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

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Tumor-immune interactions in colorectal cancer

Link between the primary tumor and circulating immune cells

Daniëlle Krijgsman

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Tumor-immune interactions in colorectal cancer

Link between the primary tumor and circulating immune cells

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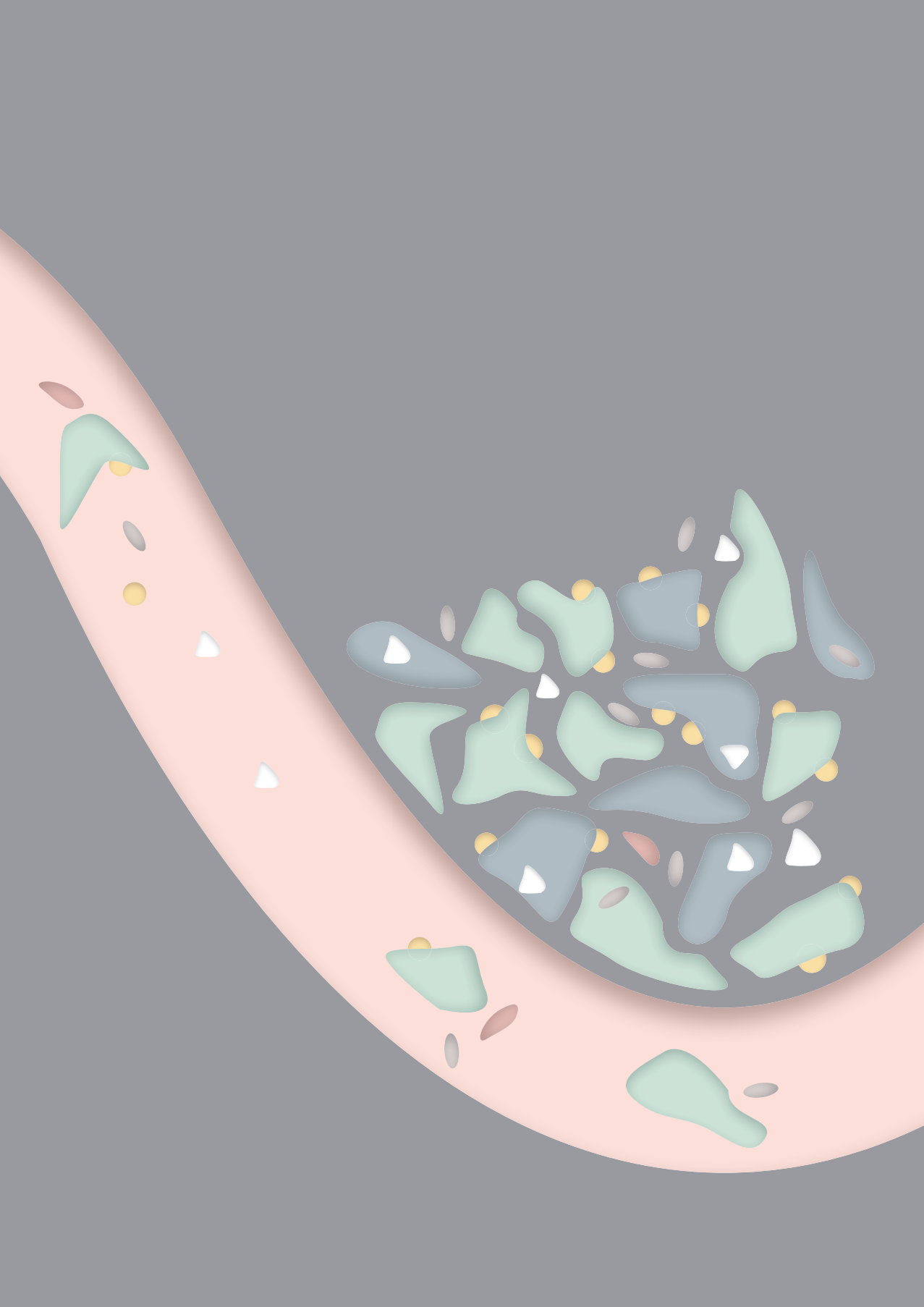
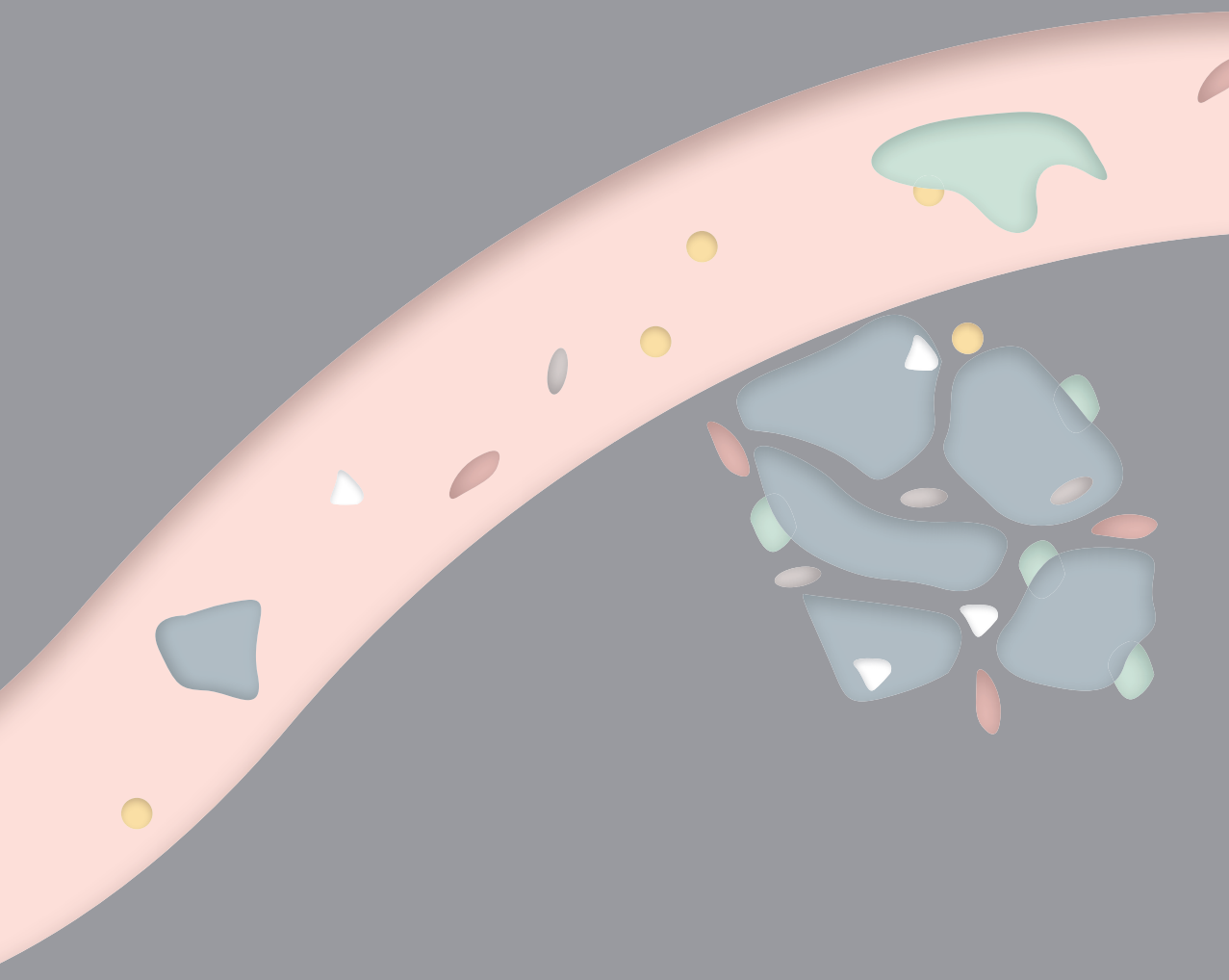


CHAPTER 1

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.

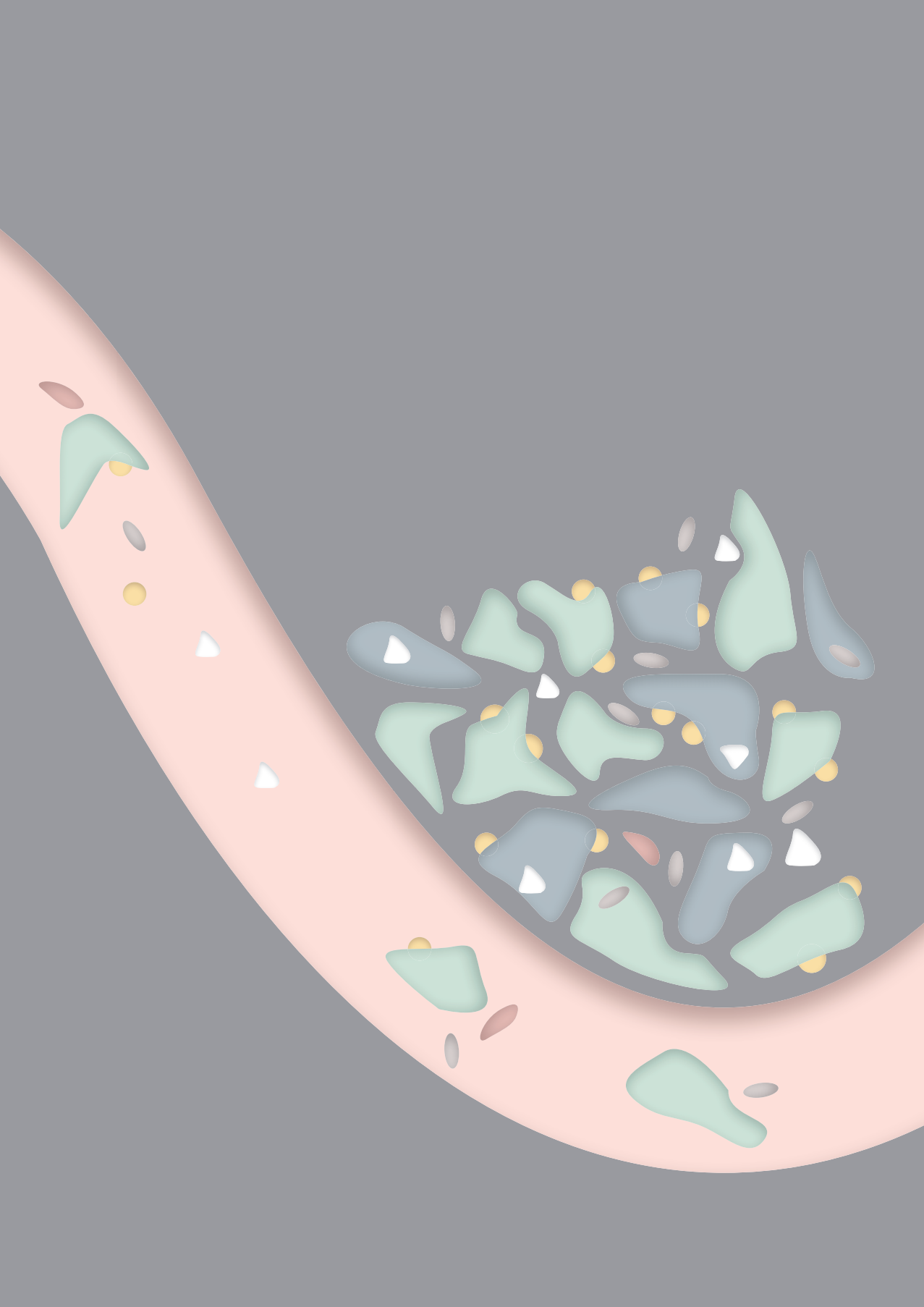
Chapter 2 describes a possible role of NKT cells in the TME and in the circulation of patients with cancer, by considering their phenotype and function. In **Chapter 3**, the immunophenotype of circulating T-, NK-, and NKT cell subsets was studied in patients with CRC and in healthy donors. Additionally, 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.

