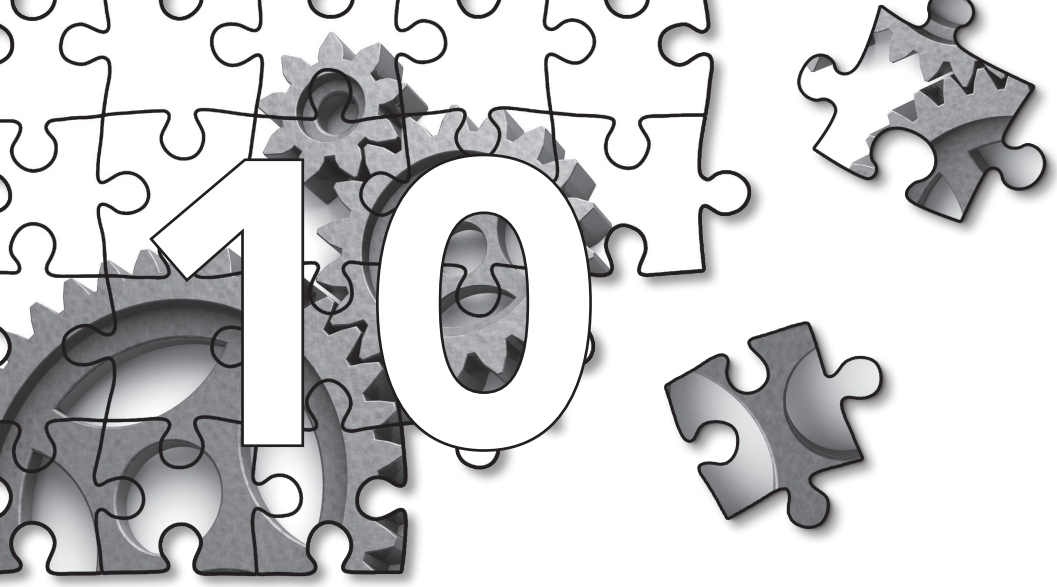

GENERAL DISCUSSION

Immunotherapy is emerging as a successful and promising treatment option for cancer patients. A rapidly increasing number of clinical studies show encouraging results with various types of immunotherapies. Checkpoint blockade is emerging as a valuable treatment option, especially for cancers with unknown or many mutated antigens. Monoclonal antibodies targeting CTLA-4 (Ipilimumab) or PD-1/PD-L1 have already shown clear clinical effects but many other immunotherapeutics are promising as well. Therapeutic vaccination against cancers with known antigens is also evolving as a treatment option. Since no central tolerance can be induced to virus and mutated antigens, therapeutic cancer vaccines preferably target these.

Side effects

While many patients benefit from immunotherapy, improvements to treatment protocols are necessary. This dissertation aimed at further understanding of the mechanisms involved in various immunotherapies and combination treatments in animal models and to translate these to the current treatment protocols. New advances in (combinatorial) interventions for HPV related tumors have been described in **chapters 6, 7** and **9**. In **chapter 6A** for example, we show that an understanding of mechanisms involved in optimally matched combinations of chemotherapy and immunotherapy, permit lowering of the chemotherapy dose resulting in decreased treatment related side effects, a phenomenon further discussed in **chapter 6B**.

Treatment related side effects are also observed in a significant proportion of the patients treated with checkpoint blockade. For example, patients treated with CTLA-4 blockade often develop immune-related adverse events such as enterocolitis, hypophysitis and hepatitis (1). We show in **chapter 8** that local treatment with one injection of low-dose CTLA-4 blocking antibodies is equally effective in tumor eradication as repeated treatment with systemic high-dose anti-CTLA-4 antibodies and results in systemic tumor-specific CD8 T cell responses, capable of controlling a distant tumor.

There are two important advantages of this local treatment modality. First, local treatment coincides with decreased treatment related side effects and may therefore be a preferred treatment modality for cancer patients. A second advantage of local low-dose therapy is that the total amount of antibody needed per patient may be decreased. Currently, there is debate about the hefty price tag for the use of immunomodulating antibodies such as Ipilimumab and PD-1/PD-L1 targeting antibodies such as Nivolumab. Immunotherapy becomes such an expensive treatment option that it may become unaffordable and inaccessible for many patients (2-4). In **chapter 8** we show that one local injection with 50 μg anti-CTLA-4 antibody is equally effective as two injections with each 200 μg antibody, indicating that local treatment decreases the number of necessary treatment cycles. This suggests that not only the total amount of antibody needed per patient can be lowered but also that the total time a patient is admitted to a hospital to receive treatment can be decreased. This implicates that local instead of systemic Ipilimumab treatment in patients could result in decreased treatment related costs. In animals the use of a lower dose and only one injection of antibody together results in an eight-time reduction in the amount of anti–CTLA-4 used. Currently, clinical trials are initiated in Europe and the USA to investigate this and first reports indicate that $1/20^{th}$ of the clinical dose may be effective (personal communication of Professor R. Levy and Professor L. Eggermont).

