Tumor immunology & the application of immunotherapy in ovarian carcinoma

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DOI:
10.33612/diss.182133606

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Tumor immunology and the application of immunotherapy in ovarian carcinoma
PhD dissertation, University of Groningen, The Netherlands

Author: Sterre T. Paijens
Cover design: Catherine Willemse
Printed by: Ridderprint

Financial support of this thesis was kindly provided by:
University Medical Center Groningen
Graduate School of Medical Sciences
Stichting Olijf
Chipsoft
Bridea Medical B.V.
Paijens-Schouten B.V.

The digital version of this thesis can be found by scanning the QR code

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Tumor immunology and the application of immunotherapy in ovarian carcinoma

Proefschrift

Ter verkrijging van de graad van doctor aan de
Rijksuniversiteit Groningen
op gezag van de
rector magnificus prof. dr. C. Wijmenga
en volgens besluit van het College voor promoties

De openbare verdediging zal plaatsvinden op
maandag 15 november om 14.30 uur

door

Sterre Tjitske Paijens
Geboren op 13 augustus 1990
te Utrecht

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Chapter 1

General introduction

Introduction

Epithelial ovarian cancer (EOC) is the most lethal gynecological malignancy with an overall 5 year survival rate of 46%. Due to the asymptomatic nature of the disease and lack of screening methods, most woman are diagnosed at an advanced stage (FIGO stage >IIB), which has a 5 year survival rate of only 29%. Each year, 230.000 woman are diagnosed with EOC worldwide, with approximately 1300 new cases within the Netherlands.[1,2] EOC can be divided into various histological subtypes, of which high grade serous carcinoma (HGSOC) is the most common. HGSOC accounts for approximately 70% of EOC and is considered to originate from the fallopian tube.[2] Other histological subtypes are endometrioïd (10%), clear cell(10%), mucinous (3-4%) and low grade serous carcinoma (<5%)[3]. Around 15% of the EOC have a hereditary cause, with mutations in the BRCA1 and BRCA2 genes accounting for approximately 70% of hereditary cases and Lynch syndrome for 10-15%.[2]

Current primary treatment is the same for all histological subtypes and consists of cytoreductive surgery and intravenously administered platinum-based chemotherapy, in most cases carboplatin combined with paclitaxel. Patients are either treated with primary debulking surgery (PDS) followed by six cycles chemotherapy or are initially treated with three cycles of neo-adjuvant chemotherapy (NACT), followed by an interval debulking surgery and three additional cycles of chemotherapy (figure 1).[4] In addition to interval debulking surgery, a subset of stage III NACT patients are also treated with hyperthermic intraperitoneal chemotherapy (HIPEC). Intraperitoneal delivery of chemotherapy eliminates residual microscopic tumor tissue via enhanced drug delivery at the peritoneal surface. In addition, the hyperthermic state induces increased sensitivity of the cancer to chemotherapy, is directly cytotoxic for the cancer cells and increases chemotherapy penetration into the peritoneal tissue.[5] Thus far, one randomized controlled phase III trial was published and has demonstrated improved progression free survival (PFS) and overall survival (OS) when adding HIPEC to interval debulking surgery. This procedure did not result in higher rates of side effects.[5,6]

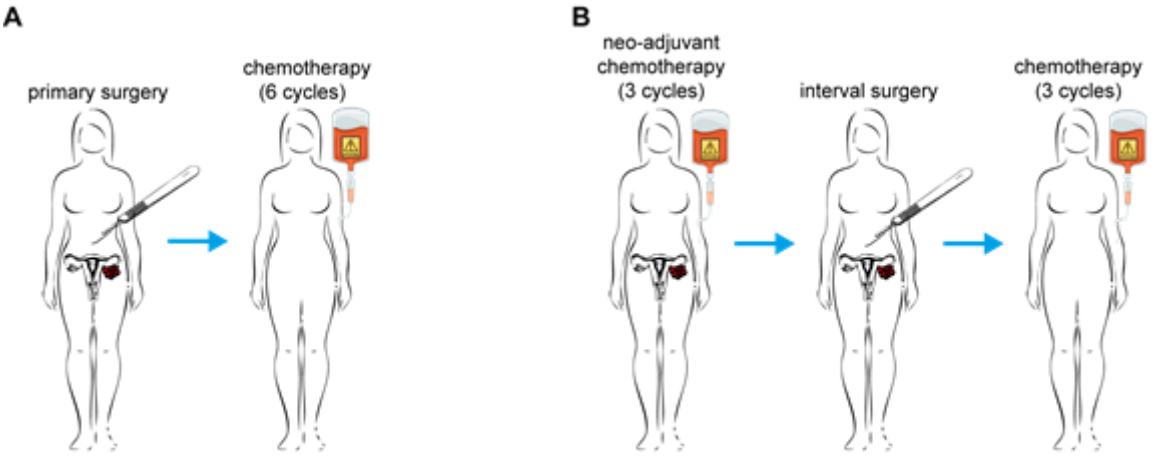

Figure 1. Primary treatment strategies for ovarian cancer. A. Ovarian cancer patient treated with a primary debulking surgery followed by six cycles of chemotherapy. **B.** Ovarian cancer patients treated with three cycles of neo-adjuvant chemotherapy (NACT), followed by an interval debulking surgery and three additional cycles of chemotherapy.

Although initial response to primary treatment is high, most patient suffer from a relapse within 2 years, become chemotherapy-resistant and succumb to their disease. Therefore new therapy strategies are needed. Over the last year, three major phase III clinical trials have been published on the application of PARP-inhibitors in EOC; as first-line and maintenance therapy, as mono-therapy and in combination with chemotherapy or bevacizumab. All strategies showed improvement of PFS in the overall EOC population, although the effect was most profound in BRCA germline mutated patients. These studies demonstrate a great potential of PARP-inhibitors, however, effect on overall survival is still pending.[7–9]

The immune system is considered to play an important role in the control of EOC. Hence, the presence of tumor infiltrating lymphocytes (TILs) represents a favorable prognostic indicator.[10] Especially, differentiation, exhaustion, and other functional parameters of intraepithelial $CD8^+$ T cells have been associated with improved OS.[11–13] In addition, the presence of both $CD4^+$ T cells and $CD20^+$ B cells have shown to influence the beneficial effect of $CD8^+$ T cells.[14,15] Moreover, T cells and B cells co-localize in well-organized tertiary lymphoid structures (TLSs) resembling activated lymph nodes. The TLSs are frequently surrounded by antibody producing B cells (plasma cells) and are also associated with improved survival in HGSOC patients.[16] Regulatory T cells and myeloid-derived suppressor cells have been associated with an unfavorable effect on prognosis.[14,17]

A number of studies have investigated the application of immunotherapy in EOC, including vaccination strategies and immune checkpoint inhibitors (ICI). Unfortunately, thus far, most studies conducted in EOC are phase I/II clinical trials. Randomized control trials (RCT) are scarce and overall success is limited. While different vaccination strategies have proven to be immunogenic, so far they lack convincing clinical response. In line with the limited success of vaccines, ICI monotherapy has also shown poor objective response rates of only 10-15%.[18,19] These trials suggest that clear cell ovarian carcinomas are potentially more responsive to ICI.[18–20] The success of immunotherapy hinges on the ability of the immune system to recognize tumor-associated antigens (TAA), such as neoantigens, and to induce an anti-tumor immune response.[21] In order for the immune system to recognize TAA, adequate antigen presentation via major histocompatibility a complex class 1 (MHC-1), is needed. MHC-1 expressed on tumor and immune cells, presents small antigenic peptides to the receptor of T cells (T cell receptor (TCR)), thereby initiating an immune response. Down regulation of MHC-1 is a well-known mechanism of immune escape in cancer and has also been described in EOC.[22–24] Moreover, EOC is characterized by a relatively low mutational burden and low numbers of TILs compared to e.g. melanoma, and lung cancer.[25,26] Consequently, the naturally occurring anti-tumor immune response is quite low in EOC. Improving immunogenicity of EOC may improve clinical response rates to immunotherapy.[21] Taken together, no major advances in the treatment of EOC have been developed and OS has only improved by 2% over the past decade.[1] Studies providing insight into EOC tumor immunology are therefore crucial to help understand these failures, unravel which patients might respond to immunotherapy and to which type of immunotherapy. Advances in the field of immunotherapy application in general have further highlighted the importance of patient selection, timing of therapy administration and combination strategies in order to optimize response rates. This thesis elaborates on the makeup of the tumor immune environment in EOC, the application of immunotherapy regimens, and highlight differences between primary debulking surgery and neo-adjuvant chemotherapy treated EOC patients.

Outline of the thesis

It has become abundantly clear that a successful anti-tumor immune response requires the presence, activation, and co-stimulation of all lymphoid components of the immune system, including $CD8^+$ T cells, $CD4^+$ T cells, B cells, and innate lymphoid cells. This is especially demonstrated by the discovery of tertiary lymphoid structures (TLSs), which represent well-organized clusters of TILs and give rise to an advanced immune response. Interestingly, not only TIL presence but also TIL differentiation and localization have been shown to determine clinical outcome. In **chapter 2** we discuss the recent advances in the understanding of TIL biology, their prognostic benefit, as well as their predictive value for therapy. Herein, we particularly address the recently identified role of tumor-resident memory cells and T cell exhaustion as key cellular effectors of immune surveillance and therapy. In addition, we elaborate on the role of TLSs and B cells as crucial supportive regulators in immune tumor control.

In the next chapters we investigate the tumor immune environment in EOC. **Chapter 3** describes the impact of chemotherapy on the general immune contexture of EOC patients by analysis of immune cell populations in a series of primary tumors, tumor-draining lymph nodes (tDLN) and circulating T cells. To further dissect the effect of chemotherapy on the EOC immune environment a tissue micro-array (TMA) was constructed including 281 HGSOC patients from two hospitals. In **chapter 4,** digital quantification of $CD8^+CD103^+$ T cell subsets was explored to see whether this could improve upon existing clinical survival prediction and for which patients. Next, we investigated the immune profile of HGSOC patients treated with PDS and NACT.

The following section of the thesis focuses on the clinical application of vaccination strategy as immunotherapy in OC. **Chapter 5**, is a systematic review describing the clinical efficacy of antigen-specific active immunotherapy for the treatment of OC. In line, with the review, we are currently evaluating the effect of the BNT115 vaccination in a first-in-human phase I clinical study in OC patients, as described in **chapter 6**.

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Chapter 2

Tumor infiltrating lymphocytes in the immunotherapy era

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Cellular & Molecular Immunology 2020

Abstract

The clinical success of cancer immune checkpoint blockade (ICB) has refocused attention on tumor-infiltrating lymphocytes (TILs) across cancer types. The outcome of immune checkpoint inhibitor therapy in cancer patients has been linked to the quality and magnitude of T cell, NK cell, and more recently, B cell responses within the tumor microenvironment. State-of-the-art single-cell analysis of TIL gene expression profiles and clonality has revealed a remarkable degree of cellular heterogeneity and distinct patterns of immune activation and exhaustion. Many of these states are conserved across tumor types, in line with the broad responses observed clinically. Despite this homology, not all cancer types with similar TIL landscapes respond similarly to immunotherapy, highlighting the complexity of the underlying tumor-immune interactions. This observation is further confounded by the strong prognostic benefit of TILs observed for tumor types that have so far respond poorly to immunotherapy. Thus, while a holistic view of lymphocyte infiltration and dysfunction on a single-cell level is emerging, the search for response and prognostic biomarkers is just beginning. Within this review, we discuss recent advances in the understanding of TIL biology, their prognostic benefit, and their predictive value for therapy.

Introduction

It has become abundantly clear that a successful antitumor immune response requires the presence, activation, and costimulation of all lymphoid components of the immune system, including $CD8^+$ T cells, $CD4^+$ T cells, B cells, and innate lymphoid cells. This is especially demonstrated by the discovery of tertiary lymphoid structures (TLSs), which represent well-organized clusters of TILs and give rise to an advanced immune response. Interestingly, not only TIL presence but also TIL differentiation and localization have been shown to determine clinical outcome. To translate these relationships into a usable diagnostic tool to predict prognosis and determine treatment strategy, state-of-the-art advanced computational techniques are making their way into the clinic. Within this review, we discuss recent advances in the understanding TIL of biology, their prognostic benefit, and their predictive value for therapy. Herein, we particularly address the recently identified role of tumor-resident memory cells and T cell exhaustion as key cellular effectors of immune surveillance and therapy. In addition, we elaborate on the role of TLSs and B cells as crucial supportive regulators in immune tumor control.

Search strategy

Studies relevant to the subject were searched for via PubMed. Several high-impact journals were searched specifically for the literature of interest, including Cell, Nature, Nature Communications, Nature Medicine, Clinical Cancer Research and Cancer Immunology Research. Diverse search terms were used, including *"tumor-infiltrating lymphocytes", "T cells", "B cells", "natural killer cells", "innate lymphoid cells", "TCRαβ+", "TCRγδ+", "T helper cells", "CD4 T cells", "follicular helper T cells", "tissue-resident memory cells", "bystander cells", "effector memory T cells", "T cell exhaustion", "progenitor stem-like exhausted cells", "CD103", "survival", "cancer", "tertiary lymphoid structures", "stromal-infiltrating lymphocytes", "digital immune scores", "immunotherapy", "checkpoint inhibition", "microsatellite instability", and "adoptive T cell transfer"*.

When possible, studies published from 2018 to the 1st of June 2020 were used, when no studies were available, older literature was used. Searches were updated until the 1st of June 2020.

Tumor-infiltrating lymphocytes

T cells

T cells are broadly classified according to their T cell receptor (TCR) subunits, as well as the core lineage markers CD8 and CD4. The αβ TCR complex endows T cells with the capacity for recognition of peptides presented on the cell surface in the context of major histocompatibility complex (MHC) class I (CD8 T cells) or class II (CD4 T cells). By contrast, the γδ TCR subunit is thought to function largely independent of MHC class I and II. In general, $CD8^+$ and $CD4^+$ TCRαβ T cells are the most abundant T cell subsets in tissues, including tumor tissues (Table 1).

Table 1. Phenotype and functional properties of tumor infiltrating lymphocytes.

	Phenotype	Functional properties
T cells		
$TCR\gamma\delta^+$	Express NK-cell markers such as NKG2D. Two main subsets; Vδ1 γδ T cells and Vγ9Vδ2 T cells.	Display both innate and adaptive immune features and are described to exhibit both effector like and regulatory like functions.
$TCR\alpha\beta^+$ $CD4^+$ $CD8^+$ *Double positive T cells*	Effector memory like phenotype Four main subpopulations: $CD4^{high}CD8^{low}$, $CD4^{high}CD8^{high}$, $CD4^{med}CD8^{high}$, $CD4^{low}CD8^{high}$.	Cytokine production, expression of inhibitory receptors (PD1, TIM3, TIGIT) and activation markers (HLA-DR, CD38, 4-1BB, Ki67).
$TCR\alpha\beta^+$ $CD4^-$ $CD8^-$ *Double negative T cells*	Regulatory-like and/or effector memory-like phenotype.	Different functional properties which might reflect differences between circulating double negative T cells from healthy donors versus tumor infiltrated double negative T cells.
$CD3^+$ T cell Memory subsets		
Stem cell like memory (TSCM)	$CD45RO^-$ $CD45RA^+CCR7^+CD62L^+CD27^+CD28^+$ $IL7R\alpha^+CD95^+IL2R\beta^+$	Self-renewal, high proliferative capacity, circulation through lymphoid organs, cytokine production.
Central memory (TCM)	$CD45RA^-CD45RO^+CCR7^+CD62L^+$ Diverse CD27CD28 expression.	Reduced self-renewal and multipotentcy compared to TSCM. Circulation through lymphoid organs. Limited effector functions.
$CD3^+$ T cell Memory subsets		
Effector memory (TEM)	$CD45RA^-CD45RO^+CCR7^-CD62L^-$, Diverse CD27CD28 expression.	Exhibit pro-inflammatory effector functions. Preferentially traffic through peripheral tissue.
effector memory RA^+ (TEMRA)	$CD45RA^+CCR7^-$ $CD27^-CD28^-$	Terminally differentiated effector T cell. Exhibit cytolytic capacity.
$CD8^+$ TCRαβ subsets -Cytotoxic T lymphocytes (CTL)		
Tissue resident memory (TRM)	$CD103^+CD39^+$	Cancer-specific CTL that reside in the tumor epithelium. Often co-express inhibitory receptors such as PD-1.

	Phenotype	Functional properties
Bystander	$CD103^+CD39^-$	Non cancer specific CTL that reside in the tumor epithelium. Capable of inducing anti-tumor immune response.
Progenitor stem-like exhausted (T_{PE})	$TCF1^+Slamf6^+CXCR5^+PD1^+$ $CD39^-CX3CR1^-$	Maintain antigen specific immune response, persist long-term, self-renewal, differentiation into T_{EX}.
Exhausted (T_{EX})	$CX3CR1^+CD39^+PD1^+Tim3^+$ $TCF1^-CXCR5^-$	Exhibit high cytolytic and cytotoxic function.
$CD4^+$ $TCR\alpha\beta^+$		
Helper (T_{HC})	STAT activated	Direct lysis of tumor cells, inducing $CD8^+$ T cells activation and expansion. Improvement the antigen-presenting capacity of dendritic cells
Follicular helper (T_{FH})	CXCR5, BCL-6 expression	Promoting B cell activation, expansion and differentiation into plasma cells. CXCL13 production.
Regulatory (Treg)	$FOXP3^+CD25^+$	Production of suppressive cytokines, modification of antigen presenting cells, nutrient deprivation, IL-2 exhaustion and cytolysis.
B cells	$CD19^+CD20^+$	
Antigen-presenting	MHC-mediated	Antigen presentation to T cells
Plasma cells (PC)	$CD20^-CD38^+CD138^+CD79a^+$	Production of antibodies
Regulatory (Breg)	Lack of phenotypic markers	Production of suppressive cytokines IL-10, IL-35 and TGFβ.
Germinal center	Bcl-6^+, activation-induced deaminase (AID^+), Ki67 expression	Enables recombinant class switching of the constant region from IgM/IgD to IgG, IgE or IgA, and somatic hyper mutation of the BCR resulting in increased antigen affinity.
Class-switched	IgG, IgA or IgE	Contain affinity-matured antibodies; give rise to a highly advanced immune response.
Innate lymphoid cells		
Natural killer cells (NK)	$CD16^+NKp30^+NK46^+NKp44^+NKG2D^+NKG2A^+$	Pro-inflammatory; high cytolytic capacity; release of granzymes, perforins and IFNγ production.
Helper-like innate lymphoid cells (ILC)		
ILC group 1 (ILC1)	$NK1.1^+$ and $NKp46^+$	NK-like cells. Production of IFNγ.
ILC group 2 (ILC2)	IL33 receptor ST and $CD127^+$	GATA3 dependent function. Pro-inflammatory.
ILC group 3 (ILC3)	$ROR\gamma t^+CD127^+$	Controversial role; both pro-inflammatory and immune regulatory.

TCRαβ+ T cells

TCRαβ+ T cells of all states of differentiation have been observed in tumors, including nonclassical TCRαβ+CD4−CD8− and TCRαβ+CD4+CD8+ T cells. 'Double-positive' (CD4+CD8+) TCRαβ+ T cells have been identified in multiple tumors, including melanoma and lung, colon, and renal cancer. These double-positive T cells can be broadly subdivided into four major subpopulations, $CD4^{high}CD8^{low}$, $CD4^{high}CD8^{high}$, $CD4^{med}CD8^{high}$, and $CD4^{low}CD8^{high}$, although many studies assess them as a single subset.[1] To date, most work has focused on $CD4^{low}CD8^{high}$ T cells, the subset that is thought to develop from peripheral CD8 T cells, which coexpress low levels of CD4 after activation.[2] In renal cell carcinoma, $CD4^{low}CD8^{positive}$ T cells have a CD8-like effector memory phenotype (CD45RO+CCR7−) with expression of the inhibitory receptors PD1, TIM-3, and TIGIT and the activation markers HLA-DR, CD38, 4-1BB, and Ki67.[3] In melanoma, transcriptome analysis revealed a gene signature closer to that of CD8 single-positive T cells than that of CD4 single-positive T cells for $CD4^{low}CD8^{positive}$ T cells. However, the cells shared functional similarities with CD4 single-positive cells, including reduced cytolytic potential[2]. These findings were confirmed in urological cancers, in which both $CD4^{high}CD8^{low}$ and $CD4^{low}CD8^{high}$ T cells showed an effector memory-like phenotype, along with the production of the classical Th2 cytokines IL-4, IL5, and IL-13.[4–6]

'Double-negative' (CD4−CD8−) T cells are the subject of conflicting reports. Some studies in healthy donors have ascribed a regulatory-like phenotype to these cells[7,8], consisting of both CD45RA+CCR7+ and CD45RA+CCR7− cells, with an intermediate maturation stage (CD27+CD28−), high expression of CD95 and lack of activation markers such as CD25 and CD69.[7] Other work argues specifically for the use of double-negative T cells from healthy donors as a source for adoptive cellular therapy due to their observed phenotype which is more consistent with that of effector memory cells: expression of CD45RA, CD44, and CD49d and low expression of CCR7, CD62L, CD127 and the inhibitory molecules ICOS, CTLA-4, and PD1.[9] These differences may reflect changes in double-negative T cells infiltrating tumors. Indeed, a study comparing the reported phenotypes of double-negative cells across tumors found a comparable phenotype across human melanoma, renal cell carcinoma and glioblastoma, and the TILs in cancer tissues were phenotypically distinct from the double-negative T cells found in nonmalignant tissues. Interestingly, the double-negative population seemed to expand shortly after initiation of BRAF inhibitor treatment.[10]

CD8+ TCRαβ T cells

CD8+ TCRαβ T cells, referred to as CD8+ T cells, are mostly known for their exquisite antiviral and antitumor functions and are often referred to as cytotoxic T lymphocytes (CTLs). CTLs have the ability to produce high levels of antitumor cytokines and cytotoxic molecules, such as interferon-γ (IFNγ), tumor necrosis factor-α (TNFα), perforin, and granzymes.[11] Accordingly, CD8+ CTLs are associated with improved prognosis in almost all types of cancer. Under physiological conditions and following elimination of their targets, CTLs generally form a number of memory subsets that provide long-term protection against reinfection after the resolution of the immune response. These memory T-cell subsets form a heterogeneous compartment and range from cells exhibiting a more naive-like phenotype to cells presenting an effector-like phenotype, they roughly follow along the line of stem cell-like memory T (TSCM), central memory T (TCM), effector memory T (TEM), and effector memory RA+ T (TEMRA) cells.[12,13] TSCM cells are arguably the most naive cells (CD45RA+CCR7+CD27+) and have consistent recirculation patterns in vivo, mostly localized in the lymph nodes and to a lesser extent in the spleen and bone marrow but rarely found in peripheral mucosae. TSCM cells maintain their own pool through self-renewal. In addition, TSCM cells retain the ability to proliferate rapidly and release inflammatory cytokines. TCM cells differ from TSCM cells in their CD45RA−CD45RO+ phenotype and a reduced capacity for self-renewal and multipotency.[14] However, TCM cells do possess naive-like functions and express lymph node-homing molecules such as C–C chemokine receptor type 7 (CCR7) and CD62L. TCM cells are also thought to have limited direct effector functions.[12] TEM cells are characterized by cell surface expression of CD45RO+CCR7−CD62L−, and although these cells can (re)circulate through the blood, they preferentially traffic to peripheral tissues. In addition, these cells exhibit proinflammatory effector functions upon secondary antigen encounter with a cognate antigen and have diverse expression of CD27 and CD28.[15] In line with their role in long-term protection, early work in colorectal cancer (CRC) demonstrated that the presence of a TEM cell immune infiltrate correlated with less advanced tumor stage and no signs of metastatic disease or lymph node involvement. Accordingly, the presence of TEM cells in the tumor was an independent prognostic factor for overall survival.[16] Other studies around the same time highlighted the role of TCM cells in tumor control, as they possess high proliferative capacity and are suitable for adoptive T cell transfer, especially when combined with a tumor-antigen

vaccination.[17] Accordingly, a recent study highlights the correlation of CD45RO$^+$ TILs with overall and disease-free survival in breast cancer.[18]

More recently, the role of peripheral tissue-resident memory (TRM) CTLs in tumor immunity has come into focus. After resolution of an immune response, TRM cells normally stay in the peripheral tissues without recirculating, providing a first line of defense against reinfection. TRM cells in peripheral tissues expresses canonical markers CD103, also known as integrin αE; CXCR6, which is involved in TRM development; CD49a, which is needed for retention and cytotoxic function; and CD69, an inhibitor of S1PR1 that mediates T cell recirculation.[19–21] In tumors, TRM cells are also characterized by the expression of CD103. CD103 complexes exclusively with integrin β7, forming the αEβ7 complex; this complex interacts with E-cadherin, which is often expressed on tumor cells. Accordingly, CD103$^+$ CTLs have been correlated with improved survival in a multitude of solid tumors, including several gynecological malignancies, lung cancer, breast cancer, melanoma, CRC and several head and cancers.[22–26] In cancer mouse models, loss of E-cadherin or CD103$^+$ CTLs was associated with loss of tumor control.[27] Importantly, a recent study identified coexpression of CD103 and the immunosuppressive molecule CD39 as definitive markers of cancer-specific CTLs in tumors, further supporting the key role of the TRM cell subset.[28]

Finally, bystander TRM cells, which are not specific for tumor antigens but for epitopes unrelated to cancer, have also been identified in multiple solid tumors. These cells have diverse phenotypes but lack CD39 expression, which distinguishes them from the tumor-specific TRM cell population.[29] Interestingly, it has been demonstrated that although unspecific for tumor antigens, these bystander cells are capable of contributing to the antitumor response. For instance, intratumoral viral-specific CTLs can be activated via the delivery of adjuvant-free viral peptides, which induce a broad immune response evidenced by accumulation and activation of CD8$^+$ T cells and natural killer (NK) cells, increased expression of markers associated with dendritic cell (DC) activation and upregulation of PDL1. Consequently, tumor-bearing mice are more susceptible to PDL1 blockade when it is combined with viral peptide therapy than when it is used as a monotherapy.[30]

CD4+ TCRαβ+ T cells

$CD8^+$ T cells do not function in isolation; there is also a well-established role for conventional $CD4^+$ helper TCRαβ T (THC) cells in the antitumor immune response.[31–34] THC cells promote $CD8^+$ T cell priming through stimulation of CD40 on DCs via the expression of CD40 ligand (CD40L), resulting in the release of cytokines, such as IL-12, IL-15, and IFNγ, the upregulation of costimulatory ligands such as CD70, recruitment of B cells and naive $CD8^+$ T cells and increased antigen presentation. In this two-step process, $CD4^+$ and $CD8^+$ T cells first independently interact with DCs in different areas of the lymphoid organs, whereas in the second-step of priming, both $CD4^+$ and $CD8^+$ T cells recognize their cognate antigens on the same DCs.[34] In addition to a helper role in priming, THC cells can also possess cytolytic mechanisms that enable them to directly lyse tumor cells.[33–35]

In addition to these conventional THC cells, recent work has also identified T follicular helper (TFH) cells as crucial cells supporting B cell activation, expansion, and differentiation into plasma cells (PCs) and memory B cells in multiple human tumors.[36] The presence of these TFH cells has been associated with improved prognosis in breast cancer and CRC. The $CD4^+$ TFH cells, are characterized by CXCR5 expression, which is indispensable for T cell migration from T zones towards CXC chemokine ligand 13 (CXCL13)-rich B cell follicles, where they activate B cells through interactions with CD40 ligand (CD40L) and the production of interleukin (IL) 21. In addition, they are characterized by high expression of B cell lymphoma 6 (BCL-6). They also possess the capacity to produce CXCL13 and seem to be involved in the formation of TLSs, via which they shape intratumoral $CD8^+$ T cell and B cell responses.[37,38] Also capable of CXCL13 production are $CD4^+$ TRM cells, which have a phenotype comparable to that of $CD8^+$ TRM cells, including the capacity for the production of cytokines such as IFNγ and TNFα.[21]

In contrast to THC and TFH cells, $CD4^+$ regulatory T (Treg) cells are known as tumor-promoting $CD4^+CD25^+FoxP3^+$ T cells and have been shown to counteract tumor-specific immune responses by suppressing $CD8^+$ cells, amongst other cell types.[39] Consequently, Treg cells have been associated with poorer survival in multiple solid tumors, including pancreatic, ovarian, gastric, cervical, breast, and colon cancers.[40–45] Several mechanisms exist by which Treg cells limit an effective antitumor response. Treg cells are known to produce immune-suppressive cytokines, including IL-10, IL-35, and

TGFβ, but can also suppress productive immunity through nutrient deprivation, IL-2 exhaustion, and cytolysis.[39] Complementary research specifically implicated the role of IL-10 and IL-35 in promoting BLIMP1-dependent inhibition of $CD8^+$ TILs.[46] Cell–cell-mediated suppression can also occur by CD28 costimulatory competition. Treg cells constitutively express CTLA4, which has a high affinity for CD80 and CD86 expressed on antigen-presenting cells (APCs). As CD80/CD86 also interacts, at a lower affinity, with the costimulatory receptor CD28 on T cells, Treg cells inhibit T cell activation by competitive CTLA4-CD80/CD86 binding.[39,47] In addition, Tim3-positive Treg cells have displayed a superior capacity to inhibit naive T cell proliferation compared to Tim3-negative Treg cells, which is partially reversed by IFNγ production.[39,48] Moreover, IFNγ was shown to drive the fragility of Treg cells, which in turn boosts antitumor immunity.[49] Perhaps counterintuitively, two studies in gastric cancer and four in CRC demonstrate a good prognosis for patients with tumors with high densities of Treg cells (summarized by Fridman et al. in ref. 50). These results might be explained by the technical difficulties surrounding Treg cell quantification, the inability to detect multiple relevant markers at the same time, and the concomitant infiltration of other immune cells such as $CD8^+$ TILs.[50]

TCRγδ⁺ T cells

$TCR\gamma\delta^+$ T cells are mostly negative for CD4 and CD8, but these cells coexpress NK cell markers such as NKG2D. Consequently, $TCR\gamma\delta^+$ cells have been proposed as a link between the innate and adaptive immune systems. Two main subsets have been described, the Vδ1γδ T cells and the Vγ9Vδ2 T cells, both displaying innate and adaptive immune features to differing extents.[51,52] γδ T cells are described with different phenotypes, including CD4 T cell-like effector-like and regulatory phenotypes.[53] In mice with lupus, it was demonstrated that a subset of γδ T cells express CXCR5 after activation. These $TCR\gamma\delta^+$ $CXCR5^+$ cells can then present antigens to naive $CD4^+$ T cells and can induce follicular helper T cell differentiation, which in turn can induce a B cell response.[54,55] Effector-like functions such as cytokine production have also been attributed to γδ T cells. Interestingly, TGF-β signaling upregulated the expression of CD54, CD103, IFNγ, and granzyme-B in Vγ9Vδ2 T cells, augmenting their cytotoxic effector activity.[56]

T cell exhaustion

Upon persistent antigen stimulation, T cells show a gradual decrease in various effector functions known as T cell exhaustion, which is characterized by a decrease in proliferative and cytolytic capacity and upregulation of multiple inhibitory signals, including PD1, LAG-3, CD160, 2B4, TIM-3, and TIGIT.[13] Although characterized as an exhausted phenotype, these T cells can retain their cytolytic and proliferative capacity. The identification of this T cell phenotype led to the theory that exhaustion is a gradually developing state with various functional and phenotypic substates. Exhausted $CD8^+$ T cells are thought to comprise both progenitor stem-like exhausted (T_{PE}) cells and terminally exhausted T (T_{EX}) cells, in a scheme that is similar to the classical T cell differentiation described above(Fig. 1).[13,57,58] The classic view of T cell differentiation using TSCM, TCM, TEM, and TEMRA phenotypes is thus giving way to a T_{PE}/T_{EX}-based classification.

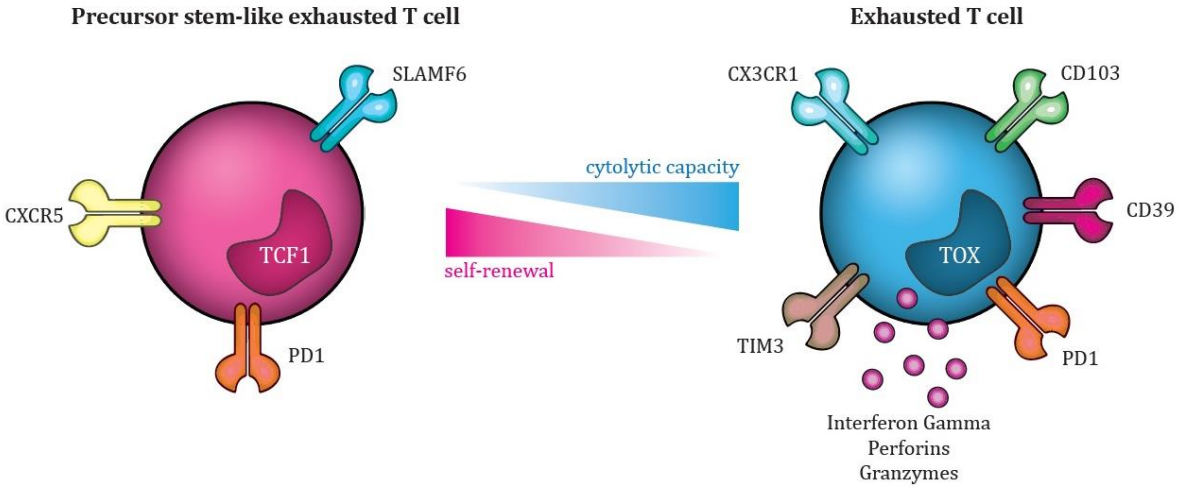

Figure 1. $CD8^+$ T cell exhaustion states. $CD8^+$ T cell exhaustion is thought of as a gradual developing, with various functional and phenotypic states, including T_{PE} and T_{EX}. The T_{PE} phenotype is characterized by the expression of TCF1, which is lost upon differentiation into T_{EX}. Cell surface markers identified on T_{PE} include Slamf6, PD1 and CXCR5 and their functional capacity comprises the ability to maintain an antigen specific immune response, persist long-term, the capability of self-renewal and eventually the differentiation into T_{EX}. On the contrary, T_{EX} express mostly co-inhibitory cell-surface receptors and transcription factors associated with effector and exhausted cells, including the expression of CX3CR1, PD1, CD39 and TIM3, which reflects the functional capacity of TEX that exhibit mostly cytotoxic functions.
T_{EX}: terminally exhausted T cells, T_{PE}: progenitor STEM-like exhausted cells, TCF1: transcription factor 1

T_{PE} cells are known to maintain antigen-specific immune responses, persist long-term, be capable of self-renewal and eventually differentiate into T_{EX} cells.[57,59] The T_{PE} cell phenotype is characterized by the expression of the transcription factor T cell factor

(TCF1), encoded by the gene Tcf7, which is lost upon differentiation into T_{EX} cells and is essential for the stem-like functions of T_{PE} cells.[57,58,60] Differential gene expression analysis has recently identified a $CD39^{-}Tim3^{-}Slamf6^{+}Tcf1^{+}PD1^{+}CD8^{+}$ cell phenotype to identify precursor states of exhaustion.[57,59] In addition, CD127 and killer cell lectin-like receptor subfamily G member 1, a protein critical for T cell homeostasis and involved in the lysis of tumor cells, are found to be nearly absent on $PD1^{+}CD8^{+}$ T_{EX} cells in breast and melanoma tumors.[13] A recent report has also suggested CXCR5 as a marker for T_{PE} cells that is coexpressed with Tcf1 in the absence of Tim3, and cells with CXCR5 expression showed similar functionality and persistence to cells with Tim3 expression.[61] Interestingly, CXCR5 expression on $CD8^{+}$ T cells has also been used to define follicular $CD8^{+}$ T cells, which are able to migrate into B cell follicles and promote B cell differentiation. These cells also express lower levels of inhibitory receptors and exhibit more potent cytotoxicity than $CXCR5^{-}CD8^{+}$ cells, similar to the T_{PE} cells phenotype.[62] Considering the recent insights into ectopic B cell follicles in human tumors, the role and localization of $CXCR5^{+}$ T_{PE} cells might be of particular interest (see also corresponding sections below).

T_{PE} cells have relatively high transcript levels of genes encoding cytokines, costimulatory molecules, and survival/memory molecules compared to T_{EX} cells. T_{EX} cells mostly expresses coinhibitory cell surface receptors and transcription factors associated with effector and exhausted cells. These differences are reflected in the functional capacity of both subsets: T_{PE} cells contain the ability to proliferate, generating $Tcf1^{+}PD1^{+}$ and differentiated $Tcf1^{-}PD1^{+}$ cells, and T_{EX} cells exhibit mostly cytolytic functions. Together, these data suggest that a delicate balance of both T_{PE} cells and T_{EX} cells is required for an effective antitumor immune response.[57,58] Accordingly, data from mouse models of chronic viral infection have demonstrated that both T_{PE} and T_{EX} cell subsets are required for long-term viral control.[59]

Complementary studies used cell surface expression of CX3C chemokine receptor 1 (CX3CR1) as a marker for T cell differentiation and exhaustion.[63,64] A recent in vivo study divided $CX3CR1^{+}CD8^{+}$ T cells into three subsets ranging from less to more terminally differentiated: $CX3CR1^{-}$, $CX3CR1^{int}$ and $CX3CR1^{high}$. Indeed, the $CX3CR1^{-}$ cells were characterized by high Tcf1 expression and possessed high proliferative capability upon activation. Moreover, PD1, LAG-3, and TIGIT expression decreased when CX3CR1 expression increased. Conversely, the $CXC3C1^{high}$ population exhibited the highest

cytotoxicity. In addition, CX3CR1$^-$ cells were found to delay tumor growth and increase survival.[64] The nuclear factor TOX has also been identified as a crucial regulator of T cell exhaustion. TOX expression was increased upon chronic TCR stimulation and was low during acute infection. In the absence of TOX, T_{EX} cells do not form; T cells no longer upregulate inhibitory receptors, chromatin remains largely inaccessible, and Tcf1 expression is maintained. Although these cells are phenotypically "nonexhausted", they are still dysfunctional.[65–67] Interestingly, the aforementioned studies on TOX indicate that T cell exhaustion may be a beneficial process because it protects T cells from tumor and/or activation-induced cell death.

The T cell exhaustion phenotype appears to largely overlap with that observed for the TRM cell population. Indeed, the tumor-reactive TRM marker CD39 is a marker of persistent TCR stimulation, as demonstrated in both mice and human models.[28,68] RNA sequencing of CD39$^+$CD8$^+$ cells revealed an exhausted transcriptome with PD1, Tim-3, Lag-3, TIGIT, and 2BA highly coexpressed. In addition, these cells demonstrated impaired production of IL-2, IFNγ, and TNF.[68] Gene expression profiles of double-positive CD103$^+$CD39$^+$CD8$^+$ cells (DP CTLs) versus double-negative CD103$^-$CD39$^-$CD8$^+$ cells (DN CTLs) also identified a gene signature of DP CTLs consistent with that of cells with an exhausted, tissue-resident phenotype. This included high expression of PDCD1 (PD1), CTLA4 (CTLA-4), and HAVCR2 (Tim3) and decreased expression of T cell recirculation genes such as KLF2, SELL, and S1PR1 as well as lower expression of CCR7, CD127, and CD28 indicative of an effector memory phenotype. However, contrary to the findings of Canale et al., these DP CTLs exhibited more cytotoxic potential than DN CTLs, as more cells were granzyme-B positive, although this was not reflected in the production of IFNγ and TNFα.[28] CD4$^+$ TRM cells are also characterized by high expression of CD103, CD69, and CD49a and inhibitory molecules such as PD1, CTLA-4, and B24. Altogether, these findings support earlier observations that suggested CD103$^+$ CTLs comprise tumor-reactive CD8 T cells in ovarian and lung cancer, characterized by the expression of exhaustion markers but without complete loss of functional competence.[69–71]

B lymphocytes

Signatures for patient stratification and response evaluation in clinical immunotherapy have focused predominantly on T cell responses. However, recent work has also

identified a key role for B lymphocytes in immunotherapy, and their presence has been associated with an improved prognosis across different cancer types, including breast cancer, melanoma, renal cell carcinoma, CRC, hepatocellular carcinoma, and head and neck squamous cell carcinoma.[72–76] However, the tumor-promoting effects of B cells have also been extensively described.[77–80]

Functionally, B cells may act as APCs for T cells, promoting local tumor-associated T cell responses.[81,82] The observation of B cell clonal expansion and immunoglobulin phenotype switching across human cancers further indicates a possible role for antibody-dependent cell-mediated cytotoxicity (ADCC) in the antitumor humoral immune response, facilitated by antibody-secreting plasma B cells.[83] Tumor-infiltrating B (TIL B) cells can also kill tumor cells directly by secreting toxic cytokines such as IFNγ and granzyme B or indirectly by promoting tumor-specific T cell secretion of immunostimulatory cytokines (Fig. 2, Table 1).[76,84]

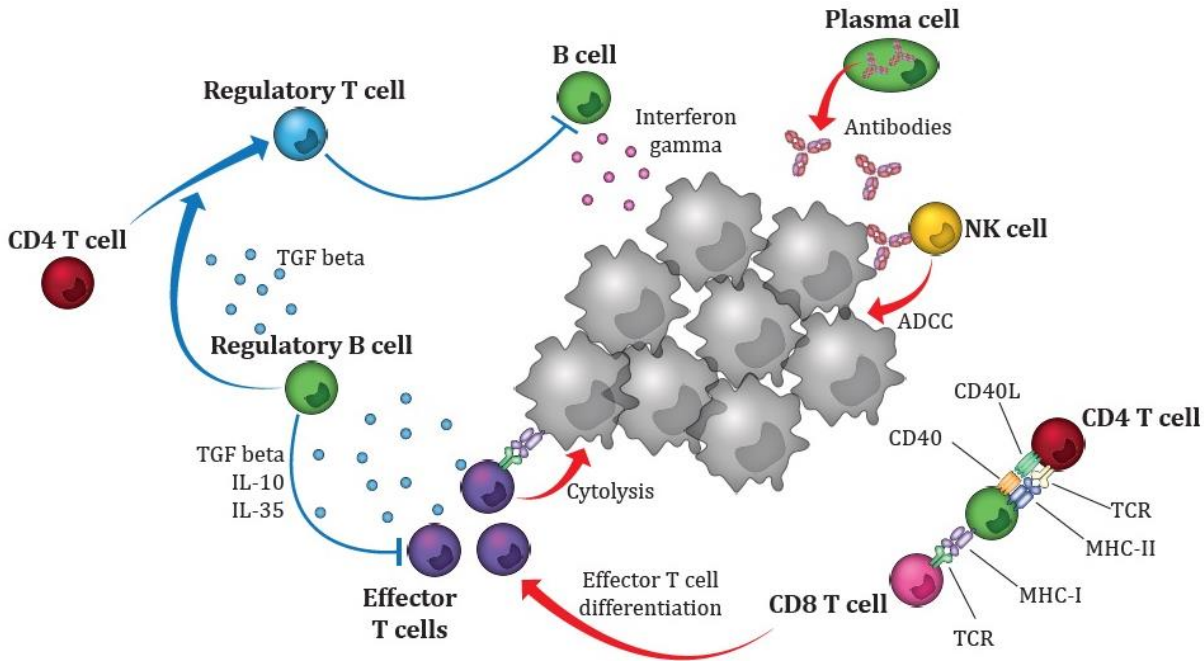

Figure 2. Anti and pro-tumor related functional properties of B cells. B cells and plasma cells have several ways to promote local tumor-associated T cell responses. Functionally, B cells may act as antigen presenting cells and facilitate tumor-antigen derived presentation to T cells. B cells also promote the anti-tumor immunity by the secretion of immunostimulatory cytokines, such as IFNγ, that drive cytotoxic immune responses, In addition, they can directly kill tumor cells by secreting toxic cytokines such as granzyme B. Plasma cells promote the anti-tumor immune response by the secretion of tumor-specific antibodies which can mediate ADCC, resulting in phagocytosis of tumor cells. On the contrary, Bregs suppress the anti-tumor immune response indirectly by the secretion of immunoregulatory cytokines IL-10, IL-35 and TGFβ and directly by inhibiting effector cells, such as cytotoxic T $CD8^+$ T cells. Furthermore, Bregs suppress anti-tumor immunity by the conversion of $CD4^+$ T cells into Treg via TGFβ.
ADCC: antibody-dependent cell cytotoxicity, Bregs: Regulatory B cells, Treg: regulatory T cells.

Antigen-presenting B cells

Professional APCs are characterized by their ability to take up antigens and load the processed antigen product onto MHC class molecules for presentation to T cells.[85] Decades ago, B cells were found to be able to act as APCs, although they seem to function less efficiently than DCs, probably due to their reduced, nonspecific antigen uptake. When B cells encounter antigens, the binding affinity is relatively high (multivalency), resulting in B cells that are more sensitive to antigens at lower concentrations than DCs.[86]

Before immunization, antigen-specific B cells are very rare compared to DCs. Therefore, it was long assumed that B cells only minimally contributed as APCs to activate naive $CD4^+$ T cells. However, by using RNA phage Qβ-derived virus-like particles as a nanoparticle antigen model, Hong et al. demonstrated that B cells, and not DCs, were responsible for the initial activation of $CD4^+$ T cells and promoted $CD4^+$ T cell differentiation into $CD4^+$ TFH cells. Additionally, a germinal center (GC) response could be induced in this model in the absence of DCs.[87] Similar results were observed when another type of immunization, a soluble protein, was used. Again, B cells acted herein as professional APCs upon immunization with inactivated influenza virus and initiated activation of naive $CD4^+$ T cells. These results suggest an important role for B cells in initiating $CD4^+$ T cell responses, with an emphasis on viral infections. However, it has also been shown in murine and human models that B cells efficiently present tumor-associated antigens (TAAs) to T cells.[88,89] For instance, TIL B cells efficiently presented TAAs to $CD4^+$ T cells in non-small-cell lung cancer patients and influenced the $CD4^+$ phenotype. Specifically, activated TIL B cells ($CD69^+HLA\text{-}DR^+CD27^+CD21^+$) were associated with a $CD4^+$ effector T cell response ($CD4^+IFN\gamma^+$), demonstrating the plausible role of B cells as professional APCs in promoting the antitumor immune response.[88]

Antibody-producing (plasma) B cells

PCs are characterized by the absence of CD20 and the coexpression of CD38, CD138, and cytosolic CD79a and are the dominant antibody-producing B cell subset. Recently, it was shown that PCs seem to have an important role in promoting antitumor immunity.

Kroeger et al. found that the prognostically favorable effects of $CD8^+$ TILs accompanied by $CD20^+$ B cells were even further enhanced by the presence of stromal

PCs.[90] In high-grade serious ovarian cancer patients, tumors infiltrated with $CD20^+$ B cells and $CD4^+$ and $CD8^+$ T cells together with PCs were associated with increased survival, with ~65% of the patients being alive at 10 years after diagnosis. Interestingly, tumors containing $CD8^+$ TILs accompanied by solely $CD4^+$ TILs, $CD20^+$ TILs, or PCs were associated with minor insignificant survival increases, suggesting the importance of interplay between these different immune subsets in promoting antitumor immunity.[90] Several studies further analyzed the association between class-switched B cells with an increased B cell receptor (BCR) diversity and clonal fraction resulting from tumor-related GC activity. Hu et al. identified widespread clonal B expansion and Ig subclass switch events in various human cancers by observing the same complementarity-determining region 3, containing both IgG1 and IgG3 isotypes (IgG3-1 sCSR).[83] These results were comparable to Kroeger et al., who detected clonally expanded PCs as well as the presence of somatic hypermutation (SMH) within VDJ families. Additionally, IgG transcripts, specifically IgG1, IgG2, and IgG3, represented the majority of immunoglobulin subtypes.[90] Increased BCR diversity and clonal expansion were also observed in tumors of melanoma patients.[75]

Of note, some autoantibodies have also been found to be tumor promoting. Coussens et al. showed that antibodies that are deposited at tumor sites in the form of immune complexes recruit myeloid cells and macrophages to become tumor promoting by binding to the immune complexes via Fcγ-activating receptors. These myeloid cells and macrophages were found to secrete proangiogenic factors and immunoregulatory cytokines, enabling tumor progression.[91]

Regulatory B cells

Regulatory B (Breg) cells are a subpopulation of B cells characterized by their unique immunoregulatory and immunosuppressive qualities, possessing an important role in peripheral tolerance.[92] Accordingly, Breg cells have been associated with worse clinical outcome in cancer.[93,94] Phenotypic markers to characterize Breg cells, other than IL-10 production, are not yet definitive, complicating in-depth analysis.

Nevertheless, it is clear that Breg cells suppress the immune response by secreting IL-10, thereby inhibiting DC differentiation, suppressing helper T1 (TH1) and helper T17 (TH17) cell proliferation, and inducing the differentiation of Treg cells.94 Accordingly, Breg suppressive immune functions are favorable in autoimmune diseases,

as the absence of Breg cells results in the exacerbation of rheumatoid arthritis (RA) and systemic lupus erythematosus.[95,96]

The antitumor immune response is likely indirectly suppressed by Breg cells secreting immunoregulatory cytokines (IL-10, IL-35, and TGFβ) but also directly suppressed by inhibition of effector cells such as cytotoxic $CD8^+$ T cells. In ovarian cancer, IL-10 secretion by Breg cells significantly suppressed the production of cytotoxic effectors, such as IFNγ, by $CD8^+$ T cells.[78] Additionally, in human hepatoma, IL-10 secretion by Breg cells supported tumor growth and suppressed tumor-specific T cells.[79] In glioblastoma, Breg cells were characterized by the immunosuppressive molecules PDL1 and CD155 and the production of IL-10 and TGF-β and were found to suppress $CD8^+$ T cell activation, proliferation and production of IFNγ and granzyme B. Furthermore, local B cell depletion in mice using CD20 immunotherapy significantly improved OS, which correlated with increased tumor-infiltrating $CD8^+$ T cells and production of granzyme B and IFNγ. Interestingly, this survival benefit was not observed in mice receiving systemic anti-CD20 immunotherapy. This suggests that B cells have different functions depending on their location and that naive B cells might differentiate into a Breg phenotype when localized in the immunosuppressive tumor microenvironment (TME).[97]

Finally, Breg cells were shown to suppress antitumor immunity by influencing the conversion of $CD4^+$ T cells into Treg cells via TGFβ, which was observed in a 4T1 breast cancer mouse model of human gastric and tongue squamous cell carcinoma.[80,98,99]

Innate lymphoid cells

Innate lymphoid cells are a more recently appreciated subset of tumor-infiltrating lymphocytes with key roles under physiological immune homeostasis. In general, these cells are characterized as NK cells, type 1 innate lymphoid cells (ILC1s), ILC2s, or ILC3s (Table 1).

Natural killer cells

Natural killer cells (NK cells) are defined by the absence of antigen-specific B or TCRs due to their lack of recombination activating genes. The majority of peripheral NK cells are $CD56^{dim}CD16^+$ and characterized by the ability to rapidly mediate cytotoxicity. In addition, the $CD56^{bright}CD16^-$ NK cell population accounts for ~10% of peripheral NK

cells and is characterized by low perforin production but normal production of IFN-γ and TNF-α.[100,101] NK cell activity is dependent on a repertoire of costimulatory and inhibitory signals that bind to their respective ligands on the cell surface. The dominant activation receptors are CD16, NKp30, NK46, NKp44, and NK group 2, member D (NKG2D). Inhibitory receptors include killer Ig-like receptors and CD94/NKG2A-B, which recognizes HLA-E molecules. When activated, NK cells exhibit antitumor activity via the release of granzymes and perforins, the induction of TNF-related apoptosis and the production of IFNγ.[100,102] In mice, indirect antitumor activity of NK cells has also been demonstrated; NK cells were recruited in lymph nodes undergoing an immune response and produced IFNγ, which was necessary for the priming of T-helper cells.[103] In addition, more recent research has demonstrated cancer immune control by NK cells through the accumulation of conventional type 1 dendritic cells (cDC1s) via the production of the chemoattractants CCL5, XCL1, and XCL2. The tumor cells were able to counteract this axis by the production of prostaglandin E2, which caused impaired chemokine production by NK cells, consequently leading to reduced intratumoral cDC1 recruitment.[104]

The activity and presence of both circulating and intratumoral NK cells have been associated with disease progression, metastatic disease development, and survival.[100,102,105] In gastric cancer, a low percentage of NK cells in the tumor was associated with poor survival and disease progression. Ex vivo studies showed impairment of NK cells through TGF-β signaling by monocytes, which resulted in decreased IFNγ, TNFα, and Ki-67 expression in NK cells.[106] Interestingly, surgical stress impairs peripheral NK cell function. In patients undergoing surgery for CRC, IFNγ production by NK cells was significantly suppressed for up to 2 months.[101] Taking into account the cytolytic potential of NK cells, there is an increased interest in the use of NK cells for immunotherapy, either in adoptive transfer therapies or reactivation strategies affecting their activation and inhibitory ligands.

