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Chapter 8

Summary and future perspectives

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SUMMARY

In this dissertation, the triad immune system, colorectal cancer and immunotherapy was explored to understand how they interact, to develop immunotherapeutic approaches and to improve prognosis of colorectal cancer patients in the future.

Immune cell infiltration and HLA class I expression in colorectal tumors

Many factors present on tumor cells and in the cancer microenvironment influence the function of the immune cells and enable tumor cells to escape from immunity. One of these might consist of down-regulation of human leukocyte antigen (HLA) class I on tumor cells, thereby prohibiting presentation of tumor antigens to cytotoxic T lymphocytes (CTL), and keeping CTL from tumor cell lysis. There are strong indications that complete absence of HLA class I expression in colorectal tumors is particularly found in colon tumors with a high level of microsatellite instability (MSI-H) ^{1;2}. Unfortunately previous studies that evaluated the prognostic impact of HLA class I expression in colorectal cancer, used cohorts consisting of both colon and rectal cancer patients including both microsatellite stable (MSS) and MSI-H tumors ^{3;4}. These studies described a survival advantage for patients with HLA class I negative tumors, probably being the MSI-H tumors ^{3;4}. To study the prognostic impact of HLA class I loss in MSS tumors, we decided to determine HLA class I expression in a population of rectal cancer patients, as rectal tumors mainly consist of MSS tumors ⁵⁻⁷. In **chapter 2** our results indicated that low expression of HLA class I in rectal tumors was associated with poor overall and disease free survival of rectal cancer patients ⁸. Therefore these results indicated that rectal cancer cells down-regulate expression of HLA class I molecules to escape CTL mediated immunity. Our results in rectal tumors might be extrapolated to patients with a MSS colon tumor. The clinical impact of HLA class I expression remains to be established for patients with a MSI-H colon tumor.

It is generally accepted that of all immune cell markers, especially presence of the T-cell markers CD3 and CD8 is positively associated with prognosis of colorectal cancer patients ⁹. Down-regulation of HLA class I surface molecules is generally thought to be a tumor immune escape mechanism aimed at evading CTL cell recognition and elimination ^{3;4;8}. Cytotoxic activity of Natural Killer (NK) cells is regulated by a balance of activating receptors and inhibitory receptors ^{10;11}. The most prominent inhibitory receptor in humans being: HLA class I. Consequently down-regulation of HLA class I potentially activates Natural Killer (NK) cells. Previously it has been shown that presence of CD8⁺ lymphocytes in colorectal cancer cells correlated with absence of HLA class I ¹². Whether intratumoral CD8⁺ cells represented CTL, NK or NK-T cells remained to be determined. Obviously, patients with tumors lacking HLA class I

expression would benefit most if these CD8⁺ T-cells represented NK cells. In **chapter 3** we showed that NK cells form only a minor fraction of the total tumor-infiltrating leukocyte population in all colorectal tumors, using CD56 to detect NK cells ¹³. A potential pitfall is formed by the expression of CD56. Two subpopulations of NK cells exist i.e.: CD56^{dim} NK cells appear to be primarily cytotoxic effector cells while CD56^{bright} NK cells have predominately regulatory functions ¹⁴. A possible explanation for the low number of NK cells might be that immunohistochemical techniques are not capable to detect CD56^{dim} cells. Therefore, a four-color-immunofluorescence staining technique was applied ¹⁵, demonstrating that tumors showing loss of MHC class I expression were more vigorously infiltrated by CD3⁺CD8⁺Granzyme B⁺ positive T-cells, confirming that tumors are poorly infiltrated with NK cells. A possible explanation for the lack of intratumoral NK cells might be that the main function of NK cells is on a systemic level, where they may be able to eliminate metastasized malignant cells ^{16;17}.

