Tumor-immune interactions in colorectal cancer: link between the primary tumor and circulating immune cells

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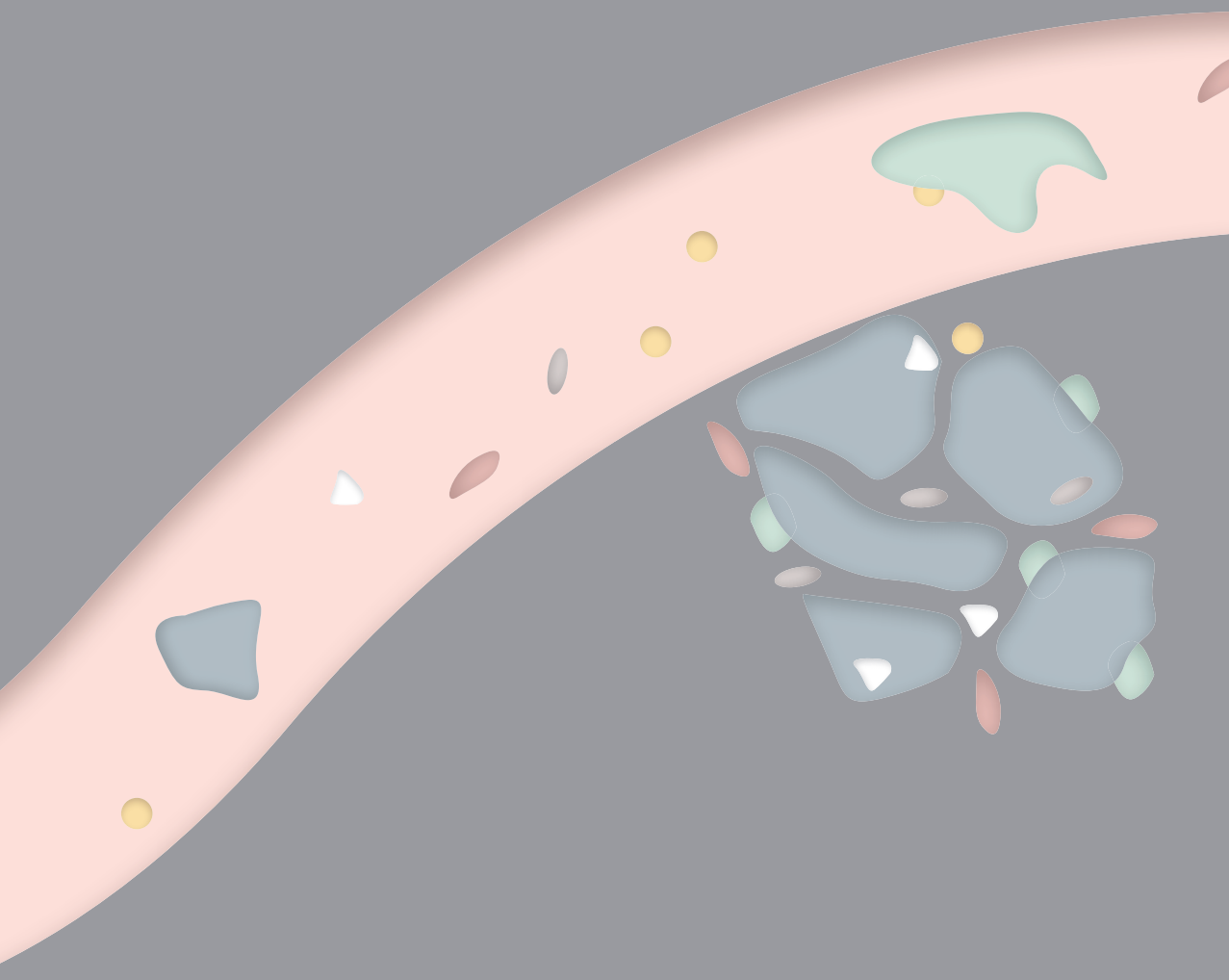
CHAPTER 1

