Unravelling The Tumour Immune-Biology Of Ovarian Cancer

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Is this the end or the beginning?

Part of Life we see daily
Is it the end or the beginning?
Immune we are to its presence

It is not in our thinking.

Is it a point on a cycle,
Is it a point on a line?
What comes before or after
Who knows the design.

What comes after it,
What was there before?
Why worry our heads
No one knows for sure.

What will be lost after it
The same we lost prior
If we worry about the former
Take comfort in the latter.

Why worry about existence
Or the lack of it after
Are we not the experts
Having done this forever.

It surrounds us everyday
In thoughts and binds
In ideas or in life
And in all the right things.

Yet life always flows back
And it binds and thinks
Through a new life or idea
And in all the right things.

We see this every day
What is it if not a beginning
It may not be the same
But is born out of the same thing

Is this the end or the beginning?

Martin Rushton

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ABBREVIATIONS

ADNEX	Assessment of Different Neoplasia's in the adnexa model
APC	Antigen presenting cells
ARRIVE guidelines	Animal Research: Reporting of In Vivo Experiments guidelines
AUC	Area under the curve
BLI	Bioluminescence imaging
BRCA	Breast cancer gene
CA125	Cancer antigen 125
CAFs	Cancer associated fibroblasts
CAR T-cells	Chimeric antigen receptor T-cells
CD	Cluster of differentiation
cDCs	Conventional dendritic cells
CFSE	Carboxyfluoresceinsuccinimidyl ester
CL	Clodronate liposomes
COX2	Cyclooxygenase 2
CSF1	Colony stimulating factor 1
ctDNA	Circulating tumour DNA
CTL	Cytotoxic T-lymphocyte
CTLA-4	Cytotoxic T-lymphocyte associated protein 4
CXCL	C-X-C chemokine ligand
CXCR	C-X-C chemokine receptor
DCs	Dendritic cells
DD	Dose dense
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPBS	Dulbecco's phosphate-buffered saline

EDTA	Ethylenediaminetetraacetic acid
EMA	European medicines agency
EOC	Epithelial ovarian cancer
ESMO	European Society for Medical Oncology
FACS	Fluorescent activating cell sorting
FIGO	Federation of Gynecology and Obstetrics
FMO	Fluorescence minus one
FOXP3	Forkhead box P3
GLOBOCAN	Global Cancer Observatory
GLUT1	Anti-glucose transporter-1
GM-CSF	Granulocyte-macrophage colony-stimulating factor
gMDSC	Granulocytic MDSC (new name: PMN-MDSC)
GR1	Granulocytic marker
HDR	Homology directed repair
HE4	Human epididymis *protein* 4
HGS(T)OC	High grade serous (tubo-)ovarian cancer
HLA-DR	Human Leukocyte Antigen – antigen D Related
HR	Hazard ratio
HRD	Homologous recombination deficiency
IDS	Interval debulking surgery
IFN	Interferon
IFNg1b	Interferon gamma 1b
IHC	Immunohistochemistry
IL	Interleukin
IOTA	International ovarian tumour analysis group
ip	Intraperitoneal
ISG	Interferon-stimulated genes
IVC cage	Individually ventilated cage

IVIS	In vivo imaging system
LAG-3	Lymphocyte-activation gene 3
LGSOC	Low grade serous ovarian cancer
LR2	Logistic Regression model-2
MCP1	Monocyte chemoattractant protein-1, also known as CCL2
MDSC	Myeloid-derived suppressor cells
MHC	Major histocompatibility complex
MIP-1 alfa	Macrophage inflammatory protein 1 alfa, also known as CCL4
M-MDSC	Monocytic myeloid-derived suppressor cells
MoDCs	Monocytic dendritic cells
moSAIC	Molecular small animal imaging centre
MRC1	Mannose receptor C type 1
Myd88	Myeloid differentiation primary response 88
NACT	Neoadjuvant chemotherapy
NCR1 (NKp46)	Natural cytotoxicity triggering receptor 1
NFKB	Nuclear factor kappa-light-chain enhancer of activated B cells
NK cells	Natural killer cells
NKT cells	Natural Killer T cells
OOR	Out of detectable range
OS	Overall survival
p/s	Photons per second
PARP	Poly-ADP ribopolymerase
PARPi	PARP inhibitor
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PD-1	Programmed cell death protein 1
pDCs	Plasmacytoid dendritic cells
PD-L1	Programmed death-ligand 1

PDX	Patient derived xenograft
PFS	Progression free survival
PLD	Pegylated liposomal doxorubicin
PMN-MDSC	Polymorphonuclear myeloid-derived suppressor cells
RMI	Risk malignancy index
RT	Radiotherapy
SEER	The Surveillance, Epidemiology, and End Results Program
sFIS assay	Serum-functional immunodynamic status assay
SPF	Specific-pathogen-free
SR	Simple Rules risk model
TAMs	Tumor associated macrophages
TCR	T-cell receptors
TGF-beta	Transforming growth factor beta
TIGIT	T-cell immunoreceptor with Ig and ITIM domains
TILs	Tumour-infiltrating lymphocytes
TIM-3	T-cell immunoglobulin domain and mucin domain 3
TME	Tumor microenvironment
TNF alfa	Tumour necrosis factor
Tregs	Regulatory T-cell
UKCTOCS	UK Collaborative Trial of Ovarian Cancer Screening
VEGF-A	Vascular endothelial growth factor A
VISTA	V-domain Ig suppressor of T cell activation
WT	Wild type

TABLE OF CONTENTS

Chapter 1

Introduction

OVARIAN CANCER: CHARACTERISTICS AND MANAGEMENT

Epidemiology and histology

Ovarian cancer originates in the ovaries or the fallopian tubes. It is the eight most common cancer type in women worldwide with an age-standardized incidence rate of 6.6 per 100.000 women [1]. In Belgium, approximately 781 cases are diagnosed each year [2]. This corresponds with a lifetime risk of developing ovarian cancer of 1 in 78 [3]. Ovarian cancer histology reveals a wide range of different histological and molecular subtypes [4]. The vast majority (90%) of ovarian cancers is of epithelial origin [5]. Non-epithelial ovarian tumours include germ cell, sex cord stromal and mesenchymal tumours. Within epithelial ovarian cancer (EOC), five main subtypes are identified according to the new 2020 World Health Organization classification of Female Genital Tumours: mucinous carcinoma (3-4%), clear cell carcinoma (6-10%), endometrioid carcinoma (10%), low grade serous ovarian carcinoma (LGSOC, 5%) and high grade serous tubo-ovarian carcinoma (HGSTOC, 70%) [5]. It needs to be acknowledged that all these subtypes are different diseases with their own histological, molecular and genetic features [4]. Clinical management of the subtypes should be (and to a certain level is) adapted to the specific subtype [6]. All malignant subtypes are staged based on the International Federation of Gynecology and Obstetrics (FIGO) staging system (See Table 1).

Table 1. The 2014 FIGO staging for malignant ovarian cancer

Stage I: Tumour confined to ovaries or fallopian tube(s)		
IA		*Tumour limited to one ovary (capsule intact) or fallopian tube; no tumour on ovarian or fallopian tube surface; no malignant cells in the ascites or peritoneal washings*
IB		*Tumour limited to both ovaries (capsules intact) or fallopian tubes; no tumour on ovarian or fallopian tube surface; no malignant cells in the ascites or peritoneal washings*
IC	*IC1*	*Surgical spill*
	IC2	*Capsule ruptured before surgery or tumour on ovarian or fallopian tube surface*
	IC3	*Malignant cells in the ascites or peritoneal washings*
Stage II: Tumour involves one or both ovaries or fallopian tubes with pelvic extension (below pelvic brim) or primary peritoneal cancer		
IIA		*Extension and/or implants on uterus and/or fallopian tubes and/ or ovaries*
IIB		*Extension to other pelvic intraperitoneal tissues*
Stage III: Tumour 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		
IIIA1		*Positive retroperitoneal lymph nodes only (cytologically or histologically proven)*
	IIIA1(i)	*Metastasis up to ten millimeter in greatest dimension*
	IIIA1(ii)	*Metastasis more than ten millimeter in greatest dimension*
IIIA2		*Microscopic extra pelvic (above the pelvic brim) peritoneal involvement with or without positive retroperitoneal lymph nodes*

IIIB	*Macroscopic peritoneal metastasis beyond the pelvis up to two cm in greatest dimension, with or without metastasis to the retroperitoneal lymph nodes*
IIIC	*Macroscopic peritoneal metastasis beyond the pelvis more than two cm in greatest dimension, with or without metastasis to the retro-peritoneal lymph nodes (includes extension of tumour to capsule of liver and spleen without parenchymal involvement of either organ)*
Stage IV: Distant metastasis excluding peritoneal metastases	
IVA	*Pleural effusion with positive cytology*
IVB	*Parenchymal metastases and metastases to extra-abdominal organs (including inguinal lymph nodes and lymph nodes outside of the abdominal cavity)*

Adapted from the FIGO CANCER REPORT 2021 by Berek S. et al. [6]

Diagnosing ovarian cancer

Diagnosing ovarian cancer still proves to be challenging because of three main hurdles. **First**, patients are asymptomatic or experience only **vague symptoms** that are not specific to ovarian cancer disease [7]. Symptoms can include, but are not limited to, abdominal pain, bloating, early satiety, profound changes in weight, increased fatigue and (post-menopausal) vaginal bleeding. As these symptoms are vague and seemingly none worrisome, most malignant cases are only diagnosed in advanced disease stages. For HGSTOC, a malignant subtype that can often disseminate extensively throughout the abdomen before causing symptoms, the vague nature of symptoms results in 52% of the patients getting diagnosed at FIGO stage III and 30% at FIGO stage IV [8]. In these advanced stages, the build up of ascites in the peritoneal cavity becomes a distinct and frequently seen symptom [9]. **Secondly**, **no screening is available**. The current standard diagnostic tool for suspected ovarian masses remains transvaginal ultrasound. Additionally, serum protein measurements of cancer antigen 125 (CA125) are often used. However, one should be cautious of the use of CA125, as this marker is not ovarian cancer specific and could be increased by various other medical issues. As these two tools are used in diagnosis, many have tried to look if they could prove useful in screening for ovarian cancer. The UK Collaborative Trial of Ovarian Cancer Screening (UKCTOCS) is the largest trial to date, that prospectively screened woman annually via CA125 and/or ultrasound tests [10]. Regrettably, they were not able to identify (advanced) ovarian diseases sufficiently earlier to impact mortality rates [10]. Other efforts made to develop a screening method include, but are not limited to, the addition of other protein biomarkers (such as HE4 [11]), genetic screening [12], [13], the monitoring of circulating tumour cells and/or circulating tumour DNA (ctDNA) [14] or improving imaging techniques [15]. To date, no real successes have been made yet, but the development of more specific and more early diagnostic methods remains ongoing. For example, in the currently ongoing BEDICA trail (Biomarkers of Early Detection of ovarian cancer – NCT03150121), uterine lavages are used as a technique in the hope to discover new biomarkers. In the DovEE phase III trial, a genomic uterine sample test was specifically developed to detect ovarian and endometrial cancer more early (NCT02288676 and NCT0489102). Currently however, it is still waiting for a real screening/diagnostic breakthrough for ovarian cancer. **The third challenge** in diagnosing ovarian cancer is to correctly **discriminate between malignant, benign or borderline tumours**. Correct discrimination is of high clinical relevance as patients with

benign lesions may be followed conservatively or undergo minimal invasive surgery with the option of fertility preservation, which is especially important in young patients [16]–[18], which is also the case for borderline tumours. On the other hand, patients with malignant disease gain from being treated by specialized physicians as it could impact their outcome (see treatment section below). To help in correct discrimination between ovarian neoplasms, different diagnostic models have been developed over the past years combining an array of clinical and ultrasound features. The oldest model is the risk of malignancy index (RMI) including the parameters menopausal status, an ultrasound score and the serum CA125 levels to make a discrimination between benign and malignant masses [19]. Many other models have followed, including those of the international ovarian tumour analysis (IOTA) group. They developed the Logistic Regression model-2 (LR2) [20], the Assessment of Different Neoplasia's in the adnexa model (ADNEX) [21] and the IOTA Simple Rules (SR) risk model [22]. Multiple studies have shown that the IOTA models performed best in preoperative characterization of any suspected ovarian mass [23]–[27]. Combinations of novel screening biomarkers together with these diagnostic models may become part of an improved strategy for more early and correct diagnosis.

Treatment

The standard of care for malignant epithelial ovarian cancer consists of tumour debulking surgery with (neo-)adjuvant platinum-based chemotherapy. **Surgery** where all macroscopic visible tumours can be removed is referred to as a R0-resection. Full chirurgical resection is an important prognostic factor in advanced disease staging as it is associated with a prolonged progression-free survival (PFS) and overall survival (OS) [28], [29]. If in advanced disease stages full removal of all macroscopic visible tumour tissue is not expected to be feasible in a primary setting (primary debulking surgery, PDS), neo-adjuvant chemotherapy treatment, where half of the predefined cycles of chemotherapy is given, can be an option. An interval debulking surgery (IDS) could be performed if the tumour is judged operable upon radiological re-evaluation [30]–[32].

Chemotherapy in first-line setting most often consists of carboplatin combined with paclitaxel, either a three weekly or a weekly schedule [33]. The majority of patients, especially HGSTOC, initially respond well to this primary treatment. However, more than 70% of patients experience a relapse in disease after their full primary treatment (surgery combined with chemotherapy) [28]. If progression occurs during or immediately after the last line of platinum-based chemotherapy, patients will not be eligible for second-line platinum-based chemotherapy [34]. Mechanisms of resistance are heavily studied as evidenced by the recent review by Machetti C *et al.* [35], but are not completely understood. No validated biomarkers or predictors of chemotherapy resistance are available at this time and response to the standard therapy is currently still evaluated by CA125 in correlation with radiological and clinical assessment.

Fairly recently, **two targeted therapies** got implemented into the primary treatment schedule for ovarian cancer patients. The first one, approved first by the European Medicines Agency (EMA) in 2005, in combination with carboplatin-paclitaxel chemotherapy in advanced EOC, is bevacizumab. In the beginning of 2021, two bio-similars of bevacizumab were approved as well. **Bevacizumab** is a recombinant, humanized, monoclonal antibody affecting blood vessel formation by targeting vascular

endothelial growth factor A (VEGF-A). Approval came after positive PFS benefits were seen in both recurrent disease (OCEANS (NCT00434642) and AURELIA (NCT 00976911)) and first-line setting (ICON7 (NCT00483782) and GOG218 (NCT00262847)) [36]–[39]. Next to PFS benefit, the AURELIA trial also reported a reduction in ascites development [36]. Additional benefit for OS was harder to obtain and only demonstrated in a retrospective, subgroup analysis of high risk patients in the ICON7 trial – defined as patients with inoperable FIGO stage III, suboptimal debulked stage FIGO stage III or FIGO stage IV [37]. More recently available data however, have indicated that bevacizumab as solo maintenance therapy is not able to translate its positive PFS effects into an OS benefit for ovarian cancer patients [40]. Attempts should be made to identify response predictors to better apply bevacizumab.

The second class of targeted drugs are the **poly-ADP ribopolymerase (PARP) inhibitors** with currently three approved inhibitors: olaparib, niraparib and rucaparib. When inhibiting the enzyme PARP, single strand breaks in the DNA persist and are forced into double stranded DNA breaks that subsequently need to be repaired correctly. This is especially detrimental in cancerous cells that often have mutations in their DNA repair pathways, such as loss of function mutations in homologous recombination repair pathway proteins like BRCA1 and BRCA2. Homologous recombination repair deficiency (HRD) renders the cancer cells unable to repair double stranded breaks correctly and will ultimately result in cell death. Indeed, first studies with PARP inhibitors focussed on ovarian cancer pathology with BRCA mutations (SOLO-1 (NCT01844986) and SOLO-2 trials (NCT01874353)) [41], [42]. Later on, in the PRIMA-1 trial (NCT02655016), a benefit for PARP inhibitor use was seen in HRD-positive patients with a PFS prolongation from 10.4 months in the placebo group versus 21.9 months in the treated group (HR 0.43, $p<0.001$) [43]. More interesting however, was the demonstration of PFS benefit with PARP inhibitor maintenance, albeit smaller but still significant, in homology directed repair (HDR)-negative patients (8.1 months versus 8.2 months, HR: 0.68, $p=0.02$). Next, the PAOLA-1 trial (NCT02477644) was the first phase III trial investigating the combination of bevacizumab and PARP inhibitors in a first-line setting [43]. After first-line therapy response with three or more cycles of bevacizumab, patients received maintenance bevacizumab in combination with olaparib or placebo. Median PFS significantly improved in the combination arm (22.1 months) versus bevacizumab maintenance only (16.6 months) (HR 0.59; [0.49-0.72]; $p< 0.001$). However, this benefit was only seen in patients with HRD mutations, leading to EMA approval for this first-line maintenance therapy only for this specific subgroup of patients with ovarian cancer. Regrettably, also for these therapies, resistance mechanisms are being discovered, which drives scientists into a continuous search for amelioration so patients keep on benefiting from these treatments. [44], [45].

Outcome

Ovarian cancer has the fifth highest mortality rate among women with cancer. However, prognosis heavily depends on the subtype and stage at diagnosis. Localized (FIGO stage I) ovarian cancer tumours have a good prognosis with five-year survival rates of 93.1% (Figure 1) [8]. Unfortunately, the clinical reality is that most ovarian cancers are diagnosed at advanced stages, where survival rates drop to an average of 30.8% [8].

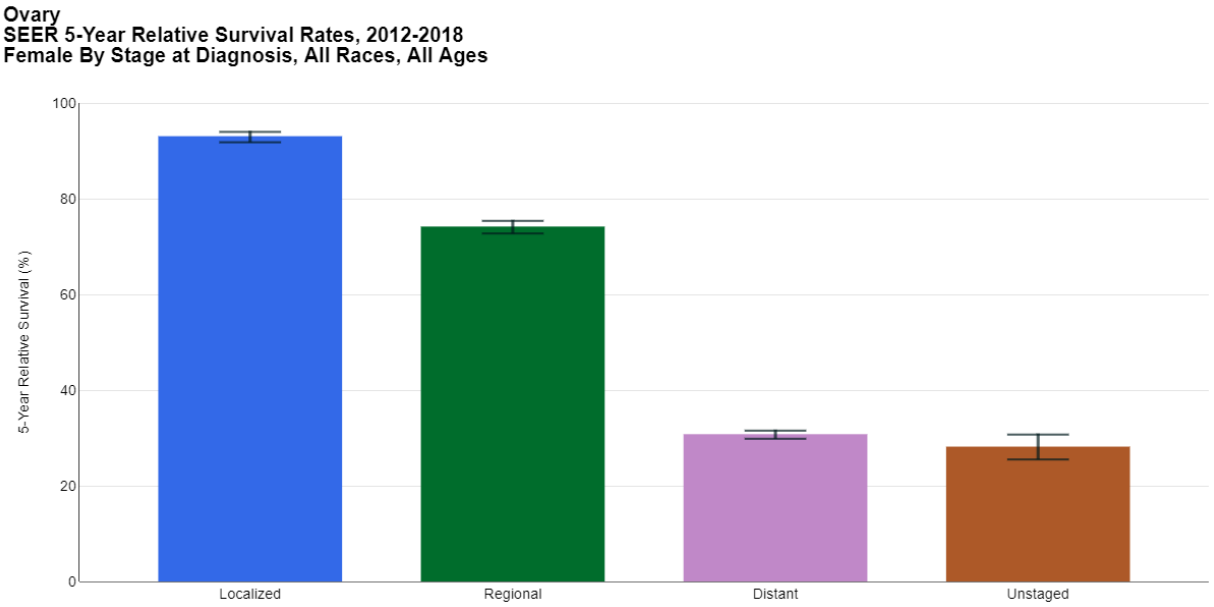

Figure 1. SEER five year survival rates (2012-2018) of patients with ovarian cancer. Localised cancer (on the left) has high survival rates of 93.1%, gradually dropping to 30.8% if cancer has distant metastases. Figure adapted from the SEER database.

TUMOUR IMMUNOLOGY

Basic concept of immunology

The immune system is a complex network of cells that can protect the body from a wide variety of pathogens. Immune cells can be found throughout the whole human body, with a few important organs such as the bone marrow, lymph nodes, thymus and spleen. The immune system comprises two components that work together: the innate and the adaptive immune system. The innate immune system is already fully developed upon birth, is non-specific in its reaction against pathogens and serves as the fast, first-line response. The adaptive immune system is further developed during life as it can be educated to generate pathogen-specific reactions. An important part of the adaptive immune system's function, is the ability to create a memory from previously encountered pathogens. In contrast to the innate immune response that is fast and immediate, it can take up to one week for the adaptive immune response to arise. Both work together to generate immunity against pathogens. Each part involves a number of specialized immune cells that perform different functions. Cells categorized as being innate immune cells are macrophages, dendritic cells (DC), myeloid-derived suppressor cells (MDSC), natural killer cells (NK cells), mast cells, basophils, neutrophils and eosinophils. Cells of the adaptive immune system are T-lymphocytes or T-cells, and B-lymphocytes or B-cells (see infra for more details).

Basic concept of tumour immunology

Tumour immunology is based on the concept that cells of the immune system can influence malignant cells and vice versa. Studying how these two systems interact can offer valuable opportunities that

could revolutionize cancer management, something which was acknowledged by the scientific community by rewarding the Nobel Prize of medicine in 2018 to James Allison and Tasuku Honjo. The underlying basic concept states that the immune system is able to discriminate healthy cells from malignant cells, just like it is able to recognize bacteria or viruses, and is subsequently able to remove the malignant cells from the body. This hypothesis was termed as "cancer immunosurveillance" and introduced in 2002 [46]. Schreiber updated this theory in 2011 to the "Three E's of Cancer Immunity" that include (a) elimination (b) equilibrium and (c) escape [47]. In the elimination phase, the immune system actively detects and eliminates malignant cells. The elimination of cancer cells composes of a series of stepwise events that are referred to as the cancer-immunity cycle [46]. However, if malignant cells are not properly eliminated due to a malfunction anywhere in the cancer-immunity cycle, the system enters the equilibrium phase. In this phase, the immune system still tries to control cancer growth but not all malignant cells are eliminated anymore. The selective eliminating of cancer cells by the immune system induces a gradual editing of the malignant cells, each time selecting and eliminating only the ones that are the most recognizable. During this phase, tumour cells that aren't as easily recognized or eliminated because they supress the immune function of cells, can continue growing. This eventually leads to the escape phase, where tumour cells can grow unrestrained by the immune system. However, one should consider that in a specific case tumour cells may not pass sequentially through the three distinct phases but that the whole process is dynamic and that the flow and direction of the phases are multi-factorially influenced. Later on, the principle of "cancer immunoediting" was added to this framework as more evidence became available that cancer cells were actively influencing immune cells. Cancer cells can edit the immune system in their surroundings such that it can become tumour-protective or even tumour-promoting [48].

Cells implicated in tumour immune-biology

The cancer-immune-axis is a concept that tries to describe the complex interactions of malignant cells with cells of the immune system.

T-Lymphocytes

T-lymphocytes are part of the adaptive immune system. $CD8^+$ cytotoxic T-cells (CTLs) are one of the primary mechanisms of tumour cell killing, but requires prior activation by antigen-presenting cells (APCs) to generate a specific antigen response. This activation is based on the presentation of relevant tumour antigens on major histocompatibility complex (MHC) class I together with co-stimulatory factors. $CD4^+$ T-cells, often give rise to $CD4^+$ "helper cells" and were believed to primarily assist in anti-tumour immunity through coordination of other immune cells and providing effector cytokines that help $CD8^+$ killing responses. However, their role as merely "helper" cells has recently been questioned as new data emerged describing their functions as essential. Some publications even reported direct cytotoxic activity [49]. Another important category of $CD4^+$ cells are regulatory T-cells (Treg). Treg in non-cancerous conditions are important for maintaining self-tolerance and cessation of an immune reaction if the pathogen in question is cleared as they are an immune suppressive cell type. In

malignant disease however, these immune suppressive capacities are often detrimental for durable anti-tumoral responses [50].

B-Lymphocytes

B-lymphocytes are, next to T-lymphocytes, an important part of the adaptive immune system as they are responsible for the production of antigen-specific immunoglobulins (Ig). Immature B-cells are formed in the bone marrow, where mature B-cells circulate in the blood or accumulate in secondary lymphoid organs. If mature B-cells are activated upon antigen recognition, they can differentiate into plasma cells or memory B-cells. B-cells have also been found to be capable of infiltrating tumour tissues. In general, the presence of infiltrated B-cells in cancerous tissues was found to correlate with a more positive prognostic effect [51]. They can support anti-tumour responses of T-cells by promoting their activation, expansion and memory formation. However, B-cells have also been reported to suppress anti-tumoral responses by various mechanisms recently reviewed by Catalán *et al.* [52].

Tumour associated macrophages (TAMs)

In cancer specific conditions, macrophages are referred to as tumour associated macrophages (TAMs). TAMs originate from bone marrow-derived blood monocytes (in case they are infiltrating) or from yolk sac progenitors (if they are tissue-resident) [53]. They are involved in most of the aspects of tumour biology including tumour and immune cell stimulation, angiogenesis, tissue remodelling, drug resistance and immune suppression [54]. However, their role and prognostic significance heavily depends on the functional phenotype of the TAMs with at the extreme end the M1 macrophages that display anti-tumoral and pro-inflammatory properties versus - at the other end - the M2 macrophages with tumour growth promoting and immune suppressive capabilities [53]. However, the phenotypical heterogeneity is found to be extremely large in TAMs. Cells seem to be subjected to high levels of plasticity and will have specific responses depending on the different stimuli present in the specific tumour microenvironment in which they are present [53]. The M1 and M2 nomenclature extremities and their use in a cancer context are therefore an oversimplification, originating and adopted improperly from *in vitro* culture studies [55]. In these studies, cytokine stimulation (independently of any tumour associated context) activates macrophages into a certain path, where interferon-stimulated macrophages are considered as “classical” activated M1 macrophages, in contrast to the IL-4 “alternatively” activated M2 macrophages [55]. Therefore, the wording M1-like TAMs and M2-like TAMs will be used in this thesis to indicate their functional abilities in respect to the wide spectrum in which they might position themselves.

Dendritic cells (DCs)

DCs are cells of the innate immune system that are an important element in generating effective antigen-specific immune responses. They originate from the bone marrow as immature DCs and scan the surroundings for pathogenic situations. Immature DCs become mature when encountering a pathogenic situation and maturate into professional antigen presenting cells, making both the antigen

and the necessary co-stimulatory molecules available for other cells of the immune system. DCs generally have been reported to be more pro-tumoral as they could promote angiogenesis [56] or inhibit influx of cytotoxic T-cells [57]. However, like macrophages, the DC population contains multiple different subsets, each with a different role in the tumoral process [58]. These different subsets were found to be conserved across different tumour types [59]. Five different subsets can be observed in cancer; two classical DC states (cDC1 and cDC2), DC3s, plasmacytoid DCs (pDCs) and monocyte/inflammatory DC (MoDC) [59]. However, the heterogeneity and role of these different DC phenotypes in cancer is currently still unclear but is a rapidly evolving field.

Myeloid-derived suppressor cells (MDSC)

MDSC comprise a heterogenous group of mainly immature, myeloid cells that are characterized by strong immune suppressive features [60]. The term represents some controversy as their origin and nomenclature is still heavily discussed. It is assumed that MDSC can only exist and expand in pathological conditions where chronic stimulation of the bone marrow progenitor cells leads to proliferation of progenitor cells but is not sufficient and/or correct to drive maturation of these cells. This explains their immature nature and also the difficulty in finding distinct markers for identifying MDSC as they comprise of a large and heterogenic group of cells that share common myeloid markers with their mature counterparts. Generally however, MDSC are subdivided into two main subtypes, polymorphonuclear MDSC (PMN-MDSC) and monocytic MDSC (M-MDSC), based on their similarity to neutrophils and monocytes respectively [61]. In humans, M-MDSC can be separated from monocytes based on the absence or very low expression levels of HLA-DR [60], [62], [63]. For PMN-MDSC, no specific surface markers are identified, but some suggest that the lower granulocytic density could be used to separate PMN-MDSC from neutrophils [60]. Currently, no surface markers allow for true discrimination between PMN-MDSC and neutrophils or M-MDSC and monocytes in mice [63]. This limits their discrimination a purely functional discrimination, based on their immune suppressive, pro-tumoral phenotype. However, their phenotypic heterogeneity also seems to be extended in their function as it is believed that their mechanisms of actions are tumour-depended, adding increased complexity to the field [64], [65].

Neutrophils

Neutrophils are the most abundant type of granulocytic white blood cells. They are important for a first, fast response at any signs of infection in the human body. They are a difficult to study cell type as they are generally short-lived. Adding to the complexity, they display a large heterogeneity that can be influenced based on the tissue in which they are studied, their maturation status and disease context [66]. Nevertheless, their challenging nature has not prohibited researchers to take a keen interest in their function in cancer; functions that appear to have at least a similar high level of heterogeneity. Indeed, they have been implicated in many phases of tumour onset and growth and have been attributed with both pro- and anti-tumoral capacities [67]. Similar to macrophages, Fridlender and colleagues proposed the use of the terminology N1-like for anti-tumoral neutrophils and N2-like for pro-tumoral macrophages [68]. Intriguingly, Fridlender later studied the transcriptomes of tumour-associated neutrophils, naïve neutrophils and PMN-MDSC and concluded that each population

considerably differed from one another [69]. However, further research on the different neutrophil populations remains necessary to better understand their origin and function.

Eosinophils

Eosinophils are a second subset of granulocytes and are found to be especially important in context of parasite infections or allergy, but are also implicated in cancer [70]. Their presence in the tumour microenvironment is generally associated with a positive outcome in numerous solid cancer types [70]. However, more recent evidence revealed a more paradoxical role for eosinophils in cancer [71]. Indeed, pro-tumoral activity has been noted in several pre-clinical studies showing effects on tumour angiogenesis, recruitment of immunosuppressive cells towards the tumour site and even the possibility of direct tumour cell killing [71]. Recently, the concept plasticity, as is now widely accepted for myeloid cells such as macrophages, was also introduced for eosinophils and can possibly explain the seemingly contradicting functions they have in different cancer studies [72]. Further investigations are needed to determine their exact role.

Basophils

Basophils are the least abundant type of granulocytes and have been (as a minority) neglected over the past years as a potential cell of interest in cancer biology. However, recent evidence suggests that it might be worth including basophils as they do seem to be implicated in several aspects of cancer development. For instance, an *in vitro* study co-culturing basophils with murine B16.F10 melanoma cells showed elegantly that if basophils were activated with IL-33, tumour cell growth was restricted, indicating that basophils might have tumoricidal properties [73]. In addition, patient data showed that peripheral basopenia was associated with a worse prognosis in colorectal cancer [74]. Nevertheless, some studies also reported that there was a possibility for basophils to support tumour growth by inhibiting anti-tumoral immune responses. For example, in human pancreatic cancer it was shown that the presence of basophils correlated with reduced survival [75], based on basophil-derived IL-4 production that contributed to M2-like TAMs polarisation. In a single cell analysis of lung adenocarcinomas, it was shown that some basophils in the TME were able to express PD-L1 [76]. These findings do confirm the presence of basophils in different cancer subtypes, yet, the exact importance and contributions that basophils have in the cancer-immunity-cycle remains to be further investigated [70].

Natural killer cells (NK)

NK cells are part of the innate immune system but can exhibit, like T-lymphocytes, cytotoxic activity against target cells such as viral infected cells or malignant cells. Therefore, they are regarded as the bridge between the adaptive and the innate immune system. In contrast to their adaptive immune system counterparts, NK cells can kill cells without the need for prior activation. They recognize their target cells in specific conditions. One such condition applicable to cancer-immunology is the downregulation of MHC I molecules on cancer cells, something that malignant cells sometimes try in order to evade adaptive immune recognition [77]. Another mechanism that NK cells can use to recognize malignant cells is when these cells have increased expression of stress ligands on their cell surface [77].

Non-immune cells

It is clear that the immune biology of cancer is a complex system of interactions between malignant cells and immune cells. It is however important to realise that the cancer-immune-axis also can be influenced by multiple other components of the tumour microenvironment. For example, one of the most abundantly present cells at the tumour site are cancer-associated-fibroblasts (CAFs). They originate from diverse cell types including smooth muscle cells, adipocytes, fibroblasts, endothelial cells and epithelial cells [78]. They have many tumour promoting features including stimulation of angiogenesis, remodelling of the extracellular matrix and remodelling the tumour microenvironment, which can alter immune cell infiltration [79]. However, CAFs can also influence immune cells more directly by expression of various cytokines that influence maturation and activation, as nicely reviewed by Lui *et al*. [80]. Another component influencing the cancer-immune-axis is the microbiome. The microbiome represents the community of all microbes such as bacteria, fungi and viruses, that live in symbiosis on or in the human body. Recently, the microbiome received increased attention as it became clear that it can influence tumour initiation, progression and response to (immune)therapy indirectly via manipulation of the immune system. Indeed, Uribe-Herranz *et al.* for example showed that the gut microbiome could inhibit DC following radiotherapy and that after vancomycin antibiotic therapy the antitumour activity of radiotherapy could be enhanced [81]. Composition of the gut microbiome was also proven to influence efficacy of immune checkpoint inhibitors in epithelial carcinomas [82]. As microbiome studies are relatively new in relation to cancer (immune)biology, further research to decipher the mechanisms in detail remains warranted. Nevertheless, its importance in cancer biology is unmistakable and therefore included by Hanahan in the most recent update of “hallmarks of cancer” [83].

Taken all together, it is clear that cancer-immune studies are of a complex nature, with many different players. An additional layer of complexity can be found in the idea that the immune biology of each cancer type is highly likely to be different [84], [85].

The importance of the immune system in ovarian cancer

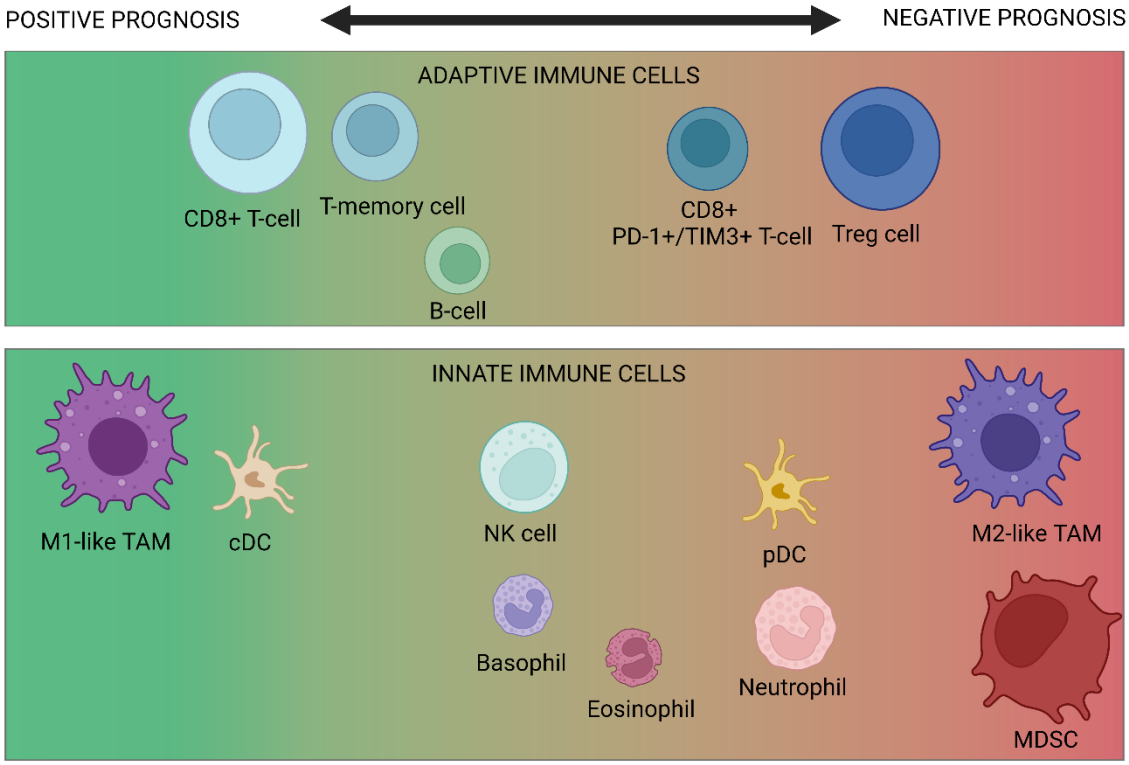

Figure 2. Illustration of prognostic relevance of different immune components in ovarian cancer. Cells illustrated more towards the left side (green) are increasingly positive prognostic, cells illustrated on the right (red) side are more negatively prognostic. Upper panel depicts cells of the adaptive immune system. Lower panel depicts cells of the innate immune system. Size of cells illustrated correspondents with magnitude of evidence provided for their prognostic effect.

T-lymphocytes

The first report of prognostic significance of immune cells in ovarian cancer dates back to 2003 where Zhang and colleagues found that elevated numbers of $CD3^+$ tumour-infiltrating lymphocytes (TILs) were associated with a favourable outcome [86]. After this, the adaptive immune system and especially TILs were extensively investigated, diversifying the $CD3^+$ field into more beneficial and more detrimental TIL subsets. Sato e*t al.* first specified that high numbers of cytotoxic $CD8^+$ T-lymphocytes correlated with a favourable prognosis [87]. Numerous studies confirmed these findings in the years after and were reviewed in a meta-analysis in 2012 [88]. However, the presence of $CD8^+$ lymphocytes was found to correlate with poor disease outcome if they had high expression of Programmed cell death protein 1 (PD-1) and T-cell immunoglobulin domain and mucin domain 3 (TIM-3) check-points, indicating that they were functionally exhausted [88]. The presence of specific subsets of (tissue-resident) memory T-lymphocytes was found to associate with a better outcome [89]–[91]. In contrast,

increased presence of Treg was generally associated with a less favourable disease outcome [92]–[96]. However, some reports found contrasting results with Treg infiltration being associated with a more favourable outcome in ovarian cancer patients [89], [97]. However, additional studies could explain this discrepancy with evidence that high Treg infiltration was indeed a negative prognostic value if they were more abundant in comparison to $CD8^+$ lymphocytes or Th17 cells [87], [98]–[100].

B-lymphocytes

In about 40% of all ovarian cancer cases, subsets of B-cells can be found [97]. Additionally, if present in the tumour microenvironment, the quantity of B-cells can vary considerably. [101]. Interesting to see is that $CD20^+$ B-cells are often found in strong association with other immune infiltrates, especially T-lymphocyte subsets [97], [102]–[105]. Generally, their presence correlates with a better prognosis. However, $CD20^+$ B-cell numbers as individual prognostic markers are not easily reported [97], [103]. Instead, they are added to other immune infiltrate statistics where they can subsequently increase the positive prognostic values [102], [104]–[107]. The prognostic effect of plasma B-cells, marked by CD138 expression (as they are terminally differentiated B-cells), is less clear and less studied in literature. Only two reports describe better responses for patients with ovarian cancer if high $CD138^+$ numbers were present, but again in association with other immune infiltrates and not as an individual prognostic marker [104], [108]. In contrast, Lundgren and colleagues reported $CD138^+$ high infiltrate numbers that correlated with reduced OS [109], something that was recently supported by a large genetic dataset considering differentially expressed genes in ovarian cancer patients [110].

Tumour associated macrophages (TAMs)

TAMs are considered to be one of the most abundantly present immune infiltrating cell types in ovarian cancer tissue and ascites [111]. Overall, higher densities of TAMs are associated with poor overall survival in many cancer types, including ovarian cancer [112]. The total number of macrophages can be correlated with histological malignant subtypes, with malignant and borderline tumours containing significantly more TAMs compared to benign tumours [113]. Their more detrimental effects can be attributed to their relevant roles in promoting ovarian cancer invasiveness, angiogenesis and metastasis [111]. Additionally, they are also reported to induce chemotherapy resistance in various manners [114], [115]. However, it is currently accepted that TAMs display a wide variety of phenotypical subtypes (see supra), but the spectrum is not yet fully characterised [116]–[118]. In ovarian cancer, the knowledge on these subtypes is still limited to the two main subtypes that can be connected to differences in prognostic activity where macrophages displaying more of an M1-like phenotype are associated with a more favourable disease outcome [53]. In contrast, high numbers of M2-like phenotypes are often associated with a poor disease outcome [104], [119], [120]. A high M1-like to M2-like TAMs ratio seems to be the best indicator of both a higher progression-free survival and a beneficial outcome for patients with ovarian cancer [121], [122].

Dendritic cells (DCs)

It has been demonstrated that DCs are an important component in ovarian cancer tissues and ascites [59], [123]. DC precursor cells can actively be recruited into the ovarian tumour microenvironment, however, infiltrating DC are modulated in different manners so that they have often been labelled as dysfunctional or even immune suppressive. DCs have been shown to promote angiogenesis and inhibit the influx of cytotoxic cells [57], [59]. However, more recent terminology has also subcategorized DCs into different phenotypes (see supra). Differences in prognostic effects of DC subsets can be found in ovarian cancer, with high numbers of conventional/mDCs being associated with a more favourable disease outcome [104], [107], [124]–[126]. Intriguingly and in line with their prognostic effects, mDCs have been found to be absent in malignant ascites in ovarian cancer [123]. In contrast, pDCs are associated with high levels of immune suppression, increased angiogenesis and a poor prognosis [123], [127]–[129].

Myeloid-derived suppressor cells (MDSC)

For ovarian cancer, almost all data currently available point to a detrimental contribution of MDSC in ovarian cancer pathology via several mechanisms [46]. Our own group reported that malignant from benign ovarian disease could be discriminated at diagnosis based on circulating MDSC, NK cells and Treg, suggesting their involvement in aggressiveness of neoplasms [130]. Cui *et al.* showed that MDSC were able to inhibit T-cell activation and induce in ovarian cancer cells increased stem cell and metastatic capacities [131]. Furthermore, higher numbers of infiltrating MDSC were found to be inversely correlated with the abundancy of cytotoxic T-cells and shorter overall survival [132]. Taki *et al.* showed that recruitment of MDSC in ovarian cancer promoted disease progression [9]. This uniformity in MDSC outcome for ovarian cancer renders them interesting therapeutic targets. However, this is not as straightforward as it seems. Lechner *et al.* illustrated that inhibition of cyclooxygenase 2 (COX2) did not decrease MDSC induction *in vitro* in ovarian cancer, although the strategy was successful in other tumour types [65]. Moreover, co-administration of COX2 and interferon (IFN)-gamma / tumour necrosis factor alfa (TNF-alfa) was even shown to hyperactivate ascites-derived MDSC [123]. Both studies suggest that targeting MDSC in ovarian cancer is very specific and successful results from other cancers cannot be implemented without substantial preclinical evidence.

Neutrophils, basophils and eosinophils

The three granulocytic cell types, neutrophils, basophils and eosinophils, are rarely studied in an ovarian cancer specific context. For eosinophils, no clear evidence on their function is available, but some small studies exist. In clear cell ovarian carcinomas, it has been shown that eosinophil infiltration is downregulated if the tumour has high PD-L1 expression [133]. Eosinophilic gene signatures have also been part of a risk score model based on tumour-immune-microenvironment associated genes as a lower risk for a negative outcome [134]. For basophils, a higher circulating number and their ability to respond to *ex vivo* stimulation were associated with a better prognosis [135]. Neutrophils have been most studied, compared to eosinophils and basophils. Most often, in the context of the neutrophil to lymphocyte ratio, which if high, has proven to result in a poor progression free and overall survival

[136]. They have also been associated with suppression of T-cell responses, promotion of an epithelial to mesenchymal transition of tumour cells and direct facilitation of metastatic spread [137]–[139].

Natural killer cells (NK cells)

In contrast to the other cells discussed above, the role of NK cells in ovarian cancer pathogenesis is still unsure. Part of the uncertainty lies in the choice of markers to identify NK cells. In studies where NK cells are defined as $CD57^+CD103^+$ cells, a positive correlation with prognosis can be found. However, these markers could also be expressed by activated $CD8^+$ cytotoxic cells and are - as discussed above - associated with survival benefits in ovarian cancer. On the other hand, if NK cells are defined by the expression of natural cytotoxicity triggering receptor 1 (NCR1, also known as NKp46), their presence does not impact survival and yields no prognostic value. This latter notion can be supported by studies indicating that NK effector functions are suppressed in the ovarian tumour microenvironment.

MODULATION OF THE IMMUNE COMPONENT IN OVARIAN CANCER

Based on the above-summarized overview of the different immune subsets and their roles in ovarian cancer prognosis, it can be assumed that ovarian cancer is an immunogenic cancer type but experiences high levels of immune suppression that hinder a functional anti-tumour immune response. Transforming the immune system into an immune-activated status is therefore the strategy forward to improve outcome.

Modulation of the ovarian immune system by the standard of care therapy

Primary debulking surgery is quite extensive. Removal of lymph nodes has been standard practice to eliminate the possibility of residual disease even if they appear normal [9]. However, a randomized trial investigating the effect of normal lymph node resection on patients outcome concluded that there was no survival or progression free survival benefit compared to women without a lymphadenectomy [140]. Results that were recently confirmed in a large database analysis comprising more than 8000 patients with ovarian cancer [141]. Nevertheless, nearly 56% of the patients in the lymphadenectomy group of the first study were found to have metastatic disease in the lymph nodes [140]. These findings suggest that residual disease possibly present in the lymph nodes does not contribute to ovarian cancer progression and death. Furthermore, women with a lymphadenectomy experience more complications during and after surgery [140]. Combining these findings with the knowledge that lymph nodes are important sites in the cancer immunity cycle, it might be of interest not to resect them and harness a potential benefit from the immune cycle if activated by immunotherapies.

Immunological changes by the effects of surgery have only limitedly been studied. After PDS, increased levels of circulating $CD4^+$ helper and $CD8^+$ cells were found [142], in contrast to decreasing levels of $CD4^+$ naive, Treg and NK cells [142]–[144]. Noteworthy, reduction of circulating Treg levels was less if the PDS was suboptimal [145] and none of the patterns could be found after IDS or secondary debulking [142], [143]. Also circulating cytokines were studied, but resulted in confounding data. For

example, IL-10 levels after cytoreductive surgery were found to increase in one study [143], but decreased in two others [142], [146]. No changes were reported for transforming growth factor beta (TGF-beta) after PDS in two studies [142], [146]. However, Nowak *et al.* reported a reduction in TGF-beta after PDS, that was less pronounced if surgery was sub-optimal [145].

Chemotherapy treatment is known for its wide variety on immune modulating properties, both immune suppressive and stimulating [147], [148]. Remodelling of the immune landscape by chemotherapy can either be via direct killing of all fast-dividing immune cells both at the tumour site or the bone marrow or via indirect stimulation of immune components by the induction of immunogenic cell death with release of possible (neo-)antigens. Especially the immune stimulation of the latter effect is something well studied in various cancer types with a variety of chemotherapies [149]. However, many previously performed studies have several limitations that require some more detailed attention. First of all, not many studies were able to define an optimal window where anti-tumoral immune responses are at their best. Secondly, almost no studies have performed head to head comparisons of different chemotherapeutics and their immune effects. Thirdly, it should be recognized that immune modulating effects of chemotherapeutics can differ in different types of cancers. This results in only small pieces of the puzzle being provided, leaving the final and overall immune modulating effects of a certain chemotherapy in a certain cancer type, especially for ovarian cancer, yet to be unravelled.

Likewise, the immunological effects of the newer targeted therapies are not fully established. Especially for anti-VEGF(R) therapies, there is little to no information available on the immune modulating properties in an ovarian cancer population. However, VEGF is implicated in several immune suppressive pathways [150], [151]. It is therefore highly likely that anti-VEGF therapy will influence the immune composition or function in the tumour microenvironment but it remains a question to what extend. For PARP inhibitors, the initial available information seems to indicate a beneficial impact on the immune composition at the tumour site [152]. PARP inhibitors are hypothesized to increase the mutational load of cancer cells as a result of their DNA repair inhibition mechanisms. In a murine model of ovarian cancer, PARP inhibition was shown to increase numbers of $CD8^+$ T-cells and NK cells and activate their pro-inflammatory IFN-gamma and TNF-alfa cytokine production [153]. Additionally, Ding *et al.* showed in a BRCA1-deficient mouse model that PARP inhibition induced both adaptive $CD4^+$ and $CD8^+$ T-cell responses, but also positively affected APC in a stimulator of interferon genes (STING)-dependent type I IFN mediated manner [153]. This, together with PARP immune modulating evidence in other cancer types, has been the rationale for combining PARP inhibitors with immunotherapies in ovarian cancer [152]. However, also here the exact immune modulating properties remain to be investigated. Nevertheless, this information is of interest if one wants to obtain the best possible outcome for immunotherapies added onto the standard of care.