Helper-like innate lymphoid cells

Based on function, cell surface markers and transcription factors, ILCs have been categorized into three groups: group 1 (ILC1s), group 2 (ILC2s), and group 3 (ILC3s). Overall, the role of helper-like lymphoid cells in cancer remains poorly understood, with these cells having high plasticity and seemingly occupying controversial roles.[107]

The ILC1s are most comparable to NK cells, as both require the transcription factor Tbet to function; both express NK1.1 and NKp46, and they mostly produce IFNγ. Unlike NK cells, ILC1s are not dependent on Eomes expression.[108] In mice, it has been demonstrated that ILC1s can arise from NK cells as a result of TGFβ signaling. NK cells are known to limit tumor growth and metastatic outgrowth. However, their conversion into ILC1s leads to inferior tumor control.[109] Indeed, complementary research in mice suggested that SMAD4 impeded the conversion of NK cells into ILC1s via TGFβ signaling.[110] This was recently confirmed in in vitro human cell cultures.[111] In melanoma patients, ILC1s were found to be an enriched subset, although dysfunctional, as demonstrated by impaired IFNγ production. Follow-up experiments in mice identified the production of adenosine (ADO) and kynurenines by melanoma cells as possible causes of ILC1 disruption and impaired IFNγ production. These data suggest that the exploration of targeting IDO and the adenosinergic immunosuppressive axis in melanoma patients is warranted.[112] Overall, these data suggest that at least part of the ILC1 subset emerges from NK cells. In addition, the function of ILC1s should be further explored to investigate their role in tumor immunology and therapy.[109,110,112]

ILC2s are mostly described as proinflammatory, although some studies highlight tumor-promoting characteristics. Their function and development are GATA3-dependent, and the cells are characterized by the expression of the IL-33 receptors ST2 and CD127.[113] ILC2s have been detected in multiple tumor types, including breast, pancreatic, gastric, bladder, and prostate cancer.[114] As ST2 is highly expressed on ILC2s, it was demonstrated that they are dependent on IL-33 for their expansion and cytokine production. Furthermore, IL-33-activated ILC2s are implicated in the priming of tissue-specific $CD8^+$ T cells, as ILC2 expansion is accompanied by increased cytokine capacity and PD1 upregulation in $CD8^+$ T cells, implicating a possible role of ILC2s in the antitumor response to PD1 blockade.[113,115] In contrast, in acute leukemia, ILC2s have been shown to promote myeloid-derived suppressor cells through the production of IL-13.[116] In mice, ILC2s were shown to activate Treg cells through IL-9 production, although this was in the context of chronic inflammation where treatment with IL-9 induced resolution of the inflammation.[117]

The overall role of ILC3s seems controversial, and they have been described as both proinflammatory and immune regulatory. They are characterized by the expression of RORγt and CD127. In non-small-cell squamous lung cancer, ILC3s were found to

accumulate and produce the proinflammatory cytokines TNFα and IL-22. Moreover, ILC3s were specifically found at the edge of TLSs, suggesting that they may contribute to the formation of protective tumor-associated TLSs.[118] In contrast, another study in squamous cell lung carcinoma demonstrated tumor immune evasion by the conversion of ILC1s into ILC3s via IL-23 production by tumor cells. The converted ILC3s were capable of IL-17 production, which promoted tumor growth and was associated with poorer prognosis.[119] In addition, in breast cancer, increased numbers of ILC3s were correlated with the likelihood of developing lymph node metastasis, and in consecutive mouse experiments, depletion of ILC3s was sufficient to decrease lymph node metastasis.[120]

Organization of TILs in tumors

The presence of TILs in tumors has been associated with improved clinical outcomes. However, the type and function of TILs (e.g., CTL versus Treg cell) as well as the TME localization of different TILs are key with respect to eventual tumor control or tumor progression.[121] Therefore, a deeper analysis of the spatial organization of TILs in the TME, e.g., marginal zone versus tumor stroma, is needed to provide a better understanding of antitumor immunity and to discover potentially new biomarkers.[122]

Early histopathological analyses of tumor samples already demonstrated varying TIL distribution across tumor types and showed that different types of immune cells are found in different locations, around and inside the tumor. Specifically, the distribution of TILs was found to be not random but well organized in specific areas. B cells, for instance, are mainly found in the invasive margin and clustered inside TLSs, close to the tumor, with NK cells mainly found in the stroma.[121]

TIL infiltration of tumors

The initial step in the formation of TILs from circulating lymphocytes requires the migration of immune cells from the blood to the tumor across the tumor endothelial barrier. The tumor endothelium is often disturbed and able to directly suppress T cell function, thereby preventing tumor infiltration. For instance, proangiogenic growth factors such as VEGF-A impair lymphocyte adhesion due to an associated defect in

vascular cell adhesion molecule (VCAM-1) and intracellular adhesion molecule (ICAM-1).[123] Proangiogenic factors can also induce overexpression of the endothelin B receptor (ETBR), which is associated with a lack of TILs in ovarian cancer patients.[124] These changes are therapeutically targetable, as in vitro inhibition of VEGF-A and ETBR resulted in a restored amount of TILs and an improved response to immunotherapy.[124,125] Similarly, FasL (CD95L or CD178), a pro-apoptotic cell surface protein, might also be targeted, as it is frequently overexpressed on endothelial tumor cells of humans and mice.[126,127] To address this, Motz et al. studied FasL expression in tissue microarrays (TMAs) of human breast, renal, bladder, colon, prostate and ovarian adenocarcinomas and control TMAs derived from healthy tissues.[128] Normal vasculature tissue did not express FasL, whereas the blood vessels of primary and metastatic tumors did, which was associated with reduced $CD8^+$ T cell infiltration. VEGF-A, IL-10 and prostaglandin E2, three tumor-derived factors, together induced FasL expression, resulting in the elimination of $CD8^+$ CTLs. Treg cells were resistant to FasL-mediated apoptosis due to their higher levels of the anti-apoptotic gene c-FLIP, which resulted in decreased levels of intratumoral $CD8^+$ T cells and accumulation of intratumoral Treg cells. Conversely, FasL suppression resulted in increased infiltration of $CD8^+$ T cells in tumors, improving the $CD8^+$ T cell/Treg cell equilibrium, leading to reduced tumor volumes in mice. Of note, vessels carrying circulating lymphocytes were largely absent from the tumor core, localizing in the surrounding stroma and/or invasive margin. This suggests a direction of travel from vessels to the stroma by cancer cells and highlights a key role for the stroma in tumors.[128]

Tumor stroma

The stroma surrounding the tumor cells is an important component of the TME and harbors a cellular immune component including various innate and adaptive immune cells (B cells, T cells, macrophages, DCs, myeloid-derived suppressor cells, and NK cells) and a nonimmune cellular component (fibroblasts, endothelial cells, pericytes, and mesenchymal cells). Stromal cells in the TME can be either tumor promoting or tumor suppressing. Physiologically, in most nonmalignant tissues, stromal cells are suppressive, regulating the proliferation and migration of differentiated epithelial cells, as well as maintaining the structure and size of organs.[129] Immunologically active

cytokines, comprising growth factors, chemokines, angiogenic factors, and interferons, are major driving forces in tumor-stroma interactions.[130]

Stromal TILs, such as B and T cells, serve as key immune organizers in the TME through the secretion of cytokines. One of the most relevant and well-characterized chemokines in the structural organization of the immune cell cluster is CXCL13, which induces chemotaxis of CXCR5-expressing B cells and T cells towards the invasive margin, where they cluster together in well-organized structures, referred to as TLSs. This invasive margin represents the first line of defense against cancer metastasis. In CRC, the immune cell density is even higher at the tumor boundary than in the tumor core. As in other solid malignancies, CRC patients who exhibit TLSs in the invasive margin, also known as a 'Crohn's-Like reaction (CLR)', have better OS than CRC patients who exhibit only diffuse inflammatory infiltration (DII).[131,132] Accordingly, the existence of immune infiltrates in TLSs at the invasive margin was associated with a decreased presence of early metastatic processes such as vascular, lymphatic, and perineural invasion in CRC.[133] In endometrial cancer, the number of TLSs is directly correlated with specific tumor mutations, such as the ultramutated POLE exonuclease domain or hypermutated microsatellite unstable (MSI) mutations.

Tertiary lymphoid structures

Many tumors are associated with TLSs, de novo lymphoid tissue resembling secondary lymphoid organs (SLOs). TLSs have been observed near zones of infection and tumors and less frequently near transplanted organs and autoimmune syndromes, where there is continued need for lymphocyte extravasation.[82,134–137] In tumors, TLSs are associated with favorable prognosis and responses to immune checkpoint inhibitors.[73,138] TLSs are mostly found peritumorally in the stroma and/or in the invasive margin, creating an optimally organized immune structure where DCs, T cells, and B cells interact and activate each other, promoting a local sustained immune response, e.g., induction of effector function, antibody generation, SMH, class switch recombination (CSR), and clonal expansion. As is the case for SLOs, the chemokine CXCL13, secreted by activated T cells, plays a crucial role in the formation of TLSs.[82]

Neogenesis of tertiary lymphoid structures

The neogenesis of TLSs starts with local production of IL-7 and CXCL13 by stromal cells or lymphocytes, which leads to the recruitment of IL-17-secreting $CD4^+$ lymphoid-tissue inducer (LTI) cells.[139] LTI cells express membrane-bound lymphotoxin α1β2 (LTα1β2), which can interact with stromal cells via the lymphotoxin β (LTβ) receptor, initiating NFκB signaling.[140] Of note, it has been shown that TLS neogenesis can occur independent of $CD4^+$ LTI cells, as B cells, T-helper 17 cells, and M1 macrophages were also found to be able to initiate TLS neogenesis.[141–144]

Together with IL-17 secretion, NFκB signaling in $CD4^+$ LTI cells results in the production of homeostatic chemokines, notably CXCL12, CXCL13, CCL19, and CCL21.82 Additionally, in stromal cells, LTα1β2-LTβ signaling leads to the secretion of adhesion molecules (VCAM1, ICAM1, and MADCAM1) and vascular endothelial growth factor C, thereby stimulating the formation of high endothelial venules (HEVs).[145] HEVs, MECA-79-expressing specialized postcapillary venules, enable lymphocyte migration and extravasation into TLSs.[82,146,147] Finally, the organization of the recruited lymphocytes results in the formation of a nodular TLS consisting of a $CD3^+$ T cell-rich zone with mature DCs in close proximity to $CD20^+$ GC-like follicle B cells intermingled with follicular dendritic cells (FDCs), and surrounded by $CD8^+CD138^+$ PCs $CD38^+CD138^+$ PCs82 (Fig. 3).

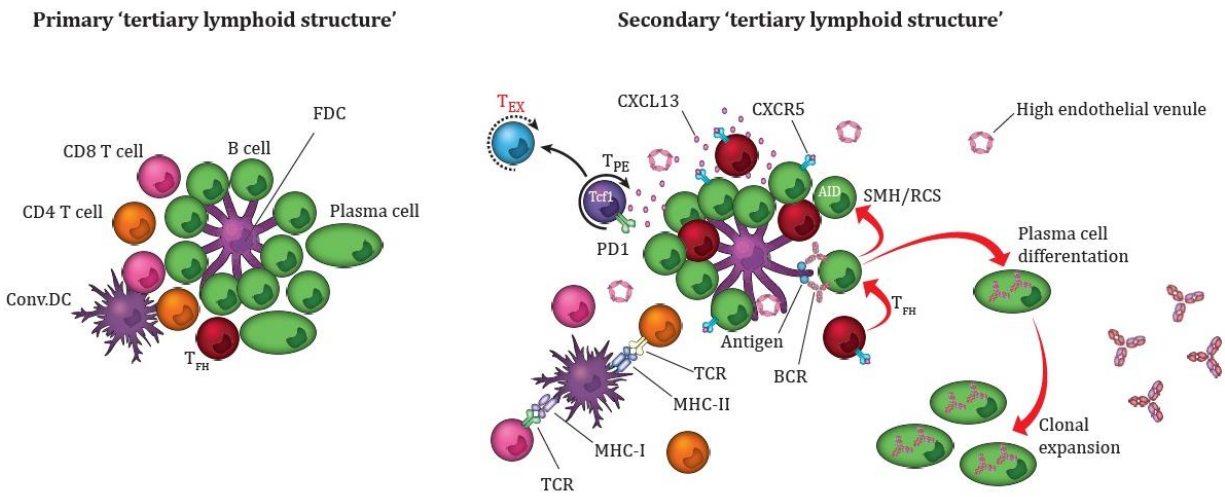

Figure 3. TLS maturation state & CXCL13. TLSs are optimally organized nodular immune structures, consisting of a $CD3^+$ T cell-rich zone with mature DCs, in close proximity to $CD20^+$ GC-like follicle B cells intermingled with FDCs and surrounded by plasma cells. The CXCL13-CXCR5 axis regulates the organization of B cells inside the follicles. CXCL13, secreted $CD8^+$ T cells induces chemotaxis by binding to the receptor CXCR5, which is mainly expressed by B cells and T_{FH} cells. Inside the TLS, B cells, T cells, DCs and FDCs interact and activate each other, promoting a local, sustained organized immune response. TLS maturation varies from dense lymphocyte aggregates to primary TLSs and secondary follicle-like mature TLSs. The difference between primary and secondary TLSs comprises the presence of germinal center activity, defined by B cells expressing AID, facilitating SHM and RCS resulting in high-affinity antibodies produced by class-switched plasma cells. In addition, mature TLS are surrounded by HEVs, facilitating lymphocyte migrations and extravasation.
TLSs: Tertiary lymphoid structures, DCs: dendritic cells, FDCs: follicular dendritic cells, T_{FH} cells: follicular helper T cells, *AID: activation-induced deaminase, SMH: somatic hypermutation, RCS: recombinant class switch, HEVs: high endothelial venules*

Cellular components, locations, and maturation of tertiary lymphoid structures

Two important subsets of the represented T cells in TLSs are TFH cells and FDCs. Differentiation of conventional $CD4^+$ T cells into TFH cells is stimulated by TGF-β, IL-12, IL-23, and Activin A signaling, followed by upregulation of the TFH cell-associated genes Bcl6, PD1, ICOS, and CXCR5.[148–153] In SLOs, TFH cells are specialized in helping B cells in helping B cells and are essential for GC formation, affinity maturation, SMH of immunoglobulin light chains and CSR of and immunoglobulin heavy chains.

FDCs facilitate long-term retention of the intact antigen in the form of immune complexes, enabling the positive selection of the SMH-mutated BCR by testing its antigen affinity.[154] Furthermore, FDCs contribute to GC B cell survival and GC affinity maturation, as demonstrated by the inactivation of FDCs by Toll-like receptor 4, which is normally upregulated during GC responses, resulting in smaller GCs and decreased antibody titers in response to immunization.[155] Finally, FDCs secrete increased levels of

transforming growth factor β1 and express increased levels of the chemokine CXCL13.[156] Although the described functional capacities of TFH cells and FDCs are mainly applicable in SLOs, the presence of these cells in TLSs has been identified, and similar functioning is assumed.[82,157–159]

TLSs are mostly located peritumorally in the stroma and/or in the invasive margin where the TLS maturation varies from dense lymphocytic aggregates (early TLSs) to primary and secondary follicle-like TLSs, depending on the presence of follicular dendritic cells (FDCs) and a GC reaction.[158,160] Mature TLSs contain GC activity, defined by B cells expressing activation-induced deaminase (AID) and the proliferation marker Ki67, and are surrounded by HEVs.[161] Interestingly, it seems that not only the presence of TLSs but also the TLS cellular components, such as T cells, B cells, FDCs, TFH cells, Treg cells, macrophages, HEVs, and chemokines, representing the TLS maturation state, are important for functionality in terms of a prosperous antitumor response. This was demonstrated in colorectal cancer (CRC) stage II and III where not TLS density, but TLS maturation was associated with disease recurrence. Tumors with mature GC-harboring TLSs (secondary TLSs) had significantly decreased risk of recurrence compared to tumors without GC-harboring TLSs (early/primary TLSs).[158] Similar results were found in chemotherapy-naive lung squamous cell carcinoma patients; only secondary TLSs, but not early or primary TLSs, were correlated with improved survival.[160]

These results are further supported by Yamaguchi et al., who demonstrated that TLSs can be categorized based on the different cellular component densities.[159] CRC samples were stained for CD3, CD8, CD20, FDC, CD68, and Bcl-6 and counterstained with DAPI, and TLSs were defined as those structures that included specific T cells (THC cells: $CD3^+CD8^-Bcl\text{-}6^-$; CTLs: $CD3^+CD8^+$; TFH cells: $CD3^+CD8^-Bcl\text{-}6^+$), B cells (B cells: $CD20^+Bcl\text{-}6^-$; GC B cells: $CD20^+Bcl\text{-}6^+$) and FDCs (FDC^+). TLS densities of $CD4^+$ THC cells and macrophages were significantly higher in patients with disease recurrence than in patients without disease recurrence. Interestingly, on multivariate analysis, there was a significant correlation between CRC recurrence and the proportion of $CD4^+$ T-helper cells ($CD3^+CD8^-Bcl\text{-}6^-$), suggesting that a high $CD4^+$ T-helper cell density hampers the antitumor immune reaction in TLSs and might be an independent predictor for CRC recurrence.[159] On the other hand, the expression of TFH cell-related genes, such as CXCL13 and IL-21, was found to predict improved survival in CRC. Indeed, loss of

CXCL13 was associated with a higher risk of relapse and lower densities of B and TFH cells in the invasive tumor margin.[162]

Because TLSs are only present in the invasive margin, Schürch et al. analyzed this region in CRC TMAs of CLR and DII patients.[163] When further exploring the spatial organization of the invasive margin, they identified "nine coordinated cellular neighborhoods (CNs)", specific areas of tissue within which every cell has a comparable surrounding neighborhood defined by the relative frequencies of cell types inside a defined radius. Similar sets of CNs were observed in both patient groups (CLR and DII), except for the follicle-enriched CN, representing TLSs, which was significantly more abundant in CLR patients.[163]

Strikingly, in CLR patients, the tumor immune compartments were isolated from the tumor compartments, but in DII patients, the immune compartments were increasingly interspersed with tumor compartments, suggesting that in DII patients, the tumor might interfere with proper development of the immune response and prevent efficient communication between CNs, which otherwise might result in the formation of follicular structures (TLSs). Furthermore, while T cells and macrophages were among the most common immune cells in the invasive margin, in DII patients, the CN1 (T cell-enriched) and CN4 (macrophage-enriched) areas were highly intertwined, having close physical contact and communication. Additionally, the CN functional states were different: in CLR patients, the CN1 (T cell-enriched) areas were more cytotoxic, and in DII patients, the CN4 (macrophage-enriched) areas were more immunosuppressive. Thus, the immune escape resulting in poor survival in DII patients might be due to factors released by the tumor, resulting in the coupling of CN1 (T cell-enriched) and CN4 (macrophage-enriched) areas and thus causing a shift towards an immunosuppressive macrophage phenotype and suppressed cytotoxic activity of the T cell-enriched CN, resulting in poor tumor immune control.[163] These results highlight the importance of understanding the underlying immune architecture in the TME. Whether this CN spatial organization is applicable across tumor types needs to be further explored.

The role of CXCL13 in tertiary lymphoid structure formation

The chemokine CXCL13 induces chemotaxis by binding to the receptor CXCR5, which is mainly expressed by B cells and TFH cells. The CXCL13–CXCR5 axis regulates the organization of B cells inside the follicles of lymphoid tissues.[164]

Interestingly, Thommen et al. showed a potential link between CXCL13-secreting exhausted $CD8^+$ T cells (high expression of PD1) and the formation of TLSs.[165] They analyzed and compared the functional, metabolic, and transcriptional signatures of $CD8^+$ TIL populations with PD1-high ($PD1^{hi}$), PD1-intermediate, and no PD1 expression ($PD1^-$) from tumor samples of non-small-cell lung cancer patients. Indeed, $PD1^{hi}CD8^+$ T cells were highly dysfunctional concerning classic cytotoxic functions such as IFNγ production compared to the other subsets, but strikingly, $PD1^{hi}CD8^+$ T cells highly expressed and constitutively secreted CXCL13. To study the function of CXCL13 in recruiting CXCR5-expressing cells towards the TME, colocalization of $PD1^{hi}$ $CD8^+$ T cells with $CD4^+$ TFH and B cells within the TME was analyzed. $PD1^{hi}CD8^+$ T cells were most represented in peritumoral and intratumoral TLSs, in close proximity to B cell infiltrates and $CD4^+$ TFH cells. In the majority of the tumors, $PD1^{hi}CD8^+$ T cells were localized at the tumor-host interface, surrounding the central B cells, suggesting an active role of $PD1^{hi}CD8^+$ T cells in recruiting immune cells and forming TLSs. Additionally, the presence of $PD1^{hi}CD8^+$ T cells was predictive of the response to PD1 blockade treatment in non-small-cell lung cancer patients and correlated with OS and durable responses, demonstrating the reinvigoration capacity of $PD1^{hi}CD8^+$ T cells upon PD1 blockade treatment.[165]

A similar relationship between exhausted CXCL13-secreting tissue-resident $CD8^+$ T cells ($CXCL13^+CD103^+CD8^+$) and TLS formation was found by Workel et al.[166] They analyzed pretreatment tumorous tissue of stage IIIC high-grade serous ovarian cancer patients (one patient received three cycles of chemotherapy prior to interval debulking surgery) and found that the phenotype of exhausted $CD8^+$ tissue-resident T cells was similar to the exhausted $CD8^+$ subpopulation of the study of Thommens et al, with both populations expressing equal PDCD1 (PD1) levels. Indeed, CXCL13 expression and secretion were observed in exhausted $CD103^+CD8^+$ TILs. Interestingly, as demonstrated by its ability to reactivate $CD8^+$ T cells isolated from peripheral blood from healthy donors in vitro, TGFβ turned out to be a specific inducer of CXCL13 and CD103 in $CD8^+$ T cells. Furthermore, the association between CXCL13-secreting tissue-resident $CD8^+$ T cells and TLS formation was assessed by analyzing TCGA mRNA expression across different tumor types, including ovarian, uterine, lung, and breast cancers. The TLS-related genes of all four tumor types correlated with the $CXCL13^+CD103^+CD8^+$ cell-related genes, suggesting that exhausted tissue-resident $CD8^+$ T cells recruit

lymphocytes towards the tumor and promote the formation of TLSs across tumor types.[166]

Similar results were found by Duhen et al., who identified CD39 and CD103 double-positive intratumoral CD8 T cells ($CD103^+CD39^+CD8^+$), which displayed an exhausted TRM phenotype (expression of PD1, CTLA-4, and TIM-3), as tumor-reactive T cells in human solid tumors.[28] Accordingly, TGFβ presence was needed for the maximum coexpression of CD39 and CD103 on $CD8^+$ T cells, and indeed, this $CD8^+$ TIL subset highly expressed CXCL13. In addition, $CD103^+CD39^+CD8^+$ TILs were associated with increased survival in head and neck squamous cell carcinoma, lung adenocarcinoma and lung squamous cell carcinoma patients.[28]

TILs in clinical practice

In clinical practice, TILs have been suggested as potential prognostic and therapeutic biomarkers, most notably in the context of immune checkpoint blockade (ICB) therapy. Interestingly, the established prognostic benefit of TILs in ovarian and breast cancer does not directly translate to therapeutic benefit for ICB treatment in these malignancies, suggesting differences in the quality of the TIL response. Nevertheless, TIL quantification is steadily making a clinical impact in combination with the traditional parameters of disease staging.

Prognostic benefit of TILs

As discussed above, intraepithelial $CD8^+$ T cells are associated with improved survival; however, some studies have also highlighted the importance and prognostic relevance of stromal TILs.[167] In epithelial ovarian cancer, stromal TILs were associated with improved OS, specifically in high-grade serous ovarian cancer.[168] In contrast, another study with similar research techniques found that increased levels of both intratumoral and stromal TILs were associated with a better prognosis, but statistical significance was only found for intratumoral TILs.[169] In HER2-positive breast cancer patients, higher levels of stromal TILs are associated with improved prognosis.[170–172] In one of the largest retrospective studies, Kim et al. assessed 1581 eligible B-31 cases for TILs in the NSABP trial and analyzed the association between infiltration of stromal TILs and clinical outcome in early-stage HER2-positive breast cancer patients receiving combined

adjuvant trastuzumab plus chemotherapy or adjuvant chemotherapy alone. They found that higher levels of stromal TILs were associated with improved DFS in both groups. However, there was no association between stromal TILs and trastuzumab benefit. The authors concluded that "stromal TILs may have utility as a prognostic biomarker identifying HER2-positive early BC at low recurrence risk".[173]

Stromal TILs were also found to have increased prognostic value in CRC compared to intraepithelial TILs.[174] The importance of stromal TILs is reflected in the existence of a standardized methodology for evaluating TILs, designed by the International TILs Working Group (ITWG) in 2014. This methodology was initially designed to assess TILs in breast cancer, but subsequently, the ITWG also proposed a model for other solid malignancies. In short, stromal TILs residing in the stromal areas, in-between carcinoma cell islets, are scored as a percentage. The surface areas occupied by the carcinoma cell islets are not included in the total valued surface area.[175,176] Fuchs et al. assessed the efficacy of the methodology in all-stage CRC patients (n = 1034). They used the ITWG method to estimate the stromal TIL density and found that the assessed stromal TILs had a superior predictive value compared to intraepithelial TILs using a traditional system proposed in the Royal College of Pathologists of Australia protocol (using the criterion of ≥5 intraepithelial lymphocytes directly in contact with tumor cells per high-power field).[177] This study demonstrated that estimating stromal TILs, which are not in direct contact with carcinoma cells, seems to be a more adequate parameter than estimating intraepithelial TILs. This does not imply that intraepithelial TILs are not important but rather reflects the difficulties in determining intraepithelial TILs on H&E staining due to the small numbers and heterogeneous appearance of TILs. Another advantage of solely assessing stromal TILs is that the density or growth pattern of carcinoma cell islets does not affect the stromal TIL score.[174] Recent advances in machine-learning approaches may help provide new insight into the utility of stromal versus intraepithelial TILs.

Clinical quantification of TILs

Traditionally, TIL infiltration has been manually quantified by pathological assessment. However, with the ever-increasing complexity in the understanding of TIL composition and localization, novel quantification approaches are under active development. The current development of digital immune scores, digital prognostic scores integrating

multiple immune features into a single model, provides the opportunity to translate the prognostic benefit of TILs into a clinically usable diagnostic tool to aid clinical decisions and to improve personalized therapy. Digital pathology is earning more attention due to the advent of whole-slide imaging.[178]

In colon cancer, an internationally validated immune score is predictive of time to disease recurrence independent of existing prognostic factors, such as age, sex, tumor stage, node stage, and MSI. Of all clinical parameters, the immune score had the highest relative contribution to the risk of recurrence. This immune scoring system represents the first standardized immune-based assay for the classification of cancer.[179] In the metastatic setting, response to treatment and prolonged survival were both significantly associated with high immune infiltration.[180] In addition, a prognostic score for oral squamous cell carcinoma incorporating four immune markers, including the levels of immune cells present in both the invasive margin and center of the tumor, was recently published. This seven-immune-feature-based prognostic score is significantly correlated with disease-free survival.[181] An independent study in oral squamous cell carcinoma identified the abundance, location, and spatial patterns of TILs as strong predictors of survival.[182] While both scoring methods were tested in small cohorts and need to be cross-validated in larger patient groups, these studies indicate oral squamous cell carcinoma as a promising next candidate for the implementation of digital immune scoring in the clinic. The use of immune scores has also been suggested for gastric and bladder cancer.[183,184] The accuracy of a digital immune score is dependent on the markers, regions of interest, procedures, measurements, and strategies used for quantification.[179,181] Therefore, immune scores should be evaluated per cancer, and the method should be validated in multiple independent cohorts.

It is worth noting that manual quantification of TILs may have limited application as a diagnostic tool due to interobserver variability and the lack of diagnostic reproducibility. With automatic machine learning, these limitations may be overcome by the quantification and classification of digitized tissue samples by supervised deep learning.[185] Studies presenting deep learning-based models for nuclei segmentation have been published.[178,186,187] In addition, a deep learning model to differentiate between adenocarcinoma and squamous cell carcinoma and predict commonly mutated genes was validated in lung cancer, and the classification is expected to be extended to other less common lung cancers.[188] In colon cancer, the accuracy of a deep learning

classifier to predict 5-year disease-specific survival was compared with that of visual assessment. Patients were categorized into low-risk or high-risk, and the machine learning-based method demonstrated superior accuracy in patient stratification compared to human observers.[185] The application of deep learning algorithms was also suggested for the detection of lymph node metastases in breast cancer.[189]

Overall, recent developments support the implementation of immune scores as a new component in the classification of cancer and advocate for the development and use of automatic machine learning. The use of immune scores will likely be extended to a wide variety of tumors, and the application could be extended to predict the development of metastatic disease and even the response to immunotherapy.[179,181,190,191]

TILs and response to immunotherapy

Immunotherapy, and in particular ICB, is targeted towards the reactivation of T_{EX} cells in tumors. Accordingly, gene profiling of responders includes assessment of (exhausted) T cell signatures, IFN-related genes, enrichment of both immunosuppressive checkpoints and immune signaling of T, B, and NK cells and increased cytokine and chemokine signaling. To elucidate determinants of response, one recent study compared pretreatment biopsies of metastatic melanoma patients responding and not responding to ICB. The gene profile of responders included IFN-related genes and genes related to enrichment of both immunosuppressive checkpoints and immune signaling of T, B, and NK cells and increased cytokine and chemokine signaling. In addition, there was an abundance of TRM cells in ICB responders.[192] Complementary research has demonstrated an increased prevalence of TRM cells in treatment-naive tumors versus healthy adjacent tissue, as well as demonstrating that ICB responders are characterized by $CD8^+$ TCM cell accumulation.[193] During ICB, these TCM cells develop an effector-like phenotype with a cytolytic gene signature. Characterization of nonresponders revealed increased coexpression of LAG-3, BTLA4, and PD1 during treatment.[194]

Interestingly, several recent studies have suggested that treatment with anti-PD1 therapies does not necessarily reverse the state of already T_{EX} cells but rather acts on the activation of T_{PE} cells. In human lung cancer, transcriptional analysis of $PD1^{high}CD8^+$ T cells identified that these cells had a low cytolytic capacity but high proliferative function compared to $PD1^{low/negative}CD8^+$ T cells, corresponding with a T_{PE} cell phenotype. $PD1^{high}CD8^+$ T cells were predictive for both survival and response to PD1

blockade and were shown to secrete CXCL13, indicating their involvement in the formation of TLSs (see also corresponding section above).[165] This observation is supported by several studies describing that ICB induces a proliferative response of less differentiated $CD8^+$ T cells, whereas T_{EX} cells cannot respond to anti-PD1 therapy.[57,58,64] This is supported by a high-dimensional single-cell RNA analysis of melanoma tumors treated with checkpoint inhibition, which identified $TCF7^+CD8^+$ T cells in particular to be associated with better tumor regression and overall response in checkpoint-treated patients.[60] Taken together, these findings suggest that signature genes consistent with T_{PE} cells may serve as a potential biomarker for ICB response.[57,58,60,63,64]

In addition to the role of $CD8^+$ T cells and MHC class I, Elspach et al. recently reported on a series of elegant experiments demonstrating the importance of MHC-II during ICB. In their work, mice were challenged with a sarcoma tumor cell line expressing MHC-I and/or MHC-II neoantigens in the absence or presence of administration of ICB. Interestingly, only the mice with functioning MHC-I and MHC-II were able to slow down tumor growth in the absence of ICB and were able to completely reject the tumor with the administration of ICB, thus demonstrating the requirement of MHC-II-mediated THC cell responses for optimal priming of MHC-I-restricted $CD8^+$ cells and their maturation into CTLs. Unresponsiveness to ICB in the presence of a favorable mutational burden could therefore be explained by the lack of MHC-II expression.[33] In human tumors, MHC-II expression was also associated with the response to ICB. In addition, low MHC-II expression was associated with primary resistance to ICB.[31]

ICB does not only affect beneficial antitumor immune cells. In particular, Treg cells are known to express both PD1 and CTLA-4, in addition to GITR, ICOS, and OX40.[195,196] In some cases, patients treated with an anti-PD1 antibody develop hyperprogressive disease (HPD). A recent study revealed a markedly increased proliferation of Treg cells in HPD patients, while there was a reduction in Treg cells in patients with no HPD. This suggests that Treg cell depletion before anti-PD1 therapy may help prevent the induction of HPD.[196] Accordingly, the success of anti-CTLA-4 treatment seems to be at least partially attributable to the depletion of intratumoral Treg cells.[195]

Mutational load, immune infiltrates, and immune checkpoint blockade

As mentioned, both the quantity and quality of TILs are likely factors in determining prognostic and therapeutic benefits. In general, the quality of T cell responses is determined by the antigen recognized through their cognate TCR. In a recent study of ICB nonresponders and responders, TCR sequencing from the tumor, normal adjacent tissue and peripheral blood revealed expansion of T cell clones in the periphery, normal adjacent tissue and tumor. Moreover, the expansion of both peripheral and intratumoral T cells was correlated with the response to ICB. This suggests a relationship between peripheral clonal expansion and tumor infiltration. Interestingly, peripherally expanded T cells infiltrating the tumor acquired a TRM-like phenotype during successful ICB responses, reconfirming the observed link between T cell exhaustion and TRM phenotypes. Considering this link, liquid biopsies to identify peripheral expanded T cell clones may help predict ICB response.[193] Accordingly, single-cell RNA and TCR sequencing from site-matched tumors after anti-PD1 treatment revealed expansion of $CD8^{+}CD39^{+}$ T cells, yet these T cell clones did not derive from pre-existing intratumoral T cells, suggesting that they were derived from peripheral T cells.[197] Similarly, high TCR clonality but lack of TCR diversity in pretreatment liquid biopsies was associated with longer PFS and good response to PD1 blockade but a poor response to CTLA4 inhibition. Multivariate regression models confirmed both TCR clonality and diversity as independent predictive factors for response.[198] Altogether, these studies suggest that TCR specificity and cognate antigens are key determinants of the quality of the TILs and the corresponding ICB response.

During successful ICB, TCR specificity is mainly directed against neoantigens and mutation-induced changes in (generally nondriver) cancer cell proteins. Accordingly, tumor mutational burden is directly related to immune infiltration and is associated with response to ICB. A subset of cancers is characterized by mismatch repair deficiency (dMMR), which leads to the accumulation of mutation-associated neoantigens (MANAs) and TAAs that stimulate the activation, differentiation, and infiltration of TILs and are associated with a better prognosis.[199,200] In endometrial cancer, increased immune infiltration and improved clinical outcome are seen in molecular subtypes harboring more mutations, such as POLE-mutant, MSI, and p53-mutant tumors.[201–203] Similar results were shown in MSI CRC, which showed increased immune infiltration and a superior prognosis compared with microsatellite-stable (MSS) CRC.[204,205] Interestingly,

the results of the study presenting a validated colorectal immune score showed better prognostic benefits in highly infiltrated MSI and MSS tumors than poorly infiltrated MSI and MSS tumors. In line with the literature, relatively more MSI tumors than MSS tumors are infiltrated with a high number of TILs, indicating that mutational load does not directly contribute to improved survival but rather that high immune infiltration occurs as a consequence of high mutational load.[179]

Tumors with a high mutational load and thus increased immune infiltration have a favorable response to ICB. A recent meta-analysis including 939 patients with MSI advanced cancer from 14 studies demonstrated a response rate of 41.5%, a disease control rate of 62.8% and a 1- and 2-year overall survival of 75.6% and 56.5%, respectively.[206] Earlier work showed a response rate of 53% and a complete response rate of 21% in ICB-treated dMMR tumors. Moreover, the expansion of MANA-specific T cells in the peripheral blood was documented as early as 2 weeks after the start of treatment.[207] The application of neoadjuvant ICB has also been successfully demonstrated in multiple tumors characterized by high mutational burden, such as melanoma and small-cell lung cancer, demonstrating impressive response rates of 78% and 45%, respectively.[208,209] In addition, neoadjuvant administration of ICB produces a superior response in MSI CRC, as demonstrated by the 100% pathological response rate in dMMR tumors and only 27% response rate in mismatch repair-proficient tumors.[210] Taken together, these data demonstrate that mutational burden is highly predictive of the pathological response to ICB and highlight a role for neoadjuvant ICB administration.[208–211]

In tumors with low mutational burden, treatment with ICB has relatively poor response rates.[212–214] A recent phase II trial compared anti-CTLA4-treated prostate cancer patients with a favorable outcome to those with an unfavorable outcome, demonstrating a gene signature enriched for the IFNγ response and CTL pathways, which was confirmed by the increased immunohistochemistry staining of CD3, CD8, granzyme-B, and PD1 in the favorable cohort. Interestingly, although there was no difference in total mutational burden between the cohorts, 8/9 patients in the favorable cohort showed peripheral T cell expansion in response to TAAs/MANAs, whereas this was only demonstrated in 4/10 patients in the unfavorable cohort. These data demonstrate that despite a low mutational burden, some mutations are capable of inducing antigen-specific T cell responses that facilitate the ICB response.[215] To improve

the ICB response in tumors with a low mutational burden, strategies to elicit immune induction are being explored, including vaccination, chemotherapy, and radiation strategies that target either T cells alone or a combination of TIL subsets.[216–218]

Reactivation of T cells coactivates NK cells

Some cosignaling receptors are expressed on both NK cells and T cells and have been of interest due to their therapeutic potential of simultaneously activating both cell types.[219–221] For example, TIGIT is expressed on both tumor-infiltrating T cells and NK cells and has been associated with tumor progression. Interestingly, TIGIT expression was found to be significantly higher on intratumoral NK cells than on peritumoral NK cells. In mice, TIGIT blockade was capable of reversing NK cell exhaustion and delaying tumor growth. Moreover, NK cells were proven critical for therapeutic effects with TIGIT blockade, PD1 blockade and combination therapy.[219]

Another receptor expressed on ~50% of peripheral NK cells and on a subset of activated $CD8^+$ T cells is NKG2A. NKG2A is an inhibitory receptor that binds to HLA-E; upon engagement, NK cells transmit intracellular signals, preventing its activation. In tumor-bearing mice, treatment with the anti-NKG2A antibody monalizumab resulted in both T and NK cell effector functions.[220,222] A first-in-human trial of MSS CRC treated with monalizumab and durvalumab demonstrated preliminary activity and manageable toxicity.[220] In head and neck cancer, overall response rates to cetuximab improved when it was combined with monalizumab, and the combination resulted in manageable side effects.[222] Finally, monotherapy with monalizumab in advanced-stage gynecological malignancies demonstrated no dose-limiting toxicities and manageable adverse events. However, no clinical effects were elicited.[221] This could suggest that NKG2A inhibition is more suitable for combination strategies. Overall, these studies indicate an important role of NK cells in the successful application of immunotherapy, advocating the dual targeting of both $CD8^+$ T cells and cytotoxic NK cells.[219–221]

Adoptive T cell transfer

In addition to immune checkpoint inhibition, adoptive cell therapy (ACT) has emerged as a promising cancer immunotherapy. ACT involves three different strategies using either TILs or genetically modified T cells with novel TCRs or chimeric antigen receptors (CAR-T).[223] For the first approach, naturally occurring TILs are harvested, expanded ex

vivo, and subsequently transfused back into the patient to induce a robust immune-mediated antitumor response. In addition to TILs, peripherally obtained T cells can be genetically modified in vitro to T cells expressing TCRs that recognize specific TAAs expressed by tumor cells. However, recognition of the TAA by the TCR requires antigen presentation via MHC, which is often downregulated in cancer cells.[224] ACT with CAR-T cells circumvents this problem because the CAR molecules, containing an extracellular antigen-binding domain and intracellular signaling and cosignaling domains, possess the properties to facilitate binding of CAR-T cells to their target and subsequently activate CAR-T cells independent of MHC.[225] The overall application of ACT with TILs has been mainly investigated in solid tumors, especially in the context of metastatic melanoma, and multiple independent studies have demonstrated durable responses, including in patients resistant to ICB.[226,227] In addition, adoptive transfer of autologous lymphocytes specifically targeting somatic mutations has elicited objective responses in gastrointestinal, colon, and breast cancer.[228–230] To optimize ACT using TILs, selection of the TIL subtype most suitable for expansion and reinfusion with the highest antitumor response is crucial. A phase II clinical trial in melanoma showed a correlation between the total number of $CD8^+$ T cells and clinical response, whereas nonresponders were characterized by higher percentages of $CD4^+$ TILs. During more in depth analysis into CD8 differentiation memory status, especially the more differentiated effector memory T cells compared to TCM were found in responding patients compared to TEM and TCM.[231] However, other studies suggested TCM cells as a suitable candidate for ACT as they possess high proliferative capacity.[17] More recently, γδ T cells have been of interests because they have been reported to possess effector-like functions.[53,232] One study successfully expanded γδ T cells from PBMCs and demonstrated that adoptive cell transfer of these cells in an ovarian carcinoma mouse model was capable of suppressing tumor growth via specific cytotoxic activities.[233] However, although generally well tolerated, no impressive therapeutic response was seen in the initial clinical trials.[232,234,235] In the context of CAR-T cells, high effectivity has been demonstrated in B cell malignancies targeting CD19. In addition, CAR-T cells have been investigated in multiple myeloma and leukemia. However, the treatment of solid tumors with TAA-specific CAR-T cells has achieved limited success, mostly due to restricted antitumor activity or severe toxicity.[236]

B cells in the response to immunotherapy

In addition to T cells and NK cells, a subset of B cells, ICOSL$^+$ B cells, emerges after chemotherapy treatment and boosts antitumor immunity. Pretreatment breast cancer samples were enriched with high IL-10 and low complement-receptor 2 (CR2) expression, while postchemotherapy samples showed an increase in ICOSL- and CR2-expressing B cells, but IL-10$^+$CD19$^+$ B cells were dramatically decreased.[237] In a mouse model, ICOSL blockade significantly inhibited chemotherapy efficacy and was accompanied by increased Treg cells and decreased levels of cytotoxic CD8$^+$ T and TH1 cells in the tumors.[237] Interestingly, Griss et al. observed similar results in melanoma patients, in which tumor-infiltrating CD8$^+$ and CD4$^+$ T cells diminished after anti-CD20 treatment.[238] Moreover, ICOSL$^+$ B cells were associated with improved therapeutic efficacy and improved DFS and OS in breast cancer patients receiving neoadjuvant chemotherapy, except the HR$^+$Her2$^-$ subtype.[237]

Pretreatment TIL B cells by themselves are also associated with improved immunotherapy responses and better survival.[75,239,240] Helmink et al. observed significantly higher expression levels of B cell-related genes in tumor samples from melanoma and renal cell carcinoma patients who responded to ICB treatment than in those from nonresponding patients. Moreover, increased BCR diversity, clonal expansion, and switched memory B cell signatures were more frequently observed in tumor samples from ICB responders than in those from nonresponders.[75] Accordingly, Petitprez et al. found that a B cell lineage signature correlated with improved survival in soft tissue sarcoma patients, regardless of the amount of tumor-infiltrating CD8$^+$ T cells. Furthermore, tumors enriched with B cell lineage signatures exhibited the highest response rate to ICB treatment.[240] Comparable results were found in tumor samples of metastatic melanoma patients.[239]

In short, TIL B cells can suppress antitumor immunity, although accumulating evidence shows that TIL B cells support antitumor immunity and promote immunotherapy responses by acting as APCs, producing high-affinity antibodies and secreting antitumor cytokines. These conflicting observations might be caused by the heterogeneity of B cell subsets as well as the various responses of these B cells to different anticancer treatments.[241] Future research and new immunotherapy strategies should focus on TIL B cells and how to exploit plasma B cells to promote lymphocyte

infiltration and stimulate cytotoxic T cell activation to increase the antitumor immune response.

Of note, while corticosteroids are often used to treat the side effects of chemotherapy, radiotherapy, and immunotherapy, in addition to other cancer patient comorbidities, in lung squamous cell carcinoma patients, it was recently suggested that GC formation inside TLSs, reflecting a mature (secondary) TLS, could be impaired by corticosteroid treatment.[160] Tumors of chemotherapy-naive lung squamous cell carcinoma patients treated with corticosteroids before surgery, either systemically or locally, showed a significantly lower TLS density and size of GCs than those of noncorticosteroid-treated patients. Using a mouse model, the causal role of corticosteroids in TLS impairment was studied. TLSs were induced in mice by intranasal administration of alum, a chemical compound, with or without Ova as an antigen, followed by systemic low-dose dexamethasone treatment. Interestingly, TLS development was observed in both models, but high TLS density and GC formation were only found in mice challenged with Ova antigen, suggesting that GC formation requires antigen-dependent interactions between TLS-residing lymphocytes.[160] Supporting their hypothesis, dexamethasone treatment did not affect TLS density but significantly reduced the development of mature GC-bearing TLSs. Altogether, these data suggest that corticosteroids negatively influence TLS maturation in the lungs. The use of corticosteroid treatment in cancer patients deserves further exploration.

Perspectives

It is evident that $CD8^+$ TILs are crucial for an effective antitumor immune response. With the rise of single-cell sequencing, it has become clear that $CD8^+$ T cells are divided into a wide variety of subsets ranging from more naive-like and proliferative to more differentiated and cytolytic immune cells. In particular, $CD103^+CD39^+$ TRM cells seem capable of tumor control. Interestingly, $CD8^+CD103^+CD39^-$ bystander T cells, although unspecific for tumor antigens, seem capable of contributing to the antitumor response. In our review, we further emphasize the importance of B cells that promote immune surveillance and improve the ICB response as a single-cell type. But even more when

clustered together with T cells in well-organized TLSs, initiated by chemokine CXCL13. TLSs give rise to a highly advanced immune response, reflected by the production of affinity-matured antibodies secreted by plasma B cells and created in GC-harboring TLSs near tumor areas. Interestingly, the presence of a TLS itself is associated with improved clinical outcome and response to ICB; however, when TLSs harbor GC activity, reflecting a mature TLS, this survival benefit is even further increased. Accordingly, proper identification of the presence and location of TILs and their spatial organization and identification of highly advanced immune structures such as TLSs is important to accurately predict prognosis, including the development of metastatic disease or recurrence, and response to ICB. A valuable tool to realize this could be found in automatic digital machine learning. Automatic machine learning enables the assessment of complex TIL composition and localization using multiple defining markers simultaneously, without interobserver variability. The success of ICB, especially anti-PD1 therapy, is undeniable, especially in a subset of solid tumors harboring a high mutational load. This clinical efficacy has been mostly attributed to the reactivation of T_{EX} cells. However, the current literature suggests that anti-PD1 therapy does not necessarily reverse the exhausted state of T cells but rather acts on the activation of T_{PE} cells, as T cell exhaustion seems to be a gradual developing state. Tcf7-expressing T_{PE} cells persist long term, are capable of self-renewal and can eventually differentiate into T_{EX} cells exhibiting high cytolytic capacity. In this overview, we show that T_{PE}-like cells are especially associated with better tumor regression and overall response in ICB-treated patients. We believe that combinatorial immunotherapy regimes are the key to successful optimization of response rates and clinical outcomes of immunotherapy-treated cancer patients. Such strategies include the immune checkpoint inhibitors simultaneously targeting NK cells and T cells that are now entering the clinic, including anti-NKG2A and anti-TIGIT agents. In addition, strategies priming patients prior or during ICB treatment to induce an immune response via vaccination, chemotherapy, or radiotherapy also exist. More research into the recently discovered helper-like innate lymphoid cells might reveal new opportunities for the application of immunotherapy. Finally, we believe that future research should focus on the development of new immunotherapy strategies that can induce and exploit TLS formation.

In conclusion, tumor-infiltrating lymphocytes play a significant role in the tumor immune environment. No individual lymphocyte subset is responsible for tumor immune control; rather, the location, clustering, interplay, and costimulation of all lymphocyte subsets are required for a successful antitumor immune response.

Declarative statement on findings

Tumor-infiltrating lymphocytes play a significant role in the tumor immune environment. Individual lymphocyte subsets are not solely responsible for tumor immune control. Rather, the proper location, clustering, interplay and costimulation of all lymphocyte subsets are required for a successful antitumor immune response.

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Chapter 3

Deep immune profiling of ovarian tumors identifies minimal MHC-I expression after neo-adjuvant chemotherapy as negatively associated with T-cell-dependent outcome

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Oncoimmunology 2020

Abstract

Epithelial ovarian cancer (EOC) is the most lethal gynaecological malignancy and has limited curative therapeutic options. Immunotherapy for EOC is promising, but clinical efficacy remains restricted to a small percentage of patients. Several lines of evidence suggest that the low response rate might be improved by combining immunotherapy with carboplatin and paclitaxel, the standard-of-care chemotherapy for EOC. Here, we assessed the immune contexture of EOC tumors, draining lymph nodes, and peripheral blood mononuclear cells during carboplatin/paclitaxel chemotherapy. We observed that the immune contexture of EOC patients is defined by tissue of origin, independent of exposure to chemotherapy. Summarized, draining lymph nodes were characterized by a quiescent microenvironment composed of mostly non-proliferating naïve $CD4^+$ T cells. Circulating T cells shared phenotypic features of both lymph nodes and tumor-infiltrating immune cells. Immunologically 'hot' ovarian tumors were characterized by ICOS, GITR, and PD-1 expression on $CD4^+$ and $CD8^+$ cells, independent of chemotherapy. The presence of PD-1^+ cells in tumors prior to, but not after, chemotherapy was associated with disease-specific survival (DSS). Accordingly, we observed high MHC-I expression in tumors prior to chemotherapy, but minimal MHC-I expression in tumors after neo-adjuvant chemotherapy, even though there were no differences in the number of TIL in both groups. We therefore speculate that TIL influx into the chemotherapy tumor microenvironment may be a consequence of the general inflammatory nature of chemotherapy-experienced tumors. Strategies to upregulate MHC-I during or after neo-adjuvant chemotherapy may thus improve treatment outcome in these patients.

Introduction

Epithelial ovarian cancer (EOC) is the most lethal gynecological malignancy and the fifth leading cause of cancer mortality in women. Almost all EOC patients present with advanced stage of disease and relapse rates are high with a 5-year survival of only 40%.[1] The poor prognosis for women with EOC has not improved in decades and new therapies are urgently needed. A new approach for the treatment of EOC may be immunotherapy.

The immune system is considered to play an important role in the development and control of EOC. The number of intraepithelial $CD8^+$ T cells is strongly associated with prolonged survival across studies.[2–4] In addition, differentiation, exhaustion, and other functional parameters of intraepithelial $CD8^+$ T cells have been associated with prognosis, as has the presence of regulatory T cells, macrophages, B cells, myeloid-derived suppressor cells and other immune cell subsets.[5–9] The immune checkpoint programmed death 1 (PD-1) and its ligand PD-L1 are also associated with prognosis in EOC, although controversy on the direction of this effect remains.[10–14] Initial trials using blocking antibodies (immune checkpoint blockade; ICB) targeting PD-1 or PD-L1 in EOC have demonstrated clinical effect, albeit in a small percentage of patients.[15] One potential strategy to increase the efficacy of immunotherapy, including ICB, is to combine treatment with other modalities, such as standard chemotherapy.