General introduction and thesis outline



Colorectal cancer

Colorectal cancer (CRC) is a major contributor to cancer-related morbidity and mortality, accounting for approximately one million new cases per year throughout the world, with more than half a million deaths annually [1]. Around 25% of these patients present with unresectable metastatic disease at the time of diagnosis, often in the liver, and around 30% of early stage patients develop recurrence or dissemination of the disease following surgery [2]. In order to reduce the risk of relapse and disease progression, (neo)adjuvant chemotherapy and radiotherapy are often considered in addition to surgical resection of the tumor. However, prevention of metastasis is not always achieved and a substantial price has to be paid in terms of treatment-associated morbidity and mortality [3]. In the scope of personalized medicine, it has become increasingly important that patients with a high risk for disease recurrence or progression, and patients who would benefit most from additional therapies, are identified. Biomarkers may play a major role in clinical decision making by providing information on the risk of metastasis development in patients, and prediction of therapy response.

Biomarkers

Oncological biomarkers can broadly be categorized into two groups: 1. Tumor-associated biomarkers and 2. Systemic biomarkers. In carcinomas, tumor-associated biomarkers are studied in tumor epithelium or stroma within the primary tumor or metastases. These tumor-associated biomarkers are usually studied at a fixed time point in tumor development, because they are evaluated in resection material, including tumor biopsies. Due to the invasive procedure, obtainment of tumor biopsies can pose risks to the patient, such as bleeding, perforation, and infection, and are therefore usually obtained only for diagnostic purposes. Analysis of hematoxylin-eosin (HE)-stained and immunohistochemical (IHC)-stained tissue sections are the standard diagnostic procedures. Importantly, assessment of primary tumor-associated biomarkers by HE and IHC relies on visual microscopic inspection, manual annotation procedures and inter-observer agreement. Therefore, these methods are prone to subjective criteria and will always be qualitative rather than quantitative. Since biomarker research is aimed at development and improvement of personalized cancer treatment in a clinical setting, objective and quantitative assessment of tumor biomarkers is crucial. Computer-assisted image analysis is a promising technique that might be used to overcome these problems and to justify treatment decisions based on biomarkers.

Systemic biomarkers are measured in the circulation of patients, for instance in peripheral blood, serum or plasma, and can, in contrast to biomarkers in primary and metastatic tumor tissue, rather easily be followed in patients over time. Many studies have focused on primary tumor-associated biomarkers. Although it is important to study biomarkers and oncogenic processes in primary tumors, it must be realized that CRC patients die from the consequences of metastatic disease, and usually not due to primary tumor growth. The primary tumor is usually successfully removed during surgery, and, therefore, not present in patients when they develop metastases in the months or even years after surgery. Systemic biomarkers may provide insight in the dynamic process of metastases development since they can be monitored over time. In this thesis, we specifically focused on systemic biomarkers related to the immune system.

Immunogenicity of colorectal cancer

Over the past decades, the focus of cancer research has changed. Whereas tumor growth was previously studied in context of tumor cells only, especially for their ability to escape from cell cycle control mechanisms, the focus nowadays is on studying tumor growth in context of its tumor microenvironment (TME). The TME consists of different cell types and structures, including fibroblasts, signaling proteins, extracellular matrix, blood vessels, and, importantly, immune cells [4]. Due to their genetically unstable nature, tumor cells acquire mutations during tumor progression which generate possibly immunogenic antigens. Tumor cells may also acquire mutations which result in downregulation of proteins that are crucial for an immune response, for instance human leukocyte antigen (HLA) class I. Due to this process, also called “immunoediting”, tumor clones selectively grow out that are insensitive for immune control. Interestingly, up to 20% of sporadic CRC is observed to have DNA microsatellite instability (MSI) which results in high mutational load [5]. Patients with MSI CRC are observed to have numerous tumor-infiltrating lymphocytes [6], and showed reduced metastasis rate and significantly better clinical outcome compared to patients with microsatellite stable (MSS) CRC [7]. This suggests an important role for tumor-immune interactions in tumor development and progression of CRC. Different types of tumor-immune interactions can be identified in CRC patients, both in the primary tumor, the circulation, and in distant metastases [8].