Targeted modulation using immunotherapies as anti-cancer strategy

Immunotherapies, focused on improving the patient's own immune system, could provide a promising new anti-cancer strategy. Checkpoint inhibitors are the most extensively used immunotherapeutic

strategy in ovarian cancer clinical trials, partly due to the great success they have reached in other cancer types. Different checkpoints and ligands are expressed by infiltrating immune cells in ovarian cancer tissues, including PD-1, TIM3, cytotoxic T-lymphocyte associated protein 4 (CTLA-4), lymphocyte-activation gene 3 (LAG3), T cell immunoreceptor with Ig and ITIM domains (TIGIT) and V-domain Ig suppressor of T cell activation (VISTA) [154], [155]. These checkpoints function as breaks of the immune system, that upon activation by their respective ligands will drive an immune cell to suppress others and tolerate cancerous cells rather than stimulating others or attacking their target. By targeting these checkpoints using antibodies, the checkpoints cannot be activated, rendering protection to active immune cells. This strategy has been widely explored in clinical trials for ovarian cancer with currently 82 studies registered in the ClinicalTrials.gOV database (accessed 16/05/2022), initially as monotherapy strategies. Despite a strong rationale, success of checkpoint inhibitor therapy remained absent with low response rates ranging from 10 to 15% in ovarian cancer [156]. To increase the potential of checkpoint inhibitor therapies, multiple combinatorial strategies have been set-up of which three have results. In the JAVELIN Ovarian 100 trial (NCT 02718417), previously untreated patients with EOC were randomized either into the standard chemotherapy arm or the interventional arms combining chemotherapy and the anti-programmed death-ligand 1 (PD-L1) checkpoint inhibitor avelumab. Avelumab was either sequentially added after chemotherapy or added during chemotherapy and given subsequently as maintenance therapy. Although none of the avelumab arms reported any safety concerns, first line treatment with either avelumab maintenance or combination of avelumab plus chemotherapy followed by maintenance did not increase progression free survival [157]. Also the JAVELIN Ovarian 200 trial (NCT02580058) testing avelumab monotherapy, avelumab plus pegylated liposomal doxorubicin (PLD) compared to PLD alone in patients with platinum-resistant or refractory ovarian cancers failed to induce significant improvements in progression-free survival or overall survival at data-cut-off [158]. The IMAGYN050 trial, testing the checkpoint inhibitor avelumab in combination with chemotherapy and bevacizumab, also could not achieve the anticipated beneficial results [159].

Also other immunotherapeutic strategies have been tested in ovarian cancer albeit to a lesser extent. One such strategy is the genetic modification of T-cell receptors (TCR) or generation of chimeric antigen receptor T-cells (CAR T-cells), designed to emit a stronger immune response to pre-determined tumour antigens. In a clinical phase I study with CAR T-cells against folate receptor alpha, an accepted safety profile was noted [160]. However, further clinical trials were not set up, after results of a large phase III trial using farletuzumab, a monoclonal antibody against the folate receptor alpha, failed to increase progression free survival [161]. Also CAR T-cells directed against mesothelin were tested in patients with ovarian cancer, showing a good safety profile but limited clinical activity [162], [163]. Another immunotherapeutic strategy is to use DC vaccines. Multiple clinical trials have investigated DC vaccines in ovarian cancer and have been reviewed by Zhang and colleagues [164]. So far, DC treatments have only been evaluated in small phase I and II studies. Overall, most trials seem to generate promising effects as their safety profile is generally well accepted and modest disease modulation can be found [164]. For instance, a cohort of patients with relapsed ovarian cancer in remission after standard of care therapy showed a significant longer progression free survival after vaccination with a mucin-1 dendritic cell vaccine (>13 months) compared to patients receiving the

standard of care alone (5 months, p=0.04, HR = 0.32) [165]. In the study of Chu *et al.*, monocyte-derived DC vaccination was combined with low-dose cyclophosphamide [166]. Although there were no added benefits of cyclophosphamide, this DC vaccine suggested promising survival rates with overall survival being 90% at the three year cut-off [166].

To summarize, the overall results of immunotherapy (dominated by immune checkpoint inhibition) in ovarian cancer remain unsatisfactory. The limited success is most likely multifactorial, but a few things certainly add to the complexity. The immune system in ovarian cancer is notoriously heterogenous with every metastatic spot showcasing a different immune composition [167]–[169]. Additionally, ascitic fluid adds an extra level of complexity where cells and cytokines of the immune system can be disseminated more easily. But also, the immune behaviour is not or only partially taken into account (*e.g*. only a PD-L1 staining on (often) one archival biopsy) when including a patient in an immunotherapy trial. Therefore additional studies deciphering the dynamic immune changes in patients with ovarian cancer could help identify the best possible window of opportunity and can help design the best possible therapeutic (combination) strategy.

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Chapter 2

Objectives

OBJECTIVES

Current insights provide ample evidence of immune reactivity in ovarian cancer, however, recent clinical studies investigating immunotherapies in these patients did not meet expectations. This highlights the need for improving our understanding of the immune biology in ovarian cancer. In turn, this will increase the establishment of successful combinatorial (immune)therapies for patients with ovarian cancer. Therefore, the aims of this dissertation are threefold:

PART A. Deciphering the immunobiology of ovarian cancer

The primary goal of this part was to study the immunobiology of ovarian cancer more in depth. We wanted to identify the immune cells impacting ovarian cancer disease progression most significantly. To answer this question:

We manipulated the immune system in the ID8-fLuc ovarian cancer mouse to evaluate the effects on survival and ascites development (Chapter 3).

We explored the innate immune biology in tumour biopsies of ovarian cancer patients (Chapter 4).

PART B. Development of therapeutic combinations

The goal of this part was to develop and test combinatorial immunotherapy strategies to manipulate the immune system, based on the results of PART A. However, evidence on how to best combine different therapeutic strategies and their impact on the immune system in ovarian cancer patients was missing. Especially evidence about immune modulating properties of the standard of care treatments was scattered. Therefore, to answer this question:

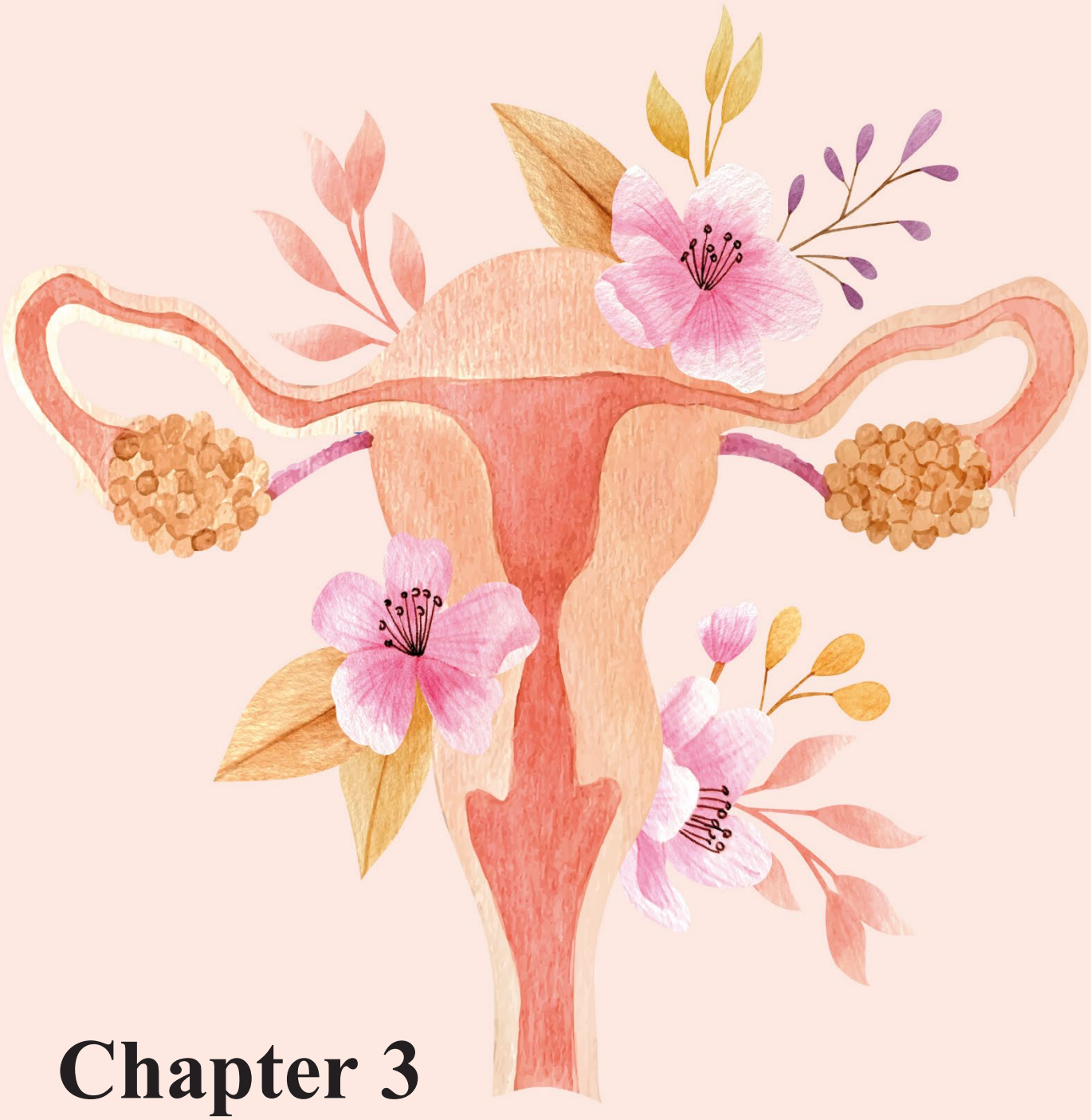
We investigated the immune changes in a mouse model, induced by six different but commonly used chemotherapeutics (Chapter 5).

We tested novel treatments in an ovarian cancer mouse model, combining the standard of care with immunotherapy (Chapter 6).

We explored survival differences in a mouse model for ovarian cancer if the order and timing of therapies given in combination differed (Chapter 7).

PART C. Implementing immune biology into the clinical setting

Multiple studies, including some by our own group, have shown that the interactions of the immune system in ovarian cancer are dynamic in nature. Monitoring immune changes throughout the patients treatment process at a systemic level could offer valuable information that is currently often missing. To facilitate implementing regular monitoring of immune changes in patients, we collaborated with imec in a proof of concept study to test a disposable microfluidics chip (Chapter 8). With this, we envision to provide a basis for the further development of a point of care instrument that allows for a fast and easy read-out of the immune status in patients without the need for highly trained staff.



Chapter 3

Myeloid Derived Suppressor Cells: Key Drivers of Immunosuppression in Ovarian Cancer.

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ABSTRACT

The presence of tumor infiltrating lymphocytes (TILs) is associated with a longer overall survival in advanced stage epithelial ovarian cancer. Despite the prognostic impact of TILs, response to checkpoint-inhibitors and antigen-specific active immunotherapy is limited in ovarian cancer. The goal of our study was to investigate the interaction between ovarian cancer and the innate and adaptive immune system in the ID8-fLuc syngeneic ovarian cancer mouse model. For the *in vivo* experiments C57BL/6, B6.129S7-Rag1tm1Mom/J, and B6.129P2(SJL)-Myd88tm1.1Defr/J mice were inoculated with ID8-fLuc. *In vivo* depletion experiments were performed using clodronate liposomes (CL), anti-CD8a, anti-GR1, anti-colony stimulating factor 1 (anti-CSF1), and TMβ1 (anti-CD122). Immune read out was performed by fluorescent activated cell sorting analysis for effector T cells, regulatory T cells, natural killer cells, B cells, macrophages, and myeloid derived suppressor cells (MDSC), immunohistochemistry for MDSC and tumor-associated macrophages (TAMs) and immunofluorescence for M1-like and M2-like TAMs in the vascular context. The effect of MDSC on T cell proliferation and phenotype were studied *in vitro*. We discovered that the absence of T and B cells did not influence tumor growth or survival of B6.129S7-Rag1tm1Mom/J mice compared to immunocompetent C57BL/6 mice. CL-induced macrophage depletion promoted tumor proliferation and shortened survival in C57BL/6 mice ($p = 0.004$) and in B6.129S7-Rag1tm1Mom/J mice ($p = 0.0005$). During CL treatment, we observed a clear increase of pro-inflammatory cytokines ($p \leq 0.02$) and monocytic MDSC ($p \leq 0.01$). Selective depletion of MDSC by anti-GR1 improved survival, certainly in comparison to mice treated with anti-CSF1 ($p = 0.01$—median survival 91 vs. 67.5 days). B6.129P2(SJL)-Myd88tm1.1Defr/J mice displayed a longer median survival compared to C57BL/6 mice (90 vs. 76 days). MDSC activated by ID8-fLuc conditioned medium or ascites of tumor-bearing mice showed Tcell suppressive functions *in vitro*. Based on these findings, we conclude that the adaptive immune system does not efficiently control tumor growth in the ID8-fLuc model. In addition, we discovered a prominent role for MDSC as the driver of immunosuppression in the ID8-fLuc ovarian cancer mouse model.

INTRODUCTION

Ovarian cancer is the 5th leading cause of cancer death for women in developed countries (1). Standard treatment for advanced stage epithelial ovarian cancer is cytoreductive surgery in combination with platin-based chemotherapy (2). Despite radical surgery and excellent responses to first line chemotherapy, most patients diagnosed with advanced ovarian cancer do not survive beyond 5 years after diagnosis because of treatment-resistant recurrences (3). Ovarian cancer can be subdivided into four subtypes based on mRNA and miRNA expression, DNA copy number, DNA promotor methylation and whole-exome DNA sequence analysis: immunoreactive, differentiated, proliferative and mesenchymal (4). The immunoreactive subtype, which is characterized by increased expression of CXCL11, CXCL10 and CXCR3, displays the most favorable overall survival (OS) compared to the other subgroups (5). In line with this evidence, Zhang *et al*. demonstrated that the presence of tumor infiltrating lymphocytes (TILs) significantly correlates with improved survival in advanced epithelial ovarian cancer (6). In 2015, the Ovarian Cancer Action meeting suggested to study the interaction between ovarian cancer and the immune system, in order to develop strategies aimed at potentiating the anti-tumor immune response (7). Despite these efforts, only a limited number of ovarian cancer patients have responded to checkpoint-inhibitor therapy (8–10). In addition to this, no significant survival benefit was observed in ovarian cancer patients receiving antigen-specific active immunotherapy to date, most likely due to an overwhelming immunosuppression (11, 12).

Unlike the adaptive immune system, the innate immune system has not been extensively studied in the context of ovarian cancer, where it might be a key driver of immunosuppression. In previous studies, a high number of alternatively activated M2-like tumor-associated macrophages (TAMs) in ascites has been linked to poor clinical outcome. Furthermore, given the positive effects of anti-vascular endothelial growth factor (VEGF) treatment in ovarian cancer and the evidence that TAMs are an important source of VEGF, targeting TAMs could also be an interesting therapeutic option in this context (13–15). In addition, Cui *et al*. demonstrated that a high number of CD33+ cells in the tumor microenvironment was prognostic for shorter PFS ($p = 0.006$) and OS ($p = 0.02$) (16). The role of other innate immune cells, such as natural killer (NK) cells, dendritic cells, etc., remains unclear in ovarian cancer.

In this study, we discovered that depleting immune effector cells of the adaptive immune system (CD8+ T cells) does not increase tumor growth or influence survival in the ID8-fLuc model. We therefore explored the role of the innate immune system in the inhibition of the adaptive immune response. We observed a key role for (monocytic) myeloid derived-suppressor cells (mMDSC) in immune surveillance in the ID8-fLuc model.

MATERIALS AND METHODS

Mice

Six- to eight-week-old mice were used. C57BL/6 and C57BL/6/BrDCHsd-Tyrc mice were obtained from Harlan/Envigo (Horst, Netherlands) or from an internal colony at KU Leuven. C57BL/6J-Tyrc-2J/J, B6.129S7-Rag1tm1Mom/J and B6.129P2(SJL)-Myd88tm1.1Defr/J mice were obtained via Charles River

from The Jackson Laboratory (Bar Harbor, ME, USA). For the *in vivo* experiment, only female mice were used. C57BL/6/BrDCHsd-Tyrc and C57BL/6J-Tyrc-2J/J are albino C57BL/6 mice, lacking all pigment from skin, hair and eyes.

B6.129S7-Rag1tm1Mom/J are immune deficient mice with a C57BL/6 background, lacking for mature T or B cells (17). B6.129P2(SJL)-Myd88tm1.1Defr/J are C57BL/6 mice that have a defect in the Myd88 cytosolic adapter, a protein which plays a central role in dendritic cell metabolism and in the immunosuppressive function of MDSC by activating NADPH oxidase and arginase-1 (18, 19).

Ovarian cancer was induced in the mice by intraperitoneal (IP) administration of 5 × 106 ID8-fLuc cells dissolved in 100 µL cold Phosphate-Buffered Saline (PBS). The ID8-fLuc cell line was transducted by the Laboratory of Molecular Virology and Gene Therapy and Leuven Viral Vector Core in our institute. All *in vivo* experiments were performed with 5–6 mice per group and passages 2–4 of the ID8-fLuc cells. No systematic mycoplasma testing was performed. Severely ill animals were euthanized following humane endpoints as previously described by our group (20). All animals were housed and treated according to the Federation for Laboratory Animal Science Associations guidelines (21). Ethical approval was obtained from the local Ethical Committee (p075/2014 and p125/2017).

Bioluminescence Imaging (BLI)

Non-invasive bioluminescence imaging (BLI) was used to evaluate tumor burden in albino C57BL/6/BrDCHsd-Tyrc and C57BL/6J-Tyrc-2J/J mice. As read-out, we used the maximum luminescence after administration of D-Luciferin (Promega, Madison, WI, USA) as a measure of viable tumor load. Image analysis was performed on the IVIS Spectrum Preclinical *in vivo* Imaging System (PerkinElmer, Waltham, MA, USA) at the Molecular Small Animal Imaging Centre (moSAIC) at the KU Leuven (22). The first scan was performed 1 week after tumor challenge in order to obtain a baseline of tumor engraftment. Subsequent measurements were performed once a week until 6 weeks after inoculation. In the CD8 T cell depletion experiment mice were scanned only twice (week 1 and week 6 after tumor inoculation).

In vivo Depletion Experiments

Clodronate Liposomes (CL) were purchased from Liposoma (Amsterdam, The Netherlands). We started treating the mice 1 week after tumor challenge with CL IP twice a week at a dosage of 0.05 mg/g bodyweight. As a control, PBS liposomes were used in preliminary experiments. Depletion of CD8+ T cells was achieved using anti-CD8a (clone 53-6.72) purchased from BioXCell (West Lebanon, NH, USA). Three weeks after tumor inoculation, we administered a loading dose of 0.5 mg per mouse IP on 3 consecutive days after which we performed weekly maintenance IP injections of 1 mg in accordance to manufacturers' protocol. For the depletion of NKp46+ NK cells we used TMβ1 (anti-CD122 monoclonal antibody), which was a kind gift of Ben Sprangers and Mark Waer (Lab of experimental transplantation, KU Leuven, Belgium). TMβ1 was produced in house by using the hybridoma technique. TMβ1 was administered IP at a dosage of 1 mg per mouse starting 1 day before tumor inoculation and continued at the same dosage twice a week. Depletion of MDSC was achieved using anti-GR1 (Clone:RB6-8C5) purchased from BioXCell (West Lebanon, NH, USA). The monoclonal antibody was

administered IP, at a dose of 10 mg/kg body weight, 3 times per week starting 1 week after inoculation. A monoclonal antibody targeting colony stimulating factor 1 (CSF-1) (Clone:5A1) was used for the selective depletion of macrophages. Both the depleting antibody and the control antibody were bought from BioXCell (West Lebanon, NH, USA) and were administered IP. After a loading dose of 1 mg per mouse at day 21 after tumor challenge, a maintenance dose of 0.5 mg of anti-CSF1 or control antibody was administered once every 6 days IP.

Immunohistochemistry (IHC)

Tumor tissue from metastatic disease was stained for the presence of Ly6C. In brief, paraffin-embedded tissue slices were deparaffinized and rehydrated using graded ethanol. Endogenous peroxidase activity was blocked by 0.5% H2O2 in methanol. After washing, heat-mediated antigen retrieval was carried out at 37°C in hydrogen chloride buffer containing pepsine 0.04% during 10 min. After cooling down and washing, non-specific binding was blocked and sections were incubated overnight at 4°C with rat anti-mouse Ly6C primary antibody (1:200 dilution; Thermo Fisher, Merelbeke, Belgium). After washing, sections were incubated during 30 min with goat anti-rat biotinylated secondary antibody (dilution 1:100; Abcam, Cambridge, UK), followed by another 30 min with streptavidin/peroxidase (dilution 1:1,000; DAKO/Agilent, Haasrode, Belgium). Staining was performed using 3,3-diaminobenzidine (DAB) during 10 min. Sections were counterstained with Mayer's Hematoxylin solution, dehydrated with ethanol and mounted in DePex medium. Images were acquired on Zeiss Axio Scan.Z1 using a x20 objective and ZEN2 software (Zeiss). Four random fields at 20x magnification were chosen and used to manually count positive cells. The mean of the four values was used for downstream analyses. IHC was scored by AVK using Image J software [National Institutes of Health and the Laboratory for Optical and Computational Instrumentation (LOCI, University of Wisconsin)].

Immunofluorescence Staining

Mice were sacrificed 33 days after tumor inoculation and peritoneal biopsies were taken. Tumor biopsies were prepared as 200 µm-thick vibratome sections, blocked and permeabilized in TNBT buffer [0.1 M Tris pH 7.4; NaCl 150 mM 0.5% blocking reagent from Perkin Elmer (Waltham, Massachusetts, USA), 0.5% Triton X-100] for 4 h at room temperature. Tissues were incubated overnight at 4°C with the following primary antibodies diluted in TNBT buffer: anti-glucose transporter-1 (Glut1) (Millipore, Burlington, Massachusetts, USA; 1:200 dilution), anti-Glut1 (Abcam, Cambridge, UK; 1:200 dilution), anti-major histocompatibility complex II (MHC-II) (Thermo Scientific, Waltham, Massachusetts, USA; 1:100 dilution) or anti-mannose receptor C type 1 (MRC1) (R&D Systems, Minneapolis, Minnesota, USA; 2 ug/ml). Next, slides were washed in TNBT buffer and incubated overnight at 4°C with the appropriate secondary antibody coupled with Alexa 488/555 (Life Technologies, Carlsbad, California, USA; 1:200 dilution) diluted in TNB Triton buffer. Tissues were washed and mounted on slides in fluorescent mounting medium (Dako/Agilent, Santa Clara, California, USA). Images were acquired using a Leica TCS SP8 confocal microscope. Semi-automated quantification analyses were performed using Fiji software (23).

Immune Monitoring

The immune status of mice was evaluated at predefined time points, as described in the specific experimental set-ups. Mice were anesthetized with 80 µL ketamine [100 mg/mL; Nimatek (Eurovet, Bladel, Nederland)] and blood was collected from the retro-orbital plexus using glass capillaries. Blood was centrifuged at 8,000 rcf for 10 min. Serum was collected and stored at 80°C for further analysis. Next, the animals were euthanized by cervical dislocation. Peritoneal washing with 10 mL of PBS was performed to collect the circulating immune cells in ascites and from the peritoneal lining. Peritoneal washings were centrifuged for 5 min at 500 rcf and resuspended. Supernatant was collected and stored at −80°C for cytokine analysis. Using a Lymphoprep (Stemcell technologies, Vancouver, Canada) gradient, immune cells were isolated from the cell suspension and analyzed with flow cytometry (FACS). Using flow cytometry, dead cells were excluded via eFluor780 fixable viability dye staining (Affymetrix Inc. San Diego, CA, USA). Immune cells were stained for myeloid cells, T cells and B cells using antibody panels, which are available as Supplementary Material (Supplementary Tables 1–3, respectively). For the myeloid panel, the cells were permeabilized using Leucoperm (Bio-Rad Laboratories Inc., Kidlington, UK) in accordance to manufacturers' protocol and stained for CD206. Permeabilization in the T cell panel was achieved using the eBioscience Foxp3/Transcription Factor Staining Buffer Set (ThermoFisher scientific, Waltham, Massachusetts, USA) and cells were then stained for FoxP3. Samples were acquired on the BD LSRFortessa (BD Biosciences, San Jose, CA, USA) and the analysis was performed using FlowJo Analysis software (Flow Jo, LLC, Ashland, Oregon, USA).

Cytokines in serum and ascites were determined using cytometric bead assay technique (BD Biosciences, San Jose, CA, USA). Both serum and peritoneal washings/ascites were used undiluted. The analysis was performed in accordance to the manufacturers' protocol using flex sets for IL-1 β, GM-CSF, IL-6, IL-10, MIP-1α, MIP-1β, TNFα, and IFNγ. Samples acquisition was performed on the BD LSRFortessa (BD Biosciences, San Jose, CA, USA) and the analysis was performed using FCAP Array Software v3.0 (BD Biosciences, San Jose, CA, USA).

In vitro Experiments

MDSC were derived from bone marrow progenitor cells and splenocytes of C57BL/6 mice. Bone marrow progenitor cells were isolated from bone marrow by flushing the long bones with PBS. For splenocytes, a single cell suspension was generated by passaging spleens through a 70 µm nylon strainer. From both splenocytes and bone marrow cells, dead cells were removed by the dead cell removal kit (130-090-101, Miltenyi Biotec, Bergisch Gladbach, Germany) in accordance to manufacturers' protocol. Next, MDSC were selected with the MDSC cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany), which provides two fractions based on relative GR1 expression: the MDSC-DIM corresponding to mMDSC and the MDSC-HIGH corresponding to gMDSC. For the T cell fraction, CD8+ T cells were selected from a single cell suspension of splenocytes using the CD8+ T cell isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). T cells were activated by CD3/CD28 coated beads and cultured in medium supplemented with recombinant interleukin-2 (IL-2). Purity of all isolated cell types was verified by FACS.

In the first *in vitro* experiment naïve MDSC were exposed to ID8-fLuc conditioned medium. For this purpose, ID8-fLuc cells were grown in 96-well plates with trans well inserts (CoStar, Washington, D.C., USA), while MDSC were cultured in the inserts. Next the activated MDSC were co-cultured with CD8+ T cells. We evaluated the proliferation of T cells by quantification of the CFSE (Affymetrix Inc. San Diego, CA, USA) dilution.

In the second experiment MDSC were cultured in the presence of supernatant derived from ascites of tumor bearing mice to investigate the role of soluble factors in ascites. Subsequently, the stimulated MDSC were co-cultured with activated T cells and stained for FACS using the staining panel in Supplementary Table 4. Dead cells were excluded from the analysis by use of the eFluor780 fixable viability dye (Affymetrix Inc. San Diego, CA, USA). Cells were acquired on the BD Canto-II (BD Biosciences, San Jose, CA, USA). Analysis was performed using FlowJo Analysis software (Flow Jo, LLC, Ashland, Oregon, USA).

Statistical Analysis

Prism 6 (GraphPad Software, Inc., San Diego, CA, USA) was used for statistical analysis and graphics. To evaluate statistical significance, α was set at 0.05. D'Agostino & Pearson omnibus normality test was used to evaluate normality. For continuous variables, data are presented as mean ± SD or medians (interquartile ranges) as appropriate. Between-group comparisons used the Mann–Whitney U-test or t-test depending on the sample size for continuous variables. In cases where more than two groups are compared, one-way ANOVA test was performed, followed by Turkey's multiple comparison test if $p < 0.05$. Log-rank testing was performed to compare survival curves.

RESULTS

Adaptive Immune Tolerance

We compared tumor growth of ID8-fLuc cells in B6.129S7-Rag1tm1Mom/J mice to tumor growth in C57BL/6 mice using BLI. As shown in Figure 1A, there was no significant difference in tumor burden between immunocompetent mice (C57BL/6) and mice lacking mature T and B cells (B6.129S7-Rag1tm1Mom/J). The B6.129S7-Rag1tm1Mom/J mice developed ascites at approximately the same moment as the C57BL/6 mice. There was no significant difference in survival between the two groups (Figure 1B). To investigate the specific role of CD8+ T cells in immune surveillance in the ID8-fLuc ovarian cancer model, we performed a depletion experiment by which we inoculated C57BL/6 mice with ID8-fLuc and started treating the mice with anti-CD8 20 days after tumor inoculation (onset of exponential tumor growth phase, as demonstrated earlier) (20). In this experiment, we did not observe a difference in tumor burden 6 weeks after inoculation between anti-CD8 treated and control mice, which corresponds to the results obtained with B6.129S7-Rag1tm1Mom/J mice (Figures 1C,D). Based on these findings, we can conclude that in the ID8-fLuc model the adaptive immune system has developed a tolerance against the tumor since knock-out or depletion of the adaptive immune system does not significantly influence tumor growth or survival. We therefore hypothesize that the innate immune system could play a role in rendering the effector cells of the adaptive immune system unfit for cancer immune surveillance in our model.

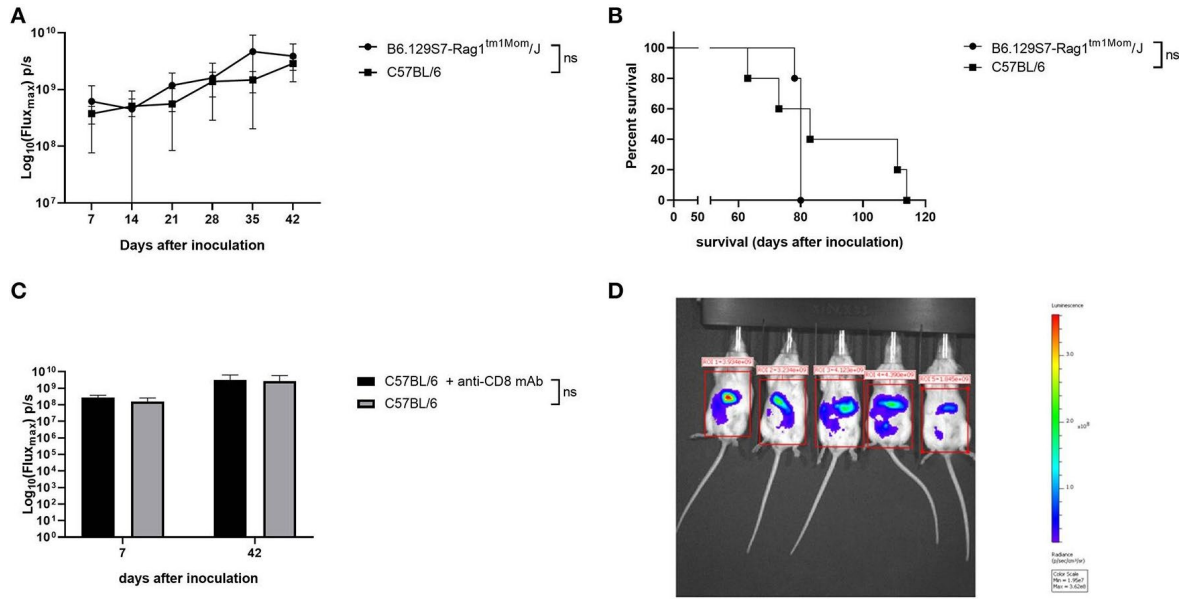

FIGURE 1 | Lack of cancer immune surveillance by the adaptive immune system in the ID8-fLuc model. (A) Evaluation of tumor growth using BLI, Log10 transformation of maximal flux in photons per second (p/s) are shown as mean with standard deviation. We observed no significant (ns) difference in tumor growth in the mice lacking mature T cells and B cells (B6.129S7-Rag1tm1Mom/J) compared to the immunocompetent C57BL/6 mice. (n = 5 mice per group). (B) Kaplan-Meier curve showing the survival of B6.129S7-Rag1tm1Mom/J mice compared to immunocompetent C57BL/6 mice. Median survival is 80 days for B6.129S7-Rag1tm1Mom/J and 83 days for C57BL/6 (n = 5 mice per group). (C) Follow-up of tumor growth using BLI in wild type mice that received CD8 depletion (C57BL/6 + anti-CD8a mAb) compared to untreated mice (C57BL/6). Imaging was performed 1 and 6 weeks after tumor inoculation. No significant differences between the groups were observed. (n = 6 mice per group). (D) Representative picture of BLI imaging taken with the IVIS Spectrum Preclinical *in vivo* Imaging System.

Influence of Macrophages on Tumor Growth and Survival: the ID8-fLuc Model

In order to target innate immunosuppression, we treated the immunocompetent C57BL/6 model and in the B6.129S7-Rag1tm1Mom/J mice with CL (24). Compared to controls, the administration of CL led to a non-significant increase in tumor growth independent from the presence of T cells and B cells (Figure 2A), and to a significantly shorter survival of the mice (for C57BL/6 mice, p = 0.004; for B6.129S7-Rag1tm1Mom/J mice, p = 0.0005; Figure 2B). Administration of CL also reduced the incidence of ascites, both in B6.129S7-Rag1tm1Mom/J and in the C57BL/6 mice (16 and 33% of the B6.129S7-Rag1tm1Mom/J and C57BL/6 mice treated with CL developed ascites, respectively; in comparison to 90% of the untreated B6.129S7-Rag1tm1Mom/J and C57BL/6 mice) (20).

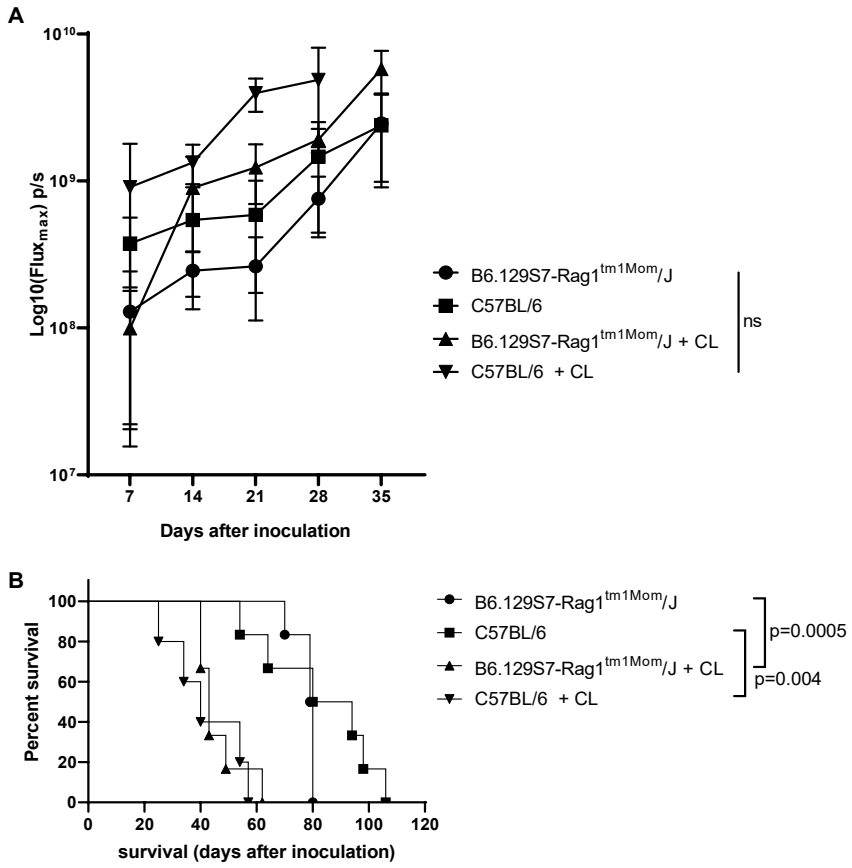

FIGURE 2 | Influence of the innate immune system on tumor growth. (A) Evaluation of tumor growth using BLI, Log10 transformation of maximal flux in photons per second (p/s) are shown as mean with standard deviation. Tumor growth was increased both in C57BL/6 mice and B6.129S7-Rag1tm1Mom/J mice when treated with clodronate liposomes (CL) (n = 6 mice per group). (B) Kaplan-Meier curve showing the survival of B6.129S7-Rag1tm1Mom/J mice and C57BL/6 mice treated with CL. In both groups treatment with CL led to a significant reduction in survival of the mice (p = 0.0005 and p = 0.004, respectively) (n = 6 mice per group).

Next, we studied the immunological changes during CL treatment to investigate the underlying mechanisms in detail. Using flow cytometry, we analyzed the immune cells present in peritoneal washings of C57BL/6 mice treated with CL and compared with PBS-treated controls at two predefined time points (T1 and T2, respectively, 23 and 30 days after tumor inoculation). Macrophages were reduced to <1% of CD11b+ cells after the administration of CL, demonstrating their high efficacy of CL in depleting TAMs in the ID8-fLuc ovarian cancer model (Figure 3A). In accordance to literature, we observed no significant changes in CD4+ T cells, regulatory T cells (Treg) or conventional dendritic cells (cDC) following CL administration (Figures 3B–D) (25). At the first time point, we observed a higher amount of CD8+ T cells and NK cells in CL-treated mice; however, this effect was lost at the second time point (Figures 3E,F). The number of CD11b+ cells was significantly reduced upon treatment with

CL at the first time point (Figure 3G). CL led not only to a significant decrease of TAMs, but also a reduction in granulocytic MDSC (gMDSC), plasmacytoid DC (pDC) and B cells (Figures 3H–J). Monocytic MDSCs (mMDSC) were the only cell population, which were significantly increased at both time points upon CL treatment (Figure 3K). Additionally, we observed a clear significant increase in pro-inflammatory cytokines in ascites, such as interleukin-1β (IL-1β), IL-6 and tumor necrosis factor-α (TNFα) and interferon-γ (IFN- γ) (Figures 4A–D). This effect was not limited to ascites; we observed similar findings in serum of mice treated with CL (Figure 4E). As an additional readout, we performed IHC staining for Ly6C peritoneal biopsies of mice treated with CL or PBS. At the second time point, we observed an increase of Ly6C+ cells ($p = 0.05$), demonstrating the increased presence of intra-tumoral Ly6C+ MDSC upon CL treatment (Figures 4F–H).

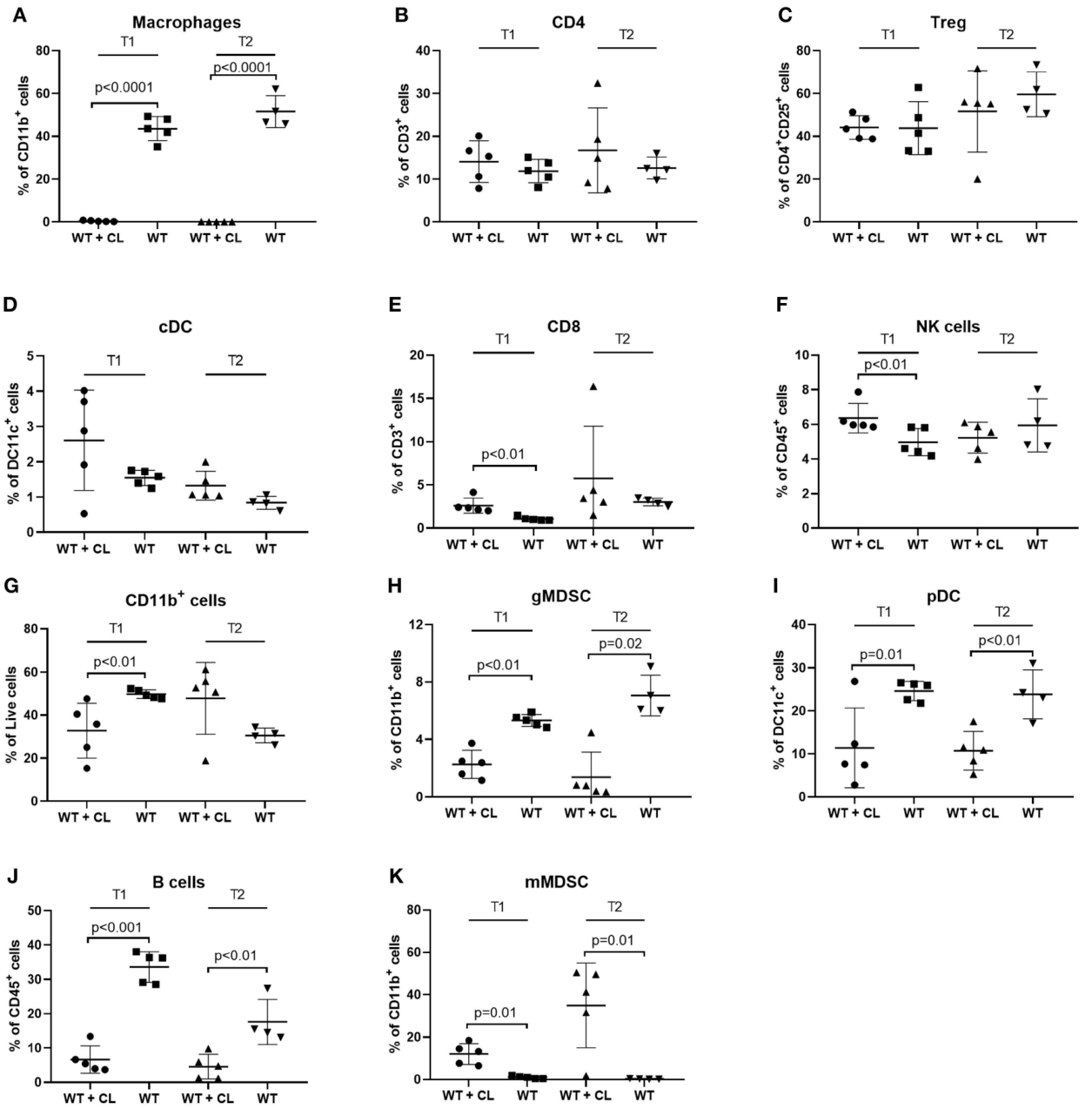

FIGURE 3 | Effect of Clodronate liposomes (CL) on immune cells in the peritoneal cavity of tumor-bearing mice measured by FACS. Changes in immune cells in peritoneal washings during treatment with CL. Immunocompetent animals (C57BL/6) treated with CL (WT + CL) are compared to untreated wild type animals (WT) at two time points (T1 = 23 days after inoculation-T2 = 30 days after inoculation). (n = 5 mice per group). (A) Treatment with CL led to a relevant depletion of macrophages after treatment with CL to <1% of CD11b+ cells ($p < 0.0001$ for both time points). (B–D) No significant changes in CD4+ T cells, regulatory T cells (Treg) or conventional dendritic cells (cDC) were observed. (E–G) For CD8+ T cells, natural killer (NK) cells and CD11b+ myeloid cells significant differences [increase of CD8+ T cells and NK cells upon treatment with CL ($p < 0.01$ in both cases) and a reduction in CD11b+ cells in CL treated mice ($p < 0.01$)], were observed on the first time point only. (H–J) On both time points we observed a significant decrease in granulocytic myeloid-derived suppressor cells (gMDSC) (T1 $p < 0.01$-T2 $p = 0.02$), plasmacytoid dendritic cells (pDC) (T1 $p = 0.01$-T2 $p < 0.01$) and B cells (T1 $p < 0.001$-T2 $p < 0.01$). (K) Monocytic myeloid derived suppressor cells (mMDSC) increased after treatment with CL ($p = 0.01$ for both time points).

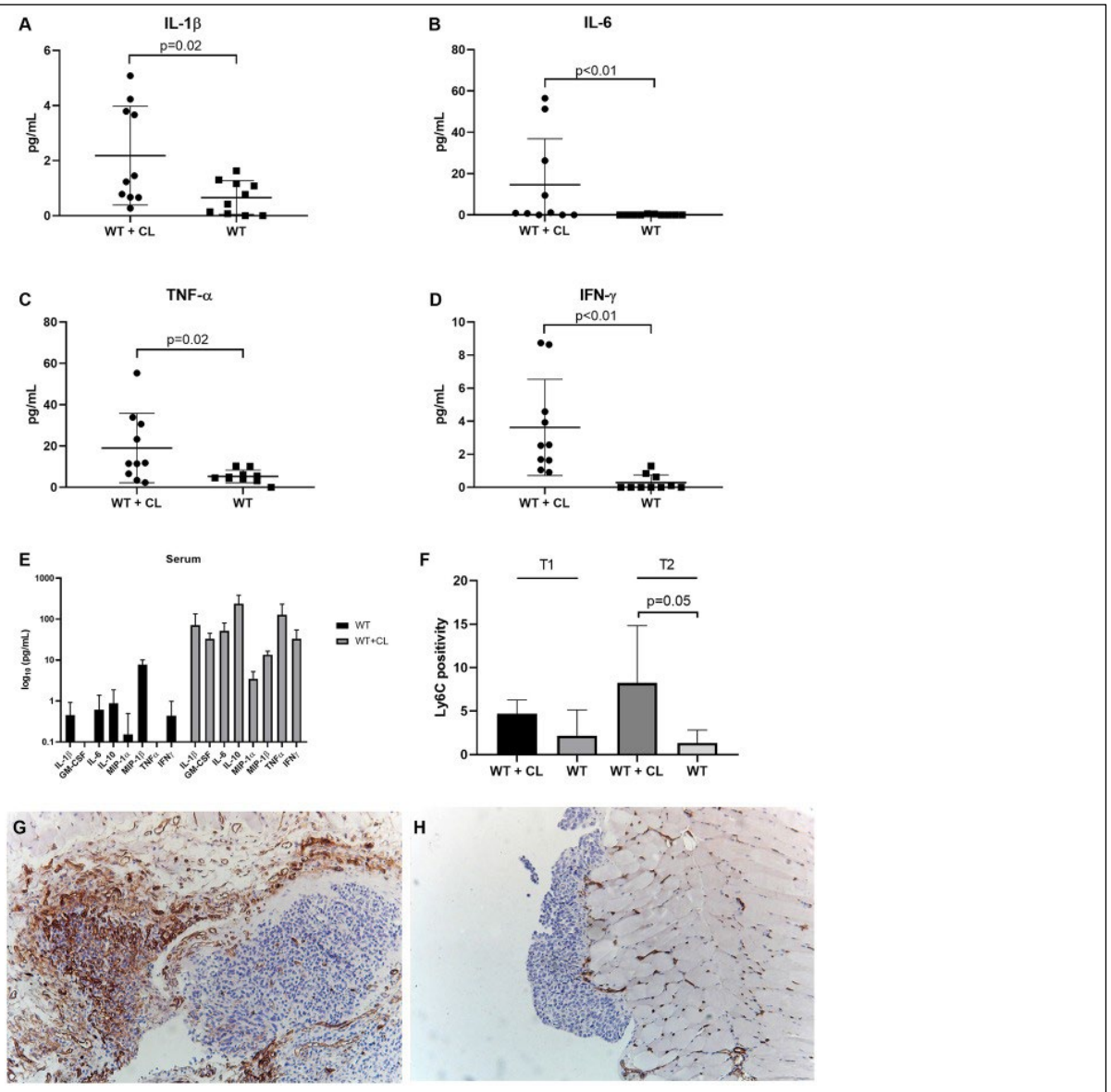

FIGURE 4 | Effect of Clodronate liposomes (CL) on cytokines and tumor-infiltrating MDSC in tumor-bearing mice. Measurement of cytokines in peritoneal washings and serum of C57BL/6 mice treated with CL (WT + CL) are compared to untreated mice. (A–D) Changes in cytokines in peritoneal washings due to CL treatment: We observed a significant increase in pro-inflammatory cytokines such as IL-1β (Interleukin), IL-6, tumor necrosis factor-α (TNF-α), and interferon-γ (IFNγ). (E) Changes in cytokines in serum due to CL treatment at the second time point (day 30 after inoculation). We observed a significant increase in cytokines such as GM-CSF ($p = 0.0004$), IL-6 ($p = 0.004$), IL-10 ($p = 0.006$), Microphage inflammatory protein 1 (MIP1) α ($p = 0.003$), MIP1β ($p = 0.009$), and IFNγ ($p = 0.008$). (F) Using immunohistochemistry, we evaluated the percentage of Ly6C positivity during treatment with CL. Immunocompetent animals (C57BL/6) treated with CL (WT+CL) are compared to untreated wild type animals (WT). At the second time point, 30 days after inoculation we observed a significant higher number of Ly6C+ MDSC cells in the tumor upon treatment with clodronate liposomes ($p = 0.05$). ($n = 5$ mice per group). (G,H) Representative Ly6C staining of Immunocompetent animals (C57BL/6) treated with CL (WT+CL) (G) and untreated wild type animals (H) at the second time point. Magnification 10x.