References

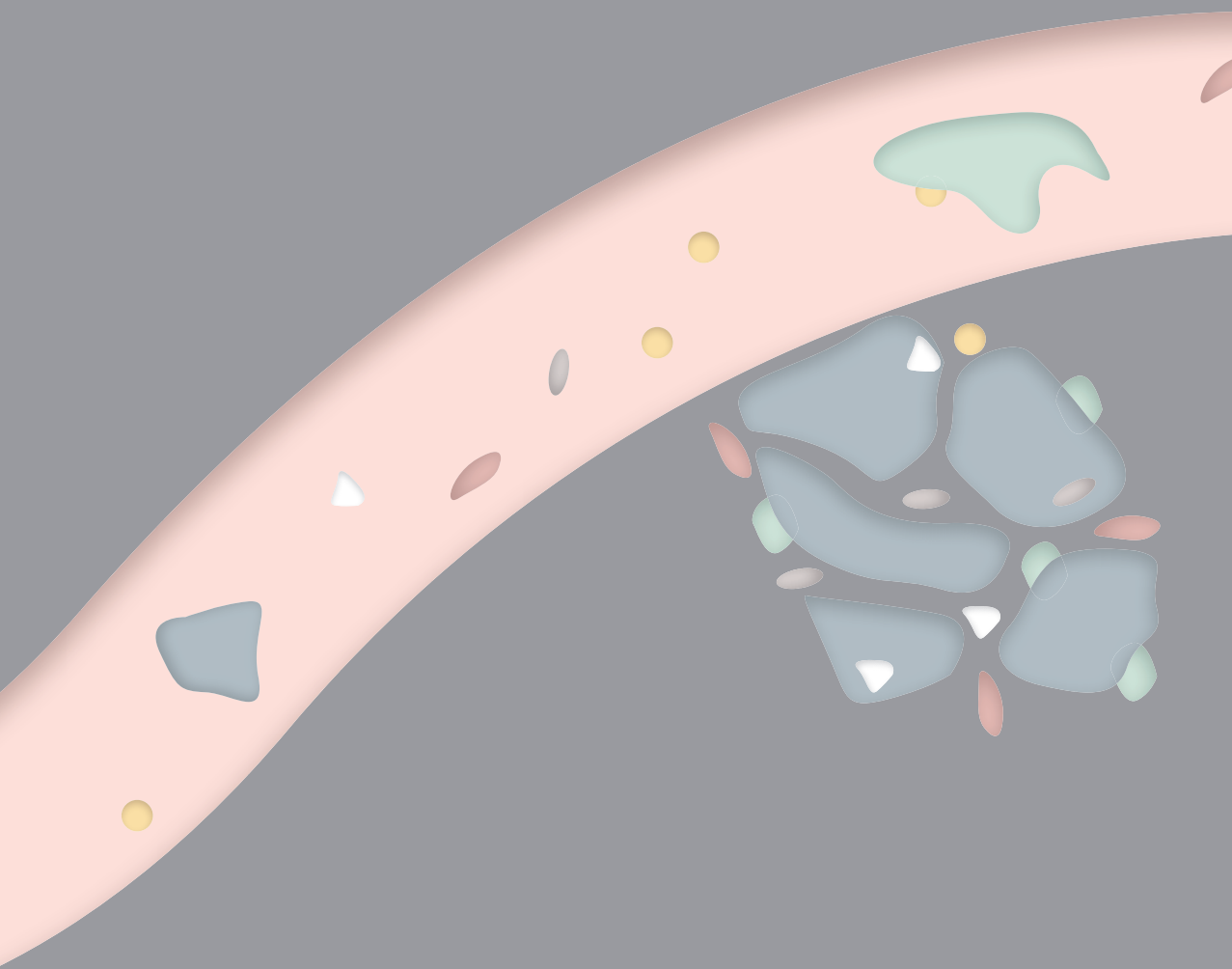
1. Torre, L.A., et al., *Global cancer statistics, 2012.* CA Cancer J Clin, 2015. **65**(2): p. 87-108.
2. Van Cutsem, E., et al., *Metastatic colorectal cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up.* Ann Oncol, 2014. **25 Suppl 3**: p. iii1-9.
3. Yu, I.S. and W.Y. Cheung, *Metastatic Colorectal Cancer in the Era of Personalized Medicine: A More Tailored Approach to Systemic Therapy.* Can J Gastroenterol Hepatol, 2018. **2018**: p. 9450754.
4. Peddareddigari, V.G., D. Wang, and R.N. Dubois, *The tumor microenvironment in colorectal carcinogenesis.* Cancer Microenviron, 2010. **3**(1): p. 149-66.
5. Boland, C.R. and A. Goel, *Microsatellite instability in colorectal cancer.* Gastroenterology, 2010. **138**(6): p. 2073-2087 e3.
6. Smyrk, T.C., et al., *Tumor-infiltrating lymphocytes are a marker for microsatellite instability in colorectal carcinoma.* Cancer, 2001. **91**(12): p. 2417-22.
7. Popat, S., R. Hubner, and R.S. Houlston, *Systematic review of microsatellite instability and colorectal cancer prognosis.* J Clin Oncol, 2005. **23**(3): p. 609-18.
8. de Vries, N.L., et al., *The Immunogenicity of Colorectal Cancer in Relation to Tumor Development and Treatment.* Int J Mol Sci, 2016. **17**(7).
9. Galon, J., et al., *Type, density, and location of immune cells within human colorectal tumors predict clinical outcome.* Science, 2006. **313**(5795): p. 1960-4.
10. Mlecnik, B., et al., *Histopathologic-based prognostic factors of colorectal cancers are associated with the state of the local immune reaction.* J Clin Oncol, 2011. **29**(6): p. 610-8.
11. Galon, J., et al., *Towards the introduction of the 'Immunoscore' in the classification of malignant tumours.* J Pathol, 2014. **232**(2): p. 199-209.
12. Pages, F., et al., *International validation of the consensus Immunoscore for the classification of colon cancer: a prognostic and accuracy study.* Lancet, 2018. **391**(10135): p. 2128-2139.
13. Zeitoun, G., et al., *The Immunoscore in the Clinical Practice of Patients with Colon and Rectal Cancers.* Chirurgia (Bucur), 2019. **114**(2): p. 152-161.
14. Menon, A.G., et al., *Down-regulation of HLA-A expression correlates with a better prognosis in colorectal cancer patients.* Lab Invest, 2002. **82**(12): p. 1725-33.
15. Watson, N.F., et al., *Immunosurveillance is active in colorectal cancer as downregulation but not complete loss of MHC class I expression correlates with a poor prognosis.* Int J Cancer, 2006. **118**(1): p. 6-10.
16. Zeestraten, E.C., et al., *Combined analysis of HLA class I, HLA-E and HLA-G predicts prognosis in colon cancer patients.* Br J Cancer, 2014. **110**(2): p. 459-68.
17. Sandel, M.H., et al., *Natural killer cells infiltrating colorectal cancer and MHC class I expression.* Mol Immunol, 2005. **42**(4): p. 541-6.
18. Swets, M., et al., *HLA-G and classical HLA class I expression in primary colorectal cancer and associated liver metastases.* Hum Immunol, 2016. **77**(9): p. 773-9.
19. Chang, C.C., M. Campoli, and S. Ferrone, *Classical and nonclassical HLA class I antigen and NK Cell-activating ligand changes in malignant cells: current challenges and future directions.* Adv Cancer Res, 2005. **93**: p. 189-234.
20. Caligiuri, M.A., *Human natural killer cells.* Blood, 2008. **112**(3): p. 461-9.
21. Vivier, E., et al., *Functions of natural killer cells.* Nat Immunol, 2008. **9**(5): p. 503-10.
22. Menon, A.G., et al., *Immune system and prognosis in colorectal cancer: a detailed immunohistochemical analysis.* Lab Invest, 2004. **84**(4): p. 493-501.
23. Sconocchia, G., et al., *NK cells and T cells cooperate during the clinical course of colorectal cancer.* Oncoimmunology, 2014. **3**(8): p. e952197.

24. Tachibana, T., et al., *Increased intratumor Valpha24-positive natural killer T cells: a prognostic factor for primary colorectal carcinomas.* Clin Cancer Res, 2005. **11**(20): p. 7322-7.
25. Menon, A.G., Janssen-van Rhijn CM, Morreau H, Putter H, Tollenaar RA, van de Velde CJ, et al., *Immune system and prognosis in colorectal cancer: a detailed immunohistochemical analysis.* Lab Invest, 2004. **84**(4): p. 493-501.
26. Sconocchia, G., Eppenberger S, Spagnoli GC, Tornillo L, Droeser R, Caratelli S, et al., *NK cells and T cells cooperate during the clinical course of colorectal cancer.* Oncoimmunology, 2014. **3**(8): p. e952197.
27. Lissbrant, I.F., et al., *Tumor associated macrophages in human prostate cancer: relation to clinicopathological variables and survival.* Int J Oncol, 2000. **17**(3): p. 445-51.
28. Koide, N., et al., *Significance of macrophage chemoattractant protein-1 expression and macrophage infiltration in squamous cell carcinoma of the esophagus.* Am J Gastroenterol, 2004. **99**(9): p. 1667-74.
29. Tsutsui, S., et al., *Macrophage infiltration and its prognostic implications in breast cancer: the relationship with VEGF expression and microvessel density.* Oncol Rep, 2005. **14**(2): p. 425-31.
30. Shabo, I., et al., *Macrophage Infiltration in Tumor Stroma is Related to Tumor Cell Expression of CD163 in Colorectal Cancer.* Cancer Microenviron, 2014. **7**(1-2): p. 61-9.
31. Waniczek, D., et al., *Tumor-Associated Macrophages and Regulatory T Cells Infiltration and the Clinical Outcome in Colorectal Cancer.* Arch Immunol Ther Exp (Warsz), 2017.
32. Shibutani, M., et al., *The peripheral monocyte count is associated with the density of tumor-associated macrophages in the tumor microenvironment of colorectal cancer: a retrospective study.* BMC Cancer, 2017. **17**(1): p. 404.
33. Forssell, J., et al., *High macrophage infiltration along the tumor front correlates with improved survival in colon cancer.* Clin Cancer Res, 2007. **13**(5): p. 1472-9.
34. Zhou, Q., et al., *The density of macrophages in the invasive front is inversely correlated to liver metastasis in colon cancer.* J Transl Med, 2010. **8**: p. 13.
35. Gulubova, M., et al., *The density of macrophages in colorectal cancer is inversely correlated to TGF-beta1 expression and patients' survival.* J Mol Histol, 2013. **44**(6): p. 679-92.
36. Koelzer, V.H., et al., *Phenotyping of tumor-associated macrophages in colorectal cancer: Impact on single cell Invasion (tumor budding) and clinicopathological outcome.* Oncoimmunology, 2016. **5**(4): p. e1106677.
37. Biassoni, R., et al., *Human natural killer cell receptors and co-receptors.* Immunol Rev, 2001. **181**: p. 203-14.
38. Bendelac, A., P.B. Savage, and L. Teyton, *The biology of NKT cells.* Annu Rev Immunol, 2007. **25**: p. 297-336.
39. Peng, Y.P., et al., *Comprehensive analysis of the percentage of surface receptors and cytotoxic granules positive natural killer cells in patients with pancreatic cancer, gastric cancer, and colorectal cancer.* J Transl Med, 2013. **11**: p. 262.
40. Rocca, Y.S., et al., *Altered phenotype in peripheral blood and tumor-associated NK cells from colorectal cancer patients.* Innate Immun, 2013. **19**(1): p. 76-85.
41. Tan, D., et al., *Prognostic significance of lymphocyte to monocyte ratio in colorectal cancer: A meta-analysis.* Int J S urg, 2018. **55**: p. 128-138.
42. Song, W., et al., *Prognostic value of the lymphocyte monocyte ratio in patients with colorectal cancer: A meta-analysis.* Medicine (Baltimore), 2016. **95**(49): p. e5540.
43. Ijsselsteijn, M.E., et al., *Revisiting immune escape in colorectal cancer in the era of immunotherapy.* Br J Cancer, 2019. **120**(8): p. 815-818.



CHAPTER 2

The role of Natural Killer T cells in cancer – a phenotypical and functional approach



Daniëlle Krijgsman, Marianne Hokland, Peter J.K. Kuppen

Front Immunol. 2018 Feb 27;9;367

Abstract

Natural killer T (NKT) cells are a subset of CD1d-restricted T cells at the interface between the innate and adaptive immune system. NKT cells can be subdivided into functional subsets that respond rapidly to a wide variety of glycolipids and stress-related proteins using T- or NK cell-like effector mechanisms. Because of their major modulating effects on immune responses via secretion of cytokines, NKT cells are also considered important players in tumor immunosurveillance. During early tumor development, T helper (T_H)1-like NKT cell subsets have the potential to rapidly stimulate tumor-specific T cells and effector NK cells that can eliminate tumor cells. In case of tumor progression, NKT cells may become overstimulated and anergic leading to deletion of a part of the NKT cell population in patients via activation-induced cell death. Additionally, the remaining NKT cells become hyporesponsive, or switch to immunosuppressive T_H2-/T_{reg}-like NKT cell subsets, thereby facilitating tumor progression and immune escape. In this review, we discuss this important role of NKT cells in tumor development and we conclude that there should be three important focuses of future research in cancer patients in relation with NKT cells: (1) expansion of the NKT cell population, (2) prevention and breaking of NKT cell anergy, and (3) skewing of NKT cells towards T_H1-like subsets with anti-tumor activity.

Introduction

The immune system is a host defense mechanism that plays a pivotal role in the protection against pathogens and cancer [1]. It comprises multiple specialized subsets of cells that differentiate from a common pluripotent progenitor, the hematopoietic stem cell [2]. These subsets include natural killer T (NKT) cells that feature characteristics of both conventional T cells and natural killer (NK) cells. Upon activation, NKT cells are able to kill target cells either directly [3-5] or indirectly by influencing both myeloid- and lymphoid-derived immune cells [6]. Moreover, NKT cells are potent immune regulators since they can skew immune responses towards both inflammation and tolerance very quickly by secreting either T helper (T_H)1-, T_H2-, T_H17-, T regulatory (T_{reg})- or follicular helper (T_{FH})- cell-associated cytokines [7]. Because of their major modulating effects on immune responses, NKT cells have also been considered important mediators of tumor immunosurveillance [8]. The role of NKT cells in relation to cancer has therefore been the focus of recent studies. In this review, we discuss the role of NKT cells in cancer in relation to their phenotype and function. We focus on non-haematological malignancies i.e. carcinomas, sarcomas, melanomas and neuroblastomas. First, the development and function of NKT cells are addressed in healthy individuals. Thereafter, the role of NKT cells is discussed in the development and progression of cancer. Finally, available NKT cell-based immunotherapies are presented and possibilities for future research are discussed.

