Anti-colorectal cancer immunity : control ‘the force’!

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Chapter 1

General introduction

INTRODUCTION

Main objective of this thesis is to explore factors involved in especially the T-cell mediated anti-tumor immune response and to understand and control the force of the immune system to effectively search and destroy tumor cells.

COLORECTAL CANCER

Colorectal adenocarcinoma is the third most common cancer and accounts for a significant number of cancer deaths worldwide [1-3]. Colorectal cancer has a lifetime risk of about 5-6% with a peak incidence in the 7th decade. Surgery is treatment of choice when the disease is only confined to the bowel wall. However, 30–40% of patients have loco-regionally advanced or metastatic disease on presentation which cannot be cured by surgery alone [4]. Adjuvant radiation therapy, chemotherapy, or both are beneficial in selected patients [4-6]. Despite intended curative therapy still a large proportion of the patients eventually die of their disease leaving room for new treatment modalities such as T-cell mediated immunotherapy [7].

CANCER AND THE IMMUNE SYSTEM

Both spontaneous and therapeutic induced tumor specific immune responses require induction of cell-mediated immunity, to attack and eliminate tumor cells. This calls for close collaboration between cells of the innate immune system, in particular antigen presenting Dendritic Cells (DCs), and cells of the adaptive immune system, notably B-cells, CD4$^+$ T-helper cells (T_H) and CD8$^+$ cytotoxic T cells (CTL). Despite scientific progress, the interaction between the immune system and cancer remains elusive. Growth of tumor cells that escaped the immune system may implicate selective pressure of the immune system. These mechanisms include active down-regulation of immune responses by the tumor by producing immunosuppressive agents, altered expression of major histocompatibility complex (MHC) and/or tumor-associated antigens (TAAs) by tumor cells, altered expression of adhesion molecules by tumor and/or DCs, and the use of host immune responses to the advantage of the cancer. Better understanding of mechanisms of tumor immune evasion may improve immunotherapeutic strategies.

TUMOR INFILTRATED LEUKOCYTES REPRESENT THE PRESENCE OF AN ONGOING ANTI-TUMOR RESPONSE

Presence of both myeloid and lymphoid cells in different intra- and peri-tumoral compartments in colorectal cancer represents one of the most evident witnesses of an active involvement of the immune system in cancer growth and progression. Immunohistochemical techniques comprise one of the most frequent techniques used to study infiltration of leukocytes in colorectal tumors. These studies determined the clinical impact of many different leukocyte subpopulations such as dendritic cells, macrophages and different (sub-)populations of lymphocytes [8-23]. However, there is still a lot unclear about the exact type and role of leukocytes that infiltrate into tumors. Only the infiltration of intra-tumoral or more precise intra-epithelial located CTLs is without doubt associated with good prognosis in colorectal cancer patients [8;10;12;16;18;19;23]. In addition, several studies showed that intra-epithelial compared to stromal $CD8^+$ T-cells express more molecules involved in target cell killing such as higher expression of Granzyme B and TIA-1 and showed higher proliferative activity, suggesting that intra-epithelial $CD8^+$ T-cells are active effectors [8;12;24]. Limitation of most immunohistochemical techniques is that in general per staining only one antigen is identified. Unfortunately most leukocytes characterized with one antigen fulfill different and even opposing functions. This is one of the explanations why it is difficult to assess the clinical impact of leukocytes using immunohistochemical techniques. Studies using different techniques revealed that especially tumor-specific $CD4^+$ T_H1 cells are associated with a supportive cancer microenvironment that is beneficial to the prognosis of colorectal cancer patients [25-27]. It has been well documented that $CD4^+$ T-cells not only license the priming of $CD8^+$ T-cells but are important to sustain their fitness [28], and also enhance $CD8^+$ T-cell proliferation and cytolytic function [29]. Expression of the IL-17-associated genes in colorectal cancer patients correlated with poor prognosis [30]. The expression of T_H2- and regulatory T cells has no or opposing effects on clinical outcome [30-33].