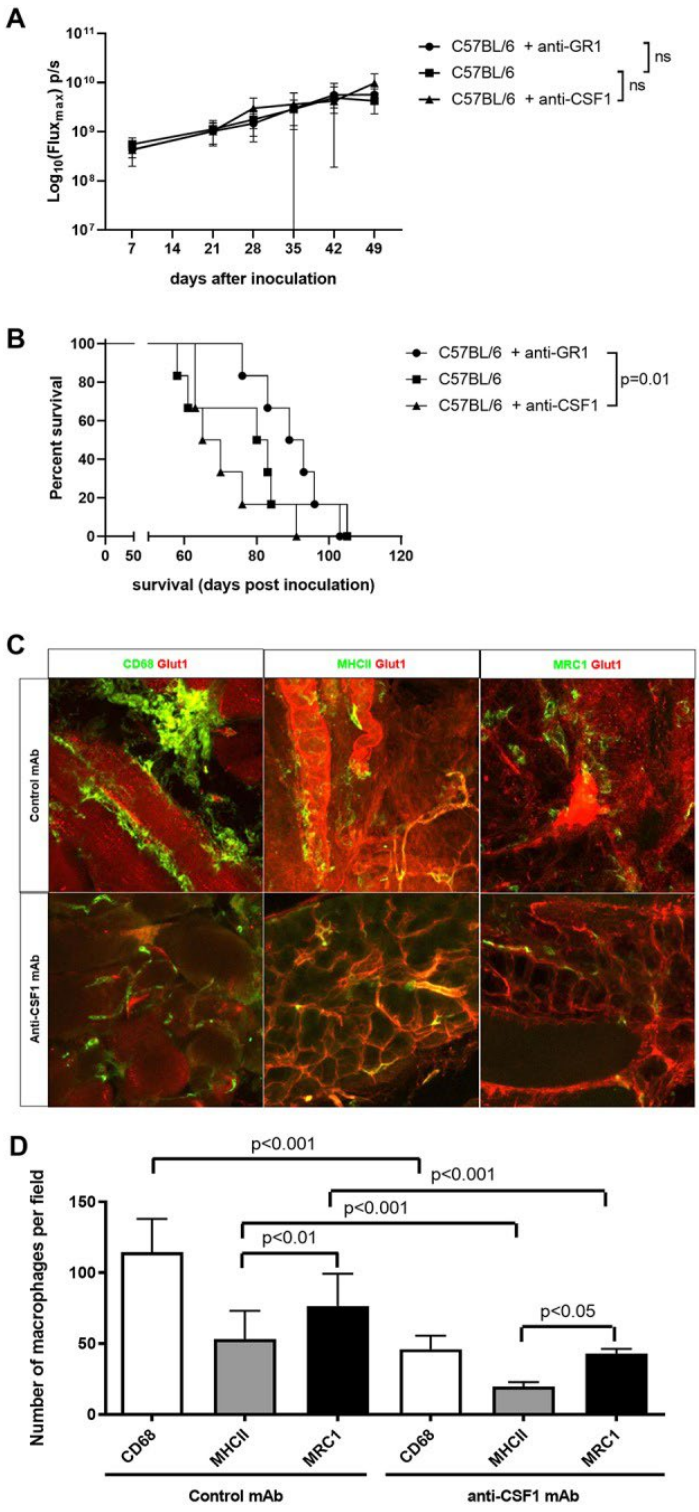

FIGURE 5 | Selective depletion of innate immune cells using monoclonal antibodies (mAb). (A) Evaluation of tumor growth using BLI, Log10 transformation of maximal flux in photons per second (p/s) are shown as mean with standard deviation. We observed no significant differences in tumor load between the untreated immunocompetent mice (C57BL/6) and the MDSC-depleted mice (C57BL/6 + anti-GR1) or the macrophage depleted mice (C57BL/6 + anti-CSF1) (n = 6 mice per group). (B) Kaplan-Meier curve of untreated immunocompetent mice (C57BL/6) and the MDSC-depleted mice (C57BL/6 + anti-GR1) or the macrophage depleted mice (C57BL/6 + anti-CSF1). We observed a significantly improved survival in the mice treated with anti-GR1 compared to the mice treated with anti-CSF1 (p = 0.01) (n = 6 mice per group). (C) Immunofluorescent images of tumor biopsies of mice treated with anti-CSF1 or control mAb. In all panes blood vessels were stained for Glut1 in red. In the left pane, CD68 in green was used to stain total macrophages. In the middle pane, green MHC-II staining was used for M1-like macrophages and on the right pane, MRC1 staining in green was used for M2 macrophages. The images at the top represent the mice treated with the control antibody, while the images at the bottom represent the mice treated with anti-CSF-1 (scale bar: 50µm) (n = 6 mice per group). (D) Quantitative evaluation of macrophages using immunofluorescent staining. Total macrophages were reduced to less than half due to anti-CSF1 (5A1). Both M1-like and M2-like macrophages were reduced in the same proportion following pan-macrophages mAb induced depletion (n = 6 mice per group).

Selective Depletion of Innate Immune Cells Using Monoclonal Antibodies

Based on these findings, we performed a selective depletion of TAMs, MDSC and NK cells using depleting monoclonal antibodies (mAb). In none of these experiments, we were able to detect significant differences in tumor growth using BLI (Figure 5A). Depletion of MDSC using anti-GR1 led to an increase in median survival from 81.5 to 91 days compared to untreated mice. The mice, which received anti-GR1, showed a significant survival advantage compared to the anti-CSF1 treated mice ($p = 0.01$; Figure 5B). Selective depletion of TAMs using anti-CSF1 (5A1) led, similar to treatment with CL, to a non-significant reduction in median survival from 81.5 days (untreated mice) to 67.5 days (anti-CSF1 treated mice). Of note, treatment with anti-CSF1 depleted ~70% of TAMs (Figures 5C,D), which was less profound (61.5% reduction of TAMs after treatment with anti-CSF1 compared to the control

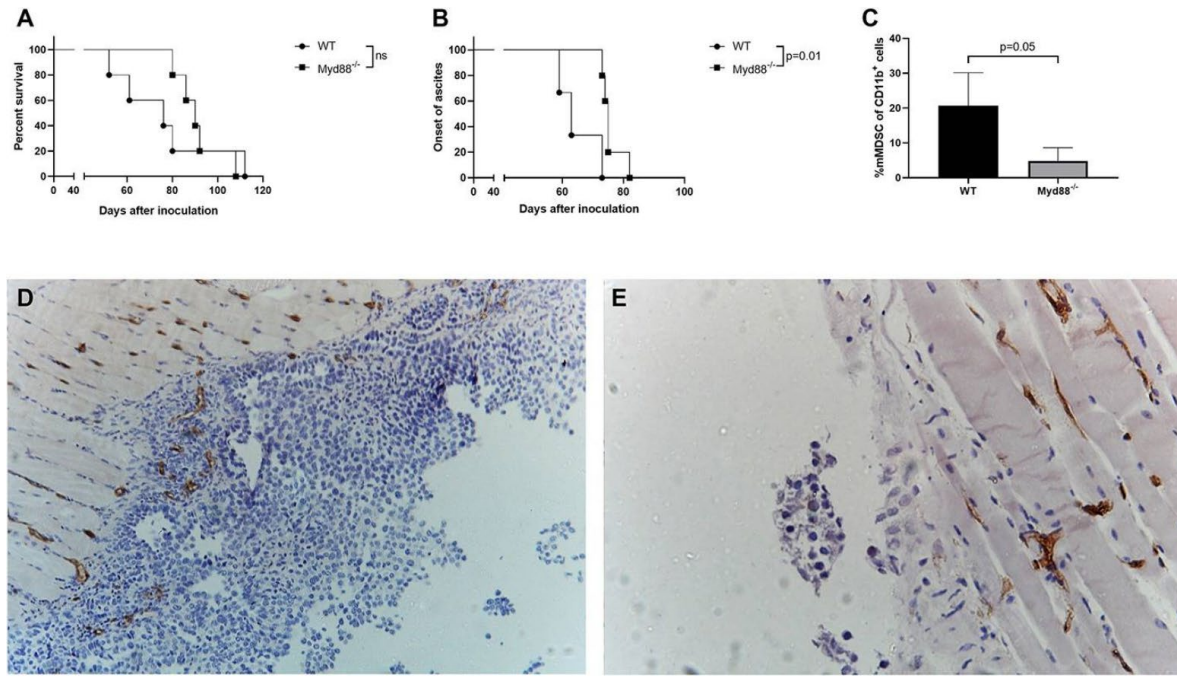

FIGURE 6 | ID8-fLuc induced ovarian cancer has a more indolent nature in Myd88 knockout mice [B6.129P2(SJL)-Myd88tm1.1Defr/J] compared to wild type (C57BL/6). (A) The B6.129P2(SJL)-Myd88tm1.1Defr/J (Myd88−/−) mice had a median survival of 90 days compared to 76 days for C57BL/6 mice (WT). This difference in survival was not statistically significant. (n = 5 mice per group). (B) Onset of ascites was used here as a surrogate marker for onset of disease symptoms and here we observed a significant longer latency period ($p = 0.01$) (n = 5 mice per group). (C) Using FACS we observed significantly less mMDSC in the B6.129P2(SJL)-Myd88tm1.1Defr/J mice compared to C57BL/6 mice. (D,E) Representative Ly6C staining of C57BL/6 (D) and B6.129P2(SJL)-Myd88tm1.1Defr/J (E) mice 8 weeks after inoculation. The C57BL/6 mice displayed macroscopically more peritoneal carcinosis compared to the B6.129P2(SJL)-Myd88tm1.1Defr/J mice. In addition, the peritoneal biopsies showed a higher Ly6C positivity in the tumor in the C57BL/6 mice compared to the B6.129P2(SJL)-Myd88tm1.1Defr/J mice.

antibody) compared to the depletion achieved by CL administration (near complete depletion of TAMs). In addition, treatment with anti-CSF1 did not lead to a more favorable macrophage polarization (cytotoxic M1-like vs. tumor supportive M2-like TAMs ratio remained unchanged). Depletion of NK cells using anti-CD122 (TMβ1) did not influence tumor growth or survival of the mice.

Ovarian Cancer (ID8-fLuc) Has a More Indolent Nature in B6.129P2(SJL)-Myd88tm1.1Defr/J Mice

In order to confirm our hypothesis that MDSC-mediated immunosuppression stimulates tumor growth and reduces survival in the ID8-fLuc model, we inoculated B6.129P2(SJL)-Myd88tm1.1Defr/J mice and C57BL/6 mice with ID8-fLuc cells. The goal of this experiment was to observe the *in vivo* effect of reduced MDSC-mediated immunosuppression. The Myd88 knock-out mice have a mutation in the Myd88 cytosolic adapter protein, which leads to an impaired immunosuppressive function of MDSC (18, 19). In these B6.129P2(SJL)-Myd88tm1.1Defr/J mice we observed a longer median survival after inoculation with ID8-fLuc compared to the wild type mice (C57BL/6) (90 days vs. 76 days, respectively) (Figure 6A). The B6.129P2(SJL)-Myd88tm1.1Defr/J mice also had a significant delay in the onset of ascites compared to C57BL/6 mice (75 days vs. 63 days, respectively,−p = 0.01) (Figure 6B). We also observed, in addition to the known reduced function of MDSC in Myd88−/− mice, a significantly reduced presence of mMDSC in peritoneal lavage fluid of B6.129P2(SJL)-Myd88tm1.1Defr/J mice (Figure 6C). Using IHC we observed a larger tumor volume in wild type mice compared to the B6.129P2(SJL)-Myd88tm1.1Defr/J counterparts. In addition, we also found a reduced infiltration of Ly6C+ MDSC in the tumor of B6.129P2(SJL)-Myd88tm1.1Defr/J mice compared to C57BL/6 mice (Figures 6D,E). Based on these findings, we can conclude that MDSC support tumor growth and have a negative influence on survival of tumor-bearing mice.

Monocytic MDSC Increase As Tumor Develops In ID8-Fluc Model And Suppress Effector T Cell Functioning.

Next, we studied the natural evolution of MDSC in the ID8-fLuc ovarian cancer model by assessing the relative numbers of MDSC in ascites over time in tumor-bearing mice and healthy controls. As anticipated from literature, we observed higher numbers of mMDSC in tumor-bearing mice compared to naive mice (26). Additionally, we observed significantly more mMDSC in ascites of mice with end stage disease compared to early stage disease (p = 0.02) (Figure 7A). Using immunohistochemistry, we observed an absolute reduction in TAMs (p = 0.004) and an increase in absolute number of Ly6C+ MDSC (p = 0.04) in the tumor over time (Figures 7B–G). To study the immunological role of mMDSC in ovarian cancer further, we performed *in vitro* experiments. In these experiments, we evaluated the T cell suppressive capacities of MDSC after stimulation by soluble factors derived from ID8-fLuc cell culture or ascites. Activation of mMDSC by conditioned medium of ID8-fLuc cell culture reduced T cell proliferation (p = 0.05), as measured by CFSE (Figure 8A). Both mMDSC and gMDSC reduced the number of T cells in co-culture when activated by filtered ascites of tumor bearing mice (Figure 8B). Next, we explored the suppressive effect of MDSC on the different T cell subsets using FACS. Co-culture of T cells with mMDSC and gMDSC, led to a reduction in the percentage of CD8+ T cells in the T cell population, even without activation of the MDSC (Figures 8C,D). In addition, the number of regulatory

T cells (Treg) increased during co-culture with activated gMDSC (Figure 8E). Co-culture of T cells with MDSC also led to a strong reduction in de CD8+/CD4+ T cell ratio, irrespective of the activation status of the MDSC (Figure 8F). Based on these *in vitro* experiments we can conclude that MDSCs activated by soluble factors present in ascites of ID8-fLuc tumor bearing mice induced an unfavorable immune profile with increased regulatory T cells and decreased effector T cells.

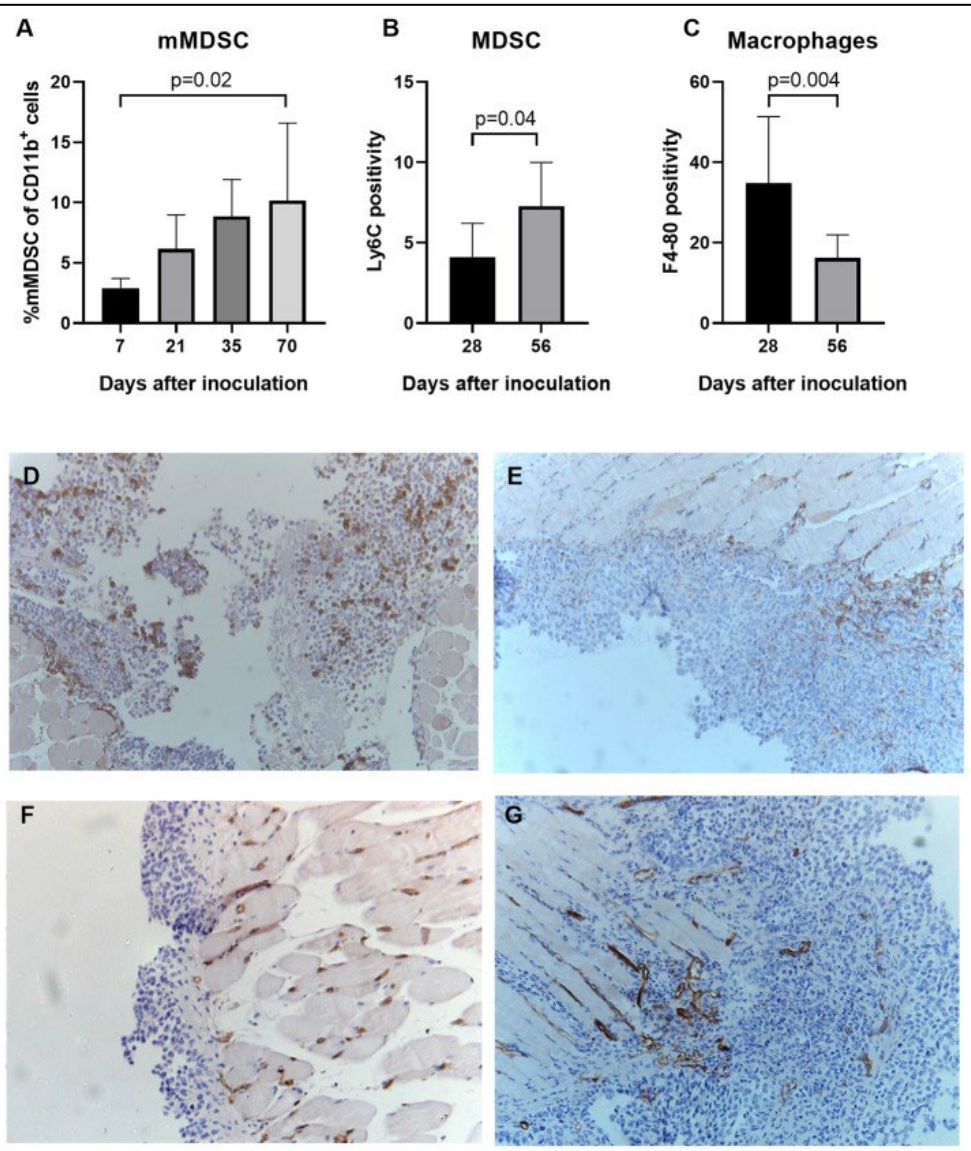

FIGURE 7 | MDSC increase as tumor develops in ID8-fLuc model. (A) Using fluorescent activated cell sorting (FACS) we measured the relative number of mMDSC at different time points during tumor development. We observed a significant higher number of monocytic MDSC mice with end stage disease (10 weeks after tumor challenge) compared to mice with early stage disease (1 week after tumor challenge) ($p = 0.02$) ($n = 3–6$ mice per group). (B,C) Based on immunohistochemistry for F4-80 and Ly6C we evaluated the presence of, respectively, macrophages and MDSC in the tumor over time. (B) We observed a significant decrease in the number of total tumor-associated macrophages over time ($p = 0.004$). (C) Parallel to the results using FACS, we observed a significant higher number of Ly6C+MDSC 8 weeks after tumor challenge, compared to 4 weeks after tumor challenge ($p = 0.04$). (D,E) Representative images of the F4-80 staining 28 days (D) and 56 days (E) after inoculation. Magnification x10. (F,G) Representative images of the Ly6C staining 28 days (F) and 56 days (G) after inoculation. Magnification x10.

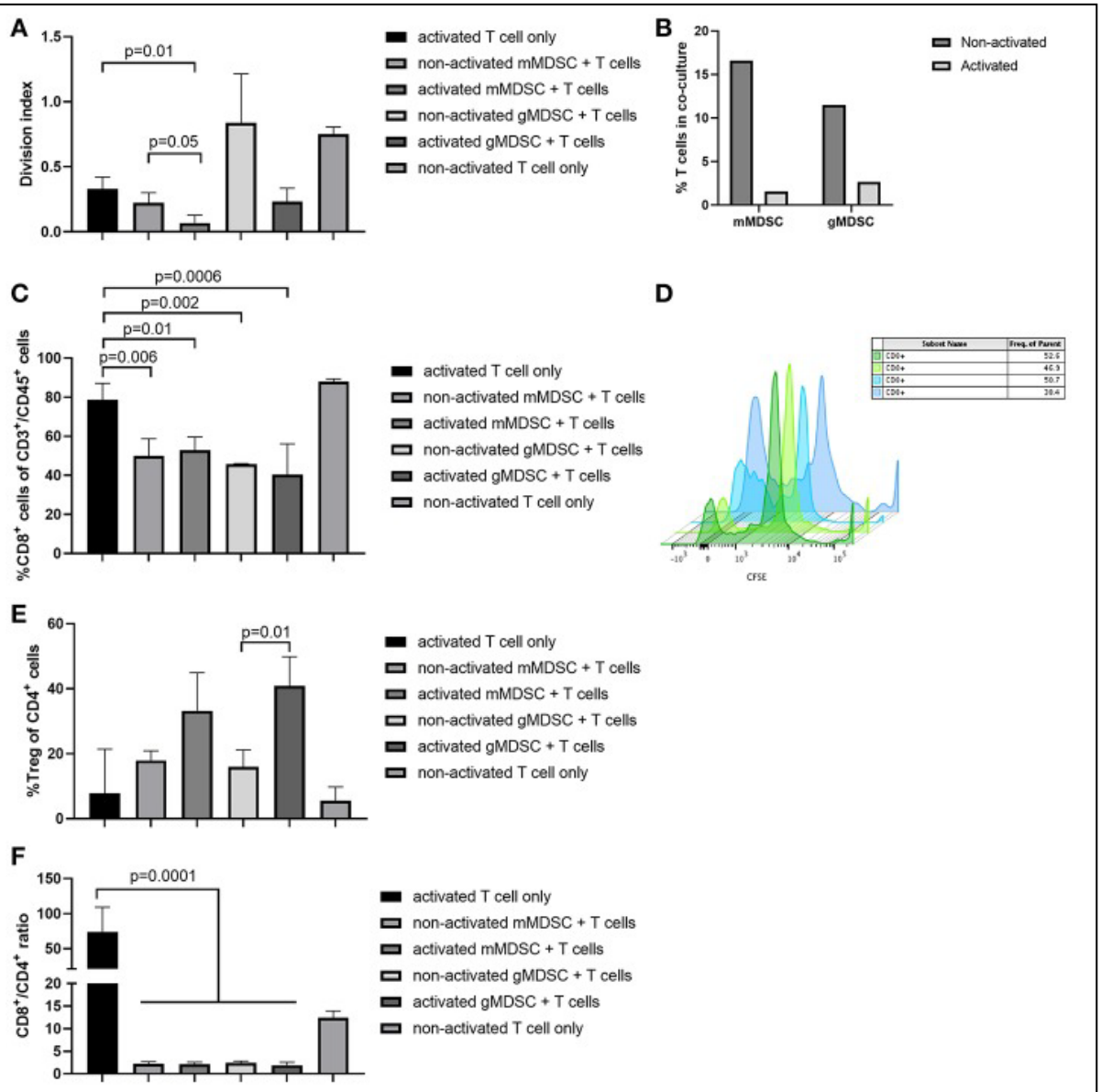

FIGURE 8 | MDSC activated *in vitro* can suppress T cell proliferation and skew the T cell compartment toward a reduction in cytotoxic T cells. (A) mMDSC activated by conditioned medium of ID8-fLuc cell culture reduced T cell proliferation significantly ($p = 0.05$) as shown by carboxyfluoresceinsuccinimidyl ester (CFSE). (B) Activation of mMDSC and gMDSC by filtered ascites led to a strong reduction in the number of live T cells in co-culture. (C) FACS analysis of T cells co-cultured *in vitro* with MDSC. Co-culture of mMDSC and gMDSC led to a significant reduction of CD8 positivity. (D) MDSC activated by soluble factors in ascites induce a significant reduced T cell proliferation as shown by CSFE dilution. T cells in the presence of non-activated MDSC (blue) are capable of multiple divisions; in contrast T cells co-cultured with activated MDSC display a reduced proliferation (green). (E) Activation of gMDSC by filtered ascites led to an increase in regulatory T cells (Treg) ($p = 0.01$). (F) Co-culture of both mMDSC and gMDSC skewed the T cell phenotype toward CD4, which led to a reduction in the CD8+/CD4+ T cells ratio even before activation of MDSC ($p = 0.0001$).

DISCUSSION

In this paper we studied the interaction between ovarian cancer and the immune system in the ID8-fLuc ovarian cancer mouse model. In short, we demonstrated that tumor growth and survival of tumor bearing mice is not controlled by the adaptive immune system in the ID8-fLuc model. Tumor growth in B6.129S7-Rag1tm1Mom/J mice, which lack T cells and B cells, was similar to tumor growth in immunocompetent C57BL/6 mice. Survival did not significantly differ between both mice strains. Additionally, depletion of CD8+ T cells did not significantly influence tumor growth in the ID8-fLuc model. There are two main possible explanations for these findings. The first being lack of immunogenicity of the model itself. This is unlikely as multiple studies have shown the antigenicity and immunogenicity of the ID8 model (27–29). Therefore, we hypothesized that the adaptive immune system in the ID8-fLuc model could be rendered anergic. As the behavior of the tumor was very similar in both the specific CD8+ T cell depletion and the B6.129S7-Rag1tm1Mom/J mice, we hypothesized that the innate immune system might play a role in the immunosuppression exerted on the adaptive immune system.

In order to study the role of the innate immune system, macrophages more specifically, we explored the effect of CL in the ID8-fLuc model. Treatment with CL led to a shorter survival both in C57BL/6 mice as in B6.129S7-Rag1tm1Mom/J mice. As CL is considered a dirty drug, which effects are not limited to macrophages only, we investigated the effect of CL treatment on the immune system in the ID8-fLuc model. CL effectively depleted macrophages in the peritoneal cavity of tumor bearing mice. In addition to this, we observed a significant increase in mMDSC and proinflammatory cytokines, which might explain the poor survival of mice treated with CL. We hypothesize that the strong reduction in TAMs (to <1% of CD11b+ cells) disrupts the homeostasis of the tumor microenvironment in the ID8-fLuc model. The observed cytokine reaction could explain the increase in mMDSC, since IL-6 is a known inducer of mMDSC expansion in humans and IL-1b correlates with mMDSC in blood of ovarian cancer patients (30, 31). We also observed a significant increase in Microphage inflammatory protein 1 (MIP1) α and MIP1β, which might have contributed to the recruitment of highly immunosuppressive CCR5+mMDSC (32). The activation of MDSC can lead to an increase in IL-6, IL-10, IL-1β and IFNγ, creating a feedback loop (32). Based on the assumption that the rise in MDSC caused by the depletion of macrophages by CL was responsible for the detrimental effect on survival of the mice, we performed a more selective depletion experiment. We compared survival and tumor growth of mice treated with anti-CSF1 (selectively TAMs depletion), anti-GR-1 (depletion of MDSC) and untreated tumor bearing mice. Selective reduction of GR-1+ MDSC led to a small survival benefit, as was demonstrated previously by others (33). Survival of mice treated with anti-GR-1 was significantly longer than survival of anti-CSF1 treated mice. Depletion of macrophages by anti-CSF1 was less efficient compared to depletion achieved by CL, which might explain why the effect of anti-CSF1 on survival and tumor growth is less pronounced compared to the CL. To support our hypothesis that MDSC have a negative impact of survival on tumor bearing mice in the ID8-fLuc model, we induced ovarian cancer in B6.129P2(SJL)-Myd88tm1.1Defr/J mice. These mice carry a deletion of exon 3 of the myeloid differentiation primary response gene 88 locus, which leads to a reduced (immunosuppressive) function of MDSC. Median survival was longer in the B6.129P2(SJL)-Myd88tm1.1Defr/J mice compared

to wild type C57BL/6 mice. In addition, onset of disease symptoms (ascites) was significantly delayed in the B6.129P2(SJL)-Myd88tm1.1Defr/J mice, supporting our hypothesis.

Next, we investigated the immunosuppressive effects of MDSC on T cells in the context of the ID8-fLuc model *in vitro*. In these experiments, we demonstrated that mMDSC and gMDSC activated by conditioned medium of ID8-fLuc cell culture or filtered ascites, led to a reduction in T cell proliferation and reduced the relative number of effector T cells in co-culture. These findings are supported by Horikawa *et al*. who demonstrated that MDSC suppress the CD8 T cells in the tumor microenvironment (34), suggesting that MDSC-induced immunosuppression might be one of the drivers of adaptive immunotolerance in ovarian cancer.

It should be noted, however that in an attempt to study the interaction between ovarian cancer and the immune system in a comprehensive way, we decided to use relatively nonspecific tools such as clodronate liposomes and B6.129S7-Rag1tm1Mom/J mice. As immunocompetent models for ovarian cancer are scarce, we limited ourselves to the ID8-fLuc model, this is of course also a possible bias. However, we used different methods, which all pointed toward an important role for (monocytic) MDSC in tumor-associated immunosuppression. In addition, the importance of the innate immune system, MDSC in particular, as a source of immunosuppression is being increasingly recognized in ovarian cancer. Cui *et al*. were the first to demonstrate a prognostic role for intra-tumoral MDSC in ovarian cancer (16, 34).

In addition, our study underscores the plasticity of the innate immune system and the balanced relationship between the different innate immune cells. A large part of the tumor stroma consists of TAMs; therefore, it is not surprising that rash depletion of TAMs, leads to a cytokine reaction, which attracts other innate cells to fill this niche. It is also important to note that macrophages and mMDSC originate from the same immature myeloid cells in bone marrow and that mMDSC can differentiate into macrophages (35). Therefore, it is not surprising that such interaction between the innate immune cells exist. Upon treatment with CL mMDSC were attracted to the tumor microenvironment, which led to worse survival of the mice, probably due to a detrimental effect on tumor immune control.

Until recently, tumor immunology research in ovarian cancer has focused mainly on the influence of the adaptive immune system on antitumor immunity (36). Only a limited number of studies have investigated the role of the innate immune system in ovarian cancer. We are the first to demonstrate that the presence of T cells was irrelevant for tumor growth and survival in the ID8-fLuc model. These results suggest that immunosuppression dominates the adaptive immune response in the ID8-fLuc model. In addition, we showed that MDSC are an important source of immunosuppression in ovarian cancer. This is an important finding as clinical immune oncology trials in ovarian cancer are currently focusing on the adaptive immune system. In ovarian cancer, the success has currently been limited to a small number of patients. Targeting MDSC might be a possible strategy to increase the number of patients who respond to immunotherapy. Preclinical studies have detected several possible strategies to deplete or inhibit MDSC, *e.g*., gemcitabine, 5-FU, ATRA, sunitinib, aspirin, etc. (37). However, we believe that further preclinical and translational research is needed to design rational immunotherapeutic approaches in ovarian cancer.

CONTRIBUTION TO THE FIELD STATEMENT

Ovarian cancer is the second most lethal type of gynecological cancer in women with an incidence rate of 12.5 per 100,000 women. Standard therapy consists of extensive surgery in combination with chemotherapy. As tumor-infiltrating lymphocytes have a positive prognostic impact in ovarian cancer, immune checkpoint inhibitors have been put forward as a new treatment modality. However, response was only 10% in monotherapy. According to our findings, this might be explained by underlying immune biology in ovarian cancer. In this paper, we demonstrate that the adaptive immune system is unable to control tumor growth in an ovarian cancer mouse model. We hypothesized that the innate immune system suppresses the adaptive immune response. We show that myeloid-derived suppressor cells (MDSC), increase during the disease course and that MDSC are able to suppress the T cells of the adaptive immune system. In addition, we show that suppression of MDSC function positively influences survival of mice with ovarian cancer. Therefore, we argue that MDSC play an important immunosuppressive role in ovarian cancer and that future studies on immunotherapy should consider combining agents that optimize the T cells response to strategies targeting innate immunosuppression.

ETHICS STATEMENT

All animals were housed and treated according to the Federation for Laboratory Animal Science Associations guidelines (21). Ethical approval was obtained from the local Ethical Committee at KU Leuven (p075/2014 and p125/2017).

ACKNOWLEDGEMENTS

All authors helped write and approved the submitted version of the manuscript. TB designed the study, wrote the manuscript, set-up and performed the *in vivo* experiments, analyzed the data, and performed the statistical analyses. AV helped with the *in vivo* experiments, performed *in vitro* experiments, and scored the IHC. MR provided technical assistance. GT and AVH performed FACS staining and acquisition. TM performed immunofluorescent staining. IV added to the concept of the study. AC supervised the experiments, helped write the manuscript, and conceived the study. This work was supported by Kom Op Tegen Kanker (Stand up to Cancer), the Flemish cancer society (2016/10728/2603 to AC); the Olivia Fund (2017/LUF/00135 to AC); Amgen Chair for Therapeutic Advances in Ovarian Cancer (2017/LUF/00069 to IV); Research Foundation – Flanders (FWO) (12F3114N to AC).

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SUPPLEMENTARY TABLES

Supplementary table 1. *Myeloid panel*

Monoclonal Ab	Fluorophore	Supplier
CD11b	PerCPCy 5.5	BD Biosciences (San Jose, CA, USA)
CD11c	AF700	BD Biosciences (San Jose, CA, USA)
B220	V500	BD Biosciences (San Jose, CA, USA)
CD8a	PE CF595	BD Biosciences (San Jose, CA, USA)
Ly6C	AF647	BioLegend (San Diego, CA, USA)
Ly6G	FITC	BD Biosciences (San Jose, CA, USA)
CD193/CCR3	PE	eBioscience (San Diego, CA, USA)
F4/80	BV421	BD Biosciences (San Jose, CA, USA)
I-A/I-E	BV650	BD Biosciences (San Jose, CA, USA)
CD206/MMR	PE-Cy7	eBioscience (San Diego, CA, USA)

Supplementary table 2. *T cell panel*

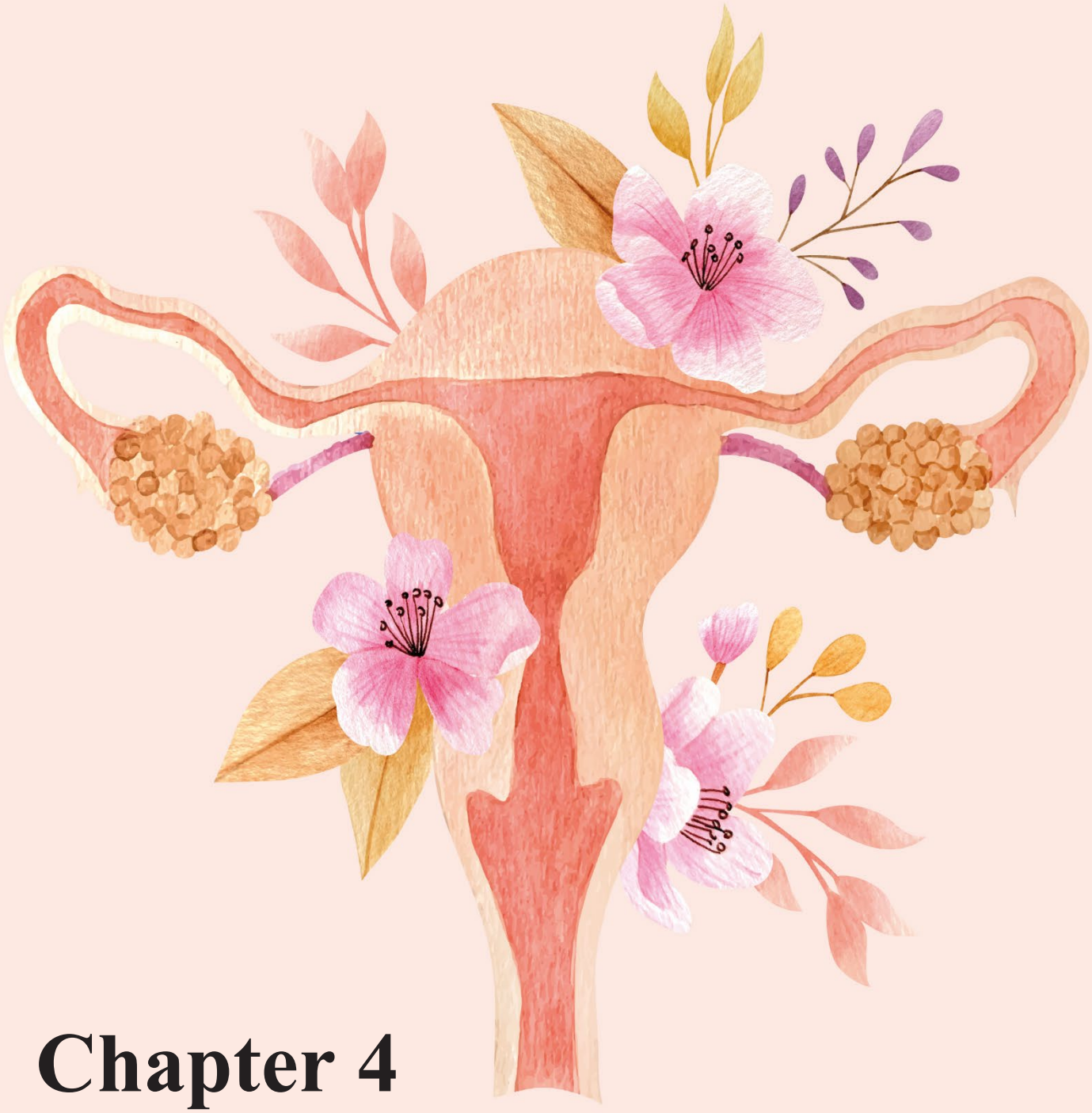
Monoclonal Ab	Fluorophore	Supplier
CD45	AF700	eBioscience (San Diego, CA, USA)
CD3	BV510	BD Biosciences (San Jose, CA, USA)
CD4	PerCP Cy5.5	eBioscience (San Diego, CA, USA)
CD8	BV421	BD Biosciences (San Jose, CA, USA)
CD25	PE	BD Biosciences (San Jose, CA, USA)
FoxP3	AF488	BD Biosciences (San Jose, CA, USA)

Supplementary table 3. *B cell panel*

Monoclonal Ab	Fluorophore	Supplier
CD45	AF700	eBioscience (San Diego, CA, USA)
CD3	BV510	BD Biosciences (San Jose, CA, USA)
CD19	AF647	BD Biosciences (San Jose, CA, USA)
CD20	PE	eBioscience (San Diego, CA, USA)
NK1.1	BV421	BD Biosciences (San Jose, CA, USA)
NKp46	PerCP Cy5.5	BioLegend (San Diego, CA, USA)

Supplementary table 4.*T cell panel in vitro* experiments

Monoclonal Ab	Fluorophore	Supplier
CD45	APC	eBioscience (San Diego, CA, USA)
CD3	APC eFluor780	eBioscience (San Diego, CA, USA)
CD4	PerCP Cy5.5	eBioscience (San Diego, CA, USA)
CD8	BV421	BD Biosciences (San Jose, CA, USA)



Chapter 4

Opposite Macrophage Polarization in Different Subsets of Ovarian Cancer: Observation from a Pilot Study

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The content of this chapter was published in Cells *(2020), 9, 305,* but slightly modified for this thesis based on jury comments and the changed macrophage landscape over the past two years.

ABSTRACT

The role of the innate immune system in ovarian cancer is gaining importance. The relevance of tumor-associated macrophages (TAMs) is insufficiently understood. In this pilot project, comprising the immunofluorescent staining of 30 biopsies taken from 24 patients with ovarian cancer, we evaluated the presence of total TAMs (cluster of differentiation (CD) 68 expression), M1-like TAMs (major histocompatibility complex (MHC) II expression) and M2-like TAMs (anti-mannose receptor C type 1 (MRC1) expression), and the blood vessel diameter. We observed a high M1-like/M2-like TAMs ratio in low-grade ovarian cancer compared to high-grade tumors, more total TAMs and M2-like TAMs in metastatic biopsies and a further increase in total TAMs and M2-like TAMs at interval debulking, without beneficial effects of bevacizumab. The blood vessel diameter was indicative for M2-like TAMs tumor infiltration (Spearman correlation coefficient of 0.65). These data mainly reveal an immune beneficial environment in low-grade ovarian cancer in contrast to high-grade serous ovarian cancer, where immune suppression is not altered by neoadjuvant therapy.

1. INTRODUCTION

Ovarian cancer has the fifth highest mortality rate among women diagnosed with cancer in Europe [1]. Based on its histopathologic and genetic profile, epithelial ovarian cancer (EOC) can be subdivided into different subtypes: endometrioid, clear-cell, mucinous and serous carcinomas [2]. Serous ovarian cancer is the most common subtype, accounting for ±75% of all EOC, and it can be further subdivided into low-grade and high-grade serous ovarian cancer [3]. Due to the absence of distinct symptoms, the majority is diagnosed at an advanced disease stage (International Federation of Gynecology and Obstetrics (FIGO) stage III (51%) or stage IV (29%)) [4]. The backbone of first-line treatment consists of a primary debulking surgery (PDS) and platinum-based chemotherapy. However, patients that are not eligible for PDS can instead receive neoadjuvant chemotherapy (NACT) followed by interval debulking surgery (IDS) [5,6]. Despite this extended first-line treatment, the majority of patients relapse. Overall, patients in an advanced disease stage have a poor five-year survival of only 25% [4,7].

The immune system has an important role in ovarian cancer initiation, progression, and prognosis. However, most data in this regard exist on the adaptive immune system [8,9]. Nevertheless, our group recently provided evidence on the importance of the innate immune system in ovarian cancer [10,11]. The major players within this innate immune system are dendritic cells, myeloid derived suppressor cells, and macrophages. Macrophages infiltrating the tumor microenvironment (TME) are defined as tumor-associated macrophages (TAMs). Although they are known to have a high degree of plasticity and heterogeneity, the majority of macrophages can be classified into two phenotypes based on their activation status. The classically activated macrophages (M1, type I) express a broad series of proinflammatory and immunostimulatory effector molecules, such as interleukin-1-beta and tumor necrosis factor alpha, and they possess a strong anti-tumoral activity. This is in contrast to the alternatively activated macrophages (M2, type II) which are characterized by a tumor promoting phenotype, as well as expression of a wide array of anti-inflammatory effector molecules such as interleukin-10 and tumor necrosis factor beta, thereby contributing to a more immunosuppressive TME [12]. However, very recent data showed that the gene expression profile between tumor-resident macrophages and TAMs is different and that TAMs cannot always be dichotomized into a pure M1 or pure M2 phenotype [13]. In fact, this dichotomized wording can only be applied to TAMs generated in *in vitro* systems where macrophages are stimulated with lipopolysacharide (LPS)/interferon or interleukin (IL)-4. *In vivo*, TAMs cannot be categorized as they represent a continuum along a spectrum of phenotypes in responses to stimuli present in the TME. Therefore, we should rather refer to TAMs as M1-like or M2-like TAMs.

Previous studies already demonstrated the importance of TAMs in ovarian cancer. TAMs in the ascites of ovarian cancer patients were shown to express B7-H4+, resulting in a dysfunctional phenotype and suppressing T-cell anti-tumor immunity [14]. A study by Zhang *et al.* (2014) demonstrated that an increased M1-like/M2-like TAMs ratio correlated with improved prognosis in ovarian cancer patients [15], indicating the importance of this balance between more immune stimulatory M1-like and more tumor-promoting M2-like macrophages.

In addition to the relevance of the immune system in ovarian cancer development, angiogenesis is another important hallmark [16]. Angiogenesis is defined as the expansion of vascular endothelial cells and the development of new blood vessels in order to provide sufficient supply of essential nutrients such as oxygen. As tumor cells are rapidly dividing, they have an increased need for nutrients and, therefore, they induce angiogenesis by secreting various growth factors such as vascular endothelial growth factor (VEGF) [17]. However, the newly formed vasculature network in tumors is highly irregular and often comprises leaky blood vessels [17,18]. This dysfunctional vascularization results in chronic hypoxia and contributes to metabolic changes in tumor cells, making them more resistant to therapy. Moreover, they facilitate the dissemination of cancer cells to metastatic sites [19]. In ovarian cancer, bevacizumab (anti-VEGF, a monoclonal antibody targeting VEGF) is one of the few targeted therapies available for patients, inhibiting the binding of VEGF to its receptor and, therefore, regulating and normalizing the blood vessels [20,21]. However, VEGF is a factor produced by a variety of cells, including macrophages. The presence of VEGF stimulates both the migration of macrophages to the TME and their polarization toward a more M2-like TAMs phenotype [17]. Furthermore, macrophages were shown to facilitate resistance to anti-VEGF treatment [22].

In this exploratory pilot study, we evaluated different ovarian cancer tumor biopsies for the presence of total TAMs (cluster of differentiation (CD) 68 expression), M1-like TAMs (major histocompatibility complex (MHC) II expression), and M2-like TAMs (anti-mannose receptor C type 1 (MRC1) expression), and glucose transporter-1 (Glut1) for the blood vessel diameter. Our results highlight differences between low-grade and high-grade tumor, as well as between metastases and primary tumor biopsies, and they underscore the relationship between the blood vessel diameter and macrophage infiltration. We also explored if platin-based chemotherapy and bevacizumab altered the macrophage composition.

2. MATERIALS AND METHODS

2.1. Patients and Clinical Data

We prospectively collected 30 tissue samples from 24 patients diagnosed with ovarian cancer at the University Hospitals Leuven (Belgium) in the OV-IMM-2014 study. Patients with known immune diseases or taking immunosuppressive drugs at diagnosis, infectious disease (human immunodeficiency virus (HIV), hepatitis B and C) or concurrent non-ovarian cancer at the moment of diagnosis were excluded from the study. Samples were collected between June 2014 and April 2016. We collected tumor samples at diagnosis (treatment-naïve, PDS) and/or during IDS (after chemotherapy and/or bevacizumab treatment). The study was approved by the ethical committee of the University Hospitals Leuven (s56311). All patients included gave written informed consent.

2.2. Immunofluorescence Staining

Tumor biopsies were fixed with 4% paraformaldehyde for 24 h, washed with DPBS (Dulbecco's phosphate-buffered saline) and stored at 4°C until further processing. Samples were prepared as 200-µm-thick vibratome sections, blocked and permeabilized in TNBT buffer (0.1 M Tris pH 7.4; NaCl 150 mM 0.5% blocking reagent from Perkin Elmer (Waltham, Massachusetts, USA), 0.5% Triton X-100) for

four hours at room temperature. Tissues were incubated overnight at 4°C with the following primary antibodies diluted in TNBT buffer: anti-glucose transporter-1 (Glut1) (Millipore, Burlington, Massachusetts, USA; 1:200 dilution), anti-CD68 (Abcam, Cambridge, UK; 1:200 dilution), anti-MHC-II (Thermo Scientific, Waltham, Massachusetts, USA; 1:100 dilution), or anti-MRC1 (R&D Systems, Minneapolis, Minnesota, USA; 2 ug/mL). Next, slides were washed in TNBT buffer and incubated overnight at 4°C with the appropriate secondary antibody coupled with Alexa 488/555 (Life Technologies, Carlsbad, California, USA; 1:200 dilution) diluted in TNBT buffer. Tissues were washed and mounted on slides in fluorescent mounting medium (Dako/Agilent, Santa Clara, California, USA). Images were acquired using a Leica TCS SP8 confocal microscope. Semi-automated quantification analyses were performed using Fiji software [23] to assess the number of CD68+, MHCII+ and MRC1+ cells per field. Five fields were assessed per sample, and the average was used in all subsequent analyses.

2.3. Statistical Analyses

The expression of three biomarkers (CD68, MHCII, MRC1) and the blood vessel width (blood vessels visualized by Glut1 staining) were compared between FIGO stages (FIGO stage I, FIGO stage III, and FIGO stage IV), bioptic sites (primary tumor and metastatic tumor), tumor grades (low-grade ovarian carcinoma and high-grade ovarian carcinoma), moment of surgery (PDS and IDS) and administered therapy (chemotherapy and chemotherapy + bevacizumab). For this, we visualized the data with box plots. Spearman's rank-order correlation was used to correlate MRC1 with Glut1. We performed this exploratory analysis using R version 3.5.1.

3. RESULTS

3.1. Description of Patient Samples

Tumor biopsies were collected for 24 patients. Patient characteristics are displayed in Table 1.

Table 1. Patient characteristics

Characteristic		Result (Absolute/Percentage)
Age (median (years), range)		63 (47–85)
Histological subtype	Serous	21 (88)
	Endometrioid	1 (4)
	Clear-cell carcinoma	1 (4)
	Carcinosarcoma	1 (4)
Differentiation grade	High-grade	21 (88)
	Low-grade	3 (12)
Stage	I	3 (12)
	III	11 (46)

	IV	10 (42)
Surgical treatment	Upfront primary debulking	11 (46)
	Interval debulking surgery	13 (54)
Chemotherapy treatment	Carboplatin-paclitaxel	10 (42)
	Carboplatin monotherapy	2 (8)
	Carboplatin-paclitaxel + bevacizumab	10 (42)
	Other (letrozole, interruption of chemo because of toxicities)	2 (8)
Outcome	No evidence of disease	5 (21)
	Alive with evidence of disease	8 (33)
	Death of disease	11 (46)
Progression-free survival	Median (months), range	21 (6–60)
Bioptic site	Total amount of biopsies	30
	Primary tumor at diagnosis	11 (37)
	Metastasis at diagnosis	6 (20)
	Primary tumor at interval debulking	9 (30)
	Metastasis at interval debulking	4 (13)
	Paired samples (primary tumor + metastasis)	5 (17)
	Paired samples (before and after neoadjuvant chemotherapy)	1 (3)

3.2. TAMs at Ovarian Cancer Diagnosis

At diagnosis, metastatic tumor sites showed more total TAMs and more M2-like TAMs compared to the primary tumor. This was true for a comparison in bulk, but also for a paired sample comparison (Figure 1 and Figure S1, Supplementary Materials). Low-grade ovarian cancer showed less total TAMs, more M1-like TAMs, and less M2-like TAMs compared to high-grade ovarian cancer at diagnosis in the primary tumor (no metastatic biopsies available of low-grade tumors). M2-like TAMs were less abundant in stage IV ovarian cancer, compared to stage III in metastatic tumors, while there were no changes in primary tumors (Figure 2). Representative fluorescent pictures are shown in Figure 3.