Development and localization of NKT cells

NKT cells constitute a unique, but highly heterogeneous, subset of immune cells that arise in the thymus from $CD4^+CD8^+$ cortical thymocytes that have undergone T cell receptor (TCR) gene rearrangement, as is the case with conventional T cells [9]. TCRs are composed of an α- and a β-chain, each containing a variable and constant domain. The TCR α-chain is generated by recombination of the variable (V) and joining (J) segments, whereas the β-chain also requires diversity (D)-segment recombination. Based on their TCR repertoire, two NKT cell subsets have been described: type I and type II NKT cells. Type I NKT cells were first identified in mice in 1990 as a unique T cell population expressing the Vα14Jα18 invariant TCR α-chain. The type I NKT cell subset recognizes the glycosphingolipid α-galactosylceramide (α-GalCer) or its synthetic analogs when presented by major histocompatibility complex (MHC) class I-like CD1d molecules [10-12]. Four years after the identification of the invariant Vα14Jα18 TCR α-chain in mice, the human counterpart Vα24Jα18 was discovered which predominantly pairs with the Vβ11 TCR β-chain [13-16]. In addition to type I NKT

cells, type II NKT cells are described with a more diverse and less well-defined TCR repertoire recognizing non-α-GalCer molecules (primarily sulfatide) presented by CD1d molecules [12,17-19].

Development of type I NKT cells in mice

The development of type I NKT cells has been thoroughly studied in mice. During positive selection in the murine thymus, T cells expressing TCRs that are capable of binding to MHC class I or II molecules on cortical thymic epithelial cells are selected to undergo lineage commitment [9]. This process leads to maturation of $CD4^+$ or $CD8^+$ T cells that recognize MHC-presented peptides. Alternatively, type I NKT cells that express the randomly rearranged invariant Vα14Jα18 chain are positively selected upon binding to CD1d molecules expressed by cortical thymocytes [9,20-22]. As a result of this alternative positive selection, they recognize lipid-derived antigens presented by CD1d molecules [10]. During the maturation process, a part of the type I NKT cell population retains expression of the T cell-associated marker CD4, resulting in two major populations in mice: $CD4^+CD8^-$ and $CD4^-CD8^-$ (double negative, DN) type I NKT cells [23,24]. Additionally, Type I NKT cells acquire expression of the NK cell receptor (NKR) NK1.1 during maturation [9].

Development of type I NKT cells in humans

Whereas the thymic development of type I NKT cells is well-defined in mice, it has not as yet been studied in details in humans. It has been reported that NKT precursor cells can be identified in thymic tissue derived from human embryos and young children [25,26]. Similar to type I NKT cell development in mice, human type I NKT cells express cell surface markers that are usually associated with both T- and NK cells. For instance, a part of the human type I NKT cell population retains expression of the T cell-associated markers CD4 or CD8 during maturation, resulting in three major populations in humans: $CD4^+CD8^-$, $CD4^-CD8^+$ and DN type I NKT cells [27,28]. In addition, a part of the human type I NKT cells acquires expression of the NK cell-associated marker CD161 (the human counterpart of NK1.1 in mice), the classical NK cell marker in humans CD56 [27-29], and various other NK cell-associated receptors [27-31].

Localization of type I NKT cells in humans

After development and maturation in the thymus, NKT cells migrate to the periphery. In general, human type I NKT cells are present in small numbers (<0.1% of total T lymphocytes) in peripheral blood (PB), lymph nodes, spleen, thymus, lung, and bone marrow [32-34]. Whereas larger type I NKT

cell populations reside in the liver, colon, kidney (~1% of total T lymphocytes) [35-37], and omentum (~10% of total T lymphocytes) [38]. Importantly, it has to be taken into account that NKT cell numbers vary substantially among healthy individuals. For instance, circulating type I NKT cells have been reported to comprise more than 5% of the total T lymphocyte population in some individuals [39].

Morphology of NKT cells

Despite the fact that NKT cells are derived from the T cell lineage, their morphology resembles NK cells more closely. NK and NKT cells are both referred to as large granular lymphocytes, whereas T cells are described as small and non-granular [40-42]. In addition, NKT cells were reported to have a low nuclear-to-cytoplasmic ratio and their nucleus contained dispersed chromatin, similar to NK cells [41,43-45].

Characterization and identification of human NKT cell subsets

In addition to classification of NKT cells in type I and type II NKT cells based on their TCR repertoire, human NKT cells can also be classified into functional subsets based on their cytokine secretion pattern upon activation, using a similar approach as for T_H-cell subsets [7].

Functional type I NKT cell subsets

At the moment, type I NKT cells can be divided into five different functional subsets (Figure 1). T_H1-like type I NKT cells have been identified in healthy individuals producing T_H1-associated cytokines such as IFN-γ and TNF-α upon stimulation [7,30,46]. The majority of these type I NKT cells are DN and are thought to exert limited cytotoxic function [30,46]. They are able to induce an effective pro-inflammatory immune cascade through cytokine signaling. Furthermore, a second T_H2-like type I NKT cell subset with regulatory properties has been described secreting IL-4 and IL-13 upon activation [7,30,46]. This type I NKT cell subset mainly consists of $CD4^+CD8^-$ cells which are able to suppress immune responses in various disease models [47-49]. Recently, three additional minor type I NKT cell subsets were identified. T_H17-like type I NKT cells have been described, secreting the pro-inflammatory cytokines IL-17, IL-21 and IL-22 when activated [50,51]. Additionally, FOXP3 expressing T_{reg}-like type I NKT cells secreting the immunosuppressive cytokine IL-10 have been identified [52], as well as T_{FH}-like type I NKT cells secreting IL-21 upon activation [46,53]. Interestingly, murine studies showed that functional type I NKT cell subsets (Figure 1) express unique transcription factors and the "choice" to become a certain subset appears to be set in the thymus during fetal development [54].

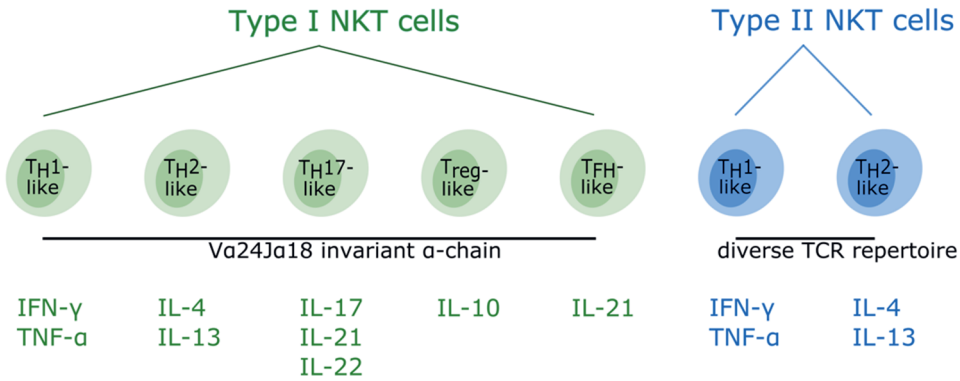


Figure 1. Overview of the different functional human NKT cell subsets. CD1d-restricted human NKT cells can be divided into subsets based on their TCR repertoire and cytokine profile. Type I NKT cells express the invariant Vα24Jα18 TCR α-chain and can be subdivided into five distinct functional subsets (indicated in green). Additionally, type II NKT cells express a diverse TCR repertoire and can be subdivided into two functional subsets (indicated in blue). Upon activation, NKT cells secrete a unique pattern of cytokines, indicated for each subtype. Type I and type II NKT cells are able to switch between different functional subsets upon interactions within the TME. Abbreviations: NKT (natural killer T), TCR (T cell receptor), T_H (helper T), T_{reg} (regulatory T), T_{FH} (follicular helper T), TME (tumor microenvironment).

The fate of type I NKT cells might, however, not be permanently determined at this time since their cytokine production upon activation can be influenced by the microenvironment [27,55], similar to T_H-cell subsets [56]. For instance, the cytokine secretion pattern of type I NKT cells is altered by the presence of immunosuppressive cytokines and/or immune cell subsets in the tumor microenvironment (TME) [57], as well as co-stimulation via CD28 [58], thereby implying plasticity of type I NKT cell subsets.

Identification of type I NKT cell subsets

NKT cells can be identified from human peripheral blood mononuclear cells (PBMC) with flow cytometry using monoclonal antibodies (mAb) and multimers as thoroughly described by Metelitsa *et al*. [59]. For instance, type I NKT cells have often been identified by co-staining with anti-Vα24 (clone C15) and anti-Vβ11 (clone C21) mAb [27,29,60,61]. However, this mAb combination leads to overestimation of type I NKT cell numbers since conventional T cells can also express Vα24 and Vβ11 TCR subunits [62,63]. Alternatively, type I NKT cells can be detected with anti-Vα24Jα18 (Clone 6B11) mAb [28,31]. Furthermore, α-GalCer-loaded CD1d dimers [64] and tetramers [30,65-67] can be used to specifically detect CD1d-restricted type I NKT cells, for instance in combination with anti-CD3, anti-

Vα24 or anti-Vβ11 mAb. Importantly, Sag *et al.* reported on detection of cytokines in type I NKT cells upon stimulation, which enables accurate identification of different functional NKT cell subsets in future studies [68]. Since this approach has not been used in NKT phenotype studies yet, no information is available on the phenotype of the different functional NKT cell subsets.

Phenotype of type I NKT cell subsets

Type I NKT cells constitutively express a variety of T cell markers such as the TCR signaling complex CD3, and co-stimulatory receptors CD4, CD8 and CD28 [27-31]. CD4 is expressed by 15-80% of the type I NKT cell population [27-29,31], and is sometimes used to subdivide type I NKT cells into $CD4^-$ and $CD4^+$ populations. Besides, type I NKT cells constitutively express a variety of receptors that are usually observed on NK cells, such as the adhesion molecule CD56 and the activating NKR CD161 [27-30]. Seventeen to 70% of the $CD4^-$ type I NKT cells express CD56, in contrast to only a small fraction of the $CD4^+$ type I NKT cell population (3-11%) [29]. Interestingly, type I NKT cells acquire a memory-activated phenotype before birth (unlike NK- and T cells), reflected by high expression of CD45RO and low expression of the homing receptor CD62L [28,29,31,69]. This might indicate that these NKT cells have been sensitized and activated during fetal life by encountering a natural ligand [69], which contributes to the ability of NKT cells to respond fast upon meeting the antigen.

In addition, type I NKT cells have the ability to induce expression of a number of phenotypic markers upon activation and/or interactions within the microenvironment. For instance, type I NKT cells can upregulate CD62L expression upon α-GalCer-mediated activation and expansion which can serve as a marker for NKT cells with superior survival and proliferative capacity [70]. In addition, $CD4^+$ and DN type I NKT cells express CD69, which is involved in lymphocyte proliferation [28,71]. Upon cytokine-mediated activation, type I NKT cells upregulate CD69 expression. Furthermore, $CD4^+$, $CD8^+$ and DN type I NKT cells express CD27, a co-stimulatory immune-checkpoint molecule involved in the control of T cell immunity [28,29,31]. Remarkably, expression of CD27 seems to be downregulated on type I NKT cells upon activation with α-GalCer, whereas its expression is upregulated in activated T cells [72,73]. This downregulation could be related to the fact that NKT cells already constitute a memory phenotype, and therefore do not require CD27 to generate NKT cell immunity and maturation upon first antigen encounter. α-GalCer-activated type I NKT cells also express the co-stimulatory molecule CD40L, and the activation marker CD38 [28,30,31,71]. In addition, type I NKT cells express the inhibitory NKR NKG2A, the low affinity Fc receptor CD16, and the activating NKRs DNAM-1, NKG2D, NKp30, NKp44, NKp46, and 2B4, that are usually expressed by NK cells [27-31]. The

proportion of type I NKT cells expressing specific NKRs is highly variable among healthy individuals (1-85%) and can be altered upon interactions within the TME [31,56,74-78]. In addition to the expression of NK- and T cell-associated cell surface markers, type I NKT cells express a wide range of inducible cytokine- and chemokine receptors enabling them to respond to various signals [28-31,79-81]. For instance, type I NKT cells induce expression of the IL-2 receptor chain CD25 (IL-2RA) upon α-GalCer-mediated activation [28,29,31], primarily in the $CD4^+$ type I NKT cell population [30]. A different pattern is observed regarding the chemokine receptors CCR5, CCR6, CCR7, and CXCR6, which are all higher expressed on $CD4^-$ type I NKT cells compared to $CD4^+$ type I NKT cells [27,29,30]. Finally, type I NKT cells express a variety of markers that are involved in a wide range of functionalities such as granzyme B, perforin and CD95L, which play important roles in cytotoxicity [29,31,82].

Functional type II NKT cell subsets

So far, two distinct functional type II NKT cell subsets have been identified (Figure 1). T_H1-like type II NKT cells secrete the pro-inflammatory cytokines IFN-γ and TNF-α upon stimulation, whereas T_H2-like type II NKT cells secrete the regulatory cytokines IL-4 and IL-13 [19,83-85]. Murine studies showed that the cytokine profile of type II NKT cells can be influenced in the same way as has been observed for type I NKT cells [83], suggesting plasticity of type II NKT cell subsets as well.

