Thesis summary

Epithelial ovarian cancer (EOC) is the most deadly gynecological malignancy with a 5-year survival rate of no more than 40% (1). Only limited advances have been obtained with regard to treatment strategies over the past decades with minimal improvement in survival rates (2). In EOC, the prognostic value of tumor-infiltrating lymphocytes (TIL) has previously been shown (3–5) and immunotherapy to further enhance the anti-tumor immune response might be a viable option as a new treatment modality. However, success of immunotherapy in ovarian cancer has been limited so far (6,7). In order to increase success rates of immunotherapeutic strategies there is an urgent need to better understand the immune response towards these tumors. Immunity profiles might give clues on I) how to stratify patients for strategies, II) select patients to be included in clinical trials, and III) implementation and integration of immunotherapy with standard care for this disease. In this thesis, results are presented on the analysis of immune infiltrate in EOC, in relation to clinicopathological characteristics, treatment regimen and clinical outcome.

While CD3+ and CD8+ TIL have been associated with longer survival (3–5), other TIL subsets such as B cells may also aid in the anti-tumor response. In EOC, CD20+ cells present with a phenotype of mature memory B cells and are often found in close proximity of CD4 and CD8+ T cells in so-called tertiary lymphoid structures (TLS). They are thought to act as an antigen-presenting cell to these CD4 and CD8 TIL subsets, aiding the anti-tumor response (8). In Chapter 2, we show the presence of CD20+ T cells in TLS, a phenotype that might result from B cell/T cell interaction through the process of trogocytosis. In vitro experiments revealed that mixing T and B cells led to transfer of HLA-DR and CD20. In vivo, a small number of CD3+ T cells co-express CD20 in healthy tonsil as well as TLS-like structures in ovarian tumors. CD20+ T cells derived from PBMCs of patients produced IFN-γ, and rarely interleukin-4 (IL-4) or IL-17 after stimulation, representing a Th1/Tc1 effector memory T cell (Tem) phenotype. This population was significantly enriched in the ascites of patients, suggestive of antigen-presentation of B cells to TIL in the tumor microenvironment (TME).

Next to cell subsets that induce or enhance the anti-tumor response, multiple suppressive mechanisms also take place in the TME. IL-6 signaling has been implicated in the regulation of inflammation, cell differentiation and proliferation, and carcinogenesis. In chapter 3, we analyzed the prognostic value of the IL-6 signaling pathway by determining the expression levels of IL-6, its receptor (IL-6R) and pSTAT3 in a large cohort of EOC patients. IL-6 expression in tumor epithelium was associated with a worse prognosis, confirming data in which high expression levels of IL-6 in serum and ascites were correlated to worse outcome (9,10). IL-6R was correlated with early stage, low-grade disease, but also proved to be an independent prognostic marker for better outcome in EOC. Since IL-6 signaling might affect the infiltration and polarization of myeloid cell subsets, we also studied the presence of
CD14+, CD33+, and CD163+ tumor-infiltrating myeloid cells (TIM) in these tumors. Most tumors were highly infiltrated with CD163+ cells, suggestive of an immuno-suppressive environment. Most infiltration with myeloid cells was found in the stromal regions and stromal IL-6 was associated with CD14+ cell influx. Based on the markers of the IL-6 signaling pathway, TIL and TIM infiltrates, a clustering analysis was performed subdividing patients on the basis of their immune profile. Patients with an immune profile with high TIL infiltration and low suppressive cell infiltration (FoxP3+ and CD163+ cells) clearly showed a better prognosis as compared to those patients who had high levels of these TIL and TIM subsets. Further confirming the suppressive role of CD163+ TIM, a high CD8+/CD163+ ratio was predictive of a better outcome.