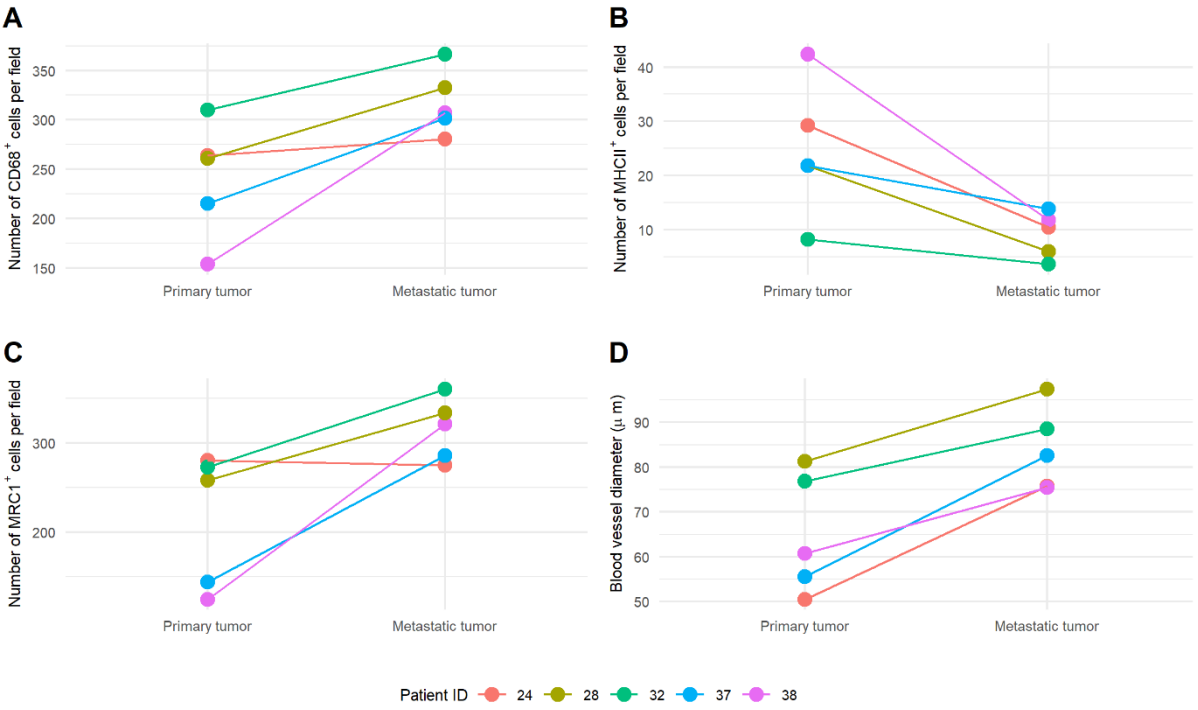

Figure 1. Comparison of macrophage profile and blood vessel diameter in matched biopsies at primary and metastatic tumor sites. The amount of (A) cluster of differentiation (CD) 68+, (B) major histocompatibility complex (MHC) II+, and (C) anti-mannose receptor C type 1 **(**MRC1)+ cells for five patients with matched biopsies and (D) blood vessel diameter (µm) based on glucose transporter-1 (Glut1) expression. Samples of patients 37 and 38 were collected at primary debulking surgery (PDS), while samples of patients 24, 28 and 32 were collected at interval debulking surgery (IDS).

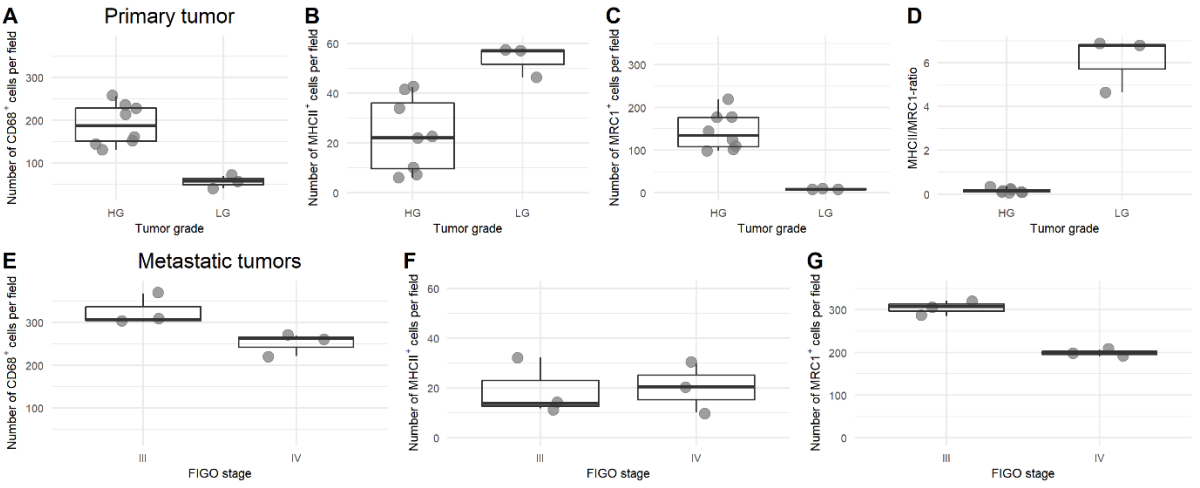

Figure 2. Comparison of macrophage profile in low-grade versus high-grade tumors and in International Federation of Gynecology and Obstetrics (FIGO) stage III versus IV. (A–C) Number of CD68+ (A), MHCII+ (B) and MRC1+ (C) cells per field in primary high-grade and low-grade tumor samples. Ratio between MHCII+/MRC1+ (i.e., M1-like/M2-like) in high-grade vs. low-grade ovarian

cancer (D). (E–G) Number of CD68+ (D), MHCII+ (E), and MRC1+ (F) cells per field in metastatic biopsies of stage III and stage IV ovarian cancer.

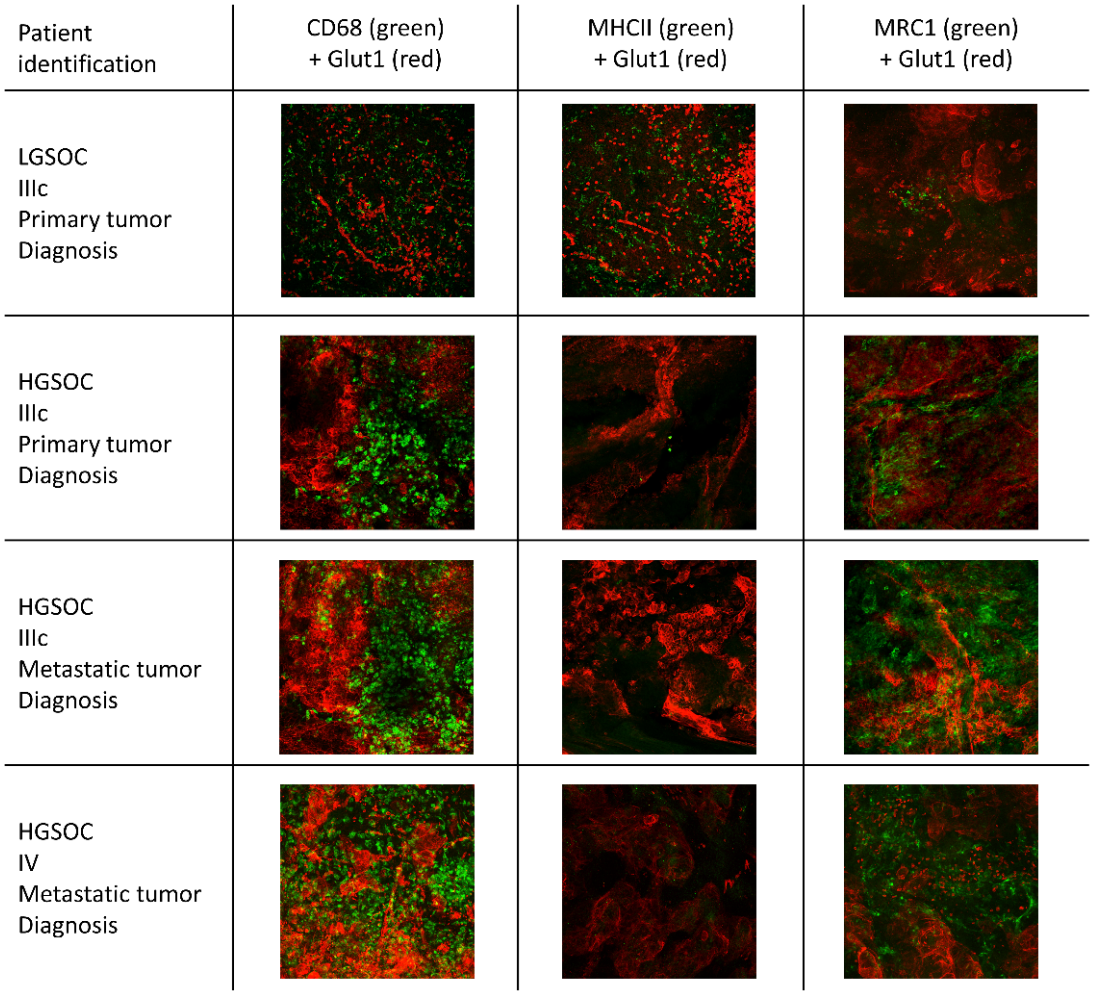

Figure 3. Comparison of macrophage infiltration between different subsets of ovarian cancer. Immunofluorescence staining for CD68, MHCII, MRC1, and Glut1 in low-grade serous ovarian cancer (LGSOC) and high-grade serous ovarian cancer (HGSOC), in both primary and metastatic tumors at diagnosis. Each image is 500 µm × 500 µm.

3.3. Effect of Platin-Based Chemotherapy and Bevacizumab on the Presence of TAMs in High-Grade Serous Ovarian Cancer Biopsies

Platin-based chemotherapy was associated with an increased amount of total and M2-like macrophages in HGSOC. This was most pronounced in biopsies of the primary tumor (Figure 4A–C) and in stage IV ovarian cancer patients (Figure 4D–F). The additional use of bevacizumab compared to paclitaxel-carboplatin alone seemed to increased the number of total TAMs and M2-like when evaluated in interval debulking samples at the primary tumor site (Figure 4G–I).

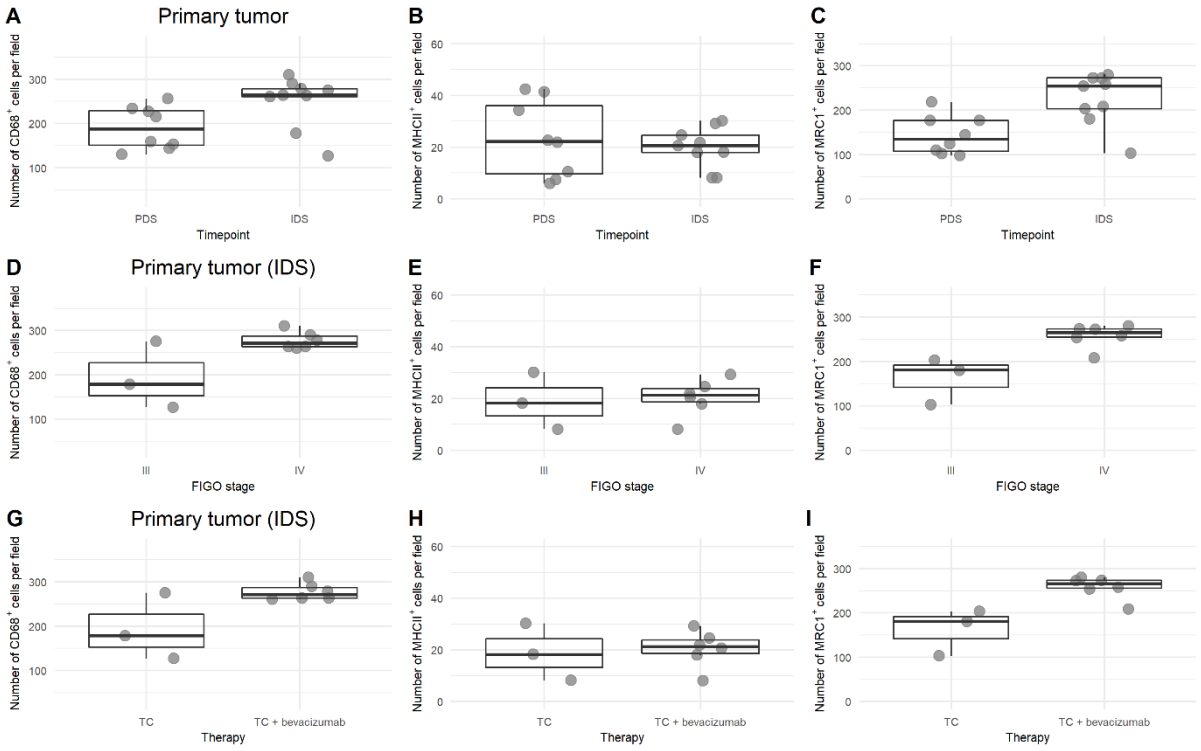

Figure 4. Comparison of macrophage profile after platin-based chemotherapy and bevacizumab in HGSOC. (A–C) Number of CD68+ (A), MHCII+ (B) and MRC1+ (C) cells per field in primary tumor samples at diagnosis (PDS = primary debulking surgery and at IDS = interval debulking surgery, i.e., after receiving platin-based chemotherapy). (D–F) Number of CD68+ (D), MHCII+ (E) and MRC1+ (F) cells per field in stage III vs. stage IV ovarian cancer after platin-based chemotherapy. (G–I) Number of CD68+ (G), MHCII+ (H) and MRC1+ (I) cells per field in primary tumor samples at IDS, after receiving platin-based chemotherapy alone or in combination with bevacizumab.

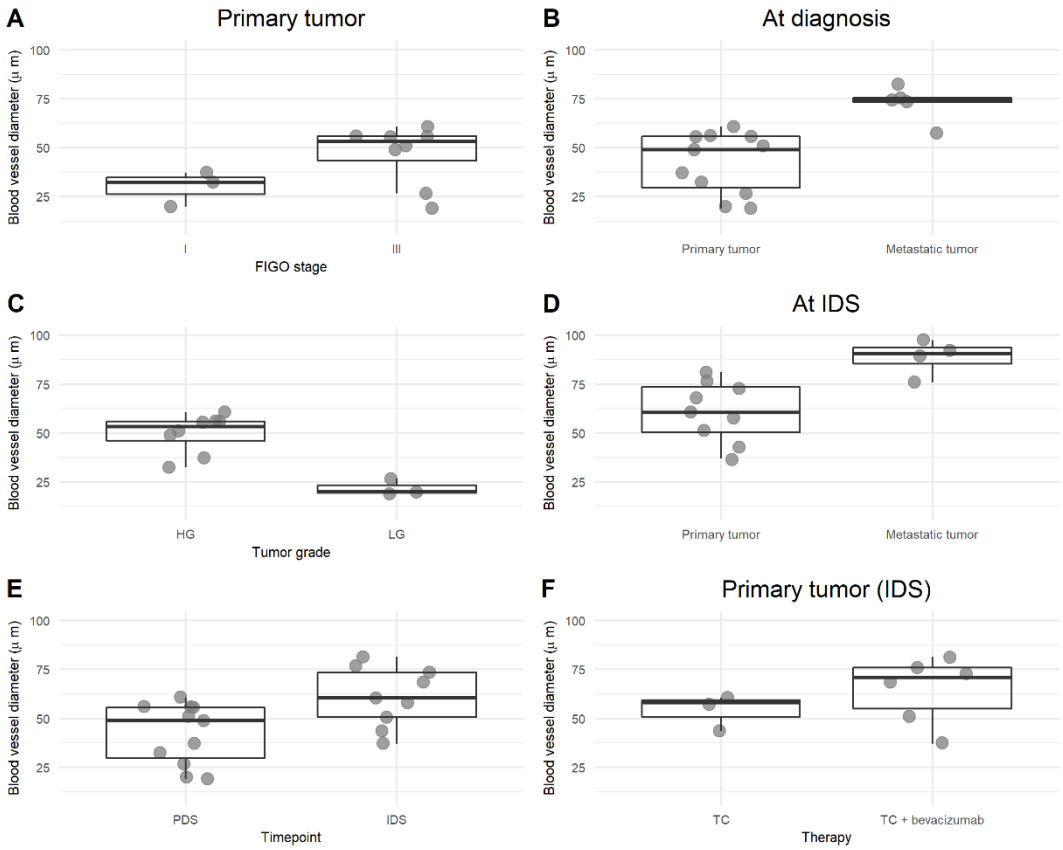

Figure 5. Comparison of blood vessel diameter (µm). Blood vessels were stained for Glut1. Diameter of the visualized blood vessels was measured subsequently, and results are presented on the Y-axis. Comparison of primary tumor samples of early vs. late stage ovarian cancer (A), of primary vs. metastatic tumor samples at diagnosis (B), of high-grade (HG) versus low-grade (LG) tumors (C), after platin-based chemotherapy (D,E), and after the addition of bevacizumab (F).

3.4. Blood Vessel Abnormality in Ovarian Cancer and Its Relationship with M2-like Macrophages

Blood vessel diameter increased with increasing stage and grade and was also larger in metastatic tumors compared to primary tumors, both at diagnosis and at interval debulking. The use of chemotherapy and bevacizumab could be linked to an increased blood vessel width (Figure 5). Wider blood vessels could be associated with more M2-like macrophages (Figure 6). Representative pictures are presented in Figure 7.

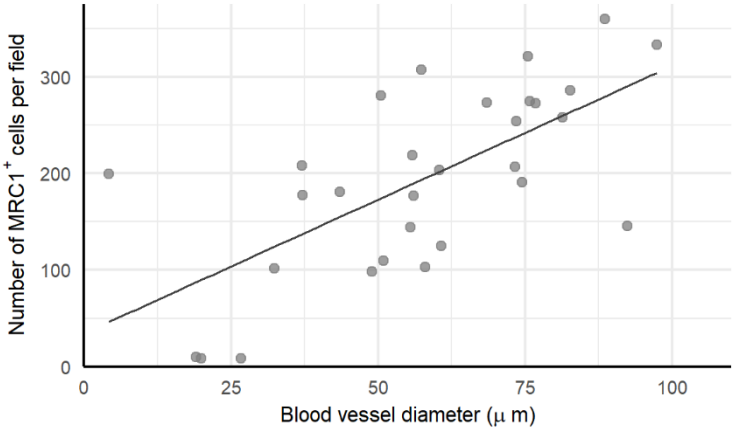

Figure 6. Correlation between blood vessel width and M2 macrophages. A larger blood vessel denotes more M2-like macrophages infiltrating the tumor (Spearman correlation coefficient of 0.65).

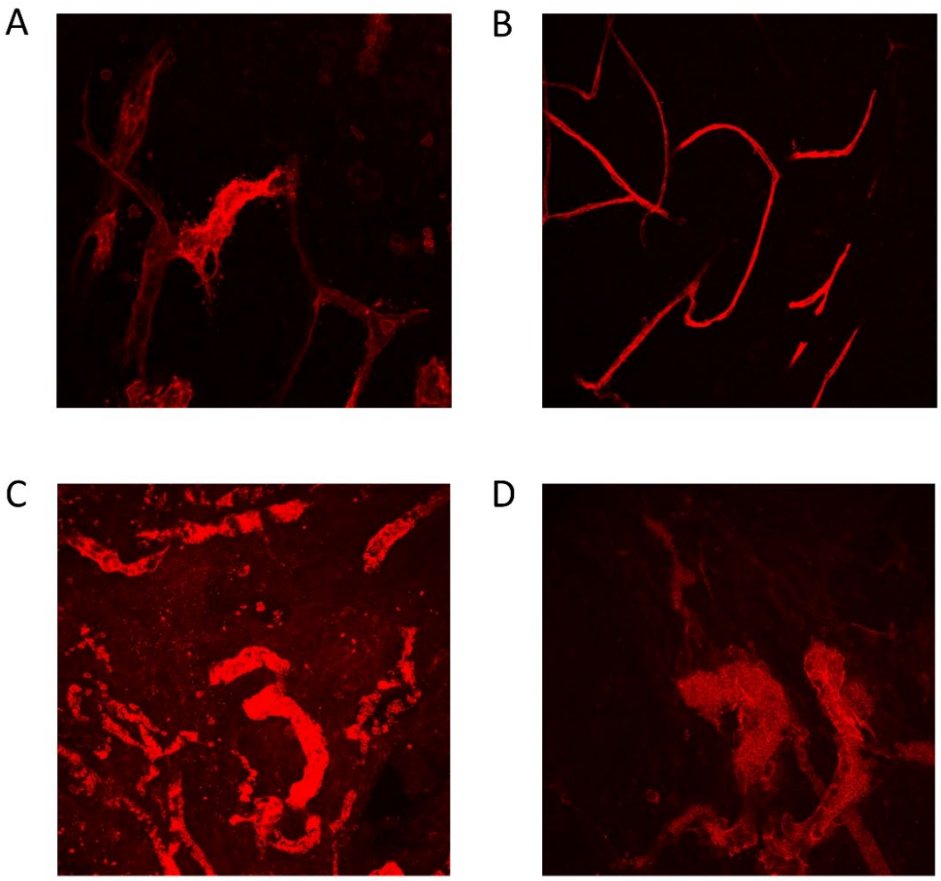

Figure 7. Comparison in blood vessel width (Glut1) between high-grade serous ovarian cancer (HGSOC) and low-grade serous ovarian cancer (LGSOC) (A,B) and between primary and metastatic tumor (C,D). (A) Primary tumor biopsy of a stage IIIC HGSOC. (B) Primary tumor biopsy of a stage IIIC LGSOC. (C) Primary tumor biopsy of HGSOC stage IIIC (different patient than (A)). (D) Metastatic tumor biopsy of the same patient as (C). Each image is 500 µm × 500 µm.

4. DISCUSSION

This exploratory pilot experiment aimed to visualize the presence of TAMs in different subsets of ovarian cancer. At diagnosis, we found that the M1-Like/M2-like TAMs ratio was high in low-grade ovarian cancer and that, in metastatic sites, more M2-like TAMs were present compared to primary tumor sites. We also discovered that neoadjuvant chemotherapy tends to increase these M2-like TAMs and that bevacizumab does not alter this effect. Visual inspection revealed a striking association of co-location of intratumoral blood vessels and TAMs, an increase of M2-like TAMs with increase blood vessel abnormality (measured by blood vessel width), and an increase of this abnormality by neoadjuvant chemotherapy and bevacizumab.

Our findings at diagnosis where M2-like TAMs are increased at metastatic sites is in line with previous literature data showing that M2-like TAMs increase the invasiveness of ovarian cancer [24] and that

TAMs behaviour, number, and composition differ according to the tumor area (demonstrated in different solid tumors, but not yet in ovarian cancer [25]). Although often treated similarly, low-grade tumors respond differently to chemotherapy compared to high-grade tumors [26], differ genetically [27], and have a different immune biology. The group of Yang *et al*. demonstrated, based on the retrospective analysis of micro-array datasets, that the gene signature of immune cells is nearly opposite in high-grade serous ovarian cancer compared to low-grade serous ovarian cancer [28]. The expression of B7-H4 (regulating T-cell immunity) was significantly reduced in low-grade serous ovarian cancer compared to high-grade serous ovarian cancer [29]. Our group recently demonstrated a reduced innate immune suppression in blood samples of low-grade ovarian cancer patients compared to high-grade ovarian cancer [11]. The current pilot study adds a high M1-like/M2-like TAMs ratio at the protein level to the immune knowledge of low-grade ovarian tumors. This less immune suppressive landscape might be an advantage for the success rate of immunotherapy in this type of tumor. However, few clinical studies addressed this niche (e.g., NCT02923934 focusing on immune checkpoint inhibitor therapy in rare cancers, including low-grade ovarian cancers).

The evaluation of tumor biopsies after neoadjuvant chemotherapy revealed an increase in vessel width, TAMs, and M2-like TAMs. We could demonstrate that blood vessel width is correlated with M2-like presence, but the cause and the consequence are not so clear. *In vitro* experiments showed that paclitaxel-carboplatin increases CCL2 (chemokine (C-C) motif ligand 2) [30]. CCL2 is known to attract macrophages. In murine ovarian cancer experiments, inhibition of CCL2 increased the response to carboplatin-paclitaxel [31]. However, the immune system is much more than only macrophages and, to evaluate the immune-stimulating or immune-suppressive effects of carboplatin-paclitaxel in ovarian cancer, the whole immune system has to be taken into account. Our group highlighted in this regard that immune suppression measured in ascites is reduced after neoadjuvant chemotherapy [32], and that carboplatin-paclitaxel can render the cytokine profile in serum of ovarian cancer patients less immunosuppressive, through a decrease in interleukin-10, VEGF, transforming growth factor-β, and arginase [33]. Moreover, taking into account that the immune system is most likely different in different metastatic biopsies of ovarian cancer patients [34–36], we have to be cautious in interpreting data after treatment based on one tumor biopsy.

Furthermore, the data on bevacizumab have to be considered with this in mind. Anti-VEGF therapy, which was proven beneficial in ovarian cancer [20], is indeed known to modify the immune contexture [37] and is expected to reduce the amount of M2-like macrophages. On the other hand, there is evidence that the presence of M2-like macrophages can be an indication of resistance to anti-VEGF therapy [22,38,39]. It inhibits angiogenesis but can also promote the recruitment of TAMs, immature monocytes, and other vascular modulators to the tumor site [39]. Furthermore, M2-like TAMs are known to produce a wide variety of proangiogenic factors such as VEGF to further enhance tumor vascularization, eventually resulting in therapy resistance [40,41]. Moreover, MMP-9 (matrix metalloproteinase 9) secreted by macrophages can increase the bioavailability of VEGF to its receptor [42,43]. Therefore, our observation that bevacizumab in combination with carboplatin-paclitaxel increases the blood vessel diameter and the amount of TAMs and M2-like in comparison to chemotherapy alone might (albeit having only one biopsy) be relevant to take into account in

combinatorial immunotherapy design (already ongoing in, for example, the IMagyn050 (NCT03038100) and DUO-O (NCT03737643) studies). Increase in immunosuppression can alter the response to immunotherapy.

We did not report on the correlation of macrophages and survival, notwithstanding the fact that we have survival data on all patients. The number of patients was too small to perform reliable statistics. Nevertheless, in the literature, there is compelling evidence that M2-like TAMs worsen the prognosis of ovarian cancer patients (meta-analysis of Yuang *et al*. in nine studies on 794 patients) [44].

Of course, this study had limitations, such as the small sample size, the lack of several metastatic biopsies of one patient to compare [34–36], and the substantial variation between macrophage counts in biopsies under treatment. Another important limitation of this study was the use of a single marker to define the M1-like and M2-like TAMs subsets, MHCII and MRC1, respectively. Macrophages are a very diverse set of cells that constantly shift their functional state and consequently also display altered expression levels of markers [12]. Therefore, multiple expression markers will be required in future studies to be able to distinguish more subsets of macrophages such as M2a, M2b, and M2c subtypes [45]. However, our pilot study was designed to detect possible trends worth further investigating in the future and not to perform a full characterization of different TAMs subsets.

The most important finding of this pilot project is without any doubt the clear discrepancy between high-grade and low-grade ovarian cancer, with a less immune suppressive profile in low-grade ovarian cancer. This might open up possibilities for altered therapeutic management in this less common subtype of ovarian cancer.

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Conceptualization, A.C. and T.B.; methodology, T.M., A.C. and T.B.; software, J.C.; formal analysis, J.C.; investigation, A.C and T.B.; resources, A.C.; data curation, T.M. and T.B.; writing—original draft preparation, R.W., A.V. and A.C.; writing—review and editing, A.V., R.W., T.M., J.C., T.B., A.V.H., H.G., I.V. and A.C.; visualization, R.W., A.V. and J.C.; supervision, A.C. and I.V.; project administration, A.C.; funding acquisition, A.C. and I.V. All authors have read and agreed to the published version of the manuscript. This work was supported by Kom Op Tegen Kanker (Stand Up to Cancer), the Flemish Cancer Society under grant 2016/10728/2603 to A.C., the Olivia Fund under grant 2017/LUF/00135 to A.C., Amgen Chair for Therapeutic Advances in Ovarian Cancer under grant 2017/LUF/00069 to I.V., and Research Foundation Flanders (FWO) under grant 12F3114N to A.C.

CONFLICTS OF INTEREST:

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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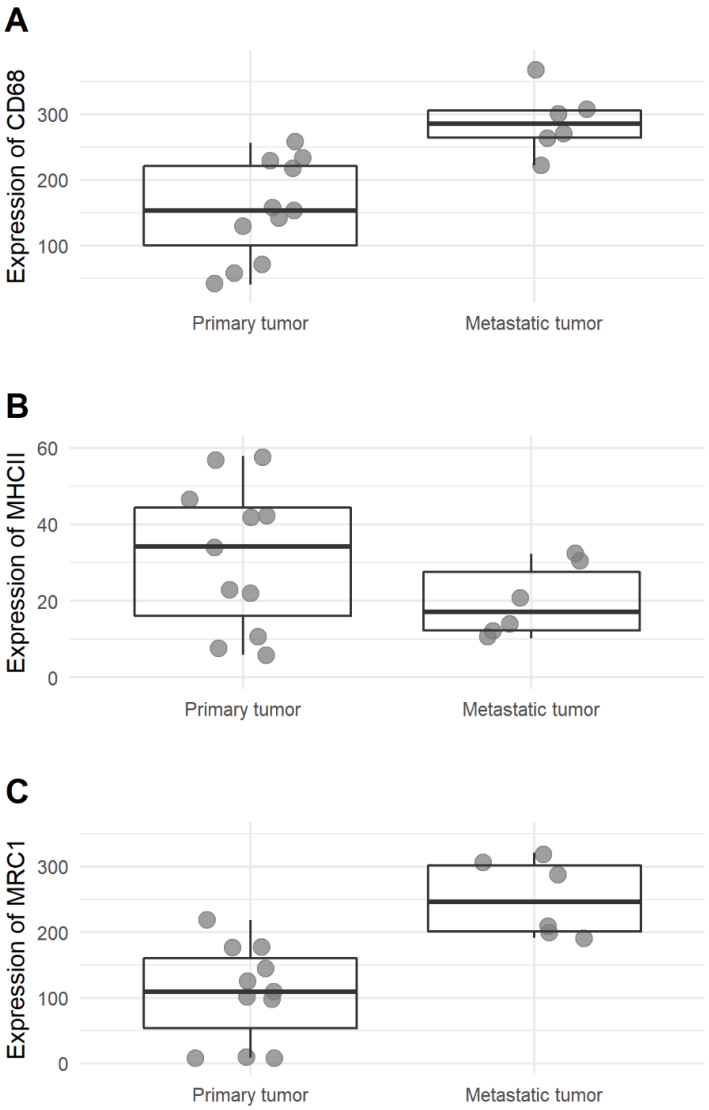
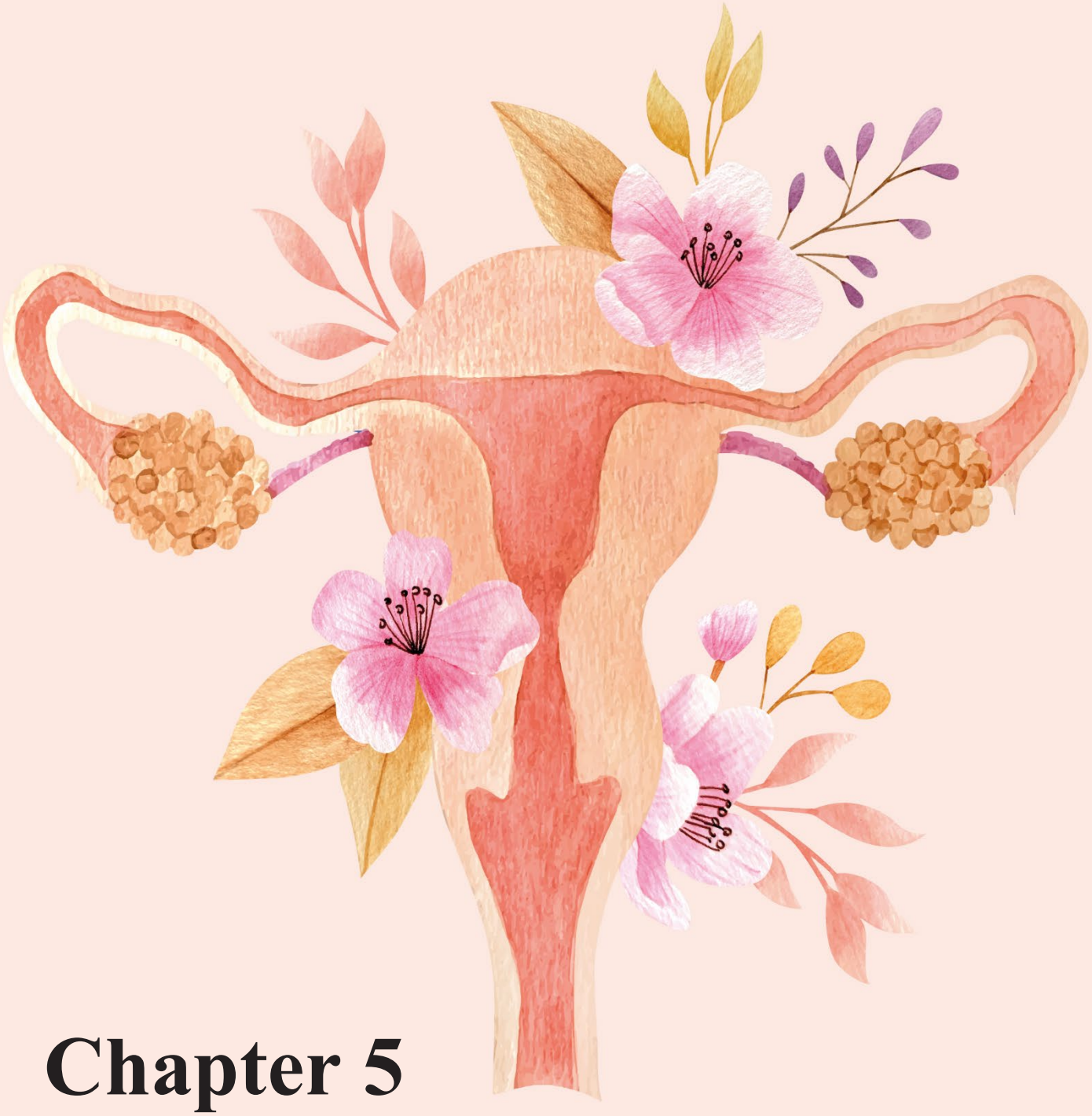


Supplementary Figure 1. Bulk analysis of primary and metastatic tumor site biopsies stained for (a) CD68, (b) MHCII, (c) MRC1.



Chapter 5

Type Of Chemotherapy Has Substantial Effects On The Immune System In Ovarian Cancer

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ABSTRACT

Chemotherapy induces a variety of immunological changes. Studying these effects can reveal opportunities for successful combining chemotherapy and immunotherapy. Immuno-chemotherapeutic combinations in ovarian cancer are currently not generating the anticipated positive effects. To date, only scattered and inconsistent information is available about the immune-induced changes by chemotherapy in ovarian cancer. In this study, we compared six common chemotherapeutics used in ovarian cancer patients (carboplatin, paclitaxel, pegylated liposomal doxorubicin, gemcitabine, carboplatin-paclitaxel and carboplatin-gemcitabine) and studied their effects on the immune system in an ovarian cancer mouse model. Mice received a single chemotherapy or vehicle injection 21 days after tumor inoculation with ID8-fluc cells. One week after therapy administration, we collected peritoneal washings for flow cytometry, serum for cytokine analysis with cytometric bead array and tumor biopsies for immunohistochemistry. Carboplatin-paclitaxel showed the most favorable profile with a decrease in immunosuppressive cells in the peritoneal cavity and an increase of interferon-gamma in serum. In contrast, carboplatin-gemcitabine seemed to promote a hostile immune environment with an increase in regulatory T-cells in tumor tissue and an increase of macrophage-inflammatory-protein-1-beta in the serum.

INTRODUCTION

Ovarian cancer (OC) is a highly aggressive cancer responsible for the death of 28 253 woman in Europe each year (Globocan). Standard-of-care consists of tumor debulking surgery and platin-based chemotherapy. Despite major advances in immunotherapy, ovarian cancer patients have disappointing response rates to immunotherapies in monotherapy and even in combinations [1–3]. Despite this failure, chemotherapy is still deemed as a useful alley to boost the efficacy of immunotherapies because of the immune-manipulating properties of chemotherapeutics. Chemotherapy can induce a wide range of effects on multiple levels that are already extensively investigated and reviewed elsewhere [4, 5]. Generally, we can summarize the effects into three main categories. A first and major effect of chemotherapy is the depletion of fast dividing cells, including the bone marrow progenitor cells and the active, proliferating immune cells at the tumor site. This can result in both immune suppression by inducing lymphopenia as well as immune activation since the immune suppressive network of both tumor cells and (immune suppressive) immune cells can be disrupted. A second effect of chemotherapy is the increased induction of (neo)antigens and the release of antigenicity during cell death. However, for ovarian cancer, O'Donnell *et al*. showed only a marginal effect of chemotherapy on increase of neoantigen expression of only 5% from the total of 78% more expressed neoantigens, indicating that other processes (e.g. mutagenesis) play a larger role in neoantigen formation [6]. A third effect is the induction of immunogenic-cell death, an increased availability of appropriate immunostimulatory signals to stimulate the anti-tumor immunity response [7].

However, the immunological changes induced by chemotherapy are not as straightforward as they may seem. The first difficulty is that there are different immune-effects when using different dosages of chemotherapeutics [8]. In a HM-1 ovarian cancer mouse model, it was shown that a dose dense chemotherapy (DD) schedule, compared to the maximum tolerated dose regimen, could preserve CD8+, CD4+ and CD11b+ cells, increase F4/80+ cell recruitment into the tumor and reduce the numbers of myeloid derived suppressor cells (MDSC) [9]. The immune related effect of DD schedule could also be correlated with an improved control of tumor growth. In patients with advanced or recurrent OC, the DD schedule was well-tolerated and had a better outcome, while maintaining a constant level of leucocyte numbers [10, 11]. The fact that chemotherapy will not only modulate the immune system but also its direct environment, makes the situation more complex. Moreover, the effect on the immune cells is also dual, since it can be an effect on the number of cells present but also an effect on the activity of the immune cell. Moschella *et al*. highlighted this in their study where they profiled genetic responses of peripheral blood mononuclear cells (PBMC) of OC tumor-bearing mice treated with cyclophosphamide and found in the bone marrow PBCM alone 1123 differentially regulated genes, added with another 1083 and 868 genes in respectively blood and spleen PBMC [12]. A last problem lies in the fact that the effects of one chemotherapeutic agent in one type of cancer cannot be generalized to other cancer types. A comparison of cisplatin or carboplatin treated ovarian and cervical cancer cell lines by Dijkgraaf *et al*. showed that the ovarian cancer cell lines COV413B, CAOV3, and cervical cancer cell lines HELA, CC8, CSCC7 upregulated their interleukin-6 (IL-6) expression upon treatment and subsequently skewed monocytes towards an M2-like phenotype. In contrast, the same

treatment on the other ovarian cancer cell lines (SKOV3, OVCAR3, A2780) or cervical cancer cell lines (CCSCC1, CAKI) did not increase IL-6 production or the skewing of monocyte differentiation [13]. Lastly, we and others have shown that not only attention should be given to the type of therapies combined, but also the order and timing, is crucial [11,14]. These remarks exemplify that a deeper knowledge of the mechanisms behind chemotherapy-driven immune changes is required. In ovarian cancer, this information is scattered and set-up of experiments are too heterogenous to draw conclusions (for review, see supplementary Table 1).

The goal of this research was to investigate and compare the immune modulating effects of the most commonly used chemotherapies in ovarian cancer patients. To this end, we used the ID8-fLuc ovarian cancer mouse model [15]. Like this, we hope to identify positive effects that can be used when designing immunotherapy-chemotherapy combinations.

MATERIAL AND METHODS

Ovarian cancer mouse model

Six to eight-week-old, female C57BL/6 mice (Envigo, Horst, The Netherlands) were intraperitoneally (ip) injected with 5 x 106 ID8-fLuc cells and randomly labelled. Approval of the ethical committee was obtained (P075/2014) and ethical standards (NIH guidelines for the Care and Use of Laboratory Animals) as well as the ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines were strictly followed [16,17]. Sample size was determined via a statistical power analysis, where five mice per treatment group had a power of 0.7320 to detect differences between groups. Mice were co-housed per five mice in individually ventilated cages (IVC) at the Specific-pathogen-free (SPF) facilities of the KU Leuven. An overview of the design of the experiment is given in Figure 1.

Chemotherapeutic treatments in mice

Different treatments were randomly allocated to the mice. All drugs were administered ip in the right flank of the mouse abdomen, three weeks after tumor inoculation. Controls were injected with Dulbecco's phosphate-buffered saline (DPBS, Thermofisher). If combinations of chemotherapies were required (for example carboplatin-paclitaxel), two separate administrations of each chemotherapeutic were given at the same moment. Carboplatin (Hospira, ONCO-TAIN) was dissolved in glucose 5% and administered ip in a dose of 100 mg/kg. Paclitaxel (AB, Aurobindo Pharma B.V) was diluted in NaCl 0,9% and administrated ip in a dose of 10 mg/kg. Gemcitabine (Hospira Benelux BVBA – BE390476) was diluted in NaCl 0,9% and administrated ip in a dose of 50 mg/kg in monotherapy and in a dose of 120 mg/kg when combined with carboplatin. Pegylated liposomal doxorubicin (PLD, Caelyx®, Janssens Cilag International NV)) was administered ip in a dose of 6 mg/kg. Animals which became severely ill due to chemotherapy toxicities (e.g. weight loss, diarrhea and cachexia) were sacrificed prematurely and were excluded from data analysis.

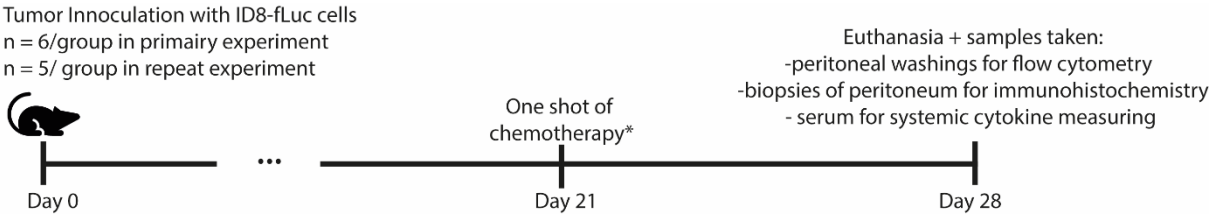

Figure 1: Overview of set-up of the experiments. *Group 1: carboplatin + gemcitabine, group 2: carboplatin + paclitaxel, group 3: Pegylated Liposomal Doxorubicin, group 4: carboplatin, group 5: paclitaxel, group 6: gemcitabine

Sampling of blood, peritoneal fluid and tumor tissue for immune monitoring

Immunological changes were evaluated one week after chemotherapy administration. This time point was chosen to study delayed immune effects, rather than the immediate effect as well as to increase the translational relevance of our experiments in correspondence to certain combinatorial immunotherapy trials where chemotherapy is started prior to immunotherapy (e.g. DUO-O (NCT03737643)). Mice were anesthetized with pentobarbital (200 mg/ml, Dolethal, Vetoquinol) prior to blood collection. Whole blood was centrifuged for ten minutes at 8000 rcf to obtain serum. Serum samples were aliquoted and stored at -80°C until cytokine quantification with cytometric bead array. Next, mice were euthanized and a peritoneal lavage was performed using 10 mL DPBS. The peritoneal washings were centrifuged for five minutes at 500 rcf. The cell pellet was resuspended and used in fluorescent activating cell sorting read out. Biopsies of the peritoneum for immunohistochemical staining were taken immediately after euthanasia of the mice and fixed by 4% paraformaldehyde.

Fluorescent-activated cell sorting (FACS)

The cell pellet was stained with eFluor780 fixable viability dye (Affymetrix Inc. San Diego, Ca, USA) in order to exclude dead cells. Next, surface markers were stained for myeloid cell markers and T-cell markers with monoclonal antibodies as described in supplementary table 2. In addition, intracellular markers were stained. For the myeloid panel, cells were permeabilized using Leucoperm (Bio-Rad Laboratories Inc., Kidlington, UK) in accordance to manufacturers' protocol and in addition stained for the intracellular marker CD206. Fluorescence Minus One technique was used to control for the gating of the marker CD206. For the T cell panel, cells were permeabilized using eBioscience Foxp3 / Transcription Factor Staining Buffer Set (Thermofisher Scientific, Waltham, Massachusetts, USA) and were stained for the intracellular marker FoxP3. All antibodies used were titrated for optimal concentration. Acquisition was performed using FACS DIVA software on the BD Canto II (BD bioscience). FlowJo Single Cell Analysis software (TreeStar, Inc., Ashland, OR, USA) was used to analyze the data.

Immunohistochemistry

Immunohistochemistry single marker stains were performed for CD8+ cytotoxic T-lymphocytes, FoxP3+ regulatory T-lymphocytes, Ly6C+ myeloid derived suppressor cells and F4/80+ macrophages as described earlier [18]. Rat anti-mouse CD8a clone 4SM15 (e-Bioscience 14-0808-82) was used in a 1:100 dilution. Rat anti-mouse Foxp3 (e-Bioscience 14-5773-82) was used in a 1:100 dilution.

Monoclonal Rabbit anti-mouse F4/80 (D2S9Rxp) (Bioké, Cell Signaling 70076s) was used in a 1:250 dilution. Rat anti-mouse Ly6C, clone ER-MP20 (ThermoFisherScientific MA1_8189) was used in a 1:200 dilution. Microscopic images were digitalized using the Zeiss Axio Slide Scanner using a x20 objective and ZEN2 software (Zeiss). Qupath was used for digital, manual analysis [19]. Per slide, four different region of interest (ROI) containing tumor tissue were selected. Positive cells were counted per ROI and a ratio was made of positively counted cells divided by the ROI in μm^2.

Cytometric bead assay

Serum samples were analyzed for cytokine expression. The following cytokines were measured using the cytometric bead assay technique (BD Biosciences, San Jose, CA, USA): interleukin (IL-) 1β, IL-6, IL-10, IL-12p70, monocyte chemoattractant protein-1 (MCP-1), granulocyte-macrophage colony-stimulating factor (GM-CSF), macrophage inflammatory protein 1α (MIP-1α), MIP-1β, interferon gamma (IFNγ), tumor necrosis factor (TNF). Undiluted samples were analyzed according to manufacturers' protocol using flex sets. Acquisition was performed on the BD Fortessa (BD Biosciences, San Jose, CA, USA). Cytokine analysis was performed using FCAP Array Software v3.0 (BD Biosciences, San Jose, CA, USA). In some treatment groups the concentration of the measured cytokines was too low to detect following the CBA-standard curve and manufactures threshold levels. Values below this threshold were excluded for statistical analysis.

RESULTS

Gemcitabine, paclitaxel and carboplatin-paclitaxel reduce immunosuppression in ascites

After administration of carboplatin, paclitaxel, gemcitabine and paclitaxel as a monotherapy, we observed that with carboplatin the amount of immune suppressive monocytic MDSC (mMDSC) increased 3,5 times. In contrast, the polymorphonuclear MDSC (PMN-MDSC) showed an almost equal decrease of 3,7x. In addition, carboplatin monotherapy caused 3,2x more of type 2 pro-tumoral macrophages (M2-like) present in the peritoneal cavity compared to control-treated tumor bearing mice (Fig 2A). Paclitaxel monotherapy induced a 12,6x decrease in type I anti-tumoral macrophages (M1-like TAMs), displaying an inflamed phenotype, compared to control-treated tumor bearing mice (Fig 2B). Upon treatment with gemcitabine, a decrease of approximately 3x fewer M1-like TAMs and 2,3x fewer Treg were detected (Fig 2C). PLD induced a decrease of 5,9x less M1-like TAMs and 2,4x less Treg (Fig 2D). The combination of carboplatin-gemcitabine treatment resulted in on average 5,7x more mMDSC and 5,2x less M1-like TAMs (Fig 2E). The combination of carboplatin-paclitaxel did not induce any significant changes in the immune composition (Fig 2F). In addition, we observed that carboplatin, PLD and gemcitabine in monotherapy resulted in a significant decrease of the M1/M2-like TAMs ratio (Fig 3D-F). None of the chemotherapeutic agents were able to induce a relevant change in the CD8/Treg ratio (Fig 3A-C).