Migration of leukocytes into the cancer microenvironment

Effective anti-tumor immunity requires contact between cells of the immune system and tumor cells. Immune effector cells that developed in lymphoid organs and entered the circulation have to leave the vasculature and enter the cancer microenvironment. Homing of activated effector T-cells into the tumor consists of different steps. At the site of the tumor, endothelial cells are activated to express ligands for leukocyte adhesion. Once leukocytes attach to these ligands they have to pass the endothelium and enter the extravascular cancer microenvironment. From here, depending on their function, they have to migrate into the nests of tumor cells. The mechanisms governing homing of effector cells into tumors remain poorly understood, but this whole process is affected and coordinated by cytokines. One group of cytokines influencing the migration of leukocytes comprises of chemokines. In **chapter 4** we showed, using a rat tumor model that low expression of the chemokine CXCL5 in tumor cells resulted in rapid tumor growth and increase in the number of metastases, while *in vitro* no difference was found in proliferation rate between clones with either high or low expression of CXCL5 ¹⁸. The relevance of these results for humans was confirmed, as low expression of CXCL5 in cancer cells was significantly associated with poor prognosis in a population of colorectal cancer patients. Finally a positive correlation between expression of CXCL5 and presence of intra-tumoral CD8⁺ T-cell infiltration in humans was found. These results indicated that expression of CXCL5 is associated with intraepithelial infiltration of specific leukocyte subtypes, resulting in tumor regression, tumor specific immunity and better prognosis ¹⁸. This concept has also been described for other chemokines in various types of tumors ¹⁹⁻²⁷. Together, these data argue that tumor cells themselves play a

key role in shaping the tumor-immune microenvironment and thereby clinical course of patients²⁸. To finally influence the type of immune cells trafficking towards tumor cells it is important to determine the correlation between colorectal cancer phenotype and type of immune cell infiltrate in the cancer microenvironment.

Colorectal cancer vaccines

One of the most unique features of the immune system consists of its capacity to specifically search and destroy targets. As such, many have discussed if tumor cells represent one of the regular targets of the immune system and in addition if the patient's own immune system can be used to specifically destroy tumor cells once tumor cells escaped immune surveillance²⁹. Subsequently, many have tried to reinforce the immune system to cure cancer patients, using different approaches. Here we focused on induction of tumor specific T-cells against predefined antigens. Distinction should be made between MSI-H and MSS tumors for immunotherapeutic purposes, as MSI-H colon tumors express neo-antigens "foreign" to the immune system while immunotherapy against MSS colorectal tumors depends on tumor associated "self"-antigens.

MSI-H tumors: frameshift mutated products, a unique class of tumor-specific antigens

Despite many years of work, the number of antigens recognized by TILs of colorectal cancer identified is limited³⁰⁻³³. Consequently, vaccines so far have been developed on the basis of proteins that are selectively expressed by tumor cells but for which immunity can be blunted or may lead to autoimmunity^{34;35}. The exception comprises MSI-H tumors that, due to numerous of frameshift mutations in microsatellites express neo-antigens. MSI-H is a molecular feature of tumors associated with the familial Lynch or hereditary non-polyposis colorectal cancer (HNPCC) syndrome, accounting for approximately 5% of all colorectal cancer cases and for approximately 15% of all sporadic colorectal cancers³⁶⁻⁴⁵. Since frameshift mutated protein products (FSPs) are foreign to the immune system, they represent a unique group of tumor-specific antigens. No tolerance and consequently strong T-cell responses are expected against these FSPs. A few studies have been performed to predict the immunogenic behavior of a selection of frameshift mutated genes which are frequently detected in MSI-H cancers^{33;46;47}. Unfortunately, relatively little is known on the immunogenic behavior of most of the FSPs³³. Therefore we developed a methodology, described in **chapter 5** for predicting their immunogenic behavior that is based on accumulation and MHC class I presentation⁴⁶. Our data indicated that, out of the 15 FSPs examined in our study, 4 (TGF R2-1, MARCKS-1, MARCKS-2 and CDX2-2) are of primary interest⁴⁶. Four additional antigens (TAF1B-1, PCNXL2-2,

TCF7L2-2 and Bax α +1) are of moderate interest for further tumor immunological research ⁴⁶. The data of others suggested that FSP-specific T-cells may be present in the circulation of patients with MSI-H colorectal cancer, healthy HNPCC syndrome mutation carriers, but not in patients with microsatellite stable (MSS) colorectal cancer or in healthy donors ^{47;48}. In general, most FSPs consist of a relatively small number of amino acids downstream of the frameshift mutation, suggesting that the FSPs may contain a sequence that can only be presented by a limited number of HLA class I or HLA class II molecules. In order to treat patients, knowledge on which HLA class I and II molecules can present epitopes comprised by the FSPs should be obtained. Although MSI-H tumors comprise only about 15% of all colorectal tumors, patients with a MSI-H tumor are very interesting vaccination candidates because: 1) strong effector responses are expected after vaccination using non-self-antigens; 2) colorectal cancer is one of the major cancers in the western world; and 3) many families with Lynch or HNPCC syndrome at risk for a MSI-H tumor have been identified. The latter group may be amenable for prophylactic vaccination to prevent the outgrowth of MSI-H tumors. Hence, a rapid identification of the immunogenic non-self-segment of the frameshift products is required.