In Chapter 4, a new homogeneous cohort of advanced stage high-grade serous ovarian cancer (HGSC) patients was created in which the immune response could be assessed in relation to treatment regimen and surgical outcome. Patients were subdivided in two cohorts, one in which patients had received primary surgery (PS) followed by chemotherapy, and one in which patients received neo-adjuvant chemotherapy (NACT) followed by interval surgery and the remaining courses of chemotherapy. Tissue was obtained during primary or interval surgery and the surgical result was well reported as being complete (no residual tissue), optimal (<1cm residual tissue) or incomplete (>1 cm). We found a clear difference in prognostic value of TIL in these patients based on treatment group and surgical outcome. CD8+ TIL were only significantly associated with better prognosis in patients that received a complete cytoreduction during primary surgery. The CD27+ TIL subset, on the other hand, also proved to be beneficial in patients with an incomplete removal of the tumor. CD27 was co-expressed on a subset of CD8+ TIL and presented a CD45R0+ CCR7+/− T_{em} or central memory (T_{cm}) phenotype. Furthermore, we found that the majority of TIL expressed PD-1 and that the CD27+ subset was enriched for CD137+ expression, suggestive of an activated, antigen-experienced, yet naive-like phenotype. In an attempt to further profile the immune response in these tumors, we analyzed a panel of lymphoid and myeloid cell populations as well as PD-L1 expression in Chapter 5. Most cell populations show a similar expression pattern in both the PS and the NACT cohort, however CD14+ and CD11c+ cells were more abundantly present in tumors obtained after NACT. PD-L1 was expressed on the tumor epithelium and on immune cells in the tumor stroma. Expression of PD-L1 was associated with better prognosis and strongly correlated with lymphoid and myeloid cell infiltration. Further, combined analysis of PD-L1 expression and infiltration by PD-1+ TIL revealed PD-L1 as a defining marker for a favorable prognosis in HGSC patients. Therefore, PD-L1 might serve as a biomarker for an active immune response and PD-L1 and PD-1 expression might be used for stratifying patients for immunotherapeutic approaches.

The last part of this thesis focuses on identification of tumor antigens that may serve as treatment targets and/or diagnostic tools for ovarian cancer.
claudin (CLDN) family of proteins represents 27 members that form components of tight junctions, of which expression patterns are often found to be dysregulated in cancers (11). In Chapter 6, we first determined the expression of CLDN6 in various gynecological malignancies. HGSC tumors highly expressed this protein and, importantly, expression patterns were not different when comparing tumor tissue obtained during primary or interval surgery. It is likely that standard treatment does not affect expression of this protein and therefore therapeutic targeting of this protein could potentially be implemented before, together with, or after chemotherapy courses. Furthermore, selection of patients that express this protein can be performed on surgically removed tumors. Another TAA from the CLDN family is the splice variant of CLDN18; namely CLDN18.2. This protein was analyzed for expression patterns in gynecological malignancies in Chapter 7. Herein we show that this protein is expressed by 76.7% of the mucinous ovarian tumors analyzed, and therefore represents an interesting therapeutic target in this subtype. Furthermore, in differentiating primary mucinous tumors from ovarian metastases of gastrointestinal origin, CLDN18.2 might be used for diagnostic purposes. In comparing this marker with other standard markers, the highest likelihood ratio, sensitivity and specificity was reached when a combination of CK7, DPC4 and CLDN18.2 was analyzed for the presence of primary ovarian tumor.
Discussion

In order to choose the most effective immunotherapeutic approach for ovarian cancer, knowledge of the pre-existing immune response is of major importance. The high variation in immune infiltrate in patients suggests that distinct approaches might be necessary to tackle the different immune environments.

Targeting the TME

Our studies revealed specific infiltration patterns of tumor-infiltrating lymphocytes (TIL) and myeloid cells (TIM) in ovarian tumors, which correlated with survival of patients. While a high infiltrate of CD8+ and more specifically CD27+ TIL is correlated with improved prognosis, a TIM infiltrate can suppress this effect (Chapter 3, 4, and 5). Furthermore, the expression of PD-L1 was associated with high TIL infiltrate and improved outcome and therefore PD-L1 expression might serve as a surrogate marker for a reactive immune response. Next to the suppressive effects of TIM and FoxP3+ TIL (Chapter 3 and 5), other factors in the TME may also suppress the T cell function, such as expression of IDO, CD73 and upregulation of PD-L1 on tumor and immune cells (Chapter 5 and (12,13)). These factors can be targeted by immunotherapy and are likely to be most effective in those patients that have a strong infiltrate of TIL. FasL expression on endothelial cells in the tumor may actively select for Treg infiltration by actively suppressing the intraepithelial infiltration of CD8+ TIL (14). Targeting this molecule may therefore shift the balance of the CD8 and Treg ratio in the tumor and create an anti-tumor effect. Furthermore, several strategies could be employed in order to deplete Treg, such as treatment with cyclophosphamide or blockade of the IL-2 receptor CD25 on these cells (15–17). As such strategies may also lead to depletion of some CTL subsets, combination with properly timed strategies in which the cytotoxic T cell response is stimulated is needed. Depletion of suppressive TIM or manipulations of the TME to lower expression of cytokines are other strategies to boost the anti-tumor response. The most abundantly present subtypes of TIM in ovarian cancer are the tumor-associated macrophages. These cells are drawn to the TME as myeloid precursor cells and depending on clues they receive from cytokines in the TME polarize to either classically-activated M1 macrophages which are tumoricidal, or alternatively to M2-polarized macrophages, which exert anti-inflammatory suppressive effects. Depletion of these cells might be achieved by blockade of B7-H4 or CSF-1R (18,19), interference with the IL-6 signaling pathway (20) and gemcitabine treatment (21). However, the modulation of these cells into M1 macrophages by appropriate immunotherapeutic approaches may be more attractive (22).
Standard treatment
Chemotherapy

