytokine function in health and ovarian cancer.

Response, cytokine* (72)	Function in normal tissue	Function in OC immune evasion	Significance for OC prognosis
Promoting cytotoxic immune responses (Th1)			
IFN-γ	Important in innate and adaptive immunity and crucial for tumor control. Contributes to: • Macrophage activation • Differentiation of naïve $CD4^+$ T cells into Th1 cells • NK cell activation and subsequent production of IFN-γ and IL-12 • Upregulation of MHC class I and II expression • Inhibiting IL-4 production • Inducing expression of genes involved in apoptosis (Fas, FasL and caspases) • Maintaining Th1 response by induction of IL12 production, causing a positive feedback loop (164, 165)	As all cytokines, IFN-γ has a dual role. It also exerts immunosuppressive effects to protect normal tissues, this can be used by the tumor (166). For example, it Induces the expression of PD-L1 on OC cells (167). Tumor cells can become unresponsive to IFN-γ through various mechanisms (165).	Patients with high levels of IFN-γ gene expression in the malignant ovarian tissue had significantly longer progression-free and overall survival (168).
IL-12	• Differentiation of naïve T cells into Th1 effector cells • Upregulation of MHC class I and II - promoting antigen presentation • CTL and NK cell stimulation, enhancing their cytolytic capacity • Downregulation of VEGF and MMPs, inhibiting angiogenesis • The most potent inducer of IFN-γ gene transcription Its immunostimulatory effect has also been seen in OC (169)		
IL-15	• Stimulates the differentiation and proliferation of B, T and NK cells • Enhances cytolytic activity of CTLs and induces the formation of $CD8^+$ memory T cells • Stimulates the secretion of proinflammatory cytokines • Shares many functions with IL-2 but does not stimulate Treg cells and seem to be a more potent inducer of anti-cancer immunity (170)		
Promoting humoral immune responses (Th2)			
IL-4	• Involved in the differentiation of T cells and causes naïve $CD4^+$ T cells to develop into Th2 cells • Involved in B cell maturation into plasma cells and induces class switching to IgE and IgG1 - an important role in allergy and protection against parasites • Upregulates MHC class II on monocytes • Involved in inflammation (171)	The Th2 profile is adverse for tumor immunity. This is reinforced by IL-4: • Directly inhibiting CTL cytotoxicity by down-regulating the expression of IFN-γ, CD8, perforin and granzyme (Th1 response) (171) • Activating TAMs and MDSCs (172) IL-4 and its receptor, along with other Th2 cytokines, is upregulated in several cancers, including OC (171, 173)	
IL-13	Is structurally and functionally related to IL-4 and exhibits mainly the same biological functions, both in normal and pathological conditions (172)		A mouse model showed increased invasion, metastasis and an association with poorer prognosis (174).
Proinflammatory			
IL-6	Produced in the initial stage of inflammation, contributing to: • Stimulation of acute phase responses • Hematopoiesis • Immune reactions (175)	• Promotes proliferation by expressing anti-apoptotic regulators (176) • Enhances migration and growth (176) • Activates TAMs (177, 178) • Stimulates the expression of B7-H4 molecules, associated with immune suppression (179) • Induces angiogenesis (180)	IL-6 is associated with chemoresistance (181) and an increased serum level is associated with poorer prognosis (182).
TNF-α	Signaling via TNF-α typically induces genes involved in inflammation and cell survival, where it: • Recruits and activates monocytes and neutrophils to/at the inflammation site • Enhances the expression of adhesion molecules for immune cells on endothelial cells and increases the permeability of blood vessels • Acts anti-apoptotic • Induces MMPs (183, 184)	Several mechanisms for inflammation are used: • Increased permeability of blood vessels • Upregulation of adhesion molecules • Enhanced MMP production (184) Also stimulates the secretion of other cytokines (IL-6), chemokines, angiogenic factors and MMPs (185), thus sustaining the tumor-promoting cytokine network.	An increased level of TNF-α is associated with poorer prognosis in OC patients (186).

IL-1β	Involved in inflammation and is together with TNF-α the most potent acute phase cytokine. There is a crucial balance between harmful and beneficial effects, all depending on the microenvironment and the amount of signaling. IL-1β secreted at a low dose in the local environment can function to eradicate tumors by priming the immune system, both the innate and adaptive branch (187).	Excessive IL-1β secretion leads to tumor promoting effects by triggering the secretion of: • Growth stimulatory and angiogenic factors (VEGF, IL-8) • Factors promoting invasiveness (MMPs) • Other inflammatory cytokines, causing an amplification loop (187-189)	
IL-8	A proinflammatory chemokine. An activator and chemoattractant of neutrophils, but also other immune cells (190).	• Favors angiogenic processes and proliferation of endothelial cells, upregulates VEGF • Increases proliferation, adhesion and migration of OC cells, upregulates MMPs (190, 191)	Elevated levels seen in blood, cystic fluid, ascites and tumor tissue of OC patients are associated with poorer prognosis (191).
LTA/TNF-β	• Development and organization of lymphoid tissue and organs • Regulation of inflammation and immune responses, like trafficking, migration and organization of immune cells in tissues • Upregulation of adhesion molecules (192)	Elevated levels are seen in OC tissue, inducing chemokine (CXCL11) expression in CAFs, promoting metastasis and tumor growth (193).	
Immunoinhibitory (Th3, Tr1)			
TGF-β	• Immune regulation – peripheral tolerance • Embryonic development • Wound healing • Tumor suppression through regulation of cell growth and division (194, 195)	• Induces immunosuppressive and tumor-promoting immune cell phenotypes, for example by converting CTLs to Tregs and inducing the formation of CAFs, TAMs and tumor-associated DCs (196, 197) • Antagonizes IFN-γ production in NK cells and CTLs • Suppresses IL-12 and IL-2 production (184) • Enhances invasiveness and metastasis through induction of MMPs (198) and EMT (199) • Stimulates angiogenesis (200) Tumors evade the suppressive effects of TGF-β by mutations leading to inhibition of the entire TGF-β pathway, as seen in OC, or loss of the "suppressor arm" of the pathway (195).	A higher concentration of TGF-β in ascites of OC patients is associated with early relapse (201).
IL-10	An anti-inflammatory cytokine, limiting the effect of other immune cells to avoid tissue damage. Its main targets seem to be monocytes/macrophages, indirectly limiting other immune cells: • Inhibits MHC class II and the expression of co-stimulatory molecules • Downregulates the production of proinflammatory and Th1 cytokines and chemokines • Enhances phagocytosis It can also act directly on $CD4^+$ T cells, inhibiting proliferation and cytokine production (both from Th1 and Th2 cells) and may induce adaptive Tregs from $CD4^+$ T cells (202).	Many of the immunoinhibitory effects of IL-10 are used by the tumor (203). In addition, it: • Works in a positive feedback loop with TGF-β • Downregulates NKG2D expression on tumor cells • Upregulates immune checkpoint molecules on immune cells (204)	A higher serum level is significantly correlated to shorter survival in OC patients (205).
Other cytokines			
IL-2	Specifically expressed on immune cells and is a mediator of intercellular communication between cells of the immune system. Its importance in the development of the cells of the immune system, immune response and clonal expansion cannot be overestimated. In thymus, IL-2 is needed for the development of effector and memory CTLs and in the combination with TGF-β it will promote the differentiation of $CD4^+$ T cells into a Treg phenotype, thus important for immune tolerance. It is also involved in Th differentiation by regulating cytokine receptor and transcription factor expression and by this directing the Th response (206).		

* Cytokines may exhibit additional functions

Extracellular microvesicles (EVs)

Extracellular microvesicles represent a heterogenous group of cell-derived vesicles, produced by virtually all cells in the body, participating in both normal and pathological processes. They can be found in blood and all kinds of bodily fluids, also in malignant effusions. They are all enclosed by a lipid bilayer carrying various biomolecules (207). EVs can roughly be divided into four groups; exosomes, microvesicles, shed microvilli and apoptotic bodies. EVs of different subtypes have some overlapping biochemical and physical characteristics that make it difficult to isolate them from each other in pure form and there is no specific marker separating them (208). The main characteristics of the different EVs are summarized in Table 4.

Table 4. A summary of the characteristics of the different extracellular microvesicles (adapted from (208)).