Combination therapy: how to (mis-)match them?

To gain insight in the potential combinatorial use of vaccines and checkpoint-blocking antibodies (**appendix**) we also used the HPV16 E6 and E7 expressing TC-1 tumor model. We blocked the interaction between PD-1 and PD-L1 and combine this with long peptide vaccination to try to improve survival. Previously others have used this tumor model to test the feasibility of the combination of other HPV targeting vaccines and blockade of PD-1/PD-L1 interactions and documented improved survival upon the addition of blockade of PD-1 (5) or PD-L1 (6) to vaccination. Indeed, we showed that vaccination results in infiltration of high numbers PD-1 expressing T cells, capable of IFN-γ production. This infiltration was associated with a strongly enhanced expression of PD-L1 on both myeloid cells and tumor cells. However, blocking the interaction between PD-1 and PD-L1 had, depending on the timing, no or minimal effect on survival of tumor bearing mice. Multiple factors potentially can explain the discrepancy between our and previous studies. First, the antibodies used in the **appendix** are of a different isotype then those used in the papers that described an improvement of the vaccine effect upon combination of PD-1 or PD-L1 blocking antibodies. The effect of various immunomodulating antibodies, including PD-1 and PD-L1, is affected by the FCγR-bindings capacity (7-9). PD-L1 antibodies with optimal FcγR binding capacity augment the anti-tumor activity of the antibody (9). In contrast to Badoual and collegues (6), we use a PD-L1 antibody of the IgG2a isotype with optimal FcγR binding capacity, suggesting that we have been using the most optimal PD-L1 blocking antibody and the lack of PD-L1 mediated (improved) anti-tumor responses is not related to the FcγR binding capacity of the antibody.

On the other hand, for PD-1 FcγR engagement can diminish the effect of PD-1 blockade, presumably by ADCC-mediated killing of effector T cells (9). As our PD-1 antibody is also of the IgG2a isotype, it may therefore have negatively affected the anti-tumor effect. Indeed, in a recent paper that combines intranasal vaccination with HPV E6/E7 peptides with α-galactosylceramide with the same αPD-1 clone as we used, PD-1 blockade had also no effect on vaccine efficacy (10). However, since the overall effect of vaccination with anti–PD-1 is similar to that with anti–PD-L1, the contribution of FcγR mediated effects to the overall survival is probably small and other explanations, such as discussed below, are more relevant.

Tumor regressions require a unique composition of immune cells in the tumor.

Synthetic long peptide (SLP) vaccination as a stand-alone treatment results in tumor regression, however many other groups that use the TC-1 tumor model show that other vaccines, such as short peptide vaccines in combination with anti-CD40 and GM-CSF or E7 subunit vaccines induce tumor delay but often no or hardly any tumor regression, let alone cure (5, 6, 10-13). Apparently, the quality and the (poly-) functionality of HPV16 $E7_{49-57}$ specific T cells induced by synthetic long HPV16 $E7_{43-77}$ peptide is better than that of T cells induced by many other vaccines such as those used in the studies that observe benefit from combined vaccination and PD-1/PD-L1 blocking therapy (5, 6). PD-1 blockade functions primarily through restoration of T-cell effector function, thereby increasing their fitness in the tumor microenvironment (14).

SLP vaccines induce apparently polyfunctional CD8 T cells with a high killing capacity resulting in tumor regressions. These T cells seem thus to function optimally and therefore require no PD-1 blockade. Therapies that are combined with SLP vaccination are probably most synergistic when their mode of action to target the tumor is via other mechanisms than through T cell activation. The data presented in **chapter 6** shows that chemotherapy is a good option but other cell death-inducing modalities may be considered as well (reviewed in **chapter 9**).