In our cohort of HGSC patients, we found major differences in survival between patients treated with PS or NACT, and a lack of prognostic benefit of TIL infiltrate in the NACT cohort. In a recent randomized trial comparing the two regimens, no differences in survival were found between the patient groups treated with either NACT or PS as primary regimen (23). In this trial, patients receiving NACT had less co-morbidities and improved cytoreductive results, which both, surprisingly, had no impact on survival. It would be of interest to determine the immune profiles in these patients, to see whether an explanation for this can be found in a differential immune response after neo-adjuvant chemotherapy. The results of Chapter 4 do not point in the direction of differences in CD8+ or CD27+ TIL infiltration. Data from Chapter 5 suggest that there are differences in some myeloid cell populations between the two cohorts, with an increase in CD14+ and CD11c+ cell infiltration in the NACT cohort. It needs to be noted, however, that this is not a direct comparison before and after chemotherapy within one patient. To differentiate the effect of chemotherapy on the immune infiltrate, a direct comparison between tissues obtained before and after chemotherapy is a necessity. These samples are, however, not often obtained since secondary surgery is not considered beneficial in ovarian cancer (24). In order to decipher the role of chemotherapy on the immune response, and the best timing for immunotherapy it is crucial to study tumor samples before and after chemotherapy. Furthermore, for adoptive cell transfer (ACT) studies the phenotype and tumor-reactivity of TIL will need to be studied in primary and recurrence tumor tissue in order to determine what the best timing is for obtaining TIL for this approach.

Surgery

The amount of residual tumor tissue after surgery is highly predictive for survival and affects the prognostic effect of TIL (Chapter 4, (25,26)). Intrinsic biologic mechanisms might be responsible for the differences in survival and surgical result. Some gene signatures have been directly correlated to status of cytoreduction and might predict for incomplete cytoreduction (27). Furthermore, in serous and endometrioid ovarian cancers several molecular subtypes were identified and validated by genomic analyses of these tumors (28–30). For HGSC, 4 main subtypes have been identified, with clear differences in survival and, interestingly, also in immune responses. The ‘immunoreactive’ subtype has high expression of immune related genes and analyses of tumor tissue revealed a high infiltration of CD3+ TIL in tumor as well as in stroma. Two other subtypes (mesenchymal and differentiated subtype) show only limited infiltration with TIL, which are mostly restricted to the stromal compartment and appear to exert only a limited effect on prognosis. The last subtype that can be differentiated is the ‘proliferative’ subtype, which is associated with a low immune profile. Interestingly, the mesenchymal subtype, charac-
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Characterized by activated stroma, is associated with worse prognosis, which might be due to or the cause of the fact that this subtype is associated with incomplete surgical resection (31). Therefore, genetic profiles in these tumors might explain differences we find in prognostic effect of TIL in the cohorts and between samples of completely or incompletely resected tumors.

Patient stratification

As we have shown in Chapter 4, in order to study the prognostic value of TIL it is necessary to take the treatment regimen and surgical result into account. The fact that we see differential prognostic value of immune populations between these subgroups of patients also suggests that stratification of patients for immunotherapy trials should be based on these parameters. Furthermore, the infiltrate itself, per patient group, could be a way of stratifying patients for each treatment regimen. It is clear that a subset of patients has a high infiltration of TIL, while the majority of patients fails to elicit a proper immune response towards their tumors. In order to define the proper treatment regimen for each tumor type, it is necessary to differentiate which mechanisms are responsible for such differences in immune response.

It is likely that in patients that have high TIL infiltrate it would be necessary to re-activate these cells in combination with inhibition of other immune cells populations or tumor/stromal factors that induce suppression. In tumors in which only stroma is infiltrated, it needs to be determined whether these populations reflect TIL with tumor-reactive potential that are trapped in the stromal regions (32), or whether these cells are so-called bystanders drawn to the TME by its inflammatory character. If stromal TIL are tumor-reactive, suppressive mechanisms of the stroma could provide new clues for therapy. Alternatively, if these cells are bystanders that do not exert tumor-reactivity, or in patients that have tumors without TIL infiltrate, therapy should be focused on a general activation of the immune response. The immunoreactive subtype might respond best to checkpoint inhibition, co-stimulation, or adoptive cell transfer (ACT) with TIL. For the other subtypes it is of interest to determine the underlying suppressive mechanisms behind the differences in immune response in these tumors. In these tumors blockade of suppressive mechanisms, or activation of the immune response by vaccination strategies or chimeric antigen receptor T cell (CAR-T) ACT are treatment options.