Characteristics	Exosomes	Microvesicles	Shed microvilli	Apoptotic bodies
Size	30-150 nm	0.1-2 μm	>400 nm	0.1-3 μm
Flotation density	1.13-1.19 g/ml	Undetermined	Undetermined	1.16-1.28 g/ml
Sedimentation (g)	100 000-110 000	10 000-100 000	10 000	1500-100 000
Morphological shape	Cup shaped, electron translucent	Various shapes, electron-dense and/or electron translucent	Various shapes, round, elongated and cylinder-like	Irregular and heterogenous in shape
Lipid membrane composition	Cholesterol-, sphingomyelin- and ceramide-rich lipid rafts, expose phosphatidylserine	Exposed phosphatidylserine, some enriched in cholesterol ad diacylglycerol, some undetermined	Undetermined	Undetermined
Specific markers(s) for identification	Tetraspanins (CD63, CD9, CD83), ESCRT complex members	Integrins, selectins, CD40 and others, depending on cell type	Various, depending on the cell type	Histones, DNA
Origin in the cell	Endosomal compartment – multivesicular bodies (MVB)	Plasma membrane	Plasma membrane	Fragments of dying cells, undetermined
Mechanism of sorting	Ceramide and ubiquitin dependent	Unknown	Unknown	Fragments of dying cells, undetermined
Biogenesis	Inward budding of MVB's limiting membrane	Fragmentation and detachment of the cytoskeleton due to cleavage by activated serine proteases causes destabilizing of the plasma membrane	Detachment from the plasma membrane	Fragments of dying cells, undetermined
Mode of release/secretion	Exocytosis by fusion of the MVB with the plasma membrane	Plasma membrane blebbing	Plasma membrane blebbing	Plasma membrane blebbing and cellular fragmentation

Exosomes – nanovesicles of endosomal origin

The current definition of exosomes is: secreted extracellular-vesicles of endosomal origin, with the following characteristics: 1) a size of 30-150 nm (measured by nanoparticle tracking analysis (NTA), up to 100 nm by electron microscopy); 2) a spherical form but are seen as cup-shaped in transmission electron microscopy, i.e. an artifact of the preparation; 3) a buoyant density of 1.13-1.19 g/ml when isolated through ultracentrifugation on a sucrose gradient and 4) a detergent-resistant membrane rich in tetraspanins, cholesterol and sphingophospholipids (208, 209). Different proteins have been proposed as exosome markers but research show that they are rather exosome enriched than specific (210). Tetraspanins are proteins with four transmembrane domains, highly enriched in exosomes, like CD63, CD81 and CD9. CD81 has been proposed as the most specific protein in separating exosomes from other EVs (211, 212).

One specific feature of exosomes compared to other EVs is that they arise from inward budding of the membrane of late endosomes, thus forming small intraluminal vesicles (ILVs), accumulating inside. These late endosomes are called multivesicular bodies (MVBs). In many cases these MVBs fuse with lysosomes, resulting in degradation of its content. Though the MVBs can also fuse with the plasma membrane of the cell, releasing ILVs into the extracellular matrix as exosomes, packed with information to be exchanged between cells (209, 213).

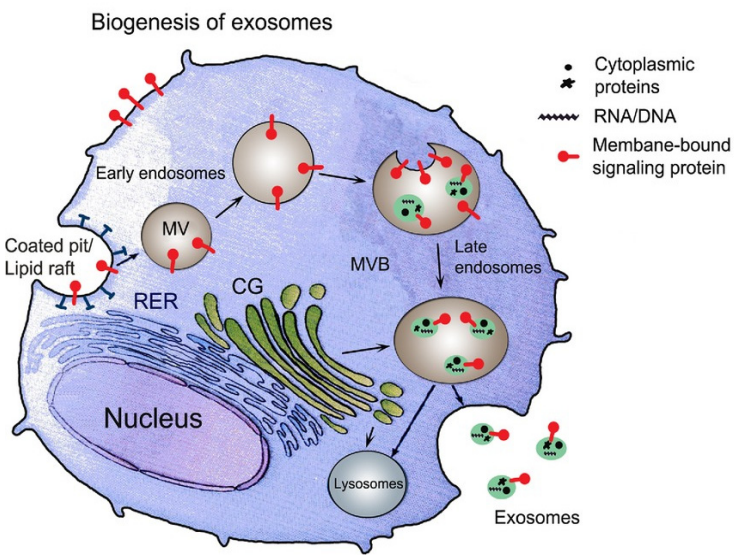

Figure 2. Biogenesis of exosomes (208). Exosomes are formed from inward budding of the limiting membrane of the MVBs. This ensures the same orientation of membrane proteins on the exosomes as on the mother cell plasma membrane. RER, rough endoplasmic reticulum; CG, complex Golgi; MVB, multi vesicular body.

How the ILVs are formed, the sorting of their content into microdomains at the limiting membrane of the endosome, the inward budding of the membrane and release into the endosomal lumen and the subsequent fusion of the MVB with the plasma membrane, releasing the ILVs as exosomes, is not clearly understood. The process seems to be dependent on cell type and the current state of this cell (207). Different pathways are described, involving one or more proteins, like different members of the Endosomal Sorting Complex Required for Transport (ESCRT), tetraspanins or small integral membrane protein of the lysosome/late endosome (SIMPLE). A "lipid pathway" is also described, involving raft-based microdomains high in sphingolipids from which ceramide form, both enriched in exosomes, causing a membrane curvature and triggering ILV formation (209).

Both tetraspanins and Rab GTPases are thought to be involved in the trafficking of the MVB. Different Rabs seem to be responsible for the intracellular transport and docking at, and fusion of the MVB with, the plasma or lysosomal membrane (214, 215).

Since exosomes resemble their cell of origin, there are differences in their composition, although there are some proteins that seem to be common for all exosomes. Exosomes are enriched with cytoskeleton proteins and cytosolic proteins (like tubulin, actin, annexins, heat shock, Rab and ESCRT proteins) and plasma membrane proteins (like tetraspanins, MHC class I and II and adhesion molecules). In addition to proteins and lipids, various nucleic acids can be found; mRNA, miRNA, lncRNA and DNA, with the potential to genetically influence the recipient cell (216). The exosome composition will of course mirror its function: elimination of unwanted molecules, communication or exchange of material between cells, transmission of pathogens, stimulation or downregulation of the immune system, antigen presentation and much more (216).

To summarize, exomes are important for intercellular communication and are produced and secreted by virtually all cells in the body. They can be found in saliva, urine, nasal and bronchial lavage fluid, amniotic fluid, breast milk, plasma, serum and seminal fluid (208). Tumor cells also secrete and use exosomes to influence their surroundings, not least in creating an immunosuppressive TME.

Exosomes in ovarian cancer

EOC cells constitutively produce exosomes that are secreted in ascites and plasma and participate in the cross-talk between cells in the TME (217-220). Some of their tumor-promoting effects are summarized in Figure 3. The level of exosomes in plasma is significantly higher in OC patients compared to women with benign ovarian tumors, suggesting that the tumor itself secrets high amount of exosomes, or influences other cells to exosome secretion (221). These exosomes were shown to induce formation of immunosuppressive immune cells, like Treg cells (222), tumor-promoting M2 macrophages (223) and to activate MDSCs through HSP70 (224). They also express immunosuppressive cytokines, like IL-10 and TGF-β1

(222) and have the ability to induce secretion of proinflammatory cytokines such as IL-6, TNF-α and IL-1β (225). Effector T cell activity can be disrupted by suppression of the ζ-chain in the TCR (226). It was recently shown that OC exosomes carry arginase-1 and by DC uptake cause inhibition of antigen-specific T cell proliferation (227). EOC exosomes have the ability to enhance apoptosis of effector lymphocytes by expressing FasL and TRAIL (217). Tumor-derived exosomes from many cancer types have been shown to express NKG2D ligands, interacting with the major NK-cell activating receptor NKG2D, impairing its function and the killing ability of cytotoxic NK- and T cells ((228-230) reviewed in (231)). To our knowledge this has not previously been investigated in OC.

Apart from contributing to the immunoinhibitory TME, OC exosomes aid in tumor progression in several ways, including angiogenesis, invasion, growth and drug resistance (120, 123).