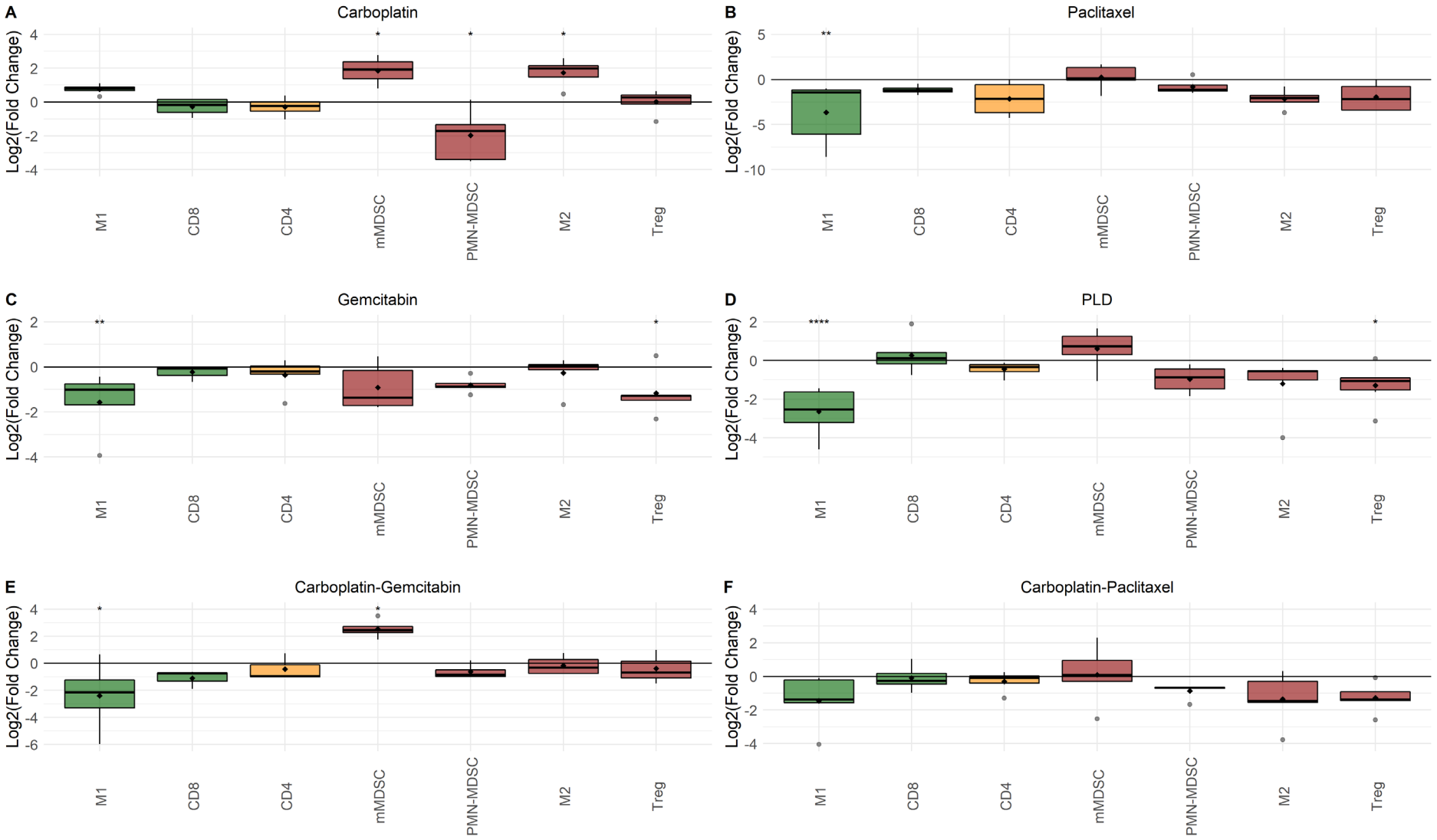

Figure 2: Fold change (transformed with base log2) of immune profile one week after chemotherapy administration versus tumor bearing non-treated mice. [continued next page]

Figure 2 [continued]: A: Carboplatin induces an increase in mMDSC (mean = 1,8, *p<0.05), a decrease in PMN-MDSC (mean = -1,9, **p<0.01) and an increase in M2-like (mean = 1,7, * p<0.05). B: Paclitaxel induces a mean 3,65-fold decrease in M1-like TAMs (**p<0.01). C: Gemcitabine induces a mean 1,57 decrease in M1-like TAMs (**p<0.01) and a mean 1,17 decrease in Treg (*p<0.05). D: PLD induces a 2,647-fold decrease in M1-like TAMs (****p<0,0001) and a 1,29-fold decrease in Treg (*p<0.05). E: Carboplatin-Gemcitabine shows a significant 2,54-fold increase in mMDSC (*p<0.05) and a 2,4-fold decrease in M1-like TAMs (*p<0.05). F: Carboplatin-Paclitaxel shows no significant differences in immune profile. The fold change is calculated by dividing the amount of immune cells (as a percentage of their parent-population) in mice one week after chemotherapy (individual values) by the amount of immune cells in control mice (mean of the group). PLD = Pegylated liposomal doxorubicin. All tests are one-way ANOVA. Grey dots represent outliers, black squares in bar plot represent mean, line represents median. (N=6 per group in first experiment, N=5 per group in repeat experiment).

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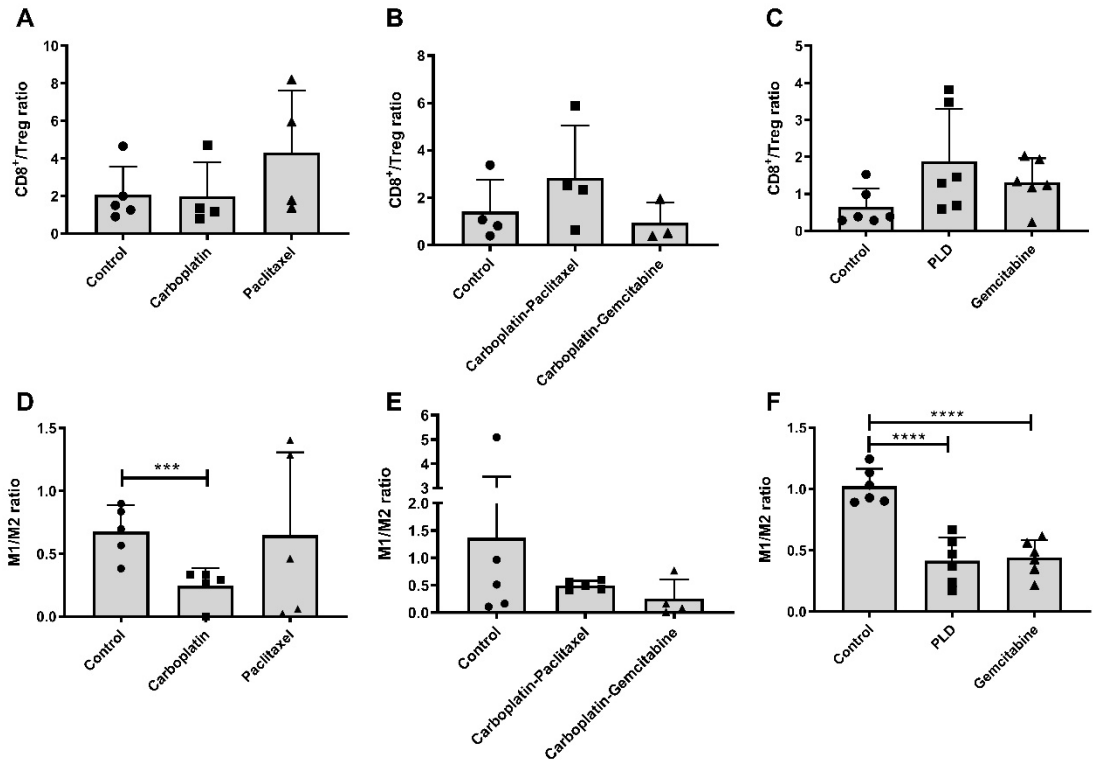

Figure 3: CD8+/Treg and M1-like/M2-like TAMs ratios to evaluate immune profile after chemotherapy administration evaluated by flow cytometry. A-C: no significant differences when the CD8+ /Treg ratio is made for all the different chemotherapies. D: Carboplatin shows a significant lower M1-like/M2-like TAMs ratio (p=0,0079, Mann-Whitney U test). E: no significant differences in M1-like/M2-like TAMs ratios for the chemotherapeutic combinations of Carboplatin-Paclitaxel and Carboplatin-Gemcitabine. F: Significant lower M1-like/M2-like TAMs ratio when PLD is compared to control-treated mice (p<0,0001, unpaired student t-test) and Gemcitabine is compared to control

treated mice (p<0,0001, unpaired student t-test). PLD = Pegylated liposomal doxorubicin. Ratios were calculated using number of immune cells as percentage of parent-population. (N=6 Per group in first experiment, N=5 per group in repeat experiment (results of repeat experiment shown)).

Carboplatin-gemcitabine increases regulatory T cells in tumor tissue

Apart from carboplatin-gemcitabine that induced a significant increase in FOXP3+ cells, we recorded no statistically significant changes in the intratumoral immune composition, after administration of any chemotherapy regimens (Figure 4).

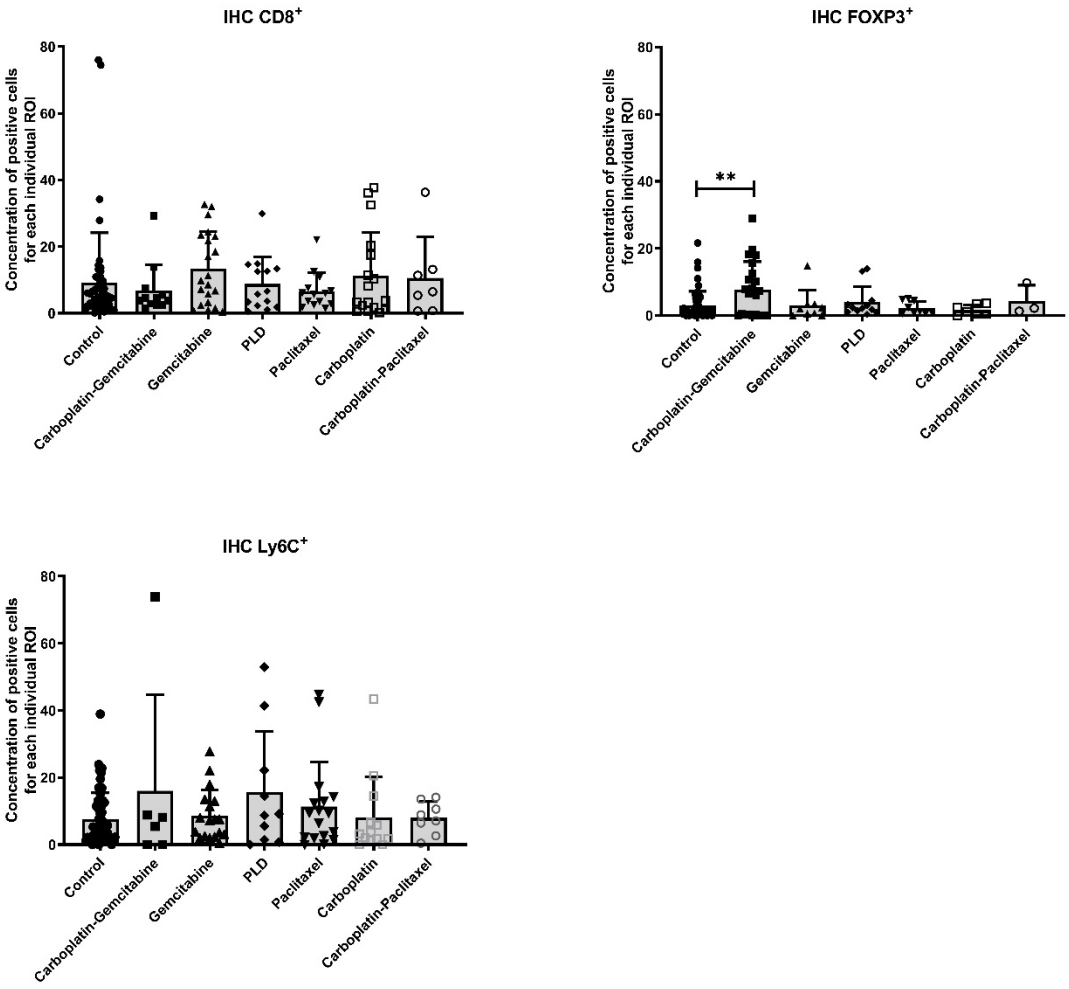

Figure 4: Immunohistochemical analysis of peritoneal biopsies. Carboplatin-Gemcitabine treated mice show an increase in intratumoral FoxP3+ cells (p-adj=0,0014, one-way ANOVA). No significant intratumoral differences could be noted in CD8+ and Ly6C+ cells. PLD = pegylated liposomal doxorubicin. (N=6 per group in first experiment, N=5 per group in repeat experiment, four different regions of interest where measured in each individual sample (results of initial experiment shown)).

Carboplatin-gemcitabine increases the concentration of Macrophage Inflammatory Protein 1 beta

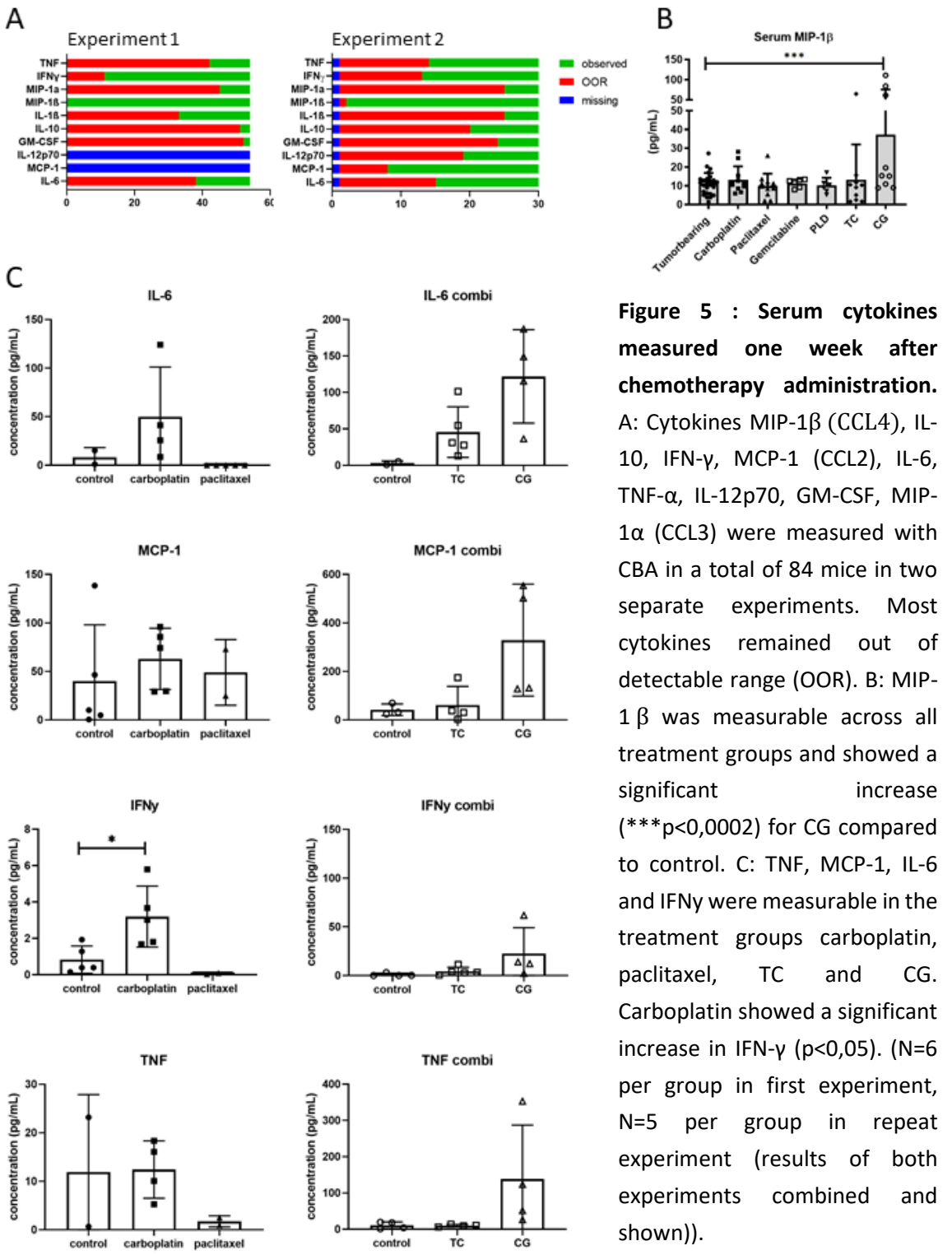

Figure 5 : Serum cytokines measured one week after chemotherapy administration. A: Cytokines MIP-1β (CCL4), IL-10, IFN-γ, MCP-1 (CCL2), IL-6, TNF-α, IL-12p70, GM-CSF, MIP-1α (CCL3) were measured with CBA in a total of 84 mice in two separate experiments. Most cytokines remained out of detectable range (OOR). B: MIP-1 β was measurable across all treatment groups and showed a significant increase (***p<0,0002) for CG compared to control. C: TNF, MCP-1, IL-6 and IFNy were measurable in the treatment groups carboplatin, paclitaxel, TC and CG. Carboplatin showed a significant increase in IFN-γ (p<0,05). (N=6 per group in first experiment, N=5 per group in repeat experiment (results of both experiments combined and shown)).

P-values were computed by comparing treated cytokine values to control group, via one-way ANOVA.

Serum cytokines were measured in a total of 84 mice over two independent experiments. However, most cytokine concentrations remained out of detectable range (OOR) (Fig 5A). The cytokines MIP-1a, IL-1B, IL-10, GM-CSF and IL-12p70 were not detectable in over half of the samples and therefore excluded from further statistical analysis. The only cytokine measured repeatedly was MIP-1b, which showed a significant increase in mice treated with carboplatin-gemcitabine (Fig 5B). Cytokines TNF, MCP-1, IL-6 and IFNy could be detected in the majority of mice treated with carboplatin, paclitaxel, carboplatin-paclitaxel and carboplatin-gemcitabine. Overall, carboplatin-gemcitabine treatment induced a major increase in all studied serum cytokines, whereas paclitaxel barely induced any changes. Carboplatin in monotherapy induced a significant increase IFNy.

DISCUSSION

To the best of our knowledge, information on the effect of chemotherapy on the immune system in ovarian cancer bearing mice was only limitedly available. In our study, we evaluated the effects at the tumor tissue level, in ascites and in blood. Our results demonstrate favorable changes of the immune system by carboplatin-paclitaxel (decrease of immunosuppressive cells in ascites and increase of IFNy in serum) and a worsening of the immune microenvironment by carboplatin-gemcitabine (increase in Treg in tumor tissue and increase in MIP1 alpha in serum) (Figure 6). The effects of chemotherapy in monotherapy are either absent or only present at one read out level and therefore represent most likely a less strong influence on the immune system. These findings are crucial in our understanding of the immune system in ovarian cancer and for the design of preclinical experiments, combining chemotherapy and immunotherapy, that should precede clinical trials.

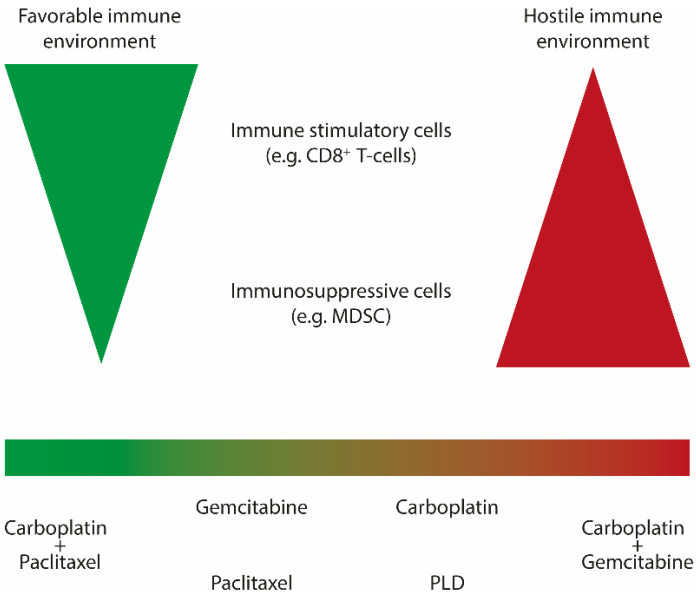

Figure 6. Outline displaying the compared chemotherapeutics and their overall immune effect.

To date, no immunotherapeutic strategies have been implemented successfully in the routine treatment of ovarian cancer, despite prove that ovarian cancer is indeed an immune responsive malignancy. A first milestone was set by Zhang *et al*. who showed that T-cell infiltration correlated with an improved 5-year survival of 73.9% versus only 11.9% for patients without. Both the KEYNOTE-100 (Phase II in advanced recurrent ovarian cancer) and the JAVELIN Solid tumor (ovarian cohort) trial using pembrolizumab and avelumab were therefore promising candidates, but ended up with disappointing results, yielding overall response rates of less than 10% in monotherapy [20,21]. Part of this failure could be attributed to the high levels of immune suppression present in ovarian cancer, represented by Treg, MDSC and M2. Indeed, in ovarian cancer, this influx of innate immune suppressive cells has been correlated with worsened survival [18,22]. Moreover, these cells are not the subject of current immunotherapeutic strategies. . Therefore, part of the solution could lie in a strategic combination of chemotherapy, immunotherapy and targeted therapy combinations to overcome immunosuppression. Knowledge about the immune-effects of chemotherapy is crucial in designing these combinations. Our data show that carboplatin-gemcitabine seems to create an increase in

immune suppression as it endorses the innate immune suppressive cells mMDSC and decreases the numbers of M1-like TAMs. In addition, carbo-gemcitabine increases the serum protein levels of MIP-1β. Giuntoli *et al*. found elevated levels of MIP-1β in both ascites and serum samples of patients at the time of primary cytoreductive surgery [23]. Taken this together, carboplatin-gemcitabine does not seem to be the chemotherapeutic of choice to be used in combination strategies. For PLD chemotherapy, we found an induction of a low M1-like/M2-like TAMs ratio. Clinical data in ovarian cancer has demonstrated that a low M1-like/M2-like TAMs ratio was associated with a worse prognosis [24], making these effects also undesirable. Gemcitabine in monotherapy decreased the number of Treg, but also exhibited a lower M1-like/M2-like TAMs ratio. This unfavorable lower M1-like/M2-like TAMs ratio, could impede the positive effects of lower Treg numbers, making also this chemotherapeutic a lesser choice in combination strategies. Also carboplatin in monotherapy showed the same unfavorable lower M1-like/M2-like TAMs ratio while in serum we detected increased IFNγ levels. Although IFNγ is generally regarded as a proinflammatory cytokine, it has been shown that IFNγ upregulates the expression of PD-L1 in ovarian cancer cells and thereby can inhibit an anti-tumoral immune response [23]. An additional important observation is that the immune modulating effect of dual chemotherapies (carboplatin-paclitaxel or carboplatin-gemcitabine) is not a summation of these effects of the chemotherapies in monotherapy.

In contrast to literature, we do not see a toxicity towards MDSC under gemcitabine treatment in mice [25–27]. One explanation for this discrepancy is that the mouse models used in these studies were subcutaneous tumor models of breast cancer and pancreatic cancer, where they studied mostly the presence of MDSC at the level of the spleen, which is in contrast to our work, based on an orthotopic mouse model and the evaluation of MDSC in ascites. However, it is also true that not all tumors respond equally and this highlights once more the important differences between the immune biology of the different tumors. In contrast to Peng *et al*., we did not found an increase in CD4+ and CD8+ cells after paclitaxel treatment. In general, we did not see many immune changes on an intratumoral level. Only the carboplatin-gemcitabine treatment could induce an increase in FOXP3+. A possible explanation for these contrasting results is that both the paclitaxel dose and the time point of analyses differs [28]. Another explanation can be found in the fact that the immune composition is different between different metastatic biopsies in ovarian cancer, as is demonstrated in three recent papers [29–31]. Therefore, we should be cautious with interpreting and extrapolating results from a single and relatively small tumor biopsy, as it may not be representable for the whole tumor or the general immune condition of the patient.

We would like to acknowledge some important limitations to this study. We recognize the absence of data on the activation status and functional capacities of the immune cells, as our study focused on exploring shifts in numbers of immune cells caused by the different chemotherapies, and that the immune system was only evaluated at one time point. This time point was chosen to see the delayed immune effects of the chemotherapy, since evidence already exists that chemotherapy can directly stimulate effector functions of several immune cells [32]. However, more time points should be investigated when looking for the optimal therapeutic windows. Another limitation is that we

investigated the effect of only one chemotherapy administration, while in a clinical setting, patients will receive multiple cycles of chemotherapy.

We believe our findings are crucial to design combinatorial immunotherapy trials. At present, it is not known how to combine chemotherapy and immunotherapy. Preclinical evidence is lacking and clinical immunotherapy studies have been stopped prematurely or were negative [20,21,33]. Besides the fact that immune monitoring in clinical immunotherapy trials will be crucial to understand how the immune biology of patients is altered [34], it will be necessary to preclinically test combination regimens. To successfully combine therapies, the knowledge of the immune modulating effects of chemotherapy is crucial.

In conclusion, we have provided information on the immunological effects of chemotherapy in an ovarian cancer mouse model. Based on these results, carboplatin-paclitaxel induced a superior immune profile to that of paclitaxel, carboplatin, gemcitabine or carboplatin-gemcitabine and therefore could be considered for future combined chemotherapy-immunotherapy treatment regimes. We are thus hopeful and patiently awaiting results of some of the newer trials (e.g. DUO-O (NCT03737643), FIRST (NCT03602859) or ATHENA (NCT03522246)), who are studying in a first-line setting the combination of checkpoint inhibitors with carboplatin-paclitaxel.

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SUPPLEMENTALS

Supplementary Table 1.					
Chemotherapeutic agent	Human / mouse	Setting	Analysing method	Effect	Author
paclitaxel (10 mg/kg) and carboplatin (32.5 mg/kg)	mouse	*In vitro* studies on MA-148 and SKOV3 - *in vivo* nude mice bearing i.p MA-148 tumors	Reverse transcription PCR + Immunohistochemistry	*In vitro* MA-148 and SKOV-3 cells treated for 24 hours with paclitaxel, carboplatin or a combination showed increased expression of (MCP-1). Mice treated with one dose of paclitaxel and carboplatin showed also an increase of MCP-1, compared to control. However, no correlation could be found on IHC regarding macrophage infiltration or vessel density.	Geller *et al.* 2010
cisplatin	mouse	2F8 and 2F8 cisplatin resistant mouse model	immunohistochemistry	2F8 mice displayed decrease in CD8+ cells as well as PD-1/L1 expression.	Grabosch *et al.* 2019
carboplatin (15mg/kg) + paclitaxel (20mg/kg)	mouse	orthotopic mouse model ID8-VEGF-defb29 received a single shot of chemo 8 days after inoculation	flow cytometry and gene expression	Acute immune suppression and increase of PMN-MDSC population.	Hartl *et al.* 2019
paclitaxel/ docetaxel or carboplatin/cisplatin	mouse	ID8 mouse model	immunohistochemistry	Increase in CD4+ and CD8+ cells.	Peng *et al.* 2015

platinum-based NACT	human	pre- and posttreatment omental biopsies and blood samples from 54 OC patients undergoing platinum-based NACT	immunohistochemistry	Comparison of pre- versus post-NACT. No changes in CD3+ cells percentage of CD45+ cells or CD4/CD8 ratio. More omental CD4 and CD8 cells were able to produce IFNγ compared with peripheral T cells from healthy controls.	Böhm *et al.* 2016
carboplatin + paclitaxel	human	Advanced epithelial OC (III or IV)	flow cytometry of PBMC samples	CD3+CD62L- cells were increased during chemotherapy. Relative proportions of naïve, central and effector memory T-cells remained unchanged during chemotherapy.	Coleman *et al.* 2005
cisplatin or carboplatin	human	*In vitro* cell culture	flow cytometry	Co-culturing tumor cells and monocytes in platinum-treated conditions resulted in an increased percentage of M2-like macrophages.	Dijkgraaf *et al.* 2013
6 cycles of gemcitabine 1000 mg/m2	human	SOC (FIGO stage II-IV), platin resistant patients	Flow cytometry on PBMC	2/3 patients completed the treatment. Both patients showed a decrease after already 2 cycles of treatment but also at the end, in HLA-DR- myeloid cells and MDSC.	Dijkgraaf *et al.* 2015
Topotecan (A/ only, B/ in association with carboplatin and taxanes, C/ as second line after platinum-based CT)	human	14 EOC patients either at diagnosis or relapsed after platinum-based CT	flow cytometry of PBMC samples	At baseline: CD2+, CD3+ and CD4+ lymphocyte subsets were significantly lower in CT pre-treated patients, but not in CT-naive patients compared to a control cohort of 20 healthy donors. In addition, CT-pre-treated patients showed decreased CD4/CD45RA+ and CD4/CD45RO+ subsets, while again normal in CT-naïve patients. During and after discontinuation of topotecan CT, no relevant changes could be reported in either groups.	Ferrari *et al.* 2002

Carboplatin + paclitaxel (possibly with PARP inhibitor or bevacizumab n=7/21)	human	Five NACT OC patients (compared with patients how underwent primary surgery n=16)	immunohistochemistry	No differences in PD-L1 expression in TAMs between patients who received primary surgery versus NACT. 4/5 patients treated with NACT possessed PD-L1+ TAMs in both primary tumor or metastatic implant.	Gottlieb *et al*. 2017
38/39 patients platinum based (cisplatin or carboplatin, with cyclophosphamide or paclitaxel), 1/39 paclitaxel + cyclophosphamide	human	previously untreated, EOC (FIGO I-IV) patients undergoing primary surgery and show decreased granulocyte count after first chemotherapy course	flow cytometry of PBMC samples	A decrease in number and activity of natural killer (NK) cells in PBMC at day 7, 14 and 21 compared to baseline. The Th1/Th2 ratio decreased significantly from baseline at day 17 and 21 after chemotherapy. Superoxide anion production by granulocytes showed a decrease at day 14 after chemotherapy.	Hidaka *et al*. 2003
paclitaxel (135 or 175 mg/m2)	human	Mix of ovarian (n=6) and breast (n=15) cancer patients	PBMC numbers	Estimated time course of leukopenia showed a decline of WBC until day 11 after treatment, gradually increase until day 23 to reach pre-treatment levels. However, high inter-individual variation is shown.	Karlsson *et al.* 1998
"chemotherapy"	human	44 EOC patients (FIGO stage I-IV)	flow cytometry of PBMC samples	plasmacytoid dendritic cells (pDC) blood levels restored after 4 cycles of chemotherapy if patients, which remained in patients with complete response, but decreased again in patients with progressive disease.	Labidi-Galy *et al*. 2011
carboplatin and paclitaxel	human	retrospective tumor samples pre- and post NACT from omentum,	immunohistochemistry	increase in T- and B-cell infiltrates as well as PD-1+ TILs	Lo *et al*. 2017

		pelvic or extra-pelvic sites (e.g. uterus, colon)			
in 97% of patients carboplatin-paclitaxel	human	pre-NACT, post-NACT or relapse tumor samples	immunohistochemistry	Increase in stromal TILs, intraepithelial TILs remained unchanged.	Mesnage *et al*. 2017
Carboplatin (n=6), taxol (n=1), carboplatin+taxol (n=54, *3 also treated with bevacizumab, 7 also enrolled in the ICON8 rial)	human	Unmatched omental biopsies pre- and post chemotherapy of HGSOC (FIGO III-IV).	immunohistochemistry	No difference in number of B-cells in pre- and post-NACT omental biopsies, but a significant increase in the proportion of class-switched memory B cells (CD27+IgM-) in metastases from NACT treated patients versus treatment naïve patients. In addition, IgG3 immunoglobulins were increased in post- versus pre-treatment biopsies.	Montfort *et al*. 2017
carboplatin-based chemotherapy	human	mixed histology (of relapsed) OC patients (FIGO III-IV)	flow cytometry of blood samples	Both relapsed or NACT treated patients interval debulking surgery (IDS) showed a significant decrease in regulatory T-cells compared with non-tumorbearing controls who underwent surgery. CD8 T cells before surgery were similar in all groups and no significant modifications could be observed in the post-operative period, while an increase in CD8+ cells was observed in woman treated with NACT	Napoletano. *et al*. 2010

carboplatin or cisplatin, or etopside, or gemcitabine or paclitaxel	human	35 of 114 samples were collected after exposure to chemotherapy; 14 are matched with an untreated sample from the same patient.	RNA-seq on immune infiltrate	Samples taken before or after chemotherapy were similar in immune infiltrate.	O'Donnell et al. 2018
6 cycles of Carboplatin (AUC5) with paclitaxel (175 mg/msq), every three weeks	human	5 OC patients, each with different histology	flow cytometry on PBMC	Over the different courses, the total T-cell fraction increased from course one, highest levels after course four. Compared to healthy control, OC patients displayed high numbers of Treg which fluctuated during the courses but overall, never decreased. No significant changes could be noted pre versus post chemotherapy in the ratio's: CD4+ effector/CD4+ Treg, CD38+CD4+ effector/CD38+CD4+ Treg, CD38+CCR4+CD4+ effector/CDCD38+CCR4+CD4+ Treg, CD8+ effector/Treg, CD8+ memory/CD4+ Treg.	Park *et al.* 2012
carboplatin (AUC 5) and docetaxel (75mg/m2) prior	human	ovarian epithelium specimens prior and after NACT	immunohistochemistry	low level of homogeneity and broad inter-individual differences for stained cell density. The mean number of infiltrating CD4, CD8 and granzyme B cells enhanced after NACT.	Pölcher *et al.* 2010

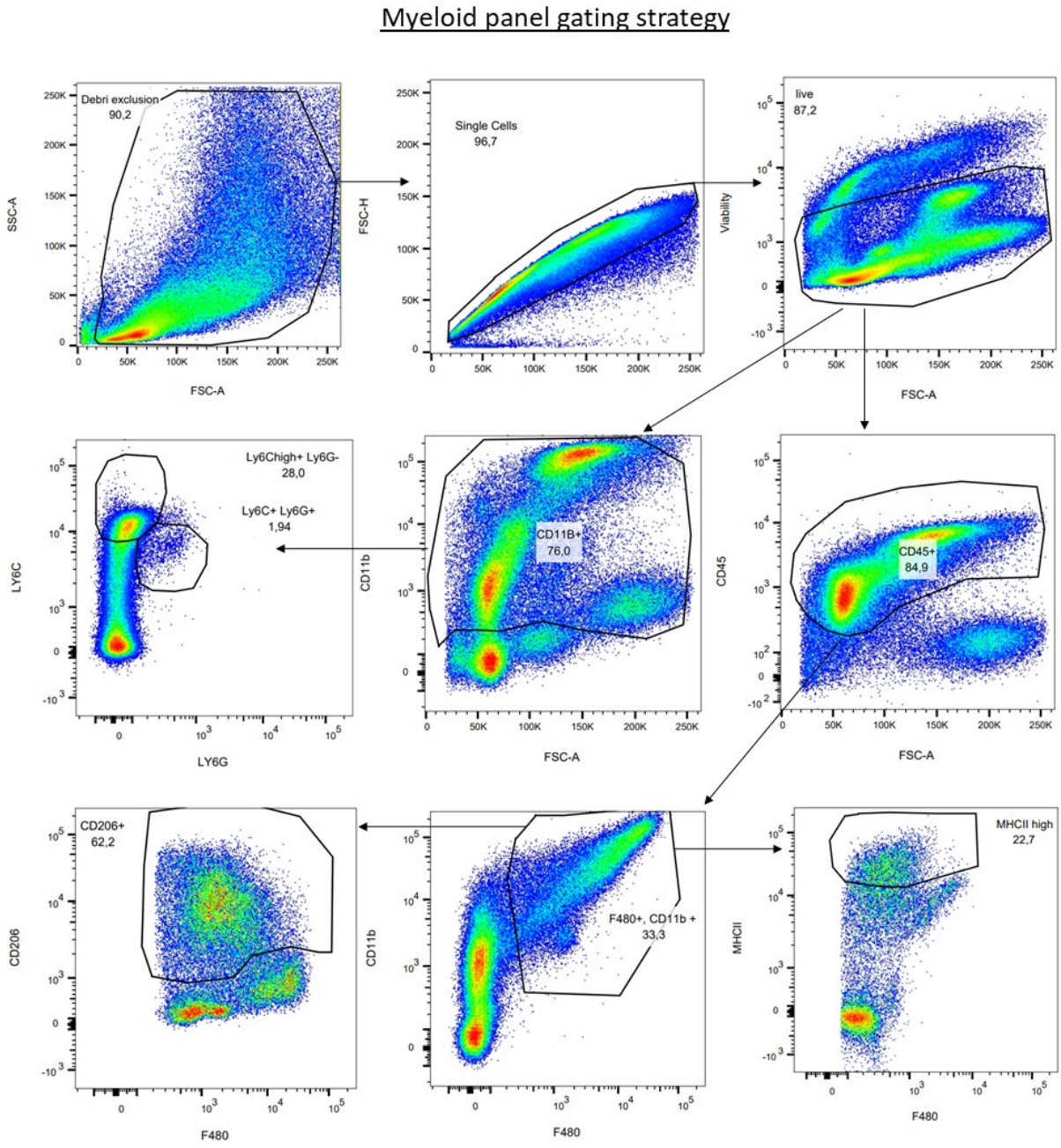
platinum-taxanes first line and taxane-carboplatin or anthracyclines-cisplatin or topotectan as second line	human	82 mixed-histology OC patients (FIGO II-IV)	immunohistochemistry	CD4+/CD8+ ratio decreased gradually from baseline 1,69, to 1,06 after two years, as does the total lymphocyte/mm3.	Recchia *et al.* 2005
18/23 carboplatin-paclitaxel, 5/23 received carboplatin only	human	HGSOC (FIGO I-IV) patients	immunohistochemistry	No significant increase in tumor-infiltrating-lymphocytes was seen in the NACT-treated patients compared to patients receiving primary cytoreductive surgery.	Scurry *et al.* 2018
platinum-based chemotherapeutic regimen	human	HGSOC (FIGO II-IV) patients receiving NACT (n=84)	immunohistochemistry	Comparison the NACT cohort to patients receiving primary cytoreductive surgery revealed no difference in CD8+ infiltration, nor any differences in CD27+ cells per mm2 tumor.	Wouters *et al.* 2016
carboplatin (AUC 5) + paclitaxel (175mg/m2)	human	13 primary EOC III/IV patients	flow cytometry of blood samples at different points pre and post chemotherapy	Two weeks after chemotherapy compared to before treatment: decrease in Treg cells and increase in proportions of Th1, Tc1, CD45RO memory T, NKT cells and IFN-γ secreting CD8+ cells. No differences were found on other time points.	Wu *et al.* 2010

Abbreviations: neoadjuvant chemotherapy (NACT), peripheral blood mononuclear cells (PBMC), interferon-gamma (IFNγ), Programmed cell death protein 1 (PD-1), Programmed death-ligand 1 (PD-L1), polymorphonuclear myeloid-derived suppressor cells (PMN-MDSC), International Federation of Gynecology and Obstetrics (FIGO), tumor infiltrating lymphocytes (TILs), Natural Killer T (NKT) cells

Supplementary Table 2: FACS panel for immune monitoring

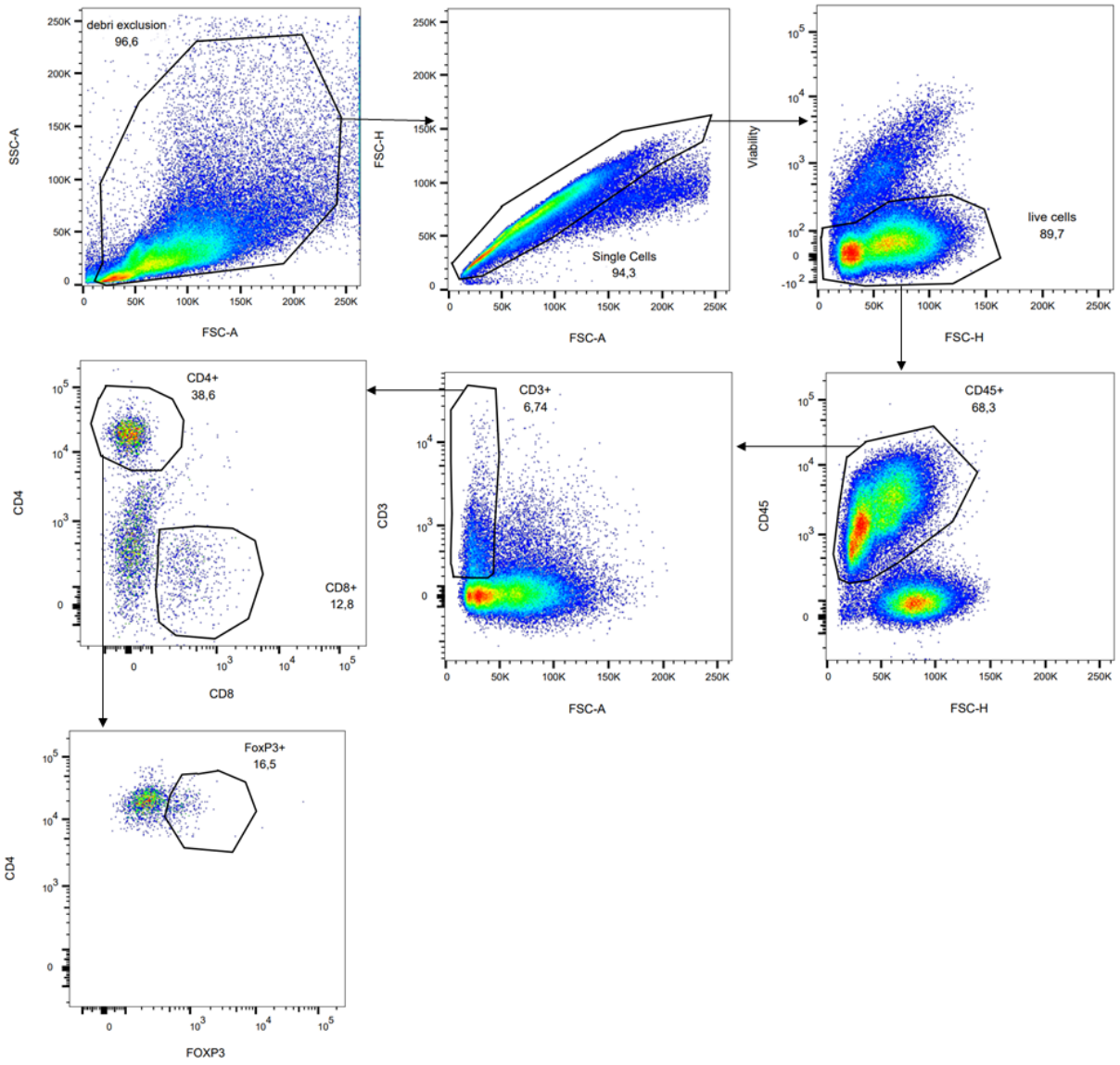
Myeloid derived suppressor cells and macrophages			
Target	Clone	Fluorophore	Supplier
CD11b	M1/70	PerCp Cy5.5	BioLegend (San Diego, Ca, USA)
CD45	104	APC	eBiosciences (San Jose, CA, USA)
Ly6G	1A8	Fitc	BD biosciences (San Jose, CA, USA)
Ly6C	HK1.4	APC-efluor780	eBiosciences (San Jose, CA, USA)
MHC II (I-A/I-E)	M5/144.15.2	PE-Cy7	eBiosciences (San Jose, CA, USA)
F4/80	T45-2342	BV421	BD biosciences (San Jose, CA, USA)
CD206	C068C2	PE	BioLegend (San Diego, Ca, USA)

T-cells			
Target	Clone	Fluorophore	Supplier
CD45	104	APC	eBiosciences (San Jose, CA, USA)
CD3	145-2C11	APC eFluor780	eBiosciences (San Jose, CA, USA)
CD4	RM4-5	PerCP Cy5.5	eBiosciences (San Jose, CA, USA)
CD8	53-6.7	BV421	BD biosciences (San Jose, CA, USA)
FoxP3	R16-715	AF488	BD biosciences (San Jose, CA, USA)

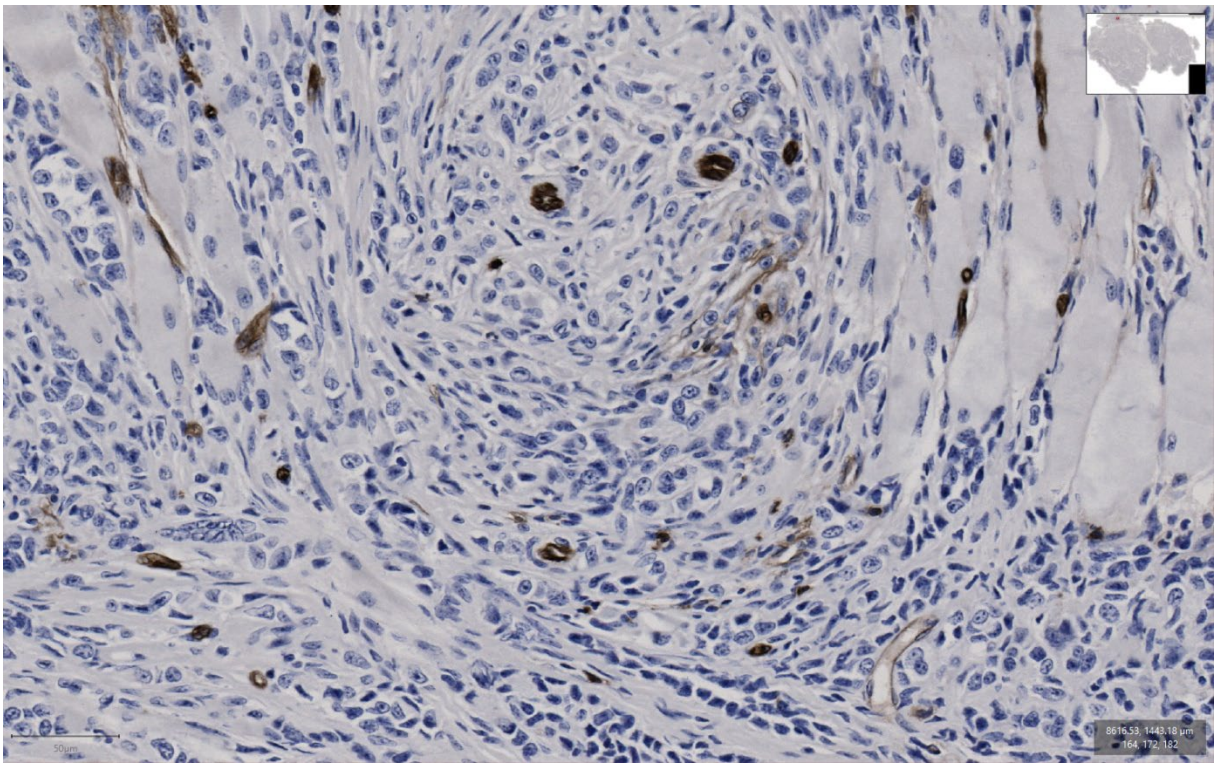


Supplementary Figure 1. Example gating strategy for myeloid cell analysis of flow cytometry data in FlowJo.

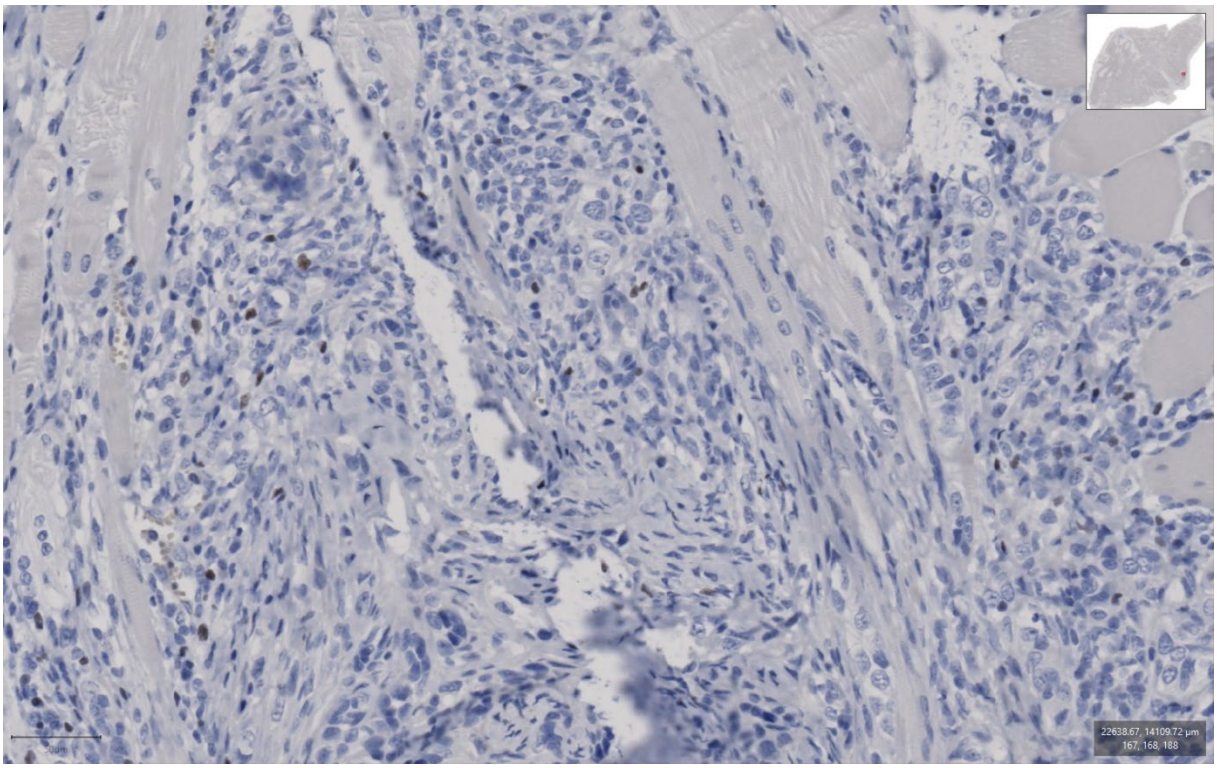
Tcell panel gating strategy



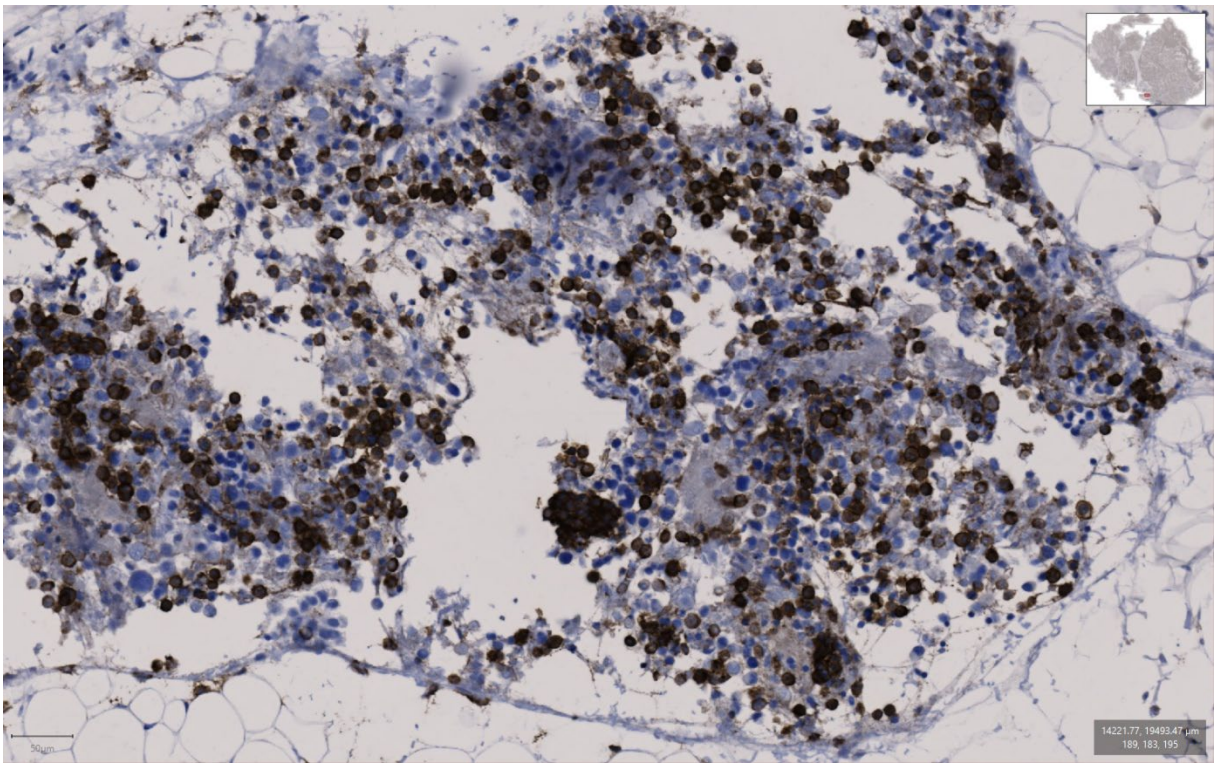
Supplementary Figure 2. Example gating strategy for Tcell analysis of flow cytometry data in FlowJo.