ROLE OF HUMAN LEUKOCYTE ANTIGEN CLASS I IN COLORECTAL CANCER

Expression of MHC class I, for humans also called Human Leukocyte Antigen (HLA) class I, presenting TAAs on the tumor cell surface, is considered as a prerequisite for effective T-cell mediated immunity [34]. As a consequence, tumor cells with down-regulated HLA class I expression might escape this immune response, resulting in a selective outgrowth of these tumor cells. HLA class I molecules comprise the classical (class Ia) HLA-A, -B, and -C alleles, and the non-classical (class Ib) HLA-E, -F, and –G alleles. In this section we focus on the role of classical HLA class I molecules.

They form a trimolecular complex consisting of a highly polymorphic heavy chain, a peptide antigen, and the non-polymorphic ß2-microglobulin (ß2m) light chain [35]. The heavy chain molecules are encoded by genes located within the HLA region on chromosome 6, whereas ß2m is encoded by a gene mapped on chromosome 15. HLA class I is constitutively expressed by many cells, although the intensity of expression varies between different tissue types. Peptides presented in the context of HLA class I molecules are generated from degraded proteins by the antigen processing machinery. After processing, the peptide is associated with the heavy chain and ß2m and expressed on the cell surface to present the antigen to CTL.

In addition to T cell-induced tumor cell killing, tumor cell lysis can also be induced by activated NK cells. NK cell activation is regulated by a balance between signals mediated through activating and inhibitory receptors [36]. HLA class I is a ligand for inhibitory receptors on NK cells. Loss or down-regulation of HLA class I is a possible strategy to escape T cell control [37], and is frequently found in colorectal cancer [38;39]. Loss or down-regulation of HLA class I might however activate NK cells and induce tumor cell lysis [40]. Defects in one of the processes that are involved in antigen presentation, will lead to loss of expression of HLA class I molecules on the cell surface. Complete loss of HLA class I is usually caused due to loss of ß2m expression or TAP deficiency [41;42]. This is mostly found in microsatellite unstable (MSI-H) tumors when compared to microsatellite stable (MSS) tumors [41;42]. Loss of one of the HLA heavy chains (A, B or C alleles) is usually caused by chromosomal aberrations of chromosome 6 [43]. Only limited studies have reported on the clinical impact of HLA class I expression in colorectal cancer using mixed cohorts of genetic instability and reporting contrasting results [44-47]. None of these studies determined the prognostic inpact of HLA class I expression with regard to genetic instability.

LEUKOCYTE TRAFFICKING IS COORDINATED BY CHEMOKINES

Chemokines are a superfamily of small secreted cytokines that were initially characterized through their ability to coordinate trafficking of leukocytes [48]. Chemokines bind to specific cell surface transmembrane receptors coupled with G proteins, whose activation leads to formation of intracellular signaling cascades that prompt migration toward the chemokine source. To date, studies have identified in humans, more than 50 chemokines and 20 chemokine receptors [48-50]. Chemokines coordinate migration of all types of cells including tumor cells, influencing tumor development and organ selective metastases [51-53]. The role of chemokines in gastrointestinal disorders and cancer has been extensively reviewed [49;54]. As described, high T-cell infiltration in colorectal cancer is associated with good prognosis and might protect from tumor growth. Chemokines regulate trafficking of immune cells and might

therefore represent an important factor in coordinating an anti-tumor immune response. This concept that (over-)expression of specific chemokines causes tumor infiltration by distinct leukocyte subsets, resulting in tumor regression and tumor specific immunity has been described in several tumor models [55-61]. However, understanding this complex network of factors involved in trafficking of leukocytes in the cancer microenvironment remains further exploration [62].