When both TC-1- and C3-tumor bearing animals were treated with the HPV16 $E7_{43-77}$ SLP vaccine (**chapter 3**) we observed that vaccination results in the infiltration of cytokine producing CD8 T cells in the TC-1 tumors, but not the C3 tumors. This T-cell infiltration is crucial for a coordinated infiltration of tumors with high numbers of various types of myeloid cells. IFN-γ and TNFα, produced by vaccine-induced intratumoral CD8 T cells can enhance the production of chemokines such as *CCL2, CCL5,* and *CXCL10* by tumor cells. The altered vaccine-related chemokine profile within the tumors then results in the attraction of different types of myeloid cells, predominantly inflammatory macrophages (15-17). Both tumor cell lines display a similar chemokine-expression profile upon incubation with IFN-γ and TNFα *in vitro*. Since the cytokine producing T cells infiltrate only the TC-1 tumor, these tumors are also increasingly infiltrated with tumor-rejection associated myeloid cells.

Interestingly, when we dissected the effect of other treatments on myeloid cell infiltration we observed that TC-1 tumors and C3 tumors respond *in vivo* in a similar way to the maximum tolerable dose of cisplatin and contain exclusively an enhanced number of inflammatory macrophages but no alterations in the frequencies of DC-like macrophages, tumor associated macrophages or granulocytic myeloid cells (**chapter 4**). This alteration is already observed four days after cisplatin treatment; a moment when it is too early to expect effects of potentially treatment-induced T cells. This suggests that, unlike vaccine-related infiltration of inflammatory macrophages, chemotherapy-induced infiltration of inflammatory macrophages is not related to the infiltration of cytokine producing T cells. The chemokine CCL2 can be involved in the recruitment of inflammatory macrophages to tumors (17, 18). The enhanced CCL2 levels in cytokine producing T cell-infiltrated tumors suggest that CCL2 is involved in the attraction of the inflammatory macrophages upon vaccination (**chapter 3**). In fact, the vaccine-induced myeloid infiltration of TC-1 tumors is decreased in CCR2-deficient mice and also the vaccine efficacy is reduced, suggesting a crucial role for this cytokine in myeloid infiltration (19). However, *in vitro* treatment of TC-1 tumor cells with cisplatin does not result in enhanced levels of CCL2 (Figure 1), suggesting that cisplatin-induced inflammatory macrophage infiltration is not a direct effect of cisplatin on tumor-produced CCL2 induction. We noted that *CCL5* levels of tumor cells are 10 times enhanced upon *in* vitro cisplatin treatment, and enhance even further when TNF or TNF and IFN-γ are added (Figure 1). Therefore, these data suggest that CCL5 may be involved in the infiltration of inflammatory macrophages (20, 21). However, we cannot exclude that other mechanisms play a role too and further studies are warranted to dissect the mechanisms involved.

In **chapter 3** we show that when treatment with the tyrosine kinase inhibitor PLX3397 is combined with SLP vaccination, survival is reduced compared to vaccination alone. In contrast, other studies that combine immunotherapeutics with PLX3397 have described synergy (**chapter**

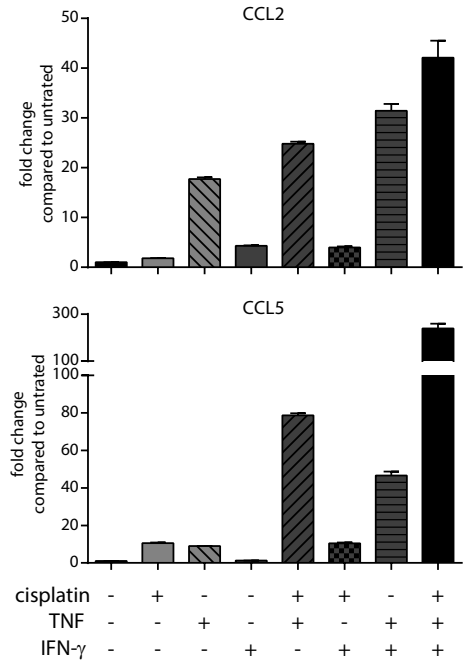

Figure 1. Chemokine induction upon treatment with cytokines and cisplatin.
Gene expression profiling by qPCR of the chemokines CCL2 and CCL5 in TC-1 tumor cells after 22 hours of exposure to TNFα, IFN-γ and/or 2 μg/ml cisplatin. Data is represented as fold-change over untreated tumor cells. Means and standard deviations are depicted.