Identification of type II NKT cell subsets

In contrast to type I NKT cells, no specific methods exist to identify the entire type II NKT cell population due to the lack of specific markers. Although, several mouse models have been developed in order to study the role of type II NKT cells in cancer *in vivo*. These models include $J\alpha18^{-/-}$ mice, without type I NKT cells, and $CD1d^{-/-}$ mice that lack both type I and type II NKT cells [86-88]. Another approach to study type II NKT cells in both mice and humans is by using sulfatide-loaded CD1d multimers [19,84,89,90]. However, this approach has not been widely used due to the unstable nature of sulfatide-loaded CD1d complexes. Furthermore, since not all type II NKT cells are sulfatide-reactive, this method excludes a significant proportion of type II NKT cells [83,89]. As a result, the phenotype and function of type II NKT cells remain largely elusive, and new methods are essential in order to characterize this cell population in further detail.

NKT-like cells

In many studies, NKT cells are identified with flow cytometry using a combination of anti-CD3 and anti-CD56 mAb [31,81,91-95]. Although it is likely that the $CD3^+CD56^+$ cell population includes CD1d-restricted NKT cells, it has to be taken into consideration that conventional T cells have been reported to express NK-cell markers as well, including CD56 [17,31,79,80]. Since it is unclear whether $CD3^+CD56^+$ cells are CD1d-restricted, this population is often referred to as "NKT-like". An additional marker is essential in order to determine which part of the NKT-like cells are true NKT cells and which are not. Besides, only a small part of the type I NKT cell population expresses CD56 [29]. Hence, a significant proportion of type I NKT cells is excluded from analyses when using the combination of anti-CD3 and anti-CD56 mAb. NKT-like cells express co-stimulatory-, cytokine- and chemokine receptors, and NKRs that are also expressed by type I NKT cells [27-31,81]. Exceptions are killer-cell Ig-like receptors (KIRs) that provide either inhibitory or stimulatory signals upon interaction with human leukocyte antigen (HLA) molecules [31,96,97]. KIRs are primarily expressed by NKT-like cells, and not by type I NKT cells.

In conclusion, different functional NKT cell subsets can be identified within the type I and type II NKT cell populations. Whereas type I NKT cells are characterized in detail, type II NKT cells are not due to lack of specific markers. Studies so far suggest that the expression levels of cell surface markers on type I NKT cells are highly variable among healthy individuals. Interestingly, murine studies showed that expression patterns of type I NKT cell surface markers are modulated upon cell-cell interaction and/or interaction with signaling molecules [74,76]. These phenotypical modulations adapt the functional capabilities of the NKT cells, including the production of specific cytokines upon activation. These data indicate a high degree of NKT cell plasticity and that type I NKT cells (and probably type II NKT cells as well) are able to switch between different phenotypical/functional subsets. Studies on human NKT cells are needed to support this hypothesis.

Activation of NKT cells

Due to expression of both NK- and T cell-associated functional molecules, NKT cells can be activated by mechanisms utilized by both NK- and T cells.

Activation via T cell-like mechanisms

First, NKT cells can be activated via their TCR in a T cell-like manner via recognition of glycolipids in the context of CD1d molecules [61,98]. CD1d is primarily expressed by antigen-presenting cells (APC) but has also been reported to be expressed by some epithelial, parenchymal, and vascular smooth muscle cells [99,100]. Importantly, APC are able to present both exogenous and endogenous glycolipids in the context of CD1d (Figure 2). Exogenous microbial- and non-microbial-derived glycolipids enter APC via different mechanisms as thoroughly reviewed by Bendelac *et al.* [101] and

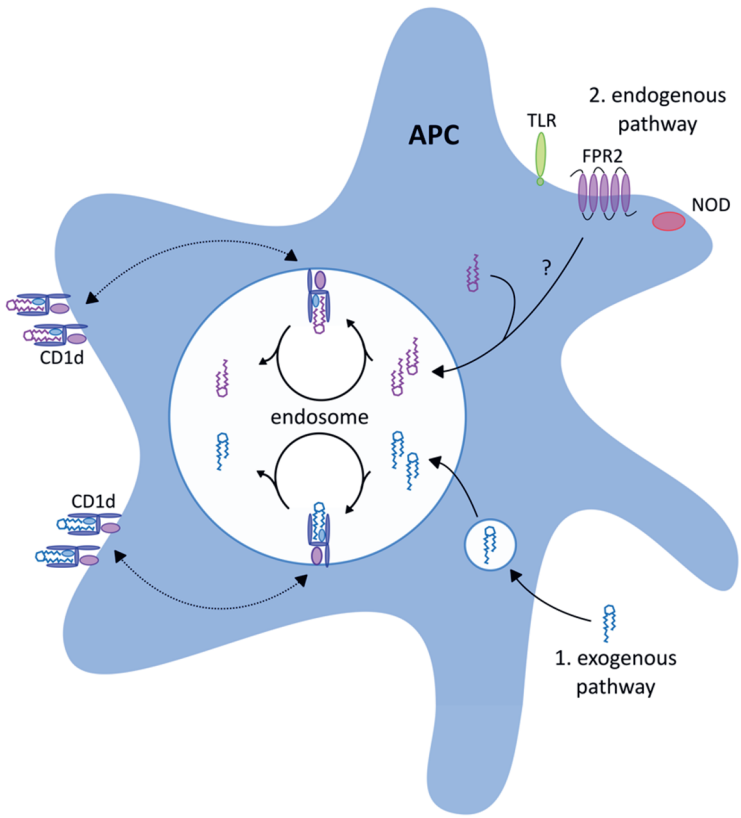


Figure 2. CD1d-presented glycolipids in APC. APC present exogenous and endogenous glycolipids in the context of CD1d. During endosomal trafficking, CD1d molecules relocate from the cell membrane towards a late endosome where the bound glycolipid antigens are removed from CD1d and replaced by new glycolipid antigens. Exogenous glycolipids (indicated in blue) enter the APC late endosome via endocytosis or phagocytosis (1). Endogenous glycolipids (indicated in purple) enter the late endosome as a result of NOD, FPR2 or TLR signaling (2), but the exact mechanism is unknown. The exogenous and endogenous glycolipids are loaded into CD1d molecules upon which they relocate back to the cell membrane. Abbreviations: APC (antigen presenting cells), NOD (nucleotide-binding oligomerization domain), FPR2 (formyl peptide receptor 2), TLR (toll-like receptor).

Barral *et al.* [102]. For instance, exogenous glycolipids can be captured by the mannose receptor, or alternatively, insert themselves directly into the cell membrane of APC, upon which they undergo endocytosis. Furthermore, exogenous glycolipids may enter APC with very low-density lipoprotein (VLDL) particles via the low-density lipoprotein (LDL) receptor, or via phagocytosis. Finally, scavenger receptors can mediate internalization of apoptotic cells and modified LDL, which also leads to entering of exogenous glycolipids into APC. During endosomal trafficking, CD1d molecules relocate from the cell membrane towards a late endosome where the bound glycolipids are removed from CD1d and replaced by new glycolipids (Figure 2) [103,104]. Thereafter, the CD1d molecules relocate back to the cell membrane. Additionally, APC also present endogenous glycolipids in the context of CD1d (Figure 2). For instance, activation of nucleotide-binding oligomerization domain (NOD) -1 and -2 intracellular pattern recognition receptors by bacteria, or activation of formyl peptide receptor 2 (FPR2) by serum amyloid A-1 (SAA-1), results in loading of endogenous glycolipids into CD1d molecules during endosomal CD1d trafficking (Figure 2) [105,106]. Furthermore, Toll-like receptor (TLR) signaling upon stimulation with lipopolysaccharide (LPS) was suggested to result in the loading of endogenous glycolipids into CD1d molecules [101,107]. The exact mechanism of how these signaling pathways lead to the loading of endogenous glycolipids into CD1d is, however, unknown.

In contrast to TCRs on conventional T cells that recognize specific peptides presented by MHC class I or II, the Vα24Jα18 TCR present on type I NKT cells recognizes a diversity of glycolipids that are presented by CD1d molecules. For instance, type I NKT cells recognize glycosphingolipids, α-galactosyldiacylglycerols, diacylglycerols, and phospholipids derived from mycobacteria in addition to α-GalCer [108-112]. Furthermore, type I NKT cells can be activated upon encountering the self-glycolipids isoglobotrihexosylceramide (iGb3) and β-glucosylceramide (β-GluCer) [11,113]. Additionally, type II NKT cells have been reported to recognize the self-glycolipids sulfatide and β-glucopyranosylceramide (β-GlcCer), as well as lysophospholipids and microbial lipids [19,83,89,114,115]. As a consequence of their diverse TCR repertoire, different type II NKT cell subsets exist, recognizing different lipids [19,85].

Activated NKT cells are able to kill tumor cells directly in a CD1d-dependent manner [116]. This antigen-specific cytotoxicity is CD95/CD95L dependent, unlike NK- and T cells that predominantly use perforin/granzyme-mediated mechanisms [116]. Upon activation via their TCR in a CD1d-dependent manner, NKT cells rapidly expand and secrete a range of cytokines [68,117-121]. Crowe *et al.* reported ~10-fold expansion of type I NKT cell numbers in the murine spleen 2-3 days after injection with 2 μg α-GalCer in mice [117]. Besides, ~7-fold and ~3-fold type I NKT cell expansion was reported in the liver and bone marrow of mice 2-3 days after α-GalCer injection, respectively [117]. Due to their

memory-activated phenotype [69], NKT cells have the ability to respond quickly upon encountering an antigen. Within an hour after injection with α-GalCer, a burst of cytokines can be detected in mice. For instance, studies reported maximal levels of IFN-γ^+ and IL-4^+ murine liver- and splenic-derived type I NKT cells within 2 hours after α-GalCer activation *in vivo* [117,118]. Additionally, high IFN-γ (400 pg/ml) and IL-4 (1500 pg/ml) levels were detected in the serum of these mice 90 minutes after injection with 100 ng/ml α-GalCer [118]. Although the percentage of IL-4^+ splenic-derived type I NKT cells dropped to baseline levels 16 hours after injection of mice with 2 μg α-GalCer, elevated IFN-γ^+ type I NKT cells could still be detected after 72 hours [117]. In conclusion, NKT cells rapidly secrete a range of cytokines following activation with α-GalCer.

Activation via NK cell-like mechanisms

NKT cells seem to behave similar to NK cells when it comes to their activation. Like in NK cells, activation is dependent on the balance between inhibitory and stimulatory signals obtained via NKRs and KIRs [122,123]. As discussed above, phenotype studies showed that NKT cells also express a wide range of these receptors [27-31]. Activating NKRs are able to recognize a variety of MHC-like molecules and cellular targets often referred to as “stress proteins”. For instance, the NKG2D receptor recognizes MHC class I-like molecules (MIC) A and B and unique long binding proteins (ULBPs) [124], whereas DNAM-1 recognizes the poliovirus receptor (PVR) and Nectin-2 [125]. Besides, NKT cells express KIRs, with less-well defined ligands, that provide activating signals [126-129]. Furthermore, NKT cells express NKRs and KIRs that provide inhibitory signals upon binding with HLA molecules [130-135]. When the balance of signals is shifted towards activation, an NKT cell is activated, resulting in cytokine production as well as direct killing of tumor cells in a CD1d-independent manner (Figure 3). Interestingly, studies showed that inhibitory signals provided by KIRs and/or NKRs were able to interrupt TCR signaling in conventional T cells without CD1d restriction [31,131,132,134]. Since NKT cells express similar functional receptors, it is likely that similar interruption of TCR signaling also occurs in NKT cells (Figure 3). Additionally, a part of the NKT cell population expresses the low affinity Fc receptor CD16 which is known to induce antibody-dependent cytotoxicity (ADCC) when present on NK cells [136]. This phenomenon has, however, not been studied as yet in relation to NKT cells. In conclusion, NKT cells can be activated via different NK- and T cell-associated mechanisms that lead to immediate killing of tumor cells and secretion of large amounts of cytokines that have a major influence on the immune system.

Finally, studies showed that NKT cells - like NK cells - can also be activated by IL-12 in combination with IL-18 via cytokine receptors in a CD1d-independent manner (Figure 3) [137-139]. These cytokines are secreted by i.e. active macrophages or dendritic cells (DC) [140-142]. Upon interaction with IL-12 and IL-18, NKT cells secrete high amounts of IFN-γ, as also observed after CD1d-dependent and NKR-mediated activation [128,137,138,143].