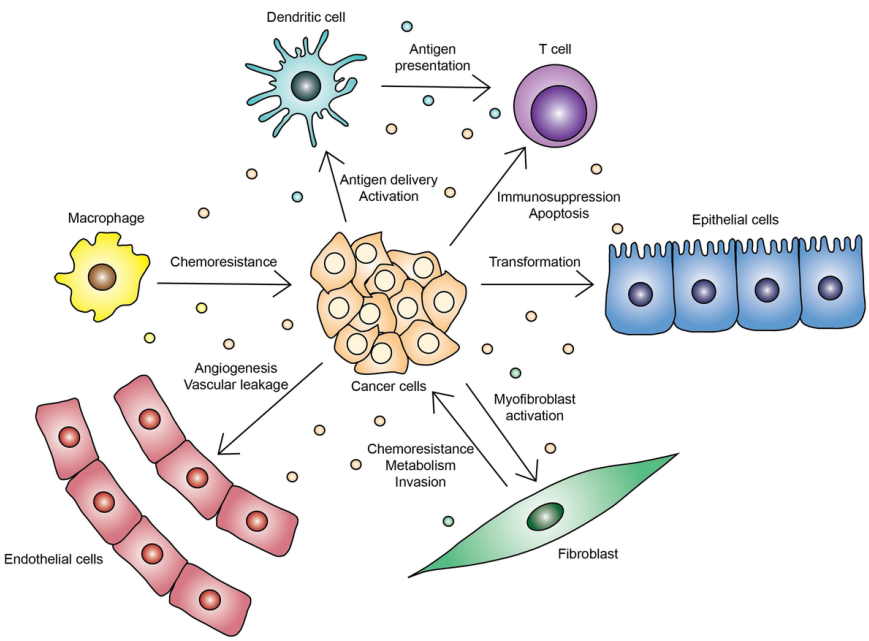

Figure 3. Some of the tumor promoting mechanisms carried out by exosomes secreted by virtually all cell types in the TME (232).

Exosomes in diagnosis and treatment

Important goals in the time-consuming effort of exosome mapping is to find specific markers for different tumors and henceforth to develop therapies targeting immunosuppressive tumor exosomes, or to use exosomes to deliver therapeutic agents. There are databases where researchers can share their findings, for example ExoCarta, to sooner reach these common goals (233).

In the search for biomarkers, exosomes have several advantages: 1) their accessibility, circulating in blood and can be found in numerous body fluids, making the sample collection easy and with minimal discomfort for the patient; 2) exosomes are stable under various conditions; 3) they are abundant; 4) can express specific surface proteins of the parental cell which can help in identifying tumors or metastasis; 5) contain biological information of the parental cell, more representative than cell-free DNA with a higher mutation frequency and prognostic value and 6) are rather straight forward to identify (234). In OC exosomes, several proteins and miRNAs (220, 221, 235, 236) have been mapped as potential biomarkers.

Considering the previously mentioned biological properties of exosomes, to use modified exosomes in treatment is an appealing idea. Exosomes in therapy are used mainly to deliver antitumor element or as vaccines, to activate the immune system (237). Exosomes for delivery of therapeutic agents would be beneficial in theory since it is possible to target them for certain tissues, thus minimizing the high doses used of non-specific drugs, they have a long half-life, are endogenous and thus have low amount of side effects and low immunogenicity (238). Exosomes have been modified in several ways to be made immune stimulating; genetically engineered through immune boosters, tumor-associated genes or using genetic material like siRNA. Clinical trials are evaluating the use of exosomes for immunotherapy in different malignancies, with promising results (237).

RATIONALE OF THE PROJECT

This project comprises research on the border of two disciplines – cancer and the immune system. On one hand, due to modern sequencing methodologies, our understanding of cancer biology has broadened. On the other hand, recent progress in oncoimmunology has rightfully acknowledged the immune system as an essential factor with an important role in the curability, prognosis and outcome of cancer disease, opening novel pathways for diagnosis and immunotherapy. The rationale of this project was to study the interactions between EOC and the immune system in the tumor microenvironment and peripheral blood of EOC patients to better understand its pathophysiological mechanisms, the impact on the host immune system and furthermore, how these interactions are affected by surgery. Such knowledge would hopefully contribute to better diagnostics and future novel treatments.

AIMS

The overall objective of this research project was to investigate tumor-mediated mechanisms that modulate the immune system in patients with epithelial ovarian cancer.

The specific aims were:

- To assess the cytokine mRNA profile in EOC tumor microenvironment and peripheral blood mononuclear cells in order to elucidate its role in host immune suppression and tumor immune escape.
- To investigate the correlation between cytokine mRNA and protein expression by EOC cells.
- To survey the composition and function of EOC-derived exosomes and their effect on the NKG2D cytotoxic pathway.
- To study the influence of surgery on circulating EOC exosomes and the NKG2D cytotoxic pathway.

ETHICAL CONSIDERATIONS

This thesis is part of a collaborative project studying the pathogenesis of ovarian cancer. All studies were performed in accordance with the Declaration of Helsinki and the project was approved by the Human Ethics Committee of the Medical Faculty, Umeå University (D. nr. 09-108M). All patients donated samples after written informed consent. They were informed that their participation is voluntary and that they are free to withdraw at any time without affecting their planned medical care. Each sample was labelled with a unique code and the personal data and the code keys were kept separate.

All experiments were performed on donated blood, tumor/ovarian tissue or ascites. No experiments were performed on/with patients. Tumor/ovarian tissue and ascites were collected in connection with planned surgery for the patient's medical care and obtained via the Department of Clinical Pathology at Laboratory Medicine, Umeå University Hospital after material for diagnosis was secured. In cases with small amounts of tissue, diagnostic procedures were always prioritized. The vast majority of blood samples were collected when samples for the patients' clinical care were taken. Only a few of the postoperative blood samples used in Paper IV, were taken separately from the collection of samples for clinical care. The procedure of venous blood sampling can be painful, time consuming and there is a small risk of infection. To minimize the risk and discomfort for the patient, the samples were collected at a health care unit by trained health care professionals.

STUDY SUBJECTS, METHODS AND METHODOLOGICAL CONSIDERATIONS

The experimental methods used in the individual studies are presented in detail in Papers I-IV which are the basis of this thesis. An overview of the study subjects, methods and statistical analyses used is presented below in Table 5.

Table 5. Overview of the study subjects, methods and statistical analyses in Papers I to IV.

Paper	Material	Methods	Statistical analyses
I	Women with HGSC of the ovary - tumor tissue/blood samples, supplemented with heparin or EDTA (n=22/14) Women with benign ovarian conditions - ovarian tissue/blood samples supplemented with heparin or EDTA (n=18/18) Women with normal ovaries - ovarian tissue/blood samples supplemented with heparin or EDTA (n=8/7)	Isolation of peripheral blood mononuclear cells (PBMCs) Total RNA extraction Real-time RT-qPCR	Independent samples t-test
II	Women with HGSC of the ovary - blood samples (n=14) Cell lines: OVCAR-3, SKOV-3, Jurkat	Heat shock stress of cell cultures Real-time RT-qPCR Multiplex protein analyses (Luminex®)	Spearman's rank correlation coefficient
III	Women with HGSC of the ovary - tumor tissue/ascites (n=7/6) Women with benign ovarian conditions - ovarian tissue (n=4) Healthy donors - blood samples supplemented with heparin or EDTA (n=6) Cell lines: OVCAR-3 and SKOV-3	Tumor explant cultures - supernatant collection Cell cultures – supernatant collection Isolation of PBMCs Exosome isolation Nanoparticle tracking analysis, TEM, IEM Western blot Immunoflow cytometry Total RNA extraction Real-time RT-qPCR Degranulation of cytotoxic cells Cytotoxicity assay with K562 target cells	Independent samples t-test
IV	Women with HGSC of the ovary - blood samples supplemented with EDTA and serum samples collected pre- and postoperatively (n=18) Healthy donors - blood samples supplemented with EDTA and serum samples (n=4)	Double immunofluorescence staining and immunoflow cytometry Exosome isolation Nanoparticle tracking analysis, TEM, IEM Cytotoxicity assay with K562 target cells	Wilcoxon signed-rank test and Mann-Whitney *U* test

Study subjects and cell lines

Selection of ovarian cancer patients

Ovarian cancer is heterogeneous in nature and its classification is based on histology. We chose to study patients with epithelial ovarian cancer, the most common type comprising about 90% of all ovarian tumors. In particular, we studied HGSC, the most malignant type of EOC. The great majority of patients were diagnosed with HGSC of the ovary, but considering the uncertainty regarding cellular origin and the difficulties in determining the primary site of HGSC, cases of HGSC of the peritoneum and fallopian tube were also included, based on their molecular and clinical similarities. This is also in line with the latest FIGO staging, where these diseases are considered collectively (4).