Clinical and therapeutic implications

Pre-existent anti-tumor immunity can be unleashed and amplified to mediate tumor regression by either blockade of negative immunoregulatory molecules or engagement of agonistic receptors on T cells. Another method to enhance the anti-tumor immune response is the use of ACT strategies. In patients with a pre-existing immune response, TIL therapy may be successful. TIL therapy would however
only be successful if tumor-reactive T cells are present. The amount of tumor-specific mutations resulting in the presentation of neoantigens on tumor cells is likely of influence of that. Success of these treatments might therefore be most present in those tumor types with a high mutational load. Indeed, in melanoma it has been shown that best responses to such strategies occur in patients with TIL directed against neoantigens (33). Ovarian cancer might have lower responses in that regard due to lower predicted expression of neoantigens (34), although T cell activity against nonmutated antigens can also be distinguished and therefore the mutational load does not necessarily predict immunotherapy success (35). In patients lacking immune infiltrate, peripheral T cells may be used for CAR-T strategies or vaccination strategies could be examined.

**Checkpoint inhibition**

Selection of patients likely to respond to checkpoint inhibition, in which the PD-1/PD-L1 axis is blocked, can be based on PD-L1 expression or infiltration of PD-1+ TIL. Recently, in a phase II trial in which ovarian cancer patients were treated with αPD-1, tumor PD-L1 expression was not correlated to success of treatment (7). However, the evaluation of PD-L1 protein expression by immunohistochemistry is challenging because of its heterogeneous expression pattern and the large variation in reproducibility of antibody reagents (36). Furthermore, not only tumoral expression of PD-L1, but also expression of PD-L1 on immune cells in the microenvironment might predict for a successful treatment, and should be taken into account (37). In melanoma patients, the responders to anti-PD-1 treatment had a pre-existing immune response, which after treatment was increased compared to the group of patients that progressed on treatment (38). Therefore, it is likely that selection of patients for checkpoint inhibition should be based on a pre-existing immune response. Based on the results obtained in Chapter 4 and 5, the best biomarkers for this are high intratumoral infiltration of CD8+CD27+ cells in pre-treatment tumor samples, as well as expression of PD-L1 on tumor and stromal immune cells.

**Co-stimulation**

Another option to enhance the anti-tumor TIL response is by agonistic activating monoclonal antibodies. The development of these strategies has suffered from safety issues, with first trials using CD28 and CD137 stimulatory antibodies causing severe toxicity (39,40). Changes in the targets, antibodies, dosing and the exploration of combination treatments have made co-stimulatory antibody treatment a viable option. In Chapter 4 we show the importance of less-differentiated CD27+ TIL for prognosis in HGSC. The expression levels of CD27 provide rationale of co-stimulatory treatment with agonistic α-CD27. Preclinical studies have shown safety and treatment efficacy of a humanized α-CD27 antibody in preclinical models (41,42), as well as a synergistic effect of combinatorial antibody treatment with blockade of the PD-1/PD-L1 axis (43). This antibody is currently in phase I and II
trials for ovarian cancer patients as a single agent (Clinicaltrials.gov identifier: NCT01460134) or in combination with α-PD-1 (NCT02335918) or ONT-10, a MUC-I peptide vaccine (NCT02270372). In preclinical models for ovarian cancer, agonistic antibodies targeting TNF receptor family members CD137, OX40, and GITR have also led to strong CTL responses, especially in combination with checkpoint inhibition and chemotherapy (44–47). It will have to be determined which of these strategies is most successful and safe in clinical practice for EOC.

**Antibody treatment directed against tumor targets**

As shown in Chapters 6 and 7, interesting new targets for ovarian tumors are members of the claudin family of tight junction proteins. High expression of CLDN6 was found on HGSC tumors and for primary mucinous ovarian cancer CLDN18.2 is a target of interest. Both proteins can be used as a therapeutic target for monoclonal antibody treatment, which induces immune effects as antibody-dependent cell cytotoxicity (ADCC) or complement-dependent cytotoxicity (CDC). Combination of such strategies with checkpoint blockade and/or co-stimulatory antibody treatment may further enhance effects, though the toxicity profile of such combination strategies needs to be considered.