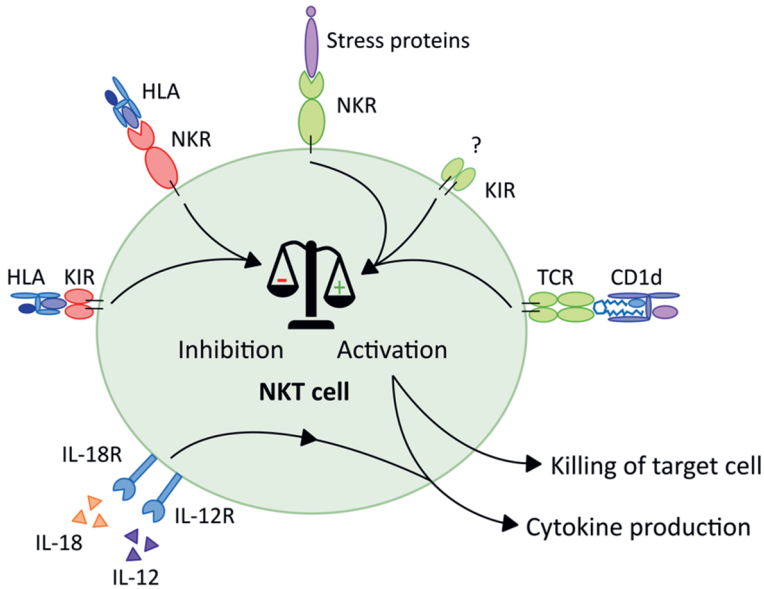


Figure 3. Activation of NKT cells. Activation of NKT cells is dependent on a balance between activating and inhibitory signals. First of all, NKT cells receive activating signals via their TCR that recognize glycolipids in context of CD1d. Secondly, NKT cells receive activating signals in a CD1d-independent manner via NKRs upon interaction with stress proteins such as MIC-A and MIC-B, ULBPs, Nectin-2, or MLL5 proteins. Additionally, NKT cells receive activating signals in a CD1d-independent manner via KIRs with merely unknown ligands. Inhibitory NKRs and KIRs that recognize HLA molecules provide inhibitory signals that are able to disrupt both TCR and NKR signaling. When the balance is skewed towards activation, NKT cells produce and secrete high amounts of cytokines. In addition, NKT cells that are activated via this mechanism are able to kill target cells directly via activation of their TCRs and NKRs. Finally, NKT cells - like NK cells - can be activated by a combination of IL-12 and IL-18 which leads to production of cytokines. Abbreviations: NKT (natural killer T), TCR (T cell receptor), NKR (natural killer receptor), MIC (major histocompatibility complex I-like molecules), ULBP (unique long binding protein), MLL5 (mixed-lineage leukemia-5), KIR (killer-cell Ig-like receptor), HLA (human leukocyte antigen), NK (natural killer).

NKT cell anergy

Importantly, murine studies have indicated that overstimulation and chronic activation of type I NKT cells with α-GalCer via TCR-CD1d interaction may result in NKT cell death and induction of anergy

[117,144-148]. This resembles the response of conventional T cells upon activation in the TME in presence of co-inhibitory stimuli or checkpoint molecules like programmed death-ligand 1 (PD-L1) [149,150]. Upon binding with α-GalCer in the context of CD1d, type I NKT cells downregulate their TCR and NKR expression and upregulate the inhibitory molecules programmed cell death protein 1 (PD-1) and B- and T-lymphocyte attenuator (BTLA) [145,147,151], resulting in hyporesponsiveness. Furthermore, chronic stimulation of type I NKT cells with α-GalCer *in vivo* resulted in activation-induced cell death (AICD) via upregulation of the death receptor CD95, thereby contributing to active depletion of type I NKT cells [152,153]. This is most likely a feedback mechanism used by NKT cells in order to prevent tissue damage. Anergy induced by α-GalCer also resulted in impaired proliferation and production of IFN-γ by type I NKT cells upon α-GalCer restimulation. In contrast, anergic type I NKT cells retained their capacity to produce T_H2-associated cytokines [145]. Moreover, pretreatment with α-GalCer skewed type I NKT cells towards a T_H2 or T_{reg}-like profile [154-157]. α-GalCer pretreated type I NKT cells acquired characteristics of regulatory cells *in vivo*, including production and secretion of IL-10, which is known to induce and maintain an immunosuppressive TME [154-156]. Chronic stimulation of NKT cells in the TME might therefore contribute to immune escape in cancer patients.

The regulatory function of NKT cells

Depending on which functional NKT cell subsets are involved, both type I and type II NKT cell subsets are able to either skew the immune response towards inflammation or towards tolerance. Activated NKT cells shape the TME via modulation of cells from both the innate and adaptive immune system (Figure 4), thereby implementing an important regulatory function.

NKT cells are unique in the sense that they can activate and induce full maturation of DC [140,141,158]. This maturation requires direct interaction of DC with NKT cells via the TCR-CD1d complex in combination with CD40/CD40L co-stimulation. As a result, DC produce IL-12, which further drives IFN-γ production by T_H1-like NKT cell subsets. In contrast, IL-13 and IL-4, produced by T_H2-like NKT cell subsets, indirectly suppress T cell function and drive T_H2 differentiation, respectively [49,159-161]. IL-10, produced by T_{reg}-like type I NKT cells drives T cell differentiation towards T_{regs}, thereby contributing to the establishment of an immunosuppressive TME [162]. Finally, the IL-21 producing T_{FH}-like type I NKT cell subset interacts directly with B cells that present the same glycolipid in context of CD1d as used to activate the NKT cells, resulting in fast immunoglobulin production and affinity maturation [53,163-166].

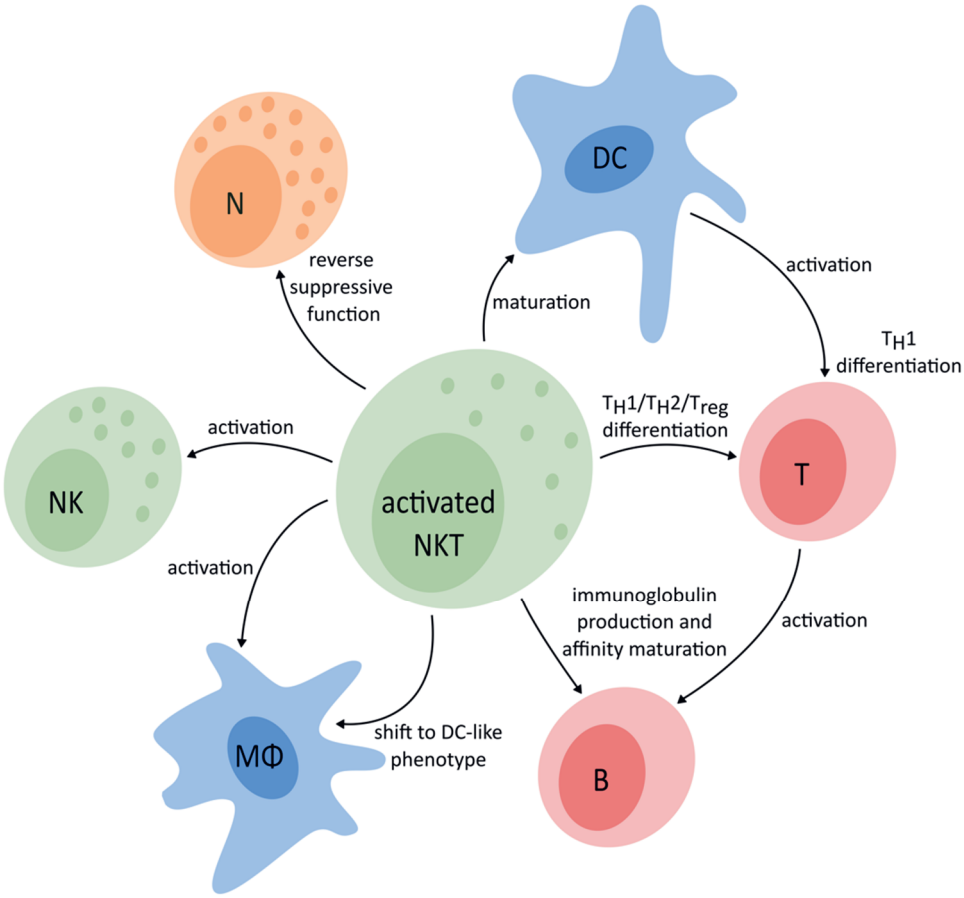


Figure 4. The regulatory function of activated NKT cells. Upon activation, NKT cells have a major influence on other immune cells. NKT cells can induce full maturation of DC upon which they activate T cells and induce T_H1 differentiation. Activated T cells interact with B cells resulting in their activation, as well as production of immunoglobulins and affinity maturation. The T_{FH} type I NKT cell subset provides cognate help for B cells which promotes the production of immunoglobulins and affinity maturation. Additionally, NKT cells can reverse the phenotype of immune suppressive neutrophils. Upon activation, NKT cells are also able to activate NK cells and macrophages, and induce a functional shift of monocytes towards a DC-like phenotype. Finally, NKT cells can drive T cell differentiation to either a T_H1-, T_H2- or T_{reg}-profile, dependent on the NKT subsets involved. Abbreviations: NKT (natural killer T), DC (dendritic cells), T_H (helper T), T_{FH} (follicular helper T), NK (natural killer), T_{reg} (regulatory T), N (neutrophil), MΦ (macrophage).

In addition to production of large amounts of cytokines as discussed above, NKT cells secrete a range of chemokines upon activation, including RANTES, Eotaxin, MIP-1α and MIP-1β, that lead to the attraction of NK cells, neutrophils, and monocytes towards the inflammatory microenvironment

[167]. IFN-γ secreted by T_H1-like NKT cell subsets then leads to the local activation of NK cells, neutrophils and macrophages [168-170]. Furthermore, granulocyte macrophage colony-stimulating factor (GM-CSF), IFN-γ and IL-4 secreted by NKT cells may shift the functional capacity of monocytes towards a more dendritic cell-like phenotype which contributes to the activation of T cells and, indirectly, B cells [171,172]. NKT cells are also able to reverse the phenotype of immune suppressive neutrophils by reducing secretion of IL-10 and enhancing IL-12 production in a CD1d-dependent manner [106].

In conclusion, NKT cells are able to rapidly respond to a wide variety of glycolipids and stress proteins using T- and NK cell-like mechanisms, respectively. Although NKT cells comprise a minor immune cell subset in most organs, they have a major effect on immune regulation since they can skew an immune response towards inflammation or tolerance in a very short time by secreting pro- or anti-inflammatory cytokines. Besides, NKT cells have the ability to kill tumor cells directly upon activation but, probably reflected by their relative low numbers, NKT cells primarily have a regulatory function. Based on this information, it is clear that NKT cells are not just cells with NK- and T cell properties: by combining characteristics of both cell types, NKT cells are able to add unique functions to the immune response. NKT cells may play a uniquely central role during the very first steps in the initiation of an anti-tumor immune response. The main reasons are the ability of NKT cells to respond fast by influencing other immune cells, resulting in amplification or dampening of the immune response.

The *in vitro* and *in vivo* anti-tumor activity of NKT cells

Type I NKT cells in tumor immunity

Twenty years ago, it was first reported that the glycolipid α-GalCer, discovered in marine sponges, had potent anti-tumor activity *in vivo* [173-175]. Mice that were intravenously inoculated with B-16 (melanoma) or intraperitoneally inoculated with EL-4 (lymphoma) cells showed a significantly prolonged lifespan after injection with α-GalCer, with a stronger potency than the typical chemotherapeutic agent mitomycin C [173]. A role for NKT cells in this anti-tumor activity was suggested a few years later when it was discovered that α-GalCer is recognized by type I NKT cells via their TCR in the context of CD1d, leading to their activation [176]. Thereafter, studies showed that type I NKT cells were the key effectors of anti-tumor responses in a murine B-16 melanoma metastasis model [177-179]. For instance, Toura *et al.* reported that activation of type I NKT cells via injection with α-GalCer-pulsed-DC resulted in complete eradication of established B-16 melanoma liver

metastases [179]. Administration of α-GalCer to activate type I NKT cells even prevented primary tumor formation in different *in vivo* models [180]. In contrast, mice lacking type I NKT cells were more prone to chemical or p53 loss-induced tumor development [181-183]. Recently, it was reported that type I NKT cells also play a role in preventing metastatic disease in a 4T1 mammary carcinoma model [184]. Upon resection of the primary breast tumors, treatment with α-GalCer-pulsed-DC limited formation of tumor metastases, prolonged survival, and provided curative outcomes in ~45% of the mice. Thereafter, it was shown that α-GalCer-pulsed-DC could also be combined with the chemotherapeutics cyclophosphamide or gemcitabine in order to enhance survival of mice with metastatic disease [185]. Importantly, studies showed that the anti-metastatic effect of α-GalCer was impaired in NK cell-depleted or IFN-γ-deficient mice [186,187]. Smyth *et al.* showed that IFN-γ production by type I NKT cells and subsequent IFN-γ production by NK cells was crucial for α-GalCer-mediated tumor protection [178]. In line with these results, it was observed that the T_H2-skewing synthetic α-GalCer-analogue OCH provided less tumor protection in the CT26 mouse model compared to α-GalCer [188]. In contrast, studies reported that analogues of α-GalCer, which skewed the cytokine profile of type I NKT cells towards T_H1, provided superior protection against metastases formation compared to α-GalCer [189-191]. This implicates a crucial role for type I NKT cells with a T_H1 cytokine profile in anti-tumor activity.

After the discovery of the important role of activated type I NKT cells in anti-tumor responses *in vivo*, studies focused on the mechanisms used by these NKT cells in order to eradicate tumor cells. As mentioned above, CD1d is primarily expressed by APC, although malignant haematopoietic cells have also been reported to express CD1d on their cell membrane [4,192-194]. Additionally, there is evidence that solid tumors also express CD1d, including renal cell and colorectal carcinomas [195,196]. Upon activation with α-GalCer, type I NKT cells were able to kill $CD1d^+$ tumor cells in a CD1d-dependent manner [4,192-194,196]. In order to kill tumor cells directly via CD1d interaction, they need to present glycolipids that can be recognized by NKT cells. There is evidence from murine studies that type I NKT cells can be activated by tumor-derived glycolipids that are cross-presented by APC in the context of CD1d [197-200]. However, until now, the nature of tumor-glycolipids that are recognized by NKT cells remains poorly elucidated. Since the cytolysis and eradication of tumor cells via type I NKT cells was shown to be dependent on CD1d expression on their cell surface, it was suggested that CD1d expression might be a predictor of whether α-GalCer-activated type I NKT cells are able to eradicate tumor cells or not [3,4]. However, other studies showed that $CD1d^-$ haematopoietic cells could also be killed directly by type I NKT cells, for instance via NKG2D activation [5,201,202]. This illustrates that type I NKT cells, like NK cells, are able to kill tumor cells via

NKR activation, even in the absence of CD1d. Moreover, although type I NKT cells are capable of killing tumor cells directly, they primarily mediate anti-tumor activity via the activation of downstream immune effector cells as demonstrated by human and mouse studies [4,178,203,204]. Especially T_H1-like type I NKT cells play an important role in this anti-tumor activity via secretion of large amounts of IFN-γ, which leads to generation of tumor-specific $CD8^+$ cytotoxic T cells, and rapid activation of NK cells [4,178,189,203-205].