Tumor-immune interactions in the primary tumor

In order to infiltrate into a primary tumor, immune cells have to navigate from blood vessels, located in the tumor stroma, into the tumor epithelial tissue in an active process mediated by cytokines and

chemokines secreted by cells in the TME. Hence, an effective primary tumor-immune response is heavily dependent on the ability of immune cells to infiltrate and migrate within the tumor. Over the last decade, a new scoring system based on T cell infiltration in the TME has been defined, also known as the "immunoscore" [9-11]. CRC patients with high density of intratumoral $CD3^+$ and $CD8^+$ T cells were observed to have a significantly better clinical outcome compared to patients with low density of intratumoral $CD3^+$ and $CD8^+$ T cells [9-11]. Recent studies suggested implementation of the immunoscore as biomarker as a new component in the classification of CRC in addition to the tumor-node-metastases (TNM) classification [12, 13]. However, a significant number of CRC patients with tumors with high density of tumor-infiltrating T cells, and, therefore, with a relatively good prognosis, develop progression of their disease in clinical follow-up [9, 10, 12]. It is hypothesized that CRC tumors may escape immune recognition by cytotoxic $CD8^+$ T cells via downregulation of HLA class I expression [14-18]. These tumors are potential targets for NK- and NKT cells, that are able to recognize and kill cells with downregulated HLA class I molecules through "missing-self" recognition [19-21]. Previous studies showed an association between NK- and NKT cell tumor infiltration and prolonged survival in patients with CRC [22-24]. However, NK- and NKT cells are generally scarcely infiltrated in CRC and might, therefore, only play a minor role in primary tumor-immune interactions [17, 24-26]. In addition to NK- and NKT cells, tumor-associated macrophages (TAMs) might also play important roles in the TME. Although TAMs have been reported as prognostic biomarkers in different epithelial cancer types [27-29], their role in CRC is still unclear as high TAM density has been reported to correlate with both unfavorable [30-32], and favorable clinical outcome [33-36]. In summary, different immune cell subsets play a role in tumor-immune interactions in primary CRC tumors. Aberrant HLA class I expression and other key proteins in T cell responses may be important immune escape strategies for primary CRC cells.

Tumor-immune interactions in the circulation

During the process of disease progression, CRC cells detach from the primary tumor and intravasate into the circulation. In contrast to the situation in the primary tumor, tumor cells in the circulation can directly interact with immune cells, not limited by the context of the TME of the primary tumor, which may result in a different tumor-immune response. For instance, whereas NK- and NKT cells scarcely infiltrate primary tumors, they are more abundantly present in the circulation [20]. The activation of NK- and NKT cells is dependent on a delicate balance between activating and inhibitory signals from cell surface receptors [37]. NK cell inhibitory receptors include natural killer group 2-A (NKG2A), and