A combination of carboplatin and paclitaxel chemotherapy is part of the standard-of-care for treatment of EOC patients with advanced disease worldwide. Carboplatin and paclitaxel are DNA intercalating and cell cycle inhibitors, respectively, used frequently in combination for the treatment of ovarian, endometrial, lung, and breast cancers. For EOC patients, carboplatin/paclitaxel is administered in 6 cycles of 3 weeks and combined with cytoreductive surgery performed either prior to chemotherapy, or at the interval (i.e. after 3 cycles of chemotherapy). Previously, we demonstrated that the number and differentiation of $CD8^+$ tumor-infiltrating lymphocytes (TIL) did not differ between tumors that were carboplatin/paclitaxel-naïve when compared with tumors isolated after 3 cycles of chemotherapy.[8,16] Lo et al. recently reported an increase in the number of TIL after carboplatin/paclitaxel chemotherapy in a subset of patients [17]. Nevertheless, little data exists on the systemic immune cell status of EOC patients undergoing carboplatin/paclitaxel chemotherapy.

We studied the impact of chemotherapy on the general immune contexture of EOC patients by analysis of immune cell populations in a series of primary tumors, tumor-draining lymph nodes (tDLN), and peripheral blood mononuclear cells (PBMC). In addition, the presence of PD-1 positive cells, MHC-I expression and its correlation with survival was explored.

Methods

Patients

We selected patients diagnosed with advanced stage (FIGO ≥IIB) EOC at the University Medical Center Groningen (UMCG). Patient in the PDS cohort received primary debulking surgery and thereafter 6 cycles of platinum-based chemotherapy. Patients in the NACT cohort received 3 cycles of chemotherapy prior to the cytoreductive debulking surgery and thereafter an additional 3 cycles of chemotherapy. Ovarian tumor tissue (n=16) and tumor-draining lymph nodes (tDLN) (n=13, for three patients 3 nodes were collected) were collected during cytoreductive surgery from 20 ovarian cancer patients (Supplementary Table S1A). Tissue was obtained at the time of primary cytoreductive surgery (n=12) or during interval surgery after 3 cycles of platinum-based neo-adjuvant chemotherapy (n=8). PBMC from 7 EOC patients were isolated from peripheral blood and obtained prior to chemotherapy, 1-3 weeks after 3 cycles of chemotherapy, and 4-6 weeks after completion of all 6 cycles of chemotherapy (Supplementary Table S1A). Written informed consent was obtained from all patients.

Selection of retrospective tumor material from patients with advanced stage (FIGO ≥IIB) high grade serous ovarian carcinoma (HGSOC) was described previously (Supplementary Table S1B)[8]. Briefly, formalin-fixed paraffin-embedded tissue (FFPE) was collected at time of the primary debulking surgery (n=83) or 1-3 weeks after 3 cycles of chemotherapy at the time of interval cytoreductive surgery (n=79). Construction of the tissue microarray (TMA) was described previously.[8]

Processing of tumor material, tDLN and PBMC

Tumor tissue and lymph nodes were cut into pieces of <1 mm^3 and placed in a T75 culture flask (Nunc™ EasYFlask™ Cell Culture Flasks, cat. no. 156499, ThermoScientific)

with digestion medium, consisting of RPMI (Gibco, Paisley, UK), 10% fetal bovine serum (FBS, Gibco, Paisley, UK), collagenase type IV (1 mg/mL; Gibco, Grand Island, USA), and 12.6 µg/mL recombinant human DNase (Pulmozyme, (Roche, Woerden, the Netherlands) for overnight digestion at room temperature. After digestion, the suspension was strained through a 70 µm filter and washed with PBS. Cells were centrifuged over a Ficoll-Paque gradient (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) and lymphocytes were isolated from between the two layers. After a wash with PBS, cells were pelleted. Total cell pellet was suspended in 1ml FBS with 10% dimethylsulfoxide (Merck, Darmstadt, Germany), and stored in liquid nitrogen until further use. Peripheral blood was centrifuged over a Ficoll-Paque gradient (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) and PBMC were isolated from between the two layers. After a wash with PBS, cells were pelleted. Total cell pellet was suspended in 1ml FBS with 10% dimethylsulfoxide (Merck, Darmstadt, Germany), and stored in liquid nitrogen until further use.

Flow cytometry

Cryopreserved cell suspensions from peripheral blood, tumor tissue, and tDLN were thawed on ice, washed with RPMI medium (Gibco, Paisley, UK) with 10% FBS and centrifuged at 1000 x *g*. The total cell pellets were resuspended in RPMI with 10% FBS, and cells were incubated with the indicated antibodies (Supplementary Table 2A). The Zombie Aqua Fixable Viability Kit (BioLegend, Uithoorn, The Netherlands) was used for live/dead staining according to the manufacturer's instructions. For intracellular staining, cells were permeabilized and fixed using the FoxP3 Transcription Factor Staining Buffer kit (A25866A, Thermofisher Scientific) according to the manufacturer's protocol. All flow cytometry analyses were performed on a BD FACSVerse (BD Biosciences) using BDFACSuite CS&T Research Beads (BD Bioscience), BD™ CompBeads Set anti-mouse Ig, κ/negative control compensation particles set (552843, BD Bioscience), and UltraComp eBeads Compensation Beads (01-2222-42, eBioscience, ThermoFisher Scientific). Samples were analyzed with Premium Cytobank software (cytobank.org) (Supplementary figure S1).

Where indicated, PBMC were activated prior to phenotyping using Dynabeads® (2 µL/1x10^5 cells, T-activator CD3/CD28 beads, 11131D, Gibco, Oslo, Norway and Vilnius, Lithuania).

Immunohistochemistry

FFPE slides were de-paraffinized and rehydrated in graded ethanol. Antigen retrieval was initiated with a preheated 10 mM citrate buffer (pH=6) and endogenous peroxidase activity was blocked by submerging sections in a 0.45% hydrogen peroxide solution. Slides were blocked in PBS containing 1% human serum and 1% BSA and incubated overnight with primary antibody at 4°C (Supplementary Table 2B). Subsequently, slides were incubated with a ready-to-use peroxidase-labeled polymer for 30 minutes (Envision+/HRP anti-mouse or Envision+/HRP anti-rabbit, 2 drops, cat. number K4001/K4003, Dako, Carpinteria, USA). Signal was visualized with 3,3'diaminobenzidin (DAB) solution, and slides were counterstained with hematoxylin. Appropriate washing steps with PBS were performed in-between incubation steps. Sections were embedded in Eukitt mounting medium (Sigma Aldrich, Steinheim, Germany), and scanned on a Hamamatsu digital slide scanner (Hamamatsu photonics, Hamamatsu, Japan).

PD-1 staining was performed by the use of Ventana Discovery Ultra Platform for automatic staining, using a mouse-anti-human PD-1 antibody. Furthermore, a sequential dual staining was performed for tumor-associated macrophages (TAMs) on the Ventana Discovery Ultra platform to identify $CD163^{+}$ cells using a mouse anti-human CD163 antibody with DAB chromogen, and $CD68^{+}$CD163- cells using a mouse anti-human CD68 with Discovery purple chromogen. Immunohistochemistry for CD8, CD3 and CD27 was performed previously in this cohort [8,16].

Immunofluorescence

FFPE slide preparation and antigen retrieval were performed as described above. Next, double immunofluorescent staining of HLA-B/C and cytokeratin was performed. Slides were incubated overnight at 4°C with primary antibody (mouse anti-human HLA-B/C) and subsequently incubated with the appropriate secondary antibody for 45 minutes at room temperature (Supplementary Table S2B). Specific signal was amplified using the TSA Cyanine 5 (Cy5) detection kit (Perkin Elmer, NEL705A001KT, Boston, USA). To allow multiple amplifications on the same slide, primary HRP labels were destroyed between incubations by washing with 0.01 M hydrochloric acid for 10 minutes. Next, slides were incubated overnight at 4°C with primary antibody (mouse anti-human cytokeratin) and subsequently incubated with the appropriate secondary antibody for 45 minutes at room temperature (Supplementary Table S2B). Specific signal was

amplified using the TSA Cyanine 3 (Cy3) detection kit according to manufacturer's protocols. Appropriate washing steps with PBS containing 0.05% Tween20 (Sigma-Aldrich, Missouri, USA) were performed during the procedure. For embedding, Prolong Diamond anti-fade mounting medium with or without DAPI was used (Invitrogen/Thermo Fisher Scientific, P36962 and P36961, Oregon, USA). Finally, slides were scanned at room temperature using the TissueFAXS acquisition software and microscope (TissueGnostics, Vienna, Austria) with the following specifications: Zeiss EC "Plan-Neofluar" 40x/1.30 Oil, DIC objective, CMOS-color camera PL-B623 Pixelink (3.1 Megapixels), EXFO Excite 120 PC fluorescence illumination and Chroma ET Dapi (49000), Chroma ET CY3 (49004), Chroma ET Cy5 (49006), and Chroma FITC (49011) filter sets. Overlay images were produced using Adobe Photoshop software. MHC-I scoring was performed manually by two individuals blinded for clinicopathological data. Cores were categorically scored as low (<1% of CK^+ cells MHC-I positive), intermediate (>1-<80% of CK^+ cells MHC-I positive) or high expression (>80% of CK^+ cells MHC-I positive). Patients were included if at least two cores contained >20% tumor epithelium.

Statistics

Heatmaps were constructed in R (version 3.3.1) with package pheatmap. Differences in the percentage of immune cell subpopulations between clusters were determined using a non-parametric Kruskal-Wallis test, followed by Dunn's post-hoc analysis. Differences in the number of tumor-infiltrating immune cells on FFPE slides were determined by two-tailed Mann Whitney test. Differences in the immune cell density between MHC-I groups were determined using a non-parametric Kruskal-Wallis test, followed by Dunn's post-hoc analysis. Differences in disease specific survival were determined by a logrank test. Variables associated with disease specific survival were entered into a multivariate analysis using the Cox proportional hazards model. All statistical analyses were performed using IBM SPDSS 24 (SPDSS inc., Chicago, USA) or GraphPad Prism (GraphPad Software Inc., CA, USA). A p-value of <0.05 was used as a cut-off for significance.

Results

Immune contexture is defined by tissue of origin, independent of chemotherapy

We analyzed the immune contexture of a series of tumors, tDLN, and PBMC samples from EOC patients before, during, and after chemotherapy. We determined the expression of activation and inhibitory markers on $CD4^+$ and $CD8^+$ T cells, $CD14^+$ monocytes, and lineage-negative $HLA\text{-}DR^+$ DCs. Cluster analysis of all markers across samples revealed four main clusters based on tissue of origin: tumor tissue (tumor 1 and tumor 2), tDLN and PBMC clusters. All samples clustered independent of chemotherapy status (Fig 1A and B).

In tumor cluster 1, both $CD4^+$ and $CD8^+$ cells were characterized by a dominant $CD45RO^+$ phenotype with heterogeneous expression of CCR7 (C-C chemokine receptor type 7). CD28 expression was heterogeneous in $CD8^+$ T cells, but expressed on most $CD4^+$ T cells present in the tumor (Figure 1A, Supplementary Table S3A, median 42.4% vs 87.7%). The phenotype of T cells in the tumor microenvironment displayed an activated and exhausted phenotype, with both $CD8^+$ and $CD4^+$ T cells co-expressing PD-1, ICOS, GITR, and HLA-DR (Figure 1A). Cluster 2 contained tumor samples that appeared to be significantly less activated than tumor cluster 1 with a lower percentage of CD8 cells expressing PD-1 (median 15.4% vs 65,1%, $P<0.001$) or ICOS ($P<0.001$) and a lower percentage of CD4 cells expressing PD-1 ($P<0.001$), ICOS ($P<0.001$) or GITR ($P<0.001$).

TLDN and PBMC clusters were characterized by a marker expression pattern more consistent with a quiescent and non-proliferative immune phenotype (Supplementary Table S3A). TDLN were almost exclusively characterized by expression of CCR7, CD28, and CD45RO on $CD4^+$ and $CD8^+$ T cells, consistent with a resting naïve-like phenotype (Figure 1A). By contrast, PBMC were characterized by $CD4^+$ and $CD8^+$ cells with heterogeneous expression of CCR7, CD28 and CD27. The memory T cell marker CD45RO was variably expressed on $CD4^+$ and $CD8^+$ T cells in both tDLN and PBMC, indicating the presence of both naïve and memory cells (Figure 1A). The expression of $CCR7^+$, $CD45RO^+$, $CD27^+$ and $CD28^+$ is indicative for the presence of a central memory T cell population. Most $CD8^+$ and $CD4^+$ T cells from tDLN and PBMC were negative for exhaustion marker PD-1 (supplementary figure S2). In addition, PBMC were characterized by the presence of $CD1c^+$ and $CD11c^+$ myeloid dendritic cells while

the tumor samples were largely devoid of these populations (figure 1A, Supplementary table S3A).

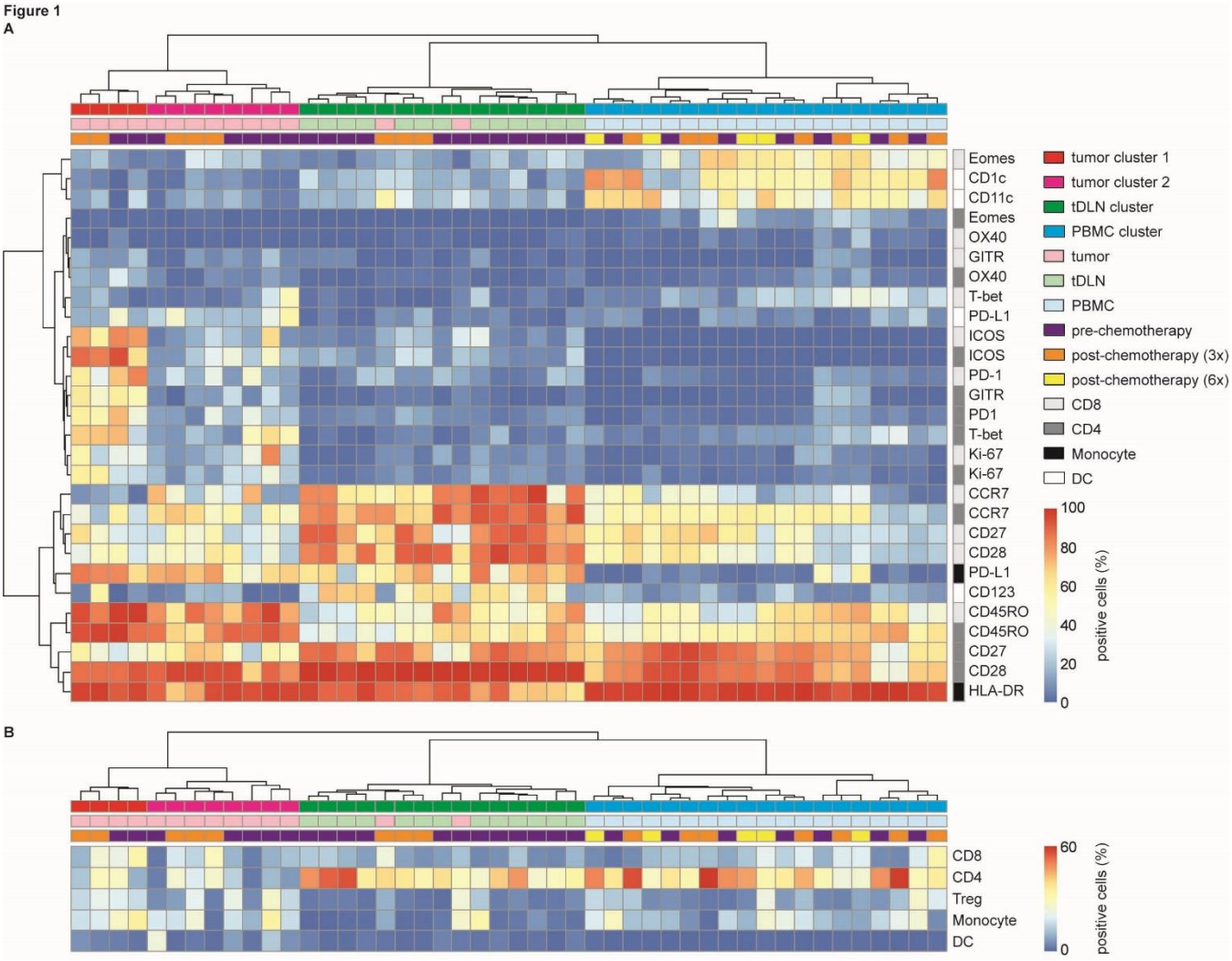

Figure 1. Immune contexture is defined by tissue of origin, independent of chemotherapy.
A, Heatmap displaying the immune contexture of fresh tumor tissue (N=16), tDLN (N=13) and PBMC (N=19) collected pre-and post-chemotherapy. Flowcytometry was used to define the immune contexture by first assessing the expression of dendritic cells, monocytes, $CD4^+$ and $CD8^+$ cells. $CD4^+$ and $CD8^+$ subsets were further defined by different immune markers including; CCR7, CD45RO, CD27, CD28, PD-1, PDL-1, ICOS, GITR, OX40, proliferation marker Ki-67 and transcription factors Eomes and T-bet. Hierarchical cluster analysis of all samples displayed four main clusters. For each sample, tissue of origin and chemotherapy status is defined. The heatmap displays the percentage of positive immune cells. Gating strategy is shown in supplementary figure S1. An overview of the determined immune cells is specified in supplementary table S3B. **B**, Heatmap displays set clusters determined in figure 1A, shows the percentage of monocytes, $CD8^+$, $CD4^+$, T-regulatory and dendritic cells.

Chemotherapy as a treatment was not associated with distinct tumor, lymph node or peripheral blood sample clusters. Thus, we speculated that chemotherapy would have a modest effect on the immune contexture of these tissues in EOC patients. To test this hypothesis, we performed immunohistochemical analysis of an independent cohort (N=162) of high-grade serous ovarian cancer (HGSOC) tumor samples obtained either prior to chemotherapy or after 3 cycles of chemotherapy (i.e. at the time of interval debulking). We included only HGSOC patients, to avoid the potential bias from differences between histological subtypes (Supplementary Table S1B). We analyzed immune markers for which commercial antibodies were available and for which we could optimize a reproducible staining protocol (Supplementary figure S5, Supplementary table S2B). There were no statistically significant differences between the immune cell infiltration of either epithelium or stroma when comparing tumor samples obtained pre- or post-chemotherapy (Figure 2A-F, Supplementary table S2C). The median density for individual immune markers showed an almost perfect correlation (R^2=0.92 P<0.0001) between the pre- and post-chemotherapy cohort (Figure 2G).

Taken together, we observed a tissue-dependent immune contexture in EOC patients. In addition our data suggests that chemotherapy does not have a major effect on the immune cell infiltration.

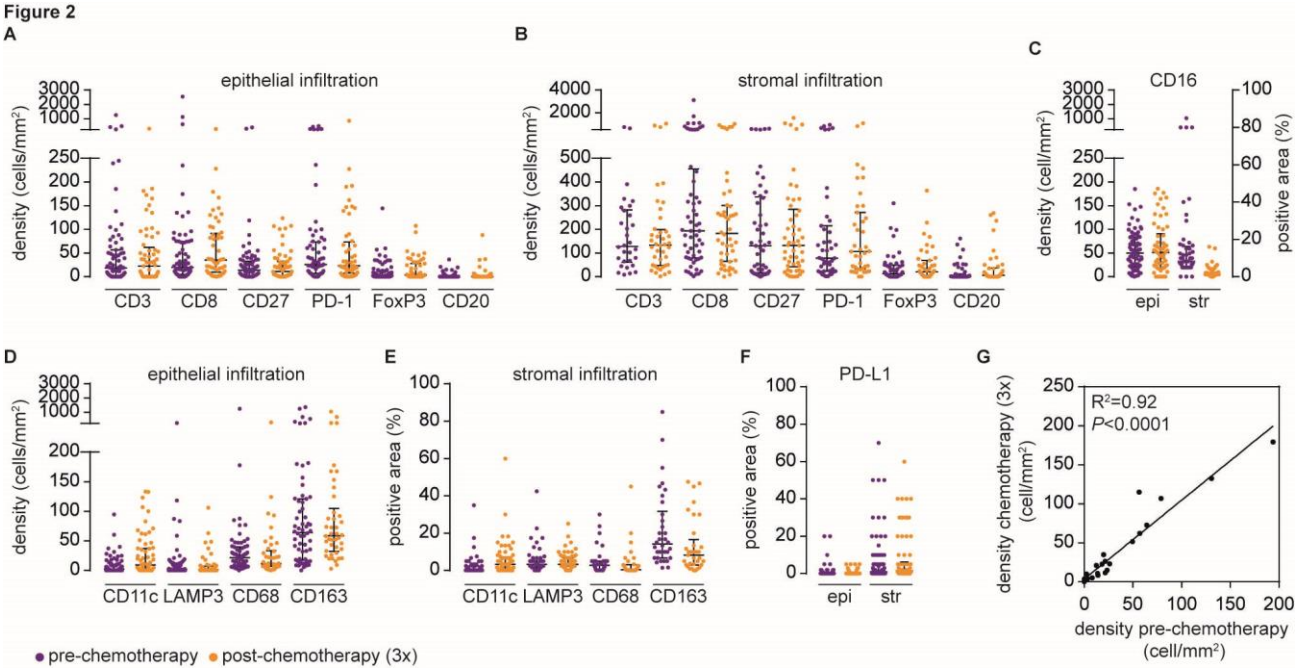

Figure 2. Immune cell distribution is comparable between pre- and post-chemotherapy tumors. **A**, Epithelial infiltration of CD3⁺, CD8⁺, CD27⁺, PD1⁺, FoxP3⁺ and CD20⁺ cells in pre- and post-chemotherapy tumors. Density is defined as cells/mm². **B**, Stromal infiltration of CD3⁺, CD8⁺, CD27⁺, PD1⁺, FoxP3⁺ and CD20⁺ cells in pre- and post-chemotherapy tumors. Density is defined as cells/mm². **C**, Epithelial and stromal infiltration of CD16⁺ cells in pre- and post-chemotherapy tumors. Epithelial infiltration was determined by density in cells/mm², stromal infiltration was determined by percentage of positive area. **D**, Epithelial infiltration of CD11c⁺, LAMP3⁺, CD68⁺ and CD163⁺ cells in pre- and post-chemotherapy tumors. Density is defined as cells/mm². **E**, Stromal infiltration of CD11c⁺, LAMP3⁺, CD68⁺ and CD163⁺ cells in pre- and post-chemotherapy tumors. Stromal infiltration is determined by percentage of positive area. **F**, Epithelial and stromal infiltration of PD-L1⁺ cells in pre- and post-chemotherapy tumors. Infiltration was determined by percentage of positive area. **(A-F)** Representative samples are depicted in supplementary figure S5. Differences in the number of tumor-infiltrating immune cells on FFPE slides were determined by two-tailed Mann Whitney test. N-numbers are described in supplementary table S3C. **G**, Median immune cell density of all epithelial and stromal immune cells depicted in **A-F**. Comparability between pre- and post-chemotherapy tumors was determined by a Pearson correlation test. R^2=0.92, <0.0001.

T cell differentiation is heterogeneous across tissue types

In our flow cytometry analyses, we observed differences in the expression of T cell differentiation markers between tumor, tDLN, and PBMC, as well as between individual clusters of tumor samples (Figure 1A). Previously, we found the expression of the T cell differentiation marker CD27 to also be highly heterogeneous in IHC analysis of EOC tumors, ranging from 0 to 407 cells/mm². Thus, we next aimed to define the co-expression of differentiation markers within the CD4⁺ and CD8⁺ immune subsets to assess their differentiation status (Figure 3A). We used the clusters identified by single

marker analysis and determined co-expression of CD45RO, CCR7, CD28, and CD27. A restricted co-expression for these markers was observed, with a large number of potential populations present at a frequency of <1% (Supplementary figure S3, cropped for clarity in Fig 3A). The tumor clusters displayed an activated T cell phenotype with a high percentage of all effector memory subsets (EM). For $CD8^+$ cells within tumors, the dominant phenotypes were $CD45RO^+CCR7^-CD28^-CD27^-$ and $CD45RO^+CCR7^-CD28^-CD27^+$ cells, consistent with the phenotype of effector cells. $CD4^+$ cells displayed a similar dominant phenotype except that most CD4 cells co-expressed CD28. This is consistent with previous reports demonstrating an earlier loss of CD28 during $CD8^+$ T cell differentiation when compared to $CD4^+$ T cells [18]. As anticipated, both tDLN and PBMC were characterized by a relatively high number of naïve and central memory $CD4^+$ and $CD8^+$ T cells. In addition, approximately half of the PBMC samples were characterized by the presence of EM3 ($CD45RO^+CCR7^-CD28^-CD27^-$) cells.

The combination of CD45RO, CCR7, CD27, and CD28 did not allow us to definitively distinguish between naïve and stem cell memory (SCM) T cells. Additionally, we performed a complementary analysis of CD45RA, CCR7, CD27, and CD95 co-expression in consecutive pre- and post-chemotherapy PBMC samples of EOC patients (n=7) and compared the differentiation status to age-matched controls diagnosed with a benign ovarian tumor (n=7) (figure 3B and C). Cluster analysis revealed neither distinguishing effects of chemotherapy, nor between patients with EOC and patients with a benign tumor. As observed before, a restricted pattern of marker co-expression was observed. Two EOC patients were characterized by a high prevalence of $CD45RA^+CD95^+CD8^+$ T cells, representing a terminally differentiated subset. Finally, sufficient PBMC were available from a single EOC patient and benign control, to confirm the observed loss of CD28 on $CD8^+$, but not $CD4^+$ T cells in tumors by analyzing T cell phenotype upon *in vitro* activation. In brief, PBMC of an ovarian cancer patient and a healthy control were activated with CD3/CD28 beads for 7 days and expression of CD45RA, CD27, CD28 and CD95 was analyzed (figure 3D). T cell activation was associated with a loss of CD28 from the cell surface of $CD8^+$, but not $CD4^+$ SCM T cells, independent of disease status and chemotherapy status (Figure 3D). No other phenotypic differences were observed between these two T cell subsets.

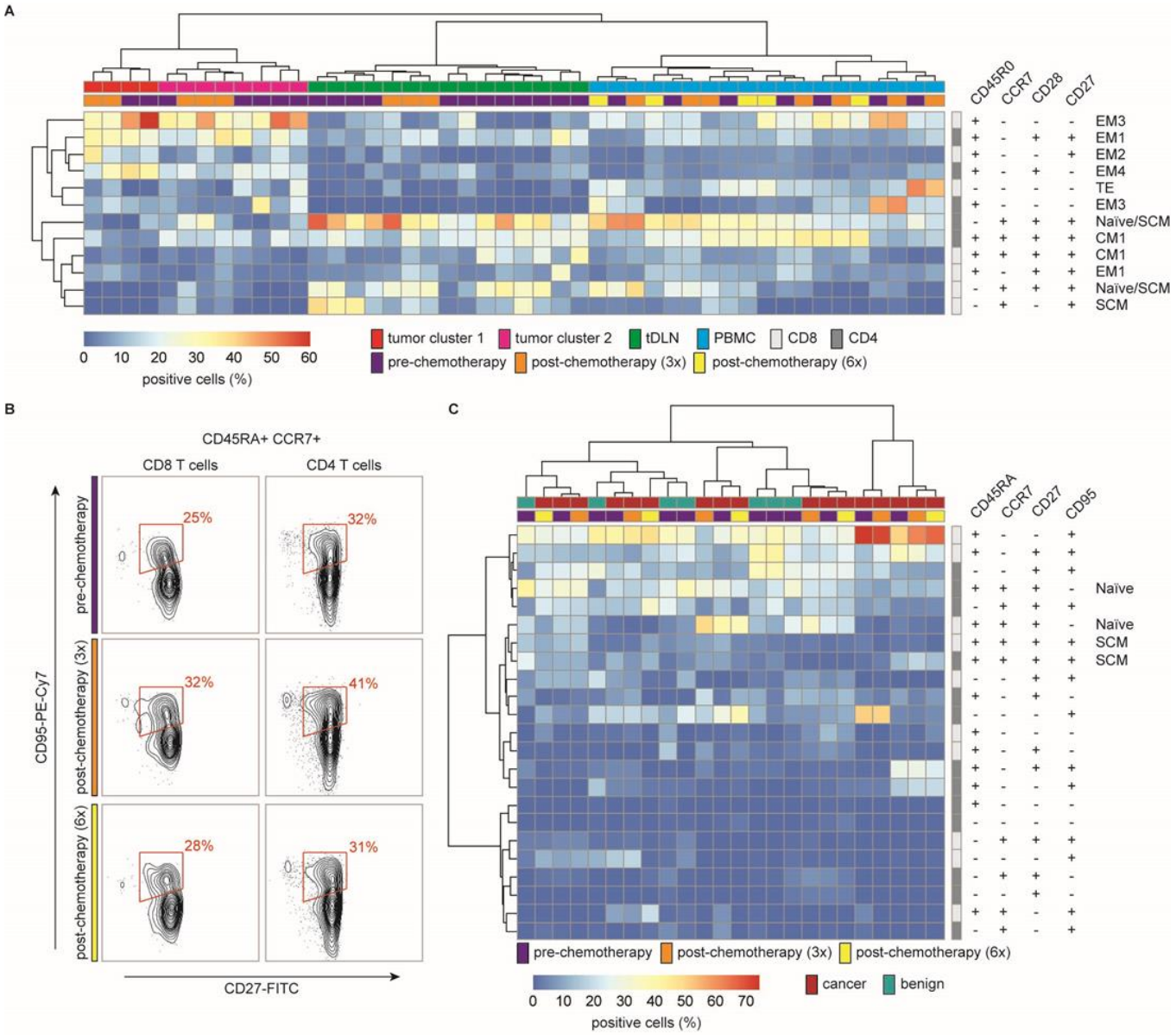

Figure 3A-C. T cell differentiation is heterogeneous across tissue types. A, The heatmap displays clusters identified by single marker analysis in figure 1A. For each sample chemotherapy status is defined. The percentage of positive cells immune subset is displayed in the heatmap. Immune subsets are determined by co-expression of CD45RA, CCR7, CD27, and CD95 on $CD4^+$ and $CD8^+$ cells. P-values are specified in supplementary table S3D. **B**, One exemplary EOC patient including before, during and after chemotherapy samples is depicted. The percentage of $CD45RA^+CCR7^+CD27^+CD95^+$ CD4 and CD8 positive cells is displayed. **C**, Heatmap displaying the percentage of naïve T-cells defined as $CD45RA^+CCR7^+CD27^+CD95^-$ and stem cell memory cells defined as $CD45RA^+CCR7^+CD27^+CD95^+$ in PBMC of EOC patients before, during and after chemotherapy (N=17) and benign controls (N=7). Hierarchical cluster analysis of all samples displayed clusters independent of disease or chemotherapy status.

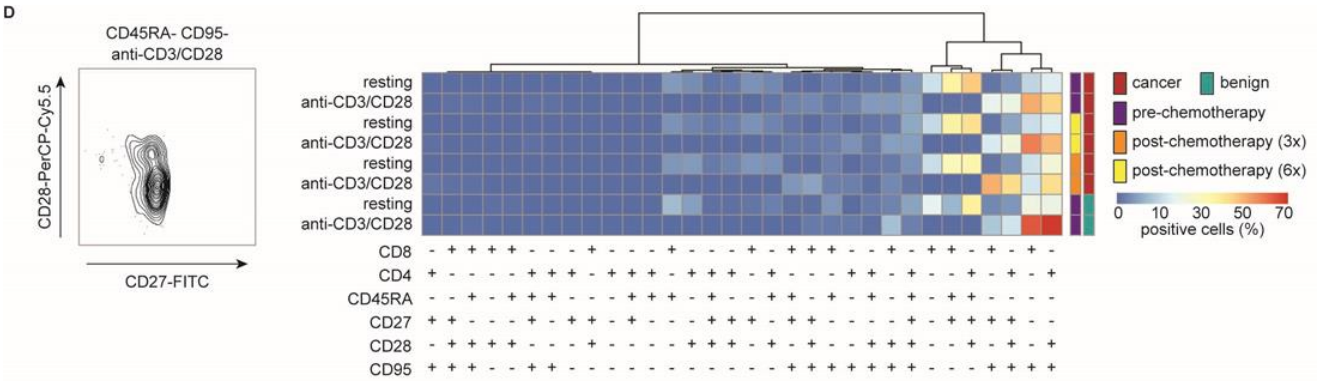

Figure 3D. T cell differentiation is heterogeneous across tissue types. D, The percentage of positive immune subsets is displayed without and after 7 day stimulation with anti-CD3/CD28 T-cell activation beads in one EOC patients before, during and after chemotherapy and in one benign controls . Immune subsets were defined by CD8, CD4, CD45RA, CD27,CD28 and CD95. T cell activation was associated with a loss of CD28 from the cell surface of $CD8^+$, but not $CD4^+$ SCM T cells, independent of disease status and chemotherapy status.

ICOS and GITR are co-expressed in ovarian cancer patients with an exhausted phenotype

Having established a comprehensive immune profile for ovarian tumors, tDLN and PBMC, we next assessed potential targets for therapeutic intervention. We observed a relatively high percentage of ICOS and GITR in tumors from cluster 1 (figure 1A). Co-expression analysis of ICOS and GITR revealed a dominant co-expression in tumor cluster 1 when compared to tumor cluster 2, mostly on T-regulatory cells, but also on $CD4^+$ and $CD8^+$ T cells (figure 4A and B). By contrast, most $CD4^+$ and $CD8^+$ cells in tumor cluster 2, TDLN and PBMC were double negative for ICOS and GITR (figure 4A and B). This difference in co-expression on $CD8^+$, $CD4^+$, and Treg cells was also evident when comparing a tumor with a draining lymph node from a patient from whom a matched sample was available (figure 4B). To confirm the observed co-expression in $CD8^+$ T cells, we also analyzed a recently published dataset of $CD8^+$ TIL from ovarian cancer[19]. *ICOS*, *TNFRSF18* (GITR), as well as in the intracellular signaling adaptor for GITR: *TRAF1* were overexpressed in $ICOS^+$ vs. $ICOS^-$ TILs (figure 4C). ICOS and GITR are also often co-expressed with immune checkpoint CTLA4 (cytotoxic T-lymphocyte attenuator 4) [19]. Therefore, we investigated the expression levels of CTLA4 within the various ICOS/GITR $CD4^+$ and $CD8^+$ T cell subpopulations (Figure 4D) and found that mean fluorescent intensities (MFI) for CTLA-4 were higher in ICOS/GITR double-positive Treg cells when compared to the other subpopulations.

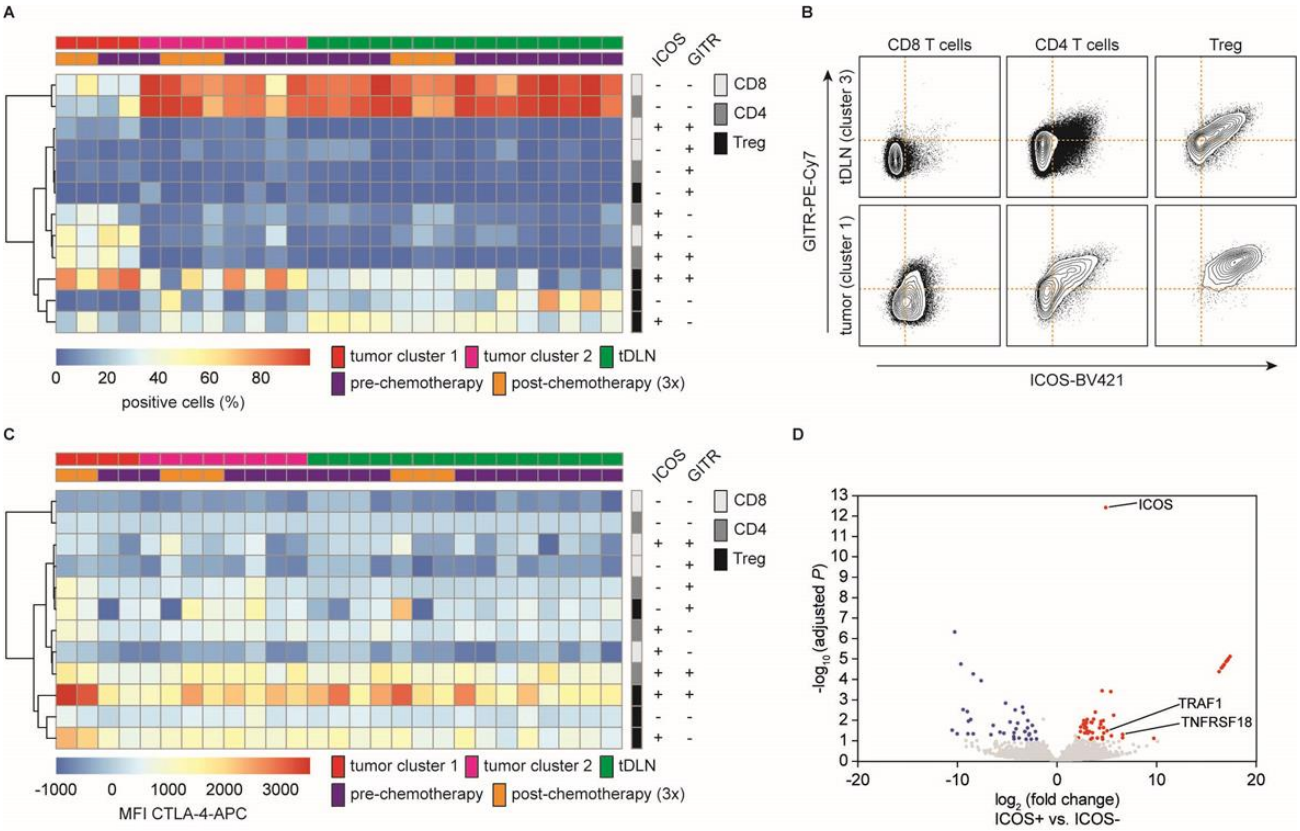

Figure 4. ICOS, GITR, and CTLA-4 co-expression. A, The heatmap displays clusters 1-3 as identified by single marker analysis in figure 1A. For each sample chemotherapy status is defined. Cell surface co-expression of ICOS and GITR on CD4$^+$, CD8$^+$ and T-regulatory cells was determined. The prevalence of the different immune cells is displayed by percentage on the heatmap. P-values are specified in supplementary table S3E. **B**, One exemplary flowcytometry graph displaying cell surface co-expression of ICOS and GITR on sorted CD4$^+$, CD8$^+$ and T-regulatory cells of a tumor sample (cluster 1) and a lymph node (cluster 3) as described for (A). **C**, The heatmap displays clusters 1-3 as identified by single marker analysis in figure 1A. For each sample chemotherapy status is defined. Mean fluorescence intensity of CTLA-4 was determined for CD8, CD4 and T-regulatory cells with differential expression of ICOS and GITR. **D**, Volcano plot of up- or downregulated genes between in ICOS$^+$ and ICOS$^-$ TILs as determined by RNA sequencing, annotated by a GITR related genes. Significance was determined as Benjamini-Hochberg FDR <0.01 and log2 fold-change >1.

Infiltration of PD-1$^+$ cells in tumor epithelium is correlated with disease specific survival in pre-chemotherapy patients only

A dominant phenotype observed in tumor cluster 1 of PD-1$^+$ CD4$^+$ and CD8$^+$ T cells compared to tumor cluster 2 (figure 1). In addition, PD-1$^+$ TIL were observed in both the pre-chemotherapy and chemotherapy group but did not differ in absolute number (Figure 2A). We tested for a potential survival benefit of high PD-1 expression in EOC. DSS was significantly longer in HGSOC patients with a higher infiltration of epithelial PD-1$^+$ cells (P=0.004) (figure 5A). This survival benefit was only observed in patients who

received primary debulking surgery (P<0.001), and not in the neoadjuvant chemotherapy cohort (figure 5A). Stromal PD-1 expression was not correlated with improved DSS in any of the cohorts (figure 5B). Next, we corrected for surgical outcome since this is a major predictor for DSS. High epithelial PD-1^{+} cell infiltrate was a predictor for DSS in case of a complete primary debulking (P<0.001) (figure 5C). Again, the survival benefit was only present in the pre-chemotherapy group, but not in the chemotherapy group. Although stromal PD-1 expression was not predictive in the entire patient group, when correcting for surgical outcome, stromal infiltration of PD-1^{+} cells did have a predictive value for DSS in the patients with a complete primary debulking (P=0.005).

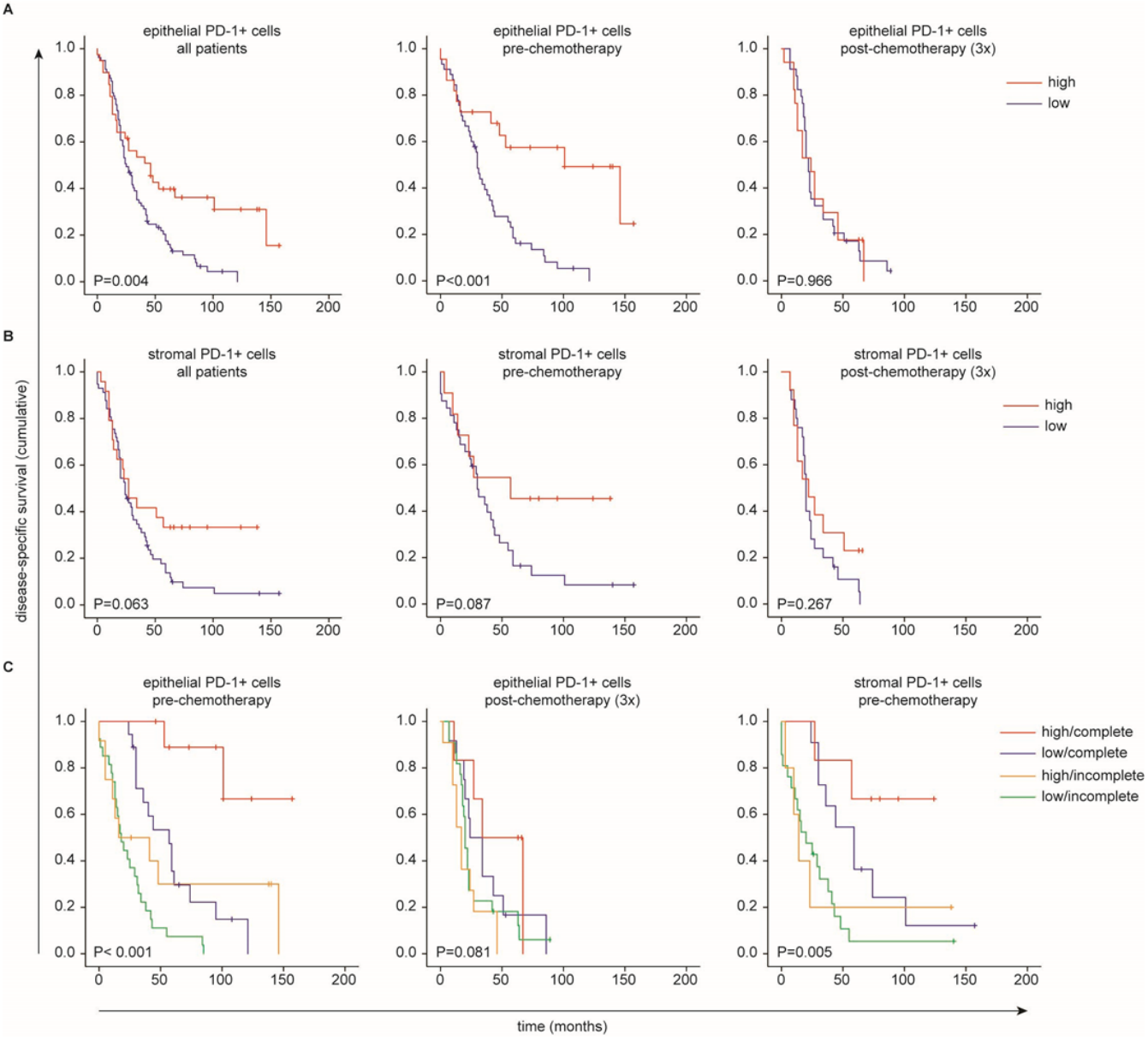

Figure 5. PD-1$^+$ cells only have a survival benefit in pre-chemotherapy tumors who were completely removed after primary surgical debulking. (A-C), Patients were stratified to high or low number of PD-1$^+$ cells in the epithelium and stroma. Differences in survival were determined by a logrank test. N-numbers are specified in supplementary table S3F. **A**, Disease specific survival (DSS) was determined in patients with high vs low epithelial PD-1$^+$ cells. DSS in all patients, P=0.004. DSS in in pre-chemotherapy tumors, P<0.001 and DSS in post-chemotherapy tumors, P=0.966. **B**, DSS was determined in patients with high vs low stromal PD-1$^+$ cells. DSS in all patients, P=0.063. DSS in pre-chemotherapy tumors, P<0.087 and DSS in post-chemotherapy tumors, P=0.276. **C**, DSS was determined in patients with high PD-1$^+$ cells/ complete debulking vs. low PD-1$^+$ cells/ complete debulking vs. high PD-1$^+$ cells/ incomplete debulking vs. low PD-1$^+$ cells/ incomplete debulking. DSS of epithelial PD-1$^+$ cells in pre-chemotherapy tumors, P<0.001. DSS of epithelial PD-1$^+$ cells in post-chemotherapy tumors, P=0.081 and DSS of stromal PD-1$^+$ cells in pre-chemotherapy tumors P=0.005.

Treatment of HGSOC patients with neo-adjuvant chemotherapy is associated with minimal expression of MHC-I

We hypothesized that the loss in prognostic benefit of epithelial PD-1$^+$ cells of patients treated with neo-adjuvant chemotherapy could be explained by inadequate antigen presentation. Therefore, we analysed the expression of MHC-I on cancer cells (figure 6A). Expression of high, intermediate and low MHC-I on cancer cells was observed in 24.1%, 45.6%, and 30.4% of tumors in the pre-chemotherapy group compared to only 1.7%, 61.7%, and 36.7% in the chemotherapy group, respectively. Next, we determined the correlation between MHC-I expression, chemotherapy and immune markers. In the pre-chemotherapy patients, a significant correlation between MHC-I expression on cancer cells was observed with all immune markers, with exception of the B-cell marker CD20 (figure 6B). A step-wise increase was observed from low, to intermediate, to high MHC-I. In the stroma a comparable trend was observed, with a significant correlation between MHC-I expression on cancer cells and both CD8$^+$ and FoxP3$^+$ cells (figure 6C). In the post-chemotherapy patients, intermediate MHC-I expression on cancer cells was significantly correlated with CD3$^+$ cells, only (figure 6D). In the stroma this correlation was only seen for CD27$^+$ cells (figure 6E).

To confirm the prognostic value of PD-1+ cell infiltration in context of MHC-I expression, treatment regimen, and surgical result we performed a multivariate Cox regression analysis, also including other known prognostic parameters FIGO stage and epithelial CD8+ cell infiltration (Supplementary Table S3H). In this model, the surgical result (hazard risk (HR): 1.338, 95% CI: 1.165-1.536) and PD-1+ cell infiltration in epithelium (HR: 0.826, 95% CI: 0.685-0.995) were the only parameters of prognostic value.

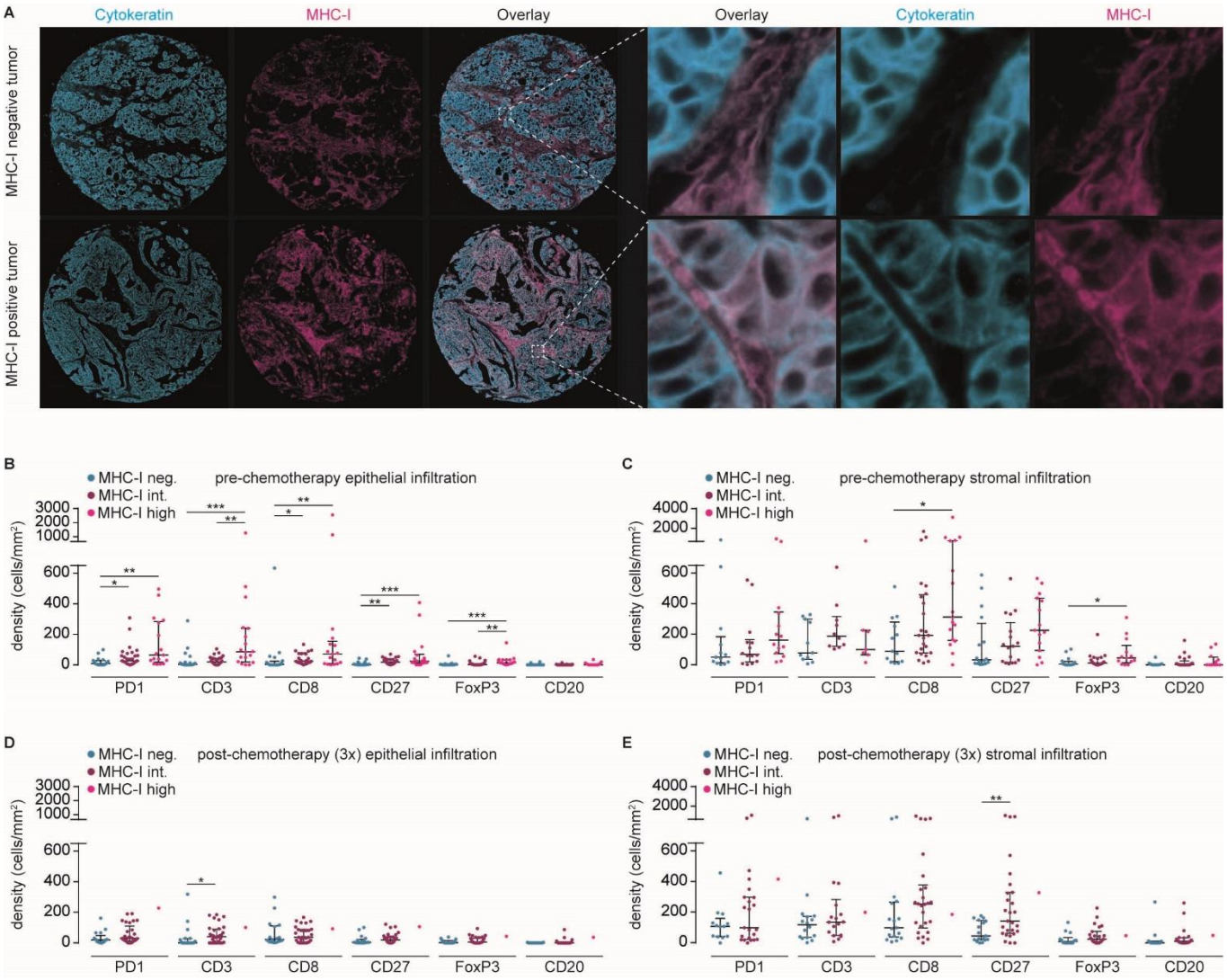

Figure 6. Treatment of HGSOC patients with neo-adjuvant chemotherapy is associated with minimal expression of MHC-I. A, Exemplary epithelial MHC-I immunofluorescent staining negative and positive staining. **(B-E)**, Epithelial and stromal infiltration of PD1, CD3, CD8, CD27, FoxP3 and CD20 positive cells in pre- and post-chemotherapy tumors stratified according to MHC-I score. Density is defined as cells/mm^2. Significance was determined by non-parametric Kruskal-Wallis test, followed by Dunn's post-hoc analysis. *P<0.05, **P<0.01, ***P<0.001. N-numbers are specified in supplementary table S3G. **B**, pre-chemotherapy epithelial infiltration. **C**, pre-chemotherapy stromal infiltration. **D**, post-chemotherapy epithelial infiltration. **E**, post-chemotherapy stromal infiltration.

Discussion

We report on the immune profile of ovarian carcinoma patients treated with carboplatin and paclitaxel chemotherapy. We demonstrated that the phenotype and the presence of immune subsets are highly variable within individual patients and are more defined by tissue of origin than by exposure to chemotherapy. Tumor draining lymph nodes were characterized by a quiescent microenvironment composed of mostly non-proliferating naïve $CD4^+$ T cells. Circulating T cells shared phenotypic features of both lymph node and tumor-infiltrating immune cells. Immunologically 'hot' ovarian tumors were characterized by ICOS, GITR, and PD-1 expression on $CD4^+$ and $CD8^+$ cells, independent of chemotherapy.