Type II NKT cells in tumor immunity

In contrast to type I NKT cells, only limited information is available regarding the role of type II NKT cells in cancer. Although, some *in vivo* models provided important information. In general, type II NKT cells are associated with immunosuppression and tumor progression. For instance, murine carcinoma and lymphoma models showed that the tumor burden of $CD1d^{-/-}$ mice, without type I and type II NKT cells, was lower compared to $J\alpha18^{-/-}$ mice that lack type I NKT cells only [86-88]. Injections with sulfatide increased the number of tumor nodules in a CT26 colon carcinoma lung metastasis mouse model, via activation of type II NKT cells [206]. Besides, administration of sulfatide abrogated the protective effect of α-GalCer-activated type I NKT cells against tumor development. Type II NKT cells were reported to produce IL-13 through the IL-4R-STAT6 pathway, which was necessary for downregulation of tumor immunosurveillance in a 15-12RM fibrosarcoma mouse model [49]. Thereafter, it was shown that IL-13 induced TGF-β-secreting myeloid derived suppressor cells (MDSC) *in vivo* that inhibited tumor-specific T cells [159,207]. A role for MDSC in inhibition of anti-tumor immunosurveillance was supported by the study of Renukaradhya *et al.* that showed large numbers of these cells at the tumor site of B cell lymphoma-bearing $J\alpha18^{-/-}$ mice without type I NKT cells [87]. This implicates an important role for type II NKT cells in suppression of immunosurveillance in cancer. Although, Zhao *et al.* showed in an *in vivo* murine model that activation of type II NKT cells with CpG oligodeoxynucleotides resulted in anti-tumor activity of these cells via the production of IFN-γ [83,208]. The involvement of IFN-γ implies that type II NKT cells are able to contribute to anti-tumor responses, but only when the T_H1-like subset is involved.

In conclusion, a crucial role is implicated for type I NKT cells with a T_H1 cytokine profile in anti-tumor activity. In addition, although it is generally accepted that the type II NKT cell population promotes tumor growth, there is evidence that T_H1-like type II NKT cells can be involved in anti-tumor responses. Hence, the role of NKT cells in malignancies is highly dependent on which functional type I or type II NKT cell subsets are involved.

The function and phenotype of NKT cells in patients diagnosed with cancer

Several human studies have addressed the presence, function and/or phenotype of NKT cells in cancer patients. Here, we will focus on both tumor-infiltrating and circulating NKT cells.

Tumor-infiltrating NKT cells

Studies showed a difference in the presence of NKT cells between tumor tissue and non-tumor tissue. The frequency of type I NKT cells was reported to be higher in intrahepatic malignant tumors and colorectal carcinomas compared to normal liver tissue and normal mucosa, respectively [209,210]. The opposite pattern was reported by Kenna *et al.* who showed a significantly lower presence of liver-infiltrating type I NKT cells in colorectal liver metastases compared to healthy liver tissue [35]. In addition, several studies showed a correlation between infiltrating NKT cell numbers and clinical outcome. High numbers of tumor-infiltrating type I NKT cells correlated with a relatively good clinical outcome in patients diagnosed with colorectal cancer and neuroblastoma [210,211]. High density of NK/NKT cells was also associated with prolonged overall survival in periampullary adenocarcinoma (including pancreatic cancer) patients [212]. Accordingly, absence of infiltrating type I NKT cells and low numbers of infiltrating NKT-like cells correlated with poor patient survival and disease progression in neuroblastoma and gastric cancer, respectively [91,213].

The function and phenotype of infiltrating type I NKT cells was addressed in studies on hepatocellular carcinoma, colorectal cancer and neuroblastoma [35,210,213]. Lower expression of CD56 and CD161 was reported on infiltrating type I NKT cells in tumor-bearing livers compared to normal livers [35]. Additionally, in a study on colorectal cancer, expression of the activation markers CD69L and FasL was reported on a larger fraction of infiltrating type I NKT cells in tumor tissue compared to normal mucosa [210]. Tumor-infiltrating type I NKT cells expressed IFN-γ and granzyme B, but the authors did not compare the expression of these markers to that of type I NKT cells in normal mucosa [210]. Additionally, it was observed that type I NKT cell infiltration in neuroblastomas was associated with CCL2 expression on tumor cells, indicating that expression of homing receptors on tumors was essential for infiltration of type I NKT cells in neuroblastoma [213]. Furthermore, in two studies [91,92] the function and phenotype of infiltrating NKT-like cells in tumors were described. Peng *et al.* reported impaired effector function of infiltrating NKT-like cells in gastric cancer-derived tumor tissue compared to non-tumor tissue, characterized by decreased expression of IFN-γ, TNF-α, granzyme B and Ki-67 [91]. Furthermore, this study also showed decreased expression of the lymphocyte proliferation marker CD69, the homing receptors CXCR3 and CCR5, and the NKRs NKG2D

and DNAM-1 on NKT-like cells in tumor tissue compared to non-tumor tissue. In addition, a study on patients with hepatocellular carcinoma showed that NKT-like cells in tumor tissue expressed FOXP3 and lost expression of IFN-γ and perforin compared to NKT-like cells in non-tumor tissue [92].

In conclusion, tumor-infiltrating type I NKT cells and NKT-like cells may express less activating receptors, homing receptors and proliferation markers, and produce lower amounts of T_H1-associated cytokines compared to type I NKT cells and NKT-like cells in healthy tissue, indicating tolerance and not anti-tumor activity.

Circulating NKT cells

In addition, studies also showed altered function of circulating type I NKT cells in cancer patients. For instance, the number of circulating type I NKT cells was significantly decreased in patients with different cancers compared to healthy controls [214-222]. In line with the results on infiltrating type I NKT cells, low circulating type I NKT cell numbers correlated with poor clinical outcome in patients with head and neck squamous cell carcinoma [215,216]. Interestingly, late-stage cancer patients presented with lower type I NKT cell numbers than early-stage cancer patients with oral squamous cell carcinoma or laryngeal cancer [219,222], suggesting cancer-mediated depletion of NKT cells. After resection of the primary tumor, type I NKT cell numbers did not increase in patients with different cancer types [214,221]. In contrast, circulating NKT-like cell numbers were not decreased in patients diagnosed with laryngeal cancer, gastric cancer or hepatocellular carcinoma [91,92,221].

Besides being reduced in numbers, circulating type I NKT cells are often functionally impaired in patients [217,219,220,223-225]. For instance, circulating type I NKT cells derived from patients with prostate cancer or oral squamous cell carcinoma had a T_H2-biased cytokine profile [219,220]. Furthermore, type I NKT cells obtained from patients with advanced cancer stages showed impaired cytokine production and proliferative capacity upon *ex vivo* activation with α-GalCer [217,220,223]. In accordance with this observation, lower numbers of IFN-γ-producing type I NKT cells were observed in patients with colon carcinoma, head and neck cancer, breast cancer, or renal cell carcinoma compared to healthy controls [214]. These changes in cytokine profile imply that type I NKT cells switched from a T_H1- towards a T_H2-like NKT cell subset.

In conclusion, reduced frequency of circulating NKT cells and altered phenotype, resulting in altered function, of both infiltrating and circulating NKT cells are often observed in cancer patients, especially in patients with late-stage disease. Since altered function was not observed in infiltrating NKT cells in healthy tissue, it can be argued that this altered function of NKT cells is cancer/TME-

mediated: tumors may suppress the immune system, and skew the cytokine profile of NKT cells from T_H1 towards T_H2 in order to escape from recognition and elimination. Although the mechanisms behind the cancer/TME-mediated altered function of NKT cells are not fully understood, studies suggested a role for metabolic derivative lactic acid [226], the production of soluble factors by tumors such as sMIC [227], and the expression of CD1d by tumors [195,228].

Shaping the TME by NKT cells

Shaping the TME by T_H1-like NKT subsets

As discussed above, T_H1-like NKT cells are promising candidates to initiate effective anti-tumor immune responses. T_H1-like NKT cells might play an important role in anti-tumor responses by shaping the TME (Figure 5). For instance, they have been reported to co-localize with tumor-associated macrophages (TAM) with an M2-polarized phenotype that promote tumor growth and progression [156,229]. This co-localization resulted in CD1d-dependent killing of TAM that cross-presented tumor-derived glycolipids *in vivo* [198,230]. Furthermore, T_H1-like NKT cells and secondary activated NK cells contributed to the inhibition of tumor angiogenesis by IFN-γ via suppression of M2-polarized TAM [231,232]. Finally, Courtney *et al.* showed that type I NKT cells were able to kill $CD1d^+$ M2 TAM or polarize M2 TAM towards an M1-polarized phenotype via GM-CSF production [233]. Hence, the presence of T_H1-like type I NKT cells might minimize the presence of tumor growth-promoting M2-polarized TAM in the TME. On the other side, an immature tolerogenic DC subset has been described that produces reduced amounts of IL-12 and high amounts of IL-10, resulting in an immunosuppressive TME [234]. As a result, tolerogenic DC skew differentiation of naïve T cells into T_{regs}, which might lead to immune escape of tumor cells [235-237]. Since T_H1-like NKT cells are able to fully mature DC, the presence of immature tolerogenic DC might be minimized in tumors where sufficient numbers of these NKT cells are present. Importantly, T_H1-like NKT cells are able to stimulate both tumor antigen-restricted T cells that recognize tumor cells with HLA expression and effector NK cells that eliminate tumor cells with low or absent HLA expression [235]. In this way, immune escape of tumor cells might be prevented. Additionally, as discussed above, tumors have been reported to express CD1d on their cell membrane [195,196], and might therefore be killed in a CD1d-dependent manner. Besides, tumors are reported to express high cell surface densities of stress-related proteins that activate the NKRs NKG2D and DNAM-1 [238,239], suggesting that these cells can also be killed in a CD1d-independent manner. HLA class I loss or downregulation has often been reported in tumors

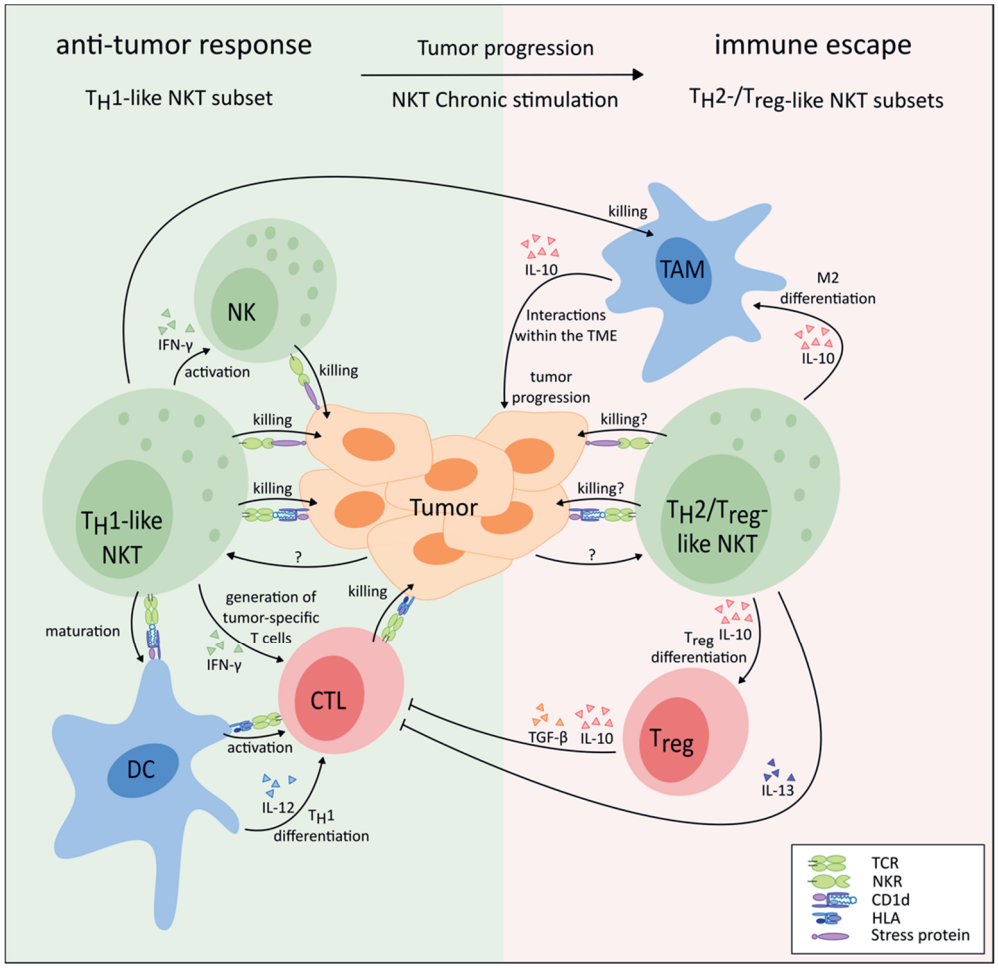


Figure 5. The dual role of NKT cells in cancer. During early tumor development, T_H1-like NKT cells may induce an effective anti-tumor response via direct killing of tumor cells upon interaction with stress proteins and CD1d molecules, respectively. Additionally, activated T_H1-like NKT cells secrete high amounts of IFN-γ, which may lead to generation of tumor-specific $CD8^+$ cytotoxic T cells and rapid activation of NK cells that kill tumor cells. Besides, activated NKT cells can induce maturation of DC in a CD1d-dependent manner, resulting in T_H1 differentiation and activation of tumor-specific $CD8^+$ T cells. Finally, activated T_H1-like NKT cells can kill M2-polarized TAM in a CD1d-dependent manner, thereby preventing their tumor growth-promoting effects. During tumor progression, T_H1-like NKT cells can become anergic and may switch to T_H2-/T_{reg}-like NKT cell subsets that facilitate tumor progression and immune escape. For instance, T_{reg}-like NKT cells can promote differentiation of M2-polarized TAM and T_{regs}. T_{regs} inhibit tumor-specific T cells via cell-cell interactions, and secretion of IL-10 and TGF-β. Additionally, T_H2-like NKT cells can inhibit tumor-specific T cells via production of large amounts of IL-13. T_H2- and T_{reg}-like NKT cell subsets might be able to kill tumor cells, either via CD1d-dependent or CD1d-independent mechanisms. However, overstimulated NKT cells produce large amounts of immunosuppressive cytokines, resulting in a net effect of immunosuppression. Receptors that are known to be involved in NKT cell-mediated anti-tumor responses are indicated in the figure. The functional NKT cell subsets indicated in the figure can be both type I and type II NKT