Freshly collected and biobank-collected patient samples

Blood, serum, tumor tissue and ascites were collected either fresh from the enrolled patients or retrieved from a biobank. A biobank stores human biological samples for future research (239). A cohort at Biobanken Norr at Umeå University Hospital comprises consecutively collected ovarian tissue and blood samples since 2005 from women removing one or both ovaries for any reason. The participation is voluntary and all samples are collected after written informed consent. To retrieve samples from Biobanken Norr, an ethical permission and a written application giving information on the research project is required.

Information on histopathologic diagnosis, set by a specialist in gynecologic pathology at the Department of Clinical Pathology, Umeå University Hospital according to the World Health Organization classification (240), and other clinically relevant information, was extracted from the medical records of the participating patients. Exclusion criteria for all studies included in this thesis was a history of any other tumor disease (according to the patient's medical record).

Cell lines

EOC cell lines were used as controls for the methods we used in Paper III and in kinetic experiments in Paper II. We have never used EOC cell lines as a model specifically for HGSC and we have never compared results obtained from the participating patients with results obtained from cell lines. We chose to work with two classical EOC cell lines, OVCAR-3 (HTB-161) and SKOV-3 (HTB-77), that were purchased from ATCC and cultured according to their recommendations. The reasons for our choice was that these cell lines are the most widely used (in 90% of published papers) and thus, our results could be tested/reproduced by other groups and compared to the results of others. Recently, 47 ovarian cancer cells lines were evaluated and compared to HGSC of the ovary to find suitable candidates for HGSC research (241). While SKOV-3 has low resemblance to

HGSC, OVCAR-3 possesses TP53 mutations and substantial copy-number changes, key characteristics of HGSC (241).

Methods

Sample collection

Frozen blood (Paper I and II) and tissue (Paper I and III) samples were retrieved from Biobanken Norr. For Paper III, ascites and fresh tumor tissues for explants cultures were collected via the Clinical Pathology Laboratory, Umeå University Hospital. In Paper I, a group of patients suffering from benign ovarian conditions was included for comparison with the EOC patient group. The groups of women with normal ovarian tissue (Paper I and III) comprise women where risk reducing salpingo-oophorectomy was performed or surgery was performed for benign conditions in other parts of the female genital tract, such as myoma of the uterus, uterine polyps, endometrial hyperplasia and benign cysts in the contralateral ovary. For Paper IV, blood samples taken before and after surgery were collected from HGSC of the ovary patients from Västerbotten County between 2014-2019. Preoperatively EDTA blood and serum samples were collected. The same patients were asked to give a second sample of EDTA blood and serum 1-3 months after surgery. Only patients with a set of paired preoperative and postoperative samples were included in the study.

Short-term tissue explant cultures

Tissue explant cultures were set up to obtain supernatants enriched in exosomes secreted from cancer cells. The tumor tissues were extensively washed in Hank's solution, cut into pieces of 5-10 mg wet weight and cultured in RPMI1640 with 0,5% BSA, ascorbic acid and antibiotics. The explants were incubated at 37°C with 5% CO2 for 24h only and subsequently the supernatants were collected. The 24-hour tissue culture was chosen to keep the explants in maximally good condition, ensuring that the exosomes in the culture supernatants were obtained from natural exosome secretion and not released from dying cells.

Exosome isolation

Exosome isolations in Paper III and partly in IV were performed according to protocol (242). In brief, after sequential centrifugation steps for removing cellular debris and larger extracellular vesicles and particles, a filtration through a 0,2 µm-filter was applied, followed by a sucrose gradient ultracentrifugation at 110 000 × *g* for 2h. For the serum samples, due to limited available material and the need of higher exosomal yield for receptor expression- and functional experiments, the sucrose density gradient step, which is a critical step where exosomal yield is lost, was substituted by pelleting at 110 000 × *g* for 2h followed

by filtration. Comparison of the characteristics and purity of exosomes between these two methods gave similar results.

Estimation of exosomal concentration, purity and morphology

After isolation, the mean exosomal size, size distribution, concentration, yield and purity were analyzed using NTA with the instruments NanoSight NS300 (Malvern, UK, in Paper III) and ZetaView (Particle Metrix GmbH, Germany, in Paper IV). Negative staining and transmission electron microscopy were used for estimation of the morphology of the isolated exosomes, including estimation of purity. In addition, exosomal tetraspanin markers (CD63 and CD81) and other markers of interest (CA-125, CD24, BerEP4, EpCAM and NKG2D ligands MICA/B and ULBPs) on the exosomal surface were studied by immunoelectron microscopy (IEM) of immunogold-stained exosomes. Isolated exosomes, not used directly, were stored at -20°C or -80°C in sterile PBS supplemented with protease inhibitors (Roche Diagnostics) to avoid cleavage of surface proteins by MMP activity.

Western blot

Samples were separated on an 8-12% SDS PAGE, transferred to polyvinylidene difluoride membranes (GE Healthcare) and blocked in 3% ECL blocking reagent and 0.05% Tween 20 in ECL/PBST for 1h at room temperature. Appropriate concentrations of mABs/Abs were applied and membranes were incubated at 4°C overnight, followed by a washing step with PBST. Peroxidase-conjugated Abs were applied for 1h at room temperature and after washing, bands were developed by chemiluminescent HRP substrate (Thermo Scientific).

Real-time RT-qPCR

We used real-time RT-qPCR for estimation of the relative expression of mRNA encoding cytokines (Paper I and II) and NKG2D and DNAM-1 ligands (Paper III). RNeasy Mini Kit was used for RNA extraction and random hexamers, MuLV transcriptase and dNTP mix for reverse transcription. Isolated total RNA was protected from degradation by RNase inhibitors and was shortly after transcribed to cDNA that was kept at -80°C until use. Multiplexed RT-qPCR was performed detecting the target gene and 18S rRNA as an endogenous control on an ABI PRISM 7900HT Sequence Detection Instrument. To be able to compare the results between samples and groups, the RNA load for each reaction was equalized. The comparability of the analyses between samples was highlighted by the uniform and stable level of the endogenous control.

Comparative Ct (ΔΔCt) method was applied for computing relative quantities (RQ). An average RQ (aRQ) was calculated for each study group. The results are presented as a fold difference, which is the result of division of a test group aRQ by a reference group aRQ.

Multiplex protein analysis

For cytokine protein analysis in Paper II, multiplex protein array analysis was selected. The advantage of a multiplex bead array assay, such as Luminex®, is its efficiency as a high throughput multiplex method analyzing several cytokines at the same time.

NKG2D receptor downregulation experiments

Receptor downregulation of NKG2D expression with exosomes was done as described (243). PBMCs from healthy donors (Paper III and IV) or from HGSC of the ovary patients (Paper IV) were incubated in absence/presence of native EOC-exosomes isolated from HGSC patient ascites (Paper III) or serum (Paper IV), or in the presence of the same type of exosomes treated with specific mAbs against NKG2D ligands or CD63. After incubation, the cells were stained for NKG2D receptor expression with monoclonal antibodies. The NKG2D receptor expression was assessed by mean fluorescence intensity (MFI). Ten thousand events/sample, corresponding to 10 000 cells/sample were collected in Accuri 6C Flow Cytometer (BD Biosciences) and analyzed by CFlow Plus (BD Biosciences).

Cytotoxic degranulation experiments

To analyze NK cell degranulation (Paper III), the CD107a expression on NK cells from healthy donors was measured by flow cytometry (Accuri 6C Flow Cytometry) before and after incubation with EOC ascites-derived exosomes. OVCAR-3 cells were used as target cells.

Assessment of the NKG2D-mediated cytotoxic pathway

To assess the cytotoxic potency of NK cells, we used CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega) in combination with the erythroid cell line K562 as target cells. K562 cells are deprived of MHC class I and class II molecules and thus, experiments with K562 as target cells exclusively test cytotoxicity raised by the NKG2D receptor.