T-CELL MEDIATED IMMUNOTHERAPY

In search for new treatment options to cure patients from colorectal cancer, much effort has been put in exploiting the immune system and evoking tumor-specific immune responses using T-cell-mediated immunotherapy. The unique advantage of this type of treatment is that theoretically the immune system is able to specifically target and destroy tumor cells. Despite great progress in the field of tumor immunology, clinical application of T-cell-mediated immunotherapy yielded only limited success [63]. So far cellular immunotherapy is not part of the clinical routine to treat colorectal cancer patients. However, recent studies have revealed the dawn of a new era in which the activation of tumor-specific T-cells starts to make a difference. Sipuleucel-T is the first therapeutic cancer vaccine to demonstrate effectiveness in Phase III clinical trials by prolonging the life of advanced or late stage metastatic, asymptomatic hormone refractory prostate cancer patients (HRPC) [64;65]. The vaccine was approved by the U.S. Food and Drug Administration to treat patients with HRPC [66]. Treatment with Ipilimumab, a monoclonal antibody that targets the immune regulatory molecule CTLA-4 represents the first modality that had a significant impact on the overall survival of patients with metastatic melanoma [67]. These results are the first positive demonstration that blockade of a T-cell activity inhibitory pathway can be an effective cancer treatment. Also adoptive T-cell therapy (ACT) has been found to be effective in the treatment for metastatic melanoma patients [68-70]. Last but not least, vaccination with a synthetic long-peptide (SLP) vaccine against the HPV-16 oncoproteins E6 and E7 resulted in the complete regression of human papillomavirus-16-positive, grade 3 vulvar intraepithelial neoplasias in 47% of the patients [71]. Complete responses in this study were correlated with the strength of HPV-16-specific immunity [71]. These encouraging results in patients with different types of carcinomas positively stimulate research on immunotherapy of colorectal cancer patients.

FRAMESHIFT-MUTATED GENE PRODUCT-DERIVED PEPTIDES, A CLASS OF TUMOR-SPECIFIC ANTIGENS

Despite many years of work, the number of antigens recognized by tumor infiltrated lymphocytes (TILs) of colorectal cancer identified is limited [40;72-74]. Consequently, vaccines so far have been developed on the basis of proteins that are selectively expressed by tumor cells. A possible unique group of TAAs comprises MSI-H tumors that, due to numerous of frameshift mutations in microsatellites express neo-antigens (Figure 1). MSI-H is a molecular feature of tumors associated with the familial Lynch syndrome also known as hereditary non-polyposis colorectal cancer (HNPCC) syndrome, accounting for approximately 5% of all colorectal cancer cases [75-77] and for approximately 15% of all sporadic colorectal, gastric and endometrial cancers, as well as at lower frequencies for various other sporadic cancers [78-82]. MSI-H colorectal tumors are predominantly localized in the proximal colon, comprising 50% of all proximal colon tumors [83;84]. Since frameshift-mutated products (FSPs) are foreign to the immune system, they represent a unique group of tumor-specific antigens. As no tolerance and consequently strong T-cell responses are expected against the non-self-segment encoded by sequences downstream of the mutation, they are considered promising candidates for prophylactic vaccination of subjects with Lynch syndrome or HNPCC, or as adjuvant therapy in combination with surgery for patients

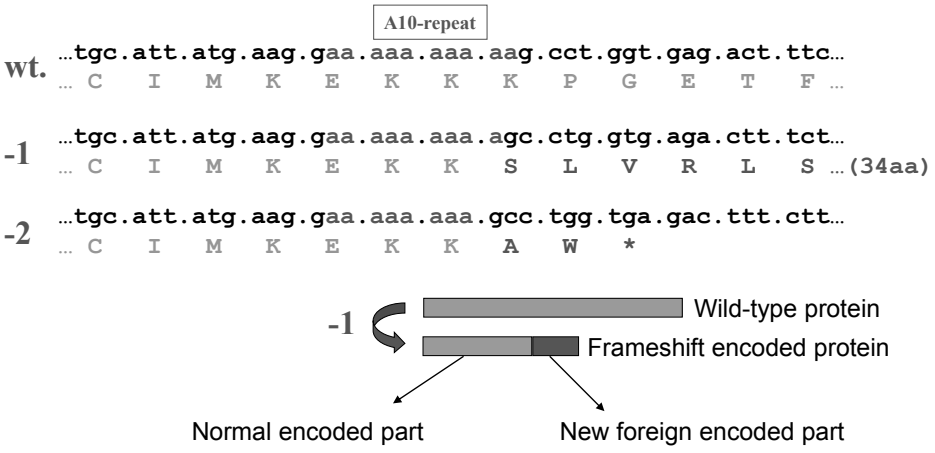

Figure 1. Insertion or deletion of mutations in microsatellites result in frameshift mutations and if 'translated' in proteins with a 'foreign' part.
A part of the TGFβR2 gene and corresponding amino-acid translation is depicted of the wild-type (wt.), and containing a -1 or -2 deletion in the microsatellite (red). As shown a -1 deletion in the microsatellite results in a new foreign encoded part after the frameshift mutation and a new stop after 34 amino acids (aa), while a -2 deletion results in a frameshift mutation and a new stop 2 amino acids after the microsatellite.

with (sporadic) MSI-H tumors. Unfortunately, relatively little is known on the immunogenic behavior of most of the FSPs [40].