cells as illustrated in figure 1. Abbreviations: NKT (natural killer T), T_H (helper T), NK (natural killer), DC (dendritic cells), TAM (tumor-associated macrophages), T_{reg} (regulatory T), TME (tumor microenvironment), NKR (natural killer receptor), TCR (T cell receptor), HLA (human leukocyte antigen).

including carcinomas, sarcomas, neuroblastomas and melanomas [240-243]. Since inhibitory NKRs and KIRs prevent NKT cell activation upon interaction with HLA molecules (Figure 3), NKT cells might be able to directly kill tumor cells with low HLA expression, similar to NK cells. NKT cells may have, however, primarily a regulatory function, suggesting that the anti-tumor activity mediated by direct killing of tumor cells is of lesser importance.

Shaping the TME by T_H2-/T_{reg}-like NKT subsets

As discussed in the previous chapter, the phenotype and function of T_H1-like subsets is frequently altered in patients. The NKT cell population in patients is skewed towards a T_H2 profile, proliferative impaired and, additionally, reduced in size. These data indicate many similarities with overstimulated/anergic NKT cells. During cancer progression, NKT cells may be exposed to chronic stimulation, which is known to induce anergy and skew NKT cells towards immunosuppressive subsets. Moreover, chronic stimulation of NKT cells activates AICD, which might explain the reduced NKT cell population observed in cancer patients. Based on this hypothesis, we propose that T_H1-like NKT cells induce an effective anti-tumor response during early tumor development and perhaps prevent further tumor development in many cases. However, in some cases, at some point during tumor progression, NKT cells become overstimulated. As a result, a part of the NKT cell population is deleted in cancer patients via AICD. Additionally, the remaining NKT cells become hyporesponsive, or switch to T_H2-/T_{reg}-like NKT cell subsets, thereby facilitating tumor progression and immune escape (Figure 5). T_H2- and T_{reg}-like NKT cell subsets do not produce IFN-γ which is responsible for most of the anti-tumor effects of T_H1-like NKT cells as discussed above. In contrast, T_H2-/T_{reg}-like NKT cells produce large amounts of IL-13 and IL-10, respectively, that suppress the TME (i.e. via fibroblasts and MDSC), thereby indirectly stimulating tumor progression. Additionally, T_{reg}-like NKT cells promote differentiation of M2-polarized TAM and T_{regs} that are also able to suppress the TME via production of IL-10 [155,156]. In contrast to T_H1-like NKT cells, T_H2-like NKT cells are not capable of inducing DC maturation and do therefore not induce activation of tumor-specific T cells [158]. T_H2-like NKT cell subsets further inhibit the activation of tumor-specific T cells via secretion of IL-13 [49,159,160], while T_{regs} inhibit tumor-specific T cells via cell-cell interactions and secretion of IL-10 and TGF-β [244,245].

T_H2- and T_{reg}-like NKT cell subsets might still be able to kill tumor cells, either via CD1d-dependent or CD1d-independent mechanisms. However, overstimulated NKT cells produce large amounts of immunosuppressive cytokines, resulting in a net effect of immunosuppression. T_H2- and T_{reg}-like NKT cell subsets, therefore, counteract the anti-tumor effects of T_H1-like NKT cells and, additionally, actively promote tumor progression.

In conclusion, we discussed evidence supporting our hypothetical model (Figure 5) that T_H1-like NKT cells are responsible for initiating effective anti-tumor immune responses during early tumor development. When NKT cells become overstimulated and anergic due to tumor progression, a part of the NKT cell population is deleted in cancer patients. Additionally, the remaining NKT cells lose their anti-tumor function and start facilitating immune escape and tumor progression. In summary, we illustrated three problems regarding NKT cells in cancer patients. First, the numbers are lower compared to healthy individuals. Second, NKT cells are often anergic in cancer patients. Third, NKT cells are often skewed towards immunosuppressive T_H2-like subsets.

Current NKT cell-based immunotherapy for the treatment of cancer

Because of their potential to induce effective anti-tumor responses *in vivo*, several NKT cell-based immunotherapies in humans have been developed over the past years as thoroughly reviewed by Nair *et al*. [246]. These immunotherapies primarily focused on activation and expansion of the type I NKT cell population.

For instance, a phase I clinical trial was executed in which intravenous (i.v.) injections of 50–4800 μg/m^2 α-GalCer were administered to 24 patients with different solid tumors [218]. The majority of patients presented with reduced numbers of type I NKT cells at baseline (median 333 cells/ml PB) compared to healthy donors (median 1013 cells/ml PB). Following α-GalCer administration, NKT cells disappeared from the circulation within 24 hours. Although not investigated in details in this study, recovery of NKT cell numbers was not observed within a week. Furthermore, increased serum levels of TNF-α and GM-CSF were detected in 5 patients and increased serum IFN-γ levels were detected in 1 patient after α-GalCer administration. A phase II trial using administration of α-GalCer was not executed. Additionally, clinical trials were conducted in which patients were injected with α-GalCer-pulsed autologous DC [247-253]. These injections resulted in expansion of the type I NKT cell population in some patients [247-253]. For instance, Chang *et al.* reported >100-fold expansion of type I NKT cells in 5 patients upon i.v. injections with 5 x 10^6 α-GalCer-pulsed DC [248]. Besides NKT cell expansion, treatment with α-GalCer-pulsed DC also increased the systemic levels of IFN-γ in

patients [247-253]. Treatment did, however, not result in a clinical tumor response in the majority of patients [247-253]. In addition, a phase II study reported stable disease in 5/17 patients with non-small cell lung cancer (NSCL) upon i.v. administration of α-GalCer-pulsed IL-2/GM-CSF-cultured PBMC (1×10^9 cells/m^2) [252]. Patients with increased IFN-γ producing T_H1-like type I NKT cells after treatment showed a prolonged median survival time compared to non-responsive patients. These data indeed indicate a crucial role for T_H1-like type I NKT cells in anti-tumor immune responses and emphasize the essential need for expansion of this NKT cell population in cancer patients. Hence, immunotherapeutic approaches focused on skewing NKT cells towards an T_H1 profile should be developed.

In later clinical trials, *ex vivo*-activated type I NKT cells were adoptively transferred to patients diagnosed with NSCL, advanced melanoma or head and neck squamous cell carcinoma, in some cases in combination with α-GalCer-pulsed APC [254-257]. In this therapy, PBMC obtained from the patient, i.e. by leukapheresis, were cultured in the presence of IL-2 and α-GalCer to facilitate proliferation and activation of the type I NKT cell population. Thereafter, the *ex vivo*-activated type I NKT cells were administered to the patients. Phase I and II clinical trials were conducted in which patients with head and neck carcinomas received nasal submucosal injections of 1×10^8 α-GalCer-pulsed APC, in combination with intra-arterial infusion of 5×10^7 *ex vivo*-activated autologous type I NKT cells via tumor-feeding arteries [256,257]. Tumor regression and stable disease were reported in 10/10 of these patients [256]. These clinical responses did, however, not correlate with the induction of immunological responses in blood (i.e. increase in type I NKT cell numbers and/or IFN-γ-producing type I NKT cells and NK cells). Additionally, *ex-vivo*-activated type I NKT cells were adoptively transferred to patients diagnosed with advanced or recurrent NSCL ($1 \times 10^7/m^2$ or $5 \times 10^7/m^2$ NKT cells per infusion) or advanced melanoma (~4×10^6 – ~2×10^8 NKT cells per infusion) in phase I clinical trials [254,255]. Treatment was well-tolerated and resulted in stable disease in 2/9 NSCL patients and 3/9 patients with advanced melanoma, respectively. However, the majority of patients developed progressive disease. This might be due to the fact that the numbers of administered *ex viv*o-activated autologous type I NKT cells were too low in comparison to the tumor load. Obtaining sufficient numbers of type I NKT cells might be a major challenge since type I NKT cell numbers are low in general, and especially in patients with cancer.

Recently, studies focused on increasing the specificity of NKT cells by transducing them with chimeric antigen receptors (CARs) that are not HLA- or CD1d-restricted [58,70,258]. Additionally, α-GalCer/CD1d-antitumor fusion proteins were suggested as a treatment for cancer patients. For instance, α-GalCer-loaded CD1d molecules fused with an antibody fragment specific for HER2 or CEA

antigens induced potent anti-tumor activity *in vitro* and *in vivo* [259,260]. Recently, Horn *et al.* showed that CD3xPD-L1 Bi-specific T cell engagers (BiTEs) activated both T cells and NKT cells to kill PD-L1$^+$ tumor cells *in vitro* [261]. Another strategy that was suggested for treatment of patients with solid tumors is vaccination with NKT-activating agents in combination with tumor antigens. For instance, a phase I study showed detectable NKT cell activity in patients with high-risk melanoma upon treatment with cancer/testis (CA) antigen-loaded DC in combination with α-GalCer [262]. However, in our opinion, increasing the specificity of NKT cells is not the most promising method of increasing the effectiveness of NKT-based immunotherapies. The strength of NKT cells does not rest in their cytotoxic capacities, but in their regulatory function. When the appropriate subsets are activated (i.e. T_H1-like NKT cells), NKT cells might shift the tolerogenic and immunosuppressive state of both innate and adaptive cells towards anti-tumor activity. Therefore, instead of increasing the specificity of NKT cells, immunotherapies should focus on the most important function of NKT cells, their regulatory function.

In conclusion, several NKT cell-based immunotherapies have been tested in clinical trials. To date, a beneficial effect in a minority of cancer patients has been reported. These clinical trials were mainly based on the activation and expansion of type I NKT cells with α-GalCer. As addressed in our hypothetical model (Figure 5), NKT cells may switch to immunosuppressive functional subsets or become anergic due to chronic stimulation during cancer progression which might explain the absence of beneficial clinical responses in patients upon treatment with α-GalCer. We propose that it is essential to prevent and break NKT cell anergy in cancer patients, and skew NKT cells in cancer patients towards T_H1-like subsets with anti-tumor activity in addition to expansion of the NKT cell population.

Focus of future NKT cell-based immunotherapies

In this review, we discussed the role of NKT cells in cancer and conclude that NKT cells play a central role in anti-cancer treatment due to their important regulatory function. In order to improve NKT cell-based immunotherapies for treatment of cancer patients, several aspects of the current treatment strategies need further attention.

Expansion of the NKT cell population

T_H1-like NKT cells (either type I or type II) have the potential to induce effective anti-tumor responses. Combined with the fact that their numbers are decreased in cancer patients, it is essential to expand this cell population in patients. For instance, induced pluripotent stem cells (iPSCs) might be used to

expand the numbers of autologous NKT cells in patients *ex vivo* [263-265]. Furthermore, culturing methods aiming at obtaining high numbers of NKT cells must be optimized. At the moment, according to the Clinical Trials registry, multiple clinical trials (Available at: https://clinicaltrials.gov/ct2/show/NCT02562963, https://clinicaltrials.gov/ct2/show/NCT03198923, https://clinicaltrials.gov/ct2/show/NCT01801852) are ongoing that study the safety and clinical efficacy of adoptive type I NKT cell transfer in patients with solid tumors. As discussed in this review, this infusion should be accompanied by a protocol that prevents induction of NKT cell anergy and generation of immunosuppressive NKT cell subsets.

Prevention and breaking of NKT cell anergy

Until now, only a limited number of studies focused on prevention or breaking of NKT cell anergy. Parekh *et al.* showed that blockade of the interaction between PD-1 and its ligands prevented the induction of type I NKT cell anergy *in vivo* [266]. Blockade of the PD-1/PD-L1 axis was, however, unable to reverse established NKT cell anergy [266,267]. Additionally, *in vitro* and *in vivo* studies showed that stimulation of type I NKT cells with IL-2 overcomes anergy and restores their capacity to proliferate [145,147]. The proliferative capacity of patient-derived type I NKT cells was also reported to increase upon COX-2 inhibition or culture with G-CSF [223,268]. It might, therefore, be an option to treat patients with a combination of anti-PD-1 antibody, such as nivolumab, combined with IL-2/G-CSF or COX-2 inhibition in order to prevent and reverse NKT cell anergy.