Phenotyping of peripheral blood lymphocytes and assessment of receptor and ligand expression

Double immunofluorescence staining and flow cytometry were used for phenotyping of the blood lymphocytes of patients in Paper IV. The following CD markers were used: CD19 for B cells, CD3 for T cells, CD4 for T helper cells, CD8 for T cytotoxic/suppressor cells, CD56 for NK cells and CD16 for cells expressing the Fcγ receptor. The expression of the NKG2D receptor and its ligands and other cytotoxic receptors were measured by geometric mean of fluorescence intensity. Importantly, to come as near the *in vivo* situation as possible, all flow cytometric data of the paired blood samples in Paper IV were performed on freshly isolated PBMCs with the same instrument settings.

Statistical analysis

In Paper I and III, independent samples t-test was used for comparisons between groups. In Paper II, Spearman's rank correlation coefficient was used to assess the relationship between cytokine mRNA and protein expression. In Paper IV, Wilcoxon signed-rank test was used to compare samples collected pre- and postoperatively from the same patients. Mann-Whitney *U* test was used for comparisons between unrelated samples.

Methodological considerations

Sample collection

For Paper IV, blood samples taken before and after surgery were collected. Out of 43 women having surgery for HGSC of the ovary that donated a preoperative blood sample during the sample collection period, 18 donated a postoperative sample. The reasons for the poor patient compliance are unknown. Some of the sample losses might have been avoided if there was a research sample coordinator that could maintain contact with the patients and ensure that a second sample was delivered, but unfortunately, such a coordinator was not available during the time period for the sample collection.

Exosome isolation

The challenges of exosome isolation are many, but, at large, three points can be mentioned: 1) exosomes are extremely small in size (nanovesicles of 30-150 nanometer); 2) they are almost always present in blood and bodily fluids in a mix together with other, larger extracellular vesicles with similar biophysical properties and 3) so far, there are no markers exclusively expressed on exosomes that could help to identify these vesicles. These points roughly summarize the difficulties in isolation of exosomes of high-grade purity and well-preserved

structure from various bodily fluids, with a yield giving adequate amounts for performing phenotypic and functional experiments. It should also be mentioned that efforts to obtain the highest possible purity will limit the isolation yield and vice versa. Many different attempts, ways and commercial kits to isolate exosomes exist. However, until now, the best way to isolate exosomes of good quality that preserve their properties and can be used in functional experiments is the classical standard reference way of using ultracentrifugation, a sucrose gradient and ultrafiltration. We used the following exosome isolation methods: 1) the reference method of sucrose gradient ultracentrifugation according to our own protocol published in Current Protocols in Immunology (242) for all supernatants from tumor explant cultures and ascitic fluid; 2) the same reference method with omission of the sucrose gradient ultracentrifugation step, and introducing a filtration step with a 200-nanometer filter for isolation of exosomes from patient blood samples taken before and at 1-3 months after surgery. The selection of the latter method for peripheral blood exosomes was made because it was less laborious and time consuming and probably provided a higher yield since a lot of exosomes are lost in the sucrose gradient ultracentrifugation procedure. Comparison of the purity of exosomes between these two methods gave similar results. Furthermore, whenever comparisons between samples were made, only exosomes isolated by the same isolation procedure were used.

Analysis of cytokines

A considerable part of our work was concentrated on assessment of cytokine profiles in the TME. Cytokines are short signaling proteins/peptides sensitive to degradation. Many of them have a short half-life with the degradation process starting directly after collection, making it pivotal to minimize the time between collection and freezing (244). Cytokines act locally in the body and are very seldom/not present in the blood circulation in healthy individuals. High amounts of inflammatory cytokines appear in the peripheral blood circulation during severe infections such as sepsis. Considering the instability of the cytokine proteins, their sensitivity to degradation, thawing, freezing and transportation, and their short time of action, mainly acting locally, it is important to decide what analytical method to use when assessing cytokine profiles. RT-qPCR is a method with high specificity, sensitivity and stability; therefore, we chose to use this method for mRNA analysis instead of a protein expression assay. The reason for this was that the samples were retrieved from Biobanken Norr and we did not have complete control over the collection procedure and thus could not be certain how quickly the samples were handled after collection, with regard to centrifugation, freezing, thawing and transportation. When sampling for cytokine protein analysis, several aspects have to be considered. It is not known if translation and/or secretion has taken place at all or if the amount of cytokine is sufficient to be measurable in the biological system of interest, for example peripheral blood. In this thesis, we did not aim to investigate protein concentration of cytokines, instead we aimed to investigate the cytokine profiles

that prevail in the TME and what effect these profiles could have on the immune system of the patients. Taking all this into consideration, our choice fell on cytokine mRNA profiles assessed by real-time RT-qPCR.

Kinetic cytokine mRNA and protein expression experiments (Paper II)

Cytokines are key signaling molecules and their major role in the immune response in health and disease is indisputable. Therefore, we wanted to further investigate the importance of the choice of analytical method in the cytokine research presented in Paper I. We assessed cytokine mRNA expression and protein expression in three cell lines - OVCAR-3 and SKOV-3, and Jurkat, a T cell-derived cell line that was used as a control to the OC cell lines (Paper II). Cell lines were used to perform kinetic experiments, thus being able to control the experimental conditions. Activation of cytokine gene expression was achieved by thermal stress. The success of thermal stress was evaluated by assessing the mRNA expression for heat shock proteins in each cell line used in the kinetic experiments. Thermal stress was chosen for estimation of transcriptional activation, since it is simple to perform, easy to control and we had previous experience of the method (229). Time-points were chosen to capture cytokine mRNA- and protein production peaks (245). We chose to analyze a set of proinflammatory cytokines because of their mRNA upregulation seen in HGSC compared to controls (Paper I).

RESULTS AND DISCUSSION

The goal of this thesis was to study how tumors interact with the immune system to impair its function, escape its attack and make room for tumor establishment and metastasis. We have focused on the role of cytokines and tumor-secreted exosomes in patients suffering from EOC.

The role of cytokines in the tumor microenvironment of EOC

Strengths and limitations in our cytokine studies

For the investigation of cytokine mRNA expression profiles in EOC patients, a panel of 12 cytokines, Il-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-15, IFN-γ, TNF-α, TNF-β/LTA, TGF-β and GM-CSF, was selected to distinguish between cytokine profiles responsible for four major functions of the immune defense: 1) the ability to kill infected or transformed cells, regulated by a cytotoxic Th1 response, 2) the ability to mount an antibody response, regulated by a humoral Th2 response, 3) the ability to suppress the immune system and prime Treg cells, regulated by a Th3/Th1 response and 4) the ability to elicit an inflammatory response.

The benefits and contributions of our study (Paper I) can be summarized as follows: 1) In paired samples, we investigated the cytokine mRNA expression profiles locally in the tumor tissue and systemically in PBMCs (Fig. 1 and 3, Paper I). Thus, we could compare the local and the systemic cytokine response in the same patient, a comparison that could provide a better understanding of the interaction between the tumor and the immune system at the local and systemic level; 2) We introduced two groups for comparison - one group of women with benign ovarian conditions and another group of women with normal ovaries. To our knowledge, so far, this has not been done before. The group with normal ovaries provided a baseline of cytokine expression in the normal situation (Fig. 1a and 2, Paper I). Furthermore, our studies of benign ovarian conditions and EOC (Fig. 2 and 3, Paper I) indicated that a similar but less prominent cytokine mRNA response was found in the benign conditions, while EOC caused a profound and characteristic cytokine mRNA profile, collectively driving towards suppression of the immune system's anti-tumor responses and creating a convenient TME for tumor growth; 3) Using a real-time RT-qPCR method, we simultaneously investigated 12 cytokines, which gave us the possibility to discriminate between the four responses mentioned above, and to obtain a better and broader picture of the local and systemic cytokine response in EOC patients. Several previous studies investigating the cytokine mRNA network in EOC were performed on a limited number of cytokines simultaneously and/or were using

semi-quantitative methods (186, 246-248); 4) In our methodological studies of cytokine mRNA contra protein expression (Paper II) we show that EOC cells themselves, represented by the EOC cell lines OVCAR-3 and SKOV-3, contribute to the cytokine profile in the TME by their own production of mRNA and protein for the inflammation-promoting cytokines IL-6, IL-8 and TNF-α.