MSS tumors: p53 vaccination in colorectal cancer patients

In **chapter 6** the safety and immunogenicity of a p53 synthetic long peptides (p53-SLP®) vaccine were investigated in patients treated for metastatic colorectal cancer ⁴⁹. The vaccine proved to be safe and highly immunogenic. However, mainly p53-specific CD4⁺ T cells were induced after vaccination. Since the p53-specific CD8⁺ T-cell, but not the CD4⁺ T-cell repertoire is known to be severely restricted by self-tolerance and might only consist of lower affinity p53-specific CD8⁺ T cells, these results confirmed previous studies ^{34;50}. The presence of tumor-specific CD4⁺ T cells is important in cancer immunotherapy because IFN- γ secreting CD4⁺ Th1-cells play an important role in orchestrating and sustaining the local immune attack by CD8⁺ CTL and innate effector cells ⁵¹⁻⁵⁴. Unfortunately, the overall production of pro-inflammatory cytokines such as IFN γ by the p53-SLP® vaccine-induced T-cell population in our trial was low. Therefore a new study was designed (**chapter 7**) to modulate the induced p53-specific CD4⁺ T-cells by combining the p53-SLP® vaccine with Interferon-alpha (IFN α). This study clearly illustrated that addition of an adjuvant such as IFN- α injection to the vaccine safely modified both the vaccine-induced p53-specific humoral and T-cell responses. Addition of IFN- α to the p53-SLP® vaccine significantly improved p53-immune response against a broader range of peptide pools and also induced a larger number of vaccine-specific IFN- γ ⁺ T-cells. These results confirmed that IFN- α is able to modulate a vaccine-induced Th1 response.

FUTURE PERSPECTIVES

Altogether this dissertation reports on the relation between the immune system, colorectal cancer and immunotherapy. This knowledge can be used to further optimize immunotherapeutic strategies to treat cancer patients. For colorectal cancer only a few trials focused on clinical efficacy, this comprised phase III trials using irradiated tumor samples^{55;56}. These trials suggested some clinical benefit in selected subpopulations but overall results were rather disappointing^{55;56}. Most of the vaccination trials for colorectal cancer patients have been designed to test safety and immunogenicity but have yet not resulted in the design and execution of phase III trials⁵⁷. Although in most trials no serious vaccine related adverse events were noted, lack of clinical results suggests that the vaccine-induced T-cell responses against these antigens are at this point not robust enough or of sufficient quality to confidently progress to efficacy trials. The most recent vaccine developments suggest that some of the current cancer vaccine strategies do harbor the capacity to induce strong immune responses in cancer patients even to self-antigens^{49;58-64}. While these vaccines may still have to be optimized, the data suggest that the vaccine-induced activation of tumor-specific T-cell reactivity is no longer an issue of concern. However, other relevant questions remain:

- What are the tumor antigens recognized by tumor-infiltrating T-cells, and which antigens would be most appropriate in colorectal cancer?
- Which immune cells are to be induced during vaccination and does vaccination only enhance effector T-cells or also suppressive T-cells?
- Which adjuvants should be combined with vaccines to optimize the induced vaccine response?
- Do vaccine-induced tumor-specific leukocytes migrate to the tumor and mediate an antitumor effect?
- Which cancer patients are most likely to benefit from immunotherapy?

What are the tumor antigens recognized by tumor-infiltrating T-cells, and which antigens would be most appropriate in colorectal cancer?

New vaccine strategies have resulted in vaccines that are able to efficiently induce vaccine specific immune responses. However, vaccine strategies in colorectal cancer still suffer from a lack of antigens that may be used for vaccination. Whereas for other types of tumors the reactivity of tumor-infiltrating T-cells validate the choice of antigen used in the vaccines for that type of cancer⁶⁵, this is still limited in colorectal cancer and calls for more in-depth studies on the specificity of T-cells infiltrating the tumor or present in metastatic lymph nodes. In view of the increasing knowledge on

the role of role of CD4⁺ T-cell help to the induction, sustainment and migration of CD8⁺ T-cells, it is advisable to screen not only for tumor-specific CD8⁺ T-cell responses but also for tumor-specific CD4⁺ T cells.

Which immune cells are to be induced during vaccination and does vaccination only enhance effector T-cells or also suppressive T-cells?