VACCINES TARGETING P53-OVEREXPRESSING COLORECTAL TUMORS

Defined antigens to be used as vaccine candidates should ideally be overexpressed in the context of HLA at the cell surface of tumor cells and not (or at very low) levels by other cells of the human body. FSPs are a unique example of tumor specific antigens. Unfortunately only a minority of the colorectal tumors comprises MSI-H tumors that express these FSPs. The majority of the colorectal cancers are chromosomal unstable (CIN). CIN tumors lack tumor specific antigens to be used in vaccination trials. Antigens used in vaccination studies for colorectal cancer comprise TAA and consequently are likely to be expressed by normal cells [85-87]. Different TAA such as: p53, CEA, MUC1, Sialyl-Tn, 5T4, SART3, MAGE have been applied in clinical trials to vaccinate colorectal cancer patients [85-89]. The use of antigens potentially expressed by normal cells bears the risk of immune tolerance. Indeed, tolerance too many TAA such as p53, CEA and MUC1 has been found [90-96]. These results indicate that tolerance forms a potential hurdle for immunotherapies of cancer when using TAA.

One of the TAA frequently used in cancer vaccination trials and much studied in the Leiden University Medical Center comprises p53. Due to a mutation, p53 is overexpressed, while wildtype (wt) p53 in normal cells is not or in very low levels expressed [97-100]. The most common way to disrupt the p53 pathway is through a point mutation that inactivates its capacity to bind specifically to its cognate recognition sequence, and often results in overexpression of p53 [101]. The aberrant expression of the p53 protein in tumor cells versus the low expression in non-tumor cells provides an immunological window for the use of p53 as a tumor antigen for immunotherapeutic intervention against cancer [102]. P53 is mutated and overexpressed in approximately 34-45% of all colorectal cancers [103].

The presence of humoral and proliferative immunity against p53 in the blood of humans has been described for a long time. Both IgM and IgG type antibodies against p53 have frequently been detected in the sera of cancer patients, including patients with colorectal cancer [104;105]. Because p53 is not expressed at the cell surface, only p53-specific T-cell mediated immunity is likely to exert therapeutic antitumor effects. T-helper responses have been described in humans especially in cancer patients [25;106-108]. However, there are strong indications that the p53-specific $CD8^+$ T-cell repertoire is severely restricted by self-tolerance [90;91;109], as high-avidity self-reactive T cells are suspected to be deleted in the thymus [110]. Most of the described human p53-specific CTLs have been generated after *in vitro* culture [111-115]. Although vaccination

against p53 might mainly induce p53-specific $CD4^+$ T cells, these are important in cancer immunotherapy because IFNγ secreting $CD4^+$ T_H1-cells play an important role in orchestrating and sustaining the local immune attack by $CD8^+$ CTL and innate immune effector cells [116-118].

Several different antigen delivery systems have been tested to immunize patients against p53. In previous studies adenoviral vector encoding wt.p53 [119], recombinant canarypox virus encoding wt.p53 [108;120], and adenoviral vector encoding wt.p53 transfected DCs [121] were used. These modalities were safe and capable of stimulating p53-specific T-cell responses in some of the vaccinated patients. Unfortunately, presence and enhancement of anti-vector immunity were found in almost all patients, which may have hampered the induction of a truly effective p53-specific T-cell response. In addition, DC pulsed with known p53 HLA-A2.1 binding peptides have been used and this resulted in safe induction of specific T-cell responses against p53 peptides in some of the treated patients [122]. This concept but has the disadvantage that patients with other HLA types cannot be treated [109].

Synthetic long peptides (SLP®) can also be used as vaccines [28;123]. When injected, these SLP® are predominantly taken up by DC resulting in the presentation of both helper T-cell epitopes and CTL epitopes that are present in the SLP® [124;125]. A SLP® vaccine for the induction of p53-specific T-cell immunity was developed. Injection of p53-SLP® resulted in a strong p53-specific $CD4^+$ T-cell response to three different epitopes in mice [91]. This p53-SLP® vaccine is to be tested for its safety and immunogenicity cancer patients.