**TIL selection for ACT therapy**

While TIL are considered to be enriched for tumor-reactive T cells compared to peripheral blood, the total pool of isolated TIL from a tumor also contains exhausted T cell populations and suppressive subsets such as Treg. Therefore, markers defining the tumor-reactive subset to select for ACT strategies need to be considered. In melanoma the PD-1 positive fraction of CD8+ TIL was found to produce more IFN-γ upon tumor stimulation (48). However, as we and others have shown, PD-1 is expressed highly on most ovarian TIL and therefore does not seem to define a specific subset (Chapter 4, (49,50)). When looking at the CD8+ TIL population, those cells present in the tumor epithelium predict for prognosis better than do stromal TIL. Therefore, the specific intratumoral marker CD103, a α/β7 integrin molecule, is of interest (51), of which the CD27+ subset would arguably have most potential (Chapter 4). CD27 represents a marker for T cells with high proliferative potential in response to antigen exposure, that persist longer after re-administration and provides for tumor regression (52–54). As an alternative, CD137, a marker for the tumor-reactive subset in ovarian cancer, could also be used as a selection criterion for the population of interest (49). However, it remains to be determined whether this population is associated with improved prognosis and a major issue with selecting this population for ACT is the low cell yield, with expression on 0.9-20% of TIL (49). Since the CD27+ population is enriched for CD137+ and associated with improved tumor control, selection of CD103+CD27+ TIL for ACT in ovarian cancer should be considered.
Chimeric antigen receptor T cell (CAR-T)

Since only a subset of patients presents with a significant number of intratumoral T cells, TIL therapy will not be applicable in a large group of patients. Another option for a T cell based approach is ACT with CAR-T using peripheral blood T cells. In this approach a chimeric T cell receptor targeting a tumor antigen is inserted in autologous T cells and expanded to sufficient quantities to infuse. Selection of T cells with the highest proliferative potential is warranted for proper persistence and proliferation of these cells after infusion. T cells with high proliferative and engraftment capacities include naïve T cells (Tn), T memory stem cell (Tscm) and the central memory (Tcm) populations (55). Therefore, to achieve longest persistence and most proliferative potential of CAR-T the Tn and Tscm populations would be first choice to use. The success of CAR-T treatment is largely dependent on the right choice of target on the tumor cells and the availability of a properly cell-specific marker at the tumor cell surface. Various tumor-associated surface antigens have been associated with ovarian tumors, with highly variable expression between patients (56). In this regard, the finding that CLDN6 is highly expressed on HGSC tumors and CLDN18.2 on primary mucinous ovarian tumors while being absent on healthy tissues provides a rationale to target these cell-surface proteins with this approach. Next to selection of T cell population and selection of target on tumor cells, it is of importance to make sure that the CAR-T will be able to survive the hostile environment of the tumor. Therefore, also here combination strategies targeting the suppressive populations such as FoxP3+ and myeloid cells as described earlier are of interest.

Future directions

In order to validate TIL as a biomarker for prognosis or for stratifying patients for immunotherapy strategies, further studies are warranted comparing samples during all phases of treatment. Studies determining differences in responses to chemotherapy on TIL infiltration, function, and the TME in different subtypes of tumors are warranted. The study of tissue from recurrent and metastatic disease can reveal whether immune profiles change during progression of disease, with potential implications for determination of timing of treatment strategies. Subsequently, in clinical trials for immunotherapeutic strategies it is of interest to determine which immune characteristics and factors in the TME are most informative to predict the success of treatment strategies.

Further studies on genetic profiles, mutational load, and microenvironmental factors can provide information on what has occurred in those patients that have low or no immune infiltrate and whether an immune reaction can still be unleashed. Combined efforts studying genetic, environmental, and immune factors will be necessary to understand the underlying mechanisms of the immune response in ovarian tumors.
Conclusion

Epithelial ovarian cancer is a heterogeneous disease, with variable histological, genetic, and immune profiles. These profiles will be of importance in order to stratify patients for the best possible treatment regimen. The prognostic effects of immune infiltrates provide rationale for immunotherapy, and selection of patients based on their immune profiles might be the next step towards successful treatment of this malignancy.

References

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54. Rosenberg SA, Yang JC, Sherry RM, Kammula US, Hughes MS, Phan GQ, et al. Durable Com-
plete Responses in Heavily Pretreated Patients with Metastatic Melanoma Using T-Cell Transfer Immuno-
55. Klebanoff CA, Gattinoni L, Restifo NP. Sorting through subsets: which T-cell populations me-