Earlier studies on the effect of carboplatin and paclitaxel chemotherapy assessed pre and post NACT samples within the same patient.[17,20,21] The largest study included 83 paired pre- and post- NACT EOC tumor samples and demonstrated an overall significant increase in the level of tumor-infiltrating lymphocytes after NACT. TIL were defined as the percentage of intra tumoral occupation by mononuclear cells on H&E stained fixed tissue, but lymphocyte subsets were not further defined [20]. Two additional studies included smaller sample sizes, but studied a broader panel of immune markers using IHC. Both studies demonstrated an increase in $CD8^+$ cells after NACT. However, these studies showed contradicting results on $CD4^+$ and Granzyme B^+ expression, highlighting the heterogeneity of ovarian carcinoma.[17,21]

In our study, no differences in the immune phenotypes were observed between tumors collected during primary surgery debulking and tumors collected during interval debulking after 3 cycles of NACT. However, no matched pre- and post-chemotherapy samples were available for our study. Therefore, we cannot exclude the potential of NACT patients with a low baseline of TILs that had increased upon chemotherapy treatment. The past decade has seen changes in the standard-of-care for patients with ovarian cancer in the Netherlands. In particular, a higher number of patients are treated with neo-adjuvant chemotherapy. Before 2008, ~30% of patients were treated with NACT, but by 2013 this increased to ~60%.[22] If NACT patients would be characterized by a lower baseline of epithelial TILs compared to PDS patients, a higher median density of epithelial $CD8^+$ T cells would thus be observed in the NACT cohort after the change in standard-of-care (e.g. <2008 vs >2008). However, no difference was observed in total

epithelial T cells between the NACT cohort 2002 – 2008 and NACT 2008 – 2012 cohort (Supplementary figure S4).

The data demonstrate a tDLN phenotype which is consistent with quiescent lymph nodes from healthy individuals.[23,24] Few studies to date have comprehensively examined the immune phenotype of tDLN in human cancer.[24,25] In one of the most extensive studies, Heeren et al. reported on the composition of tumor-negative and tumor-positive DLN in patients with cervical cancer. The phenotype for tumor-free DLN in their study is almost identical to what we have observed for ovarian tDLN [25], in line with a quiescent nature of tDLN of ovarian cancer patients. The observed quiescent nature of the tDLN suggests a lack of lymph node involvement in the antitumor immune responses in ovarian cancer. Therefore, novel immunotherapeutic treatment strategies may need to specifically target this site for enhanced antitumor efficacy. Herein, one approach of particular interest may be the use of vaccines targeted at myeloid $CD11c^+$ dendritic cells for the direct uptake and presentation of antigens. Such vaccines have recently proved effective at eliciting strong antitumor immunity in mice and humans, and have shown tentative signs of clinical activity, particularly when combined with PD-1 blockade.[26,27]

In concordance with our findings, Wu et al. observed an unchanged level of circulating immune cells in blood samples collected before chemotherapy and blood samples collected 3-4 weeks after chemotherapy administration [28]. However, at 12-14 days after chemotherapy they found a decrease in T-regulatory cells and an increase in cytotoxic $CD8^+$ T cells.[28] Importantly, standard-of-care carboplatin and paclitaxel chemotherapy is often combined with pre-medication, including dexamethasone, to reduce chemotherapy side effects. While, dexamethasone has a clearly described immune-attenuating effect, the precise mode-of-action on the circulating lymphocytes remains ambiguous. In both mice and humans, administration of dexamethasone was associated with an upregulation of circulating lymphocytes in the lymph nodes and circulation.[29,30] Considering the biological half-life of dexamethasone (36-72 hours), it is conceivable that immune modulating effects earlier than ~12 days (4x biological half-life) after chemotherapy/corticosteroid application can be contributed to the administration of dexamethasone and not chemotherapy treatment.

Importantly, we observed no deleterious effects of carboplatin/paclitaxel on differentiation, activation, and/or proliferation of T cells, confirming previous reports

that certain chemotherapeutic regimes can be effectively combined with T cell-targeting immunotherapy.[31–33] Early data from clinical trials in triple-negative breast cancer combining chemotherapy with PD-1/PD-L1 checkpoint inhibition are currently ongoing and early data suggest that the combination is relatively safe and improves response rates.[33–35]

A striking observation was the difference between the two defined tumor clusters. We observed immunologically 'hot' ovarian tumors characterized by ICOS, GITR, and PD-1 expression on both $CD4^+$ and $CD8^+$ cells. ICOS and GITR are immune checkpoint receptors known to be co-expressed on the cell surface of exhausted T cells together with CTLA-4.[19] The relative overexpression of CTLA-4, ICOS and GITR on regulatory T cells in our data is in line with a recent report on Treg phenotype in melanoma, non-small cell lung cancer (NSCLC), and RCC. The association of $ICOS^+$ Tregs with poor survival was previously described in ovarian cancer and more recently renal cell cancer (RCC).[36–38] CTLA-4, ICOS, and GITR may thus be targets for depletion of suppressive Tregs via antibody-dependent cell-mediated cytotoxicity (ADCC), either as monoclonal antibody or in bispecific format.

Tumor epithelial infiltration by $PD\text{-}1^+$ cells was predictive for DSS in patients who received a complete primary debulking surgery and not in NACT patients. A possible bias is introduced due to the fact that overall survival is better for patients receiving a primary debulking compared to NACT patients. Checkpoint inhibition targeting PD-1 has been successful for the treatment of several solid malignancies.[39] Therapeutic efficacy in ovarian cancer, however, has been limited. In a phase I trial, an objective response was observed in 3 out of 26 patients[40] and in a phase II trial, 2 complete responses out of 20 patients were reported.[41] For both studies, the studied population consisted of patients with platinum-resistant recurrences with heterogeneous histological and clinicopathological characteristics. In our study, the prognostic benefit of epithelial PD-1 expression was absent in the chemotherapy group, which can potentially be explained by the lack of proper antigen recognition via MHC-I, as high MHC-I expression was only seen in the pre-chemotherapy group. We did not observe differences in the number of TIL in both groups, even though MHC-I expression was reduced. We therefore speculate that TIL influx into the chemotherapy tumor micro-environment is not the result of tumor antigen-specific recognition, but may be a consequence of the general inflammatory nature of chemotherapy-experienced tumors. As such, these TILs may

therefore be unable to exert tumor-specific cytotoxic effects, in line with their limited prognostic benefit with regards to long-term patient survival. Determining how chemotherapy influences MHC-I expression in these tumors may lead to new opportunities to improve treatment outcome in patients treated with neoadjuvant chemotherapy. A recent paper described loss of MHC-I antigen presentation in cancer cells through transcriptional silencing of the MHC-I antigen processing pathway by conserved function of polycomb repressive complex 2.[42] In breast cancer methylation of MHC-I genes was shown to suppress MHC-I expression which could be reversed by DNA methyltransferase inhibition.[43] MHC-I could be upregulated via interferon-γ which can be induced by use of e.g. vaccination strategy or other immune stimulating therapies. However, in EOC it is firstly important to validate whether the low MHC-I expression is upfront or whether it occurs due to the NACT. In conclusion, our findings show reduced amount of MHC-I on tumors after chemotherapy, which might explain the loss of prognostic benefit of TILs in these patients. Upregulating MHC-I in ovarian tumors might therefore augment chemo-immunotherapeutic strategies.

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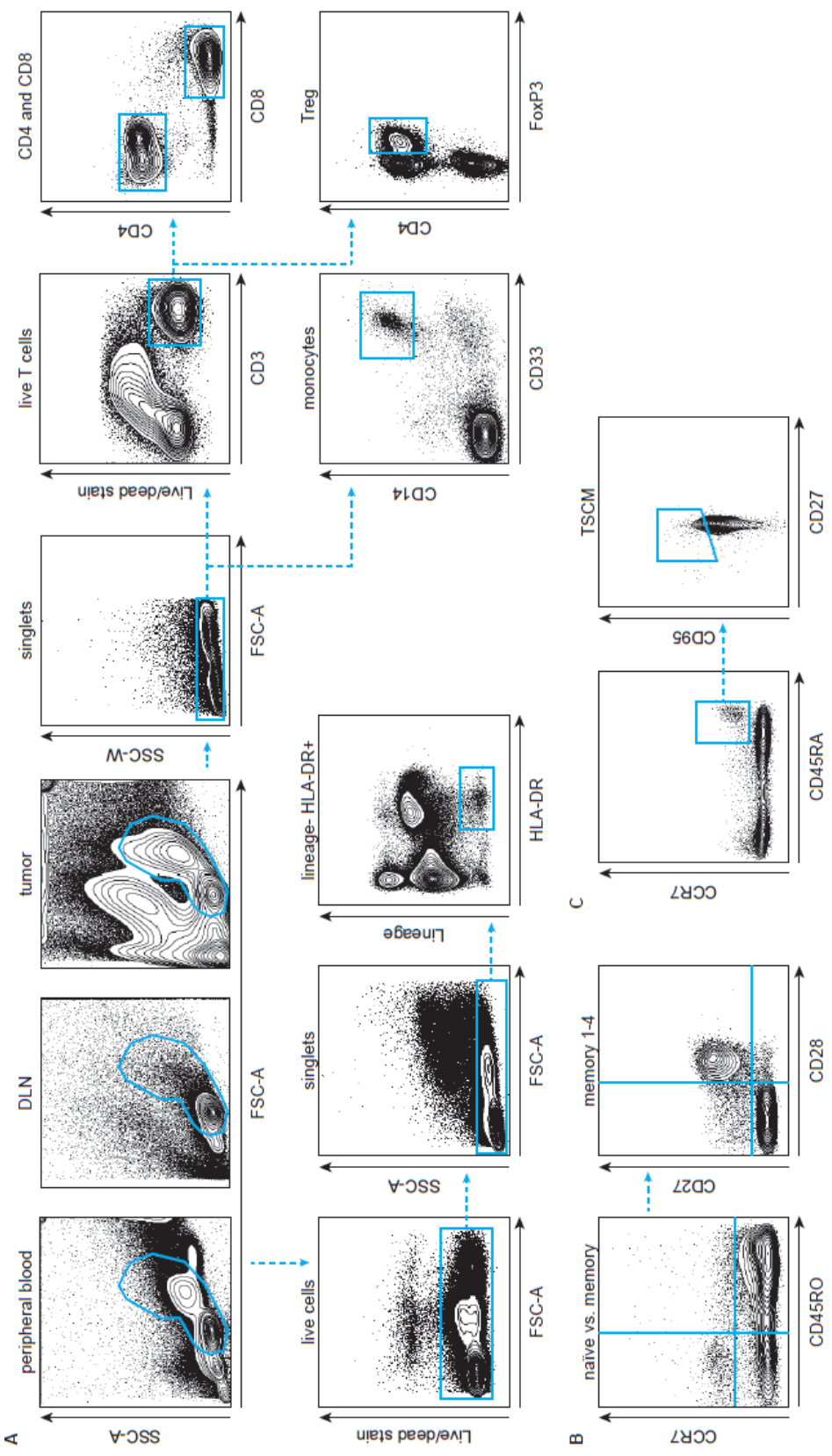
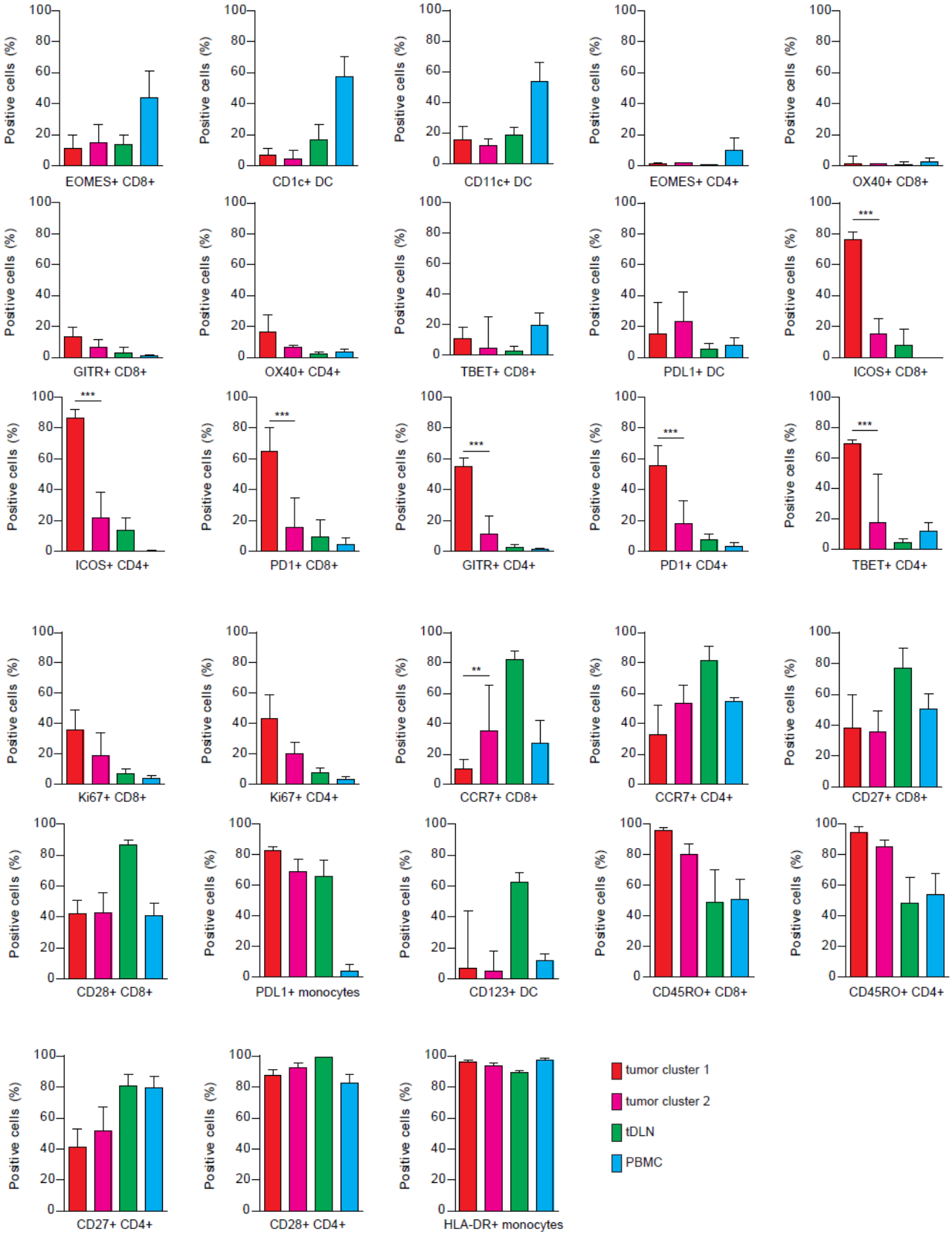
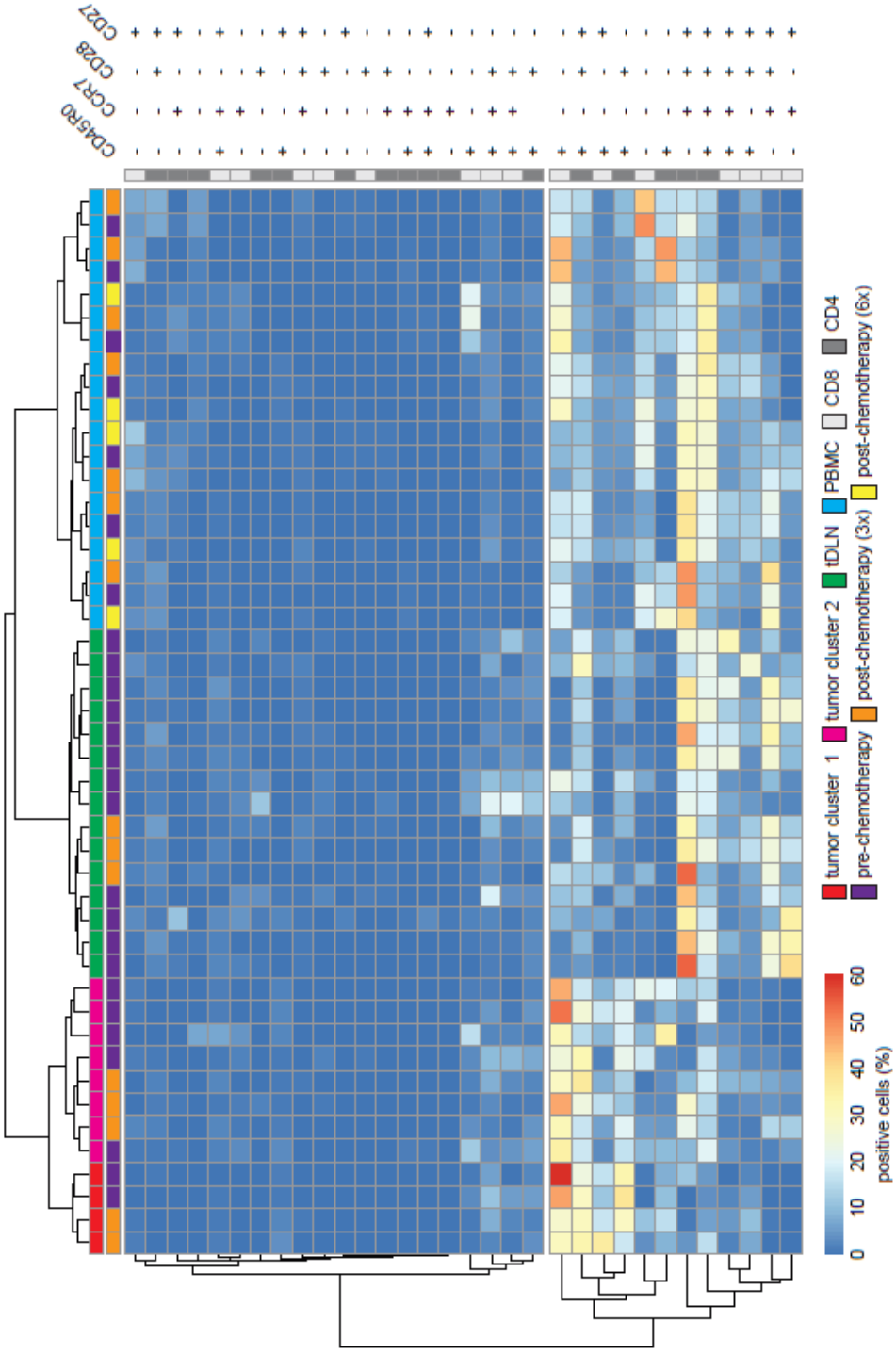


Supplementary figure S1. Gating strategy used for flowcytometry analysis in fig. 1 and 3. (A-C), Gating strategy used for peripheral blood, draining lymph nodes and tumor samples in flowcytometry analysis in figure 1 and 3. **A**, Gating strategies used in all samples figure 1 for $CD4^+$ and $CD8^+$ T-cells according CD3, CD4 and CD8 expression, for dendritic cells according lineage and HLA-DR expression, for monocytes according CD14 and CD33 expression and for Tregulatory cells according CD3, CD4 and FoxP3 expression. **B**, Gating strategy used in all samples in figure 3A for $CD4^+$ and $CD8^+$ immune subsets according CD3, CD4, CD8, CCR7, CD45RO, CD27 and CD28 expression. C, Gating strategy used in figure 3C in peripheral blood samples for naïve T-cells and stem cell memory cells according CD3, CD4, CD8, CCR7, CD45RA, CD95 and CD27 expression.

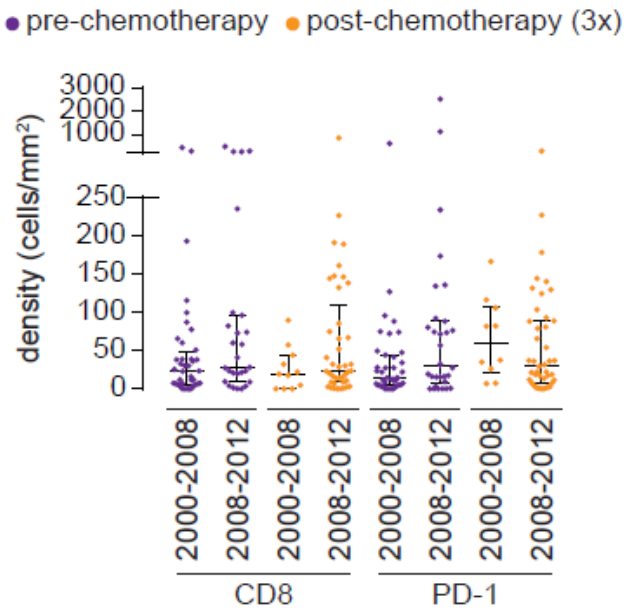


Supplementary figure S2. Percentage of positive immune cells in all four clusters.

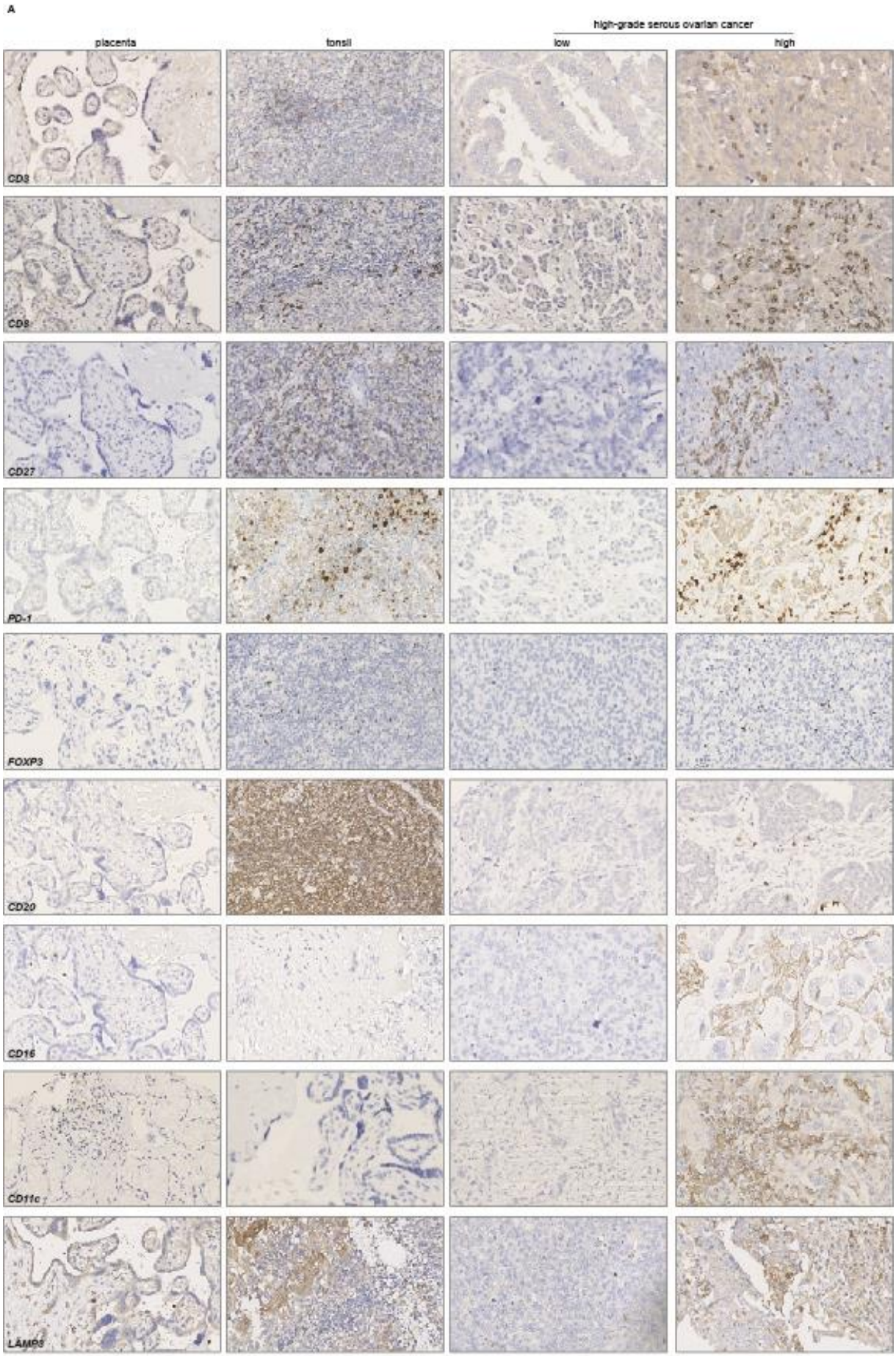
Percentage of positive immune cells depicted in figure 1A. for all four clusters. Significance between tumor cluster 1 and 2 was seen for the immune expression of ICOS$^+$CD8$^+$, ICOS$^+$CD4$^+$, PD1$^+$CD8$^+$, PD1$^+$CD4$^+$, GITR$^+$CD4$^+$ and TBET$^+$CD4$^+$ cells. *P<0.05, **P<0.01, ***P<0.001.

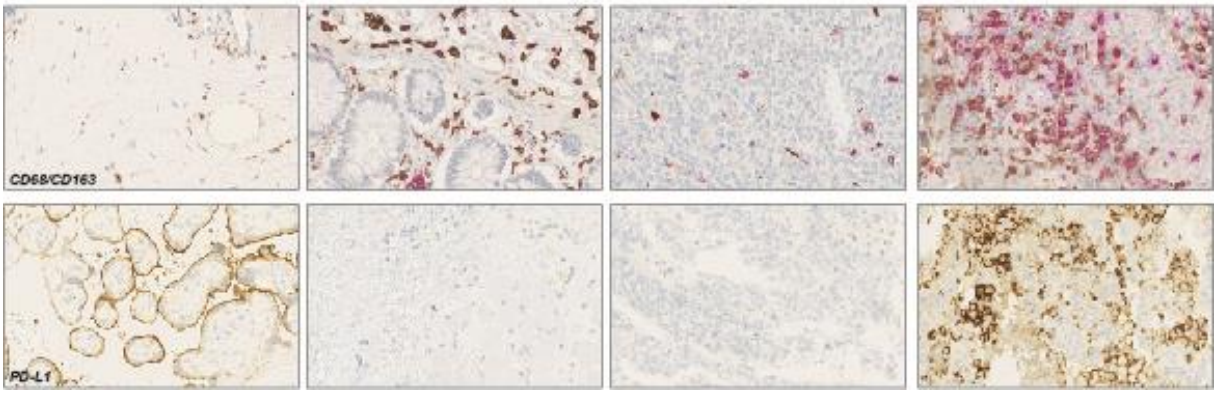


Supplementary figure S3. T cell differentiation is heterogeneous across tissue types
The heatmap displays clusters identified by single marker analysis. For each sample chemotherapy status is defined. The percentage of positive cells immune subset is displayed in the heatmap. Immune subsets are determined by co-expression of CD45RA, CCR7, CD27, and CD95 on $CD4^+$ and $CD8^+$ cells.



Supplementary figure S4. Epithelial CD8⁺ and PD-1⁺ cell density overtime in the pre- and post-chemotherapy cohort. Epithelial infiltration of CD8⁺ and PD-1⁺ cells in pre-chemotherapy tumors and post-chemotherapy tumors in the period 2002-2008 and 2008-2012. No difference in overall expression was observed between cohorts and time period. Density is defined as cells/mm².


A
placenta
tonsil
high-grade serous ovarian cancer
low
high
CD3
CD8
CD27
PD-1
FOXP3
CD20
CD16
CD11c
LAMP3



Supplementary figure S5. Examples of representative stainings of markers assessed by IHC. Representative samples of all markers assessed by IHC of low and high infiltrated HGSOC tissue is shown. In addition examples of tonsil and placenta control samples are included for all markers. Additional control tissues were included in the IHC analysis (*data not shown*).

Supplementary Table S1A. Patient characteristics FFPE tissue

Patient #	Age	FIGO stage	Histology	Tissue for analysis	CA125 pre-NACT	CA125 pre-surgery	Primary treatment	Surgical outcome	FU	DSS	PFS
172	80	IIIC	HGS	Tumor & TDLN	x	130	PDS	complete	NED	*alive at FU*	32
371	69	IIC	HGS	Tumor & TDLN	x	2373	PDS	complete	NED	*alive at FU*	10
302	76	IIIC	LGS	Tumor	x	234	PDS	incomplete	ED	1	x
173	55	IIIC	Carcino -sarcoma	Tumor & TDLN	x	968	PDS	complete	ED	*alive at FU*	20
291	49	IV	HGS	Tumor	2925	338	NACT	optimal	ED	*alive at FU*	12
286	75	IV	HGS	Tumor	471	27	NACT	complete	ED	*alive at FU*	8
254	54	IIIC	HGS	Tumor & TDLN	x	485	PDS	complete	NED	*alive at FU*	9
290	63	IIIC	Endometrioi d	Tumor	x	710	PDS	complete	NED	*alive at FU*	13
283	74	IIIC/ IV	HGS	Tumor	3830	1672	NACT	open/close	ED	13	X
299	73	IIIC	HGS	Tumor	10588	126	NACT	optimal	NED	*alive at FU*	12
324	78	IIIC	HGS	Tumor	1590	86	NACT	incomplete	ED	*alive at FU*	x
322	55	IIIC	HGS	Tumor	x	1010	PDS	complete	NED	*alive at FU*	10
200	63	IIIB	Serous borderline	Tumor & TDLN	x	308	PDS	optimal	NED	*alive at FU*	23
316	60	IV	HGS	Tumor	561	264	NACT	complete	ED	16	10
325	65	IIIC	HGS	Tumor	603	19	NACT	complete	ED	*alive at FU*	10
215	56	IIC	Endometrioï d	Tumor & TDLN (2x)	x	79	PDS	complete	NED	*lost to FU*	*lost to follo w-up*
304	50	IIIC	Endometrioi d + clearcell	TDLN	x	196	PDS	complete	ED	11	9
204	61	IIIC	HGS	TDLN	x	110	PDS	complete	ED	31	27
282	70	IIIC	HGS	TDLN (2x)	730	27	NACT	complete	NED	*alive at FU*	14
229	55	IIIC	HGS	TDLN (2x)	x	200	PDS	complete	NED	*alive at FU*	24
103	62	IIIC	HGS	PBMC (3x)	x	215	PDS	complete	ED	*alive at FU*	42
104	72	IIIC	HGS	PBMC (2x)	x	1771	PDS	complete	NED	*alive at FU*	60
110	60	IIIC	HGS + clearcell	PBMC (3x)	x	238	PDS	complete	NED	*alive at FU*	59
141	59	IIIC	HGS	PBMC (3x)	17776	20	NACT	complete	NED	*alive at FU*	51
147	64	IIIC	HGS	PBMC (3x)	x	1287	PDS	complete	ED	26	18
166	52	IIIC	HGS	PBMC (3x)	x	470	PDS	complete	ED	*alive at FU*	30
170	75	IIIC	HGS	PBMC (2x)	432	229	NACT	complete	ED	36	10

HGS: high-grade serous, **LGS:** low-grade serous
PDS (pre-chemotherapy): primary debulking surgery followed by adjuvant chemotherapy
NACT (chemotherapy 3x): neoadjuvant chemotherapy; 3 cycles carboplatin/paclitaxel chemotherapy followed by cytoreductive debulking surgery and adjuvant chemotherapy
PBMC: peripheral blood monobnuclear cell, **TDLN:** Tumor draining lymph node.
NED: no evidence of disease. **ED:** evidence of disease,
DSS: disease specific survival from first treatment in months,
PFS: progression free survival from surgery in months.
Surgical outcome:
Complete: no residual tissue after surgery ,
Optimal: residual tissue <1cm after surgery ,
incomplete: residual tissue > 1cm after surgery.

Supplementary Table S1B. Patient characteristics TMA

	PDS pre-chemotherapy (N=83)		**NACT post-chemotherapy 3x (N=79)**	
Age (mean SD)	65		66	
	N	**%**	**N**	**%**
Age				
<59	26	31.1	20	25.3
>59	57	68.7	59	74.7
Figo stage				
II	7	8.4	0	0
III	62	74.7	61	77.2
IV	14	16.9	18	22.8
Surgical outcome				
No residual tissue (complete)	37	44.6	25	31.6
≤1 cm residual tissue (optimal)	15	18.1	38	48.1
≥1 cm residual tissue (incomplete)	31	37.3	16	20.3
Chemotherapy				
No chemotherapy	5	6	0	0
Carboplatin	6	7.2	3	3.8
Cisplatin/Paclitaxel	1	1.2	0	0
Carboplatin/Paclitaxel	71	85.5	76	96.2

FIGO: International Federation of Gynaecology and Obstetrics.
PDS (pre-chemotherapy): Primary debulking surgery followed by adjuvant chemotherapy
NACT (chemotherapy 3x): 3 cycles of neoadjuvant chemotherapy followed by cytoreductive debulking surgery and adjuvant chemotherapy

Supplementary table 2A. Used antibodies flow cytometry

Antigen	Clone	Fluorophore	Vendor	Catalog no. (07-04-2019)
Lineage 1 (CD3, CD14, CD16, CD19, CD20, CD56)	SK7, 3G8, SJ25C1, L27, MφP9, NCAM16.2	FITC	BD Biosciences	340546
CD3	UCHT1	FITC	BD Biosciences	555332
CD3	OKT3	PE	Thermofisher Scientific (eBioscience)	12-0037-42
CD3	OKT3	PerCP-Cyanine5.5	Thermofisher Scientific (eBioscience)	45-0037-42
CD3	UCHT1	APC	Thermofisher Scientific (eBioscience)	17-0038-42
CD4	SK3	PE	Thermofisher Scientific (eBioscience)	12-0048-42
CD4	OKT4	PerCP-Cyanine5.5	Thermofisher Scientific (eBioscience)	45-0048-42
CD8a	RPA-T8	APC-eFluor780	Thermofisher Scientific (eBioscience)	47-0088-42
FOXP3	236A/E7	FITC	Thermofisher Scientific (eBioscience)	11-4777-41
CD14	61D3	APC	Thermofisher Scientific (eBioscience)	17-0149-42
CD1c	L161	PE-Cyanine7	Thermofisher Scientific (eBioscience)	25-0015-41
CD11c	BU15	APC-eFluor780	Thermofisher Scientific (eBioscience)	47-0128-41
CD45RA	HI100	APC	Thermofisher Scientific (eBioscience)	25-0458-41
CD45RO	UCHL1	PE-Cyanine7	BD Biosciences	560608
CD197 (CCR7)	150503	BV421	BD Biosciences	562555
CD27	L128	FITC	BD Biosciences	340424
CD28	28.2	PerCP-Cyanine5.5	Thermofisher Scientific (eBioscience)	45-0289-41
CD95	DX2	PE-Cyanine7	BD Biosciences	561636
CD279 (PD-1)	MIH4	FITC	Thermofisher Scientific (eBioscience)	11-9969-41
CD274 (PD-L1)	B7-H1	PE	Thermofisher Scientific (eBioscience)	12-5983-41
CD278 (ICOS)	DX29	BV421	BD Biosciences	562901
CD134 (OX40)	ACT35	PE-Cyanine7	BD Biosciences	563663
CD357 (GITR)	eBioAITR	PE-Cyanine7	Thermofisher Scientific (eBioscience)	25-5875-41
CD152 (CTLA4)	BNI3	APC	BD Biosciences	560938
CD123	7G3	PerCP-Cyanine 5.5	BD Biosciences	560904
HLA-DR	Tu39	BV421	BD Biosciences	564244
Ki-67	20Raj1	PE	Thermofisher Scientific (eBioscience)	12-5699-41
Tbet	4B10	eFluor660	Thermofisher Scientific (eBioscience)	55-5825-80
Eomes	WD1928	PE-Cyanine7	Thermofisher Scientific (eBioscience)	25-4877-41

Supplementary table 2B. Used antibodies TMA

Antigen	Clone	Dilution	Vendor	Catalog no. (07-04-2019)
CD3	F7.2.38	1:25	DAKO	A0452
CD8	C8/144B	1:25	DAKO	GA623
FoxP3	236A/E7	1:25	Abcam	ab20034
CD11c	EP13474	1:25	Abcam	ab52632
LAMP3	-	1:25	Sino Biological	10527-H08H
CD27	EPR8569	1:150	Abcam	ab192336
PD-1	NAT105	automatic stainer	Abcam	ab52587
PD-L1	E1L3N	automatic stainer	Cell Signaling Technology	13684
CD20	L26	1:200	DAKO	M0755
CD16	2H7	1:50	GeneTex	GTX75392
CD68	PG-M1	automatic stainer	DAKO	GA613
HLA-B/C	HC-10	1:50	prof. J. Neefjes, Dutch Cancer Institute	-
Cytokeratin	AE1/AE3	1:100	DAKO	M3515

Supplementary table S3A. median, minimum and maximum values figure 1A

	Cluster 1			Cluster 2			Cluster 3			Cluster 4		
Immune cell	Median	Min.	Max.	Median	Min.	Max.	Median	Min.	Max.	Median	Min.	Max,
CD8+CD45R0+	96,03	84,47	98,06	79,94	60,26	95,93	49,09	22,00	84,82	50,52	25,86	76,32
CD8+CD27+	37,89	28,72	64,38	35,42	23,67	51,93	77,10	30,97	91,86	50,47	18,00	71,12
CD8+CD28+	42,40	30,63	51,71	42,72	21,09	60,79	86,83	62,09	95,21	40,93	18,27	64,84
CD8+CCR7+	10,69	2,650	17,88	35,46	11,24	73,09	82,42	41,42	96,95	27,36	2,150	55,49
CD8+OX40+	1,190	0,490	7,840	1,025	0,260	1,770	0,760	0,180	4,550	2,590	1,170	22,77
CD8+GITR+	13,34	9,010	20,78	6,715	1,650	20,77	3,040	0,0	12,27	0,8100	0,340	14,04
CD8+ICOS+	76,25	56,48	82,25	14,84	4,790	65,13	7,900	4,390	35,22	0,0600	0,0	0,200
CD8+Ki67+	35,83	24,45	52,90	19,16	9,410	81,86	6,980	1,530	15,67	3,720	1,510	17,47
CD8+Tbet+	10,37	1,520	19,87	4,225	2,890	49,79	2,430	0,280	23,30	19,51	4,330	39,89
CD8+Eomes+	11,42	4,380	20,99	14,80	4,370	30,98	13,60	2,320	27,97	44,06	9,400	69,15
CD8+PD1+	65,06	34,00	83,43	15,39	10,00	43,42	9,490	2,400	21,56	4,280	0,0	16,23
CD4+CD45R0+	94,74	87,04	98,34	85,28	62,60	92,56	48,49	29,24	76,80	53,76	40,31	78,06
CD4+CD27+	40,89	30,32	56,30	51,79	22,21	71,57	80,74	36,93	90,32	79,28	35,29	95,26
CD4+CD28+	87,66	87,36	92,41	92,10	67,38	95,59	98,80	94,82	99,74	82,24	38,27	95,31
CD4+CCR7+	32,86	18,46	56,91	53,38	22,49	69,88	81,57	66,70	93,88	54,51	16,50	59,09
CD4+OX40+	16,19	13,13	31,04	6,575	1,090	12,65	1,980	0,780	6,640	3,480	0,700	16,45
CD4+GITR+	55,07	42,94	60,55	11,14	0,910	27,66	2,270	0,590	7,000	1,190	0,240	20,28
CD4+ICOS+	86,44	63,05	93,36	21,59	9,350	45,71	13,77	6,200	29,90	0,290	0,080	1,120
CD4+Ki67+	43,27	26,26	58,81	20,29	7,870	39,78	7,740	2,960	16,86	3,370	0,980	10,75
CD4+Tbet+	69,36	25,47	71,95	17,41	6,640	63,86	4,100	0,660	18,41	11,66	2,370	34,00
CD4+Eomes+	1,535	0,940	2,050	1,750	0,760	2,100	0,3400	0,100	1,300	9,860	1,770	40,81
CD4+PD1+	55,74	39,49	71,83	17,88	5,060	37,41	7,400	3,380	17,57	3,270	0,330	15,53
CD33+CD14+HLADR+	95,81	91,67	97,16	93,44	69,01	96,65	89,06	61,05	96,69	97,43	91,61	99,67
CD33+CD14+PDL1+	82,23	64,46	85,54	69,20	40,33	78,54	66,02	21,37	85,30	3,950	1,030	56,36
DC+CD1c+	6,965	0,190	12,10	4,325	2,080	14,35	16,42	3,860	27,06	57,23	15,57	82,49
DC+CD11C+	15,39	0,630	26,08	11,79	2,900	18,16	18,84	3,530	53,10	53,79	23,48	72,39
DC+CD123+	7,340	0,0	55,55	5,290	1,910	18,30	62,41	10,67	71,66	12,06	4,860	26,67
DC+PDL1+	15,52	15,07	42,35	22,98	17,77	54,72	5,330	0,7600	17,90	7,640	0,3500	24,34

Supplementary table S3B. P-values figure 1

Immune cel	cluster 1 vs cluster 2	cluster 1 vs cluster 3	cluster 1 vs cluster 4	cluster 2 vs cluster 3	cluster 2 vs cluster 4	cluster 3 vs cluster 4
CD8+CD45R0+	P > 0.05	**P<0.001**	**P<0.001**	**P<0.001**	**P<0.001**	P > 0.05
CD8+CD27+	P > 0.05	**P<0.001**	P > 0.05	**P<0.001**	P > 0.05	**P<0.001**
CD8+CD28+	P > 0.05	**P<0.001**	P > 0.05	**P<0.001**	P > 0.05	**P<0.001**
CD8+CCR7+	**P<0.01**	**P<0.001**	P > 0.05	**P<0.001**	P > 0.05	**P<0.001**
CD8+OX40+	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
CD8+GITR+	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
CD8+ICOS+	**P<0.001**	**P<0.001**	**P<0.001**	P > 0.05	**P<0.01**	P < 0.05
CD8+Ki67+	P > 0.05	**P<0.001**	**P<0.001**	**P<0.01**	**P<0.001**	P > 0.05
CD8+Tbet+	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	**P<0.01**
CD8+Eomes+	P > 0.05	P > 0.05	**P<0.001**	P > 0.05	**P<0.001**	**P<0.001**
CD8+PD1+	**P<0.001**	**P<0.001**	**P<0.001**	P > 0.05	P < 0.05	P > 0.05
CD4+CD45R0+	P > 0.05	**P<0.001**	**P<0.001**	**P<0.001**	**P<0.001**	P > 0.05
CD4+CD27+	P > 0.05	**P<0.001**	**P<0.001**	**P<0.001**	**P<0.001**	P > 0.05
CD4+CD28+	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	**P<0.001**
CD4+CCR7+	P > 0.05	**P<0.001**	P > 0.05	**P<0.001**	P > 0.05	**P<0.001**
CD4+OX40+	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
CD4+GITR+	**P<0.001**	**P<0.001**	**P<0.001**	P > 0.05	P > 0.05	P > 0.05
CD4+ICOS+	**P<0.001**	**P<0.001**	**P<0.001**	P > 0.05	**P<0.001**	**P<0.01**
CD4+Ki67+	P > 0.05	**P<0.001**	**P<0.001**	P > 0.05	P < 0.05	P > 0.05
CD4+Tbet+	**P<0.001**	**P<0.001**	**P<0.001**	**P<0.001**	P > 0.05	P > 0.05
CD4+Eomes+	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
CD4+PD1+	**P<0.001**	**P<0.001**	**P<0.001**	P > 0.05	P > 0.05	P > 0.05
CD33+CD14+HLADR+	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P < 0.05
CD33+CD14+PDL1+	P > 0.05	P > 0.05	**P<0.001**	P > 0.05	**P<0.001**	**P<0.001**
Lin-HLADR+CD1c+	P > 0.05	P > 0.05	**P<0.001**	P > 0.05	**P<0.001**	**P<0.001**
Lin-HLADR+CD11C+	P > 0.05	P > 0.05	**P<0.001**	P > 0.05	**P<0.001**	**P<0.001**
Lin-HLADR+CD123+	P > 0.05	**P<0.001**	P > 0.05	**P<0.001**	P > 0.05	**P<0.001**
Lin-HLADR+PDL1+	P > 0.05	P > 0.05	P > 0.05	**P<0.001**	**P<0.001**	P > 0.05

Supplementary table S3C. N-number, median, minimum and maximum values figure

Immune cell	Location	Cohort	N-number	Median	Minimum	Maximum
No. CD3+ cells/mm2	Tumor epithelium	pre-chemotherapy	75	18	0	1268
No. CD3+ cells/mm2	Tumor epithelium	chemotherapy	61	23	0	318
No. CD3+ cells/mm2	Stroma	pre-chemotherapy	30	129	8	731
No. CD3+ cells/mm2	Stroma	chemotherapy	40	134	2	1039
No. CD8+ cells/mm2	Tumor epithelium	pre-chemotherapy	69	20	0	2555
No. CD8+ cells/mm2	Tumor epithelium	chemotherapy	53	35	0	300
No. CD8+ cells/mm2	Stroma	pre-chemotherapy	57	194	1	3130
No. CD8+ cells/mm2	Stroma	chemotherapy	48	183	0	1008
No. CD27+ cells/mm2	Tumor epithelium	pre-chemotherapy	74	14	0	407
No. CD27+ cells/mm2	Tumor epithelium	chemotherapy	59	11	0	123
No. CD27+ cells/mm2	Stroma	pre-chemotherapy	49	131	0	588
No. CD27+ cells/mm2	Stroma	chemotherapy	50	133	0	1563
No. PD1+ cells/mm2	Tumor epithelium	pre-chemotherapy	67	26	0	496
No. PD1+ cells/mm2	Tumor epithelium	chemotherapy	52	23	0	885
No. PD1+ cells/mm2	Stroma	pre-chemotherapy	43	79	3	923
No. PD1+ cells/mm2	Stroma	chemotherapy	39	107	0	1085
No. FoxP3 cells/mm2	Tumor epithelium	pre-chemotherapy	73	3	0	145
No. FoxP3 cells/mm2	Tumor epithelium	chemotherapy	55	4	0	108
No. FoxP3 cells/mm2	Stroma	pre-chemotherapy	50	13	0	310
No. FoxP3 cells/mm2	Stroma	chemotherapy	43	21	0	365
No. CD20+ cells/mm2	Tumor epithelium	pre-chemotherapy	74	0	0	36
No. CD20+ cells/mm2	Tumor epithelium	chemotherapy	54	0	0	88
No. CD20+ cells/mm2	Stroma	pre-chemotherapy	48	0	0	161
No. CD20+ cells/mm2	Stroma	chemotherapy	42	6	0	269
No. CD16+ cells/mm2	Tumor epithelium	pre-chemotherapy	70	50	0	185
No. CD16+ cells/mm2	Tumor epithelium	chemotherapy	58	51	0	186
% CD16+ cells	Stroma	pre-chemotherapy	52	8	0	85
% CD16+ cells	Stroma	chemotherapy	68	5	0	63
No. CD11c+ cells/mm2	Tumor epithelium	pre-chemotherapy	70	2	0	95
No. CD11c+ cells/mm2	Tumor epithelium	chemotherapy	62	9	0	133

% CD11c+ cells	Stroma	pre-chemotherapy	59	0	0	35
% CD11c+ cells	Stroma	chemotherapy	74	3	0	60
No. LAMP3 cells/mm2	Tumor epithelium	pre-chemotherapy	76	2	0	239
No. LAMP3 cells/mm2	Tumor epithelium	chemotherapy	56	1	0	106
% LAMP3 cells	Stroma	pre-chemotherapy	46	3	0	43
% LAMP3 cells	Stroma	chemotherapy	63	3	0	25
No. CD68+ cells/mm2	Tumor epithelium	pre-chemotherapy	63	22	0	1250
No. CD68+ cells/mm2	Tumor epithelium	chemotherapy	42	11	0	268
% CD68+ cells	Stroma	pre-chemotherapy	34	3	0	30
% CD68+ cells	Stroma	chemotherapy	33	1	0	45
No. CD163+ cells/mm2	Tumor epithelium	pre-chemotherapy	62	64	0	1343
No. CD163+ cells/mm2	Tumor epithelium	chemotherapy	42	59	3	1051
% CD163+ cells	Stroma	pre-chemotherapy	34	14	2	85
% CD163+ cells	Stroma	chemotherapy	33	8	0	48
% PD-L1+ cells	Tumor epithelium	pre-chemotherapy	80	0	0	20
% PD-L1+ cells	Tumor epithelium	chemotherapy	74	0	0	5
% PD-L1+ cells	Stroma	pre-chemotherapy	80	1	0	70
% PD-L1+ cells	Stroma	chemotherapy	74	2	0	60

Supplementary table S3D. P-values figure 4A

Immune cells	cluster 1 vs cluster 2	cluster 1 vs cluster 3	cluster 1 vs cluster 4	cluster 2 cluster 3	cluster 2 vs cluster 4	cluster 3 vs cluster 4
CD8+ICOS+ GITR+	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
CD8+GITR+	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
CD8+ICOS+	**P<0.001**	**P<0.001**	**P<0.001**	P > 0.05	P > 0.05	P > 0.05
CD8+	**P<0.001**	**P<0.001**	**P<0.001**	P > 0.05	P > 0.05	P > 0.05
CD4+ICOS+ GITR+	**P<0.001**	**P<0.001**	**P<0.001**	P > 0.05	P > 0.05	P > 0.05
CD4+GITR	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
CD4+ICOS+	**P < 0.05**	**P<0.01**	**P<0.01**	P > 0.05	P > 0.05	P > 0.05
CD4+	**P<0.001**	**P<0.001**	**P<0.001**	P > 0.05	P > 0.05	P > 0.05
Treg+ICOS+ GITR+	**P<0.001**	**P<0.001**	**P<0.001**	**P<0.001**	**P<0.01**	P > 0.05
Treg+GITR+	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
Treg+ICOS+	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05	P > 0.05
Treg+	P > 0.05	**P<0.001**	**P<0.001**	**P<0.01**	P > 0.05	P > 0.05

Supplementary table S3E. N-numbers figure 5

	N-number PD-1+ cells	
	high	low
Epithelial PD-1+ cells all patients	39	79
Epithelial PD-1+ cells pre-chemotherapy	22	45
Epithelial PD-1+ cells post-chemotherapy	17	34
Stromal PD-1+ cells all patients	24	57
Stromal PD-1+ cells pre-chemotherapy	11	21
Stromal PD-1+ cells post-chemotherapy	13	25

	N-number PD-1+ cells and surgical outcome			
	high/ complete	low/ complete	high/ incomplete	low/ incomplete
Epithelial PD-1+ cells pre-chemotherapy	10	18	12	27
Epithelial PD-1+ cells post-chemotherapy	6	12	11	22
Stromal PD-1+ cells pre-chemotherapy	6	11	5	21

Supplementary table S3F. N-numbers figure 6

	N-number		
Immune cells	MHC-I neg.	MHC-I int.	MHC-I high
pre-chemotherapy epithelial PD-1+ cells	19	29	17
pre-chemotherapy epithelial CD-3+ cells	21	33	18
pre-chemotherapy epithelial CD8+ cells	20	30	17
pre-chemotherapy epithelial CD27+ cells	21	33	18
pre-chemotherapy epithelial FoxP3+ cells	21	32	17
pre-chemotherapy epithelial CD20+ cells	22	33	17
pre-chemotherapy stromal PD-1+ cells	13	14	14
pre-chemotherapy stromal CD-3+ cells	11	10	7
pre-chemotherapy stromal CD8+ cells	13	24	17
pre-chemotherapy stromal CD27+ cells	16	17	15
pre-chemotherapy stromal FoxP3+ cells	16	19	13
pre-chemotherapy stromal CD20+ cells	14	21	12
post-chemotherapy epithelial PD-1+ cells	17	29	1
post-chemotherapy epithelial CD-3+ cells	20	32	1
post-chemotherapy epithelial CD8+ cells	17	30	1
post-chemotherapy epithelial CD27+ cells	17	33	1
post-chemotherapy epithelial FoxP3+ cells	16	33	1
post-chemotherapy epithelial CD20+ cells	16	32	1
post-chemotherapy stromal PD-1+ cells	13	23	1
post-chemotherapy stromal CD-3+ cells	16	18	1
post-chemotherapy stromal CD8+ cells	17	26	1
post-chemotherapy stromal CD27+ cells	17	27	1
post-chemotherapy stromal FoxP3+ cells	14	23	1
post-chemotherapy stromal CD20+ cells	15	21	1

Table S3G: Multivariate Cox regression analyses of disease-specific survival

	HR	**P-value**	**95% CI**
Primary treatment (NACT)	1.041	0.894	0.575-1.885
Surgical result (residual tissue)	1.338	**<0.001**	1.165-1.536
FIGO stage	1.447	0.203	0.82-2.553
MHC-I tumor expression level	0.861	0.2	0.685-1.083
CD8 epithelium (highest tertile)	0.998	0.984	0.801-1.243
PD-1 epithelium (highest tertile)	0.826	**0.044**	0.685-0.995
PD-1 stroma (highest tertile)	1.067	0.448	0.902-1.263

Chapter 4

Prognostic image-based quantification of CD8CD103 T cell subsets in high-grade serous ovarian cancer patients

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Oncoimmunology 2021

Abstract

CD103-positive tissue resident memory-like $CD8^+$ T cells (CD8CD103 TRM) are associated with improved prognosis across malignancies, including high-grade serous ovarian cancer (HGSOC). However, whether quantification of CD8, CD103 or both is required to improve existing survival prediction and whether all HGSOC patients or only specific subgroups of patients benefit from infiltration, remains unclear. To address this question, we applied image-based quantification of CD8 and CD103 multiplex immunohistochemistry in the intratumoral and stromal compartments of 268 advanced-stage HGSOC patients from two independent clinical institutions. Infiltration of CD8CD103 immune cell subsets was independent of clinicopathological factors. Our results suggest CD8CD103 TRM quantification as a superior method for prognostication compared to single CD8 or CD103 quantification. A survival benefit of CD8CD103 TRM was observed only in patients treated with primary cytoreductive surgery. Moreover, survival benefit in this group was limited to patients with no macroscopic tumor lesions after surgery. This approach provides novel insights into prognostic stratification of HGSOC patients and may contribute to personalized treatment strategies in the future.