The history of constant interactions between tumor and immune system shapes both tumor and the immune system of an individual patient in a way that is difficult to mimic in animal tumor models. It is of utmost importance that vaccines only boost the reactivity of immune cells that mediate an antitumor effect and not that of immune cells that support tumor growth. As most tumor associated antigens are intracellular proteins and results from observational studies show that especially presence of intra-epithelial activated CD8⁺ T-cells has a positive impact on prognosis⁹, immunotherapeutic strategies start by inducing tumor-specific CD8⁺ T-cell responses. The activation of cytotoxic T-cells depends on a network of collaborating leukocytes. Consequently vaccines should create a CD8⁺ T-cell friendly and supportive cancer microenvironment. Indeed data from different studies indicate that especially a Th1 associated type of cancer microenvironment is beneficial to the prognosis of cancer patients⁶⁶⁻⁶⁸.

From immunohistochemical studies it is clear that colorectal cancers are amongst others infiltrated by both CD4⁺ and CD8⁺ Foxp3⁺ T-cells^{69;70}. The number of Foxp3⁺ Regulatory T-cells (Tregs) correlates with disease stage and survival in colorectal cancer in several studies⁷⁰⁻⁷². Notably, the analyses of the antigens recognized by colorectal cancer infiltrating Tregs revealed that they recognized colorectal cancer-associated antigens, in particular Mucin, Her-2/neu, and CEA³¹. Hence, therapeutic vaccination with these antigens may not only boost CD4⁺ and CD8⁺ effector T-cells but also the Treg population. Vaccine-induced expansion of such antigen-specific Tregs has been observed previously in a mouse tumor model⁷³ and also in humans⁵⁸. In the p53-SLP@ vaccination trial, strong p53-specific CD4⁺ T-cell responses were found but this did not coincide with the expansion of p53-specific CD4⁺Foxp3⁺ T-cells⁴⁹. This fits with the observation that the T-cell response to p53 in colorectal cancer patients is not under control of Tregs³¹.

Which adjuvants should be combined with vaccines to optimize the induced vaccine response?

It is not likely that colorectal cancer vaccines are able to induce the desired clinical responses on their own, but need to be combined with other modalities that target regulatory mechanisms in order to improve the local microenvironment. The current wealth of preclinical and clinical information predicts a future strategy in which

therapeutic vaccines, blockers of immunosuppressive mechanisms and conventional therapies are applied jointly to overcome immunological tolerance and promote tumor regression. In general, a stronger focus should be put on how to induce the strongest and best qualified leukocyte population by vaccination. Vaccines should be combined with adjuvants to induce a vaccine specific type 1 polarized response and suppress a type 2 response. At the moment many candidate adjuvants are available. Also chemotherapeutics and monoclonal antibodies comprise strong immune modulating agents that can be used to polarize a response after vaccination. Various mechanisms may explain the reported synergistic effects of chemotherapy and T-cell restricted immunotherapy. Direct effects of chemotherapy on tumor or host environment, such as induction of tumor cell death, elimination of regulatory T cells, and/or enhancement of tumor cell sensitivity to lysis by CTL may account for enhancement of immunotherapy by chemotherapy. On the other hand, immunotherapy may directly modulate the tumor's sensitivity to chemotherapy ⁷⁴. Indeed, results have suggested that a vaccine encoding the tumor antigen 5T4 can be layered on top of chemotherapy regimens in patients with metastatic colorectal cancer without any evidence of enhanced toxicity or reduced immunological or therapeutic efficacy ⁷⁵.

Monoclonal antibodies are designed to interfere with specific signaling pathways. Recently, the CTLA-4 blocking antibody Ipilimumab has been successfully used in the treatment of melanoma patients ⁷⁶. In human beings several approaches have been used to delete Tregs ⁷⁷. Notably, decreases in CD4⁺CD25⁺Foxp3⁺ cells have been detected when patients with hepatocellular cancer were treated with low cyclophosphamide ⁷⁸, as well as in metastatic melanoma patients treated with the anti-CD25 antibody Daclizumab ⁷⁹, or after using denileukin diftitox ⁸⁰. Based on their mechanisms of action it is highly likely that these antibodies will synergize with vaccines as they will block the negative feed-back on vaccine-induced tumor-specific T cells.

Do vaccine-induced tumor-specific leukocytes migrate to the tumor and mediate an antitumor effect?