We are aware that the best way to assess a certain signal substance is to prove its existence by a protein analysis. However, cytokines are proteins that act very locally, in a certain setting and although their effects are seen, they are very seldom present in peripheral blood and thus very difficult to detect in protein form. Therefore, we believe that analyzing cytokine mRNA profiles by RT-qPCR in paired blood and tissue samples and including benign ovarian conditions and normal ovaries is a good choice of method and model to study the overall picture and the changes in the cytokine responses in EOC patients.

The tumor microenvironment in EOC comprises four cytokine mRNA profiles that convey inflammation and suppression of the anti-cancer immune response

Cytokines are produced by various cells and we have analyzed the total cytokine mRNA profiles in the TME. We wanted to study the overall cytokine expression in the TME regardless of which cells have contributed since it is the overall production and effect of the cytokines that matter in the pathogenesis of OC, as well as in diagnostics and immunotherapy. We found differential cytokine mRNA expression in the tissue samples and PBMCs of HGSC patients, patients with benign ovarian conditions and women with normal ovaries. The cytokine mRNA expression was in general most prominent in HGSC patients, confirming the immunogenicity of the tumor and implying immune recognition and reaction locally in the TME and systemically in the blood (Fig. 1a and 3, Paper I).

Enhanced inflammatory response

There was a significant upregulation of inflammatory cytokines both in tumor tissue and PBMCs in the HGSC patients, compared to women with benign ovarian conditions and women with normal ovaries. Inflammation is a well-known mechanism suggested in tumorigenesis per se and is also found in the TME in EOC (13, 14). In agreement with previous studies (249-253), we found an upregulation of the inflammatory cytokines IL-1β, TNF-α and IL-6 (Fig. 1a and 3, Paper I). The mechanisms by which these cytokines influence disease progression and outcome are complex and multifactorial (14). These cytokines have been related to several cancer symptoms such as anorexia, anemia, fatigue and weight loss as well as non-responsiveness to chemotherapy (14, 254-256). It was shown that elevated IL-6 levels in EOC serum was associated with residual disease after debulking and disease recurrence (14). In contrast, in participants with benign

ovarian conditions compared to women with normal ovaries, the inflammatory cytokines were only slightly upregulated locally and slightly downregulated in PBMCs, without reaching statistical significance (Fig. 2, Paper I).

Suppressed/inadequate cytotoxic response

Most importantly, in HGSC patients, we found, locally in the TME and systemically in PBMCs, an inability to mount the crucially important IFN-γ response needed for upregulation of the cytotoxic anti-tumor response. IL-15 was upregulated in cancer patients (Fig. 1a and 3, Paper I). This might suggest a compensatory attempt to upregulate IFN-γ since IL-15 participates in IFN-γ upregulation (170).

Systemic deviation towards humoral response

In PBMCs of HGSC patients, representing the systemic immune defense, compared to women with benign ovarian conditions and women with normal ovaries, the cytokine response was oriented towards a Th2 humoral response with a significant upregulation of IL-4 (Fig. 1a and 3, Paper I), suggesting further disruption of the Th1-mediated anti-tumor defense and orientation towards antibody production, that alone, without a Th1 response, is inadequate for tumor killing.

Immunosuppressive response by T regulatory cell priming

The upregulation of the immunosuppressive cytokines IL-10 and TGF-β, combined with IL-2, in tumor tissue (Fig. 1a and 3, Paper I), suggests that TILs of T regulatory phenotype are actively produced and present in the TME. These three cytokines are instrumental for the priming, clonal expansion and immunosuppressive function of Tregs (257). In EOC, a high subset of Tregs in the TIL population is associated with metastasis (258) and a poorer prognosis (126, 131).

In summary, we have shown that in the TME of EOC patients, cytokine mRNA profiles prevail that are inflammatory and immunosuppressive, disrupting the anti-cancer immune defense. This is further shown by the absence of upregulation of the crucially important IFN-γ response, necessary for tumor cells' killing. Accompanied by a significant systemic IL-4 mRNA upregulation, the systemic immune response of the EOC patients is deviated towards humoral immunity. A summarized schematic presentation of the cytokine studies is shown in Fig. 4 below.

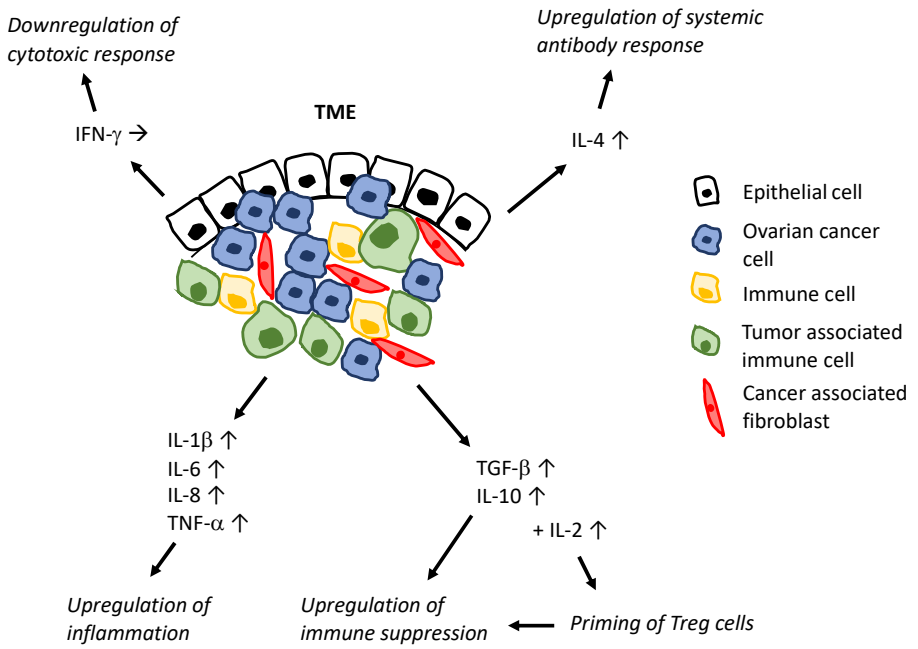

Figure 4. Schematic presentation of the role of cytokines in the TME of EOC.

The role of EOC exosomes in the derangement of the anti-tumor immune defense

EOC exosomes selectively downregulate the NKG2D receptor and impair the anti-tumor cytotoxic response

It is well known by previous studies that EOC is a great producer of exosomes, nanometer-sized extracellular vesicles of endosomal origin. We focused our efforts on investigating interferences of EOC-derived exosomes on the NKG2D receptor-ligand system, a system known to be crucial for immune surveillance against tumors (89, 259). In 2007, Carlsten et al. (96) reported that the major tumor-killing system, the NKG2D receptor-ligand pathway, was downregulated in EOC patients. Instead, the DNAM-1 receptor-ligand interaction prevailed as the main cytotoxic pathway for elimination of EOC cells *in vitro*. Our intention was to dissect the molecular mechanisms behind this observation and the results are presented in Paper III.

Using sucrose gradient ultracentrifugation, we isolated exosomes from supernatants of EOC tissue explant cultures, EOC patient's ascites, and OVCAR-3

and SKOV-3 cell lines. The purity and the exosomal nature of the isolated vesicles were confirmed using three methods – NTA, western blot (WB) and IEM staining of exosomal markers. Further, we analyzed the expression of NKG2D and DNAM-1 ligands by WB, immunoflow cytometry of exosomes captured on antibody coated latex beads and IEM. There was a variation observed in protein expression of the exosomes emanating from different tumor samples (Table 1, Paper III) reflecting the heterogeneity of EOC. While OVCAR-3 and SKOV-3 expressed DNAM-1 ligands PVR and Nectin-2 on cellular level, exosomes emanating from them lacked these ligands' expression (Table 1, Fig. 1d, Paper III), suggesting that these tumor cells did not secrete the DNAM-1 ligands through the endosomal compartment. Using WB, we found expression of NKG2D and DNAM-1 ligands in ascites and tumor explant exosomes (Fig. 3a, Paper III). However, when we investigated the exosomal surface expression of the NKG2D ligands MICA/B and ULBP1-3 and DNAM-1 ligands PVR and Nectin-1 using immunoflow cytometry and IEM (Fig. 3b and c, Paper III), only the NKG2D ligands MICA/B, ULBP1-3 were expressed on the exosome surface. The DNAM-1 ligands PVR and Nectin-2 were not. Thus, we could conclude that the DNAM-1 ligands were more sporadically expressed by EOC exosomes and if expressed, they were not associated with the exosomal membrane.