Introduction

High-grade serous ovarian cancer (HGSOC) is the most common histological subtype of epithelial ovarian cancer (EOC) and considered to originate from the fallopian tubes[1]. Advanced stage HGSOC has a poor prognosis, with a 5-year survival rate of 25-40%[2,3]. Current primary treatment consists of cytoreductive surgery and chemotherapy, in most cases carboplatin with paclitaxel[4]. Patients are either treated with primary debulking surgery followed by six cycles of adjuvant chemotherapy (PDS) or are initially treated with three cycles of neo-adjuvant chemotherapy, followed by interval debulking surgery and three additional cycles of adjuvant chemotherapy (NACT). Choice of treatment strategy is tailored for each individual patient. Patients are selected for PDS based on the estimation whether the entire tumor load can be removed during surgery, taking into account tumor location, presence of metastases and clinical condition of the patient. If not feasible, NACT is used to reduce tumor burden prior to interval debulking. The most important prognostic factors are primary treatment strategy (PDS or NACT) and surgical outcome. Surgical outcome is defined as complete (no residual macroscopic tumor tissue after surgery), optimal (residual tumor lesions <1cm after surgery) or incomplete (residual tumor lesions >1cm after surgery). Although up to 75% of all HGSOC patients initially have a favorable response to primary treatment, comprising chemotherapy and surgery, most patients relapse within 2-years, with a median progression free survival (PFS) of 12 months[5].

It has been well established that the presence of tumor infiltrating lymphocytes (TILs) represents an additional favorable prognostic indicator in many solid tumors including HGSOC[6–8]. In particular, a specific subset of $CD8^+$ T cells, known as tissue resident memory-like T cells (TRM), is associated with prognostic benefit in HGSOC[9–11]. TRM are characterized by the expression of CD103, also known as integrin αEβ7 (*ITGAE*). CD103 interacts with E-cadherin, often expressed on epithelial tumor cells, thereby facilitating the interaction between the $CD8^+$ T cells and the tumor epithelium. CD103 is therefore often used to distinguish intra-epithelial and stromal $CD8^+$ T cells[12]. Functional studies have shown that CD8CD103 TRM cells can secrete pro-inflammatory cytokines such as Interferon-γ (IFNγ), tumor necrosis factor-α, and express cytotoxic molecules granzyme A and B[13,14]. In addition, as previously demonstrated by our group,

CD8CD103 TRM cells also produce CXCL13, a crucial chemokine involved in the development of tertiary lymphoid structures(TLS)[15].

In order to translate CD8CD103 TRM quantity and location into a diagnostic tool, the development of immune scores are needed. However, manual TIL quantification by pathologists is hampered by interobserver variability and is time consuming[16]. The rise of digital pathology, including image-based quantification and machine learning algorithms, provides an opportunity to overcome these limitations. Machine learning algorithms apply statistical methods to process data and have shown to be reproducible and reliable for analysis of tissue composition in cancer[17]. The deep characterization of the tumor microenvironment through spatial analysis and multiplexing, makes image-based quantification an efficient tool to extract comprehensive information on biomarker expression levels, co-localization, and compartmentalization[18,19]. Horeweg et al., demonstrated successful application of image-based CD8CD103 TRM quantification in early-stage endometrial cancer, by demonstrating concordance between automatic machine learning and assessment by expert pathologists. The study showed greater sensitivity of automatic machine learning compared to manual quantification[20].

In this study, we applied the same innovative image-based quantification technique as Horeweg et al. to address the questions; whether CD8, CD103 or both markers need to be quantified for optimal prognostication in HGSOC; and whether all HGSOC patients or only specific subgroups of patients benefit from infiltration. We demonstrate that the prognostic benefit of CD8CD103 TRM infiltration in HGSOC is restricted to PDS treated patients with a complete debulking.

Methods

Patient selection

A recoded database was created containing information on clinic-pathological characteristics and follow-up of patients diagnosed with advanced stage HGSOC at the University Medical Center Groningen (Groningen, The Netherlands) and Isala hospital Zwolle (Zwolle, The Netherlands) between January 2008 and January 2017. Patients were staged according to international Federation of Gynecology and Obstetrics (FIGO) criteria 2014 based on World Health Organization (WHO) guidelines.

One of the gynecologic pathologist (EWD, JB, NW, HH) confirmed the histological subtype based on morphology, and when available P53 immunohistochemistry staining. Subsequently, the presence of tumor tissue was confirmed on H&E slides and representative locations with tumor tissue were selected for tissue microarray (TMA) construction. Patients were included if sufficient formalin-fixed paraffin embedded (FFPE) ovarian or omentum tumor tissue was available. Tissue was obtained either from primary debulking surgery or interval debulking surgery.

From a total of 409 patients that were screened, 268 patients (65.5%) were included, follow-up survival data was available for 240 included patients of which 2 patients had an unknown surgical outcome. Of the 141 excluded patients follow up survival data was available for 92 of the patients (Supplementary figure 1). The main reason for exclusion was the unavailability of viable tumor tissue. Approximately 80% of the excluded patients were primarily treated with NACT(Supplementary table 1). Since these excluded NACT patients might represent 'best responders to chemotherapy', we analyzed overall survival (OS) in the exclusion versus inclusion cohort. We observed a prolonged survival for the included NACT patients compared to the excluded NACT patients (Supplementary figure 2b). Within PDS treated patients, no difference in OS was observed between included or excluded patients (Supplementary figure 2a).

In total (n=268), FFPE tissue of 191 advanced stage HGSOC patients at the UMCG and 77 HGSOC patients at the Isala was available for the construction of a TMA. For 210 patients both infiltration density and survival data was available, of which 2 patients had an unknown surgical outcome (Supplementary figure 1). OS was calculated from the date of initial treatment (either primary surgery or first cycle of neo-adjuvant chemotherapy) and was last updated in July 2020.

Tissue Micro-Array

Triplicate cores with a diameter of 1 mm were taken from each FFPE block and placed in a recipient block using a tissue microarrayer (Beecher instruments, Silver Spring, USA). Both normal and tumor tissue were included as orientation cores and controls. From each TMA block, 3 µm thick sections were cut and applied to APES-coated slides (Starfrost, Braunschweig, Germany).

Immunohistochemistry staining

FFPE slides were de-paraffinized and rehydrated in graded ethanol. Antigen retrieval was initiated with a preheated 10 mM citrate buffer (pH=6). Endogenous peroxidase was blocked with a 0.3% H_2O_2 solution (0.5mL 30% H202 in 50mL PBS) for 30 minutes at room temperature. The primary antibodies against CD8 (1:50, Agilent/Dako, M710301-2) and CD103 (1:200,

CD103; ab129202) were diluted in phosphate buffered saline (PBS)(PBS + 1% BSA + 1% AB serum; total 80 µL) and, slides were incubated overnight at 4°C. Next, the slides were incubated with two secondary antibodies, first with envision+/HRP anti-rabbit (2 drops, K400311-2P), followed by secondary antibody immPRESS-AP mouse (MP-5402-15), both for 30 min at room temperature. For visualization, StayYellow/HRP (Abcam, ab169561) and Fast Red Substrate kit (Abcam, ab64254) were used according to manufacturers' instructions. Appropriate washing steps with PBS, tris-buffered saline with 0,1% Tween and demi water were performed between incubation steps. Sections were mounted with Eukitt quick-hardening mounting medium (Sigma Aldrich, Steinheim, Germany), and scanned on a Hamamatsu digital slide scanner (Hamamatsu photonics, Hamamatsu, Japan). Representative staining images are depicted in supplementary figure 3.

Image-based quantification of CD8CD103 immune cell subsets

All digital slides were reviewed by two pathologists (DL and VHK) and spots with staining artefacts, folds or less than 50% viable tissue / core were excluded from analysis. The digital image analysis was carried out using HALO digital image analysis software version v3.0.311.167 (Indica Labs, Corrales, NM, USA). Specifically, TMA slides were de-arrayed into individual spot images of each tissue sample linked to clinical annotations. To localize and quantify tumor and stroma tissue, a deep neural network algorithm was trained using the Deep Net architecture. Necrosis, erythrocyte aggregates and glass background were excluded. Graphical overlays were generated for each tissue class and the classification accuracy was reviewed. The total area of each tissue class was quantified in mm^2. Cell detection, segmentation and staining quantification for Nuclei (Hematoxylin, RGB 57, 49, 137), CD8 (Fast Red, RGB 203, 64, 122) and CD103 (StayYellow, RGB 216, 173, 81) were performed in the tumor and stromal compartment. CD8 and CD103 were classified as positive if staining intensity in the cytoplasmic compartment exceeded internal controls (non-immune cells in same tissue) as validated by pathologist review. The total tissue area in the tumor and stromal compartment and the absolute and % number of CD8 and CD103 single and double-positive positive cells were recorded (Figure 1). CD8 and CD103 infiltration density (marker-positive cells / mm^2) was calculated across all cores of each individual case and analyzed with clinicopathological parameters.

Statistical analysis

Statistical analyses were performed using IBM SPSS Statistics for Windows, version 23 (IBM Corp., Armonk, N.Y., USA) and R (version 3.6.2). For analysis, immune cell densities were log2 transformed. Clustering of cases was done by hierarchical clustering using Ward's minimum variance method in R using package pheatmap (https://cran.r-

project.org/web/packages/pheatmap/index.html). Correlations between CD8CD103 TRM cells and clinical and histopathological variables were analyzed using Multiple regression analysis in SPSS. Independent prognostic value of CD8CD103 TRM cells was analyzed using Multivariate Cox analysis in SPSS. Analyses of OS as a function of immune cell density were performed by Cox proportional hazards models in R using packages RMS (https://cran.r-project.org/web/packages/rms/index.html) and survival (https://cran.r-project.org/web/packages/survival/index.html), and plotted using package ggPlot2 (https://cran.r-project.org/web/packages/ggplot2/index.html). Proportionality of hazards was confirmed by scaled Schoenfeld residuals. Exploratory analysis of the optimal cut-off was determined in R using package Survminer https://cran.r-project.org/web/packages/survminer/index.html). Survival curves were plotted in R using Survminer by using the Kaplan-Meier method. A p-value of <0.05 was used as cut-off for significance.

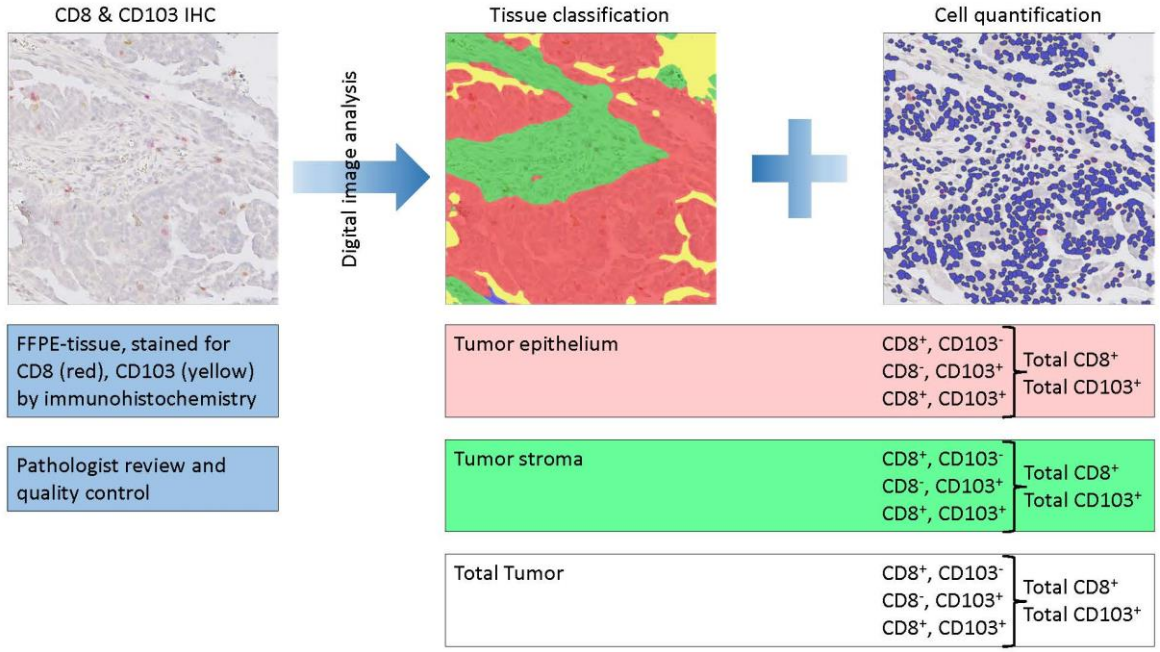

Figure 1. Schematic illustration of CD8CD103 quantification method. A digital image of CD8CD103 multiplex immunohistochemistry stained tissue was analyzed using a deep neural network algorithm, trained to distinguish the epithelial and stromal compartments. Stromal and epithelial compartments were combined to assess the unsegmented tissue (intratumoral). Quantification of $CD8^+CD103^-$ (single CD8) and $CD8^-CD103^+$ (single CD103) and double positive $CD8^+CD103^+$ (CD8CD103 TRM) cells were recorded. Single CD8 and CD103 infiltration density (marker-positive cells / mm2) was calculated across all cores of each individual case. All scores were integrated into 15 endscores.

Ethical review

Patient data were retrieved from the institutional database into a new recoded database, in which patient identity was protected by unique patient codes. According to Dutch law the institutional review board approved the use of the no-objection procedure for further-use biobank and databank(METc 2018.543).

Results

Cohort description

In total 268 advanced stage HGSOC patients were included. Patient characteristics from the two participating centers were compared and no significant difference was observed for FIGO stage, primary treatment and chemotherapy regimen (table 1). OS did not differ between the two cohorts (p=0.15; *data not shown*). *BRCA*-testing for EOC has only become standard of care since 2019 and is therefore largely unknown in our cohort and not compared for both centers. Based on the similar characteristics, both hospital cohorts were subsequently analyzed as one group.

Since patients are selected for primary treatment strategy (PDS or NACT) based on tumor burden, tumor location and health status, and therefore not comparable, the effect on OS was assessed independently for both patient groups. Additionally, we corrected for surgical outcome, since this is the main prognostic factor in HGSOC patients. Indeed, survival analysis revealed a significant benefit of the extent of cytoreductive surgery in PDS patients with survival outcomes of 58, 40 and 29 months in patients with a complete debulking versus an optimal or incomplete debulking respectively ($p<0.01$). Additionally, optimally debulked PDS patients had a significantly better survival than incompletely debulked patients ($p<0.01$). In NACT patients, patients with a complete debulking had a significantly better survival outcome as compared to patients with an optimal or incomplete debulking of 39, 29 and 27 months, respectively ($p<0.01$) (Supplementary figure 4, Supplementary table 3).

Table 1. Patient characteristics inclusion cohort

			UMCG (N=191)		Isala (N=77)		P-value
			N	%	N	%	
Mean age at diagnosis			65		63		
FIGO stage [a]	IIB/IIC		9	4.7	4	5.2	0.89
	III		142	74.3	58	75.3	
	IV		40	20.9	14	18.2	
	Unknown		0	0.0	1	1.3	
BRCA status	BCRA1/ BRCA2 mutation		12	6.3	12	15.6	N/A
	No BCRA mutation		74	38.7	12	15.6	
	Unknown		105	55	53	68.8	
Primary treatment	PDS	Complete	61	68.5	20	58.8	0.53
		Optimal	12	13.5	5	14.7	
		Incomplete	16	18.0	7	2.4	
		Unknown	0	0	2	5.9	
	NACT	Complete	34	33.3	23	54.8	0.01
		Optimal	39	38.2	9	21.4	
		Incomplete	29	28.4	9	21.4	
		Unkown	0	0	1	2.4	
NACT regime	Carboplatin/Paclitaxel		97	95.1	40	95.2	0.97
	Other/Unknown		5	4.9	2	4.8	
AC regime	Carboplatin/Paclitaxel		153	80.1	67	87.0	0.35
	No chemotherapy		13	6.8	2	2.6	
	Other/Unknown		25	13.1	8	10.4	
Disease status [a]	Evidence of disease		142	74.3	49	63.6	0.01
	No evidence of disease		27	14.1	15	19.5	
	Progressive disease during primary treatment		7	3.7	11	14.3	
	Unknown		15	7.9	2	2.6	

FIGO: Fédération Internationale de Gynécologie et d'Obstétrique.
PDS: Primary debulking surgery followed by 6 cycles of adjuvant chemotherapy; **NACT:** 3 cycles of neo-adjuvant chemotherapy. followed by an interval debulking and 3 cycles adjuvant chemotherapy.
Complete: all visible tumor lesions were removed; **Optimal**: tumor lesion left <1cm;
Incomplete: tumor lesions left >1cm.

[a] Chi-square p-value excluded "Unknown/missing".

Patterns of infiltration of the CD8CD103 immune cell subsets

Infiltration of three immune cell subsets was assessed; $CD8^+CD103^-$ (single CD8), $CD8^-CD103^+$ (single CD103) and $CD8^+CD103^+$ TRM cells (CD8CD103 TRM) in different locations; the epithelium and stromal compartments (Figure 1)[20]. Hierarchical clustering revealed that patients were clustered together based on infiltration of the various cell subsets, independent of location (Figure 2a). In addition, there was apparent heterogeneity in the degree of single CD8, single CD103 and CD8CD103 TRM infiltration with a subgroup of patient samples infiltrated by single CD8 cells or single CD103 cells, but not CD8CD103 TRM cells. By contrast, most patient samples with a high level of CD8CD103 TRM infiltration were also characterized by a strong infiltrate of CD8 and CD103 single positive cells (Figure 2a). Multiple regression analysis revealed no significant association of FIGO stage, treatment strategy, or surgery outcome with any of the clusters or cell subsets (Figure 2a). Finally, multiple regression analysis of histopathological markers determined during diagnostic workup (p53, p16, PAX8, WT1 and CK7) revealed no particular association with the CD8CD103 TRM immune clusters (Figure 2b).

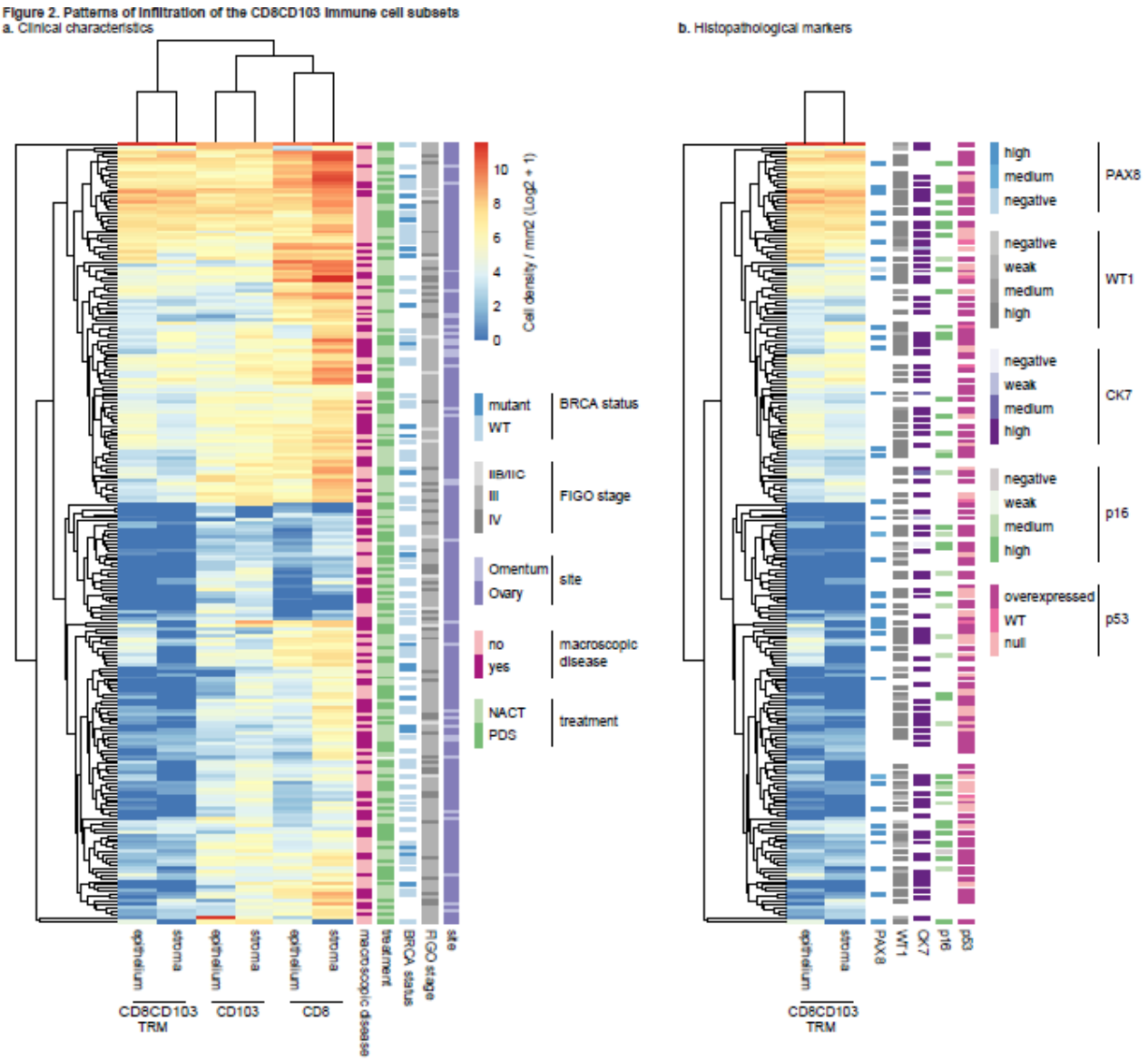

Figure 2. Patterns of infiltration of the CD8CD103 immune cell subsets. A, Heatmap displaying infiltration of the CD8CD103 immune cell subsets in the epithelium and stromal compartment. Hierarchical cluster analysis of all samples displayed three main clusters based on immune cell population; $CD8^{+}CD103^{+}$ (CD8CD103 TRM); $CD8^{-}CD103^{+}$ (single CD103) and $CD8^{+}CD103^{-}$ (single CD8). For each sample clinical characteristics are displayed including BRCA-status, FIGO-stage, site of tumor material collection, presence of macroscopic disease after surgery and primary treatment strategy. **B,** Heatmap of the CD8CD103 TRM immune cell cluster determined in figure 1A, displaying the analysis of histopathological markers determined during diagnostic workup including p53, PAX8, WT1 and CK7.

Prognostic benefit of stromal and epithelial CD8CD103 TRM infiltration

To determine which immune cell subset contributed to increased survival of the complete HGSOC patient population, we analyzed hazard ratios for all cell subsets in both the epithelial and stromal compartment (Figure 3a). Only CD8CD103 TRM in the epithelium were associated with improved survival (HR: 0.87, p=0.056 and Figure 3a

and b). Accordingly, exploratory analysis of survival at an optimal cut-off (top 15%) revealed a clear survival benefit for patients with high tumor epithelial CD8CD103 TRM infiltration (Figure 3c). In line with previous publications[21], we also assessed the survival benefit using the highest tertile for cut-off (Supplementary table 4), which revealed a survival benefit for patients with tumor epithelial high CD8CD103 TRM infiltration (p=0.01, Figure 3d).

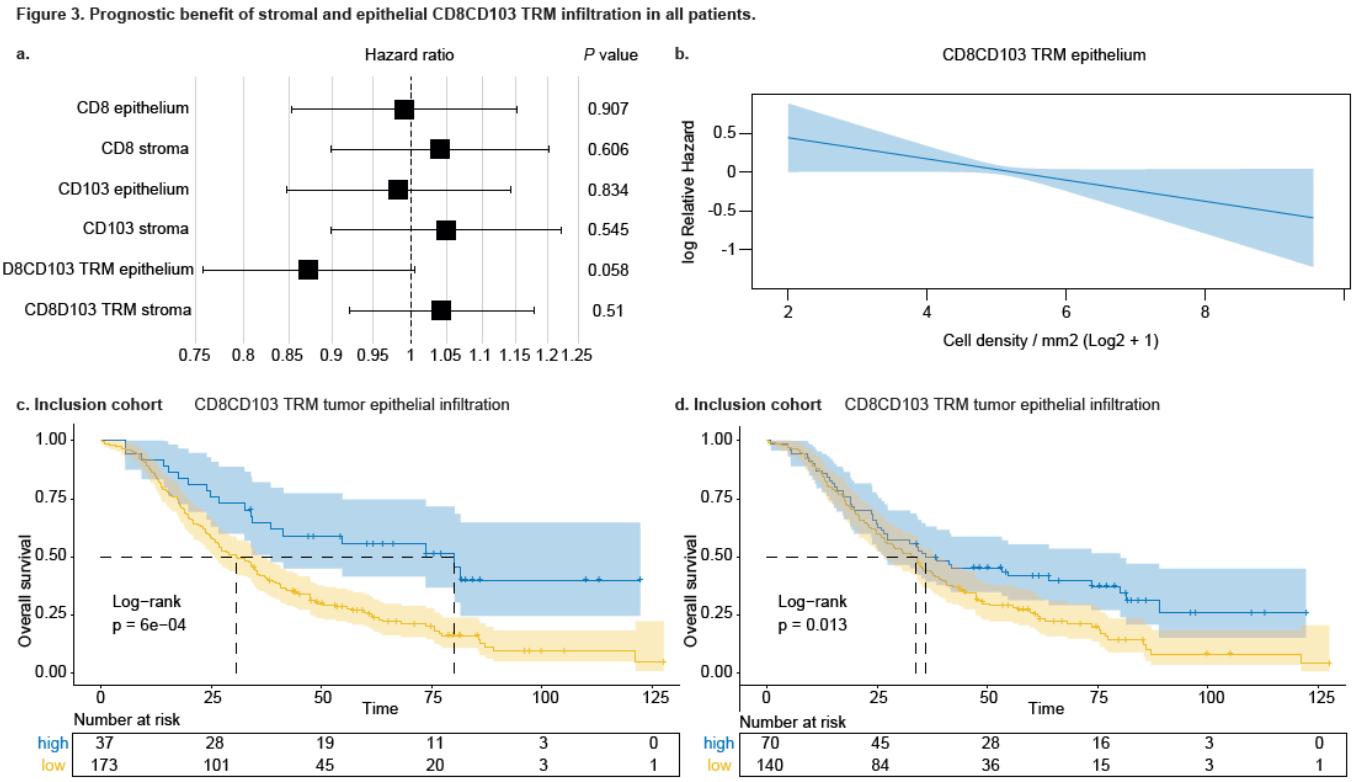

Figure 3. Prognostic benefit of stromal and epithelial CD8CD103 TRM infiltration in all patients. A, Forest plot of hazard ratios displaying stromal and epithelial infiltration of the three main CD8CD103 immune cell subsets; single CD8, single CD103 and CD8CD103 TRM. Only, epithelial CD8CD103 TRM infiltration is associated with improved survival. **B,** Plot showing hazard ratio for overall survival (OS) according to log2 transformed density of intra-epithelial CD8CD103 TRM cells. **C,** OS was determined in patients with high versus low epithelial CD8CD103 TRM infiltration based on the optimal cut-off (p<0.01). Survival differences were determined by a log-rank test. Numbers at risk are specified in the figure. **D,** OS was determined in patients with high versus low epithelial CD8CD103 TRM infiltration based on the highest tertile (p=0.01). Survival differences were determined by a log-rank test. Numbers at risk are specified in the figure.

Next, we explored survival in PDS and NACT patients as independent groups, since these two primary treatment strategies are not directly comparable. We corrected for surgical outcome through comparison of patients with no macroscopic lesions after surgery (complete debulking) and patients with macroscopic tumor lesions after surgery (optimal/incomplete debulking). To allow for sufficient numbers of patients in the sub analysis, we chose the highest tertile as cut-off. In the PDS cohort, patients with no macroscopic tumor lesions after surgery and high CD8CD103 TRM infiltration in the tumor epithelium or stroma were characterized by a significantly longer survival than patients with no macroscopic tumor lesions and low CD8CD103 TRM infiltrate (Figure 4a, 5 year survival 83% versus 52%; p=0.03 and Figure 4b, 5 year survival 77% versus 54%; p=0.01, respectively). In the NACT cohort, there was no effect of CD8CD103 TRM infiltration on OS in patients with and without macroscopic tumor lesions after surgery in stroma or tumor epithelium (Figure 4c, p=0.77 and Figure 4d, p=0.32).

Prognostic benefit of CD8CD103 TRM cell infiltration in unsegmented tissue

The pipeline used in the current study leverages both tissue segmentation and cell identification using machine learning algorithms. We next evaluated whether analysis of unsegmented tissue would provide comparable prognostic benefit and potentially accelerate future clinical workflows. Hereto, we analyzed survival of patients stratified by single CD8, single CD103 or CD8CD103 TRM in the total patient cohort. Only CD8CD103 TRM was associated with survival benefit when analyzing unsegmented tissue (p=0.01) (Figure 5a). In addition, neither total CD8 (HR 1.02 [0.92-1.1], p=0.76) nor total CD103 (HR 0.92 [0.80-1.1], p=0.23) were associated with improved survival. Analysis by log-rank test using either the optimal cut-off or the top tertile confirmed prognostic benefit for highly infiltrated patients (Figure 5c and 5d, respectively). Exploratory sub-analysis of CD8CD103 TRM in the individual patient groups again revealed the restriction of prognostic benefit to PDS patients with no macroscopic tumor tissue (Figure 5e).

Figure 4. Prognostic benefit of stromal and epithelial CD8CD103 TRM infiltration in patient subgroups.

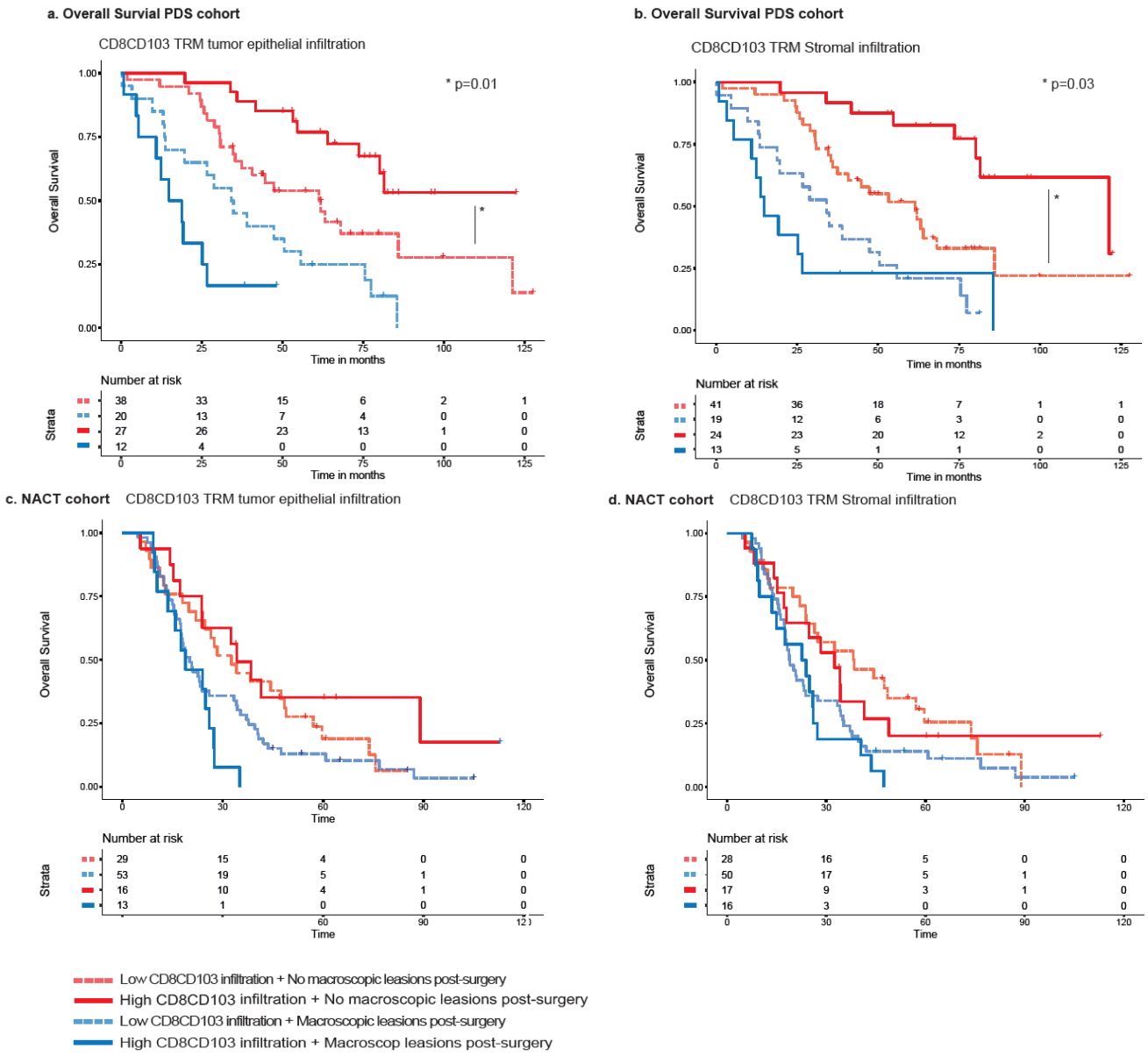

Figure 4. Prognostic benefit of stromal and epithelial CD8CD103 TRM infiltration in patient subgroups. (A-D), Overall survival (OS) differences were determined by Kaplan Meier analysis. Patients were stratified to high or low CD8CD103 TRM infiltration in the epithelial and stromal compartment using the highest tertile cut-off. Number at risk is specified in the figure. **A,** High versus low epithelial CD8CD103 TRM infiltration PDS patients with no macroscopic lesions after surgery (p=0.01) and with macroscopic lesions after surgery (p=0.06) **B,** High versus low stromal CD8CD103 TRM infiltration PDS patients with no macroscopic lesions after surgery (p=0.03) and with macroscopic lesions after surgery (P=0.428) **C,** High versus low epithelial CD8CD103 TRM infiltration NACT patients with no macroscopic lesions after surgery (p=0.32) and with macroscopic lesions after surgery (p=0.13). D, High versus low stromal CD8CD103 TRM infiltration NACT patients with no macroscopic lesions after surgery (p=0.77) and with macroscopic lesions after surgery (p=0.42).

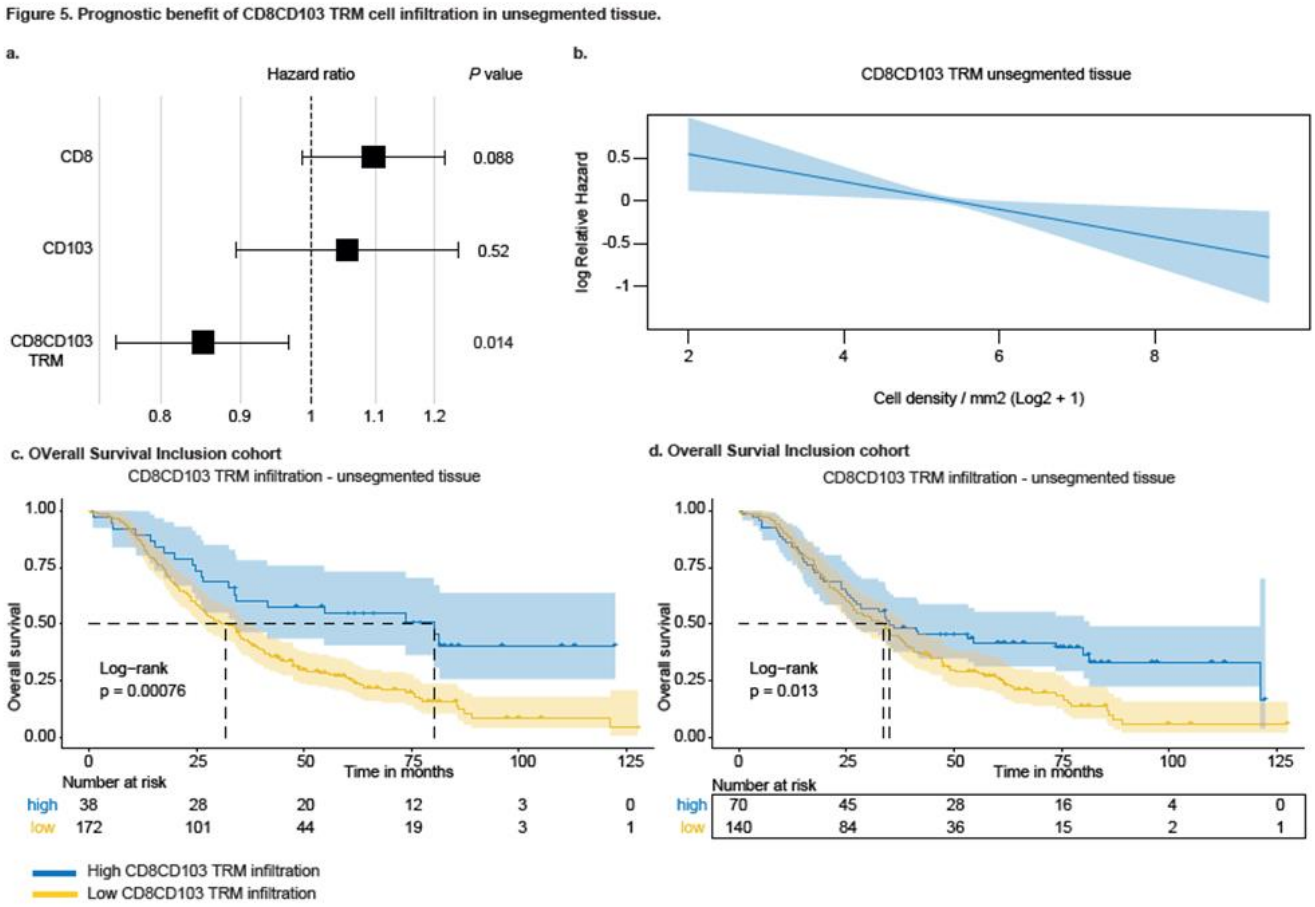

Figure 5E-F. Prognostic benefit of CD8CD103 TRM cell infiltration in unsegmented tissue. **(A-F),** Displays analysis of infiltration in unsegmented tissue of the CD8CD103 immune cell subsets. **A,** Forest plot of hazard ratios displaying infiltration of the three main CD8CD103 immune cell subsets; single CD8, single CD103 and CD8CD103 TRM. Only, CD8CD103 TRM infiltration is associated with improved survival (p=0.014). **B,** Plot showing hazard ratio for overall survival (OS) according to log2 transformed density of CD8CD103 TRM cells. **C,** OS was determined in patients with high versus low CD8CD103 TRM infiltration based on the optimal cut-off (p<0.01). Survival differences were determined by a log-rank test. Numbers at risk are specified in the figure. **D,** OS was determined in patients with high verus low CD8CD103 TRM infiltration in unsegmented tissue based on the highest tertile (p=0.01). Numbers at risk are specified in the figure.

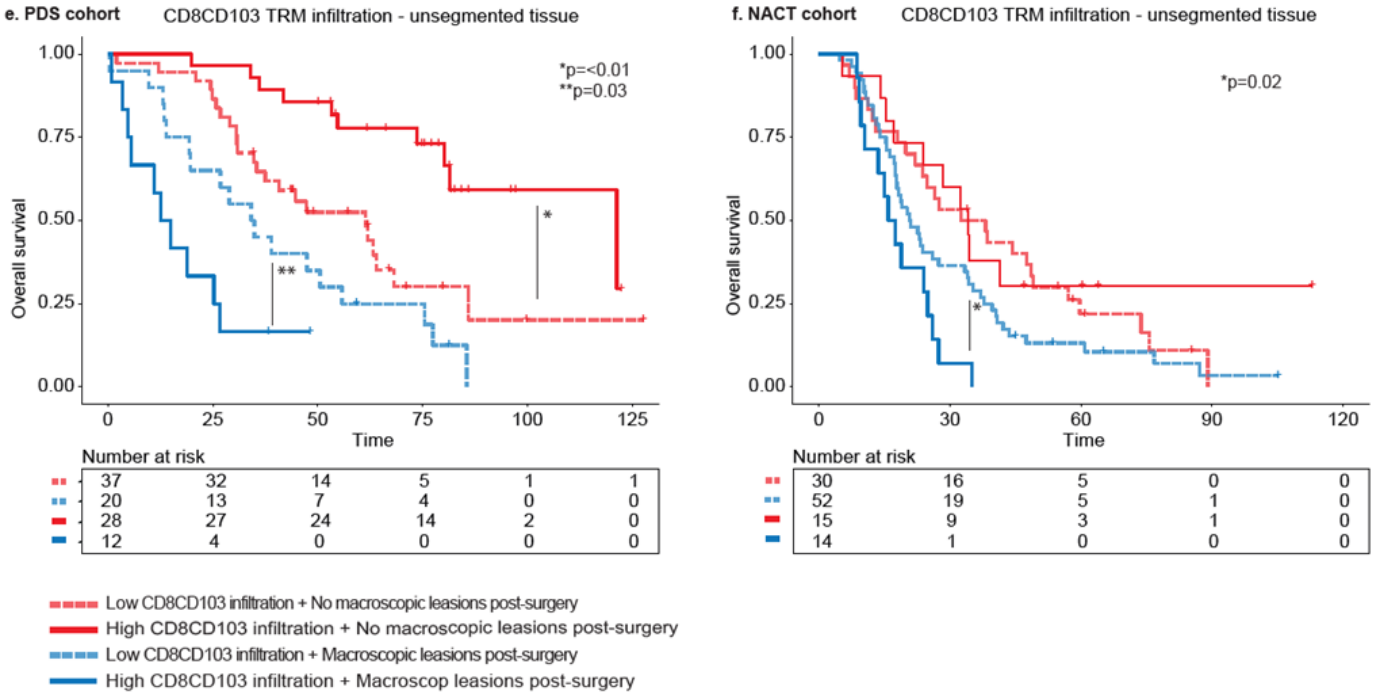

Figure 5E-F. Prognostic benefit of CD8CD103 TRM cell infiltration in unsegmented tissue. **(E-F),** Survival differences were determined by Kaplan Meier analysis. Patients were stratified to high or low CD8CD103 TRM infiltration in unsegmented tissue using the highest tertile cut-off. Number at risk is specified in the figure. **E,** High versus low CD8CD103 TRM infiltration PDS patients with no macroscopic lesions after surgery (p=<0.01) and with macroscopic lesions after surgery (p=0.03). **F,** High versus low CD8CD103 TRM infiltration NACT patients with no macroscopic lesions after surgery (p=0.59) and with macroscopic lesions after surgery (p=0.02).

Discussion

This study applies a digital quantification method[20], employing deep neural networks for tissue segmentation, to determine the infiltration patterns of single CD8, single CD103 and CD8CD103 TRM in HGSOC and to investigate the impact of the spatial distribution of immune cells in the tumor microenvironment on clinical outcomes. We demonstrate that high CD8CD103 TRM infiltration is associated with improved survival in HGSOC patients, however, this survival benefit is restricted to completely debulked PDS patients.

In line with previously published work, PDS patients with a complete debulking had the longest OS (±58 months), followed by optimally debulked PDS patients and completely debulked NACT patients (both ±40 months)[2122]. Importantly, the ~50% 5-year OS in PDS patients treated between 2008-2017 in this study was also comparable to other published data demonstrating a 44-56% 5-year OS in PDS patients treated between 2006-2013[22]. In our NACT cohort, time to recurrence and OS were approximately 13 and 31.8 months, which is a slightly inferior outcome than published by Cobb et al., who demonstrated a PFS of 16.4 and an OS of 48.2 months. However, in the recent analysis by Cobb et al., HGSOC patients were not randomly selected as they were matched to the investigated low-grade serous ovarian cancer patients. Hence, approximately two third of the NACT patients had no tumor lesions post-surgery[23], whereas in our study only one third of the NACT patients had a complete interval debulking surgery.

In general, EOC is characterized by a relatively low mutational burden and low numbers of TILs compared to e.g. melanoma, and lung cancer[24,25]. The present cohort confirms the overall low number of TILs in HGSOC with only 15% of the patients having high CD8CD103 TRM infiltrate, based on the optimal cut-off. . Our results on the restricted prognostic benefit of CD8CD103 TRM to completely debulked PDS patients are in line with Zhang et al, who also reported prognostic benefit of TILs related to surgical outcome in ovarian cancer[26].Why only this small subgroup of patients benefits from high CD8CD103 TRM infiltration remains unclear. Hypothetically, in patients with macroscopic tumor lesions after surgery, the remaining tumor load could exploit immune escape mechanisms, thereby suppressing CD8CD103 TRM activity. In NACT patients, no clear survival benefit was observed for patients with high TRM infiltration

in either the stromal or epithelial compartment, which is concordant with our previous work[21]. The absence of prognostic benefit of TILs in NACT patients has been observed in our previous research and could be explained by reduced MHC-I expression resulting in lack of CD8 T cell activation and reduced immunogenicity[27]. Indeed, chemotherapy has been associated with MHC-I down regulation in cancer[28].

As expected, CD8CD103 TRM quantification in the tumor epithelium had the strongest predictive value. However, not only epithelial but also stromal CD8CD103 TRM infiltration was predictive for improved survival. Since analysis of TMA slides provide a two dimensional assessment of tissue architecture, it cannot be excluded that the stromal CD8CD103 TRM were not still located in close proximity to the tumor epithelium just below or above the cross-section of the assessed TMA-slide. Furthermore, a fraction of the stromal CD8CD103 TRM could also represent bystander tissue resident memory T cells (*bystander*-TRM). *Bystander*-TRM are tumor-unspecific T cells, residing in lymphoid and non-lymphoid tissues and can contribute to the anti-tumor immune response via the delivery of common adjuvant viral peptides, resulting in the recruitment and accumulation of immune cells such as $CD8^+$ T cells and NK cells[29].

In unsegmented tissue, CD8CD103 TRM were only associated with improved survival in PDS patients with no macroscopic tumor tissue remaining after debulking surgery and seemed equally prognostic compared to assessment of the tumor epithelium compartment alone. Consequently, assessment of CD8CD103 TRM in unsegmented tissue, and not in individual compartments, could potentially accelerate future clinical workflows, increasing clinical applicability. Of note, an inverse correlation was observed in unsegmented tissue of both PDS and NACT patients with macroscopic lesions after surgery; low infiltration had a better survival compared to high infiltration. In the PDS cohort, the group with low infiltration consisted out of 12 optimal and 8 incomplete surgeries compared to 3 optimal and 9 incomplete in the highly infiltrated group, providing an explanation for this unexpected survival difference. In the NACT cohort, only 15 patients were characterized with high infiltration versus 53 patients with low infiltration. Unfortunately, group sizes were too small to independently analyze survival for all surgical outcomes.

The results found in this study could potentially be used to improve immune checkpoint inhibition (ICI) treatment response rates and pave the way for personalized treatment. Up to now, ICI has shown limited response rates of only 10-15% in OC[30].

However, these phase II clinical trials were performed in unstratified relapsed or platinum-resistant OC patients[31,32]. Recent studies in the primary setting suggest ICI treatment early-on might be superior compared to ICI after disease recurrence[33,34]. Thus, we would argue for the exploration of ICI maintenance therapy in HGSOC patients, in combination with standard adjuvant chemotherapy. Patients could be further stratified based on TIL infiltration, as it is well-established that ICI is most effective in tumors infiltrated by a high number of TILs[35–37]. Completely debulked PDS patients with high CD8CD103 TRM infiltrated tumors might therefore particularly benefit from ICI maintenance treatment. Whereas patients with complete PDS and low CD8CD103 TRM infiltrated tumors, might benefit more from a combinatorial treatment regimen of anti-tumor vaccination and subsequent ICI. This was recently successfully demonstrated in melanoma patients receiving an antigen-encoding mRNA vaccine, targeting non-mutated tumor-associated antigens, alone or in combination with ICI. Interestingly, response rates were not correlated with tumor associated antigen expression nor mutational burden, supporting the applicability of this combinatorial strategy in tumors with low mutational burden such as OC[38].

Overall the results provided by this study demonstrate CD8CD103 double staining as a superior tool for prognostication compared to single CD8 or CD103 and advocates the further exploration of image-based quantification of CD8CD103 TRM in HGSOC. We demonstrate that the prognostic benefit of CD8CD103 TRM infiltration in HGSOC is restricted to PDS treated patients with a complete debulking. This approach provides novel insights into prognostic stratification of HGSOC patients and may contribute to personalized treatment strategies in the future.

Declaration of Interest statements:

MJ, Advisory Board, honoraria to instituation: Merck, BMS, Novartis, Pierre Fabre, Tesaro, AstraZeneca. Clinical studies: BMS, AbbieVie, Merck, Cristal Therapeutics

VHK, V.H. Koelzer reports grants from Promedica Foundation (F-87701-41-01) during the conduct of the study, and has served as an invited speaker on behalf of Indica Labs.

MB, Outside the submitted work, dr. de Bruyn reports grants from the Dutch Cancer Society (KWF), grants from the European Research Council (ERC), grants from Health Holland, grants from DCPrime, non-financial support from BioNTech, non-financial support from Surflay, grants and non-financial support from Vicinivax; In addition, dr. de Bruyn has grants and non-financial support from Aduro Biotech, in part relating to a patent for Antibodies targeting CD103 (de Bruyn et al. No. 62/704,258).

HWN, Outside the submitted work, prof. Nijman reports grants from the Dutch Cancer Society (KWF), grants from Health Holland, non-financial support from AIMM Therapeutics, grants from DCPrime, non-financial support from BioNTech, grants and shares and non-financial support from Vicinivax; In addition, prof. Nijman has grants and non-financial support from Aduro Biotech, in part relating to a patent for Antibodies targeting CD103 (de Bruyn et al. No. 62/704,258).

There are no conflicts of interest to disclose for the remaining authors.

Acknowledgements

The study is supported by the Dutch Cancer Society: project grant RUG 2015-7235.