OPTIMIZATION OF VACCINATION STUDIES RESULTS IN CLINICAL SUCCESS

The most recent vaccine developments suggest that some of the current vaccine strategies do harbor the capacity to induce immune responses in cancer patients even to self-antigens. However, lack of clinical results in phase I/II trials in colorectal cancer patients 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. A stronger focus should be put on how to induce the strongest and best qualified leukocyte population by vaccination. A clear positive relation between survival of colorectal cancer patients and high expression of a type 1 response has been established [30]. The presence of tumor-specific $CD4^+$ T cells in the cancer microenvironment is a prerequisite for support, proliferation, recruitment and cytolytic function of tumor-specific $CD8^+$ T cells [29;126]. This unique function of the tumor-specific $CD4^+$ T cells is greatly accelerated by production of IFN-γ and IL-2 [25;29]. For example, patients with metastatic colorectal cancer receiving chemotherapy and vaccinations

against the tumor antigen 5T4 were found to have more clinical benefits when 5T4-specific IFN-γ ELIspot responses were induced. [127]. Altogether, these data suggest that clinical responses after vaccination not only depend on the induction of vaccine-specific responses, but merely require the induction of a strong and broad type 1 T-cell response. Therefore, in order to benefit from the local effect of tumor-specific T cells, vaccines should be combined with immune modulating adjuvants that specifically induce polarization of the induced immune response into a type 1 response.

A possible candidate adjuvant might be Interferon-alpha (IFN-α) as it plays a major part in the differentiation of the Th1 subset, as well as in the generation of CTL and the promotion of the *in vivo* proliferation and survival of T cells [128]. Moreover, several studies have shown that type I IFNs promote the differentiation of monocytes into DC *in vitro* and can markedly enhance DC activities [129-134]. Only one study in humans has combined IFN-α injections with peptide vaccination [135]. This study showed that the concomitant combination of a peptide-based vaccine with IFN-α was safe, resulted in a consistent enhancement of vaccine-specific $CD8^+$ T cells and yielded a general increase of the percentage of blood circulating DC precursors/ $CD14^+$ monocytes [135]. It would be interesting to study if addition of IFN-α to the p53-SLP® vaccine not only induced a stronger p53-specific but also a better polarized Th1 response.

THESIS OUTLINE

The studies described in this thesis aim to increase the knowledge on the interaction between the immune system and colorectal tumor cells, with final purpose, the design of effective T-cell mediated immunotherapy. As there are strong indications that presence of intra-tumoral $CD8^+$ T cells is associated with prognosis of colorectal cancer patients and most tumor associated antigens comprise intracellular proteins and might therefore not be accessible for antibodies, this thesis primarily focuses on T-cell mediated anti-tumor immunity.

Conflicting results have been described for the association between expression of HLA class I and prognosis in colorectal cancer patients, possibly due to the use of cohorts with mixed types of genetic instability [44-47]. Therefore in **chapter 2** we evaluated the association between HLA class I expression and prognosis in patients curatively operated for rectal cancer consisting of mainly MSS cancers. The infiltration of diverse types of NK and T-cells in the different types of tumor compartments is carefully assessed and stratified, especially in relation to HLA class I down-regulation in **chapter 3**.

Interaction of chemokines with their cognate receptors allows attraction of immune cells into a tumor, but also influences migration of disseminated tumor cells. In **chapter 4**, a specific chemokine, CXCL5 that in rats was found to be associated with aggressive growth, was studied for its association to survival and T-cell infiltration in rats and humans.

MSI-H tumors are characterized by mutations in microsatellites that result in the expression of frameshift-mutated proteins. In **chapter 5** the use of an expression system to systematically analyze the characteristics and immunogenic properties of proteins encoded by frameshift mutated genes that are commonly found in MSI-H cancers is described.

In **chapter 6** the results of a phase I trial are presented, studying both safety and immunogenicity of a vaccine consisting of a pool of synthetic long p53 peptides in patients treated for metastasized colorectal cancer. **Chapter 7** describes the results from a phase I trial that studied if addition of IFN-α to the p53-SLP® vaccine enables polarization of the induced p53 $CD4^+$ T-cell response into a strong Th1 response.

Finally, **chapter 8** provides a summary and discussion of this thesis.

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