We showed that expression of CXCL5 by tumor cells was positively related with both strong intra-epithelial infiltration of the tumor cell nests by CD8⁺ T cells and a better clinical prognosis of colorectal cancer patients. Indeed chemokine expression as well as that of endothelial adhesion molecules and extracellular matrix has been associated with the migration of leukocytes into colorectal carcinoma ^{18;81-83}. This suggests that tumor cells themselves play a key role in shaping the tumor-immune micro-environment. The tumor phenotype, i.e. the status of tumor gene expression that attracts, activates or inhibits immune defense, determines the magnitude and type of immune infiltration and thereby clinical course of patients and represents a target for innovative diagnostic and therapeutic strategies. A profound understanding of

how the trafficking of these different cell populations is coordinated can be exploited for the development of successful immunotherapeutic strategies. One can start by comparing gene profiles of colorectal tumors with a high number of tumor infiltrating leukocytes versus those with a low number of tumor infiltrating leukocytes.

Optimization of vaccination studies may result in clinical success

To gain a thorough understanding of the immunological events occurring in patients in vaccination trials it is crucial to comprehensively perform immune monitoring during vaccination trials. Results from immune monitoring make it possible to understand possible clinical effects, to guide the optimization of vaccination strategies and may even encourage investigators to move a product forward into phase III trials⁸⁴. Unfortunately, most immunotherapeutic vaccine trials mostly report on one particular aspect of the desired immune response (e.g. HLA-multimer+ cells, IFN- γ -producing cells). They do not include more detailed analyses of the total vaccine-modulated immune response⁵⁷. Therefore implementation of assays that allows correlation of a broad array of immune cells with disease parameters is a prerequisite.

Although many studies determined the induced immune response after immunization, no gold standard has been set to define clinical response after vaccination⁸⁵. Many different bioassays have been developed for immune monitoring: enzyme-linked immunosorbent spot (ELISA), carboxyfluorescein succinimidyl ester-based proliferative assays, HLA peptide multimer staining and flow cytometry-based tests. Unfortunately substantial variability in results among laboratories prohibits data reproducibility and prevents meaningful comparison among studies. Therefore initiatives have been put up to standardize immune monitoring and harmonize cellular immune assays. Harmonization will establish the use T-cell-based assays as a reproducible gold standard for immunotherapy and reliable parameter to determine the correlation between induced T-cell responses and clinical events⁸⁶⁻⁸⁸.

Which cancer patients are most likely to benefit from immunotherapy?

An important question that remains is which cancer patients are best candidates to study clinical endpoints once safety and immunogenicity of a therapeutic vaccine strategy have been established. So far most trials have included end-stage patients only. Although regression of tumor mass can be very convincing and objectively measured, vaccination of end-stage patients may present with several drawbacks that negatively influence the immunotherapeutic effect. Major drawbacks are the suppressed immune status, the general short survival period that may obscure clinical effects of therapy at later time points⁸⁹, a large immunosuppressive tumor mass, variety of treatments before vaccination, and co-morbidity. Therefore clinical endpoints might be best studied in an adjuvant rather than an end-stage setting. These

patients, who have no measurable tumor mass and a relatively normal functioning immune system are expected to respond optimally to immunization.

FINAL

There is a clear role for tumor-specific T-cell immunity in the final clinical outcome of colorectal cancer patients. Immune escape variants of tumor cells indicate the selective force of the immune system. A continued effort will be put to exploit this force in the development of vaccines and vaccine strategies against colorectal cancer. Despite that some of the current vaccines are able to induce strong antigen-specific immune responses in the absence of serious adverse events, there is hardly any evidence generated to show the clinical impact of these vaccines in patients with colorectal cancer. It is not likely that colorectal cancer vaccines are able to induce the desired clinical responses on their own, but need to be combined with immune modulating modalities to redirect the force of the immune system into an effective anti-tumor response *in vivo*. Studies suggest that these modalities should primarily induce a type 1 polarized immune response and suppress a type 2 response. As chemotherapeutics are already used in the treatment of cancer patients, they should be the first tested for their immune modulating capacity. For current vaccination studies it is of utmost important to monitor and link the type of induced immune response after vaccination to clinical cancer effect, to know which immune are to be induced after vaccination. To obtain proof-of-concept, the immunotherapy of colorectal cancer may want to first concentrate on the treatment of tumors with microsatellite instability as they are known to be heavily infiltrated by T cells and express tumor-specific antigens that are derived from frameshift-mutated gene products.

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