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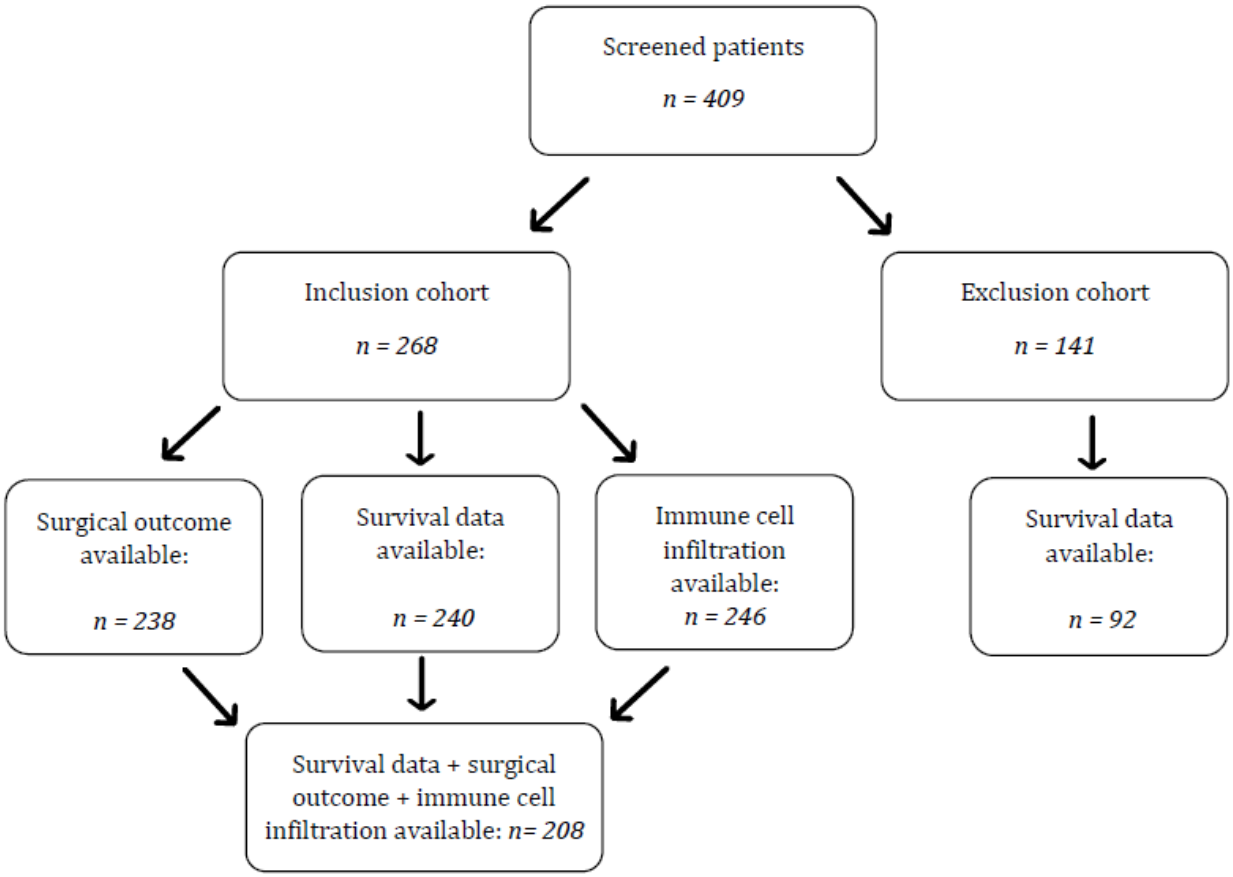
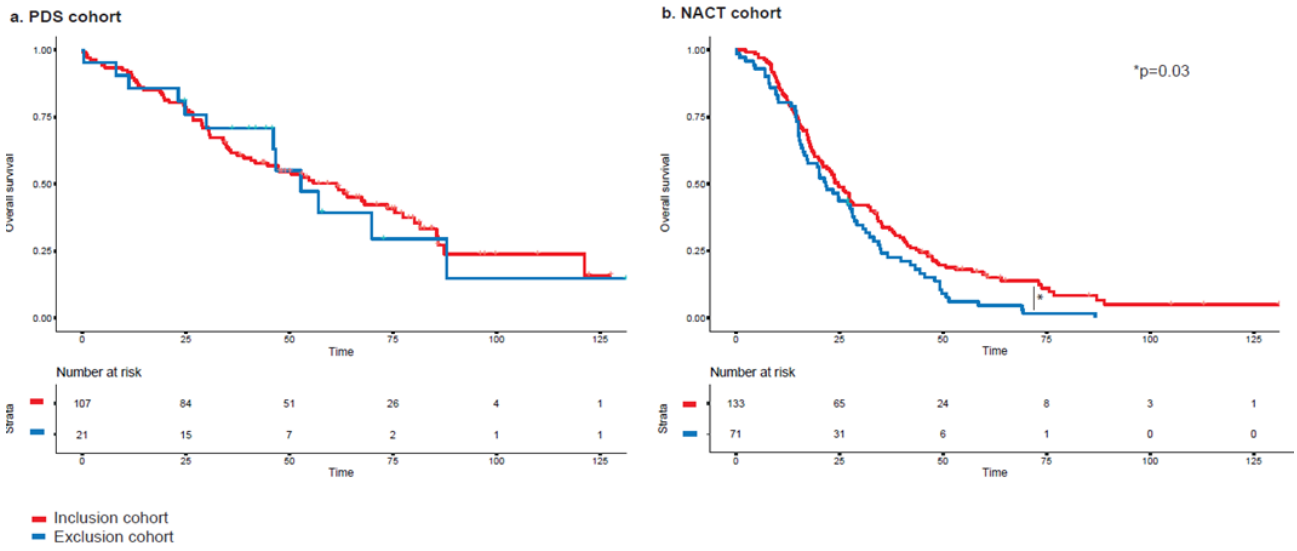
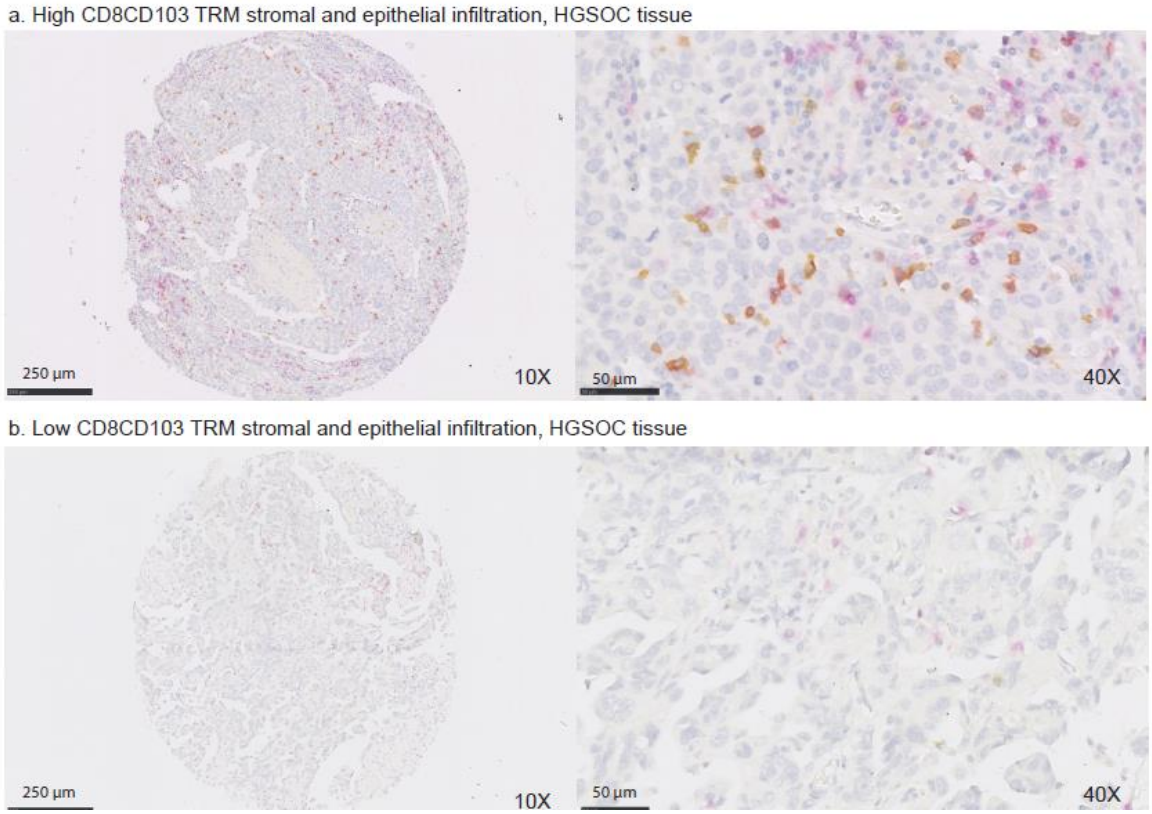


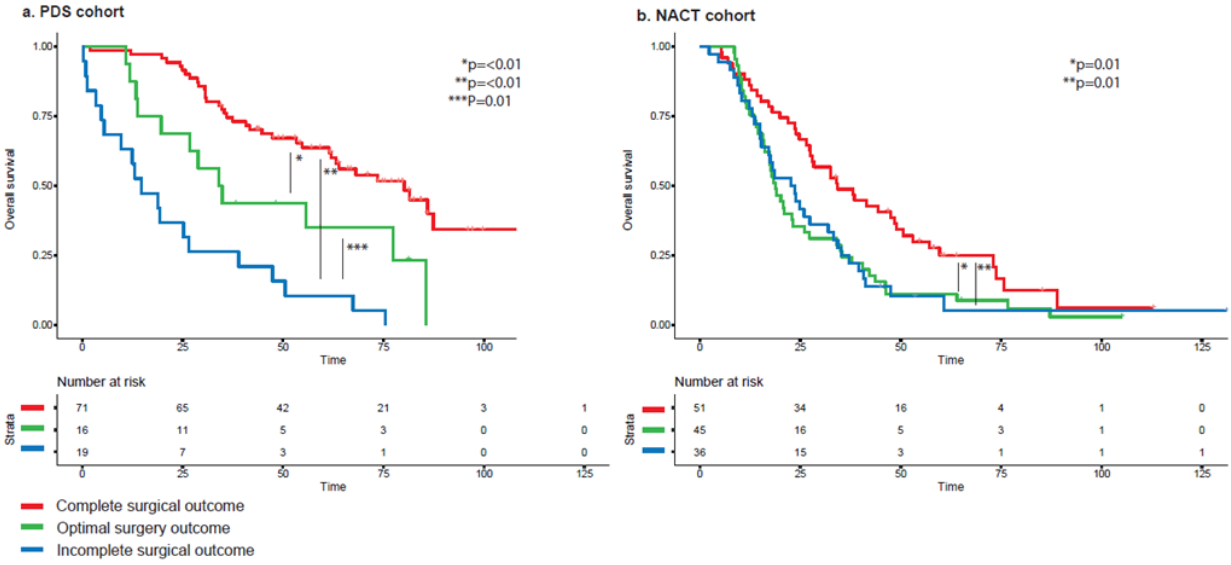
Supplementary Figure 1. Patient inclusion flowchart. Patient inclusion flowchart depicting available survival data and clinical characteristics used for survival analysis. From a total of 409 screened patients, 141 patients were excluded. From 92 excluded patients follow up survival data was available. In total, 268 patients were included for the construction of the TMA. After immunohistochemistry and quantification, immune cell infiltration was available for 246 patients. Follow-up survival data was available for 240 included patients and from 238 patients surgical outcome was known. For 210 patients both infiltration cell infiltration and survival data was available of which 2 patients had an unknown surgical outcome (n=208).



Supplementary Figure 2. Survival analysis of the inclusion and exclusion cohort. (A-B), Survival comparison between the inclusion and exclusion cohort. Overall survival differences were determined by 2-sided log-rank test. **A,** Displays survival difference of NACT-treated patients (p=0.95) **B,** Displays survival difference of PDS treated patients (p=0.03)



Supplementary Figure 3. Representative examples of CD8CD103 double staining assessed by immunohistochemistry. (A-B), Representative samples of CD8CD103 double staining assessed by IHC of low and high infiltrated HGSOC tissue is shown. Infiltration in the epithelial and stromal compartment are highlighted. Additional control tissues were included in the IHC analysis (*data not shown*).



Supplementary Figure 4. Survival analysis of primary treatment strategy in the inclusion cohort. (A-B), Displays a survival analysis of primary treatment strategy and surgical outcome within the inclusion cohort. Surgery outcome is defined as either complete (no residual tumor lesions after surgery), optimal (<1 cm residual tumor lesions after surgery) or incomplete (>1cm residual tumor lesions after surgery). Overall survival differences were determined by Kaplan-Mejer curves. **A,** Displays survival differences between surgical outcomes of PDS-treated patients. Complete versus optimal (p<0.01) , complete versus incomplete (p<0.01) and optimal versus complete (p=0.01). **B,** Displays survival differences between surgical outcomes of NACT-treated patients. Complete versus optimal (p=0.01) , complete versus incomplete (p=0.01) and optimal versus complete (p=0.81).

Supplementary Table 1. Reasons for exclusion

	PDS		NACT	
	N	Row %	N	Row %
No viable tissue available for TMA construction according to standard of care pathology report	11	39.3%	17	60.7%
No viable tissue available for TMA construction according to pathology review	1	1.6%	60	98.4%
Different histological subtype (Non-HGSOC)	7	63.6%	4	36.4%
No FFPE material available	3	10.7%	25	89.3%
Exclusion during production of TMA	1	33.3%	2	66.7%
< FIGO IIB according to follow-up data	9	90.0%	1	10.0%

Supplementary Table 2. Patient characteristics inclusion and exclusion cohort

		Inclusion (N=268)		Exclusion (N=141)	
		N	Column %	N	Column %
Mean age at diagnosis		64		67	
Hospital	UMCG	191	71.3	103	73.0
	Isala	77	28.7	38	27.0
FIGO stage	<IIB	0	0.0	7	5.2
	IIB/IIC	13	4.9	4	3.0
	III	200	74.6	93	68.9
	IV	54	20.1	30	22.2
	Unknown	1	0.4	1	0.7
BRCA status	BCRA1/ BRCA2 mutation	24	9	12	8.5
	No BRCA mutation	86	32.1	40	28.4
	Unknown	158	59	89	63.1
Primary treatment strategy	PDS	123	46.1	32	22.7
	NACT	144	53.9	109	77.3
NACT	Carboplatin/Paclitaxel	137	95.1	81	76.4
	Other/Unknown	7	4.9	25	23.6
AC	Carboplatin/Paclitaxel	220	82.1	3	75.0
	No chemotherapy	15	5.6	0	0.0
	Other/Unknown	33	12.3	1	25.0
Disease status	Evidence of disease	191	71.3	56	39.7
	No evidence of disease	42	15.7	14	9.9
	Progressive disease during primary treatment	18	6.7	10	7.1
	Unknown	17	6.3	61	43.3
Overall survival	Data Available	240		92	

FIGO: Fédération Internationale de Gynécologie et d'Obstétrique. **PDS:** Primary debulking surgery followed by 6 cycles of adjuvant chemotherapy; **NACT:** 3 cycles of neo-adjuvant chemotherapy, followed by an interval debulking and 3 cycles adjuvant chemotherapy. **Complete:** all visible tumor lesions were removed; **Optimal**: tumor lesion left <1cm; **Incomplete:** tumor lesions left >1cm.

Supplementary Table 3. Time to recurrence and overall survival inclusion cohort

		Time to recurrence (months)	Overall survival (months)
PDS	Complete	41.04	57.84
	Optimal	26.68	40.00
	Incomplete	7.54	28.53
NACT	Complete	17.05	39.30
	Optimal	9.45	28.50
	Incomplete	12.79	27.45

PDS: Primary debulking surgery followed by 6 cycles of adjuvant chemotherapy; **NACT:** 3 cycles of neo-adjuvant chemotherapy, followed by an interval debulking and 3 cycles adjuvant chemotherapy.
Complete: all visible tumor lesions were removed; **Optimal**: tumor lesion left <1cm; **Incomplete:** tumor lesions left >1cm.

Supplementary Table 4. Distribution $CD8^+CD103^+$ TRM infiltration

		$CD8^+CD103^+$ TRM/mm2 Whole tissue	$CD8^+CD103^+$ TRM/mm2 stroma	$CD8^+CD103^+$ TRM/mm2 tumor epithelium
Percentiles	25	1.1334	.0000	.6970
	33.3	2.0805	1.1527	1.7947
	50	6.9315	5.4687	7.4850
	66.6	18.5186	15.3173	20.0363
	75	27.7885	23.6956	28.7996
Mean		40.4904	43.7041	38.8221
Maximum		2311.10	2170.97	2512.93

Chapter 5

Antigen-specific active immunotherapy for ovarian cancer

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Cochrane Database of Systematic Reviews 2018

Abstract

Background

This is the second update of the review first published in the Cochrane Library (2010, Issue 2) and later updated (2014, Issue 9). Despite advances in chemotherapy, the prognosis of ovarian cancer remains poor. Antigen-specific active immunotherapy aims to induce tumour antigen-specific anti-tumour immune responses as an alternative treatment for ovarian cancer.

Objectives

The primary objective of this review is to assess the clinical efficacy of antigen-specific active immunotherapy for the treatment of ovarian cancer as evaluated by tumour response measured by Response Evaluation Criteria In Solid Tumors (RECIST) and/or cancer antigen (CA)-125 levels, response to post-immunotherapy treatment, and survival differences. In addition, we recorded the numbers of observed antigen-specific humoral and cellular responses. The secondary objective is to establish which combinations of immunotherapeutic strategies with tumour antigens provide the best immunological and clinical results.

Search methods

For the previous version of this review, we performed a systematic search of the Cochrane Central Register of Controlled Trials (CENTRAL; 2009, Issue 3), in the Cochrane Library, the Cochrane Gynaecological Cancer Group Specialised Register, MEDLINE and Embase databases, and clinicaltrials.gov (1966 to July 2009). We also conducted handsearches of the proceedings of relevant annual meetings (1996 to July 2009). For the first update of this review, we extended the searches to October 2013, and for this update, we extended the searches to July 2017.

Selection criteria

We searched for randomised controlled trials (RCTs), as well as non-randomised studies (NRSs), that included participants with epithelial ovarian cancer, irrespective of disease stage, who were treated with antigen-specific active immunotherapy, irrespective of

type of vaccine, antigen used, adjuvant used, route of vaccination, treatment schedule, and reported clinical or immunological outcomes.

Data collection and analysis

Two reviews authors independently extracted the data. We evaluated the risk of bias for RCTs according to standard methodological procedures expected by Cochrane, and for NRSs by using a selection of quality domains deemed best applicable to the NRS.

Main results

We included 67 studies (representing 3632 women with epithelial ovarian cancer). The most striking observations of this review address the lack of uniformity in conduct and reporting of early-phase immunotherapy studies. Response definitions show substantial variation between trials, which makes comparison of trial results unreliable. Information on adverse events is frequently limited. Furthermore, reports of both RCTs and NRSs frequently lack the relevant information necessary for risk of bias assessment. Therefore, we cannot rule out serious biases in most of the included trials. However, selection, attrition, and selective reporting biases are likely to have affected the studies included in this review. GRADE ratings were high only for survival; for other primary outcomes, GRADE ratings were very low.

The largest body of evidence is currently available for CA-125-targeted antibody therapy (17 studies, 2347 participants; very low-certainty evidence). Non-randomised studies of CA-125-targeted antibody therapy suggest improved survival among humoral and/or cellular responders, with only moderate adverse events. However, four large randomised placebo-controlled trials did not show any clinical benefit, despite induction of immune responses in approximately 60% of participants. Time to relapse with CA-125 monoclonal antibody versus placebo, respectively, ranged from 10.3 to 18.9 months versus 10.3 to 13 months (six RCTs, 1882 participants; high-certainty evidence). Only one RCT provided data on overall survival, reporting rates of 80% in both treatment and placebo groups (three RCTs, 1062 participants; high-certainty evidence). Other small studies targeting many different tumour antigens have presented promising immunological results. As these strategies have not yet been tested in RCTs, no reliable inferences about clinical efficacy can be made. Given the promising immunological

results and the limited side effects and toxicity reported, exploration of clinical efficacy in large well-designed RCTs may be worthwhile.

Authors' conclusions

We conclude that despite promising immunological responses, no clinically effective antigen-specific active immunotherapy is yet available for ovarian cancer. Results should be interpreted cautiously, as review authors found a significant dearth of relevant information for assessment of risk of bias in both RCTs and NRSs.

Summary of findings

Table 1. Summary of findings for the main comparison.

Patient or population: ovarian carcinoma
Setting: primary and recurrent ovarian carcinoma
Intervention: antigen-specific immunotherapy

Outcomes	Impact	Nº of partici-pants (studies)	Certainty of the evidence (GRADE)
Tumour response assessed with: RECIST	In total, 2 participants (0.01%) were defined as having a complete response, 9 (0.03%) had a partial response, and 50 (14%) had stable disease. Twelve participants (0.03%) showed no evidence of disease. Finally, 218 (61%) participants had progressive disease. The remaining 64 (18%) participants were not mentioned.	355 (17 observation-al studies)	Very low[a,b,c,d]
Tumour response assessed with: CA-125 according to GCIG criteria	In total, 8 participants (13%) were reported to have an increase in CA-125. In 22 patients, CA-125 was stable or decreasing (34%). The remaining 34 participants (53%) were considered not evaluable or were not mentioned.	64 (6 observatio-nal studies)	Very low[a,b,c,d,e]
Post-immunotherapy treatment response assessed with: survival	Two studies suggested that antigen-specific immunotherapy may lead to improved responses to future therapy. Two studies revealed no evidence of a difference.	88 (4 observation-al studies)	Very low[a,f]
Survival assessed with: overall survival	None of the 3 RCTs estimating overall survival found a significant difference in overall survival. Two studies of CA-125 monoclonal antibody vs placebo evaluated overall survival, respectively, at 57.5 vs 48.6 months (95% CI 041 to 1.25) and 80% survival for both groups.	1062 (3 RCTs)	High

Survival assessed with: progression-free survival/time to relapse	None of the 6 RCTs found statistically significant differences in progression-free survival/time to relapse, including 4 RCTs evaluating CA-125 monoclonal antibody vs placebo; time to relapse ranged from 10.3 to 18.9 months vs 10.3 to 13 months, respectively.	1882 (6 RCTs)	High
Antigen-specific immunogenicity (humoral response) assessed with: ELISA/Luminex assay	Nine studies evaluated anti-idiotopic (Ab2) humoral response, with responses ranging from 3% to 100%. Ten studies evaluated anti-anti-idiotropic (Ab3) humoral response, with responses ranging from 0% to 100%. Two studies observed no humoral response to other antigen-specific immunotherapy, and the 9 remaining studies noted large differences in percentages of participants with measurable antigen-specific antibodies (IgG: 8% to 96%).	1521 (25 observation-al studies)	Very low[a,d,g]
Antigen-specific immunogenicity (cellular response) assessed with: e.g. IFN-γ ELISPOT/proliferation assay/IFN-γ secretion assay	A total of 39 studies showed an induced cellular immune response in at least 1 cohort and to at least 1 target antigen; range of positive response varied broadly between 18% and 100%. One study retrospectively compared cellular immune response after CA-125 monoclonal antibody treatment vs placebo but showed no significant differences (31.8% intervention vs 26.3% control).	966 (40 observation-al studies)	Very low[a,d,h,g]

Ab2: anti-idiotopic; Ab3: anti-anti-idiotopic; CA: cancer antigen; CI: confidence interval; ELISA: enzyme-linked immunosorbent assay; GCIG: Gynecologic Cancer Intergroup; IFN: interferon; RCTs: randomised controlled trials; RECIST: Response Evaluation Criteria In Solid Tumors.

GRADE Working Group grades of evidence.
High certainty: we are very confident that the true effect lies close to that of the estimate of the effect.
Moderate certainty: we are moderately confident in the effect estimate: the true effect is likely to be close to the estimate of the effect, but there is a possibility that it is substantially different.
Low certainty: our confidence in the effect estimate is limited: the true effect may be substantially different from the estimate of the effect.
Very low certainty: we have very little confidence in the effect estimate: the true effect is likely to be substantially different from the estimate of effect.

[a] Most studies were uncontrolled phaseI/II trials.

[b] A large percentage of the included participants were not mentioned or were not evaluable for the analysis.

[c] Explicit descriptions of tumour responses per participant and the timepoints at which evaluations took place frequently were not available.

[d] Disease status at start of treatment differed among studies. Therefore the likelihood of clinical and immune responses to immunotherapy, especially in uncontrolled studies, which frequently include participants withrecurrentdiseaseandpreviousexposuretodifferenttypesoftherapy, is likely to be affected.

[e] CA-125 is a biomarker that serves as an indication for response; however CA-125 does not directly reflect tumour size.

[f] Although in one study participants with a complete response had strong humoral responses, similar or stronger antibody responses were observed for participants with stable or progressive disease.

[g] Between studies, there were broad difference sin (1) response definition, (2) number of treatment cycles after which immune responses were measured, and (3) targeted antigens.

[h] Explicit descriptions of immune responses per participant and the time points at which evaluations took place, types of evaluations, and when an evaluation was considered positive often were not available.

Background

Description of the condition

Ovarian cancer is the sixth most common cancer and the seventh most common cause of death from cancer among women worldwide.[1] It is the second most common gynaecological cancer and the leading cause of death from gynaecological cancers in the Western world. As most ovarian malignancies (80% to 90%) arise from the epithelium, all statements about ovarian cancer presented in the remainder of this review apply to epithelial ovarian cancer only. Worldwide age-standardised incidence rates range from 5 per 100,000 in less developed areas to 9.1 per 100,000 in developed areas.[1]

Stage of disease at presentation is the most important prognostic factor. Owing to the asymptomatic course of the disease, most participants have extensive disease at presentation (stage III to IV, according to the International Federation of Gynecology and Obstetrics (FIGO) classification[2]). Despite standard treatment, which consists of cytoreductive surgery and platinum-based chemotherapy, almost all women with advanced-stage disease at presentation will experience relapse, with median progression-free survival of only 18 months. When residual or recurrent disease manifests itself, resistance to chemotherapy often prohibits further curative therapy, resulting in disease-specific five-year survival for women with advanced-stage ovarian disease of only 10% to 20%.[3,4]

Description of the intervention

The immune system seems to play a role in ovarian cancer. This is reflected in the observation that in more than half of women with ovarian cancer, T-cells are present within tumour islets.[5,6] Women with advanced ovarian cancer, whose tumour is infiltrated by these T-cells, have better clinical outcomes than women without these tumour-infiltrating T-cells.[5–7] More specifically, higher numbers of cytotoxic T-cells, which can directly recognise and kill tumour cells, and increased ratios between cytotoxic T-cells (CD8+) and helper T-cells (CD4+) within the tumour epithelium are associated with improved survival.[8,9]

Immunotherapy is one of the novel therapeutic strategies under investigation for ovarian cancer. It aims to induce or enhance active immune responses directed towards

the tumour and to consolidate anti-tumour effects of standard therapy, delaying and possibly preventing disease progression. Antigen-specific active immunotherapy aims to activate the adaptive immune system directed towards a specific target antigen through administration of a molecularly defined antigen-specific vaccine to the patient.

How the intervention might work

An antigen is a molecule - usually a protein or a polysaccharide - that can stimulate an immune response. Tumour antigens can be subdivided into different categories such as mutated self-proteins, products of oncogenes (e.g. Her-2/Neu), mutated tumour suppressor genes (e.g. *p53*), and aberrantly expressed self-proteins (e.g. sperm protein 17, MAGE-1). Numerous tumour-associated antigens are known in ovarian cancer. To obtain a tumour-specific immune response, immunotherapy exploits the differential expression of antigens between normal and tumour cells. A major challenge related to the safety of immunotherapy lies in the prevention of autoimmunity (i.e. induction of immune cells that preferentially recognise and kill tumour cells while avoiding destruction of normal body cells). From a theoretical point of view, other possible side effects include allergic reactions to components of the vaccine and inflammatory reactions at the site of injection.

Why it is important to do this review

Researchers are now employing several immunotherapeutic strategies by using different tumour antigens. However, this research generally has not yet evolved past phase I/II studies. To our knowledge, no systematic review of antigen-specific active immunotherapy in ovarian cancer has been carried out so far.

This review evaluates the immunogenicity and clinical efficacy of antigen-specific active immunotherapy in ovarian cancer. A systematic review about this topic should prove useful for ascertaining the effectiveness of this treatment modality for ovarian cancer.

Objectives

Primary objective:

- To assess the clinical efficacy of antigen-specific active immunotherapy for the treatment of ovarian cancer as evaluated by tumour response measured by Response Evaluation Criteria In Solid Tumors (RECIST) and/or cancer antigen (CA)-125 levels, response to post-immunotherapy treatment, and survival differences.
- In addition, we recorded the numbers of observed antigen-specific humoral and cellular responses.

Secondary objective:

- To establish which combinations of immunotherapeutic strategies with tumour antigens provide the best immunological and clinical results.

Methods

Criteria for considering studies for this review

Types of studies

We had anticipated that we would identify limited randomised controlled trials (RCTs) on this topic. Therefore, we included phase I and phase II non-randomised studies (NRSs) and phase III RCTs. We realise that results from NRSs cannot readily be extrapolated to the general population, but given the lack of RCTs, inclusion of these studies in the review was justifiable.

Types of participants

We included women with a diagnosis of epithelial ovarian cancer, irrespective of stage of disease. However, as patient populations may differ substantially between different types of studies to be included in this review, we documented what t ype of participant was included in each study (e.g. women with end-stage disease, women with residual disease).

Because we anticipated that we would find few studies that included women with ovarian cancer only, we also included immunotherapeutic studies in people with cancer that included at least two women with ovarian cancer, with the additional requirement that the results for these individual women were separately identifiable from those of the study publication or could be obtained by communication with the study author, and we extracted only data on these women for inclusion in the review. We are fully aware of the vigilance necessary when conclusions are based on studies with such small numbers, but we believe that given the anticipated lack of large RCTs, inclusion of these studies in this review is justifiable.

Types of interventions

Antigen-specific active immunotherapy is defined as therapy that aims to induce an adaptive immune response directed towards the tumour through administration of a specific well-defined tumour antigen. We compared interventions against each other based on the above-mentioned characteristics.

We included all interventions that aimed to provide antigen-specific active immunotherapy, irrespective of type of vaccine, antigen, or adjuvant used; route of vaccination; and vaccination schedule.

Primary outcomes

To assess clinical efficacy, we evaluated the following.

- Tumour responses to immunotherapy (complete/partial response, stable/progressive disease), as measured by:
  - cancer antigen (CA)-125 levels according to or transposable to Gynecologic Cancer Intergroup (GCIG) criteria[10]; or
  - tumour response according to World Health Organization (WHO) criteria[11] or Response Evaluation Criteria in Solid Tumors (RECIST) criteria.[12]
- We evaluated responses to post-immunotherapy treatment, as evidence suggests that people with small cell lung cancer treated with chemotherapy after

immunotherapy have improved survival as opposed to people who do not receive immunotherapy.[13]

- We assessed survival differences, including time to relapse or progression-free survival, based on treatment with immunotherapy.

To assess antigen-specific immunogenicity We recorded the numbers of observed antigen-specific humoral and cellular responses. When possible, we separately reported responses of cytotoxic (CD8+) T-lymphocytes and/or helper (CD4+) T-lymphocytes.

Secondary outcomes

Carrier-specific immunogenicity

Given that certain immunotherapeutic strategies rely on the use of carriers that may be the target of an immune response besides the intended antigen-specific immune response, we recorded information on the induction of carrier-specific immune responses when appropriate.

Adverse events

To obtain information on the toxicity of antigen-specific immunotherapy, we extracted data on adverse events observed and reported in the different studies. We categorised adverse events as local adverse events at the site of immunisation and systemic adverse events (all other reported adverse events). We subdivided systemic adverse events into autoimmunity, allergic reactions, and other adverse events occurring after immunisation. If sufficient information was available, we classified adverse events according to the National Cancer Institute Common Terminology Criteria for Adverse Events (NCI CTCAE).[14]

Search methods for identification of studies

Electronic searches

For the original review[15], we searched the Cochrane Central Register of Controlled Trials (CENTRAL; 2013, Issue 9), in the Cochrane Library (Appendix 1), along with the Cochrane Gynaecological Cancer Group Specialised Register, in October 2013. We also searched MEDLINE (1966 to July 2009) and Embase (1974 to July 2009) according to the search strategies listed (Appendix 2; Appendix 3, respectively).

For the first update of the review, we extended the searches to October 2013, and for this update, we extended the searches to July 2017:

- Cochrane Central Register of Controlled Trials (CENTRAL; 2017, Issue 6), in The Cochrane Library;
- MEDLINE via OVID (October 2013 to June week 4 2017);
- Embase via OVID (October 2013 to 2017 week 27).

Searching other resources

We also searched the prospective trial register at www.clinicaltrials.gov.

We undertook handsearching of abstracts in the proceedings of annual meetings of the Society of Gynecologic Oncologists, the American Association for Cancer Research, and the International Society for Biological Therapy of Cancer (1996 to July 2009). The International Society for Biological Therapy of Cancer has been renamed the Society for Immunotherapy of Cancer (SITC), thus we also searched the proceedings of the annual meeting of SITC.

We checked the bibliography of each primary reference and of recent reviews on immunotherapy for ovarian cancer for additional study publications. In addition, we wrote to specialists involved in research regarding immunotherapy for ovarian cancer to ask for information about the results of unpublished and ongoing studies. We included relevant data in this review.

Data collection and analysis

Selection of studies

We downloaded to Reference Manager all titles and abstracts retrieved by electronic searching. We applied no language restrictions other than those inherent to the databases surveyed. We removed duplicates, and two review authors (HWN and NL) independently examined the remaining references. We excluded studies that clearly did not meet the review inclusion criteria and obtained copies of the full text of potentially relevant references. Two review authors (HWN and NL) independently assessed the eligibility of retrieved papers. We resolved differences by discussion or by appeal to a third review author (TD), if necessary. We documented reasons for exclusion. The second update included all titles and abstracts from October 2013 until July 2017 retrieved by electronic searches of MEDLINE, Embase, and CENTRAL. Two review

authors (STP and MB) selected and independently assessed studies using the same procedure that was used in the primary review and the first update. We resolved differences by discussion or by appeal to a third review author (HWN), if necessary.

Data extraction and management

Two review authors (HWN and NL) independently extracted data on characteristics of participants and interventions, study quality, and endpoints for included studies, and entered them onto a data extraction form specially developed for this review (Appendix 4). Two review authors (STP and MB) followed the same procedure for the second update.

When data on clinical efficacy and antigen-specific immunogenicity were missing from reports, we attempted to contact study authors to obtain the missing information. A third review author (WH or TD; or HWN during the second update) checked the results.

Assessment of risk of bias in included studies

We assessed the risk of bias in RCTs using the Cochrane 'Risk of bias' tool.

No standard tools are available to evaluate validity for non-RCTs. For these studies, we evaluated the risk of bias using the following four domains (Table 1).

- Sample definition and selection.
    - Clear definition of inclusion/exclusion criteria.
    - Representative selection.
    - Adequate description of baseline characteristics.
- Interventions.
    - Clear specification.
    - Concurrent/concomitant treatment.
- Outcomes.
    - Specifications of outcome measures.
    - Relevance of outcome measures.
    - Reporting of outcome measures.
- Statistical analysis.
    - Adequate rationale for numbers of participants included.
    - Adequate description of withdrawals/exclusions during the study.

- Adequate presentation of results.

We selected these domains as representative for, and applicable to, non-randomised non-controlled studies from a list of 12 quality domains and items deemed to be pivotal to the assessment of non-RCTs.[16]

Two review authors (HWN and NL) carried out the 'Risk of bias' assessment. We resolved discrepancies by discussion; if necessary, we consulted a third review author (WH or TD). For the second update, two review authors (STP and MB) carried out the 'Risk of bias' assessment. We resolved discrepancies by discussion; if necessary, we consulted a third review author (HWN).

Data synthesis

This review provides a narrative analysis because the included studies are highly heterogeneous in terms of intervention and outcome measures. Furthermore, publications often presented data with insufficient details (e.g. lack of standard deviations (SDs), presentation of only some of the multiple outcomes), and it was difficult for review authors to obtain additional information from report authors. Therefore we agreed that quantitative meta-analysis and calculation of effect size estimates would be neither meaningful nor appropriate for this review. We limited analysis to a structured summary and discussion of available studies and findings.

Certainty of the evidence

We assessed the certainty of the evidence for main outcomes using GRADE (Grading of Recommendations Assessment, Development and Evaluation) criteria[17], and we presented the main findings along with our judgements in a 'Summary of findings' table.

We will present the overall certainty of the evidence for each outcome according to the Grading of Recommendations Assessment, Development and Evaluation (GRADE) approach[17], which takes into account issues related not only to internal validity (risk of bias, inconsistency, imprecision, publication bias for quantitative studies) but also to external validity (directness of results).

We downgraded the evidence from 'high' certainty by one level for serious (or by two for very serious) concerns for each limitation.

- High-certainty: we are very confident that the true effect lies close to that of the estimate of the effect.
- Moderate-certainty: we are moderately confident in the effect estimate: the true effect is likely to be close to the estimate of the effect, but there is a possibility that it is substantially different.
- Low-certainty: our confidence in the effect estimate is limited: the true effect may be substantially different from the estimate of the effect.
- Very low-certainty: we have very little confidence in the effect estimate: the true effect is likely to be substantially different from the estimate of effect.

For qualitative studies, we would upgrade for large consistent effect, dose response, and confounders that only reduced the effect size.

Results

Description of studies

Results of the search

Initial version of the review.[15]

Upon completing electronic searches of MEDLINE and Embase, we selected 56 out of 311 abstracts as potentially compliant with the selection criteria of this review and retrieved the full texts. Evaluation of the retrieved full texts resulted in the exclusion of 26 papers (see Excluded studies). In addition to the 30 selected full texts, we identified another 14 abstracts by handsearching the proceedings of the periodic meetings specified in the Methods section. We contacted study authors for manuscripts but obtained no full texts for these abstracts. Together, the 44 selected full texts and meeting abstracts described a total of 35 studies. A search of the prospective trial register www.clinicaltrials.gov resulted in identification of an additional 26 studies. We could retrieve a full text or meeting abstract for only four of these and found that only one study complied with our inclusion criteria.[18] The remaining studies were either ongoing (n = 15) or completed but not yet published (n = 6). A search of CENTRAL (2009, Issue 3) yielded no additional studies. Thus, we included a total of 36 studies in this review. Generally, we selected the most recent peer-reviewed publication as the primary reference.

First update of the review.[19]

For the first update of this review, electronic searches of MEDLINE and Embase yielded 158 records, which resulted in an additional 23 included papers and 10 excluded papers (Characteristics of excluded studies). For five studies in the previous version of this review, a full-text publication, update, or additional paper was now available. A search of CENTRAL (2013, Issue 3) did not yield additional studies. A search of clinicaltrials.gov resulted in two additional published studies. Furthermore, we identified 26 relevant studies without available results (Characteristics of ongoing studies). Twelve studies are currently recruiting participants, four studies are ongoing but not recruiting, nine studies are classified as completed, and for two studies status is unknown. Overall, we included an additional 19 studies in the update of this review, resulting in a total of 55 included studies involving 3051 women (Characteristics of included studies).

Second update of the review

For the second update of the review, an electronic search of CENTRAL, MEDLINE, and Embase yielded 266 records, which resulted in an additional nine included papers and nine excluded papers (Characteristics of excluded studies). For two studies identified in the previous version of this review, a full-text publication, update, or additional paper was now available.

A search of ongoing studies identified from the last update in clinicaltrials.gov revealed four additional published studies, three of which are included in this update. In addition, five studies were completed for which no results were published, four studies are still recruiting, and for one study status remains unknown. We removed four studies from the Ongoing studies section because the study had been terminated, or because studies did not include women with epithelial ovarian cancer. Furthermore, we identified 22 relevant new ongoing studies without available results (Characteristics of ongoing studies).

Overall, we included an additional 12 studies in the update of this review, resulting in a total number of 67 included studies involving 3632 women (Characteristics of included studies).

Included studies

The 67 studies included in this updated review were all published in English (Characteristics of included studies; Table 2).

Design

As we expected, most studies were uncontrolled phase I or II studies (52/67). Only four studies were randomised placebo-controlled studies.[20–25] Eleven studies randomly allocated participants to different regimens.[26–37] Five studies retrospectively studied the immunogenicity of a previously applied immunoscintigraphic agent.[38–43]

Sample sizes

The median number of women with epithelial ovarian cancer treated per study was 20 (range 2 to 888). Twenty-one studies included fewer than 10 participants. Twenty studies also included participants with other types of cancer.[35,36,44–61] Only 13 studies provided a sample size calculation or rationale.[18,21,58,62–66,22–28,35]

Participants

As was expected, disease status at study entry varied largely between studies (Table 2). Participants with evidence of residual or recurrent disease after treatment were most frequently included (30/67).[26,35,60–63,66–71,39,72–76,40,45,49,51,56,58,59] Eight studies included participants with and without evidence of disease after prior therapy.[27,28,36,53–55,77,78] Seventeen studies included participants with complete response to therapy for primary or recurrent disease.[18,20,34,38,46,47,64,65,79–81,21–25,29,30,33]. One study administered treatment together with adjuvant chemotherapy after primary cytoreductive surgery.[27] The remaining 18 studies did not report disease status at study entry.[31,32,57,82–90,37,91,92,41–44,48,50,52]

Interventions

Most studies described antibody therapy (22/55), usually targeting cancer antigen (CA)-125 (17/22 (2347 women)). Most studies included only one target antigen in the vaccine, but 15 studies simultaneously targeted multiple antigens.[18,29,77,80,83,86,92,30,45,46,53–55,59,71] Antibodies were usually administered intravenously (12/22). For other vaccine types, subcutaneous injections were most common (29/43).

Fifteen out of 55 studies did not allow concurrent treatment with immunomodulatory drugs. In an additional 20 studies, concomitant immunomodulatory agents were not part of the studied intervention but study authors made no explicit statements in the protocol about prohibition of such drugs. For 27 studies, immunomodulatory drugs were part of the protocol (i.e. carboplatin-paclitaxel, gemcitabine, doxorubicin and decitabine, cyclophosphamide, interleukin (IL)-2 ± granulocyte-macrophage colony-stimulating factor (GM-CSF), OK-432, OPT-821, PegIntron, toll-like receptor agonist poly-ICLC or resiquimod, or diphenhydramine) and one of these allowed interruption of immunotherapy by chemotherapy for progressive disease.[89,90] Furthermore, two retrospective studies explicitly mentioned that concurrent chemotherapy was allowed at the discretion of the treating clinician.[39,42,43]

Outcomes

Information on immunological responses, clinical responses, survival, and adverse events was available for 63, 43, 44, and 54 studies, respectively.

Excluded studies

A summary of the excluded studies is given in the Characteristics of excluded studies table. Frequent reasons for exclusion were inclusion of too few participants with ovarian cancer, use of antigen non-specific immunotherapy, and the impossibility of distinguishing results for women with ovarian cancer from results for other study participants.

Risk of bias in included studies

We included GRADE ratings for all primary outcomes. We rated survival as high but all other primary outcomes as very low, as is displayed in Summary of findings table 1.

We evaluated risk of bias using the Cochrane 'Risk of bias' tool[93]. Results of individual studies (both RCTs and NRSs) are available in the Characteristics of included studies table. The fact that for four of 16 RCTs only meeting abstracts were available hindered assessment of risk of bias. The 14 trials for which we could retrieve full texts also did not report on some of the items in the 'Risk of bias' tool. This substantial lack of information means it is highly likely that included studies are subject to biases, and it is therefore difficult to make any statements about the validity of the included RCTs (Figure 1).

In addition to using the 'Risk of bias' tool, we evaluated non-RCTs using the checklist provided in Table 1. An overview of these results is provided in Table 3. Important observations from this table include lack of clearly defined inclusion/exclusion criteria in 13 out of 51 studies and serious under-reporting of baseline characteristics in 31 out of 51 studies; this combination makes it impossible to evaluate whether the study populations were representative of the true population. Although most studies carefully described the investigational interventions (47 out of 51), information on allowance or application of concomitant immunomodulatory treatment was frequently absent (24 out of 51). Albeit a clear description of outcome measures was available for 35 studies, adequate calculation of sample size based on a clearly defined primary outcome measure was available for only five studies. Furthermore, the applied checklist shows that justification for withdrawals and exclusions during the study, as well as presentation of study results, requires serious attention in the reports of these non-randomised studies.

Based on the above, the risk of bias of studies included in this systematic review cannot be neglected. Especially selection bias (selection of a treatment population not comparable to the control group or the true population), attrition bias (inadequate reporting of withdrawal and exclusions during the study, resulting in possible overestimation or underestimation of effects), and selective reporting bias are likely to affect the studies included in this review. The effects of interventions described below must therefore be interpreted with prudence.

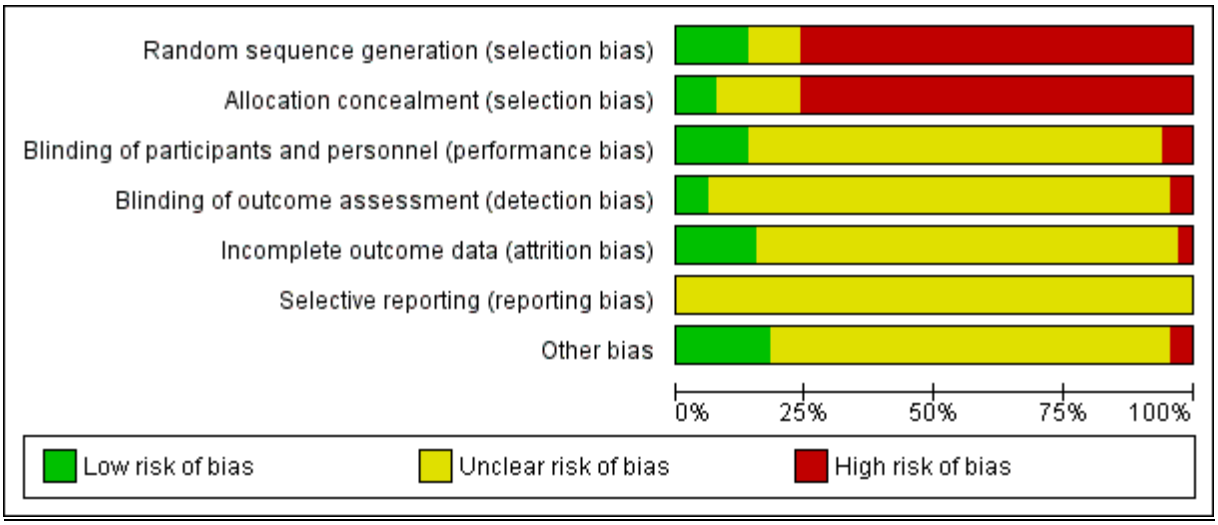

Figure 1. 'Risk of bias' graph. Review authors' judgements about each risk of bias item presented as percentages across all included studies. The high risk of selection bias in the majority of included studies is a reflection of the large number of uncontrolled studies included in this review. The risk of remaining biases could not be adequately judged for the included uncontrolled studies, thus explaining the large percentage of missing risk assessments.

Allocation (selection bias)

As can be deduced from the Characteristics of included studies table, we were unable to identify the methods of randomisation and allocation used for several randomised studies, which means that we cannot rule out a selection bias for these studies. For the remaining RCTs, selection bias does not seem likely.

However most included studies were early-phase non-randomised studies including only a single study arm. Selection bias in these studies may have occurred in two ways: (1) by selective inclusion of participants with no other treatment options owing to end-stage disease, at which point function of the immune system may also be seriously impaired, thus resulting in an underestimation of immunogenicity and possible clinical benefit of a given vaccine, or (2) via selective recruitment of fairly immunocompetent patients with no evidence of disease, resulting in a possible overestimation of immunogenicity and possible clinical benefit of a given vaccine.

Blinding (performance bias and detection bias)

Inherent to the study design, no non-RCTs blinded participants or treating (study) physicians. All participants may have derived benefit from the additional attention awarded to them as participants in a study, and thus performance bias may have influenced the results of these studies. Furthermore, it is unclear whether for these

studies, outcome assessors were aware of the clinical condition of patients; thus detection bias may have occurred in these studies.

Only five RCTs described blinding of patients, caregivers, and/or outcome assessors; all compared antibody therapy versus placebo.[20–25,29] The other RCTs compared dosage levels[26,31,32,36], administration route[28], number of gifts of a given drug[37], timing of the intervention in relation to standard chemotherapy[27], addition of an immunomodulatory drug[30], or immunotherapeutic intervention compared with standard of care.[33–35] Given these study designs, we believe that for most of these studies, risk of performance bias is low. Information on blinding of outcome assessors is frequently missing, and risk of detection bias cannot be reliably judged.

Incomplete outcome data (attrition bias)

We deemed that only one RCT had high risk of attrition bias based on differences in withdrawals between groups.[35] Risk of attrition bias was unclear for nine other RCTs[20,28,29,31–34,36–38], and risk was low for the remaining RCTs.[21–27,30]

Selective reporting (reporting bias)

None of the included studies had a publicly available registered study protocol. It is therefore unclear whether studies selectively reported outcomes.

Other potential sources of bias

Given the elapsed time since publication of the meeting abstract, a publication bias is likely to exist for two out of three RCTs for which only a meeting abstract was available.[20,31,32]

Effects of interventions

Primary outcomes

Clinical efficacy, Tumour responses

Forty-three studies evaluated clinical responses to therapy (Table 4). No RCTs evaluated tumour response.[20–25,29,34] In reports on these studies, criteria for evaluation and/or explicit descriptions of tumour responses per patient as well as the time point at which the evaluation took place were frequently not available. For studies that did mention evaluation of tumour responses, response outcomes were based on CA-125 levels

combined with tumour imaging[26,28,66–70,76,77,79,87,30,48,51,53,59,62–64], CA-125 alone[42,43,74], or imaging alone.[49,61,65,78,89,90,92] Eighteen studies explicitly mentioned evaluation of imaging according to the internationally accepted WHO or RECIST criteria[26,36,67,69,71,75,83,87,89–92,48,52,53,62–66], and only six studies evaluated CA-125 levels according to GCIG criteria or described CA-125 levels in such a way that evaluation according to these criteria was possible for at least some participants.[26,62,63,66,67,69,76] It is striking that eight studies stated that study authors evaluated tumour responses but did not provide these results in their publications.[37,42,43,57,59,78–80,89,90]. Only seven studies reported complete or partial tumour responses in a small fraction of patients with evidence of disease at study entry.[26,60,67,70,71,78,92] These results must be interpreted with caution, as two of these studies did not define criteria for response evaluation.[70,78]

Clinical efficacy, Post-immunotherapy treatment response

Although studies generally report a period of follow-up to obtain information on survival, most studies provide no report on subsequent treatment with and response to secondary chemotherapy. Nine studies mention that participants were treated with chemotherapy after immunotherapy[21,22,89,90,39,51,58,62,63,70,76,78], but only four non-comparative phase I/II studies report response to secondary chemotherapy in relation to immunological responses to immunotherapy.[58,62,63,70,89,90]

Reinartz et al. provided a preliminary report on clinical responses of 28 out of 42 participants treated with chemotherapy for clinically relevant progression during or after antibody therapy in conjunction with the induction of human-anti-mouse and anti-anti-idiotype antibodies.[89,90] Although both types of participants with a complete response had strong humoral responses, researchers observed similar or stronger antibody responses for participants with stable or progressive disease. In another study, shortly after monotherapy with a monoclonal antibody, 13 out of 20 participants received chemotherapy combined with the monoclonal antibody. Researchers in this study observed clinical responses to chemo-immunotherapy only in patients with cellular responses to CA-125 and/or autologous tumour.[70] A study of synthetic long peptides targeting *p53* showed no improvement in survival or tumour responses to secondary chemotherapy.[62,63] Finally, the authors of a study investigating plasmid DNA vaccination targeting CYP1B1 suggest that treatment has led to improved responses to

third-line therapy but included no control group, nor do we find this observation convincing when only patients with ovarian cancer are considered.[58]

Survival and time to relapse

Definitions of survival used in the different studies varied greatly (Table 5 and Table 6). Furthermore, reliable statements about survival (dis)advantages can be made only on the basis of RCT findings. Only six studies were designed to primarily evaluate survival; however, investigators found no statistically significant differences in time to relapse and/or overall survival between patients treated with a monoclonal antibody and those given placebo.[20–25] Another study compared antigen-specific immunotherapy versus a non-specific immunotherapy and noted no significant differences in progression-free survival.[29] Another study compared MUC1 dendritic cell therapy versus standard of care and reported no significant differences in progression-free survival and overall survival. However, when patients were divided into two subgroups (first and second clinical remission), a significant difference in overall survival and progression-free survival was evident among those with a second clinical remission. Researchers included a small number of participants in the trial and median overall survival of the treated group has not yet been reached; therefore these results must be interpreted with caution.[34] Many non-RCTs also evaluated survival, frequently by comparing survival of patients with robust immunological responses versus that of patients with no or weak immunological responses to treatment (Table 5 and Table 6). These results should be interpreted with great caution, as shorter survival among non-responders could merely be a reflection of the general condition of these patients and might reflect well-known clinical and pathological prognostic parameters. Patient numbers in the non-comparative groups were often too low to permit a reliable conclusion.