2, (22-24)). Again, the vaccine induced tumor regressions instead of growth delay may explain this discrepancy between our data and that of others. When tumors regress due to massive tumor cell killing by for example T cells, macrophages can phagocytose the cell debris that remains. In fact, data obtained by others that use a highly similar experimental setup as we do in **chapter 3** indicate that upon vaccination, TC-1 tumor cells can be engulfed by intratumoral macrophages at the start of the tumor regression phase. The requirement of tumor infiltrating myeloid cells for vaccine induced EG7 tumor regressions confirmed the need for myeloid cells in tumor regressions (19). Additionally, the cytotoxic capacity of macrophages isolated from tumors of vaccinated mice is higher than that of macrophages isolated from untreated tumors (19). Together these data indicate that successful vaccination alters the function of intratumoral myeloid cells. A variety of other cancer-treatments can also cause recruitment or differentiation of myeloid cells in tumors ((25, 26) and **chapter 4**). These cells may support intratumoral recruitment of CD8 T cells but the data presented in **chapter 3** indicate also that regression-inducing treatment requires multipotent myeloid cells, capable of for example phagocytosis of tumor debris. However, the underlying mechanisms remain largely unknown so far and further studies in regression models are warranted.

The data presented in **chapter 5** reveals another level of complexity regarding the role of myeloid cells in tumor progression. In this chapter we show that in mice and patients the presence

of a progressing tumor is associated with enhancing frequencies of circulating myeloid cells. Similar as in **chapter 2,** in **chapter 5** we describe a treatment-related normalization of myeloid cell frequencies that coincides with improved therapeutic efficacy in tumor bearing mice and advanced cervical cancer patients. In B16F10 bearing mice PLX3397 treatment dampens accumulation of macrophages, while the Gr-1$^+$ myeloid derived suppressor cells are unaffected. On the other hand we observe that treatment of TC-1 tumor bearing mice with carboplatin, paclitaxel (CarboTaxol) and / or therapeutic vaccination results in decreased frequencies of Gr-1$^+$ myeloid cells while macrophages and dendritic cells remain unaffected Treatment with PLX3397, however, primarily causes depletion of macrophages which is detrimental for vaccine-induced TC-1 and EG7 tumor regressions (**chapter 3** and (19)). The different outcomes in these tumor models illustrate the complexity of the intratumoral myeloid cell composition and its influence on treatment outcome. In fact, patients treated with standard CarboTaxol and vaccination, display enhance T cell reactivity to common pathogens while also the HPV16-specific responses were unusually strong, suggesting that the altered myeloid cell levels resulted in either an enhanced overall antigen presenting cell function or a decreased suppressive capacity of these cells. The selective decrease in suppressive cells may be due to the fact that fast dividing immature myeloid cells are more sensitive to chemotherapy than dendritic cells and mature macrophages. Together these data indicate that the myeloid cells removed upon this chemo-immunotherapy are not crucial for the vaccine-induced tumor regression but are in fact mmunosuppressive subsets.

"Immunogenic cell death" comes in different flavors

The expression level of costimulatory molecules on intratumoral myeloid cells, specifically on the inflammatory macrophages, is higher upon systemic cisplatin treatment when compared to the levels of these molecules on myeloid cells in tumors of untreated mice (**chapter 4**). Cisplatin related survival of TC-1– and C3–bearing animals depends on CD8 T cells and on the expression of costimulatory molecules on the myeloid cells.

Previously cisplatin and carboplatin have been described as chemotherapeutics incapable of inducing "immunogenic cell death", a process of cell death stimulating an immune response against dead-cell antigens for which calreticulin must be translocated from the lumen of the endoplasmic reticulum to the surface (27, 28). These specific characteristics can be important for the induction of a tumor-specific immune response (28), however in the context of systemic chemotherapy intended to exert a therapeutic effect, other characteristics of the chemotherapy may become even more important. The data presented in **chapter 4 and chapter 5** clearly indicates that cisplatin and CarboTaxol exert their effect via alterations in the composition of intratumoral myeloid cells expressing higher levels of costimulatory molecules, crucial for the induction and maintenance of CD8 T cell dependent anti-tumor responses. These data suggest that cisplatin induced tumor *cell death* and consequently the induction of tumor-specific CD8 T cells is not the only mechanism operative in the antitumor response implying that a more complex sequence of events, involving the attraction and alteration of various immune cells are required for effective anti-tumor responses.

Concluding remarks

This dissertation represents only a fraction of all the progress made in the field of tumor immunology in the last 5 years. It has become clear that each tumor as well as each type of therapy induces a unique composition of tumor infiltrating immune cells. These cells are capable of interacting with each other and insight in these interactions is crucial for further improvements of treatment protocols. Therapy combinations that are translated into the clinic should be based on a detailed understanding of the mechanisms involved. This will result in decreased treatment related toxicity and improved clinical responses in cancer patients.

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