killer cell immunoglobulin (Ig)-like receptors CD158a and CD158b that recognize HLA class I molecules. The activating signals are mediated by a wide array of receptors including natural killer group 2-C (NKG2C), natural killer group 2-D (NKG2D), DNAX accessory molecule-1 (DNAM-1), CD161, and natural cytotoxicity receptors (NCRs) NKp30, NKp44, and NKp46, that recognize a variety of stress-induced molecules that may be present on tumor cells. These molecules include galectin-3, recognized by the NKp30 receptor, which promotes epithelial to mesenchymal transition (EMT) of tumor cells. Additionally, NKT cells express a T cell receptor that recognizes glycolipids presented by the HLA-like molecule CD1d, which functions as an extra activating receptor [38]. Interestingly, CRC patients with downregulated HLA class I expression in their primary tumors developed fewer distant metastases in clinical follow-up [17]. This finding supports the hypothesis that NK- and NKT cells in the circulation play an active role in eliminating disseminated tumor cells in CRC patients [8]. Recently, studies showed reduced numbers of NK cells expressing activating receptors (e.g., NKG2D, NKp30, NKp46, and DNAM-1) in peripheral blood of patients with CRC [39]. Furthermore, these NK cells showed impaired IFN-γ secretion and degranulation upon activation, thereby implying impaired function [40]. This suggests impairment of NK cell activity in the circulation of CRC patients, which may facilitate the dissemination of tumor cells in the circulation, resulting in outgrowth of distant metastases. The number of NK- and NKT cells in the circulation, expression level of activating and inhibitory receptors, and presence of their ligands on tumor cells, might therefore be interesting biomarkers to predict the risk for development of metastases in CRC patients. Up till now, the underlying biology of these tumor-immune interactions is poorly understood and remains to be further explored. In contrast to TAMs, the role of circulating monocytes, the precursor cells of macrophages, seems less ambiguous. Recent meta-analyses showed that a high lymphocyte-to-monocyte ratio in peripheral blood was a significant predictor of better overall survival, disease-free survival and cancer-specific survival in CRC patients [41, 42]. In summary, different immune cell subsets play critical roles during dissemination of tumor cells in the circulation of CRC patients. Especially circulating NK cells, NKT cells, and monocytes may play important roles during this process. It is crucial that underlying biology, concerning tumor-immune system interactions in the circulation, is well understood for improvement of diagnostics, and design of efficient immune-based therapies for CRC patients.

Tumor-immune interactions in metastases

When tumor cells in the circulation escape from immune control, CRC cells mainly metastasize to the liver. Due to a different TME in the liver, tumor-immune interactions in these CRC metastases may be

different compared to tumor-immune interactions in the primary tumor or in the circulation. For instance, primary colorectal tumors that lost HLA class I expression were reported to rarely metastasize to the liver and were most often found in other organs [43]. This may be due to the abundancy of NK cells in the liver that may prevent establishment of liver metastasis by HLA class I-negative tumor cell clones. As addressed above, NK cells are also abundantly present in the circulation, and should, therefore, have recognized and eliminated HLA class I-negative tumor cells from the circulation. This suggests that other mechanisms are involved in the survival of HLA class I-negative tumor cells in peripheral blood of CRC patients. Further research is required to determine why certain circulating tumor cells are able to survive in the peripheral blood. The focus of this thesis was therefore on tumor-immune interactions in the circulation of CRC patients since the balance between immune control and immune escape may determine whether circulating tumor cells are eliminated, or alternatively, escape from immune control, leading to the formation of distant metastasis in specific organs. NK cells, NKT cells, and monocytes might be key players in these interactions and expression of activation markers on these cell subsets may, therefore, have potential as biomarkers that identify CRC patients with a high risk for development of metastases.

Outline of this thesis

The aim of this thesis was to better understand the underlying biology of tumor-immune interactions, especially in the circulation of CRC patients.

Chapter 2 describes a possible role of NKT cells in the TME and in the circulation of patients with cancer, by considering their phenotype and function. In **Chapter 3**, the immunophenotype of circulating T-, NK-, and NKT cell subsets was studied in patients with CRC and in healthy donors. Additionally, the clinical prognostic biomarker potential of circulating T-, NK-, and NKT cell subsets was studied in CRC patients. **Chapter 4** describes the influence of tumor resection and adjuvant chemotherapy on the immunophenotype of circulating T-, NK-, and NKT cells in CRC patients. In **Chapter 5**, a method for semi-automated image analysis of HLA class I tumor epithelium expression in rectal cancer is presented. In **Chapter 6**, expression of NK cell receptor ligands in primary tumors is studied in relation to the expression of NK cell receptors on circulating NK- and NKT cells of CRC patients, and to clinical outcome, using the method as described in the previous chapter. **Chapter 7** explores the clinical relevance of the macrophage-associated molecule CD163 as biomarker in CRC. In this study, CD163 was studied on circulating monocytes, TAMs in the primary tumor, and as soluble

form in the circulation (sCD163). Finally, a summary and discussion on the results of this thesis is provided in **Chapter 8**, addressing the future perspectives of immune-related biomarker research in CRC.

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