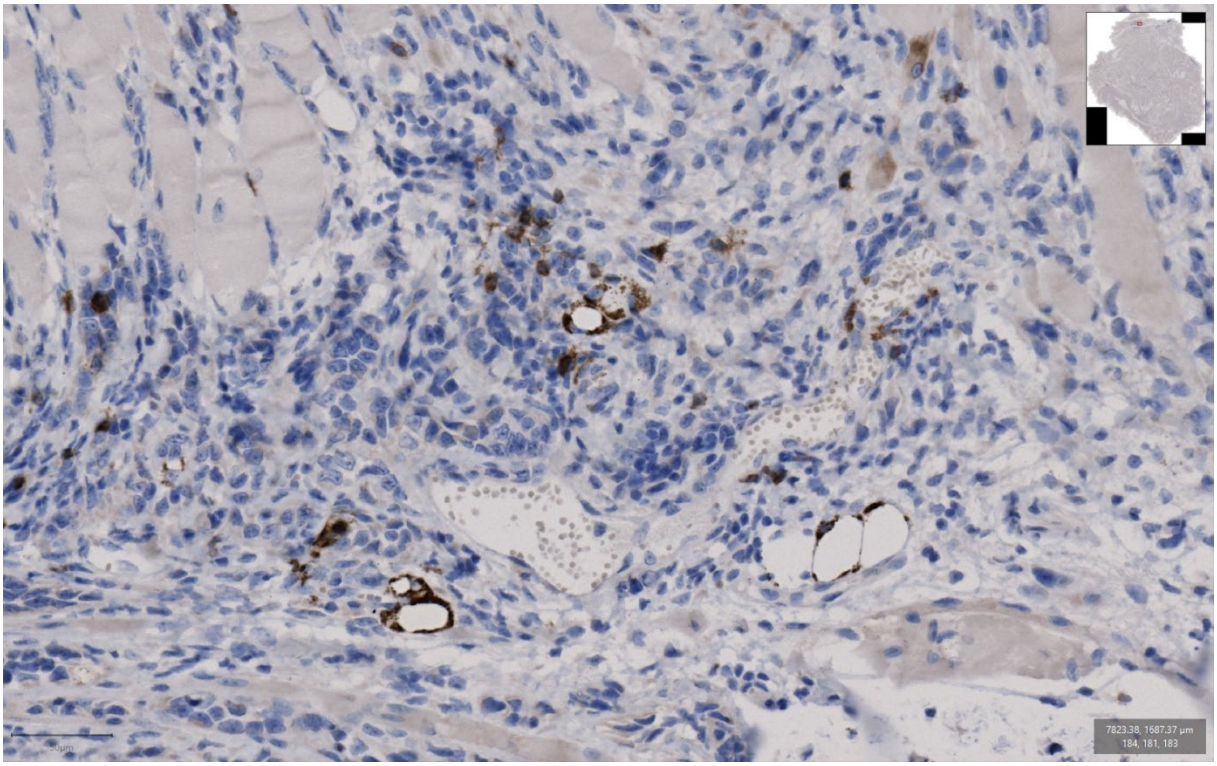
Supplementary Figure 3. Example of immunohistochemical staining of Ly6C^{+} cells.



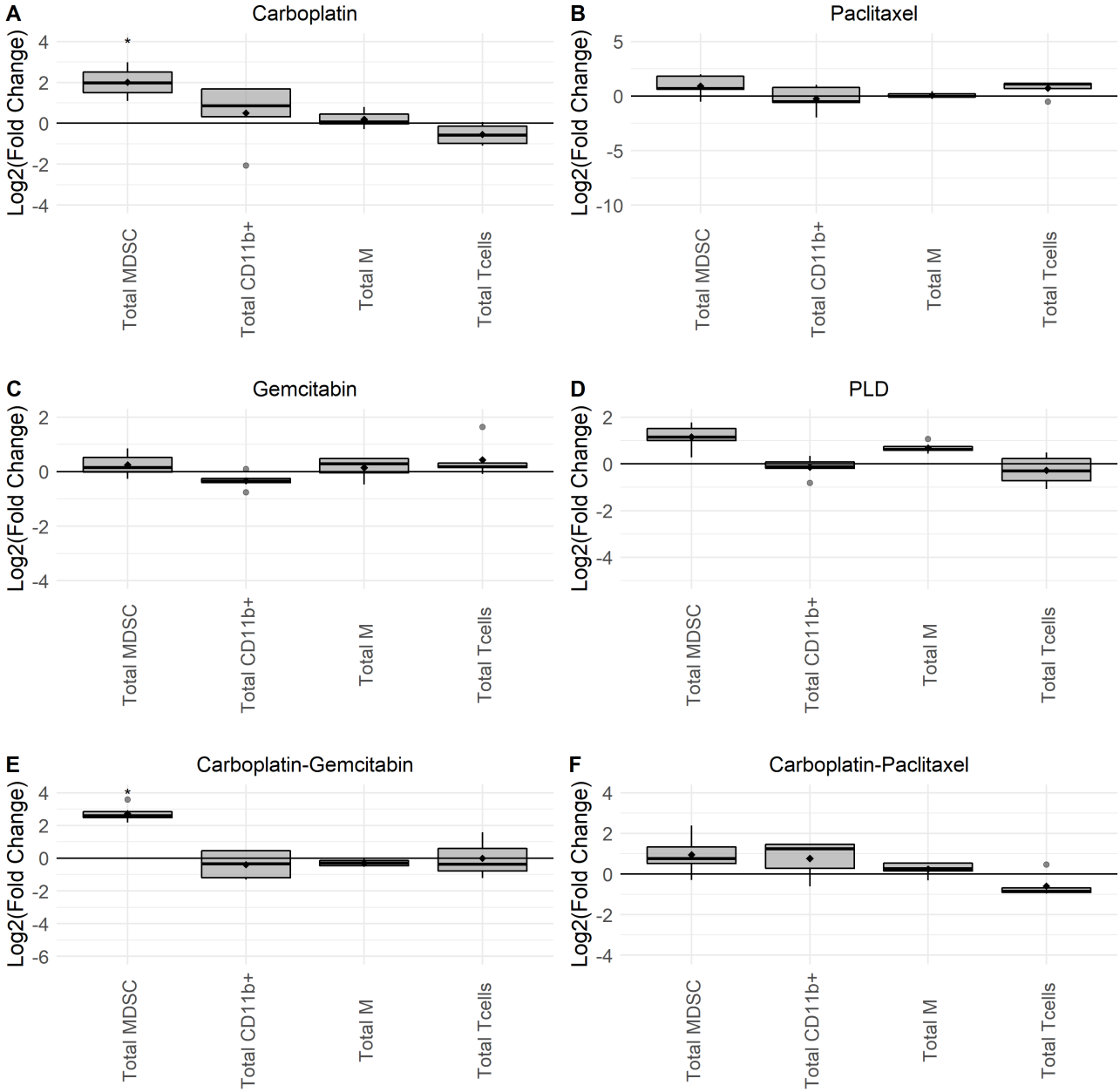
Supplementary Figure 4. Example of immunohistochemical staining of FOXP3^{+} cells.



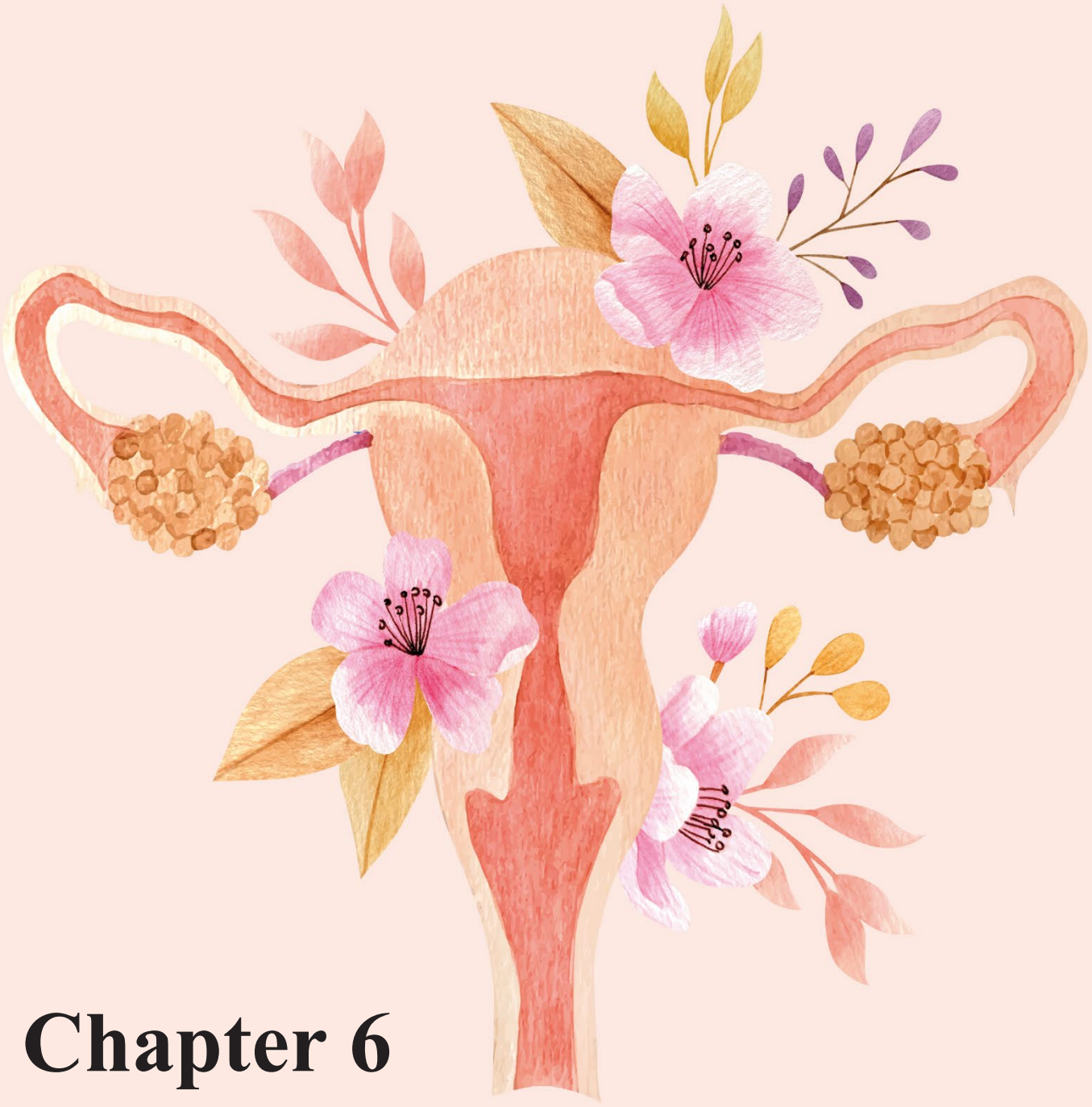
Supplementary Figure 5. Example of immunohistochemical staining of F4/80^{+} cells.



Supplementary Figure 6. Example of immunohistochemical staining of CD8^{+} cells.



Supplementary Figure 7. Fold change (transformed with base log2) of immune profile one week after chemotherapy administration versus tumor bearing non-treated mice. A: Carboplatin induces an increase in total MDSC (mean = 2.01, *p<0.05), E: Carboplatin-Gemcitabin induces an increase in total MDSC (mean = 2.73, *p<0.05). B-C-D-F: no significant changes could be detected. The fold change is calculated by dividing the amount of immune cells (as a percentage of their parent-population) in mice one week after chemotherapy (individual values) by the amount of immune cells in control mice (mean of the group). PLD = Pegylated liposomal doxorubicin. All tests are one-way ANOVA. Grey dots represent outliers, black squares in bar plot represent mean, line represents median. (N=6 per group in first experiment, N=5 per group in repeat experiment.)



Chapter 6

Development Of Rationally Designed Combinatorial Immunotherapy Strategies For Ovarian Cancer

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Part of these results are published in Sprooten J, Vankerckhoven A*, et al. J Immunother Cancer (2021);9:e003609. *Shared first author*

Chapter 6

Development of rationally designed combinatorial immunotherapy strategies for ovarian cancer

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ABSTRACT

Background and aim: Ovarian cancer is an aggressive malignancy generally treated by cytoreductive surgery in combination with carboplatinum-paclitaxel chemotherapy. The immune system has proven to be involved in ovarian cancer disease onset and progression, yet, current clinical trials have not resulted in clinical successes. Part of the explanation can be found in the complex immune biology of ovarian cancer that might require more extensive modulation than the immune checkpoint immunotherapy regimes currently investigated.

Materials and methods: Six to eight week old female C75BL6 mice were inoculated intraperitoneally with $5x10^6$ ID8-fLuc ovarian tumour cells in order to generate an orthotopic, metastatic murine model of ovarian cancer. Selected single or combined agents were administered to treatment groups, control mice received vehicle treatment. Tested therapies included carboplatinum-paclitaxel chemotherapy and anti-PD1, anti-LAG3, anti-TIM3, anti-TNF and PARP inhibitors. Via bioluminescence imaging, *in vivo* tumour growth was measured non-invasively. Development of end stage disease symptoms and survival were recorded for analysis.

Results: Monotherapy of selected targeted agents was not capable of inducing changes in disease progression. Survival benefits were noted for combinations of carboplatinum-paclitaxel chemotherapy with either anti-TNF or anti-TIM3 inhibitor therapy. Of note, altered timing of anti-TIM3 administration in combination with chemotherapy administration induced tumour hyper progression and can completely abrogate positive anti-tumour effects of chemotherapy.

Conclusion: Anti-TIM3 and/or anti-TNF inhibitor therapy in combination with carboplatinum-paclitaxel chemotherapy may offer new possibilities for ovarian cancer management. Caution is advised when designing the order of the treatments. Future trials should include in-depth monitoring of immune parameters.

INTRODUCTION

Ovarian cancer is the most lethal gynaecological cancer. High-grade serous (tubo) ovarian cancer is the most common histological subtype. It is characterized by its capability of spreading throughout the abdomen without causing specific symptoms. Therefore, most patients are diagnosed in advanced disease stages where five-year survival rates drop to 32.1% [1,2]. First-line therapy is based on a combination of cytoreductive surgery and platinum-based chemotherapy and generally induces good initial responses [3]. However, nearly all woman will suffer from tumour recurrence [3,4]. Recurred tumours often become less sensitive to platinum therapy, adding increased difficulty to adequately treat ovarian cancer [3]. Two targeted therapies have recently been implemented in the treatment of advanced ovarian cancer. These include inhibitors of vascular endothelial growth factor (VEGF) and inhibitors poly ADP-ribose polymerase (PARP) enzymes. Both therapies have shown efficacy towards prolongation of progression-free survival but not (yet) overall survival [5–9]. Therefore, efforts to find (combination) therapies with more durable responses must be maintained.

Immunotherapy has changed the landscape of cancer management dramatically. However, attempts to implement immunotherapies in ovarian cancer as a new therapeutic strategy have not yet been able to generate the expected positive results. Early clinical trials investigating immunotherapy efficacy in monotherapy in ovarian cancer, reported low response rates of only 10-15% [10]. Relative failure can be explained by multiple factors. For one, no biomarkers are available to predict responses to (immunotherapy) treatment regimes. Secondly, the cancer-immunity-axis is a dynamic aspect that changes over time and is influenced by many aspects, including the administered therapies [11,12]. A comparison study performed in mice by our group looking at immune changes induced by six commonly used chemotherapies in ovarian cancer, indicated that carboplatinum-paclitaxel induced the most favourable immune profile. We confirmed this finding in patients, based on immune data obtained from serum samples during primary treatment and additionally found that the positive effects of carboplatinum-paclitaxel were completely abrogated after surgery, suggesting only a limited window of opportunity. Monitoring these changes is thus warranted to rationally design an optimal treatment schedule [13]. Thirdly, the complexity of ovarian cancer immune biology and apparently high levels of innate immune suppression makes it highly likely that a combination strategy targeting not just one but multiple different aspects of the tumour immune biology may be necessary [14,15]. Moreover, current immunotherapies that are being tested mainly interfere with the function of the adaptive immune system and less with the innate immune system.

Therefore, the aim of this chapter of the thesis was to test preclinically several immunotherapy-based combination regimes in an orthotopic, metastatic ovarian cancer mouse model. Part of these results were published in Sprooten J*, Vankerckhoven A*, *et al*. J Immunother Cancer 2021;9:e003609 (*Shared first author).

MATERIAL AND METHODS

Ovarian cancer mouse model

Six to eight-week-old, female C57BL/6 mice (Envigo, Horst, The Netherlands) were intraperitoneally (i.p.) injected with five million ovarian cancer ID8-fLuc cells [16]. Sample size of all experiments was determined via statistical power analysis, except for exploratory pilot studies where number of mice were intentionally kept low. Mice were co-housed in groups of five in individually ventilated cages (IVC) containing wood shaving bedding and nesting materials at the Specific-pathogen-free (SPF) facilities of the KU Leuven. Examination of the animals increased from three weekly to once daily if the first signs of disease were detected. Animal care takers were not involved in administering therapeutics and were blinded from treatment groups to impartially judge onset of symptoms and progression to end stage disease. Ascites of end-stage mice, reaching 32 grams in overall body weight, was drained by means of puncturing the left abdominal flank. Severely ill mice were sacrificed based on human endpoints as previously published by our group [16]. All experiments performed were in line with the Belgian (Royal Decree, 29 May 2013), Flemish (Decision of the Flemish Government to adapt the Royal Decree of 29 May 2013, 17 February 2017) and European (Directive 2010/63/EU) regulations on the protection of animals used for scientific purposes. Approval of the local ethical committee was obtained (P125/2017) for all mice experiments prior to the start.

In vivo monitoring of tumour growth

Using bioluminescence imaging, tumour growth could be monitored non-invasively up until six weeks after tumour inoculation [16]. During the scan, mice were anesthetized with isoflurane (2L/min, IsoVet, Dechra Veterinary products n.v, Lille, Belgium). Excess hair of the abdominal and pelvic regions was removed before scanning via mechanical trimming and Veet® hair removal spray. Next, mice were injected subcutaneously with D-luciferine (126 mg/kg, Promega, Madison, US). The total photon flux (in photons per second, p/s) was measured with the IVIS Spectrum Preclinical In Vivo Imaging System (PerkinElmer, Waltham, US) using the Living Image Software at the KU Leuven core facility (Molecular Small Animal Imaging Center).

Therapeutic treatment procedures in mice

Dosages of therapeutic agents were tested beforehand in non-published monotherapy pilot-trials. Treatment groups were randomly attributed. Treatment was always started two to three weeks after inoculation to mimic metastatic spread of advanced ovarian cancer patients. Chemotherapy was administered at day 14 or 21 after tumour inoculation and consisted of two separate administrations of carboplatinum (100 mg/kg, Carbosin, Teva pharma Belgium n.v., Antwerp, Belgium) and paclitaxel (10 mg/kg, Fresenius Kabi n.v., Schelle, Belgium). Animals experiencing chemotherapy toxicities (e.g., weight loss, diarrhea, cachexia and severe hunch back) were sacrificed prematurely and were excluded from data analysis. The PARP inhibitor olaparib (AZD-2281, MedKoo Biosciences Inc., Morrisville, USA) was dissolved in a mixture of 10% dimethyl sulfoxide (DMSO) (CryoSure-DMSO, WAK-Chemie Medical

GmbH, Steinbach, Germany), 50% Polyethylene glycol 300 (PEG300) and 40% PBS. The PARP inhibitor niraparib was kindly gifted by TESARO (now GSK) and dissolved in Phosphate-Buffered Saline (PBS). Both olaparib or nirparib were administered at 50 mg/kg via oral gavage, once daily for a total of four weeks starting at day 21 after tumour inoculation. Anti-Programmed cell death protein 1 (PD1), anti-Lymphocyte-activation gene 3 (LAG3) and anti- T-cell immunoglobulin and mucin-domain containing-3 (TIM3) were a kind gift from TESARO (now GSK) and were dissolved in PBS. After GSK take-over, anti-PD1 therapy was a kind gift from Louis Boon and anti-TIM3 therapy was purchased (Clone RMT3-23, catalog #BE0115, BioXcell, Lebanon, NH, USA). Anti-PD1 was administered every other day, starting at day 21 post inoculation for five times at a dose of 2.5 mg/kg. Anti-LAG3 (17 mg/kg) was administered biweekly for three consecutive weeks starting at day 21 post inoculation. Anti-TIM3 (17.5 mg/kg) was administered biweekly for three consecutive weeks starting either at day 21 post inoculation or at day 28 post inoculation. Anti-tumour necrosis factor (TNF) alpha, kindly supplied by Louis Boon, was administered biweekly for six weeks starting 21 days post inoculation at 5 mg/kg.

Identifying feasibility of selected compounds through literature study

Anti-TIM3 and anti-LAG3 are newer checkpoint inhibitors. Therefore, we wanted to investigate their expression levels specifically in ovarian cancer (patients) before starting preclinical combination testing. A literature review was performed (as part of a master thesis project of Lotte de Veth, supervised by Ann Vankerckhoven, Roxanne Wouters and An Coosemans) to identify relevant publications. Databases PubMed, Embase and Cochrane library were searched on 02/2020 using the concepts (“ovarian cancer” AND (“TIM-3” OR “LAG3”)) and their derivatives.

RESULTS

Expression of newer immune checkpoints in ovarian cancer

Based on a literature search, a total of 45 publications (both published papers and conference abstracts) discussed immune checkpoint expression levels (Figure 1 for overview; Supplementary data Table 1 for detailed overview). Most data concerned immune infiltrate analysis on tumour biopsies of ovarian cancer patients. Despite large heterogeneity between study types, a trend could be identified. Both for TIM3 and LAG3, an average of 20% positivity in marker expression was reported, which is in contrast with the much higher expressed PD1 levels (on average by 50% of the investigated cells) (Figure 1).

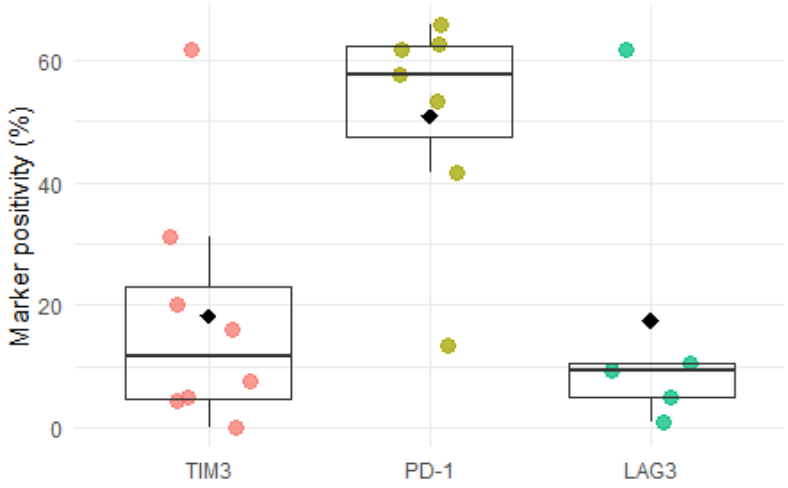

Figure 1. Summary figure of literature study on expression of TIM3, PD1 and LAG3 in ovarian cancer tissues. Dots are individual study values, diamonds represent the mean.

Combining immune checkpoint inhibitors

In a first pilot study, we explored the combination of anti-PD1, anti-TIM3 and anti-LAG3. Vehicle treated tumour bearing mice served as control. Bioluminescent measurement of *in vivo* tumour growth did not show any significant changes in disease progression between any of the tested combinations (Figure 2A). Control mice immediately succumbed to the disease, before the development of clear end stage symptoms. In the checkpoint inhibitor monotherapy groups, end stage symptoms developed with a median of 57 days post inoculation for anti-LAG3, 62 days for anti-PD1 and 72 days for anti-TIM3 (Figure 2B). For combination strategies, the median of end stage symptom development ranged from 61 (for anti-LAG3 plus anti-PD1) to 72 days (anti-PD1 plus anti-TIM3) post inoculation (Figure 2C). Similar results were obtained for overall survival. Anti-TIM3 in monotherapy and the combination group of anti-PD1 plus anti-TIM3 both had a median overall survival of 72 days post inoculation, compared to 56.5 days for controls (Figure 2E). Overall, this pilot experiment suggested that groups treated with anti-TIM3 seemed to perform better. Therefore, these were selected to continue further combination testing.

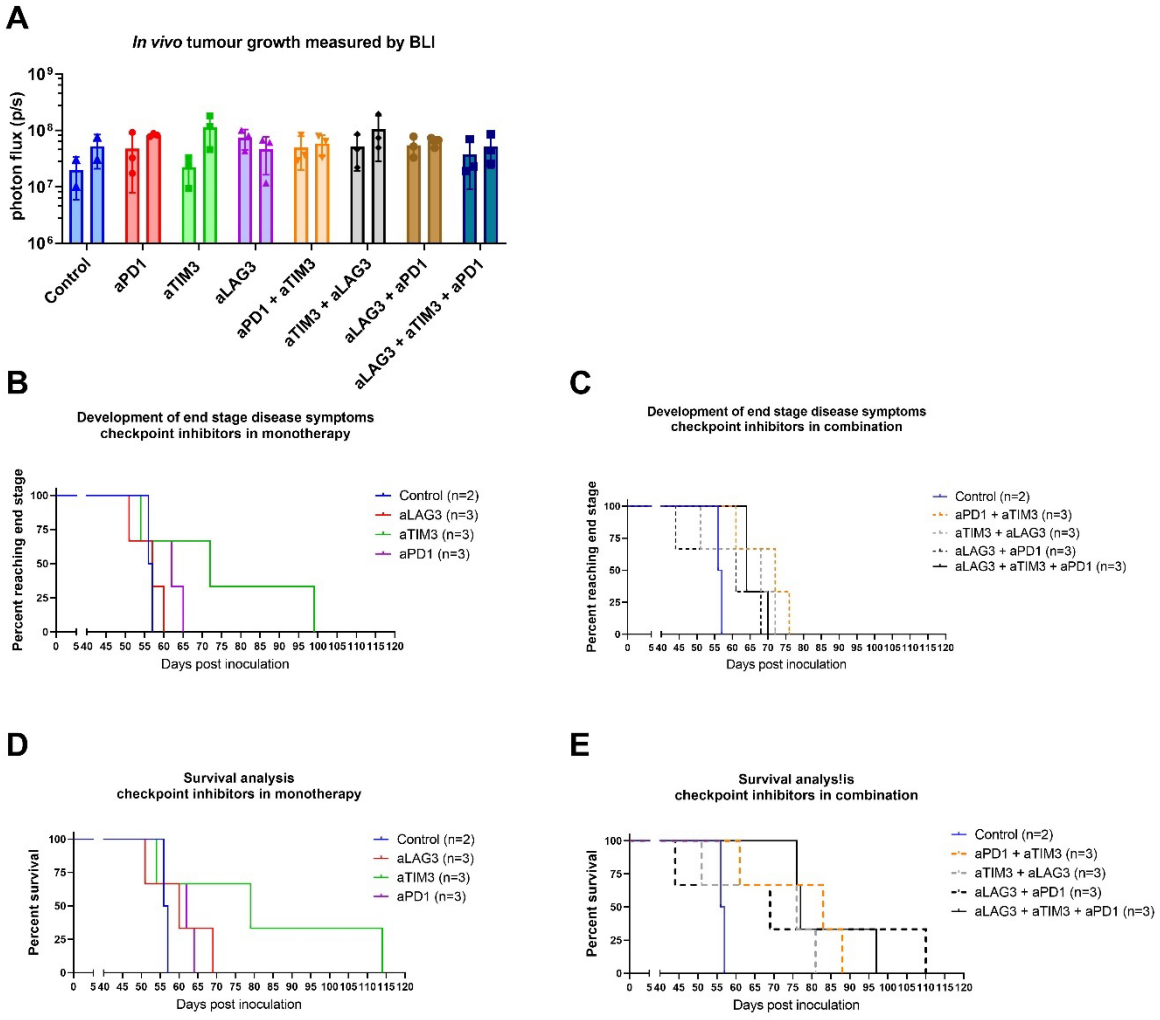

Figure 2 Testing checkpoint inhibitors *in vivo* for therapeutic efficacy. A. BLI measurements of total photon flux per seconds (log scale) performed at week one and week six post tumour inoculation did not show any major differences in tumour growth between the different treatment groups. B+C. End stage disease symptom development slightly delays in groups treated with a regime containing anti-TIM3. D+E. Survival data of groups treated with anti-TIM3 (either in monotherapy or combination) seemingly performed better. This exploratory pilot study contained three mice per group, except with control mice where one mouse died upon vehicle injection and therefore had to be excluded. No comparative static survival analysis was performed. BLI: bioluminescence imaging, p/s: photons per second, aPD1: anti-PD1, aTIM3: anti-TIM3, aLAG3: anti-LAG3, n: number.

Combining immune checkpoint inhibitors with the standard of care

PARP inhibition

In order to increase efficacy of our previously tested checkpoint inhibitor combination (anti-PD1 plus anti-TIM3), we next performed additional combination testing with the standard of care. In a first experiment, we tested the combination with the PARP inhibitor niraparib (Figure 3). Disease progression as measured by the development of end stage symptoms did not indicate differences between the different treatment groups. Controls developed end stage symptoms after a median of

59 days post inoculation, niraparib treated mice at day 53, anti-PD1 plus anti-TIM3 treated mice at day 55 and the triple combination arm of niraparib plus anti-PD1 plus anti-TIM3 at day 66. Survival analysis showed a median survival for niraparib monotherapy treated mice at 53 days post inoculation, closely followed by anti-TIM3 plus anti-PD1 treated mice at day 56. The triple combination arm extended this median survival with 15 days to day 71 post inoculation. However, control mice had a median survival of 94 days post inoculation.

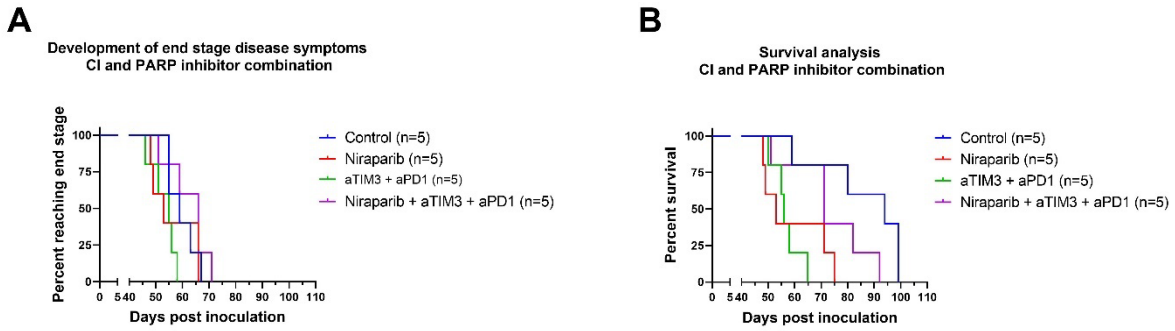

Figure 3. Combining anti-PD1 and anti-TIM3 with PARP inhibition. A. End stage symptom development did not indicate a delay in disease progression in any of the treatment groups. B. Survival analysis seemed to indicate a slight improvement of efficacy in the triple combination arm compared to niraparib or the checkpoint inhibitor combination alone, but not better than control mice. Five mice per group were evaluated. No comparative static survival analysis was performed. CI: checkpoint inhibitor, PARP: poly ADP-ribose polymerase, aTIM3: anti-TIM3, aPD1: anti-PD1, n: number.

Carboplatinum-paclitaxel

In a second experiment, we tested the addition of anti-TIM3 to chemotherapy and the triple combination where anti-PD1 plus anti-TIM3 plus chemotherapy were combined. All treatment groups including chemotherapy displayed a significant delayed end stage symptom development (****p<0.001) (Figure 4A). With median development after 65 days for vehicle treated controls and 81 days for the triple combination arm and 82 days for the chemotherapy in monotherapy or in combination with anti-TIM3. Likewise, all arms including chemotherapy experienced a significantly improved survival benefit compared to control (*p=0.044) (Figure 4B), again with the anti-TIM3 plus chemotherapy treatment arm outperforming all regimes with a median overall survival of 93 days in comparison to 71 days for control treated mice (***p=0.004) and 82 days for chemotherapy monotherapy treated mice. However, the extension of an additional 11 days for anti-TIM3 plus chemotherapy versus chemotherapy in monotherapy did not reach significance (p=0.09).

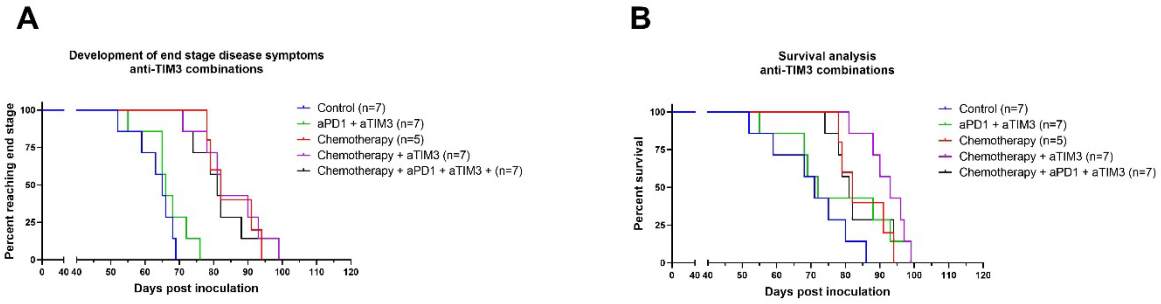

Figure 4. Testing immune checkpoint inhibitor therapy with chemotherapy in a simultaneous regimen. A. End stage symptom development was significantly delayed in all treatment groups that included carboplatinum-paclitaxel (****p<0001). B. Carboplatinum-paclitaxel plus anti-TIM3 had a superior survival. Seven mice were evaluated per treatment group, except the chemotherapy monotherapy group where two mice were excluded from analysis due chemotherapy related toxicities. Statistical test applied: Log-rank (Mantel-Cox) test. Chemotherapy: carboplatinum-paclitaxel, aTIM3: anti-TIM3, aPD1: anti-PD1

Combinations with anti-TIM3 presented above, showed moderate beneficial activity. To further exploit any possible benefits, we decided to next adjust the timing of administration of anti-TIM3 as we have previously shown that timing and order influences a treatment outcome [17]. Anti-TIM3 was either added at a simultaneous schedule with chemotherapy, comparable to earlier experiments, starting therapy at day 20, or was added in a new schedule where anti-TIM3 administration followed sequentially after chemotherapy, starting at day 28 (Figure 5A). End stage symptom development in control mice and anti-TIM3 in monotherapy treated mice (starting at day 28) had a median onset of 64 days after inoculation (Figure 5B). Chemotherapy in monotherapy treated mice developed end stage symptoms with a median of 90 days post inoculation. Adding anti-TIM3 in sequential scheme resulted in a more early onset of end stage disease, at 80 days post inoculation, while in anti-TIM3 in simultaneously schedule treated mice end stage symptoms developed 23 days later with a median of 103 days post inoculation (Figure 5B). Survival analysis showed for anti-TIM3 in monotherapy the lowest median overall survival of 69.5 days, followed by control mice with a median survival of 88.5 days after inoculation (Figure 5C). In contrast to our previous data, addition of anti-TIM3 to chemotherapy in the simultaneous scheme this time had no added benefit compared to chemotherapy in monotherapy (median survival of respectively 112 versus 109.5 days). The newly tested sequential regime of anti-TIM3 and chemotherapy however, performed worse with a median overall survival of 85.5 days, comparable to that of control mice (Figure 5C).

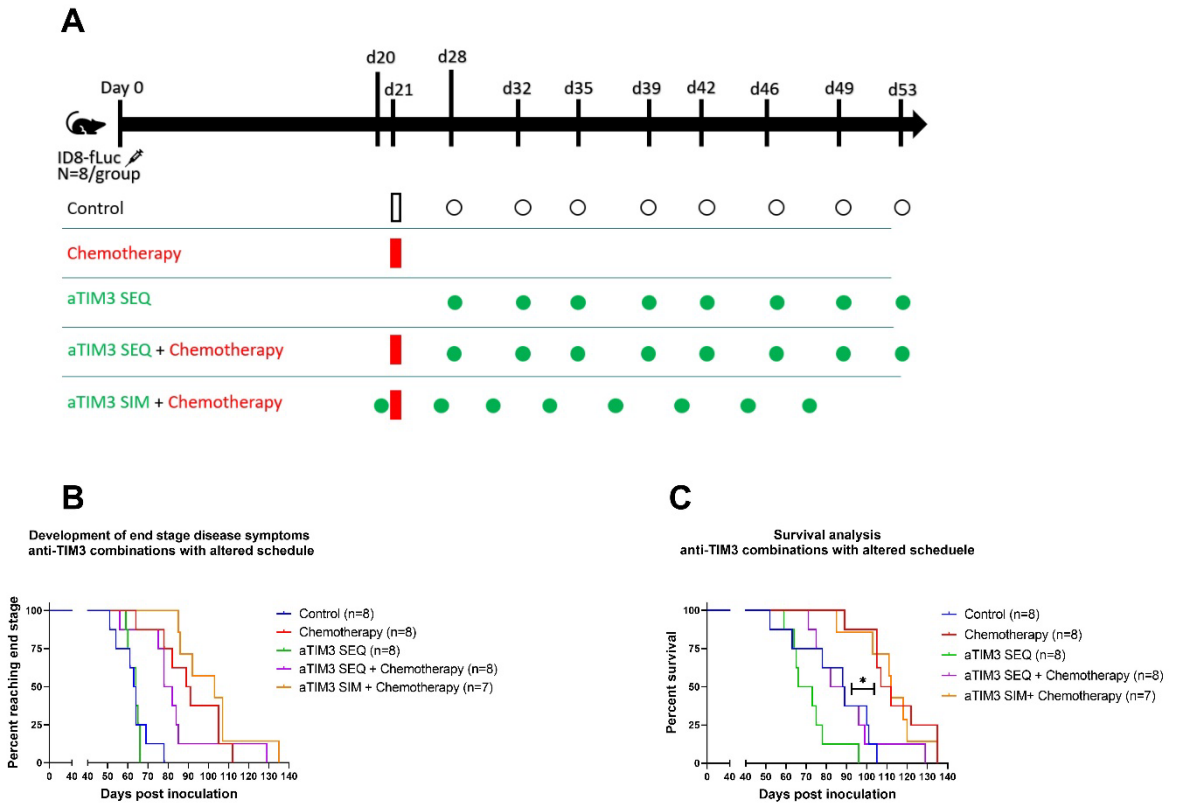

Figure 5. Head-to-head comparison of alternative timing of anti-TIM3 in combination with carboplatinum-paclitaxel chemotherapy. A. Overview of administrative schedule. B. Development of end stage symptoms was significantly delayed in all groups that included administration of chemotherapy. C. Chemotherapy in monotherapy or with administration of anti-TIM3 checkpoint inhibition in a simultaneous schedule showed significantly improved survival compared to controls and chemotherapy plus anti-TIM3 checkpoint inhibition in a sequential schedule. Log-rank (Mantel-Cox test, *p = 0.0189). Eight mice per group were evaluated, except for the chemotherapy plus anti-TIM3 simultaneous group where one mouse succumbed to chemotherapy induced toxicities. Log-Rank (Mantel-Cox) test: *p=0.018 Chemotherapy: carboplatinum-paclitaxel SEQ: sequential, SIM: simultaneous, aTIM3: anti-TIM3

Adding anti-cytokine therapy to the standard of care chemotherapy

Published data: Sprooten J*, Vankerckhoven A*, *et al*. JITC (2021). *shared first author [18]

Based on *in silico* framework performed in the lab of Prof. Abhishek Garg (KU Leuven), a serum-functional immunodynamic status (sFIS) assay was developed. Rather than quantifying serum cytokines, it seeks to provide a more functional immune profile by adding the serum to human myeloid cell responses for changes in nuclear factor kappa-light-chain enhancer of activated B cells (NFKB) or interferon (IFN)/interferon-stimulated genes (ISG) responses. In a large ovarian cancer cohort (n=699), the sFIS assay could estimate survival trends and co-estimate the risk of malignancy relative to benign/borderline ovarian lesions. High serum-induced NFKB responses over IFN/ISG responses

indicated a more negative prognosis. Next, *in silico* analysis indicated that TNF-alpha inhibitors, *e.g.* infliximab, could be an interesting therapy as TNF are robust NFKB response inducers. However, clinical trials with infliximab have been performed with limited success. Reanalysing the publicly available transcriptome of ovarian cancer patients ascites from an infliximab trial revealed that elevate NFKB responses could differentiate infliximab responding versus non-responding patients. Therefore, we wanted to validate the sFIS assay prospectively by means of the ID8-fLuc ovarian cancer mouse model (Figure 7A). Here, we evaluated anti-TNF therapy with standard of care carboplatinum-paclitaxel chemotherapy and PARP inhibitors. Serum sFIS analysis pre- and post-treatment revealed lower si-NFKB to si-IFN/ISG responses when combining carboplatinum-paclitaxel with anti-TNF therapy (Figure 7B). Indeed, this combination significantly improved survival, compared to vehicle control and anti-TNF monotherapy treated mice with a median overall survival of 112 days compared to respectively 76.5 and 83 days (Figure 7C+D).

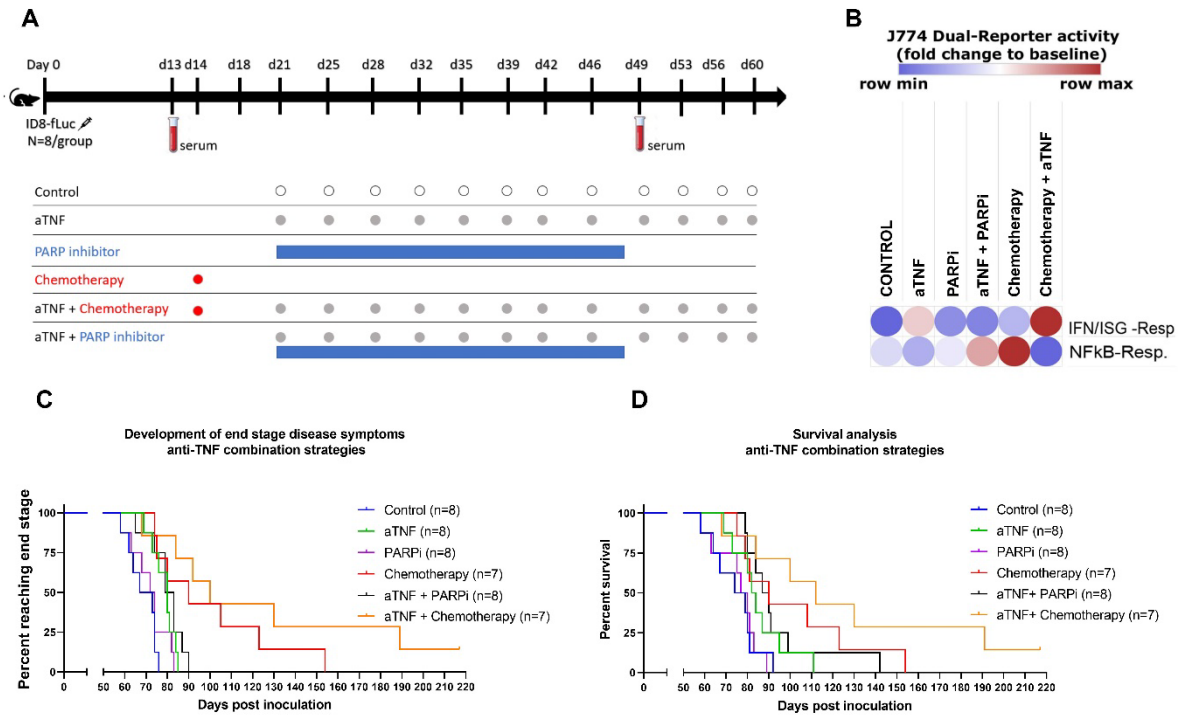

Figure 6. Testing of anti-TNF combination therapy based upon sFIS analysis prediction assay. A. Schematic representation of the treatment schedule administered *in vivo*. B. Murine sFIS assay results for IFN/ISG and NFKB responses upon serum-induction. C. End stage disease symptom development is significantly delayed when combining anti-TNF and carboplatinum-paclitaxel compared to control/anti-TNF monotherapy treated mice. (Log-Rank (Mantel-Cox) test *p=0.0211). D. Survival analysis shows significant benefit for anti-TNF plus chemotherapy treated mice, but not for chemotherapy monotherapy treated mice compared to anti-TNF monotherapy mice. (Log-Rank (Mantel-Cox) test **p=0.0067). Experiment was terminated at day 217 post inoculation. Figure is adapted from publication. Chemotherapy: carboplatinum-paclitaxel, aTNF: anti-TNF, PARPi: PARP

inhibitor (olaparib), IFN: interferon, ISG: Interferon-stimulated genes, NFKB: nuclear factor kappa-light-chain enhancer of activated B cells, resp: responses.

DISCUSSION

The goal of this part of the thesis was to test different immunotherapy based combinations in an orthotopic, ovarian cancer mouse model, with a strong focus on therapies also capable of targeting elements known to influence not only the adaptive, but also the innate immune system. As expected, results of compounds targeting more adaptive immunity failed to generate positive responses. For anti-LAG3 monotherapy, similar results were obtained in a preclinical ovarian cancer model by the group of Odunsi [19]. In addition, anti-PD1 and anti-LAG3 combination strategies did not seem to impact ovarian cancer survival. Currently, both anti-LAG3 monotherapy and the dual blockade of PD1 and LAG3 is being tested in several clinical trials, however, not specifically focussing on ovarian cancer [20].

Combinations with PARP inhibitors (both olaparib and niraparib) gave rather disappointing results. Nevertheless, many preclinical studies have indicated multiple immune modulating properties of PARP inhibitor therapy. Even for gynaecological cancers, sufficient rational can be found to set-up combination studies [21]. The clinical study MEDIOLA (NCT02734004) exploring the addition of the PD-L1 inhibitor durvalumab to olaparib in BRCA mutated solid tumours, including ovarian tumours, reported beneficial results. However, we experienced seemingly contradictory results as the combination of the PARP inhibitor niraparib plus checkpoint inhibitors anti-PD1 plus anti-TIM3 failed in our experiments. Also later on, upon combination with anti-TNF, results were negative. Here, we saw that PARP inhibitor therapy on its own, is not powerful enough to push responses towards a more beneficial profile of high NFKB over low IFN/ISG responses [18]. Moreover, combining PARP inhibition with anti-TNF therapy even seemed to delete the positive effects that anti-TNF was able to induce in monotherapy. These negative results however can potentially be explained by the absence of BRCA mutations in our ID8-fLuc cell line. Indeed, analysis from breast patient derived xenograft (PDX) models showed a dose-dependent upregulation of IFN and STING signalling, however, this was only confined to BRCA mutated PDX models and was not seen in BRCA wild type PDX models [22]. Furthermore, presented data at European Society for Medical Oncology (ESMO) congress about the combination of PARP inhibitors with anti-PD1 checkpoint therapy confirmed that the cohort of non-germline mutated BRCA ovarian cancer patients indeed did not meet predefined response targets, however, they did report that addition of anti-VEGF therapy had a more promising outcome and should be investigated further [22].