In our further analysis we investigated the impact of ascites-derived exosomes on the cognate receptor by *in vitro* studying the NKG2D and DNAM-1 receptor expression on PBMCs from healthy donors, in the presence and absences of EOC exosomes. Using flow cytometry and mean fluorescent intensity, we found that the NKG2D receptor was significantly downregulated from the cell surface of $CD8^+$, $CD56^+$ cells in the presence of EOC exosomes, while the DNAM-1 receptor remained unchanged (Fig. 4 a-d, Paper III). The downregulation could be reversed by blocking the EOC exosomes with a cocktail of antibodies against NKG2D ligands or by antibodies against the tetraspanin marker CD63. From these experiments we concluded that the NKG2D receptor was downregulated by EOC exosomes, while the DNAM-1 receptor was not. Tumors release soluble NKG2D ligands by mainly two ways, MMP-cleavage (112-115) and secretion of NKG2D ligand-bearing exosomes, the latter being the most potent way of NKG2D receptor downregulation (260, 261). In the final steps of this study (Paper III), we performed functional experiments using PBMCs from healthy donors to see the functional consequences of the observed NKG2D receptor downregulation. We measured NKG2D-mediated cytotoxicity with the MHC class I- and Fas-negative, K562 cell line as target cells, and degranulation, using OVCAR-3 cells as targets (Fig. 5b-c, Paper III). We found that the cytotoxic response of NK cells from healthy donors treated with EOC exosomes was significantly downregulated. The downregulation was comparable to that of when the targets or the receptors were blocked with monoclonal antibodies. The downregulation could be reversed in the presence of exosomes blocked by antibodies against NKG2D ligands or CD63. In contrast, we did not see any effect on the DNAM-1 receptor mediated cytotoxicity in the presence of EOC exosomes, reflecting the

fact that EOC exosomes do not carry DNAM-1 ligands on their surface and thus cannot affect the cognate receptor. Taken together, the results of Paper III demonstrate for the first time that tumor exosomes can exert a differential downregulation of cytotoxicity. The EOC exosomes only affect the NKG2D receptor-ligand pathway, leaving the DNAM-1 pathway in function. In this work, we explain why it was reported (96) that the otherwise accessory cytotoxic DNAM-1 receptor-ligand system is used in EOC as the dominant killing pathway, while the major cytotoxic pathway of the immune system, the NKG2D receptor-ligand pathway, is abrogated.

EOC exosomes isolated from patients' serum impair NKG2D-mediated cytotoxicity

Surgery is a cornerstone of OC treatment (46, 47). In Paper IV, we wanted to study the influence of surgery on circulating EOC exosomes and the NKG2D cytotoxic pathway *in vivo* by investigating exosomes isolated from EOC patients' serum. In previous studies of the influence of OC surgery on the immune status of the patients, it has been shown that there is a decrease in circulating Tregs and immunoinhibitory cytokines postoperatively, associated with a better prognosis (135, 136, 262-265). Exosomes have previously not been studied in OC surgery. We started by evaluating the phenotype of immune cells in peripheral blood before and after surgery, using flow cytometry. By studying a panel of phenotype markers separating lymphocytes into B cells, cytotoxic and helper T cells, NK cells and Fcγ receptor/CD16 expressing cells, and also cells expressing the cytotoxic NKG2D receptor, we conclude that no statistically significant differences were seen in the patient group as a whole (Fig. 1a, Paper IV). However, when studying the results more closely, we saw that within the whole group of patients, there was a group of patients that exhibited a significant upregulation of the NKG2D receptor expression postoperatively (Fig. 1b, Paper IV). Intrigued by this finding, we wanted to further investigate what could cause this change in some patients and whether it would have any effect on the cytotoxic function of the patients' NK cells. The participants showing an upregulation of the NKG2D receptor expression postoperatively were referred to as Group 1 and the rest of the patients as Group 2. We started by looking into the concentration of circulating exosomes, isolated from the patients' sera, taken pre- and postoperatively. We found that the exosomal concentration decreased postoperatively in group 1 and increased slightly, but not statistically significant, in Group 2 (Fig. 2b, Paper IV). Thus, there was a significantly lower exosomal concentration postoperatively in the serum of Group 1 patients compared to the serum of Group 2 patients.

Following this finding, we used IEM to confirm that also serum-derived EOC exosomes express NLG2D ligands on their surface (Fig. 3a, Paper IV). To further elucidate the impact of these exosomes on the NKG2D receptor-ligand system, we investigated the NKG2D receptor expression on PBMCs from healthy donors in the presence and absence of EOC exosomes, isolated from serum of patients

taken before surgery. Using flow cytometry and mean fluorescent intensity, we found that there was a downregulation of the receptor using exosomes from both groups, although the effect was more prominent for Group 1 exosomes (Fig. 3b-c, Paper IV). When blocking the exosomes with monoclonal ab against CD63, the effect was reversed, confirming that the observed downregulation of NKG2D was caused by EOC exosomes (Fig. 3c, Paper IV). To study if the decreased NKG2D receptor expression could affect the cytotoxic potential of NK cells, cytotoxic experiments were performed. PBMCs from healthy donors were used as effectors and K562 as targets, in the presence or absence of exosomes. The results show that preoperative exosomes from both groups had the ability to downregulate cytotoxicity (Fig. 3d, Paper IV). However, the downregulation was most prominent after treatment with preoperative exosomes from Group 1 and with unaffected cytotoxic function when treated with postoperative exosomes from Group 1 patients. The cytotoxic function of PBMCs was similar following treatment with pre- or postoperative Group 2 exosomes. The downregulation of the cytotoxicity was reversed when exosomes were blocked with anti-CD63 monoclonal ab.

Finally, to investigate the patients' NK cell function *in vivo*, we conducted cytotoxic experiments with the patient's own immune cells as effector cells and K562 as targets and found that the cytotoxicity was enhanced postoperatively for all patients, but more prominently in Group 1 (Fig. 4, Paper IV). These experiments suggest that by removing the source of EOC exosomes, the primary tumor, as demonstrated by the lower postoperative exosome concentration observed in Group 1 patients, the intrinsic cytotoxic ability of the patients' NK cells is enhanced/restored.

Surgery of the primary ovarian tumor had a positive effect on the postoperative cytotoxic function of NK cells

We have, in Paper III and IV, *in vitro* and *in vivo*, shown that EOC exosomes, whether derived from serum, tissue explants, ascites or cell lines, express NKG2D ligands on their surface and have the potential to downregulate the cognate receptor on healthy cytotoxic immune cells, hampering their cytotoxic function.

In summary, in this proof-of-concept study, we found that primary debulking surgery in EOC patients enhances the cytotoxic immune response. One possible mechanistic explanation could be that removal of the tumor, a main source of exosome production, induces a decrease in NKG2D ligand-bearing exosomes that otherwise could interfere with the major cytotoxic NKG2D anti-tumor response. All patients in our study underwent primary surgery for HGSC of the ovary. The material is otherwise diverse regarding surgical outcome and chemotherapeutic status before the postoperative sample collection (Table 1 and 2, Paper IV). This may affect the results. What is interesting is that the two groups don't seem to

differ in these respects, there were even more radical surgeries performed in Group 2. This suggests that there are other mechanisms at play.

Additional future studies with much larger and well-defined patient cohorts regarding treatment and type of surgery, and a longer follow-up time, are needed to proof/disproof the results of this investigation.

EOC exosomes downregulate NKG2D-mediated cytotoxicity, upregulate TRAIL- and FasL-apoptosis and promote Treg cells by surface expression of molecules that can be defined as immunosuppressive signatures

The immune system is a very potent system in the human body and it constitutively carries a potential risk to turn its mechanisms against its own host. Thus, suppression is important to keep the immune system at bay. Immunosuppression is present in health and disease and is effectuated in a variety of ways. As described above in this thesis, exosomes are produced by virtually all organs of the body, present in all bodily fluids and exert important functions not only in health and disease but also in embryogenesis and fetal development (208).