Antigen-specific immunogenicity, Humoral responses

Monoclonal antibodies may induce anti-idiotype antibodies (Ab2), directed primarily against the administered monoclonal antibody, as well as anti-anti-idiotype antibodies (Ab3), directed towards the target antigen. Anti-idiotype and anti-anti-idiotype antibodies were evaluated in 10 out of 22 studies and 9 out of 22 studies, respectively (Table 7 and Table 8). Response percentages varied greatly (Ab2: 3% to 100%, Ab3: 0% to 100%).

Twenty-one studies of other vaccine types evaluated the induction of antigen-specific antibodies as shown by enzyme-linked immunosorbent assay (ELISA) or luminex assay; however only 11 of these studies clearly defined when an antibody titre or concentration was considered positive (Table 9).[18,29,86,50,52,60,65,69,71,75,79] In addition, the study combining an NY-ESO-1 vaccine with chemotherapy and an anti-methylation agent tested humoral response with ELISA to 22 recombinant proteins that were not included in the vaccine and showed de novo serum reactivity to at least one of those proteins in all analysed participants (n = 3), suggesting that combination regimens may lead to a broadened profile of anti-tumour immune response in vivo.[75] Results show large differences in percentages of patients with measurable antigen-specific antibodies (IgG: 0% to 96%). Possible explanations for these broad ranges include differences in (1) response definition, (2) number of treatment cycles after which humoral responses were measured, and (3) targeted antigens.

Antigen-specific immunogenicity, Cellular responses

Thirteen out of 20 monoclonal antibody studies investigated induction of T-cells against the target antigen (Table 10). Investigators evaluated the presence of antigen-specific T-cells using commonly applied tests, such as interferon-gamma (IFN-γ) ELISPOT[28,37,68,70], proliferation assay[40,76,84], cytokine profiling[40,88], IFN-γ secretion assay[51], and IFN-γ intracellular staining assay.[38] One study used the leucocyte migration inhibition assay, which nowadays is rarely used.[42,43] As described above for humoral responses, response definitions were frequently lacking or inadequate. Nevertheless, results showed cellular immunity against CA-125 for 21% to 80% of participants. One study retrospectively compared cellular immune response after CA-125 monoclonal antibody treatment versus placebo but noted no significant differences (31.8% intervention vs 26.3% control)[38]. Antibody treatment targeting the membrane folate receptor did not however induce cellular responses.[76] Only two studies reported recognition of autologous tumour cells by induced T-cells, describing positive responses in five out of eight and one out of two patients, respectively.[51,70]

A total of 35 out of 44 studies evaluated antigen-specific cellular immune responses with the use of other vaccine types (Table 11). The most frequently used assay was the IFN-γ ELISPOT assay, which sometimes was used to separately analyse CD4+ and/or CD8+ cells. Again, response definitions for positive and/or vaccine-induced

responses were frequently absent or unclear (15 out of 44). Six of eight studies targeting NY-ESO-1 induced antigen-specific T-cells, with percentages of patients with NY-ESO-1-specific CD8+ ranging from 33% to 92%[47,57,65,75,78,79,85], and one study did not report the results for ovarian cancer participants separately.[57] Another study showed a positive NY-ESO-1-specific CD8+ T-cell induction by IFN-γ catch assay (1% to 5% positive CD8+ T-cells)[52]. After treatment with vaccines targeting *p53*, investigators observed *p53*-specific T-cells in 64% to 100% of patients, irrespective of the type of vaccine.[62–64,66] One study compared *p53*-specific T-cell responses between treatment with a *p53*-targeting vaccine plus chemotherapy and PegIntron versus chemotherapy and PegIntron versus chemotherapy alone. Immune response rates were 100%, 22%, and 0%, respectively[67], indicating that applying chemotherapy and PegIntron at the same time as antigen-targeted immunotherapy may induce a stronger immune response. Studies targeting multiple antigens demonstrated antigen-specific cellular immunity with varying immunogenicity of the different antigens targeted.[30,34,71,77,91,36,45,46,53–56,60] Finally, a study testing dendritic cell-based immunotherapy showed no induction of IFN-γ-specific CD4+ and CD8+ cells by flow cytometry, although tetramer staining of WT1-specific cytotoxic T-lymphocytes did show an increase in 12 out of 17 patients (70.6%).[83]

Secondary outcomes

Carrier-specific immunogenicity

Most studies using a monoclonal antibody (18/22) used a murine antibody, two studies used a trifunctional rat-mouse hybrid[26,35], and one study used a chimeric antibody construct[76]. Next to antigen-specific immunity, 16 studies assessed the induction of human-anti-mouse antibodies (HAMAs) using HAMA-specific ELISA assays (Table 12). HAMAs were present in 4% to 97% of participants immunised.[21,22,88–90,26–28,37,39,41,68,70] It seems that this large variation between studies cannot be attributed to differences in dosage but is best ascribed to different definitions of a HAMA response (i.e. some studies report only robust responses, whereas others report all responses above a certain threshold). Furthermore, the point in time at which HAMA titres were measured is of importance, as responses increase in frequency and strength with repeated administration of the antibody.[26,37,39,70]

Although eight studies investigated synthetic carbohydrate antigens conjugated to the keyhole limpet haemocyanin (KLH) carrier protein.[18,29,31,32,50,72,73,81,86] only one

study reported on KLH-specific immunity.[50] In this study, proliferative responses to stimulation with KLH and the KLH-antigen complex were substantially stronger than responses to the synthetic carbohydrate itself in all women with ovarian cancer tested, similar to what has previously been reported for viral vectors.

Five studies reported use of recombinant viruses or bacteria as vectors.[45,47,59,61,69] Three of these studies reported that they investigated anti-vector immune responses. One study used a recombinant pox-virus induced anti-vector immunity for all participants with ovarian cancer.[59] Another study used a recombinant measles virus and did not show any differences in anti-measles-antibody titres, although inclusion criteria required that included participants must be immune to measles virus.[69] In the third study, use of live-attenuated listeria did result in virus-specific T-cells in some cancer patients; however, too few patients with ovarian cancer were tested to permit any conclusions regarding this specific disease entity.[61]

Adverse events

For this review, we defined adverse events as any adverse changes in health or side effects that occurred in a clinical study participant receiving treatment, irrespective of whether the event could be attributed to the treatment received.

Although 56 studies mentioned adverse events; sufficiently detailed information on adverse events that occurred during the study was available for 43 out of 67 studies. Thirty-four studies explicitly mentioned local adverse events, all of which involved local administration of the vaccine (i.e. intradermal, intramuscular, or subcutaneous injection). When local adverse events were further specified, these were best summarised as pain at the injection site and local inflammatory responses (erythema, induration, pruritis). Researchers observed ulceration and/or abscesses at the injection site in nine of 89 participants with varying types of cancer participating in four studies.[31,32,55,58,82] One study described a patient with a grade III infection presenting with lower-limb lymphoedema at the injection site, which was attributed to the vaccine. This patient underwent a pelvic lymphadenectomy during the primary debulking surgery, suggesting in this case that women who have undergone pelvic lymphadenectomy might be less suitable for vaccination of the lower limbs.[71]

Systemic adverse events occurred in 42 studies, and four studies explicitly reported that systemic adverse events did not occur. Two studies explicitly reported

autoimmunity. In one study, a patient with strong immunological responses to the vaccine developed symptomatic hypothyroidism necessitating replacement therapy.[79] Study authors described minor induction of anti-nuclear antibodies (grade I according to Common Terminology Criteria for Adverse Events (CTCAE) v4.0[94]) for two patients receiving a multi-peptide vaccine.[77] Allergic reactions occurred in a total of 14 participants.[23,24,27,39,51,68,72,88] Allergic reactions (e.g. hypersensitivity, allergic exanthema, urticaria) were mild and were easily managed. Continuation of study treatment did not result in renewed allergic reactions.[27,39,68,88] Treatment with chemotherapy, an anti-methylation agent, and an NY-ESO-1-targeting vaccine resulted in clinically manageable adverse events.[75]

Other reported systemic adverse events, irrespective of whether attributable to the investigated drug, included haematological changes (e.g. anaemia, leucopenia), flu-like symptoms (including fatigue, myalgia, arthralgia, headache, fever, and chills), and gastrointestinal events (e.g. nausea, vomiting, diarrhoea, abdominal pain), most of which were classified as grade I or II events. Thirty-three studies reported serious (CTCAE grade III or IV) adverse events that varied from recurrent or progressive disease to local ulceration at the injection site, and from abdominal pain, neutropenia, and fever to elevated liver enzymes. One study compared standard of care versus MUC1 dendritic cell therapy. Respectively, 8% versus 27% of participants suffered an adverse event grade III or IV.[34] Another study combining vaccination with chemotherapy reported 10 high-grade adverse events, nine of which were attributed to the chemotherapy.[71] In addition, one study comparing chemotherapy alone versus chemotherapy and PegIntron versus chemotherapy, PegIntron, and *p53* vaccination reported grade III or IV adverse events in 50% of participants, with no significant differences between treatment groups.[67] A study combining chemotherapy, an anti-methylation agent, and an NY-ESO-1-targeting vaccine described three serious adverse events, which study authors did not attribute to any of the investigated drugs.[75] Twenty studies reported no serious adverse events. Ten studies did not mention lack or presence of serious adverse events.[20,39,85,40–43,50,73,80,84]

Discussion

Summary of main results

The aim of this review was to evaluate the clinical and immunological efficacy of antigen-specific active immunotherapy in ovarian cancer, whilst also obtaining an impression of the safety and tolerability of this treatment modality. The antigen-specific active immunotherapy described in this review can largely be divided into two strategies: (1) administration of antibodies targeting a specific tumour antigen and (2) administration of, or parts of, a specific tumour antigen itself. As expected, most studies were non-randomised controlled trials (NRSs).

Data suggest that almost all strategies are capable of inducing an immunological response to some extent. Furthermore, only two studies evaluated recognition of autologous tumour cells in vitro, and no studies evaluated immune responses at the tumour site. Although obtaining autologous tumour material may be burdensome, such assays would be extremely valuable, as they comprise true interactions between induced immunity and tumour cells and as such could provide important information on how immunotherapeutic strategies can continue to be improved to reach clinical effectiveness. Even though comparison between studies is difficult, it seems that most antigen-specific therapies, independent of the target, are able to induce at least a minimal immune response.

Clinical responses to immunotherapy (i.e. tumour responses, responses to post-immunotherapy treatment, and survival benefits) were observed only incidentally, and their occurrence cannot be used to draw a reliable conclusion. The indication for immunotherapeutic treatment in the adjuvant setting is supported by the observation of enhanced antigen-specific responses to immunotherapy when combined with chemotherapeutic agents currently or previously used in the primary treatment of ovarian cancer (i.e. docetaxel or cyclophosphamide).[95,96] However, four large randomised controlled trials (RCTs) using a monoclonal cancer antigen (CA)-125 antibody in the adjuvant setting after successful primary therapy did not demonstrate any differences in time to relapse and/or overall survival between treatment and placebo arms[20–25], which indicates that despite immunogenicity, CA-125-targeted monoclonal antibody therapy is clinically ineffective. For studies of other vaccine types, no such conclusions can be made at this time, as large RCTs and more studies in the

adjuvant rather than recurrent setting have yet to be performed to examine the different strategies.

Eighty per cent of studies reported adverse events in sufficient detail for interpretation. Study authors made a distinction between local and systemic events and further subdivided the latter into autoimmunity, allergy, and other adverse events. We did not evaluate whether adverse events could be or were considered attributable to the treatment studied, although for local adverse events, this is indisputably the case. Studies using intradermal, subcutaneous, or intramuscular application have frequently reported inflammatory reactions and pain at the injection site, with ulceration at the most severe side of the spectrum. Severe or life-threatening systemic adverse events occurred in approximately 50% of studies. Thirty per cent of studies explicitly described the lack of severe adverse events. For monoclonal antibody studies, researchers could identify no pattern suggestive of an underlying treatment-associated process and often considered events to be associated with ovarian cancer progression.

In summary, this review describes 67 immunotherapy studies including 3632 women with ovarian cancer. It seems that although all strategies described are capable of inducing immunological responses, be it humoral or cellular, clinical effectiveness thus far has not been convincingly demonstrated. The largest body of evidence is available for CA-125-directed antibody therapy, which has been studied in 2347 people participating in 17 studies. As only one study reported complete or partial clinical responses and four large RCTs did not demonstrate any clinical benefit of antibody treatment, we believe it is unlikely that the clinical effectiveness of CA-125-directed antibody therapy for ovarian cancer will ever be obtained. It is possible that inducing an immunological response alone is not enough to derive clinical benefit owing to immune suppressive characteristics of the tumour. To overcome this suppression, combining antigen-specific immunotherapy with other forms of immunotherapy (e.g. checkpoint inhibitors, chemotherapy, poly ADP ribose polymerase (PARP) inhibitors, anti-methylation agents) might be necessary to achieve clinical response. However, in view of the immunological responses and the usually mild side effects reported, we believe that further investigation of other antigen-specific active immunotherapy strategies in ovarian cancer is worthwhile.

Overall completeness and applicability of evidence

The most striking observations of this review unfortunately do not concern the aim of the review but address lack of uniformity in the conduct and reporting of early-phase immunotherapy studies.

According to the GRADE rating, only certainty for the primary outcome survival is assessed as 'high', whereas that for all other outcomes is assessed as 'very low' (Summary of findings table 1). Of note, most of the RCTs that were analysed for survival were investigating a CA-125 monoclonal antibody. Their results may not be applicable in a similar way for other strategies using antigen-specific immune therapy for ovarian carcinoma.

Reliability of the results for clinical response to immunotherapy was questionable because clear response definitions were lacking, and because concomitant immunotherapy or administration of additional treatment after immunotherapy often was not described. Furthermore, for studies that used a monoclonal antibody targeting CA-125, use of CA-125 as a marker for clinical response is questionable. An additional important comment regarding the likelihood of clinical response to immunotherapy, especially in uncontrolled studies, which frequently include patients with recurrent disease, is the fact that this likelihood may be affected by disease status at the start of treatment.[97]

In addition, antigen-specific humoral and/or cellular immunogenicity of different interventions showed great variation for both monoclonal antibody studies and studies examining other strategies. This variation may be attributed at least in part to variation in the immunological response definitions used by different study authors. Therefore it is not possible to reliably compare studies and infer which intervention and/or immunisation strategy is most promising for the induction of strong anti-tumour immunity.

A disturbing observation regarding adverse events is the lack of uniformity in adverse event reporting. Reporting of safety and tolerability of new treatment strategies should have high priority in all studies of investigational drugs, especially in uncontrolled phase I and II studies. To promote uniformity in adverse event evaluation and reporting, as well as comparability of adverse events between studies, in addition to the National Cancer Institute Common Terminology Criteria for Adverse Events (NCI CTCAE)[94], the Brighton Collaboration has committed itself to developing standardized,

widely disseminated, and globally accepted case definitions for an exhaustive number of adverse events following immunisation, as well as guidelines for data collection, analysis, and presentation.[98] These case definitions and guidelines are freely available, and we strongly recommend that, when applicable, they be used for all immunotherapeutic studies.

This review emphasises an aspect of immunotherapeutic studies that warrants serious attention in the immunotherapeutic scientific community, that is, lack of consensus on (1) what assays should be used to establish immunogenicity of an intervention[99], (2) what cutoffs should be used to define true immunological responses, and (3) what response definitions should be used to determine clinical efficacy. Given these large inconsistencies, it is evident that elucidation of which type of immunological response is necessary for and/or is a surrogate marker of clinical activity of an immunotherapeutic intervention is burdensome.

Quality of the evidence

We assessed the included studies for risks of bias, using the Cochrane 'Risk of bias' tool. Risk of bias items, especially selection, attrition, and selective reporting bias, are likely to affect the studies included in this review.

It is interesting to note that for 10 studies described in this review, review authors collected study information only from a meeting abstract that was several years old. The lack of full-text manuscripts, even after contact was made with abstract authors, strongly suggests the existence of a publication bias. To avoid the disappearance of negative studies, registration of trials in a prospective trial register is widely recommended and is supported by the International Committee of Medical Journal Editors (ICMJE). However, at first, in 2005, registration was requested only for RCTs. Since July 1, 2008, all trials prospectively assigning human participants to one or more health-related interventions for evaluation of their effects on health outcomes are required to be registered in a clinical trial register approved by the World Health Organization (WHO). From the ongoing studies section, it is apparent that despite registration in a prospective trial register, studies may suffer from publication bias, as several relatively small studies that began more than five years ago have not yet been published to date nor closed according to the trial register. In addition to registration in trial registers, the uniform requirements for manuscripts submitted to biomedical

journals drafted by the ICMJE encourage uniformity in reporting of clinical trials by stating ethical principles for the conduct and reporting of research and by providing recommendations related to specific elements of editing and writing. As is obvious from this review, the scientific community might benefit substantially if early-phase uncontrolled clinical trials would also strive for uniformity in trial conduct and reporting.

Potential biases in the review process

We minimised potential biases in the review process by searching the literature from a variety of sources with no restrictions on date of publication. At least two review authors independently extracted and assessed data.

To minimise the chances of error and bias, review authors adhered to Cochrane guidelines for selection of studies, extraction of data, and assessment of the certainty of evidence and potential risks of different types of biases in all included studies.

Agreements and disagreements with other studies or reviews

Our findings are in broad agreement with those presented by most systematic reviews on antigen-specific active immunotherapy for ovarian cancer.[100–102]However, the focus of current publications leans more towards immunotherapy in general (e.g. whole tumour lysate-targeting immunotherapy, immune checkpoint blockade, cytokine induction, adoptive cell transfer) and not towards antigen-specific immunotherapy alone. The general consensus is that antigen-specific immunotherapy is sufficient for eliciting an immune response, but clinical response to monotherapy is only modest.[100,102] Combining antigen-specific immunotherapy with other types of immunotherapy, especially immune checkpoint blockade, is a promising approach to be examined by future researchers.[101,102]

Authors' conclusions

Implications for practice

At this point, review authors have found no evidence of effective immunotherapy for ovarian cancer. Although promising immunological responses have been observed for most strategies evaluated, they do not coincide with clinical benefits for women with ovarian cancer. Furthermore, no immunological surrogate markers currently correlate with clinical outcomes. Therefore, until evidence of true clinical effectiveness is available, immunotherapy should not be offered as an alternative to standard therapy for primary or recurrent ovarian cancer.

Implications for research

Our primary recommendation relates to the need for uniformity in trial conduct and reporting. Not until universally accepted immunological and clinical response definitions and guidelines for adverse events reporting are adopted for immunotherapeutic studies will it be possible to make any inferences about the effectiveness of immunotherapy as a treatment for ovarian cancer. Furthermore, expanding evaluation of immunogenicity to include recognition of autologous tumour is advisable. Given the usually mild side effects and the immunological responses witnessed in most studies, we believe that further investigation of antigen-specific active immunotherapy other than cancer antigen (CA)-125-targeted antibody therapy for ovarian cancer in randomised controlled trials is worthwhile. In addition, research combining antigen-targeted immunotherapy with other forms of immunotherapy to optimise response, and perhaps induce clinical response, is of interest.

Acknowledgements

We would like to thank all members of the Cochrane Gynaecological, Neuro-oncology, and Orphan Cancers Editorial Team for their support.

This project was supported by the National Institute for Health Research, via Cochrane Infrastructure funding to the Cochrane Gynaecological, Neuro-oncology, and Orphan Cancer Group. The views and opinions expressed therein are those of the authors and do not necessarily reflect those of the Systematic Reviews Programme, NIHR, NHS, or the Department of Health.

Contributions of authors

NL selected relevant studies, assessed study quality, extracted data, and wrote the review. HWN selected relevant studies, assessed study quality, and extracted data. TD and WH checked all rejected titles and resolved disagreements on study selection and data extraction. HMB and BC provided statistical and methodological support. KM was supportive of writing the review as an expert in immunology. STP and MDB selected relevant studies, assessed study quality, extracted data, and wrote the second update of this review.

Declarations of interest

Ninke Leffers, Cornelis Melief, Toos Daemen, and Hans Nijman were investigators in two studies included in this review.[62,63,66] No potential conflicts of interest are known for the other contributing review authors (WH, BJC, STP, MDB).

Supplementary data:

Characteristics of included, excluded and ongoing studies are published in the online journal. Also, additional tables are published in the online journal and include:

Table 1. Study report to assess quality of non-randomized, non-controlled studies.

Table 2. Overview of included studies.

Table 3. Assessment of quality of non-randomized, (un)controlled studies.

Table 4. Evaluation of clinical responses to Immunotherapy.

Table 5. Definitions and results of survival and/or relapse analysis in antigen-specific antibody studies.

Table 6. Definitions and results of survival and/or relapse analysis in other antigen-specific immunotherapy studies.

Table 7. Definitions and results of anti-idiotypic (Ab2) humoral responses in antigen-specific monoclonal antibody studies.

Table 8. Definitions and results of anti-anti-idiotypic (Ab3) humoral responses in antigen-specific antibody studies.

Table 9. Definitions and results of humoral response evaluation in other antigen-specific immunotherapy studies.

Table 10. Definitions and results of cellular responses in antigen-specific antibody studies.

Table 11. Definitions and results of cellular responses in other antigen-specific immunotherapy studies.

Table 12. Definitions and results of human-anti-mouse antibody (HAMA) evaluation in antigen-specific antibody studies.

Appendices are published in the online version and include:

Appendix 1. CENTRAL search strategy

Appendix 2. MEDLINE search strategy

Appendix 3. Embase search strategy

Appendix 4. Data extraction form.

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Chapter 6

Ovarian cancer treatment with a liposome formulated mRNA vaccine in combination with (neo-) adjuvant chemotherapy:

Study protocol of the OLIVIA phase I clinical trial

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Abstract

Advanced stage epithelial ovarian cancer (EOC) is the leading cause of death from gynaecological malignancies with a 5-year survival of no more than 40%. Current treatment, comprising chemotherapy and surgery, is initially effective, but most patients suffer from chemotherapy-resistant relapse. Immunotherapy is a novel therapeutic strategy under investigation in EOC, including vaccination strategies targeting tumor associated antigens (TAA). Preclinical and clinical data support the intrinsic immunogenicity of EOC and provide a rationale for clinical therapeutic exploitation. In this first-in-human phase 1 clinical trial, an optimized liposome formulated messenger ribonucleic acid (mRNA) vaccine, , RNA-lipoplex (RNA-LPX) with three different mRNAs targeting one EOC tumor-associated antigens each (BNT115) will be tested. BNT115 vaccinations are scheduled around standard-of-care treatment with neoadjuvant as well as adjuvant carboplatin/paclitaxel chemotherapy. The primary objective is to determine the systemic induction / expansion of BNT115 antigen vaccine specific T cells. BNT115 represents a promising therapeutic vaccine for the treatment of EOC. Currently inclusion of patients is ongoing.

Trial registration: NCT04163094; NL66895.000.18

Background

Advances in chemotherapeutic or targeted treatment strategies of epithelial ovarian cancer (EOC) have remained relatively unchanged for 30 years. EOC remains the deadliest gynecological malignancy with a 5-year survival rate of only 40%. Therefore, novel approaches for treating EOC are urgently needed. Immunotherapy is a novel therapeutic strategy under investigation in EOC.

The presence of leukocyte infiltrates, mostly T lymphocytes and macrophages, is frequently described in EOC[1,2]. Higher numbers of cytotoxic CD8+ T cells and increased CD8+/CD4+ ratios within the tumor epithelium are associated with improved survival[3–6]. In addition, expression of immune-suppressive molecules such as PD-L1 are associated with a poorer prognosis.[7] Taken together, activation of the immune system directed at the tumor is a positive prognostic marker in EOC.

Tumor-associated antigens (TAA) are considered targets for immunotherapy via vaccination strategies. However, the effect of vaccines targeting TAAs has been moderate[8,9]. Partly this can be explained by the inability to effectively overcome tumor-induced immune suppression[10]. Chemotherapy may be used to overcome this obstacle by generating a tumor environment in which cancer vaccines have a better chance of success. Three mechanisms may work to enhance tumor-specific immune response elicited by a vaccine: 1. Targeting the immune system to reduce tumor-induced immune suppressive cells; 2. Targeting the tumor to increase immunogenicity (increase Major Histocompatibility Complex (MHC) or antigen expression); 3. Directly stimulating effector response by activating T cells. Standard of care (SoC) chemotherapy in EOC patients is paclitaxel/carboplatin. Preliminary studies have shown that use of paclitaxel/carboplatin chemotherapy induces a decrease in T-regulatory cells and an increase in Th1, Tc1 and natural killer cells in EOC patients[11]. In cervical cancer, paclitaxel/carboplatin treatment was associated with reduced frequency of myeloid cells in both tumor and blood, whereas frequencies of $CD4^+$ and $CD8^+$ T cells remained unchanged[12]. Combination treatment of paclitaxel/carboplatin and a therapeutic HPV16 vaccine in cervix carcinoma induced tumor immunity and demonstrated longer survival in patients with a high vaccine-induced immune response[13]. This data suggests paclitaxel/carboplatin could potentially enhance immunotherapy, and thereby enhance vaccine-induced immune response.

In order to apply successful immunotherapy strategies in the clinic, diagnostics to detect early response are needed. Positron Emission Tomography (PET) is an imaging technique that, with the appropriate tracer, allows repetitive, non-invasive assessment for immune activation, in all tumor lesions and normal organs in the patient[14]. PET uses a radiolabeled probe that is injected and binds *in vivo* to a target specifically expressed by e.g. activated lymphocytes. Upon activation, T cells start to overexpress the receptor for the cytokine interleukin-2 (IL2) on their cell membrane. To enable the tracking of tumor infiltration of T cells, we developed the PET tracer [^{18}F]FB-IL2[15,16].

In the current study, an optimized liposome formulated messenger ribonucleic acid (mRNA) vaccine, RNA-lipoplex (RNA-LPX), with three different mRNAs targeting one EOC TAA each, was developed (BNT115) to induce an immune response against EOC. In addition, the [^{18}F]FB-IL2 PET-CT will be used for the non-invasive assessment of T-cell activation. BNT115 vaccinations will be scheduled around SoC treatment with neoadjuvant as well as adjuvant carboplatin/paclitaxel chemotherapy in patients newly diagnosed with EOC. The application of vaccination before cytoreductive interval surgery also allows assessment of intratumoral accumulation of vaccine-induced T cells, an important prerequisite for successful immunotherapy.

Methods

Investigational product

Patients will be treated with the BNT115 vaccine including 3 RNA-LPX drug products (RNA DPs) each encoding one OC TAA. All target antigens are known to be immunogenic and epithelial ovarian cancers express at least one of the three antigens. The RNA DPs are optimized for the induction of strong antigen-specific immune responses[17–19]. In addition, RBLTet.1, a RNA-LPX product coding for a tetanus toxoid-derived helper sequence, is added to each RNA DP creating RNA DP mixes to further enhance the immunogenicity of the vaccine. The three RNA DP mixes are individually complexed with liposomes and separately administered in three sequential injections. The liposomes allow RNA protection from degradation by plasma RNases after intravenous application and selective RNA vaccine targeting to antigen presenting cells (APCs) predominantly in the spleen and other secondary lymphoid organs. Upon selective targeting of professional APCs in lymphoid compartments following intravenous administration, the mode of action of RNA-LPX vaccination[20] is comparable to that of intranodally injected RNA vaccines. When the mRNA is taken up by APCs via micropinocytosis, it is translated into encoded proteins. The encoded proteins are processed and presented on MHC molecules as protein-derived peptides. The anti-tumor activity derives from concomitant maturation of the APCs, induction of TLR-mediated immunomodulatory effects and the stimulation and expansion of antigen-specific $CD8^+$ and $CD4^+$ T-cell responses that elicit anti-tumor activity.

Objectives

The primary objective of the trial is to determine the systemic induction and expansion of BNT115 antigen vaccine-specific T cells.

Secondary objectives include the intratumoral induction and expansion of BNT115 vaccine antigen specific T cells. Secondly, progression free survival (PFS) of primary EOC patients treated with the BNT115 vaccine in combination with carboplatin and paclitaxel is monitored. Lastly, safety and tolerability of repetitive doses of BNT115 vaccine in combination with carboplatin and paclitaxel is determined.

In this study, the exploratory objective is the intratumoral visualization of $CD25^+$ T cells using the [^{18}F]FB-IL2 PET-CT.

Study design & interventions

This is a first-in-human, open label phase-I study in EOC patients with primary disease eligible for SoC treatment with neo-adjuvant chemotherapy, i.e. 3 cycles carboplatin/paclitaxel, interval surgery and 3 additional cycles carboplatin/paclitaxel.

EOC patients will be vaccinated prior and during neo-adjuvant and adjuvant chemotherapy with the BNT115 vaccine which will be administered by means of intravenous injections. A total of eight vaccinations will be administered with intra-patient dose escalation planned for the first two doses. The first two vaccinations are administered before start of neo-adjuvant chemotherapy with a 7 day (+/- 2 days) time lag between each vaccination. The subsequent 6 vaccinations are scheduled 15 days (+/- 3 days) after the start of each cycle of chemotherapy to avoid overlap with immune-suppressive corticosteroid pre-medication as well as with the direct side effects of chemotherapy.

Patient evaluation will be performed before, during and after each vaccination including physical examination, Eastern Cooperative Oncology Group (ECOG) performance status, toxicity scores and blood sample collection for bio monitoring analyzing biochemistry, hematology and tumor marker CA-125. To determine the vaccine specific systemic immune response (primary objective), peripheral blood mononuclear cells (PBMCs) are obtained by venous blood collection before, during and after vaccinations. To determine the intratumoral accumulation of T-cells recognizing vaccine-encoded TAAs, tumor material is collected at baseline, via tumor biopsy, and after the 5th vaccination using tumor tissue derived from interval surgery. Lastly, the [^{18}F]FB-IL2 PET-CT will be used for the non-invasive assessment of T-cell activation and correlated to immunohistochemistry tumor tissue data from pre-treatment biopsy and interval debulking surgery.

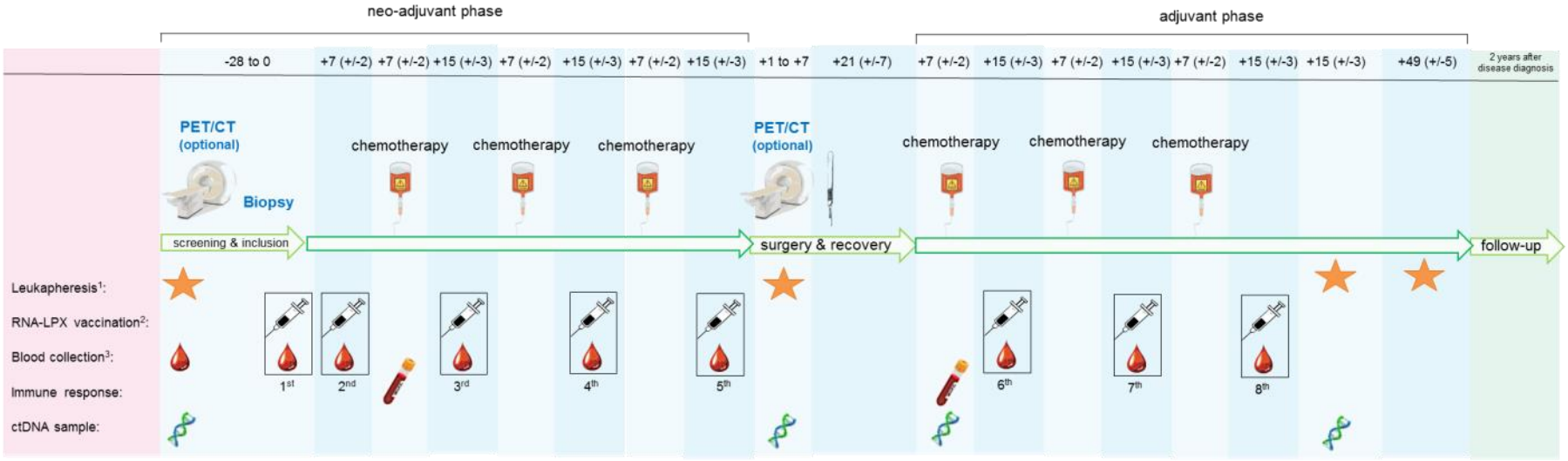

Figure 1 OLIVIA trial design.

[1] Leukapheresis can be replaced by blood draw of 100mL, if the patient refuses or has contraindication for leukapheresis. During visits 1 and 12 leukapheresis is always replaced by blood draw of 100mL.

[2] Patients will be treated with a BNT115 vaccine including three RNA-LPX drug products each encoding one EOC TAA

[3] PBMC and ctDNA collection can also be performed in combination with visits carried out for PET/CT scan, biopsy and 1st vaccination. Initial PBMC collection must take place before administration of the first vaccination.

RNA-LPX: ribonucleic acid lipoplex, EOC: epithelial ovarian cancer, TAA: tumor associated antigens, PBMC: Peripheral blood mononuclear cells, ctDNA: circulating tumor deoxyribonucleic acid.

Study population & sample size

Primary EOC patients with measurable tumor lesions by imaging techniques (Computer tomography (CT) or Magnetic Resonance Imaging (MRI)), who are intended to be treated with 3 cycles neo-adjuvant chemotherapy, interval surgery and 3 cycles adjuvant-chemotherapy will be included. Patients will only be included in this trial when they are in stable health, which will be measured by ECOG status and weight loss. Patients suffering from extensive ascites, pleural effusion and/or weight loss will not be asked to participate.

Exclusion criteria are a history of a second malignancy, except for curatively treated low-stage tumors with a histology that can be differentiated from the epithelial EOC type; ongoing or recent evidence (within the last 5 years) of significant autoimmune disease that required treatment with systemic immunosuppressive treatments; use of systemic continuous corticosteroid therapy; pregnancy or breast feeding; participation in a trial with another investigational drug within 30 days prior to the enrolment in this trial and any condition that in the opinion of the investigator could interfere with the conduct of the trial.

This study aims to establish the systemic immunogenicity of the BNT115 vaccine. There is no formal statistical sample size calculation based on the nature of this first-in-human phase I study. It is anticipated that 10 evaluable patients will be enrolled in the initial assessment. The data obtained from these 10 patients will be evaluated on the feasibility, safety, tolerability, and preliminary signs of immunogenicity and efficacy.

Immunomonitoring

To quantify the induction of a vaccine specific systemic immune response, PBMCs collected before and after vaccination will be compared. Samples will be analyzed using a validated ex vivo ELISPOT assay in a GCLP-compliant environment to screen for presence of antigen-specific T-cell responses to vaccine antigens.

Additional assays to determine the nature of the induced systemic immune response may be applied on the PBMCs collected at baseline, interim time points and follow-up to assess the influence of chemotherapy and pre-medication on T-cell response. These include:

- ELISPOT assays to screen for presence of antigen-specific T-cell responses, other tumor-associated antigens and recall antigens.

- Flow cytometry-based assays for a more detailed analysis of the phenotype and function of antigen-specific T-cell responses to vaccine antigens, other tumor-associated antigens and recall antigens.
- Flow cytometric analysis of other immune cell populations such as B cells, NK cells, myeloid–derived suppressor cells, and regulatory T cells.
- ctDNA will be isolated using standard techniques and commercially available kits. Amongst others, isolated circulating nucleic acids will be analyzed for abundance and the presence of mutations by PCR.
- TCR Profiling of T cells from peripheral blood, and TILs by next generation sequencing (NGS) to study changes in the TCR repertoire during the therapy with RNA-LPX.

To measure the induction of an intratumoral immune response, tumor samples before vaccination (biopsy) and after the 5th vaccination (surgery) are compared using immunohistochemical staining and quantification of CD8 positive cells using well-established criteria[6].

The [^{18}F]FB-IL2 PET-CT

A whole body [^{18}F]FB-IL2 PET-CT scan will be taken prior to baseline biopsy (before vaccination) and as close to interval surgery as possible (after 5 vaccinations). Patients will be intravenously injected with approximately 200 MBq [^{18}F]FB-IL2. Sixty minutes after injection of the tracer, to allow proper distribution of the PET tracer, a whole body PET scan (head to mid-femur) and a low-dose CT scan will be acquired. The low-dose CT is used for attenuation and scatter correction of the PET scan and to provide anatomical reference. For quantification of tracer uptake, regions-of-interest will be drawn around all lesions visible on the PET and CT scans. Tracer uptake will be corrected for body weight and injected dose and expressed as SUV. The results from the [^{18}F]FB-IL2 PET-CT will be compared with corresponding tumor material which will be assessed on immunological activity in the tumor, including T-cell density and IL2 receptor expression.

Statistical analysis

The primary endpoint is considered positive when at least 2 out of 10 patients have a proven vaccine induced systemic immune response.

The ELISpot Data Analysis Tool 1.0 (EDA-001 tool) will be used for statistical analysis of systemic immune response to vaccine-targets. The EDA tool has been developed based on recommendations as proposed by Moodie et al[21]. Each stimulated sample is compared to its relevant negative control using the distribution-free resampling (DRF) method with a null hypothesis of less than or equal to twofold difference between negative control and stimulated replicate means (DFR(2x)). A positive call will be issued if the stimulated sample being tested:

- is significantly different from its respective negative control
- fulfills the minimum spot count threshold (≥ 15 spots)
- passes the IRV (Intra-Replicate-Variability) threshold.

The secondary endpoint, a local immune response in the tumor, will be defined as a significant increase in CD8-cell density using immunohistochemistry (CD8-cells / mm2 cancer epithelium [cytokeratin-positive area]). A two-sided paired t-test will be used to determine significance ($p<0,05$ is considered significant).

To assess the exploratory endpoint, results from the intratumoral visualization of CD25+ T cells by the [^{18}F]FB-IL2 PET-CT imaging (expressed as standardized uptake values (SUV)) is compared to CD25+ T-cell infiltration in matching tumor material (evaluated by immunohistochemistry).

Discussion

Various immunotherapeutic strategies have been explored in early phase clinical trials in order to improve long-term survival of EOC patients[22]. These immunotherapeutic strategies aim to stimulate the immune system by stimulating cytotoxic T cells against ovarian cancer cells. Vaccines used in cancer therapy administer specific TAA via various methods. However, despite promising immunological responses, thus far antigen-specific vaccination strategies have shown limited clinical efficacy.[9] Another immunotherapeutic strategy of high interest is the use of immune checkpoint blockade (ICB). Unfortunately, ICB monotherapy as treatment for EOC has thus far shown limited response rates of only 10-15%[23–25]. Response to ICB appears to be dependent on pre-existing anti-cancer immune responses[26–28]. In EOC, such pre-existing immune

responses are often absent, rendering ICB ineffective. As such, strategies to extend the benefit of ICB to non-responding patients are focused on combination strategies that induce anti-cancer immune responses that can subsequently be 'unleashed' by ICB.

The BNT115 vaccination is an RNA-based "off-the-shelf" vaccination. In general, RNA vaccines have the advantage of delivering all epitopes of a whole antigen without the risk of integrating into the genome. The antigen-encoding RNA-based vaccine strategy has shown to efficiently elicit antigen-specific CD8+ and CD4+ T-cell responses[19,30–34] and to elicit polyepitopic humoral immune responses against surface antigens both pre-clinically and clinically[35,36] for both the prevention of infectious diseases and the treatment of cancer.

The best known RNA based vaccines currently approved are BNT162b1 and BNT162b2, designed for the prevention of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infections and the resulting disease, coronavirus disease 2019 (Covid-19)[37,38].

The most extensive data set available for the RNA-LPX vaccine platform is of the Lipo-MERIT trial that investigates BNT111, an RNA-LPX vaccine which makes use of a similar approach but encoding a set of melanoma TAA[39]. In the safety analysis population (data lock point 01-05-2020), the most frequently occurring related treatment-emergent adverse events (TEAEs) were pyrexia, followed by chills, headache, fatigue, nausea, tachycardia, vomiting, feeling cold, arthralgia, injection site pain, and hypotension. These flu-like symptoms constitute the typical safety profile of the BNT111 cancer vaccine and correlate with its mechanism of action (type I interferon (IFN) release). The majority of the flu-like events were of Grade 1 or 2 and lasted less than 3 days. The events were manageable by administration of antipyretics and analgesics without use of corticosteroids. Hypotension occurred after the first or second vaccination in up to 14% of patients and within 6 h after vaccination. Patients with hypotension responded well to fluids while no vasopressors were given or necessary to control the clinical situation. A similar safety profile is expected for the RNA-LPX cancer vaccine BNT115 used in this trial at comparable doses[20,39]. The interim analysis of the Lipo-MERIT trial shows that BNT111, alone or in combination with immune checkpoint blockade (ICB), mediates durable objective responses in patients with unresectable melanoma. Clinical responses are accompanied by the induction of strong CD4+ and CD8+ T cell immunity against the vaccine antigens[39]. Interestingly, response rates were

not correlated with tumor-associated antigen expression nor mutational burden, supporting the applicability of this combinatorial strategy in tumors with low mutational burden such as EOC[40].

Within this phase I first-in-human clinical study, vaccination is combined with SoC chemotherapy, carboplatin/paclitaxel, described to also reduce the immune suppressive environment. Patients receive dexamethasone (Dexa), a glucocorticosteroid, as supportive medicine to reduce potential side-effects of chemotherapy treatment. Given the immunosuppresive activity, Dexa is usually avoided in combination with immunotherapy. Indeed, a recent study in both mice and human PBMC shows that Dexa substantially blunts RNA-LPX vaccine-mediated immune effects. Interestingly, these effects were less when Dexa was administered as post-medication[29]. In this context, the first two vaccinations in this trial are scheduled before start of chemotherapy/Dexa treatment. Even more, the 6 booster vaccinations are scheduled approximately 2 weeks after each cycle chemotherapy/Dexa to limit the immunosuppressive effects.

In conclusion, BNT115 is intended to be developed as a therapeutic cancer vaccine for the treatment of epithelial OC and may hold promise. Currently inclusion of new patients is ongoing.

Acknowledgements

The study is supported by the Dutch Cancer Society: project grant RUG 2015-7235.

BioNTech SE is the collaborative party in the development and execution of this clinical trial.

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Chapter 7

Summary & discussion

Summary

Epithelial ovarian carcinoma (EOC) is characterized by a poor prognosis and overall survival (OS) has barely improved over the last decades. The most common histological subtype of EOC is high grade serous carcinoma (HGSOC) which accounts for approximately 70% of EOC cases. Current primary treatment consists of cytoreductive surgery and platinum-based chemotherapy, usually the DNA intercalating carboplatin, and cell cycle inhibitor paclitaxel. Patients are either treated with primary debulking surgery (PDS) followed by six cycles of chemotherapy or are initially treated with three cycles of neo-adjuvant chemotherapy (NACT), followed by an interval debulking surgery and three cycles of additional chemotherapy. Choice of treatment strategy is tailored for each individual patient. Patients are selected for PDS based on the estimation of whether the entire tumorload can be removed during surgery, taking into account tumor location, presence of metastases and clinical condition of the patient. If full surgical resection is not considered feasible, NACT is used to reduce tumor burden prior to interval debulking. The most important prognostic factor is surgical outcome, which is defined as complete (no residual macroscopic tumor tissue after surgery), optimal (residual tumor lesions <1cm after surgery) or incomplete (residual tumor lesions >1cm after surgery). Although initial response to primary treatment is high, most patient relapse within 2 years and succumb to their disease. Novel therapies for HGSOC are therefore under investigation, including immunotherapy.

Immunotherapy, such as immune checkpoint inhibition (ICI) and vaccination - aims to activate the body's own immune system to recognize and eliminate cancerous cells. Successful application of immunotherapy is dependent on a variety of factors including the tumor immune environment, mutational load, pre-existing immunity and immune evasion by the tumor. In this thesis, we explored several aspects of the tumor immune environment, the effects of standard treatment on the local and systemic immune system and the application of immunotherapy in EOC. The research performed within this thesis is to improve current immunotherapy strategies and to evaluate novel therapeutic interventions to improve the treatment and eventually the prognosis of patients with epithelial ovarian cancer.

The tumor immune environment & immunotherapy

In **Chapter 2**, we first provide a broad overview of recent insights in tumor infiltrating lymphocyte (TIL) biology, their prognostic benefit, as well as their predictive value for therapy. We review the recent data that emphasizes the importance of not just individual lymphocyte subsets as effectors of tumor immune control, but rather the location, clustering, interplay and co-stimulation of all lymphocyte subsets together.

In **chapter 3** we apply this notion to EOC by assessing the general immune contexture during standard-of-care therapy, including during carboplatin/paclitaxel chemotherapy. We analyzed immune cell populations in a series of primary tumors, tumor-draining lymph nodes (tDLN) and circulating T cells. We observed that the immune contexture of EOC patients is defined by tissue of origin, independent of exposure to chemotherapy. Summarized, draining lymph nodes were characterized by a quiescent microenvironment composed of mostly non-proliferating naïve $CD4^+$ T cells. Circulating T cells shared phenotypic features of both lymph nodes and tumor-infiltrating immune cells. Immunologically 'hot' ovarian tumors were characterized by ICOS, GITR, and PD-1 expression on $CD4^+$ and $CD8^+$ cells, independent of chemotherapy. The presence of PD-1^+ cells in tumors of PDS treated patients, but not NACT treated patients, was associated with prolonged disease-specific survival (DSS). We established that the loss in prognostic benefit of epithelial PD-1^+ cells of NACT patients could be explained by inadequate antigen presentation and observed high MHC-I expression in tumors prior to chemotherapy, but minimal MHC-I expression in tumors after neo-adjuvant chemotherapy, even though there were no differences in the number of TILs in both groups. We propose that TILs influx into the chemotherapy tumor microenvironment may be a consequence of the general inflammatory nature of chemotherapy-experienced tumors.

To further dissect the effect of chemotherapy on the EOC immune environment a tissue micro-array (TMA) was constructed including 281 HGSOC patients from two hospitals. As mentioned in **chapter 2,** while is evident that $CD8^+$ TILs are crucial for an effective anti-tumor immune response, single-cell sequencing, has made it clear that $CD8^+$ T cells are divided into a wide variety of subsets ranging from more naive-like and proliferative to more differentiated and cytolytic immune cells. In particular, $CD8^+CD103^+$ tissue resident memory T cells (TRM) seem capable of tumor control. Accordingly, the $CD8^+CD103^+$ TRM subset is associated with improved prognosis across

malignancies, including HGSOC. In **chapter 4** we made use of a digital quantification technique to assess the abundance and location of CD8, CD103 expressing immune cells in the tumor and assessed whether this was associated with improved survival. Indeed, our results suggest the prognostic benefit of T cell infiltration in HGSOC is largely restricted to $CD8^+CD103^+$ TRM. Comparable to the prognostic benefit of epithelial $PD\text{-}1^+$ described in **chapter 3**, we observed a survival benefit of $CD8^+CD103^+$ TRM only in patients treated with PDS and with no macroscopic tumor lesions after surgery. This chapter implicates image-based quantification as a diagnostic tool to improve clinical prognostication of HGSOC patients.

Vaccination strategy in ovarian cancer

Clinical success with ICI in EOC has so far been limited. In order to improve response rates and clinical outcomes of immunotherapy-treated cancer patients, it seems that combinatorial immunotherapy regimes are the key to success. Such strategies include priming patients prior or during ICI treatment to induce an immune response via e.g. vaccination or chemotherapy.

In **chapter 5,** a systematic review, we studied the current state-of-the-art in clinical efficacy of antigen-specific active immunotherapy for the treatment of OC. In total, 67 studies were included representing 3632 woman with EOC. A striking observation was the lack of uniformity in conduct and reporting of early-phase immunotherapy studies. Response definitions show substantial variation between trials, which makes comparison of trial results difficult to interpret. Also, most strategies have not yet been tested in RCTs. Thus far, no clinically effective antigen-specific active immunotherapy is available for OC. Given the promising immunological results and the limited side effects and toxicity reported, further exploration of clinical efficacy in OC may be worthwhile. As highlighted in **chapter 2**, combinatorial immunotherapy regimes could be more successful compared to mono-therapy strategies. As such, chemotherapy may also be used to generate a tumor environment in which cancer vaccines have a better chance of success. In **chapter 6**, we therefore describe a currently ongoing study on the effect of the BNT115 vaccination in combination with standard of care treatment with carboplatin/paclitaxel chemotherapy in EOC. The BNT115 vaccination is an optimized liposome formulated ribonucleic acid (RNA) vaccine (RNA-LPX) with three

RNAs, each targeting 3 OC tumor associated antigens, and was developed to induce an immune response against OC.

Taken together, the data described in this thesis contribute to our understanding of the tumor immune biology of ovarian cancer and may help guide therapeutic intervention in the near future.

Discussion

Tumor infiltrating lymphocytes in HGSOC

This thesis describes the importance of TILs in ovarian cancer and its relation to prognosis (**chapter 2-4;** figure 1). The presence of intraepithelial $CD8^+$ T cells has been widely described to be associated with a better OS in multiple malignancies including ovarian cancer, colorectal cancer, non-small cell lung cancer, renal cell cancers, hepatocellular cancers.[1] In particular, a specific subset of $CD8^+$ T cells, the CD103 expressing TRM are especially associated with prognostic benefit in a multitude of solid tumors, including OC.[2,3] In general, EOC is characterized by a relatively low mutational burden and low numbers of TILs compared to e.g. melanoma, and lung cancer.[4,5] In **chapter 4** we confirm the predictive value of intraepithelial $CD8^+CD103^+$ TRM and, in line with current literature, most HGSOC patients (85%) were characterized with overall low number of TILs.

Next to $CD8^+$ T cells, also tumor infiltrating B-lymphocyte are important for immune control in cancer..[6,7] Intratumoral $CD20^+$ B cells have been correlated with improved survival in HGSOC.[8] Further research on the functional capacity of intratumoral $CD20^+$ B cells demonstrated hallmarks of $CD20^+$ B cell activation, antigen exposure, Ig class switching and an activated memory phenotype ($IgD^-IgM^-IgG^+$).[9] A study in HGSOC omental metastases, confirmed the high proportion of B cells with a memory phenotype, which were mainly localized in lymphoid structures.[10] However, tumor-promoting effects of regulatory B cells have also been described in ovarian cancer.[11] There is a need to identify both tumor promoting and tumor suppressing B cell markers. State-of-the art single cell analysis could help identify B cell gene expression profiles and distinct patterns of immune activation and suppression, which could be exploited by developing targeted therapy.