Combinations including anti-TIM3 therapy had a tendency to perform better, although experiments should be repeated to confirm certain findings, certainly because important alterations of disease outcome were observed if the therapeutic schedule was altered. However, also others have reported good preclinical results of combination strategies with anti-TIM3 and anti-CD137 in an ovarian cancer mouse model [23]. These positive outcomes were further supported by recent data obtained from ovarian cancer patients, revealing that although PD-1, PD-L1, CTLA-4 and LAG3 are significant prognostic factors, none of them appeared to be involved enough in the clinically relevant immune

suppressive tumour microenvironment. The presence of TIM3+ immune cells however played a critical role [24]. To date (07/2022), no specific clinical trials in ovarian cancer involving anti-TIM3 are ongoing. For other cancer types, the first safety studies with a total of 16 clinical trials are registered in ClinicalTrials.Gov. Only one study evaluating the safety and dose escalation of anti-TIM3 in patients with advanced solid tumour malignancies or lymphomas has published results already (NCT03489343), reporting no dose-limiting toxicities.

Combination studies including anti-TNF also had the tendency to perform better. Treatment with anti-TNF treatment however, is not new. Studies involving the drug 'infliximab" have been performed and failed [25]. Therefore, we included a reanalysis of the transcriptome derived from ascites of patients with ovarian cancer enrolled in an infliximab trial and could separate responders from non-responders based on their NFKB and IFN/ISG response signature [18]. Using the sFIS assay and thereby capturing the peripheral immunodynamics of patients does not only give us insights in the immunobiology of the patient, but also guide us in the development of immunotherapy based trials. Nevertheless, therapeutic strategies targeting the disbalance in NKFB and IFN/ISG signalling should be further investigated.

This thesis chapter has important limitations. First, it would be of interest to not only see survival outcome but also be able to associate immune (dynamic) data with the outcome. Identifying shifts in (function of) immune cells could help guide the design of these combination regimes. The treatments tested were specifically chosen because they can have a potential impact on the innate immune system. Indeed, we observed that it were peripheral myeloid and not lymphoid cells that were associated with the strong NFKB and IFN/ISG responses and that TNF was one of the strongest inducers of a more detrimental NKFB response [18]. By means of the sFIS analysis, we could indeed show that this balance was partly restored [18]. Supplementary flow cytometry analysis that includes activation markers could offer us additional insights into the immune changes induced. Additionally, TIM3 is known to be expressed and involved in innate immune cell regulation, in contrast to its bigger brother PD1 which mainly interferes with the adaptive immune function [26,27]. However, our experiment lacks this immune data. These types of experiments are planned in the near future, but were not part of this thesis dissertation. Secondly, we noticed differences in outcomes between the first and the second experiment when anti-TIM3 was administered simultaneously with chemotherapy with in one experiment an improvement of outcome by 11 days compared to chemotherapy monotherapy and the second one with no differences in outcome. Repeat experiments, possibly with an increased number of mice could bring more conclusive results. Alternatively, one could repeat these experiments in a second ovarian cancer model. Currently, our lab is putting effort into developing a second immune-competent mouse model (HM-1 [28,29]) and an immune-competent rat model (NuTu-19, [22]) to accommodate for this limitation.

In conclusion, we tested several combination therapies in an ovarian cancer mouse model. Two regimes (one involving anti-TIM3 and one involving anti-TNF in combination with carboplatinum-paclitaxel) show promising results. In-depth monitoring of induced immune changes with these regimes as well as identifying the most optimal window and timing of administration are crucial follow-up experiments.

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SUPPLEMENTARY INFORMATION

Supplementary Table 1. Overview of studies included after literature search, ordered by subject

Publication	Publication type	Population	Samples	(Immune) cells studied	% positivity
TIM3					
Sawada *et al.* (2020)	Paper	100 ovarian cancer patients	Tumour biopsy, PBMCs	CD8+ T cells	22.8%
Li *et al.* (2018)	Paper	22 ovarian cancer patients	Tumour biopsy, PBMCs	CD4+CXCR5+ Tfh cells	69.3%
Bu *et al.* (2016)	Paper	25 ovarian cancer patients	Tumour biopsy, PBMCs	CD3+CD4+CD25+ Treg cells, CD3+CD8+ T cells	-
Wu *et al.* (2013)	Paper	52 ovarian cancer patients	PBMCs	CD4+ T cells, CD8+ T cells	CD4+ T-cells: 4.89% CD8+ T-cells: 2.91%
Guo *et al.* (2013)	Paper	Murine model	Peritoneal immune cells	CD4+ T cells, CD8+ T cells, CD4+FoxP3+ Treg cells, CD11b+Gr-1+ MDSC	-
Yan *et al.* (2013)	Paper	Not mentioned	Tumour biopsy, PBMCs	Tumor infiltrating lymphocytes, peripheral blood lymphocytes	-
Hinchcliff *et al.* (2018)	Paper	44 recurrent ovarian cancer patients	-	-	-
Curigliano *et al.* (2019)	Conference abstract	Not mentioned	-	-	-
Laeremans *et al.* (2018)	Conference abstract	30 ovarian cancer patients	Tumour biopsy	-	-
Eiva *et al.* (2017)	Conference abstract	-	-	CD137+ TIL	-

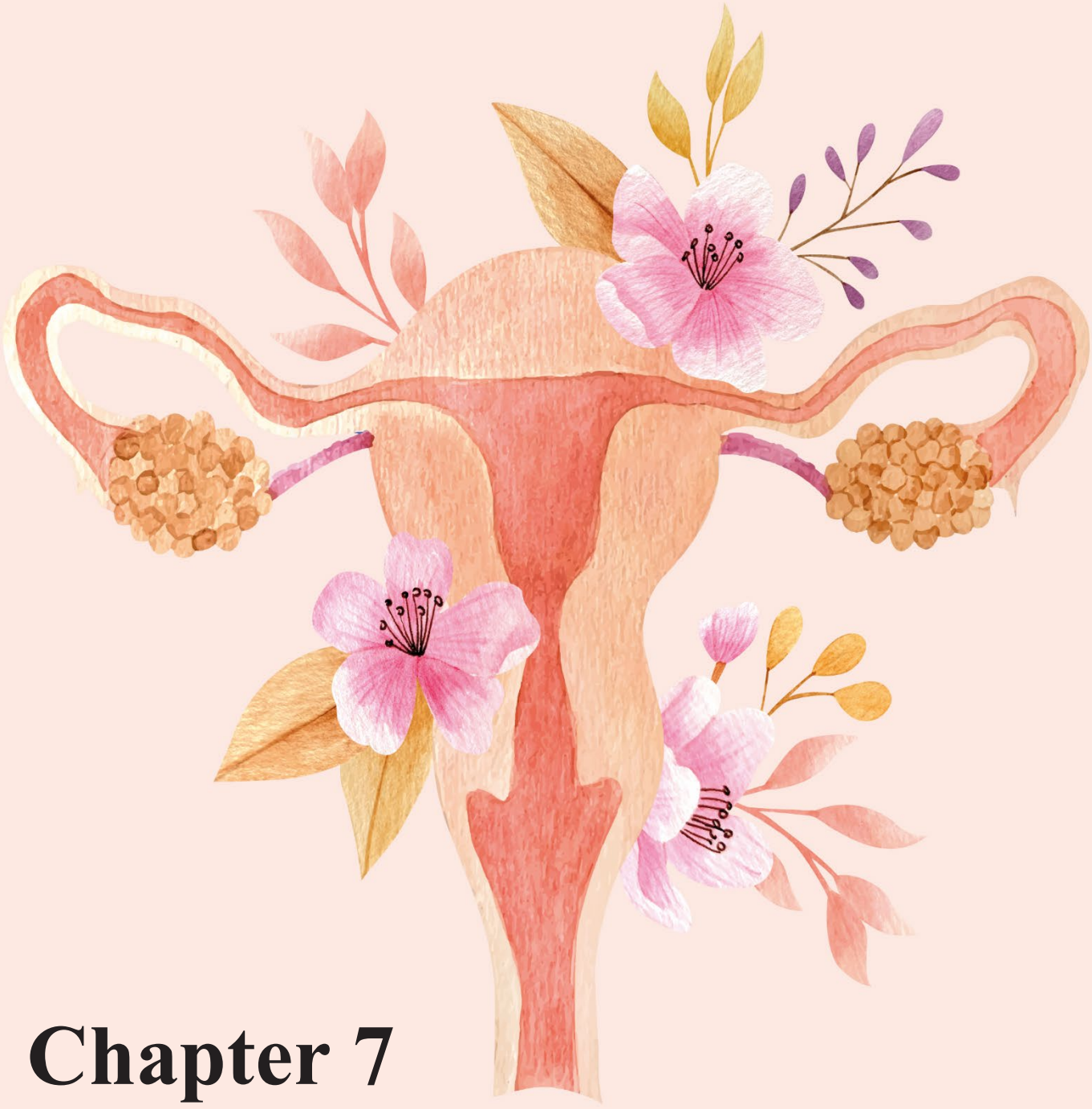
Wong *et al.* (2016)	Conference abstract	-	PBMCs	Macrophages	-
Lee *et al.* (2015)	Conference abstract	41 ovarian cancer patients	PBMCs	Tregs, MDSC, exhausted CD8+ cells	-
Harris *et al.* (2012)	Conference abstract	416 ovarian cancer patients	Existing databank	-	-
LAG3					
Tian *et al.* (2020)	Paper	589 ovarian cancer patients	TCGA databank	Th1, Th2, Th17 and 22 immune cell types	-
		220 ovarian cancer patients	Tumour biopsy, blood samples	-	-
Whitehair *et al.* (2020)	Paper	48 ovarian cancer patients	Tumour biopsy	CD8+ T cells, FOXP3+ Treg cells	-
Wu *et al.* (2019)	Paper	12 ovarian cancer patients	Cytologic cell blocks prepared from centrifuged pleural liquid sediment	CD45, CD3, CD4, CD8, CD20, CD68, MPO	-
Kim *et al.* (2018)	Paper	131 ovarian cancer patients	Tumour biopsy	CD8+ TILs, Foxp3+ TILs	Low expression: 58.2% High expression: 41.8%
Budczies *et al.* (2017)	Paper	316 ovarian cancer patients	TCGA databank	-	-
Huang *et al.* (2016)	Paper	Murine model	Tumour biopsy, ascites	Tumor-associated lymphocytes and tumour-infiltrating lymphocytes	-

Huang *et al.* (2015)	Paper	Murine model	Tumour biopsy, ascites	Tumor-associated lymphocytes and tumour-infiltrating lymphocytes	2-10%
Matsuzaki *et al.* (2010)	Paper	Not mentioned	Tumour tissue, ascites and peripheral blood	NY-ESO-1-specific CD8+ T cells, tumour-associated lymphocytes and tumour-infiltrating lymphocytes	10-40%
LaVigne *et al.* (2019)	Conference abstract	48 ovarian cancer patients	Tumour tissue	Effector T cells, macrophages and regulatory T cells	-
Jiang *et al.* (2018)	Conference abstract	Murine model	-	T cells	-
Huang *et al.* (2012)	Conference abstract	Murine model	-	CD8+ T cells	-
Combinations					
de Lima *et al.* (2020)	Paper	3 ovarian cancer patients	Tumour biopsy, blood samples	TILs	-
MacGregor *et al.* (2019)	Paper	34 ovarian cancer patients	Tumour tissue, blood samples, TCGA databank	Immune cells, stromal cells and tumour cells	-
Zou *et al.* (2019)	Paper	Ovarian cancer cell lines	-	CAR-T cells	-
Tu *et al.* (2020)	Paper	Not mentioned	Oncomine, PrognoScan, K-M plotter and TCGA database	B cells, CD8+ T cells, CD4+ T cells, neutrophils, DCs and macrophages	-

Fucikova *et al.* (2019)	Paper	80 ovarian cancer patients 20 ovarian cancer patients	Tumour tissue	CD8+ T cells, DC-LAMP+ DCs and CD20+ B cells	CD8+T-cells: 31.3% (TIM-3)
		308 ovarian cancer patients	Existing database	-	-
Rådestad *et al.* (2018)	Paper	23 ovarian cancer patients	Tumor tissue, blood, ascites	Peripheral blood lymphocytes, tumour-associated lymphocytes and TILs	CD8+ T cells: 53.3% (PD-1+) 20.2% (TIM-3+) CD4+ T cells: 41.8% (PD-1+) 15.9% (ANTI_+)
Imai *et al.* (2018)	Paper	54 ovarian cancer patients	Tumour tissue, ascites	CD4+ and CD8+ T cells	CD8+ T cells: 57.7% (PD-1) 5.0% (LAG-3) 4.9% (TIM-3) 15.7% (BTLA) CD4+ T cells: 65.8% (PD-1) 10.6% (LAG-3) 4.3% (TIM-3) 37.6% (BTLA)
Huang *et al.* (2017)	Conference abstract	Murine model	-	TILs, TALs	-
Bergamini *et al.* (2019)	Conference abstract	34 ovarian cancer patients	Tumour tissue, blood and ascites	T cells	ITTCs: 61.9% (PD-1) 61.7% (LAG-3) 61.7% (TIM-3)
Kaufmann *et al.* (2019)	Conference abstract	-	Tumour tissue	TILs	-
Anderson *et al.* (2019)	Conference abstract	Ovarian cancer cell lines	-	-	-
De May *et al.* (2019)	Conference abstract	Ovarian cancer cell lines	-	CD8+ T cells	92% (CD49d)
Laeremans *et al.* (2018)	Conference abstract	Not mentioned	Tumour tissue	T cells	-

Morgado *et al.* (2018)	Conference abstract	225 ovarian cancer patients	Tumour tissue	T cells	-
Laeremans *et al.* (2018)	Conference abstract	30 ovarian cancer patients	Tumour tissue	-	-
Harms *et al.* (2017)	Conference abstract	30 ovarian cancer patients	Tumour tissue	TILs	-
Ziello *et al.* (2017)	Conference abstract	Not mentioned	Tumour tissue	TILs	-
Gaillard *et al.* (2017)	Conference abstract	8 ovarian cancer patients	Blood and ascites	CD8+ T cells	-
Rådestad *et al.* (2016)	Conference abstract	24 ovarian cancer patients	Metastatic tumour tissue, blood and ascites	T cells	Tumor: 9.4% (LAG-3) 62.6% (PD-1) 7.5% (TIM-3) 4.0% (CTLA-4) Blood: 0.8% (LAG-3) 13.4% (PD-1) 0.1% (TIM-3) 0.7% (CTLA-4)
Peper *et al.* (2016)	Conference abstract	Not mentioned	Tumour tissue	TILs	-
Dai *et al.* (2015)	Conference abstract	Murine model	Intraperitoneal tumour nests	T cells	-

CTLA-4: Cytotoxic T-lymphocyte associated protein 4, LAG-3: Lymphocyte-activation gene 3, TIM-3: T-cell immunoglobulin domain and mucin domain 3, PD-1: Programmed cell death protein 1, TILs: Tumour-infiltrating lymphocytes, DC-LAMP: dendritic cell-lysosomal associated membrane protein, Th: T helper cell, Treg: Regulatory T-cell, TCGA: The Cancer Genome Atlas, PBMCs: Peripheral blood mononuclear cells



Chapter 7

Combining Conventional Therapy With Immunotherapy: A Risky Business?

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ABSTRACT

Because of the failure of immunotherapy as single agent in a number of cancers, current clinical trials are focusing on combining immunotherapy with other therapies. The most frequently chosen combination for immunotherapy is chemotherapy. However, almost no preclinical data on this combination is available. Some studies even showed a dismal effect of combining chemo-therapy with immunotherapy. Taken into account that each of the therapies chosen in a combination will influence the cancer cells but also immune effector cells as well as immunosuppressive cells, and that these three partners will also interact with each other, launching a combination to the patient without proper immune monitoring and preclinical evidence might be devastating.

CURRENT PERSPECTIVE

The immune system is now known to play a pivotal role in the onset and development of cancer [1,2]. The adaptive and innate immune responses are mobilized to eliminate neoplastic cells as they emerge. In some cases, elimination is not entirely successful and an equilibrium phase is established whereby the neoplastic cells enter a dormant state, side by side with the immune system. During this process, tumour cells can be edited and consequently escape this immunological control. Eventually, the immune system fails, and uncontrolled tumour proliferation occurs. Tumours themselves and/or through their interaction with the microenvironment may attract immunosuppressive cells, such as MDSC (myeloid derived suppressor cells), Treg (regulatory T cells), M2-like macrophages and others, to divert immune detection and facilitate unregulated tumour growth. Immunological evasion arises in all tumours, but the key players establishing the immune suppressive microenvironment are different per tumour, maybe even for different stages of the disease. A detailed understanding of these tumour-specific processes is gradually emerging [3], and it is essential to be aware of these as anticancer immunotherapy will change this environment. Failure or discontinuation of numerous clinical trials has high-lighted the importance of such knowledge. Immune checkpoint inhibitors have shown limited activity in the majority of tumours and can even create accelerated cancer progression, or unexpected toxicities, when combined with other immunomodulatory drugs. This was the case with pembrolizumab, a monoclonal antibody against programmed death receptor-1 (anti-PD1), in multiple myeloma (KEYNOTE 183 [NCT02576977] and KEYNOTE 185 [NCT02579863]) and also in adult T-cell leukaemia-lymphoma [4]. Based on two recent publications in Nature Medicine, it is reassuring to note that efforts are currently underway to predict sensitivity and response to immune-oncology (IO) agents before their initiation [5,6]. To add complexity, there is increasing evidence that conventional treatments (chemotherapy [7-9], radiotherapy [10] and surgery [11]) also have an impact on the immune system (Fig. 1). This re-enforces the importance of evaluating the immune status before commencing IO-containing combinations. Notwithstanding this lack of information, a recent review by Brown *et al*. [8] revealed that more than 200 ongoing or planned clinical trials are registered in which IO agents are combined with chemotherapy.

This is occurring despite a lack of preclinical data to justify the choice of combinatorial agents. Some clinical trials have shown a better outcome when chemotherapy and immune checkpoint inhibitors are administered sequentially rather than concomitantly [12]; in other studies, the administration of chemotherapy after initiation of immune checkpoint inhibitors was beneficial [13]. Recently, the JAVELIN Ovarian 100 and 200 (NCT02718417 and NCT02580058, respectively, combining anti-PDL1 with chemotherapy in ovarian cancer) were prematurely stopped because no significant differences in survival were obtained compared with chemotherapy in monotherapy [14,15]. Caution is needed for a possible devastating effect of combining chemotherapy with IO agents. As we have insufficient knowledge about the immune status of a patient at therapy initiation, incorrect sequencing of chemo-therapy and IO agents could shorten survival. This was reported ten years ago in a first-line chemotherapy study in ovarian cancer. Carboplatin and paclitaxel were administered alone or in combination with interferon gamma 1b (IFNg1b). The study was stopped prematurely because a significantly shorter overall survival was observed in the IO-containing arm ($p = 0.001$). The authors

only hypothesized about the cause (direct toxicity of IFNg1b, toxicity on bone marrow, leading to therapy reduction or an increase in Treg); however no immune monitoring was performed [16].

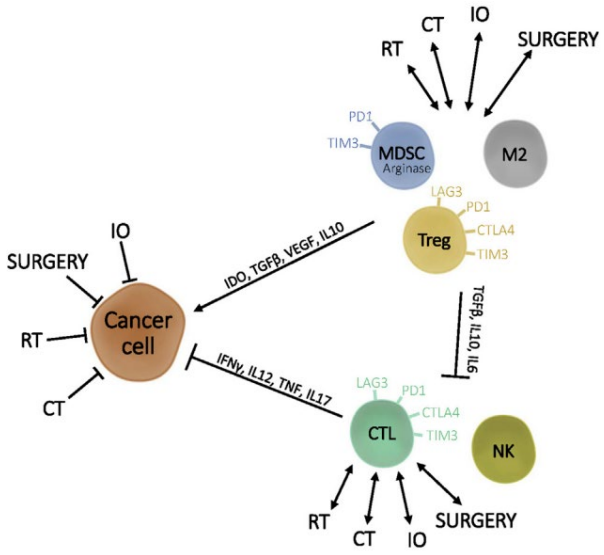

Fig. 1. Complex interplay between conventional therapies, immune-oncology agents, cancer cells and immune cells. Abbreviations: radiotherapy (RT), chemotherapy (CT), immune-oncology agents (IO), Indoleamine 2,3-dioxygenase (IDO), tumour growth factor (TGF), vascular endothelial growth factor (VEGF), interleukin (IL), interferon (IFN), tumour necrosis factor (TNF), cytotoxic T cell (CTL), programmed cell death 1 (PD1), cytotoxic T-lymphocyte associated protein 4 (CTLA4), T-cell immunoglobulin and mucin-domain containing-3 (TIM3), lymphocyte-activation gene 3 (LAG3), myeloid derived suppressor cell (MDSC), regulatory T cell (Treg), macrophage type 2 (M2).

As an example, we refer to our own experience with the ID8-fLuc serous ovarian cancer mouse model [17]. We observed a shortened overall survival depending on the sequence of immunotherapy and chemotherapy (Fig. 2). In the first experiment (A), dendritic cell immunotherapy was combined with carboplatin-gemcitabine; in the second (B), an anti-PD1 was combined with carboplatin-paclitaxel and an angiogenic inhibitor (DC101). The positive effects of synergistic administration (green curve experiment B) might be explained by the finding that PD-L1 expression tends to increase shortly after chemotherapy [18,19].

Shifts in immune cell composition induced by the different therapies separately or together should be considered as the cause of failure or success of combinations. This information can only be obtained by thorough preclinical work, investment in immune monitoring and assays that detect early response. Until we have that knowledge, we need to be careful in choosing the order, timing and dosage of combination therapies [9,20]. Inconsiderate decision-making can result in an accidently chosen beneficial combination without understanding the underlying mechanistic reason, or it can lead to a therapeutic failure with either a similar or, of greater concern, worse outcome.

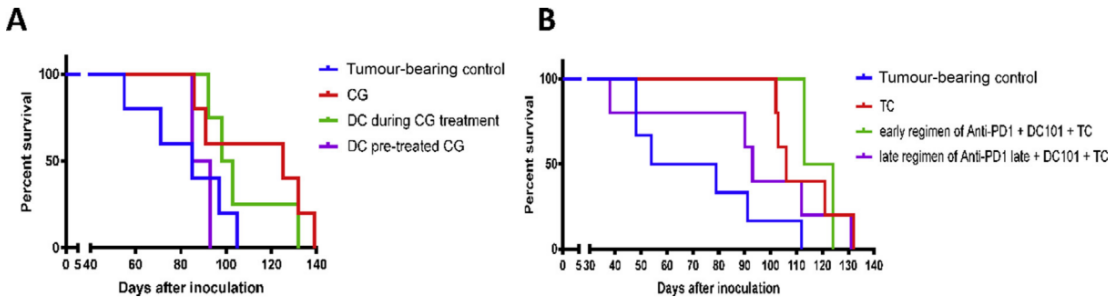

Fig. 2. Kaplan Meier survival curves on combinatorial therapies, administered in the ID8-fLuc ovarian cancer mouse model. A. The red curve represents mice receiving carboplatin-gemcitabine (CG) only, given intraperitoneally at day 21 and day 35 after tumour inoculation (n = 5). Dendritic cell (DC) immunotherapy was given subcutaneously at day 27, 34 and 41 after tumour inoculation (n = 4, 1 toxicity death) (green curve) (median survival 100.5 days). The purple curve represents mice receiving DC immunotherapy at day 1, 7 and 14 after tumour inoculation (n = 2, 3 toxicity deaths) (median survival 89 days). The blue curve represents tumour bearing controls, receiving no therapy (n = 5). B. The red curve represents the group of mice receiving only carboplatin-paclitaxel (TC), given intraperitoneally at day 21 and day 35 after tumour inoculation (n = 5, 1 toxicity death). The green and purple curve represent the mice receiving anti-PD1 (Clone RPM1-14, Bioceros BV, The Netherlands), DC101 (anti-VEGFR2) (1 mg/kg, 2 times per week, starting from day 20) and chemotherapy with administration of anti-PD1 in an early regimen (day 20-22-24-26-28 after tumour inoculation) in case of the green curve (n = 2, 4 toxicity deaths) (median survival 118.5 days) and in a late regimen (day 30-32-34-36-38 after tumour inoculation) in case of the purple curve (n = 5, 1 toxicity death) (median survival 93 days). As in figure A, blue represents the untreated tumour bearing mice (n = 6) (median survival 66.5 days). All murine experiments were performed in female mice, according to the EU directive 2010/63/EU and the ARRIVE guidelines.

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CONFLICT OF INTEREST STATEMENT

AdB (no direct COI but some relations to companies dealing with oncology products in general).

ACKNOWLEDGEMENTS

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Chapter 8

A Microfluidics Approach For Ovarian Cancer Immune Monitoring In An Out-Patient Setting

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Chapter 8

A microfluidics approach for ovarian cancer immune monitoring in an out-patient setting

Performance comparison of chip vs. flow cytometry

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ABSTRACT

Ovarian cancer has the fifth highest mortality rate among women diagnosed with cancer. Current treatment options are non-satisfactory and new/better therapies are highly needed. Immunotherapies show great promise as a cancer cure but currently have not yet reached their full potential in ovarian cancer patients. Implementation of an immune readout could offer better guidance and development of immunotherapies. However, immune profiling is often performed using a flow cytometer, which is bulky, complex and expensive. This equipment is centralized and operated by trained personnel, making it logistically cumbersome to transfer samples and acquire results in a timely fashion. We have developed a disposable microfluidics chip, capable of performing an immune readout with the sensitivity needed to guide diagnostic decision-making. Upon further system development, this microfluidics chip could enable immune-monitoring in an out-patient setting enabling fast acquisition without the need for highly trained staff. As a proof of concept, acquisition of a limited immune panel based on CD45, CD8, Programmed cell death protein 1 (PD1) and a live/dead marker was compared to a conventional cytometer (BD FACSymphony). Based on a dataset of peripheral blood mononuclear cells of 15 patients with ovarian cancer, across different stages of treatment, we have obtained a 99% correlation coefficient for the detection of $CD8^+$ $PD1^+$ T cells, relative to the total amount of $CD45^+$ white blood cells.

1. INTRODUCTION

Ovarian cancer is the fifth most lethal cancer type for women [1]. High grade serous ovarian cancer (HGSOC) is the most common subtype with five-year survival rates as low as 30% [2]. This is in part due to the paucity of early onset symptoms, causing most cases to be diagnosed in advanced disease stages. Around 52% of the women are diagnosed in FIGO stage III on top of 30% of the women diagnosed in FIGO IV [3], meaning that in 82% of the cases the cancer has spread extensively throughout the abdominal cavity, making it challenging to treat. The standard of care for these patients consists of cytoreductive surgery in combination with platinum-based chemotherapy. Unfortunately, more than 70% of the women experience relapse [4]. The implementation of two targeted therapies (anti-VEGF (vascular endothelial growth factor) and poly-ADP ribo-polymerase (PARP) inhibitors) into the primary treatment schedule has improved this situation but none of the drugs currently available are able to cure the majority of ovarian cancer patients, leaving the search for adequate treatments wide open.

The introduction of immunotherapies as anti-cancer treatments caused renewed enthusiasm as they have shown great successes in other cancer types, like melanoma. With the use of immunotherapies like checkpoint inhibitors such as ipilimumab (anti-CTLA4 (cytotoxic T-lymphocyte antigeen-4)) durable responses could be obtained with 20-26% of the patients still alive after ten years of follow-up [5]. More recently, the combination of ipilimumab and nivolumab (anti-PD1 (Programmed cell death protein 1)) in advanced melanoma patients showed excellent results where the median overall survival (OS) was not yet reached after a follow up of 60 months [6]. Following these successes, many immunotherapies were also tested in other cancer types, including ovarian cancer. Unfortunately, the immunologic situation in ovarian cancer seems to be more complex. Early clinical trials studying the efficacy of immunotherapies as a monotherapy have resulted in low response rates of only 10-15% [7]. To improve responses, new clinical trials focused on designing combination treatment schedules, often – but not exclusively – combining immunotherapies with chemotherapy or other targeted therapies. However, also these trials have not (yet) obtained the awaited positive responses [8]. This discordance could partially be explained by the fact that clinical trials have almost no biomarkers implemented beforehand but retrospectively perform exploratory biomarker analysis. Even if prospective biomarkers are used to guide therapy management, they often rely on biopsies taken prior to the treatment. Current knowledge however, clearly states that each and every metastatic spot in ovarian cancer has its own unique tumor-immune microenvironment [9–11]. In addition, multiple groups, including our own, have shown that the composition of the immune system changes throughout disease progression but also after introducing different therapies, making the design of therapeutic strategies upon biopsies taken often only once and at the beginning of treatment, a risky business [12,13]. Monitoring immune changes at a systemic level, for example via white blood cells in the blood of patients via flow cytometry, could offer valuable information that is currently missing. By diving deeper into immune changes on a regular basis during these clinical trials with an effective immune read-out, we could understand better the tumor-immune dynamics and how they change during

treatment. Furthermore, also after clinical implementation of immunotherapies, one could adjust the therapeutic strategy throughout the disease/treatment course and tailor it to the patient's need based on their patient-specific immune read-out. However, only a minority of clinical trials implement immune profiling during the treatment with immunotherapies, as the current flow cytometry systems are bulky, complex to handle and expensive. In addition, most cytometry systems are centralized in specialized lab facilities as they need to be operated by qualified personnel, making it logistically cumbersome to transfer fresh samples and acquire correct results in a timely fashion.

Microfluidics chip-based flow cytometry is a new, rapidly evolving technique that offers a solution to some challenges experienced in conventional flow cytometry [14]. For one, they have the promise of performing fast read-outs as multiple channels can be used simultaneously for cell detection. In addition, their miniaturized system would allow for a portable version that could become a point of care or bed-side application. This feature could facilitate fast diagnosis and alterations of treatment strategies based on longitudinal patient-specific data. It also eliminates the need for cryopreservation or cumbersome sample logistics to centralized laboratories. Further, these systems can be fully enclosed and be developed as disposables for a specific panel, eliminating the risk of cross sample contamination. Pre-defined panels could make the chips suited to be handled without highly educated and trained personnel. The advantages of microfluidics chip-based systems could therefore revolutionize patient-specific-care, with especially high impact for patients with cancer receiving immunotherapies. One of the challenges faced in the development of these systems is to reach the same fluorescence sensitivity for low expressing clinically relevant markers as obtained by conventional flow cytometry. Therefore, in this report, we seek to compare the analysis of an immune read-out on a conventional cytometer (BD FACSymphony) to our newly developed microfluidics-based cytometry chip. For this proof-of-concept study, we evaluated the performance of a four-color immune panel (CD45, CD8, PD1 and a live/dead marker) in a small dataset of 15 patients with ovarian cancer, across different stages of treatment.

2. MATERIALS AND METHODS

2.1 Study design

For this proof-of-concept study we prospectively collected peripheral immune cells from 15 patients diagnosed with HGSOC at various stages during their disease course. Full details on age, stage, moment of blood sampling and disease course of the patient can be found in Table 1. Samples were taken between November 2020 and August 2021. Patients with known (auto)immune diseases or receiving immune modulating drugs were excluded from this study. All patients gave written, informed consent. This study was approved by the local ethics committee (The Ethics Committee Research UZ / KU Leuven (EC Research)) (s63209) at the University Hospitals Leuven (Belgium). A schematic representation of the experimental design can be found in Figure 1.

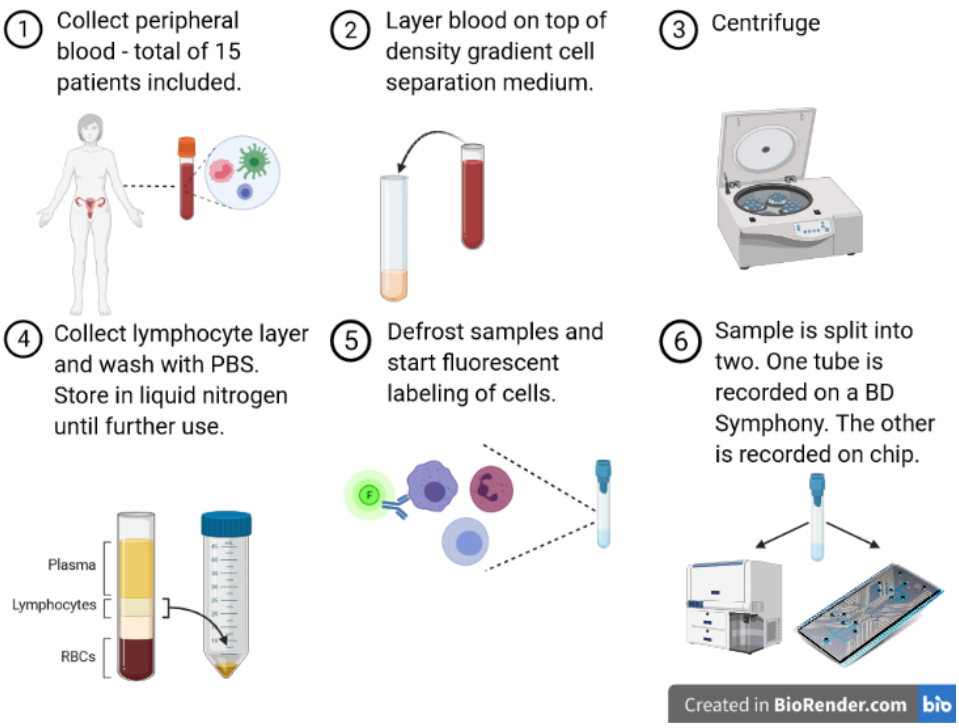

Figure I. Overview of experimental study design in a six-step process. Peripheral blood samples were taken from 15 patients with ovarian cancer. White blood cells were isolated by means of a density gradient medium and frozen until further use. Batches of four to five samples were defrosted and labeled with fluorescent dyes. Each fluorescently stained patient sample was split into two to do simultaneous but separate acquisition on a conventional flow cytometer (BD FACSymphony) and our own, silicon, microfluidics-based chip cytometer (Figure created in BioRender).

2.2 Sample preparation

Whole blood samples were taken at indicated time points (in Table 1) using Vacuette NH sodium heparin tubes (BD, ref 455051). Next, peripheral blood mononuclear cells (PBMC) were isolated using Lymphoprep Density Gradient Medium. Isolated cells were counted, frozen to -80 °C using a slow-freeze protocol (max -1 °C/min) and subsequently stored in liquid nitrogen until analysis. PBMC samples were defrosted in batches. Per batch, samples of four to five patients were simultaneously defrosted and prepared for analysis. Upon defrosting, cells were washed with 10 mL of ice-cold Dulbecco's Phosphate Buffered Saline (DPBS) and centrifuged for 10 min at 4 °C at 400 rcf. Next, cells were resuspended counted with an automated cell counter (ABX Micros 60, Axonlab) in order to be resuspended to one million cells per 100 µL for staining. In order to exclude dead cells, samples were stained with a viability dye (PE-Texas Red, Biotum, REF 32006-T) and incubated for 20 min at 4 °C in the dark. Cells were washed before adding cell surface marker stains; CD45 (PE-Cy5, clone HI30, eBioscence, REF 15-0459-42), CD8 (PE-Cy7, clone HTT8, Biolegend, REF 300914), PD1 (PE, clone REA1165, Miltenyi, REF 130-120-388). All dye concentrations were optimized via prior titration experiments. A fluorescence minus one (FMO) control for PD1 positivity was included for all patient

samples. After washing, cells were fixed with 4% paraformaldehyde for 20 min at 4 °C. Next, cells were washed again and resuspended thoroughly into 1% Bovine Serum Albumin (BSA)-DPBS before dividing each sample into two equal parts for separate but simultaneous acquisition on both applications. Both sample acquisition as well as sample analysis were performed by separate and double-blinded researchers (AVK and SL).

2.3 Flow cytometry analysis

Samples were acquired on the conventional flow cytometer BD FACSymphony at the KU Leuven FACSCore center. For these experiments, only the 561 nm Yellow-Green laser of the instrument was used. The system runs on BD FACSDiva 8.0.01 software. Specifications on detector configurations can be found in Supplementary Table 1. Analysis of raw data was performed with FlowJo Software (Version 10.7.1)

2.4 Chip cytometry analysis

Microfluidic cytometry and sorting chips have been developed by imec and are based on a microfluidic switching principle that is mild to cells and uses integrated microheaters in a sorting chamber to create local vapor bubbles using a principle that resembles inkjet printers. Those bubbles induce a pressure and force pushing cells towards a certain outlet. The bubble jet sorter chips were fabricated at imec on 200-mm silicon wafers based on the first generation described by De Wijs *et al*. [15]. Fluidic channels were fabricated in silicon to further enhance the chip fabrication process for high levels of parallelization and in order to be compatible with the mass manufacturing techniques known from the semiconductor sector. Moreover, the use of silicon channels walls and a quartz cover glass minimizes autofluorescence background signals in these chips. As depicted in Figure 2A, one microfluidic chip contained five microfluidic channels that could be addressed separately. The layout of one microfluidic channel is shown in Figure 2B. Cells are focused to the middle of the channel using 2D hydrodynamic focusing. Once in the main channel, the cells pass a narrow interrogation point after which they exit the chip through the main channel or the side channel in case the sorting chamber is activated.

In this study, we validated the cytometric aspect of the microfluidic chip. For this, an optical system was constructed based on commercially available optical components in order to detect viable CD45+ CD8+ PD1+ PBMC based on both scatter characteristics and fluorescence (Figure 2C). To visualize the fluorescence of passing cells, a 532 nm continuous wave laser (Coherent OBIS 532 LS FP, 40 mW) was focused through a 20x/0.42 NA (Mitutoyo) lens objective in the center of the 120 µm wide main channel. Beam shaping was performed to obtain a beam width of five µm. Backscattered light was collected by the objective lens and reflected by a dichroic mirror (DM LP550) to a PMT detector (BSC, Hamamatsu, H10723-01). A beam blocker (BB) was placed in the back focal plane to block reflected laser light and scatter from the fluidic channel walls. The collected fluorescence light is split by a beam splitter (BS 70:30) towards a CCD camera (Zeiss, Axiocam 503) for online monitoring of the sorting process and towards four fluorescence PMT detectors (Fl1-FL4 Hamamatsu, H10723-01) for cell detection. An optical path composed of several dichroic mirrors and bandpass filters were used as

depicted in the scheme to allow detection of PE, PE-Texas Red, PE-Cy5 and PE-Cy7. The side scatter signal (SSC) was detected on a PMT (Hamamatsu, H10723-20) through a multi-mode optical fiber with a 1000 µm core (Thorlabs M56L02). Optical signals were processed on a FPGA with integrated ADC (National Instruments USB-7855R) at a sample frequency of one million samples per second and processed by custom software (Labview, National instruments). Peak detection was set to the backscatter signal. Before samples were introduced onto the chip and in order to avoid clogging, the sample was filtered through a 30 µm mesh filter.

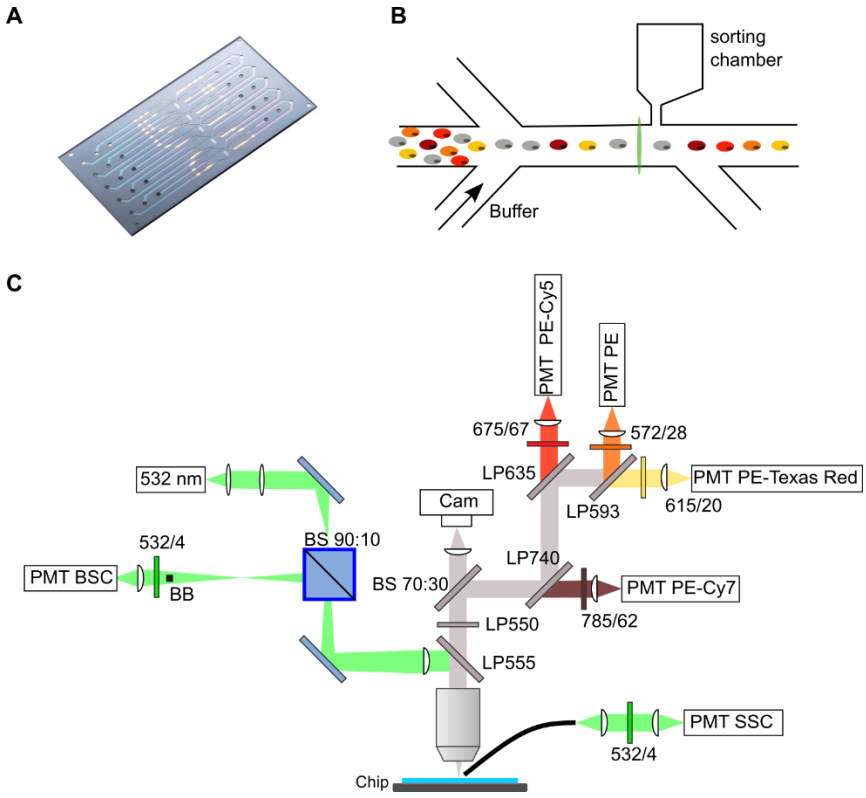

Figure 2. Description of chip cytometer setup. A/ example photograph of our chip design that contains 5 independent microfluidic channels. B/ illustration of one microfluidics system channel. Buffer fluids were used that act as sheath fluid to hydrodynamically focus cells into the center of the laser beam (green vertical line) for scatter and fluorescent detection. A sorting chamber was present but not used in current investigation. C/ illustration of optical system used in chip cytometer.

2.5 Statistics

Absolute cell numbers of $CD45^+$ cells and percentages of cell populations can be found in Supplementary Table 2. Statistical analysis was performed in Origin 2019. Linear regression analysis was performed between the measurements obtained on the BD FACSymphony and the microfluidic cytometer chip for both the $CD8^+$ $PD1^+$ and the $CD8^-$ $PD1^+$ population. Chip cytometry results were compared with flow cytometry results by means of a Pearson correlation and Bland-Altman plot.

3 RESULTS:

3.1 Patient demographics

PBMC samples were collected from 15 patients with advanced HGSOC. Patient characteristics are displayed in Table 1.

Table 1. Overview on patient characteristics

Characteristic		Detailed information	Result n (%)
Age	median (years), range		68 (49–76)
Stage	III		7 (47)
	IV		8 (53)
Moment of blood sampling	Diagnosis		4 (27)
	During primary treatment	5/8 at the moment of IDS after receiving NACT	8 (53)
		3/8 at the moment of IDS after receiving NACT in the ENGOT-OV43 study	
	After recurrence	1/3 at recurrence diagnosis	3 (20)
		2/3 during treatment for recurrence (1/3 with doxorubicine, 1/3 receiving Tisotumab-Vedotin therapy in the SGNTV-002 study	
(Peg)filgrastim	Yes		5 (33)
	No		6 (40)
	Not Applicable		4 (27)

NACT: neo-adjuvant chemotherapy (carboplatinum-paclitaxel); IDS: interval debulking surgery; ENGOT-OV43: Study of Chemotherapy With Pembrolizumab (MK-3475) Followed by Maintenance With Olaparib (MK-7339) for the First-Line Treatment of Women With BRCA Non-mutated Advanced Epithelial Ovarian Cancer (NCT03740165). SGNTV-002: A Study of Weekly Tisotumab Vedotin for Patients With Platinum-Resistant Ovarian Cancer With Safety Run-in (NCT03657043).

3.2 Gating principle of the samples: example of chip vs BD FACSymphony

Standard flow cytometry gating strategies were applied to analyze cell populations. Like in conventional flow cytometry, we excluded debris and doublets based on forward and sideward scatter information. Next, dead cells were excluded based on their positivity for the viability dye. From here, we selected the total $CD45^+$ population (versus forward scatter) and subsequently selected all $CD45^+$ $PD1^+$ cells based on FMO controls. From our viable cell population, we also gated all $CD45^+CD8^+$ versus $CD45^+CD8^-$ cells. Both cell populations were subsequently gated for their $PD1^+$ cells based on FMO controls. An example of this gating strategy on a representative sample can be found in Supplementary Figure I+2.

3.3 Correlation of PD1 populations between FACSymphony and the Chip Cytometer

Both the general $PD1^+$ populations as the more defined $CD8^+$ $PD1^+$ and $CD8^-$ $PD1^+$ populations relative to the total $CD45^+$ populations were determined. Correlation graphs of these populations measured via both methods show that the obtained data are scattered closely around the identity line, confirming that these two methods show a good similarity with a high Pearson's R value (0.99 for both $PD1^+$ and $CD8^+$ $PD1^+$ cells, 0.95 for $CD8^-$ $PD1^+$ cells, Figure 3). The agreement between the two methods was further quantified using Bland-Altman plots in which the difference between the two measurement points is plotted versus the average. For the overall $PD1^+$ population, the observed difference of the means is very close to equality (-0.31%) with a small negative bias for analysis on chip. When the $CD45^+$ population is subdivided into $CD8^+$ and $CD8^-$ populations, the resulting difference of the means for both populations are higher but remains below 2% (-0.57% in $CD8^+$ and 1.39% in $CD8^-$).

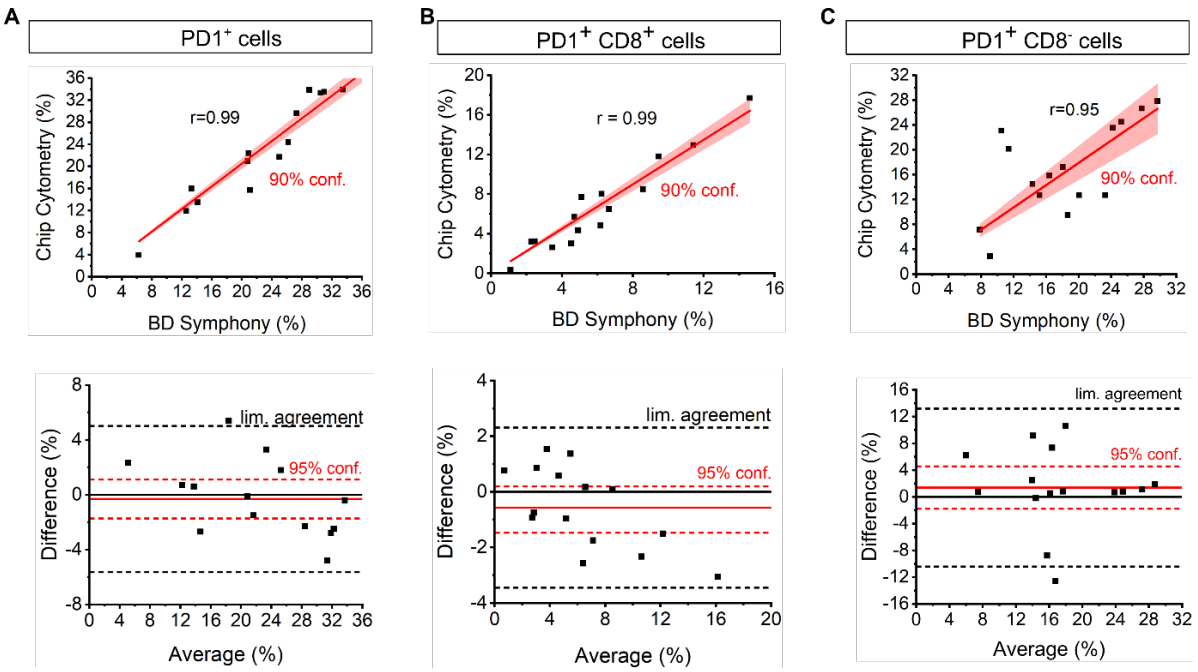

Figure 3. Pearson correlation and Bland-Altman agreement analysis for A/ total PD1$^+$ cells, B/ CD8$^+$ PD1$^+$ cells and C/ CD8$^-$ PD1$^+$ cells show high correlation and good agreement between chip cytometry and conventional flow cytometry in all three populations.

3.4 Clinically relevant patterns found on conventional cytometry are replicated through chip cytometry.

Although our proof-of-concept study only contained a limited number of patient samples, interesting patterns could be found when comparing PD1$^+$ cell populations with the clinical characteristics of the patients that were displayed both by the conventional flow cytometry data as the chip cytometry data. A first comparison was made looking at patient disease stage and the relative abundance of CD45$^+$ PD1$^+$ cells (Figure 4A+B). Strikingly, both on chip as with conventional flow cytometry, we noted the same trends when analysing CD8$^-$ PD1$^+$ and CD8$^+$ PD1$^+$ cells. Patients with FIGO stage III disease were found to have slightly more CD8$^-$ PD1$^+$ cells (Figure 4C+D). Contrary, CD8$^+$ PD1$^+$ cells were found to be less abundantly present in FIGO stage III patients (Figure 4E+F). In spite of the small dataset and large heterogeneity in the included patient population, a high abundance of total CD45$^+$ PD1$^+$ cells in the blood of patients with HGSOC, as measured by classical flow cytometry, could significantly be correlated with a shorter progression free survival (PFS) (Pearson R correlation, two-tailed, p=0.037). The same correlation could be found with our chip cytometry generated data (Pearson R correlation, two-tailed p=0.022) (Supplementary Figure 3).