Exosomes are known to upregulate or downregulate immune responses. Here, we would like to dwell on three powerful ways of exosomes-mediated immune suppression: 1) apoptosis of activated immune cells; 2) priming and activation of Treg cells and 3) downregulation of the NKG2D receptor-ligand system. These particular mechanisms are not only operating in diseases such as cancer but are also actively operating in healthy conditions such as the protection of the semiallogeneic fetus during normal mammalian pregnancy (208). It has previously been shown that EOC exosomes carry apoptosis-inducing molecules, like FasL (226, 266) and TRAIL (217), as well as immunoinhibitory cytokines, that prime T regulatory cells, on their surface (222, 225). In several cancers, it has been shown that tumor-derived exosomes also express the ligand of PD-1, aiding in the above-mentioned mechanisms for immune escape (267). It is likely that this is used also by OC. In addition, in this thesis we have provided evidence *in vitro* and *in vivo* that EOC exosomes express the NKG2D ligands MICA/B and ULBP 1-3 on their surface and thus downregulate the NKG2D receptor, disrupting the cytotoxic potency of the patients' NK cells. The role of EOC exosomes in these three powerful immune suppressive pathways could schematically be illustrated as in Fig. 5.

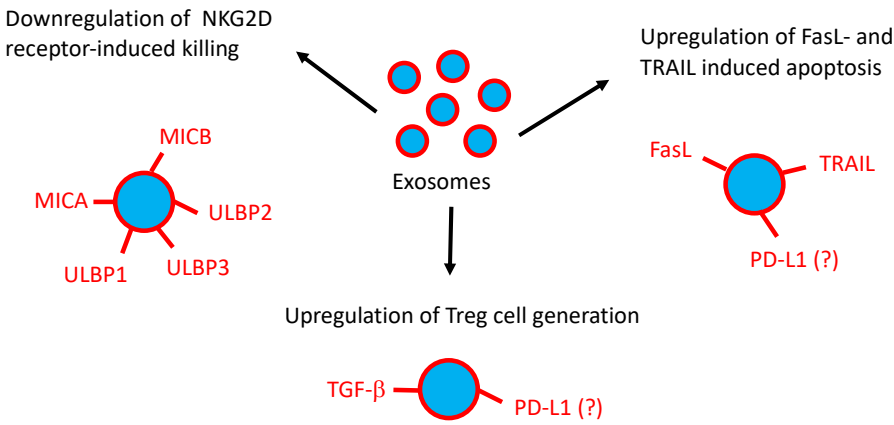

Figure 5. Schematic presentation of the role of EOC exosomes expressing immunosuppressive signatures (shown in red letters) that promote tumor immune escape by derangement of the anti-tumor immune response in patients suffering from EOC (modified after Mincheva-Nilsson, L).

Concluding remarks and future perspectives

The road from uncovering a process, or discovering a new mechanism for immune escape, to clinical applicability, the ultimate goal of all research, is often long. Our results open up for several different directions of future studies. We present novel findings of mechanistic, basic research using a smaller number of patients. These have to be confirmed in larger patient cohorts, representing more uniform patient groups, to find new approaches for diagnosis and treatment.

Both in studying cytokine mRNA profiles (Paper I) and EOC-derived exosomes (Paper III and IV), it would be interesting to correlate the results to a more detailed analyzes of the architecture of the TME and the tumor treatment used. Could cytokine mRNA profiles of the tumor offer a proxy for the overall immune status of the patient and further in determining what immunotherapeutic or other treatment would be beneficial for this patient? A study from 2017 showed that mRNA profiles, including cytokine-mRNA, of melanoma biopsies correlated to the patients PD-1-checkpoint blockade responsiveness (268). Additionally, one could speculate whether intrinsic tumor characteristics could be mirrored in the patients PBMCs, as our results suggest. Cytokines in disease are widely studied and considered as biomarkers. However, the use of cytokine mRNA profiles has to our knowledge not been considered in EOC and might offer new perspectives. Perhaps mRNA profiles of PBMCs may serve as accessible biomarkers for prognosis and monitoring of the disease in the future. Cytokines in cancer therapy have been widely studied (reviewed in (269)). Administrating cytokines systemically have often been hampered by their toxic effect. Cytokines may also be coupled to antibodies directing them towards the TME. Another way could be to block the effect of cytokines using specific antibodies. More recently cytokines

are combined with other treatments, like immune checkpoint inhibition and mAb towards other markers, e.g. VEGF, and several clinical trials have been undertaken (269).

Additionally, it would be interesting to continue to study tumor exosomes in different biological aspects to understand in detail why, for example, EOC exosomes express NKG2D ligands on their surface but not DNAM-1 ligands. Also, the expression of other immune depressing molecules, such as immune checkpoint molecules, by EOC exosomes is a scope for further investigations. It is not fully understood exactly how different molecules are sorted in the endosomal compartment to the exosomes during their biogenesis, and this is a subject of intensive research.

Potentially, exosomes could be used in diagnosis, follow-up and treatment. The immune signature of OC exosomes might in the future be used for these purposes. As previously mentioned, the expression of PD-L1 by tumor-derived exosomes has been evidenced in several cancers (267). This could offer ways of predicting responsiveness to PD-1 blockade. Considering our results of the NKG2D ligand bearing exosomes and their effect on cytotoxic function, it is a tempting idea to block these immunosuppressive exosomes, and several potential compounds are under evaluation (270). Still there is not much research on the impact of OC surgery on the immune system, and basically none regarding exosomes. Further investigations on this specific topic would be interesting. Considering the results of our study, we ask why women respond differently regarding NKG2D expression and whether this is connected to the lower concentration of serum exosomes as we speculate. As stated, we have no information on the TME of these patients. Useful information could be what cells reside in the TME, if there are tumor infiltrating lymphocytes or immune cells of a tumor favoring phenotype present, as well as the overall immunosuppressive mechanisms used by the tumor except exosomes and how the tumors differ from each other in these respects. What molecules are in total expressed by the EOC exosomes is also of interest. Of great importance is the heterogeneity of all cancers *per se*, including EOC, which is well documented to be among the most heterogeneous tumors. Heterogeneity in tumor aggressiveness of EOC may affect the production and outflow of immunosuppressive exosomes and the reason for the observed different responses to surgery might lie here. It would also be interesting to know if the difference in NKG2D expression and/or exosome concentration affects the survival of the patients, something that would have to be studied on a larger material over a longer period - is the NKG2D status or postoperative exosome concentration related to tumor aggressiveness and survival prognosis? Hopefully, this information could be used in optimizing immunotherapeutic approaches to help restore immune surveillance.

Finally, we would like to underline that the future diagnosis and treatment of cancer will be personalized, tailored based on the individual patient's distinct tumor, thus providing a specific treatment that is effective and overcomes the heterogeneity of cancer. There are already treatments that are specifically designed to treat a certain cancer type, which is diagnosed and classified by specific tumor- and immunological markers. Furthermore, together with the opportunities of genetic intervention at strategic points by CRISPR Cas9, treatments involving immune mechanisms, like immune check point inhibitions and anti-cytokine antibodies are already in use and will become more common in the future as more and more discoveries are made in the field of oncoimmunology.

CONCLUSIONS

- Proinflammatory cytokines and immunosuppressive cytokines promoting Treg cells dominate the cytokine mRNA expression locally in the EOC microenvironment and systemically in PBMCs. The absence of an IFN-γ response together with systemically elevated IL-4 levels indicates a deviation of the immune response from a cytotoxic to a humoral response.

- EOC cells can transcribe and secrete IL-6, IL-8 and TNF-α. Cytokine mRNA, instead of protein expression, can be used for analyses of cytokine profiles in studies of mechanistic pathways and in comparisons between patient groups, but cannot replace expression at the protein level.

- EOC exosomes disrupt the cytotoxic response in a differential way. The major cytotoxic NKG2D receptor-mediated pathway is suppressed by NKG2D ligand expressing EOC exosomes acting as decoys, downregulating the cognate receptor. In contrast, DNAM-1 ligands are seldom expressed and not associated with the exosomal membrane surface, leaving the accessory DNAM-1 cytotoxic pathway unaffected.

- Surgery of the primary EOC tumor has a beneficial effect on the anti-tumor cytotoxic immune response. One mechanistic explanation could be a decrease in circulating NKG2D ligand-expressing exosomes, thus improving the cytotoxic NK cell function.

- Taken together, our results unravel molecular mechanisms operating behind local and systemic immune escape in EOC, elucidating some of the reasons behind the derangement of the immune system and hopefully providing some clues for future clinical implications.

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