T cells and B cells can co-localize in well-organized TLSs resembling activated lymph nodes in cancer (**chapter 2**). The presence of both $CD4^+$ T cells and $CD20^+$ B cells have shown to influence the beneficial effect of $CD8^+$ T cells.[9,12] In this regard, $CD20^+$ B cells can function as antigen-presenting cells (APCs) to T cells, promoting local tumor-associated T cell responses. In addition to $CD20^+$ B cells, plasma B cells (PCs) can contribute to anti-tumor immunity via antibody production, triggering antibody-dependent cellular cytotoxicity and opsonize tumor antigens thereby facilitating antigen presentation (**chapter 2**). In HGSOC, the co-localization patterns of $CD4^+$ and $CD8^+$ T cells, $CD20^+$ B cells and plasma cells have been linked to the formation of TLSs.[13] The presence of all these TILs is associated with higher survival rates than tumors containing $CD8^+$ TILs alone.[13] Similar results have been reported in a variety of other cancers (**chapter 2**).[14–16] CXCL13 is one of the most relevant and well-characterized chemokines involved in the structural organization of immune cell clusters and the formation of TLSs. CXLC13 exclusively interacts with CXCR5, mainly expressed by follicular helper T cells and B cells, and regulates the organization of B cells inside the follicles of TLSs (**chapter 2**).[17] Interestingly, exhausted $CD8^+PD1^+$ T cells have shown to constitutively secrete CXCL13. In HGSOC, PD-1 expressing tissue-resident $CD8^+CD103^+$ T cells were shown to express and secrete CXCL13.[18] Subsequent analysis of

The Cancer Genome Atlas (TCGA) messenger ribonucleic acid (mRNA) expression in different tumor types, including ovarian cancer, showed a correlation between TLS-related genes and $CXCL13^+CD103^+CD8^+$ cell-related genes. Suggesting that exhausted tissue-resident $CD8^+$ T cells recruit lymphocytes towards the tumor and promote the formation of TLSs.[18] The presence of chemokine CXCL13 in HGSOC tissue has been correlated with improved progression free survival (PFS) and OS.[19]

The TLSs in HGSOC have been identified in both ovarian and omental tissue samples.[13] Kroeger et al. indicated that TLSs might be more prevalent in the omentum while TIL densities were comparable in both tissue types.[13] The prognostics advantage of TLSs in HGSOC suggests that the $CD4^+$ T cells, $CD8^+$ T cells, $CD20^+$ B cells and plasma cells work in concert to promote anti-tumor immunity. Hence, both the humoral and cellular immune system are important in immune surveillance in EOC. Knowledge on TLSs in EOC remains limited, therefore more research should be invested in the frequency, formation and anti-tumor capacity of TLSs in EOC. It is of interest to investigate why some patients form TLSs and why some do not, especially since ICI

responders are characterized by the enrichment of TLSs[20]. Identifying an unique cell surface marker capable of identifying mature TLSs could be of additive value.

The majority of the immunotherapies are designed to (re)activate T cells even though it has become increasingly clear that B cells play an important role in the anti-cancer response induced by immunotherapy. This is demonstrated by enriched B cell activation in patients responding to ICI treatment. An active humoral immune response was characterized by somatic hypermutation, IgG class switching, clonal expansion of plasma blasts and production of high-affinity antibodies.[21] The humoral immune response could be promoted through immunotherapy through e.g. the administration of recombinant antibodies combined with immune modulators mimicking the effect of PCs or through adoptive B cell transfer of tumor specific PCs.[13,21] Strategies depleting regulatory B-cells with tumor promoting capacity have also been described demonstrating contradictory results.[21] New immunotherapy strategies should focus on the simultaneous activation of both B and T cells. This could be achieved by B cell immunotherapy alone, stimulating B cell tumor infiltration and plasma B cells which will eventually promote lymphocyte infiltration and stimulate cytotoxic T cell activation to increase the anti-tumor immune response. The applicability of combining B and T cell immunotherapy should also be explored.

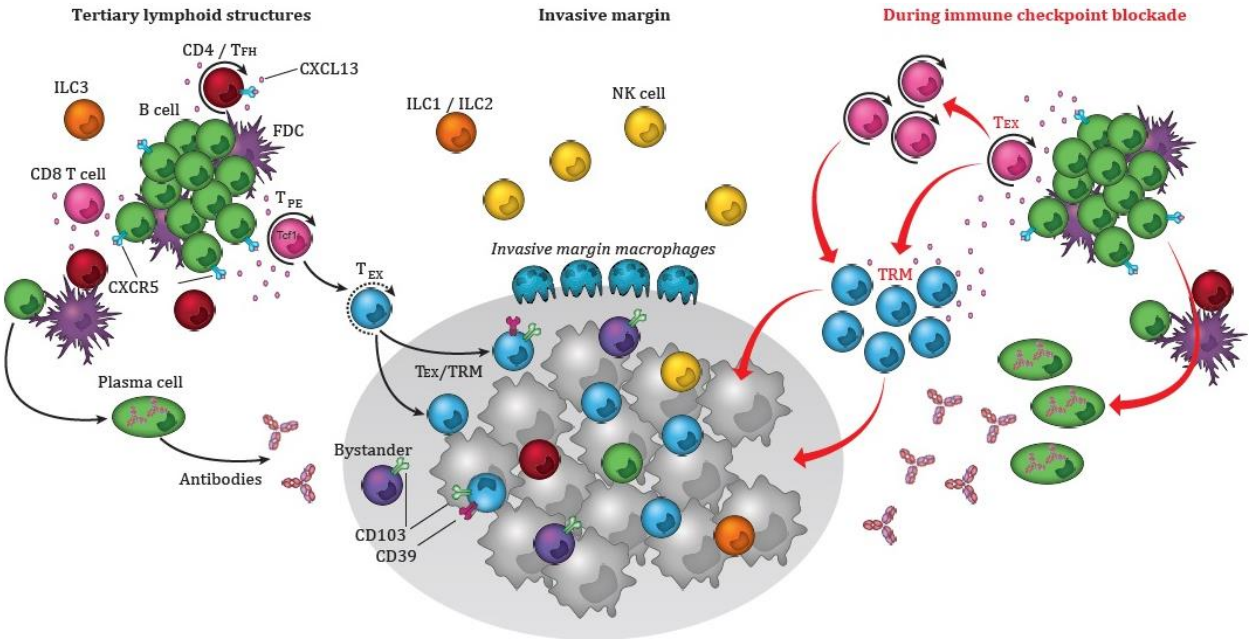

Figure 1. The tumor immune environment. The schematic representation of the tumor immune environment shows the composition and function of a TLS, who are usually found peritumorally in the stroma and/or in the invasive margin. Chemokine CXCL13, produced by $CD8^+$ T cells, induces chemotaxis by binding to receptor CXCR5, mainly expressed by B cells and T_{FH} cells and regulates the organization of B cells inside the follicles of lymphoid tissues. In the optimally organized TLS immune structure, DC, FDCs, T cells and B cells interact and activate each other, promoting a local sustained immune response including the induction of T cell effector function, antibody generation and clonal expansions. The stroma surrounding the tumor epithelium and the invasive margin further harbors cellular immune components including NK cells, macrophages and ILC1 and ILC2 and a non-immune cellular component including fibroblasts. Within the tumor epithelium ILC, NK-cells, B-cells and different T-cells subsets are present, including the $T_{EX,}$ tumor specific $CD103^+CD39^+$ TRM and $CD103^+CD39^-$ bystander TRM. Upon immune checkpoint blockade both T- and B-cells signaling is increased. TCF1 expressing T_{PE} cells, expand and differentiate into TRM/T_{EX} migrating to the tumor were they can exert their cytolytic potential. In addition, response to immune checkpoint blockade also increases B cell receptor diversity, induces clonal expansion and differentiation into antibody producing plasma cells.
TLS: tertiary lymphoid structure, T_{FH} cells: follicular helper T cells, DCs: dendritic cells, GC: germinal center, FDCs: follicular dendritic cells, ILC1-3: helper-innate lymphoid cell group 1-3, NK cells: natural killer cells, T_{EX}: terminally exhausted T cells, TRM: tissue resident memory, TCF1: transcription factor 1, T_{PE}: progenitor STEM-like exhausted cells.

Tumor immune evasion via disruption of MHC-I expression

It is evident that the presence of $CD8^+$ T cells are crucial for an effective anti-tumor immune response in HGSOC.[3,22–24] In line with previous publications[23,25], the data in this thesis demonstrate that the prognostic benefit of TILs in HGSOC is restricted to PDS patients with a complete surgery debulking (**chapter 3 and 4**). This raises the question why patients with NACT do not benefit from comparable TIL influx.

In **chapter 3** we demonstrated that the tumors collected after NACT are mostly characterized by low or intermediate (98.4%) MHC-I expression, whereas 24.1% tumors collected during PDS showed a high expression of MHC-I. The lack of proper antigen

presentation via MHC-I in the NACT cohort could potentially explain the lack of prognostic effect of TILs in these patients as they are unable to exert tumor-specific cytotoxic effects. We speculate that TIL influx into the chemotherapy tumor micro-environment is in part a consequence of the general inflammatory nature of chemotherapy induced immunogenic-cell death. The induction of immunogenic-cell death can lead to an increased availability of appropriate immunostimulatory signals that consequently stimulate an immune response. Several studies comparing matched pre- and post-chemotherapy tumor tissue have described an increase in TIL after chemotherapy.[26,27] Importantly, this study did not include matched pre- and post-chemotherapy tumor samples. Therefore, we cannot exclude that the observed low MHC-I expression was present at baseline before chemotherapy exposure. In addition, we were unable to include complete responders to chemotherapy in this study as no viable tumor tissue was left for research after NACT. These best responders could possibly represent patients with high MHC-I expression. Therefore, we would like to propose subsequent research comparing MHC-I expression and TIL influx from matched pre- and post-chemotherapy tumor tissue samples.

Downregulation of MHC-I has been identified as a mechanism of immune evasion in cancer and restoring MHC-I expression could augment immunogenic therapies.[28,29] It is possible that MHC-I expressing cells are more sensitive to chemotherapy and/or subsequent TIL influx and are therefore not detected during interval debulking (**chapter 3**). In this case, MHC-I upregulation could be accomplished via the stimulation of IFNγ production using e.g. therapeutic vaccinations. IFNγ up-regulates MHC-I expression via different mechanisms including the stimulation of NLRC5, a IFNγ inducible nuclear protein, specifically associated with the activation of the MHC-I promoters.[30,31]

Another commonly used mechanism of cancer to suppress gene function and to evade the immune system is abnormal methylation of the CpG islands in the promotor region of a gene. This mechanism could potentially be reversed by a DNA methyltransferase inhibitor (DMTi) and has been tested in platinum-resistant OC patients. Treatment with DMTi induced partial responses and was shown to restore platinum sensitivity in patients. However, severe toxicity which is probably caused by the unspecific nature of DMTi remains a concern.[32]

Burr et al., describes the loss of MHC-I antigen presentation in cancer cells through transcriptional silencing of the MHC-I antigen processing pathway by a

conserved function of the polycomb repressive complex 2 (PRC2).[33] Enhancer of zeste 2 (EZH2), a main component of the PRC2 complex is responsible for the tri-methylation of histone H3 on lysine 27 (H3K27me3), thereby inhibiting MHC-I expression.[33,34] Indeed, inhibition of PCR2 lead to restored cell surface expression of MHC-I in different cancer cell lines.[33] In ovarian cancer, EZH2 is commonly overexpressed and correlated with tumor progression.[35] In a human OC model, EZH2 was negatively associated with intratumoral $CD8^+$ T cells and patient outcome.[36] Another study using a tissue micro assay containing ovarian cancer tissue correlated $EZH2^{low}$/$H3K27me3^{low}$ with improved patient outcome and better response too chemotherapy.[37] These studies suggest restoration of antigen-presentation and increase of intratumoral $CD8^+$ T cell infiltration through EZH2 inhibiton. We would therefore argue for the exploration of combination therapy with EZH2 inhibitors and ICI.[34] This was recently demonstrated in head and neck cancer cell lines, where EZH2 inhibition enhanced antigen presentation and subsequently sensitized resistant tumors to anti-PD-1 therapy.[38] Drugs targeting EZH2 for the pharmacological inhibition of PRC2 are currently under investigation in a number of clinical trials in various cancer types including ovarian cancer.[34]

Another interesting approach, in the context of MHC-I low expressing tumors, is the stimulation and activation of natural killer (NK) cells. NK cells are defined by the absence of antigen-specific B or TCRs due to their lack of recombination activating genes. Their activity is dependent on a repertoire of costimulatory and inhibitory signals that bind to their respective ligands on the cell surface and is therefore independent of MHC-I expression. When activated, NK cells exhibit antitumor activity via the release of granzymes and perforins, the induction of TNF-related apoptosis and the production of IFNγ (**chapter 2**). Currently, several clinical trials are exploring the effectiveness of adoptive NK cell transfer in EOC.[39] Available data demonstrates that a part of the OC patients receiving NK cell therapy reached stable disease and experienced mild side effects. Better responses were seen in patients receiving repeated infusions. In addition, the additive value of intraperitoneal infusion and combination with ICI are currently under investigation.[39]

Digital quantification of TILs and personalized therapy

T cell infiltration has been extensively linked to improved prognosis. Traditionally, TIL infiltration has been manually quantified by pathological assessment. However, manual

TIL quantification by pathologists is hampered by interobserver variability, is time consuming and with the ever-increasing complexity in the understanding of TIL composition and localization, novel quantification approaches are under active development.[40–42] Digital pathology, including image-based quantification and machine learning algorithms, apply statistical methods to process data and have shown to be reproducible and reliable for analysis of tissue composition in cancer.[41] The deep characterization of the tumor microenvironment through spatial analysis and multiplexing, makes image-based quantification an efficient tool to extract comprehensive information on biomarker expression levels, co-localization, and compartmentalization.[43] The current development of digital pathology provides the opportunity to translate the prognostic benefit of TILs into a clinically usable diagnostic tool to aid clinical decisions and to improve personalized therapy (**chapter 2**).

In **chapter 4** we applied the same innovative image-based $CD8^+CD103^+$ TRM quantification technique in a HGSOC cohort as Horeweg et al. successfully demonstrated in early-stage endometrial cancer.[44] We demonstrate that the prognostic benefit of CD8CD103 TRM infiltration in HGSOC is restricted to PDS treated patients with a complete debulking. These results could potentially be used to improve ICI response rates and pave the way for personalized treatment. Patients could be stratified for treatment based on TIL infiltration, indeed, it has been well-established that ICI is most effective in tumors infiltrated by a high number of TILs.[45–47] Completely debulked PDS patients with highly infiltrated tumors might therefore particularly benefit from ICI maintenance treatment, whereas patients with complete PDS and low CD8CD103 TRM infiltration, might benefit more from a combinatorial treatment regimen of anti-tumor vaccination and subsequently ICI. This was recently successfully demonstrated in melanoma patients receiving an antigen-encoding mRNA vaccine, targeting non-mutated tumor-associated antigens, alone or in combination with ICI.[48] Interestingly, response rates were not correlated with tumor associated antigen expression nor mutational burden. Even more, vaccination administration was accompanied by IFNγ production[48]. As mentioned above, IFNγ can potentially upregulate MHC-I, thereby facilitating antigen-presentation and triggering TIL activation. This data supports the applicability of this combinatorial strategy in tumors with low mutational burden and suppressed MHC-I expression (**chapter 3**) as demonstrated in OC.

In addition to TIL quantification, identification of predictive gene signatures could add to clinical decision making and patient stratification. A recent meta-analysis identified gene expression signatures that were associated with improved prognosis in HGSOC. Among the top 5 predictive genes were TAP1, which is involved in the antigen-presenting pathway and CXCL9, a chemokine involved in the recruitment of T cells. Expression of both genes have been previously linked to OS and with high TIL counts.[49] Suggesting that CXCL9 and TAP1 expression may be indicators of immune competency and could add to predict overall response to immunotherapy.

Therapeutic vaccinations, chemotherapy and dexamethasone

The success of antigen specific vaccination strategy as a single agent has been limited in epithelial ovarian cancer as described in **chapter 5**. The ability of therapeutic vaccinations to trigger a robust anti-tumor immune response through active immunization has been demonstrated by various vaccination strategies. However, the majority of the patients do not show improved clinical outcome and efficacy of vaccination strategy is likely hampered by immune escape of the tumor.[50] Therefore, combining vaccination therapy to induce an immune response, with other agents to overcome immune escape are of interest. The combination of ICI and vaccination has been explored in melanoma and demonstrated objective response rates.[48]

Also chemotherapeutic agents could have synergistic effects when combined with a therapeutic vaccination. Platinum-based chemotherapy, which is first choice chemotherapy treatment in EOC, can induce release of tumor antigens in the microenvironment, promoting activation of antigen presenting cells, augmenting antigen processing and presentation, providing a boost for the activation and differentiation of the immune system eventually resulting in immunogenic cell death.[50] In **chapter 6** we describe an ongoing clinical trial researching the immunogenicity of the antigen specific vaccination *BNT115* administered during standard-of-care carboplatin/ paclitaxel chemotherapy in EOC. Carboplatin/paclitaxel chemotherapy was shown to induce a decrease in peripheral T-regulatory cells and myeloid cells and an increase in Th1, Tc1 and natural killer cells in OC patients.[51] Even more, carboplatin improved the capacity of peripheral $CD8^+$ T cells to produce IFNγ without negatively affecting frequencies.[52] In addition, class-switched memory B cells were enhanced after NACT in HGSOC.[10] In cervical cancer, combination treatment of carboplatin/paclitaxel and a

therapeutic HPV16 vaccine induced tumor immunity and demonstrated longer survival in patients with a high vaccine-induced immune response.[53] This suggests carboplatin/paclitaxel could potentially enhance immunotherapy, and thereby enhance vaccine induced immune response.

Importantly, standard-of-care carboplatin and paclitaxel chemotherapy is usually combined with dexamethasone, a glucocorticosteroid, as supportive medicine to reduce potential chemotherapy side effects. Dexamethasone has a clearly described immune-attenuating effect and is therefore avoided in combination with immunotherapy. Indeed, a vaccination trial in glioblastoma patients only lead to a robust de novo immune response in patients that that did not receive dexamethasone simultaneously.[54] What is more, a recent study in both mice and human PBMC shows that dexamethasone substantially blunts RNA-LPX vaccine-mediated immune effects. Interestingly, these effects were less when dexamethasone was administered as post-medication.[55] Lastly, a study investigating the in vivo effects of dexamethasone in a mouse model also demonstrated T cell suppression. Interestingly, these effects were partially reversed by the addition of a CTLA-4 antibody.[56] Taking into account the biological half-life of dexamethasone (36-72 hours), most immunotherapy regimens could be scheduled around glucocorticosteroid administration to avoid the immune modulating effects. Interestingly, a recent study also demonstrates the use of CRISPR-Cas9-mediated knockout of the glucocorticoid receptor to generate glucocorticoid-resistant T cells. These virus-specific T cells were resistant to the immune suppressive effects of dexamethasone treatment.[57] This approach is of interest in the field of immunotherapy cancer treatment.

Perspectives on immune checkpoint inhibition in HGSOC

Up to now, ICI has shown modest response rates of only 10-15% in OC.[58–60] The largest body of evidence currently published is a phase II trial examining the efficacy of pembrolizumab monotherapy in patients with recurrent disease. Patients were stratified according to previous treatments; cohort A consisted of EOC patients receiving one to three prior lines of therapy and cohort B consisted of EOC patients receiving more than three prior treatments. Overall response rates were comparable between the two cohorts and did not exceed 10%.[59] PD-L1 expression has been linked to a favorable response to ICI in EOC patients[50]. However, the clinical trials performed mostly included

unstratified relapsed or platinum-resistant OC patients.[59,61] Recent studies in lung, melanoma and colon cancer suggest ICI treatment early-on in a neo-adjuvant/primary setting might be superior compared to ICI after disease recurrence.[20,47,62,63] Thus, we would argue for the exploration of ICI maintenance therapy or neo-adjuvant ICI in combination with standard of care chemotherapy. In lung cancer, neo-adjuvant atezolizumab was combined with carboplatin and paclitaxel and showed manageable treatment-related toxic effects, no surgical delays and a response rate of 57%.[64] Currently ongoing trials are investigating the combination of ICI with platinum-based chemotherapy and bevacizumab in OC (ClinicalTrials.gov Identifier: NCT02891824, NCT03038100).[50] The JAVELIN ovarian 200 phase III trial investigated a combination of anti-PD-L1 with liposomal doxorubicin, a chemotherapeutic agent often used in patients with platinum resistant disease.[65] Unfortunately, the results showed no improvement of PFS or OS.[50] Interestingly, overall response rates to PD-L1 inhibition plus avelumab was superior in PD-L1 positive patients than in negative patients (3.4% vs 18.5%).[50] As mentioned, it is well known that ICI is most effective in tumors infiltrated by a high number of TILs.[45,66,67] Therefore we would argue for the application of ICI therapy in immunocompetent HGSOC patients. Immunocompetence could be defined by high frequency of TILs (**chapter 4**), high PD-L1 expression, gene expression of e.g. TAP1 and CXLC9 (**see corresponding section above**) or a combination. Additionally, HGSOC patient with signs of immunodeficiency, e.g. low MHC_I expression (**chapter 3**), could be (pre-)treated with e.g. vaccinations, chemotherapy or a combination (**chapter 6**) to first augment an anti-tumor immune response.

Conclusion

This thesis elucidates on the immune environment and its importance in the application of immunotherapy in ovarian cancer. Thus far, immunotherapy is moderately successful in the treatment of ovarian cancer compared to e.g. melanoma and lung cancer. To improve clinical outcome it is essential to combine the right therapies for the right patient and to administer the treatment at the right window-of-opportunity. From our data we conclude that $CD8^+CD103^+$ TRM have a strong predictive value and quantification can play an important role in determining treatment strategy for different patient groups (high vs low TIL). Furthermore, upregulation of MHC-I expression in NACT patients may restore antigen presentation and the prognostic effect of TILs, which

could eventually lead to improved response to immunotherapy in this group of patients. Finally, combining vaccination strategy with chemotherapy and/or ICI could improve the overall response rates in HGSOC patients.

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Appendices

Nederlandse samenvatting (Dutch summary)

Publicatielijst (List of publications)

Dankwoord (Acknowledgements)

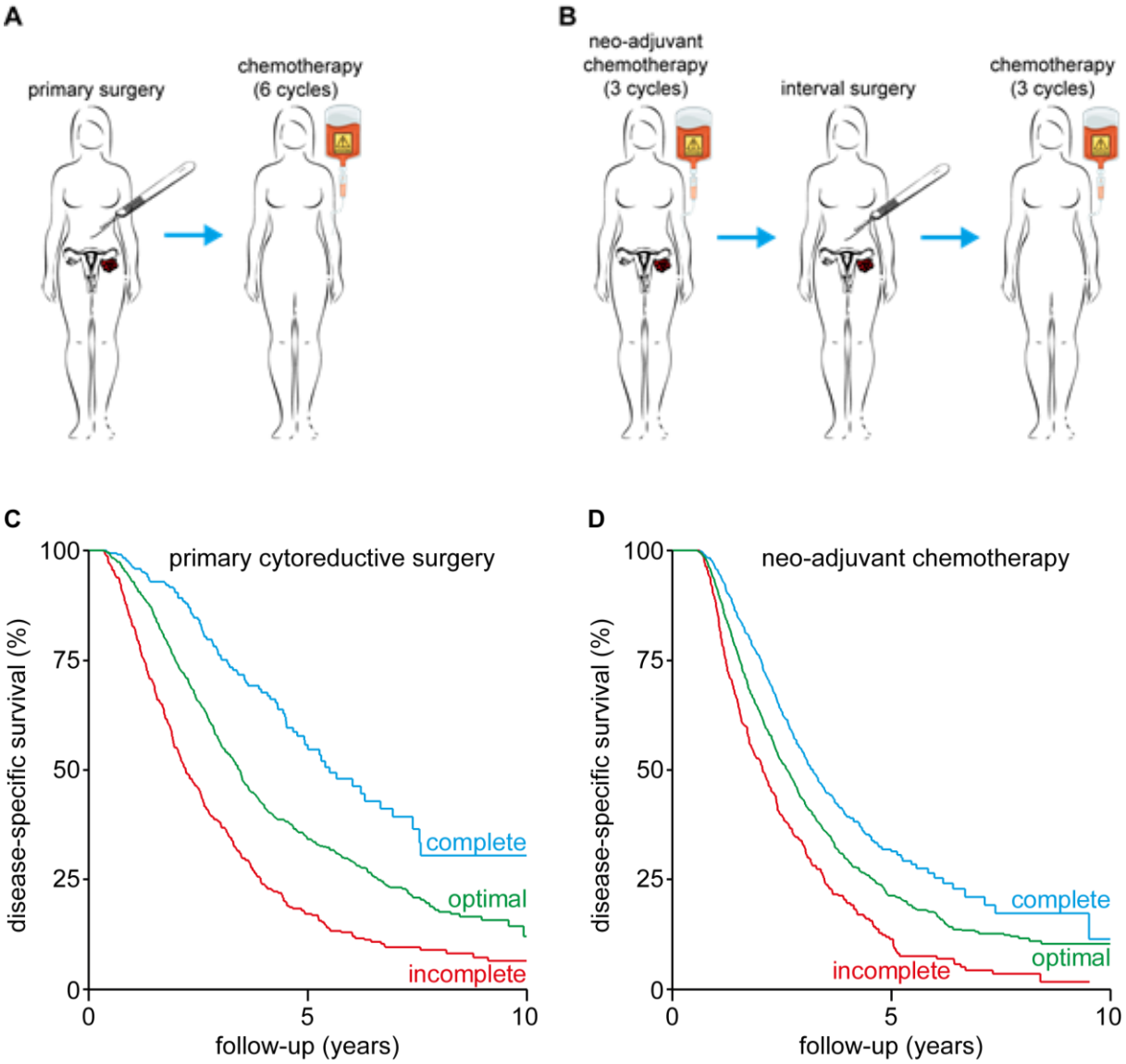
Nederlandse samenvatting

Dutch summary

Nederlandse samenvatting

Eierstokkanker is een dodelijke vorm van kanker met een gemiddelde 5-jaars overleving van slechts 40%. Eierstokkanker wordt vaak pas in een ver gevorderd stadium van de ziekte gediagnostiseerd. Dit komt mede doordat vrouwen met eierstokkanker vaak pas laat, en niet-specifieke, klachten (symptomen) ontwikkelen. Daarnaast is er geen goede screening methode beschikbaar. Het meest voorkomende histologische subtype (70%) van eierstokkanker is hooggradig sereus ovarium carcinoom (HGSOC). De eerste (primaire) behandeling bestaat uit een operatie en platinum bevattende chemotherapie, meestal een combinatie van carboplatine en paclitaxel. Patiënten worden of behandeld met primaire chirurgie (primary debulking surgery (PDS)) gevolgd door 6 kuren chemotherapie of zij worden eerst behandeld met 3 kuren neoadjuvante chemotherapie (NACT), een interval operatie (interval debulking surgery) en 3 kuren chemotherapie. De belangrijkste prognostische factor is resttumor na de operatie. Deze uitkomst wordt onderverdeeld in compleet (geen tumorrest na operatie), optimaal (tumorrest <1 cm) of incompleet (tumorrest >1 cm; figuur 1). Ofschoon in het algemeen goed gereageerd wordt op de primaire behandeling, keert bij de meeste vrouwen de ziekte binnen twee jaar terug (recidief) en overlijden zij uiteindelijk aan hun ziekte. Er is dus vraag naar verbetering van de huidige behandeling.

Het immuunsysteem speelt een belangrijke rol in het herkennen en verwijderen van kankercellen. Er zijn verschillende soorten immuuncellen waaronder de $CD8^+$ cytotoxische T cellen, de $CD4^+$ helper T cellen, de B cellen en de "*natural killer*" (NK) cellen, die allemaal nodig zijn voor een succesvolle immuunreactie tegen kankercellen. Wanneer deze immuuncellen zich in het tumorweefsel begeven worden ze tumor-infiltrerende lymfocyten (TILs) genoemd. Uit onderzoek is gebleken dat de aanwezigheid van TILs, T cellen en B cellen in het bijzonder, samenhangen met een verbeterde overleving in verschillende soorten kanker, waaronder ook eierstokkanker. In de kliniek zouden TILs gebruikt kunnen worden als biomarker om het beloop van de ziekte te voorspellen (prognose), maar mogelijk ook als aanknopingspunt om de patiënten te kunnen identificeren die baat kunnen hebben van immuuntherapie (predictie).



Figuur 1. Primaire behandeling strategie van eierstokkanker. A. Eierstokkanker patiënten worden behandeld met primaire chirurgie (PDS) gevolgd door 6 kuren chemotherapie. **B.** Indien primaire chirurgie niet haalbaar is, worden patiënten eerst behandeld met 3 kuren neoadjuvante chemotherapie (NACT), een interval operatie en daarna nog 3 kuren chemotherapie. **C.** ziekte specifieke overleving van OC patiënten behandeld met PDS gecorrigeerd voor operatie uitkomst. **D.** ziekte specifieke overleving van OC patiënten behandeld met NACT gecorrigeerd voor operatie uitkomst.

Immuuntherapie is er op gericht het lichaams eigen immuunsysteem te activeren. Globaal zijn er drie soorten immuuntherapie waar momenteel veel onderzoek naar wordt gedaan; immuun checkpoint inhibitie (ICI), therapeutische vaccinatie strategieën en celtherapie. ICI is er op gericht om inactieve T cellen te (re)activeren. De ICI halen als het ware de rem van T cellen eraf, waardoor de T cellen weer volop actief kunnen worden. Therapeutische vaccinaties zijn er op gericht om de bestaande of een nieuwe afweerreactie op te wekken of te versterken. Een dergelijk afweerreactie is bij voorkeur specifiek gericht tegen kankercellen. Bij celtherapie worden immuuncellen, bijvoorbeeld

T of NK cellen, die specifiek tegen de kanker gericht zijn uit het lichaam geïsoleerd, buiten het lichaam (*ex-vivo*) vermenigvuldigd (gekweekt) en vervolgens in grote hoeveelheden teruggegeven aan de patiënt.

De studies beschreven in dit proefschrift hebben als doel bij te dragen aan de kennis over de interactie tussen het immuunsysteem en eierstokkanker. Daarnaast evalueren we de toepassing van therapeutische vaccinaties in eierstokkanker. Deze kennis is nodig voor het optimaliseren van de toepassing van immuuntherapie bij eierstokkanker teneinde de prognose van patiënten met eierstokkanker te kunnen verbeteren.

De tumor immuun omgeving en immuuntherapie

In **hoofdstuk 2** wordt een overzicht gegeven van de verschillende soorten TILs, hun functie en de rol die ze spelen in het verwijderen van kankercellen. Daarnaast worden de meest recente ontwikkelingen besproken over de toepassing van TILs op het gebied van immuuntherapie. In kankeronderzoek is altijd veel aandacht geweest voor de $CD8^+$ T cel vanwege het vermogen om kankercellen te herkennen en uit te schakelen. De aanwezigheid van de $CD8^+$ T cel is geassocieerd met een betere overleving in vele soorten kanker. Nu is het duidelijk dat er diverse $CD8^+$ T cel subtypes bestaan met verschillende functies. Deze functies variëren van meer “naïeve” T cellen die vooral kunnen vermenigvuldigen (prolifereren) tot gedifferentieerde “cytotoxische” T cellen die juist in staat zijn om kankercellen te verwijderen. Het subtype $CD8^+CD103^+$ T cellen, ook wel bekend als tissue-resident memory cellen (TRM), staat specifiek bekend om hun vermogen kankercellen te verwijderen. De TRM bevinden zich in het kankerweefsel en zijn in staat tumorgroei te controleren. De aanwezigheid van TRM in het kankerweefsel heeft een gunstig effect op de overleving (prognose) van patiënten met eierstokkanker. Verder wordt in **hoofdstuk 2** de nadruk gelegd op het belang van verschillende soorten B cellen als onderdeel van een anti-tumor immuunrespons. Plasma B cellen kunnen kanker specifieke antilichamen produceren. Daarnaast zijn er B cellen die functioneren als antigeen presenterende cel waardoor een immuun reactie in gang wordt gezet. Wanneer B cellen en T cellen samen clusteren kunnen ze een georganiseerde immuun structuur vormen, ook wel “tertiaire lymfe structuren” (TLS) genoemd. De TLS is een lymfeknoop-achtige structuur die ontstaat in perifeer weefsel. De georganiseerde

immuun structuur creëert een omgeving waarin B cellen, T cellen en antigeen presenterende cellen elkaar stimuleren, activeren en een immuun reactie op gang brengen. De aanwezigheid van de TLS is geassocieerd met betere overleving in verschillende type kanker. Op basis van de huidige literatuur kunnen we concluderen dat voor een effectieve anti-kanker immuunreactie de locatie, clustering, samenwerking en costimulatie van alle TILs bepalend is.

Tumor immunologie in eierstokkanker

In **hoofdstuk 3** beschrijven wij de samenstelling van de verschillende immuuncellen (immuun profiel) in de tumor zelf, in de lymfeklieren en in het bloed van patiënten met eierstokkanker. Hierin maken we onderscheid tussen twee groepen; 1. Patiënten behandeld met een PDS, waar het lichaamsmateriaal dus niet is bloot gesteld aan chemotherapie en 2. Patiënten behandeld met NACT, waar het lichaamsmateriaal dus wel is blootgesteld aan chemotherapie.

Wij hebben onderzocht of het immuunprofiel er anders uit ziet bij patiënten die zijn blootgesteld aan chemotherapie dan bij patiënten die nog geen chemotherapie blootstelling hebben gehad. Om dit te onderzoeken hebben we immuuncellen geïsoleerd uit tumorweefsel, lymfeklieren en bloed voorafgaand aan chemotherapie (tijdens PDS) en na NACT (tijdens interval debulking). Uit ons onderzoek blijkt dat het immuunprofiel niet was veranderd na blootstelling aan chemotherapie. Tevens hebben we onderzoek gedaan naar de expressie van PD-1 op immuuncellen, een receptor die op geactiveerde afweercellen voorkomt. Ons onderzoek liet zien dat de aanwezigheid van PD-1$^+$ cellen in de tumor geassocieerd is met een verbeterde overleving in patiënten die zijn behandeld met PDS. Deze associatie was niet te zien in NACT patiënten, terwijl het aantal PD-1$^+$ immuuncellen in beide behandelgroepen hetzelfde is. Een mogelijke verklaring hiervoor is de aanwezigheid van major histocompatibility complex klasse 1 (MHC-1). MHC-1 komt onder andere voor op kankercellen en is verantwoordelijk voor het presenteren van stukjes eiwit (tumor antigenen) aan het immuunsysteem. Het stukje tumorantigen wordt door MHC-1 gepresenteerd aan de T-cel receptor (TCR) van de T-cel (antigeen presentatie). Hierna worden T cellen geactiveerd om te vermenigvuldigen en de cellen die het tumorantigen tot expressie brengen, in dit geval dus de kankercellen, te verwijderen. Tumor antigenen worden bij voorkeur alleen door kankercellen tot

expressie gebracht. In dit onderzoek laten we zien dat MHC-1 minder tot expressie wordt gebracht in tumoren na chemotherapie (NACT). Dit maakt het waarschijnlijk dat door gebrekkige antigeen presentatie, de PD-1$^+$ cellen niet in staat zijn de tumor cellen te herkennen en dus geen overlevingsvoordeel geven in NACT patiënten. Het is mogelijk dat de TILs aanwezig in NACT tumorweefsel het gevolg zijn van een algemene (niet tumor specifieke) immuun respons als gevolg van inflammatie veroorzaakt door de chemotherapie. Het is belangrijk om verder onderzoek te doen naar de reden dat MHC-1 minder voorkomt na NACT én of er een manier is om de expressie weer te verhogen.

Om de tumor immuun omgeving van eierstokkanker beter in kaart te brengen hebben we vervolgens een cohort samengesteld van 281 eierstokkanker patiënten waarvan tumorweefsel beschikbaar was voor onderzoek. Zoals beschreven in **hoofdstuk 2** zijn de TRM (CD8$^+$CD103$^+$ T cellen) geassocieerd met een verbeterde prognose in meerdere type kanker, waaronder HGSOC. In **hoofdstuk 4** hebben we onderzocht of dit verband geldt voor alle HGSOC patiënten of slechts voor een deel daarvan. We hebben hiervoor gebruikt gemaakt van een digitale kwantificatie techniek, op geautomatiseerde wijze het aantal TRM cellen in de tumor bepalen. Uit onze resultaten blijkt dat patiënten met relatief veel TRM cellen in de tumor een gemiddeld betere overleving hebben. Net als in **hoofdstuk 3** zien we dat dit overlevingsvoordeel alleen geldt voor PDS patiënten. Tot slot, laten we in dit hoofdstuk zien dat de gebruikte digitale kwantificatie techniek geschikt is om het aantal TRM cellen in de tumor te bepalen en dat deze techniek in de toekomst kan worden ingezet om de prognose van HGSOC patiënten beter te voorspellen.

Therapeutische vaccinatie als behandeling van eierstokkanker

Zoals eerder benoemd hebben vaccinaties als doel een immuun reactie op te wekken tegen kankercellen. Dit doen vaccins voor een belangrijk deel door tumor geassocieerde of tumor specifieke tumorantigen in MHC moleculen op het celoppervlak zichtbaar te maken voor het immuunsysteem, lees T cellen. De T cellen zullen dit complex van MHC-1 en lichaamsvreemd peptide herkennen waarna de cascade van activatie en herkenning van tumorcellen kan plaats vinden. Wanneer het immuunsysteem de kankercellen herkent, kunnen ze de kankercellen vervolgens verwijderd. In **hoofdstuk 5** is een overzicht gegeven van de klinische effectiviteit van tumor-antigeen specifieke

vaccinaties in eierstokkanker. Er werden 67 studies geïdentificeerd, gepubliceerd tussen 1966 en 2017, die samen 3632 vrouwen includeerden en behandelde met tumor-antigeen specifieke vaccinatie therapie. In de meeste studies werd de veiligheid en het vermogen van de vaccinatie om een immuun reactie op te wekken tegen de kankercellen (immunogeniciteit) beoordeeld en niet de klinische effectiviteit. Samenvattend zijn de meeste vaccinaties veilig om toe te dienen en laten slechts beperkte bijwerkingen zien. Daarnaast zijn de meeste vaccinaties immunogeen. Momenteel is de klinische effectiviteit van tumor-antigeen specifieke vaccinaties in de behandeling van eierstokkanker niet bewezen en is meer onderzoek nodig. Er wordt gedacht dat het combineren van een vaccinatie strategie met andere immuun modulerende behandelingen, zoals bijvoorbeeld ICI, radiotherapie of chemotherapie, de klinische effectiviteit van vaccinatie behandelingen kan verbeteren. In **hoofdstuk 6** wordt een momenteel lopende studie beschreven naar het effect van een tumor-antigeen specifieke vaccinatie genaamd "BNT115" in combinatie met de standaard chemotherapie behandeling (carboplatine/paclitaxel) in eierstokkanker patiënten. Eerdere studies naar de relatie tussen carboplatine/paclitaxel en het effect op het immuunsysteem hebben aangetoond dat deze chemotherapie in staat is om immuun suppressieve cellen te verminderen en immuun ondersteunende cellen te stimuleren. Tot slot, heeft een studie in baarmoederhalskanker hoopvolle resultaten laten zien bij een combinatie van een therapeutisch vaccinatie met carboplatine/paclitaxel.

Conclusie

Eierstokkanker is de meest dodelijke gynaecologische vorm van kanker, in Nederland is de gemiddelde 5-jaars overleving slechts 40%. Het immuunsysteem speelt een belangrijke rol in het herkennen en verwijderen van kankercellen en immuuntherapie is er op gericht het lichaams eigen immuunsysteem te activeren. Dit proefschrift onderschrijft het belang van het immuunsysteem in de controle en de behandeling van kanker. Tot nu toe is immuuntherapie in de behandeling van eierstokkanker in theorie veelbelovend, maar in de praktijk worden nog maar kleine successen geboekt. Dit kan verbeterd worden door de timing van toediening van behandelingen te optimaliseren, de juiste therapieën met elkaar te combineren en de juiste patiënten te selecteren voor verschillende behandelingen. Daarnaast is het belangrijk om te zoeken naar

combinatietherapieën die zowel het cellulair (T cellen) als het humoraal (B cellen) immuunsysteem stimuleren. Dit proefschrift beschrijft dat de aanwezigheid van tumor-infiltrerende cellen voorspellend is voor overleving in slechts een subgroep van vrouwen met eierstokkanker én dat tumoren blootgesteld aan chemotherapie weinig MHC-1 tot expressie brengen. Onderzoek naar de oorzaak van verminderde MHC-1 expressie en onderzoek naar methoden om de expressie van MHC te verhogen kan bijdragen aan het succes van immuuntherapie in de toekomst. De digitale kwantificatie van $CD8^+CD103^+$ TRM cellen bleek in ons onderzoek zeer geschikt als methode om de overleving te voorspellen. Digitale kwantificatie van immuuncellen kan een belangrijke rol vervullen in patiënten selectie. Tot slot, beschrijven we de stand van zaken van een nu lopend onderzoek naar de immunogeniciteit van een tumor antigeen specifieke vaccinatie (BNT115) in combinatie met de standaard chemotherapie behandeling (carboplatine/paclitaxel) in patiënten met eierstokkanker.

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*These authors contributed equally

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Dankwoord

Acknowledgements

Dankwoord (Acknowledgements)

Het schrijven van dit dankwoord hoort bij het afsluiten van dit proefschrift en een 4-jarige periode gekenmerkt door het leren uitvoeren van onderzoek, het interpreteren van wetenschappelijke data, het uitwisselen van kennis en het zoeken naar nieuwe inzichten. Dit proefschrift was niet tot stand gekomen zonder de medewerking, steun en betrokkenheid van een groot aantal mensen.

Mijn eerste dank gaat uit naar de patiënten die hebben deelgenomen aan de klinische studies beschreven in dit proefschrift.

Vervolgens mijn eerste promotor **prof. dr. H.W. Nijman**, beste Hans. Graag wil ik van deze gelegenheid gebruik maken om je te bedanken voor de mogelijkheid om deel te nemen aan de gynaecologie onco-immunologie onderzoeksgroep in het U.M.C.G. en aan het prachtige project, de OLIVIA-studie. Je laagdrempelige en betrokken houding heb ik altijd erg gewaardeerd en de spanning om op je kantoordeur te kloppen voor feedback of (meestal) een handtekening, was dan ook snel verdwenen. Door ons als onderzoekers tijdens onderzoeks- vergaderingen het vuur aan de schenen te leggen, leer je ons kritisch nadenken en kijken naar het volledige plaatje. Met je scherpe gevoel voor humor creëer je een geweldige sfeer die ik iedere onderzoeksgroep toewens. Dank je wel voor de afgelopen 4 jaar.

Geachte promotor **prof. dr. A.K.L. Reyners**, beste An. In de laatste twee jaar van mijn promotietraject zijn wij veel gaan samenwerken in de kliniek voor het includeren en het begeleiden van de studiepatiënten binnen de OLIVIA- en de PAM studie. Het was ontzettend fijn om te merken hoe we in een rap tempo patiënten wisten te includeren voor beide studies. Je was altijd bereikbaar voor vragen, zelfs tijdens de tennislessen. Ik neem een voorbeeld aan je doortastende en betrokken houding naar patiënten. Dank je wel voor de fijne samenwerking.

Dankwoord (Acknowledgements)

Geachte copromotor **dr. M. de Bruyn**, beste Marco. Wat heb jij mijn promotietraject leuk gemaakt. Je passie en enthousiasme voor onderzoek en voor de immunologie zijn aanstekelijk. Je bent altijd bereid om mee te denken, een kritische discussie te voeren en vooral veel uit te leggen, het liefst met tekeningen. Dit gepaard aan een goede dosis zwarte humor maakte dat de afgelopen 4 jaar voorbij zijn gevlogen. Ik hoop in de toekomst verder samen te werken aan mooie immunologische projecten.

Graag bedank ik de leden van de beoordelingscommissie, bestaande uit **prof. dr. J.D. Laman**, **prof. dr. J.A. Gietema en prof. dr. V.T.H.B.M. Smit** voor het beoordelen van mijn proefschrift.

Graag bedank ik alle **co-auteurs** voor onze productieve samenwerking.

De betrokken **gynaecoloog-oncologen** en **medisch-oncologen** uit het U.M.C.G. wil ik bedanken voor de fijne sfeer, het laagdrempelig overleg en de medewerking aan alle klinische studies op de afdeling. In het bijzonder, **dr. R. Yigit**, **dr. H. Arts**, **dr. M. Oonk, prof. dr. M. Mourits, dr. T. Roelofsen** en **dr. N. de Lange** zonder jullie betrokken houding waren er nooit zoveel patiënten geïncludeerd voor de VVAX, PAM, OLIVIA en Granulosa studies. Beste **dr. H. Jalving**, dank voor het altijd bereid zijn om in te springen als supervisor voor de OLIVIA studie en voor de fijne samenwerking.

Ook de **afdeling pathologie** in het U.M.C.G. wil ik graag bedanken voor de prettige samenwerking. In het bijzonder gaat mijn dank uit naar **dr. E.W. Duiker** en **dr. J. Bart**. Beste Evelien, in mijn eerste jaar hebben we samen een verschrikkelijke berg coupes beoordeeld en afgetekend. Het was heerlijk om met jou als pragmaticus deze enorme klus in relatief korte tijd te volbrengen. Het was hard werken maar vooral altijd erg gezellig. Beste Jos, onze samenwerking begon in het Isala ziekenhuis. Ook met jou heb ik een grote hoeveelheid coupes beoordeeld en afgetekend. Je bent altijd bereid te sparren over de opzet en de richting van de lopende onderzoeken. Dank je wel voor je betrokkenheid en je enthousiasme.

Dankwoord (Acknowledgements)

Beste **prof. dr. S. Scherjon,** beste Sicco, alweer in 2014 leerde wij elkaar kennen ten tijde van mijn wetenschappelijke stage in Manchester (GB) voor de studie geneeskunde. Graag wil ik je bedanken voor je betrokken begeleiding en positieve houding. Jij hebt me geënthousiasmeerd voor het onderzoek. Door jou is mijn eerste ervaring met wetenschappelijk onderzoek dusdanig positief geweest dat ik me, nu 4 jaar geleden, aan een PhD durfde te wagen.

Graag bedank ik **prof. dr. S. de Jong**, **prof. dr. M. van Vught**, **prof. dr. F. Kruyt**, **dr. B. Wisman**, **prof. dr. C.A.H.H. Daemen**, alle onderzoekers van het **MOL lab** en alle **arts-onderzoekers** binnen de **medische oncologie** voor de kritische vragen en de gezellige afdelings-activiteiten.

Ook gaat mijn dank uit naar het secretariaat van de afdeling Obstetrie & Gynaecologie, naar **Heidi**, **Janette**, **Linda** en **Jikke**. In het bijzonder bedank ik **Diana** en **Mirjam** voor de brede ondersteuning. Ik hoop dat ik binnenkort weer een souvenirtje op jullie vensterbank kan achterlaten.

Tevens bedank ik iedereen van onze onco-immunologie onderzoeksgroep, ook wel het DreamTeam: **Fenne**, **Florine**, **Kim**, **Hagma**, **Joyce**, **Arjan**, **Marta W.** & **Marta R.**, **Anneke**, **Annegé**, **Annechien** en **Nienke**. Helaas hebben we elkaar in mijn laatste jaar met name via ZOOM gezien en werd de communicatie zo nu en dan bemoeilijkt door matige internetverbindingen. Ik heb heel prettig met jullie allemaal samengewerkt, ik heb ontzettend veel van jullie geleerd maar met name veel met jullie gelachen en genoten van goede koffies & kroket-Tuesday. Nienke en Annechien, sterkte met al Marco's projecten. Martha and Martha I would like to wish you much success with your upcoming projects. Anneke & Annegé zet hem op met alle klinische studies! Joyce ontzettend veel succes met de laatste loodjes. Hagma, ik weet zeker dat je het fantastisch hebt gedaan in September. Allemaal hartstikke bedankt voor de afgelopen 4 jaar.

Lieve **Annegé**, in het laatste jaar van mijn PhD ben jij het onderzoeksteam (en mij) komen versterken. Wat heerlijk om naast samen te werken ook elke dag te kunnen roddelen en (heel af en toe) te klagen. We hebben onze mooie publicaties altijd goed gevierd en ik hoop dat we het feestje na mijn verdediging nog even goed door kunnen zetten. Ik wil je heel veel succes wensen met het afronden van de OLIVIA studie, de overige klinische studies en natuurlijk je eigen promotie. Ik zit uiteraard juichend vooraan als jij straks aan de beurt bent.

Lieve geniale lichting, lieve **Sanne** en lieve **Jossie**, het is lang geleden dat wij elkaar als kikkers en sneeuwpop leerden kennen. Destijds had ik me niet kunnen voorstellen dat we ruim 10 jaar later, 3 promoties verder zouden zijn. Maar hier zijn we dan toch aanbeland. Wat fijn dat jullie hier vandaag aanwezig zijn om dit moment met mij te delen. Ik heb ontzettend zin om de komende 10 jaar nog meer lichtingstripjes, diners en wijnavonden met jullie te plannen.

Lieve **Lotte,** Lieve **Eva**, oud-huisgenootjes, dispuutsgenootjes en partners in crime. Het was superleuk om met jullie allebei te hebben gewoond. Eva, eindelijk wonen we sinds lange tijd weer in dezelfde stad, ook al is het helaas maar van korte duur. Als waakzwijn aan jouw zijde heb ik altijd een goede avond. Je bent een van de zachtaardigste en trouwste mensen die ik ken. Lot, tussen ons is de geografische afstand helaas wat groter geworden, maar gelukkig spreken we elkaar nog steeds veel. Aan jou heb ik altijd een goede gesprekspartner en een heerlijke avond. Ik vind het ontzettend knap hoe je aan het shinen bent binnen de huisartsengeneeskunde. Elke patiënt mag straks blij zijn met jou als huisarts.

Lieve **Ammie**, **Sandra**, **Ei**, **PW** en **Deir**. Ons geweldige dames 2 jaar op GCHC was toch een van de hoogtepunten in het studentenleven. Helaas mocht deze epische samenstelling maar 1 jaar duren, omdat er een aantal de focus hadden op hockeyen en een aantal op de derde helft. Vergeet nooit dat 5 liter bier slechts 1 glas per persoon is. Dat laag gaan een pré is. Dat als de fles toch open is, je hem beter leeg kan drinken. Dat één gang, geen gang is en dat dik zijn niet erg is, als je maar lekker hebt gegeten. Ik geniet elk jaar weer van ons heerlijke weekend weg en ik hoop dat we binnenkort écht naar Wenen kunnen.

Lieve **Mart**, ik ben ontzettend blij dat je er bent en ik verwacht, omdat je er zo goed in bent, dat je tot het einde blijft. Love you.

Lieve paranifmen, lieve **Deirdre** & **Laura**. Wat geweldig dat jullie vanavond als paranimf naast mij staan! Het voelt goed en vertrouwd om met jullie aan mijn zijde mijn proefschrift te verdedigen. Mocht ik vallen, dan weet ik zeker dat jullie me overeind zullen helpen als jullie zijn uit gelachen. D, we kennen elkaar ondertussen al 11 jaar en zijn heel wat rare eetgewoontes en epische reizen verder. Ik kan altijd voor alles bij je terecht en ik weet dat ik op je ongezouten mening kan rekenen. Jij bent de enige die ik accepteer met een smiley-tas, zoals jij mij accepteert in een alto-legging. Lieve Lau, ik had nooit verwacht dat Gideon nog eens een verstandige keuze zou maken. Maar het lijkt dan toch gelukt. Ik ben ontzettend blij met jou als schoonzusje en nog meer als vriendin. Het is immers altijd keten in de zusterflat en de chardonnay is nooit te vet.

Aan het ouderlijk front, lieve **mam**, lieve **pap**, lieve **Arina** en lieve **Just**. Wat ontzettend fijn dat jullie er vandaag allemaal bij zijn. Dank jullie wel voor het warme thuisfront, de onvoorwaardelijke steun en het vertrouwen dat het uiteindelijk allemaal goed zou komen. Ik heb heel veel zin om vanavond met jullie te proosten op het proefschrift.

Lieve **Gideon**, lieve Kiniko, wat fijn dat jij uiteindelijk nog 4 jaar in Groningen bent blijven wonen om mij te ondersteunen tijdens mijn PhD. Je ging zelfs, speciaal voor mij, op slechts 140 meter afstand van het UMCG wonen zodat je me op slechte dagen kon opvangen met een koud biertje of een goed glas wijn. Ik ben ook erg benieuwd wat je gaat doen nu mijn PhD is afgerond. Gelukkig, heb je tegenwoordig je handen vol aan Laura die ook de weg naar de wetenschap is ingeslagen. Aan jou wil ik graag de laatste woorden van mijn proefschrift wijden. Je bent mijn lievelingsbroertje, ik ben gek op je en ik ben trots op je. Ik hoop dat we snel weer op Paijens-avontuur kunnen.

Sterre Paijens