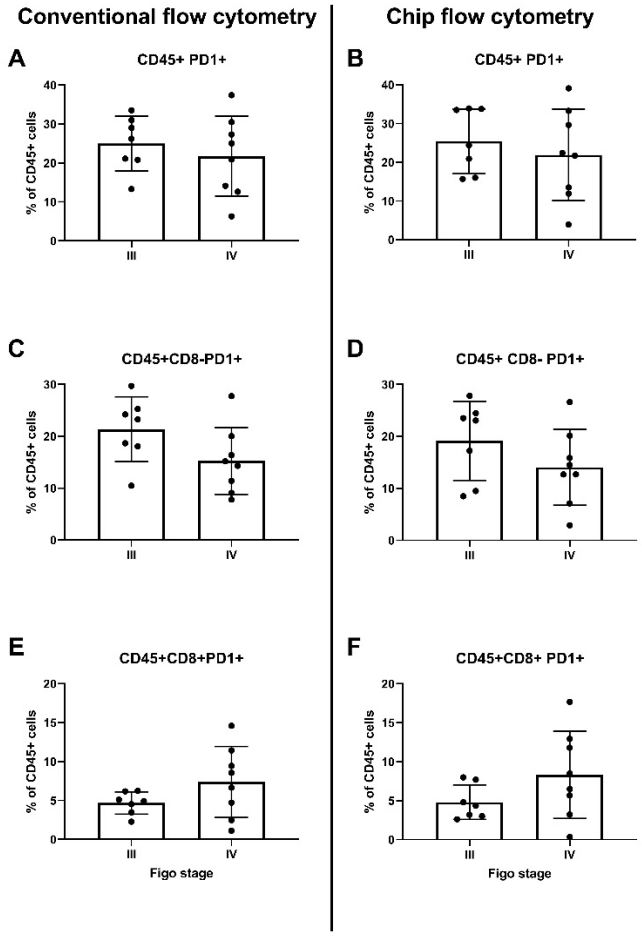

Figure 4. Clinical patterns of immune cells can be detected similarly on conventional and chip cytometry. A+B/ All $CD45^+$ $PD1^+$ cells as measured by respectively flow and chip cytometry showing a non-significant decline from stage III to stage IV. C+D/ Read-out of $CD8^-$ $PD1^+$ cells on respectively flow and chip cytometer after analysis showing a slightly higher mean of PD1 positivity in stage III (21.37% for conventional and 19.15% for chip flow cytometry) then stage IV (15.25% for conventional and 14.06% for chip flow cytometry). However, no significant difference between FIGO stages could be found. E+F/ Read-out of $CD8^+$ $PD1^+$ cells on respectively flow and chip cytometer after analysis. Mean PD1 positivity for stage III was 4.67% for conventional and 4.81% for chip flow cytometry versus respectively 7.38% and 8.33% respectively in stage IV samples. No significant difference between FIGO stages could be found.

3.5 Higher throughput acquisition

A fraction of eight samples was run on chip at a higher speed of 1 m/s to assess whether the smaller integration time would affect the sensitivity of the measurements. It must be noted that in the condition of 1 m/s, the ratio of the sample to the sheath fluid was increased, similar to increasing the speed on a conventional cytometer. This allowed for higher detection rate but also induced a higher occurrence of coincidence events (Figure 5). The measured population fraction at both speed regimes shows again a good correlation, especially for the defined $CD45^+$ $CD8^+$ $PD1^+$ population (Figure 5A).

This demonstrates that a decreased integration time for fluorescence collection at this speed regime does not hamper the measurement sensitivity. Further, it shows that the lower ratio of cell to sheath flow did not significantly affect the detection after proper doublet discrimination. Given that these chips can handle concentrations of 6.6 M/ml opposed to currently used 1 M/ml, we envision that a detection rate of 5000 cells per second per cytometric channel can be achieved. This opens perspectives towards multichannel chips that can analyse larger volume of single cells with higher throughput than conventional FACS instruments can.

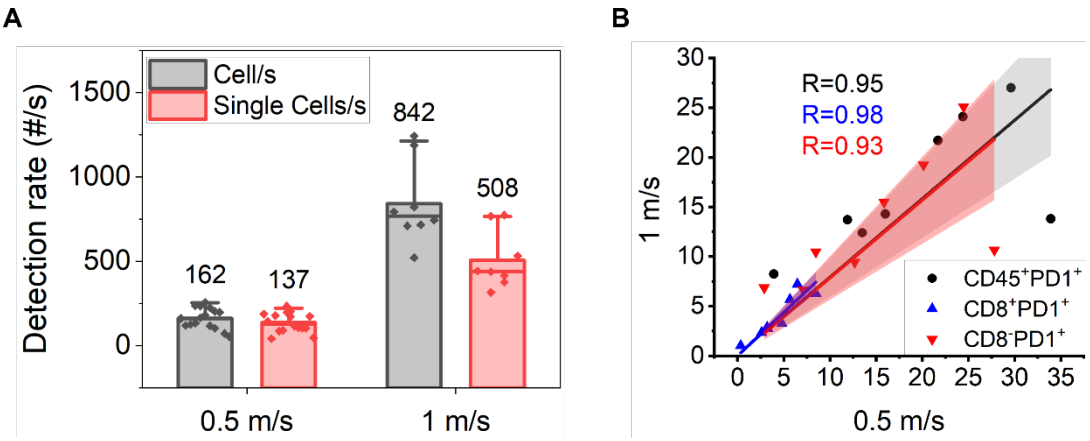

Figure 5. High speed acquisition does not hamper measurement sensitivity. A/ comparison of total detections and single cells acquired at speeds of 0.5 m/s and 1 m/s shows that the chip can handle high sample concentrations run at high speed and B/ still generates the same fluorescence sensitivity compared to lower speed acquisition as demonstrated by the Pearson corelation analysis for total $PD1^+$ cells (R = 0.94, black dots), $CD8^+$ $PD1^+$ (R = 0.99, blue, full coloured triangle) and $CD8^-$ $PD1^+$ (R = 0.92, red upside-down triangle) populations.

4 DISCUSSION

The ultimate goal of developing chip flowcytometry is to have a point of care instrument enabling fast acquisition and read-out of the immune profile in an out-patient setting without the need for highly trained staff. We performed this proof-of-concept study to investigate if our microfluidics-based chip cytometer could perform immune fluorescent data acquisition with the same accuracy and sensitivity as conventional flow cytometry. First, the enumeration of the different cell types on the chip flow cytometer corresponded well to the conventional cytometer. The success to detect dim PD1 marker expression with high sensitivity can be attributed by the absence of any auto fluorescent signal using silicon channels sealed with a quartz cover, compared to other materials frequently used in microfluidics [16]. Secondly, the same and clinically significant patterns in PD1+ cells could be picked up on the chip flow cell and the conventional cytometer.

Given the dim PD1 marker expression, these results show great promise for further development towards a compact chip cytometer for rapid, clinical decision making. However, it should be noted that current chip microfluidic flow cell has been evaluated for a limited color panel using bulk optical components. Although the flow cell itself has been optimized for the removal of any background signal, further limitation on sensitivity relies on the optical components used. Further optimization of this optical system towards larger immune panels and overall miniaturization needs to be done.

Next, in this proof of concept, the flow cytometry system was applied on a single microfluidic channel. To enable the throughput needed to compete with conventional flow cytometry, we envision to multiplex the microfluidic channels. However, there are some challenges ahead to realize the full potential of such system, especially related to parallelizing the detection as well as the sample focusing. Considering the optical challenge, we believe that the use of a silicon platform is the only option that allows for easy integration of photonics on chip to realize parallelized detection within a reasonable device format suited for an out-patient setting. Our institute has shown that a photonic circuit can be developed in silicon that is able to guide the excitation light to multiple detection points and the emitted fluorescent to the detector [17]. Another challenge is 3D sample focusing in a multichannel setting. The group of Wu tackled this issue by integrating two bulk standing acoustic waves on a glass microfluidic chip [18]. Although this approach eliminates the use of sheath fluid, the acquisition flow speed is largely limited by the length of the focusing channels thereby expanding the footprint of the device. Considering that high throughput will be needed in future applications to allow for enumeration of low abundance populations in a short time frame, we have opted to use hydrodynamic focusing. This technique allows for compact 3D focusing at a higher flow speed range.

On top of the cytometry aspect, we believe we can reach full clinical potential if downstream sorting would be integrated to this system as demonstrated by De Wijs *et al*. [15]. For example, the sorting of tumor antigen-specific T-cells with a high affinity could be used for the development of patient-specific, adaptive cell (immuno)therapy, a highly promising treatment for cancer patients – in part – hampered due to the absence of adequate cell-sorting techniques [19]. Besides our current microfluidics-based chip cytometry design, numerous other devices and approaches have been published based on mechanical, piezoelectric, dielectrophoretic and acoustic principles. The achievable throughput is different for each actuation scheme, but our bubble jet technology looks most promising in view of higher throughputs, i.e. higher speeds up to 5 kHz and higher levels of parallelization [15]. This sorting performance fits well with the observed detection sensitivity in this study. We have shown that with current optical components, we could achieve a detection rate of 5000 cells/s with a flow speed of 1 m/s and sample concentration of 6 M/ml

Comparing to detection rate in current state of the art flow cytometry, one could achieve 50000 cells/s using 10 microfluidic channels. However, this microfluidic approach could be scaled to more than ten channels per chip thanks to the silicon platform and the availability of on chip photonics [17]. In addition, microfluidics allows for future incorporation of cell staining and washings steps into one, fully automated system. Current clinical workflows are a combination of automated single steps. A different tool is used for automated antibody staining, automated washing and a final tool for automated flow cytometry analysis. All of those steps are prone to human error. Adherence to specific laboratory protocols and in depth knowledge of the conventional flow cytometers is paramount to achieve reproducible and trustworthy clinical data [20]. Currently, processing at a central lab has been the standard way of working to reduce this variability. However, it has been shown that lyophilization and sample transport on its own induce variability, especially on low abundance markers [21]. Using silicon technology, these steps could be envisioned as combined into one single device without the need for manual intervention and planning to transport the samples from one tool to the other [22,23].

In conclusion, we have paved the way for the implementation of immune profiling in clinical practice. Our lab-on-chip can produce the same immune results, compared to a conventional flow cytometer devise. More experiments with more complicated immune panels and combination into one single device are next steps to be undertaken.

FUNDING STATEMENT

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CONFLICT OF INTEREST DISCLOSURE

A.C. is a contracted researcher for Oncoinvent AS and Novocure and a consultant for Sotio a.s.. T.B. has received travel fees from MSD and Tesaro/GSK, is chair holder of an endowed chair from Roche and is a consultant for Tesaro/GSK. TVG has received honoraria for advisory boards from Eisai (Inst), OncXerna Therapeutics (Inst), AstraZeneca (Inst), GSK (Inst), MSD (Inst), research funding from Amgen (Inst), Roche (Inst) and AstraZeneca (Inst) and expense reimbursements from MSD, Immunogen, PharmaMar and AstraZeneca.

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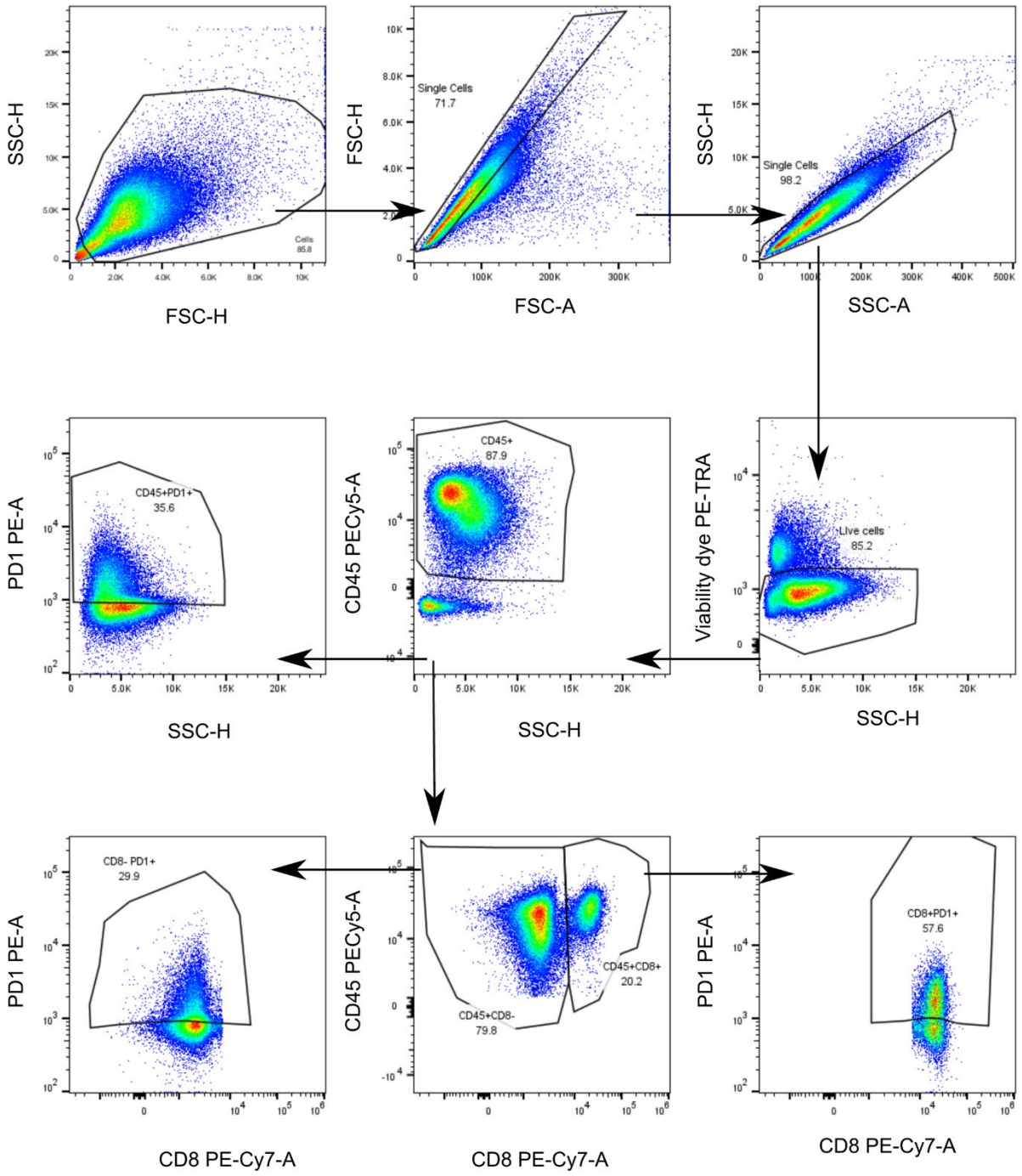
SUPPLEMENTARY INFORMATION

Supplementary Table 1. Information on filters used with the 651 nm Yellow-Green laser on the BD FACSymphony and the 488 nm Green laser on the Chip cytometry setup.

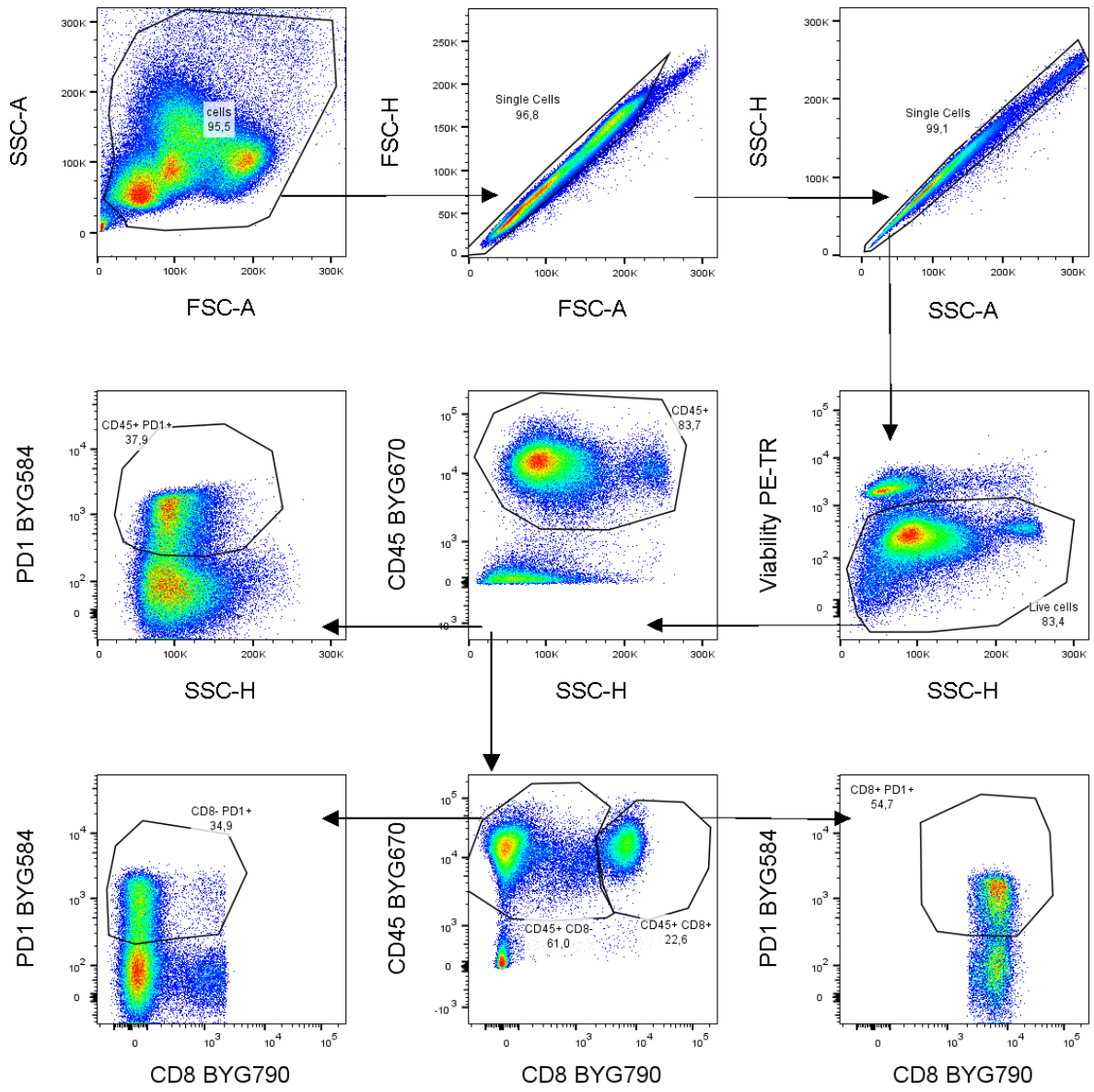
Fluorophore	BD FACSymphony		Chip cytometry setup	
	LP filter	BP Filter	LP filter	BP filter
PE	/	586/15	/	572/28
PE-Texas Red	600	610/20	593	615/20
PE-Cy5	635	670/30	635	675/67
PE-Cy7	750	780/60	740	785/62

Supplementary Table 2. Individual data per patient as recorded on BD FACSymphony and on chip cytometry setup. Total number of recorded $CD45^+$ cells are given, in addition to percentages of the total $CD45^+$ population respective populations.

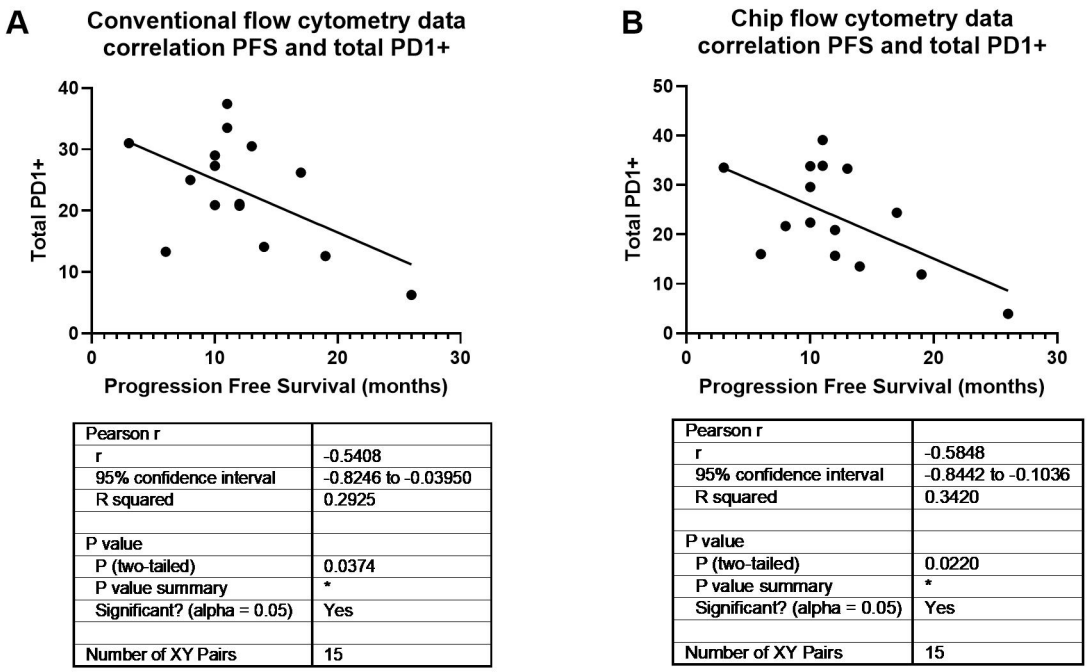
	BD FACSymphony				Chip cytometer			
	absolute cell count	percentage from CD45+ cells			absolute cell count	percentage from CD45+ cells		
Patient count	CD45+	CD45+ PD1+	CD45+ CD8+ PD1+	CD45+ CD8- PD1+	CD45+	CD45+ PD1+	CD45+ CD8+ PD1+	CD45+ CD8- PD1+
1	130737	20,9	11,4232	14,3172	66161	22,4	12,9352	14,5024
2	112849	25	8,5796	16,3944	32883	21,7	8,4942	15,8562
3	77064	12,6	4,704	7,82144	37970	11,9	5,675	7,1116
4	86969	21,1	4,5356	18,6524	28577	15,7	3,003	9,487
5	58837	33,5	6,1815	29,69225	507554	33,9	4,81164	27,80212
6	64535	26,2	3,4602	25,25508	26661	24,4	2,60307	24,46521
7	81104	20,8	4,9236	18,0544	47876	20,9	4,344	17,248
8	67803	6,27	1,10097	9,0949	47096	3,94	0,334908	2,889138
9	78491	31	6,2466	24,2018	29641	33,5	7,995	23,506
10	84055	30,5	14,6142	15,1774	40497	33,3	17,6788	12,6828
11	79259	37,4	9,4563	27,7715	60486	39,1	11,7943	26,6198
12	68849	27,3	6,6591	20,0455	86098	29,6	6,4872	20,1312
13	87501	14,1	2,46257	11,38138	62523	13,5	3,21993	12,68634
14	306322	29	5,1135	23,27	65999	33,8	7,6934	23,0533
15	100985	13,3	2,268	10,492	30647	16	3,192	8,4864



Supplementary Figure 1. Gating strategy on representative sample as acquired on chip cytometer.



Supplementary Figure 2. Gating strategy of the same sample illustrated in Supplementary Figure 1. as acquired on conventional flow cytometer.



Pearson r	
r	-0.5408
95% confidence interval	-0.8246 to -0.03950
R squared	0.2925
P value	
P (two-tailed)	0.0374
P value summary	*
Significant? (alpha = 0.05)	Yes
Number of XY Pairs	15

Pearson r	
r	-0.5848
95% confidence interval	-0.8442 to -0.1036
R squared	0.3420
P value	
P (two-tailed)	0.0220
P value summary	*
Significant? (alpha = 0.05)	Yes
Number of XY Pairs	15

Supplementary Figure 3. Similar correlation pattern of progression free survival (PFS, in months) with total number of $PD1^+$ immune cells can be found with data recorded on conventional flow cytometer (A) and chip flow cytometer (B).

Chapter 9

DISCUSSION

DISCUSSION

Immunotherapy is a powerful anti-cancer treatment option, but it is not yet working to its full potential in every cancer type, including ovarian cancer. Therefore, the goal of this dissertation was threefold: (A) deciphering the immunobiology of ovarian cancer in further depth, (B) development of therapeutic combination strategies based on information gathered in part A and (C) implementing immune-based strategies into the clinical setting.

PART A. DECIPHERING THE IMMUNOBIOLOGY OF OVARIAN CANCER

In a first study, using the ID8-fLuc ovarian cancer mouse model, the immune system was manipulated in various ways to identify cells of interest with the most significant impact on ovarian cancer disease progression (Chapter 3). Cells from the innate immune system (MDSC and macrophages) were found to impact the course of disease the most. In contrast, manipulation of the adaptive immune system failed to generate any effect [1]. To endorse the important role of the innate immune system in human ovarian cancer, we additionally set up a prospective pilot-study to explore macrophage subsets (Chapter 4). In 30 biopsies from 24 patients with ovarian cancer, we studied the presence of CD68+ cells (a protein highly expressed on monocytes and macrophages), MHCII (major histocompatibility complex II, found on professional antigen presenting cells such as tumour-associated macrophages with a more inflamed, anti-tumoral response) and MRC1 (anti-mannose receptor C type I, also known as CD206, primarily present on the surface of macrophages associated with immune suppressive capacities) [2]. Again, results suggested the importance of innate immune cells in ovarian cancer disease progression, as more total CD68+ cells and more MRC1+ cells were found in metastatic biopsies compared to matched, primary tumour samples. However, considering the limitations of biopsy based research [3–5], our research group also explored in a prospective cohort of patients with ovarian cancer, different immune subsets in blood taken at diagnosis. Here, we found that a panel of MDSC, NK cells and Treg cells could discriminate malignant from benign neoplasms with an AUC of 0.858 [6]. However, also this study lacked functional immune data. Therefore, we explored for a more systemic approach that could offer functional information as well. In collaboration with Prof. Abhishek Garg and his team (particularly Jenny Sprooten), we therefore established a new and unique assay (named sFIS, serum-functional immunodynamic status) that enables to screen for functional immune dynamics in the serum of patients [7]. In silico analysis of cytokine induced pathways in ovarian cancer showed – again – a dominant role for myeloid, and not lymphoid, driven activation of the nuclear factor kappa-light-chain enhancer of activated B cells (NFκB) pathway and interferon (IFN)-stimulated response element (ISRE) signalling. Furthermore, we could identify that higher enrichment of serum induced NFKB signalling over IFN signalling was associated with a negative prognosis. A strong inducer of NFKB

signalling is the cytokine tumour necrosis factor (TNF), suggesting that anti-TNF treatment would therefore be a valuable treatment option. Indeed, in murine experiments, anti-TNF treatment increased overall survival (Chapter 6). These findings were published in one of the leading immunotherapy journals (Sprooten J*, Vankerckhoven A*, *et al*. J Immunother Cancer 2021 - *shared first author). However, existing clinical trials using anti-TNF therapy (e.g. infliximab) resulted in mixed responses [8–10]. Intriguingly, NFKB genetic signature levels of these trials including ovarian cancer patients (available in a published gene expression dataset (GSE18681)) could separate responders from non-responders [7], meaning that a functional immune read-out of myeloid responses on patient serum cytokines can not only predict outcome but can also guide the therapy regime of patients.

The concept of the innate immune system as a key player in ovarian cancer onset and progression was not a popular idea at the start of this dissertation. Indeed, as already discussed in the introduction, the majority of evidence provided addressed the roles of different subset of T-cells. Consequently, the majority of clinical trails conducted in ovarian cancer have also focused on T-cells [11]. However, none of these have translated into a change in how we treat ovarian cancer today as many observed no or only a limited effect in overall survival. The significant and previously neglected role of the innate immune system in ovarian cancer progression could partially explain this discrepancy in results. However, research on innate immune cells in (ovarian) cancer is less mature and still overshadowed by discussions on correct identification, origin and terminology of the cells in question. MDSC are one of the most discussed innate immune cells in terms of context and classification. Originally, they have been identified as largely immature cells, derived from the myeloid lineage and exhibiting suppressive capacities [12,13]. They embody a complex and diverse innate cell population, showing large levels of plasticity. However, since the majority of these cells do not display distinct phenotypical characters to identify them, it remains up for discussion if they indeed should be classified separately instead of subdividing them as suppressive counterparts of existing innate immune cell classes [14]. As with the concept MDSC, also other myeloid cells such as macrophages are being rediscussed. In recent years, the simplified concept of macrophage polarisation with an M1 “anti-tumoral” and M2 “pro-tumoral” macrophage has been adapted [15]. The arbitrary terminology describing two opposing activities caused difficulties as the functional signature of macrophages often showed a more diverse portfolio [16]. An important but rather recent discovery and point of discussion is the idea that the “myeloid cells” we have been studying, might not at all be myeloid cells. Myeloid lineage refers to cells derived from bone marrow progenitor cells. However, increasing evidence has been provided that macrophages in the tumour microenvironment can be traced back to two or three separate lineages and are thus not all myeloid in origin [17]. This of course raises some important questions concerning cancer immunology: Are these different lineages functionally different? Do they respond to cancerous

cells in the same way? And can they be influenced by immunotherapies? An article by Ruo-Yu *et al*. showed that recruited, monocyte-derived macrophages had a greater impact at promoting bone metastasis formation of breast cancer than tissue resident macrophages [18]. But are these findings similar in every tissue/cancer type? These questions remain to be answered by future studies.

PART B. DEVELOPMENT OF THERAPEUTIC COMBINATIONS

The second goal of this dissertation was the development of rational-based therapeutic combination strategies and test them pre-clinically in our orthotopic ovarian cancer mouse model. Often, immunotherapy is combined with the standard of care, being surgery and chemotherapy in ovarian cancer, to increase its potential and thereby generate an increased survival benefit. However, also clinical combination trials up till now are mainly focussed on targeting the adaptive immune system instead of the innate immune system. Up till now, also here results remain unsatisfactory. For example, a large phase III clinical trial (IMAGYN050), testing the efficacy of atezolizumab in combination with carboplatin-paclitaxel failed to increase progression free survival, with 19.4 months in the atezolizumab group versus 18.4 months in the placebo group (hazard ratio, 0.92; 95% CI, 0.79 to 1.07) [19]. We believe these disappointing results can be explained by two different factors. First, as discussed above, the innate immune suppression is not properly addressed with most therapeutic strategies currently in trial. Secondly, our results demonstrate that standard of care induces immune changes, which are currently not taken into account when designing clinical trials. We performed in our ID8 mouse model a head to head comparison of six different chemotherapeutics often used to treat ovarian cancer [20] (Chapter 5). Carboplatin-paclitaxel was found to induce the most beneficial immune changes, which made it appealing for combinatorial use. Simultaneously, we performed a prospective study on 90 patients with ovarian cancer, where serial serum samples were collected at diagnosis and during primary treatment (De Bruyn C, Ceusters J, Landolfo C, Baert T, Thirion G, Claes S, Vankerckhoven A, *et al*. Cancers (Basel), 2021) [21]. Also here, we discovered that carboplatin-paclitaxel chemotherapy, especially in a neo-adjuvant treatment setting of nine weeks, was most efficient in reducing immunosuppression, creating a window of opportunity to administer immunotherapy. However, we also discovered that this window was very limited in time, as interval debulking surgery seemed to abrogate this positive effect. Additionally, we hope to confirm the effects of carboplatin-paclitaxel also by flow cytometry data of PBMC samples of the same cohort and on samples collected in the control arm of an international clinical trial.

Based on this knowledge, we focused on combination strategies including carboplatin-paclitaxel chemotherapy. Pre-clinical experiments, testing different immunotherapies, were performed during the scope of this dissertation (Chapter 6). Unfortunately, most of them did not result in impressive

changes in the disease course. Mostly, slight changes in ascites formation were seen but a significant overall survival benefit if compared to the standard of care remained absent. We hypothesize that this can be explained by two main issues.

On the one hand, as discussed in the introduction, we are facing different kinds of lineages of immune cells contributing to the full immune suppressive spectrum in ovarian cancer. It is rather unrealistic to assume that one immunotherapeutic strategy can sufficiently target this diverse set of immune cells. In addition to this, we most likely also work with different stages of maturation within one specific type of immune cells. Differences in maturation stages can result in difference in function and how to target them. Indeed, a study by Kitamura *et al*. shows in a lung cancer model that there are three different maturation states of myeloid cells present in the tumour microenvironment [22]. All three were able to inhibit effector T-cell function and thus contribute to the immune suppression present in the tumour microenvironment, halting an effective anti-tumoral immune response. However, as they mature into a different state, they express different levels of checkpoint receptor ligands with the highest expression in the most mature stages. In the more early stages, their immune suppression mainly is regulated by nitric oxygen or reactive oxygen species production. Therefore, concerns can be raised if one type of immunotherapy combined with the standard of care will be enough to tackle these different immune suppressive hurdles. On the other hand, we have discovered that not only the compounds which you combine should be rationally chosen, but that also the order and timing of the therapies significantly impact their effect [23]. Indeed, on multiple occasions we have shown that slight adjustments in the therapeutic schedule of the same therapies dramatically change the disease progression (Chapter 6 and 7). Underlying mechanisms of these changes remain to be identified and are part of future studies. In-depth studies of proposed schedules in preclinical models in addition to detailed exploration of (functional) immune dynamics in patients during the course of their therapy regime could help in deciphering the most optimal regimen

Altogether, these findings support the hypothesis that we most likely will need more than one immunotherapy-based strategy to boost a successful, durable, anti-tumoral response. Furthermore, pre-clinical testing of these therapy regimes will need to be carefully executed. With current techniques available, this work is highly laborious, expensive and time-consuming. Monitoring (functional) immune changes during clinical trials at a systemic level could offer better guidance in the design and testing of these strategies.

PART C. IMPLEMENTING IMMUNE BIOLOGY INTO THE CLINICAL SETTING

Monitoring changes of the immune system at the systemic level has the potential of offering an additional clinical tool for cancer diagnosis and care. Earlier, our group already showed that a blood-

based immune read out (mainly focussing (again) on innate immune suppressive cells) could discriminate malignant from benign ovarian tumours [24]. Regular in-depth monitoring throughout treatment and disease course could offer insights into the dynamic changes of the immune system could assist into the choice, timing and combination of immunotherapies. Yet, this is no common practice. An explanation for this might be that classical flow cytometry systems are complicated to handle and require specialized personnel, limiting its use only to specialized facilities. With the development of microfluidic chip based flow cytometry in close collaboration with IMEC, we aim to develop an instrument that enables a fast immune read-out at the patients bed-side, without the need for specialized personnel (Chapter 8). In a first set of 15 patients, we were able to demonstrate the performance of this technology. Our results can be the first step for future development of this chip, that could support physicians in their decision of selecting the most appropriate therapy for a patient in the future. Next steps are the combination of the different mechanical elements into one single device and the expansion to larger and more complicated immune panels.

To conclude, we have provided additional evidence that the innate immune system is the key player in driving ovarian cancer progression by contributing significantly to anti-cancer immune suppression. Therefore, effective immunotherapeutic strategies should - in part - be focused on targeting innate immune cells. Additionally, we have provided proof in pre-clinical models that combination treatments with only one immune modulating agent are not effective enough to target the multiple facets of the immunosuppression in ovarian cancer to generate a sufficient anti-cancer response. We also demonstrated that when designing a combination, the order and timing of the different compounds used is crucial. Monitoring immune dynamics during treatment may offer valuable insights, but is cumbersome at this moment. Therefore, we have provided the first proof of concept of a microfluidics chip based cytometer, that could make implementation of regular immune-read outs more accessible.

FUTURE PERSPECTIVES

The immune system is an important player in ovarian cancer disease onset and progression. Elucidating the immunodynamics – especially on a systemic level – at regular intervals during disease development and treatment can offer valuable insights. For this, our group is involved in large prospective (European) studies that monitor patients during their disease course or during new targeted treatments. To facilitate this process in a clinical setting, this thesis has contributed largely in the development of two new techniques, which have the full potential to be further explored to the full in the future: lab-on-chip cytometer and the sFIS assay. On the long run, this could then assist treating physicians in personalized cancer care, depending on the immune profile of the patient. Therefore, also the development and testing of new immunotherapies should be continued, so that for different

immune profiles, a different treatment can be proposed. Next to this, the immune system can also be an added value for ovarian cancer diagnosis or even in screening. Our group has already shown that a profile of myeloid and Treg cells can discriminate benign from malignant ovarian masses at diagnosis [24]. Developing models that can incorporate immune information could provide additional assistance. Moreover, together with the group of Professor Sarah Blagden (University of Oxford, Department of Oncology, Cancer Therapeutics and mRNA dysregulation, Oxford, UK), we are currently exploring the immune system in the pre-invasive precursors of ovarian cancer, STIC lesions (serous tubal intraepithelial carcinoma).

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Chapter 10

SUMMARY

SUMMARY

Ovarian cancer has the fifth highest mortality rate among women diagnosed with cancer. High-grade serous (tubo) ovarian cancer is the most common histological subtype. The standard of care is a combination of cytoreductive surgery and platinum-based chemotherapy. Although initial responses are good, the majority of women experience relapse and often, relapsed tumours are less sensitive to chemotherapy, making successful treatment more challenging. Recently, two targeted therapies have been approved for maintenance therapy. The first is a monoclonal antibody directed against vascular endothelial growth factor (VEGF) that targets angiogenesis in the tumour microenvironment. The second comprises inhibitors of the poly ADP-ribose polymerase (PARP) enzyme and target DNA repair mechanisms of the tumour cell. Both have shown excellent improvement of progression-free survival, but have not (yet) proven to improve overall survival. Therefore, new therapeutic strategies remain to be investigated, including immunotherapies.

Immunotherapies have revolutionized cancer management and have shown great successes in some cancer types. Also in ovarian cancer, the immune system has been identified as a key player in the disease onset and progression. However, immunotherapy trials for ovarian cancer have led, until now, to only limited responses. It appears that the immunological situation for ovarian cancer is more complex and needs better understanding in order to make immunotherapy more successful.

Therefore, we first decided to explore the immune biology of ovarian cancer better. In an orthotopic, fully immune competent, metastatic ovarian cancer mouse model, we demonstrated that not the adaptive immune system, but the innate immune system and more specifically suppressive cells like MDSC and tumour-associated macrophages (TAMs) are key in manipulating ovarian cancer disease development. Additionally, we explored the components of the innate immune system in biopsies of ovarian cancer patients. Here, we could correlate the importance of immunosuppressive M2-like TAMs with the more aggressive character of ovarian cancer.

Following these findings, we wanted to develop and test combinatorial treatment strategies that included the standard of care and immunotherapies that manipulated the (innate) immune system. However, information on how and when to combine different strategies with the standard of care was limited and scattered. To ameliorate this, we compared six commonly used chemotherapies in our ovarian cancer mouse model. Here, we found that the most commonly used chemotherapy in first line, a combination of carboplatinum and paclitaxel, resulted in the most favourable immune profile. With this in mind, we combined this chemotherapy with several immune modulating drugs in an ovarian cancer mouse model. Here, we found that combinations with monoclonal antibodies directed against the checkpoint TIM3 (T-cell immunoglobulin and mucin-domain containing-3) and the cytokine TNF-alpha (tumour necrosis factor alpha) were most successful in changing the disease course. We also could demonstrate that the order/sequence in which the different treatments were combined, altered the survival outcome of the mice.

From our observations, it is clear that the immune biology is important to take into account when designing immunotherapeutic strategies. Monitoring these immune dynamics at a systemic level on a

regular basis can offer guidance. To facilitate immune monitoring, we collaborated with Interuniversity Microelectronics Centre (IMEC) on a successful proof of concept study that tested a microfluidics based chip cytometer and compared its performance with conventional flow cytometry. This study can be the basis of easy implementable immune monitoring in an out-patient setting.

In summary, we have given new insights into the complex landscape of ovarian tumour immunology, proposed strategies to make successful combinations and provided a proof of concept to facilitate implementation of monitoring immune changes from the patient at a systemic level in order to better guide and design immunotherapeutic combination strategies.

Chapter 11

SCIENTIFIC ACKNOWLEDGEMENT, CONFLICT OF INTEREST AND FUNDING STATEMENTS

SCIENTIFIC ACKNOWLEDGEMENT

Chapter 3: Myeloid Derived Suppressor Cells: Key Drivers of Immunosuppression in Ovarian Cancer

Conceptualization: Thaïs Baert, An Coosemans, Ignace Vergote
Design of experiments: Thaïs Baert, An Coosemans
Experimental work: Thaïs Baert, Ann Vankerckhoven, Thomas Mathivet
Analysis of results: Thaïs Baert, Ann Vankerckhoven
Statistics: Thaïs Baert
Interpretation of results: Thaïs Baert, Ann Vankerckhoven
Technical Assistance: Matteo Riva, Gitte Thirion, Anaïs Van Hoylandt
Manuscript writing: Thaïs Baert, An Coosemans
Manuscript proofreading: Ann Vankerckhoven, Matteo Riva, Gitte Thirion, Thomas Mathivet Holger Gerhardt, Ignace Vergote
Supervision: Thaïs Baert, An Coosemans

All authors have read and agreed to the published version of the manuscript.

Chapter 4: Opposite Macrophage Polarization in Different Subsets of Ovarian Cancer: Observation from a Pilot Study

Conceptualization: An Coosemans, Thaïs Baert
Design of experiments: An Coosemans, Thaïs Baert, Thomas Mathivet
Experimental work: Thomas Mathivet, Thaïs Baert
Resources: An Coosemans
Analysis of results: Thomas Mathivet, Thaïs Baert
Statistics: Jolien Ceusters
Interpretation of results: Ann Vankerckhoven, Roxanne Wouters, An Coosemans, Thaïs Baert
Visualisation: Ann Vankerckhoven, Roxanne Wouters
Technical Assistance: Anaïs Van Hoylandt
Manuscript writing: Ann Vankerckhoven, Roxanne Wouters
Manuscript proofreading: Ann Vankerckhoven, Roxanne Wouters, Thaïs Baert, Thomas Mathivet, Holger Gerhardt, Ignace Vergote, An Coosemans
Supervision: An Coosemans, Ignace Vergote

All authors have read and agreed to the published version of the manuscript.

Chapter 5: Type Of Chemotherapy Has Substantial Effects On The Immune System In Ovarian Cancer

Conceptualization: Ann Vankerckhoven, Thaïs Baert, An Coosemans
Design of experiments: Ann Vankerckhoven, Thaïs Baert
Experimental work: Ann Vankerckhoven
Analysis of results: Ann Vankerckhoven, Jolien Ceusters
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Manuscript writing: Ann Vankerckhoven, An Coosemans

Manuscript proofreading: Ann Vankerckhoven, Thaïs Baert, Matteo Riva, Christine De Bruyn, Gitte Thirion, Katja Vandenbrande, Jolien Ceusters, Ignace Vergote

Supervision: An Coosemans, Ignace Vergote

All authors have read and agreed to the published version of the manuscript.

Chapter 6: Development Of Rationally Designed Combinatorial Immunotherapy Strategies For Ovarian Cancer

Conceptualization: Ann Vankerckhoven, An Coosemans

Design of experiments: Ann Vankerckhoven, Jenny Sprooten, An Coosemans, Abhishek D. Garg

Experimental work: Ann Vankerckhoven, Yani Berckmans, Roxanne Wouters, Jenny Sprooten

Analysis of results: Ann Vankerckhoven, Jenny Sprooten

Statistics: Ann Vankerckhoven, Jolien Ceusters

Interpretation of results: Ann Vankerckhoven, Jenny Sprooten

Technical Assistance: Gitte Thirion, Katja Vandenbrande, Louis Boon

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Supervision: An Coosemans, Abhishek D. Garg, Ignace Vergote

Part of these results are published. All authors have read and agreed to the published version of the manuscript.

Chapter 7: Combining Conventional Therapy With Immunotherapy: A Risky Business?

Conceptualization: An Coosemans, Ann Vankerckhoven, Thaïs Baert

Design of experiments: An Coosemans, Thaïs Baert

Experimental work: Ann Vankerckhoven, Thaïs Baert, Hanne Ruts

Analysis of results: Ann Vankerckhoven, Thaïs Baert

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Supervision: An Coosemans, Ignace Vergote

All authors have read and agreed to the published version of the manuscript.

Chapter 8: A Microfluidics Approach For Ovarian Cancer Immune Monitoring In An Out-Patient Setting

Conceptualization: Ann Vankerckhoven, Sarah Libbrecht, An Coosemans, Liesbet Lagae, Dirk Timmerman

Design of experiments: Ann Vankerckhoven, Sarah Libbrecht, Koen de Wijs, An Coosemans, Liesbet Lagae

Experimental work: Ann Vankerckhoven, Sarah Libbrecht
Analysis of results: Ann Vankerckhoven, Sarah Libbrecht
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Supervision: An Coosemans, Liesbet Lagae

All authors have read and agreed to the version of the manuscript submitted for publication.

CONFLICT OF INTEREST STATEMENT

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Chapter 12

CURRICULUM VITAE

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Research activities

01/10/2018 – 31/10/2022: PhD Project

Subject: Unravelling the tumour immunology of ovarian cancer
Promotor: Prof. Dr. An Coosemans, Co-Promotor: Prof. Stefaan Soenen

Awards and grands:

- Travel grant "First contact Initiatives" 2018-2019
- Emmanuel van der schueren scholarship by "Kom op tegen Kanker" 2019-2020

Research stays:

- Aug 2021 – Nov 2021: part time internship at imec, Leuven
- Jan 2019: research stay abroad at University of Oxford, followed by multiple short stays (2-3 days) over the course of 2019-2022)

Technical skills:

- Cell culture
- Fluorescence-activated cell sorting (FACS): panel design, staining, acquisition, data analysis
- Cytometric bead array (CBA)
- Scientific Animal Research
- Bioluminescence imaging
- Basic statistical analysis (Prism)
- Magnetic bead cell isolations
- Immunohistochemistry analysis (Qupath)
- Processing of whole blood or serum samples for white blood cell (PBMC) or protein extraction

Co-promotor/instructor of thesis
to obtain the degree of master in the Biomedical Science

- 2020-2021: Yani Berckmans

to obtain the degree of master in Medicine

- 2020-2021: Wilhelmine Verreet, Julie Kempeneers, Lotte de Veth
- 2021-2022: Janne Versonnen, Nele Vermeulen

Teaching

- Guest Lecture on "model organisms in research" at Thomas Moore (Campus Geel) (04/2021)
- Supervision of labrotation students (First master in Biomedical Sciences)
    - 2018-2019: Marta Ciwinska, Ruth Lanoo
    - 2019-2020: Sam Verwimp, Pauline Dagneau de Richecour
    - 2020-2021: Emilia Laura Bialek, Lowie Deprez
    - 2021-2022: Bo Verwimp, Loes Vanhoof, Lori Vermoesen
- Supervision of summer student investigators
    - 2019: Esma Bahar Tankus
    - 2020: Vanshika Malviya

Additional courses for personal skill development

- Good scientific conduct: Scientific integrity (2021)
- Communication: Pitch your own research (2020), Delivering presentations remotely (2020), Sosocial media academy (2021)
- Information and publication skills: Writing course for biomedical research (2018), Effective poster creating (2019), Systematic review writing (2020), Cochrane systematic review writing course (2020)
- Career development: Online Branding & LinkedIn (2019), Networking (2019), Introduction to leadership (2021), brand your career profile (2021)
- Didactic training: Supporting academic writing (2018)
- Scientific tools: Flowjo Software (2018)
- Scientific skills and certificates: Radioprotection (2018), Safety in the lab (2018),
- Felasa C: Advanced Course in Laboratory Animal Science (Module II)

September 2018: summer job as researcher

Subject: role of innate immune cells in ovarian cancer
Head: Prof. Dr. An Coosemans
Technical skill development: immunohistochemistry analysis, laboratory animal handling

2017-2018: Dissertation for Master in Biomedical Sciences

Subject: The function of MDSC in ovarian cancer
Promotor: Prof. Dr. An Coosemans, Co-promotor: Prof. Dr. Thaïs Baert
Awarded with: "Best Master thesis of Biomedical Sciences 2018 – second place".
Fast-paced research to finish project in nine months, required stress resilience and successful balancing work-life, obtained by good planning, time management and prioritizing of projects to meet deadlines.

August 2017: Student Investigator (LVSO)

Subject: role of the adaptive immune system in ovarian cancer
Promotor: Prof. Dr. An Coosemans, Co-promotor: Prof. Dr. Thaïs Baert
Fastly familiarizing with wet lab activities and quickly acquiring knowledge on the given topic of Tumor Immunology in Ovarian Cancer. Technical skill development: performing basic cell culture, basic laboratory animal handling, providing technical assistance during experiments.

Education

2018-2022 PhD student at the Doctoral School of Cancer (KU Leuven, Belgium)
2012-2018 Biomedical Sciences at Catholic University of Leuven) – Magna cum Laude (KU Leuven, Belgium)
2008-2012 Modern Languages and Science at Sint-Dimpnacollege (KOGEKA, Belgium)
2006-2008 Science and Mathematics at Sint-Aloysius institute (KOGEKA, Belgium)

Scientific Output

Publications

Vankerckhoven, A., Baert, T., Riva, M., De Bruyn, C., Thirion, G., Vandenbrande, K., Ceusters, J., Vergote, I., Coosemans, A. (2021). Type of chemotherapy has substantial effects on the immune system in ovarian cancer. TRANSLATIONAL ONCOLOGY, 14 (6), Art.No. ARTN 101076.
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