Skewing of NKT cells towards T_H1-like subsets

NKT cells in which anergy was reversed retained their T_H2-biased cytokine profile upon IL-2 stimulation and did not change back towards a T_H1-like subset with anti-tumor activity [147]. It is therefore also necessary to use agents that are able to skew the cytokine profile of activated NKT cells towards a T_H1 profile, which means a change in functional subset. For instance, culturing of patient-derived T_H2-biased type I NKT cells with IL-12 resulted in IFN-γ production of these cells in response to α-GalCer *in vitro* [220]. Additionally, Laurent *et al.* showed that chemical modification of the α-GalCer compound was able to increase T_H1-associated cytokine production by activated type I NKT cells, whereas stimulation of type I NKT cells with conventional α-GalCer resulted in production of both T_H1- and T_H2-associated cytokines [269]. Other synthetic agonists have also been described that induce a T_H1-skewed cytokine profile in type I or type II NKT cells [83,191,270,271]. Hence, the use of modified NKT

cell activating agents in cancer patients might skew the cytokine profile of NKT cells towards a T_H1 profile while simultaneously preventing the induction of anergy.

Concluding remarks

Due to their important regulatory function, NKT cells are promising candidates for immunotherapies in patients diagnosed with cancer. However, NKT cell-based immunotherapies that focus on activating NKT cells have resulted in beneficial clinical responses in a minority of patients so far. In this review, we illustrated a hypothetical model regarding the role of NKT cells in solid tumors based on their function and phenotype. During early tumor development, T_H1-like NKT cell subsets have the potential to initiate effective anti-tumor immune responses against tumors. However, when NKT cells become overstimulated and anergic during tumor progression, they lose their anti-tumor function and start facilitating immune escape. The role of NKT cells in cancer might therefore be more dynamic than initially thought. So far, studies have primarily focused on methods to activate and expand the type I NKT cell population in patients, but the contribution of functionally altered NKT cells to the failure of NKT cell-based immunotherapies has been largely ignored. In this review, we conclude that there should be three important focuses of future research in cancer patients: (1) expansion of the NKT cell population, (2) prevention and breaking of NKT cell anergy, and (3) skewing of NKT cells towards T_H1-like subsets with anti-tumor activity.

References

1. Parkin, J.; Cohen, B. An overview of the immune system. *Lancet* **2001**, *357*, 1777-1789, doi:10.1016/S0140-6736(00)04904-7.
2. Lim, W.F.; Inoue-Yokoo, T.; Tan, K.S.; Lai, M.I.; Sugiyama, D. Hematopoietic cell differentiation from embryonic and induced pluripotent stem cells. *Stem Cell Res Ther* **2013**, *4*, 71, doi:10.1186/scrt222.
3. Haraguchi, K.; Takahashi, T.; Nakahara, F.; Matsumoto, A.; Kurokawa, M.; Ogawa, S.; Oda, H.; Hirai, H.; Chiba, S. CD1d expression level in tumor cells is an important determinant for anti-tumor immunity by natural killer T cells. *Leuk Lymphoma* **2006**, *47*, 2218-2223, doi:10.1080/10428190600682688.
4. Metelitsa, L.S.; Naidenko, O.V.; Kant, A.; Wu, H.W.; Loza, M.J.; Perussia, B.; Kronenberg, M.; Seeger, R.C. Human NKT cells mediate antitumor cytotoxicity directly by recognizing target cell CD1d with bound ligand or indirectly by producing IL-2 to activate NK cells. *J Immunol* **2001**, *167*, 3114-3122.
5. Kuylenstierna, C.; Bjorkstrom, N.K.; Andersson, S.K.; Sahlstrom, P.; Bosnjak, L.; Paquin-Proulx, D.; Malmberg, K.J.; Ljunggren, H.G.; Moll, M.; Sandberg, J.K. NKG2D performs two functions in invariant NKT cells: direct TCR-independent activation of NK-like cytolysis and co-stimulation of activation by CD1d. *Eur J Immunol* **2011**, *41*, 1913-1923, doi:10.1002/eji.200940278.
6. McEwen-Smith, R.M.; Salio, M.; Cerundolo, V. The regulatory role of invariant NKT cells in tumor immunity. *Cancer Immunol Res* **2015**, *3*, 425-435, doi:10.1158/2326-6066.CIR-15-0062.
7. Brennan, P.J.; Brigl, M.; Brenner, M.B. Invariant natural killer T cells: an innate activation scheme linked to diverse effector functions. *Nat Rev Immunol* **2013**, *13*, 101-117, doi:10.1038/nri3369.
8. Robertson, F.C.; Berzofsky, J.A.; Terabe, M. NKT cell networks in the regulation of tumor immunity. *Front Immunol* **2014**, *5*, 543, doi:10.3389/fimmu.2014.00543.
9. Godfrey, D.I.; Stankovic, S.; Baxter, A.G. Raising the NKT cell family. *Nat Immunol* **2010**, *11*, 197-206, doi:10.1038/ni.1841.
10. Kinjo, Y.; Illarionov, P.; Vela, J.L.; Pei, B.; Girardi, E.; Li, X.; Li, Y.; Imamura, M.; Kaneko, Y.; Okawara, A., et al. Invariant natural killer T cells recognize glycolipids from pathogenic Gram-positive bacteria. *Nat Immunol* **2011**, *12*, 966-974, doi:10.1038/ni.2096.
11. Zhou, D.; Mattner, J.; Cantu, C., 3rd; Schrantz, N.; Yin, N.; Gao, Y.; Sagiv, Y.; Hudspeth, K.; Wu, Y.P.; Yamashita, T., et al. Lysosomal glycosphingolipid recognition by NKT cells. *Science* **2004**, *306*, 1786-1789, doi:10.1126/science.1103440.
12. Kumar, V.; Delovitch, T.L. Different subsets of natural killer T cells may vary in their roles in health and disease. *Immunology* **2014**, *142*, 321-336, doi:10.1111/imm.12247.
13. Porcelli, S.; Yockey, C.E.; Brenner, M.B.; Balk, S.P. Analysis of T cell antigen receptor (TCR) expression by human peripheral blood CD4-8- alpha/beta T cells demonstrates preferential use of several V beta genes and an invariant TCR alpha chain. *J Exp Med* **1993**, *178*, 1-16.
14. Dellabona, P.; Casorati, G.; Friedli, B.; Angman, L.; Sallusto, F.; Tunnacliffe, A.; Roosneek, E.; Lanzavecchia, A. In vivo persistence of expanded clones specific for bacterial antigens within the human T cell receptor alpha/beta CD4-8- subset. *J Exp Med* **1993**, *177*, 1763-1771.
15. Dellabona, P.; Padovan, E.; Casorati, G.; Brockhaus, M.; Lanzavecchia, A. An invariant V alpha 24-J alpha Q/V beta 11 T cell receptor is expressed in all individuals by clonally expanded CD4-8- T cells. *J Exp Med* **1994**, *180*, 1171-1176.
16. Lantz, O.; Bendelac, A. An invariant T cell receptor alpha chain is used by a unique subset of major histocompatibility complex class I-specific CD4+ and CD4-8- T cells in mice and humans. *J Exp Med* **1994**, *180*, 1097-1106.
17. Godfrey, D.I.; MacDonald, H.R.; Kronenberg, M.; Smyth, M.J.; Van Kaer, L. NKT cells: what's in a name? *Nat Rev Immunol* **2004**, *4*, 231-237, doi:10.1038/nri1309.

18. Terabe, M.; Berzofsky, J.A. The role of NKT cells in tumor immunity. *Adv Cancer Res* **2008**, *101*, 277-348, doi:10.1016/S0065-230X(08)00408-9.
19. Jahng, A.; Maricic, I.; Aguilera, C.; Cardell, S.; Halder, R.C.; Kumar, V. Prevention of autoimmunity by targeting a distinct, noninvariant CD1d-reactive T cell population reactive to sulfatide. *J Exp Med* **2004**, *199*, 947-957, doi:10.1084/jem.20031389.
20. Bendelac, A. Positive selection of mouse NK1+ T cells by CD1-expressing cortical thymocytes. *J Exp Med* **1995**, *182*, 2091-2096.
21. Coles, M.C.; Raulet, D.H. NK1.1+ T cells in the liver arise in the thymus and are selected by interactions with class I molecules on CD4+CD8+ cells. *J Immunol* **2000**, *164*, 2412-2418.
22. Wei, D.G.; Lee, H.; Park, S.H.; Beaudoin, L.; Teyton, L.; Lehuen, A.; Bendelac, A. Expansion and long-range differentiation of the NKT cell lineage in mice expressing CD1d exclusively on cortical thymocytes. *J Exp Med* **2005**, *202*, 239-248, doi:10.1084/jem.20050413.
23. Pellicci, D.G.; Hammond, K.J.; Uldrich, A.P.; Baxter, A.G.; Smyth, M.J.; Godfrey, D.I. A natural killer T (NKT) cell developmental pathway iInvolving a thymus-dependent NK1.1(-)CD4(+) CD1d-dependent precursor stage. *J Exp Med* **2002**, *195*, 835-844.
24. Gapin, L.; Matsuda, J.L.; Surh, C.D.; Kronenberg, M. NKT cells derive from double-positive thymocytes that are positively selected by CD1d. *Nat Immunol* **2001**, *2*, 971-978, doi:10.1038/ni710.
25. Sandberg, J.K.; Stoddart, C.A.; Brilot, F.; Jordan, K.A.; Nixon, D.F. Development of innate CD4+ alpha-chain variable gene segment 24 (Valpha24) natural killer T cells in the early human fetal thymus is regulated by IL-7. *Proc Natl Acad Sci U S A* **2004**, *101*, 7058-7063, doi:10.1073/pnas.0305986101.
26. Baev, D.V.; Peng, X.H.; Song, L.; Barnhart, J.R.; Crooks, G.M.; Weinberg, K.I.; Metelitsa, L.S. Distinct homeostatic requirements of CD4+ and CD4- subsets of Valpha24-invariant natural killer T cells in humans. *Blood* **2004**, *104*, 4150-4156, doi:10.1182/blood-2004-04-1629.
27. Eger, K.A.; Sundrud, M.S.; Motsinger, A.A.; Tseng, M.; Van Kaer, L.; Unutmaz, D. Human natural killer T cells are heterogeneous in their capacity to reprogram their effector functions. *PLoS One* **2006**, *1*, e50, doi:10.1371/journal.pone.0000050.
28. Montoya, C.J.; Pollard, D.; Martinson, J.; Kumari, K.; Wasserfall, C.; Mulder, C.B.; Rugeles, M.T.; Atkinson, M.A.; Landay, A.L.; Wilson, S.B. Characterization of human invariant natural killer T subsets in health and disease using a novel invariant natural killer T cell-clonotypic monoclonal antibody, 6B11. *Immunology* **2007**, *122*, 1-14, doi:10.1111/j.1365-2567.2007.02647.x.
29. Sandberg, J.K.; Bhardwaj, N.; Nixon, D.F. Dominant effector memory characteristics, capacity for dynamic adaptive expansion, and sex bias in the innate Valpha24 NKT cell compartment. *Eur J Immunol* **2003**, *33*, 588-596, doi:10.1002/eji.200323707.
30. Lee, P.T.; Benlagha, K.; Teyton, L.; Bendelac, A. Distinct functional lineages of human V(alpha)24 natural killer T cells. *J Exp Med* **2002**, *195*, 637-641.
31. Chan, W.K.; Rujkijyanont, P.; Neale, G.; Yang, J.; Bari, R.; Das Gupta, N.; Holladay, M.; Rooney, B.; Leung, W. Multiplex and genome-wide analyses reveal distinctive properties of KIR+ and CD56+ T cells in human blood. *J Immunol* **2013**, *191*, 1625-1636, doi:10.4049/jimmunol.1300111.
32. Bandyopadhyay, K.; Marrero, I.; Kumar, V. NKT cell subsets as key participants in liver physiology and pathology. *Cell Mol Immunol* **2016**, *13*, 337-346, doi:10.1038/cmi.2015.115.
33. Berzins, S.P.; Smyth, M.J.; Baxter, A.G. Presumed guilty: natural killer T cell defects and human disease. *Nat Rev Immunol* **2011**, *11*, 131-142, doi:10.1038/nri2904.
34. Matangkasombut, P.; Marigowda, G.; Ervine, A.; Idris, L.; Pichavant, M.; Kim, H.Y.; Yasumi, T.; Wilson, S.B.; DeKruyff, R.H.; Faul, J.L., et al. Natural killer T cells in the lungs of patients with asthma. *J Allergy Clin Immunol* **2009**, *123*, 1181-1185, doi:10.1016/j.jaci.2009.02.013.
35. Kenna, T.; Golden-Mason, L.; Porcelli, S.A.; Koezuka, Y.; Hegarty, J.E.; O'Farrelly, C.; Doherty, D.G. NKT cells from normal and tumor-bearing human livers are phenotypically and functionally distinct from murine NKT cells. *J Immunol* **2003**, *171*, 1775-1779.

36. Wingender, G.; Kronenberg, M. Role of NKT cells in the digestive system. IV. The role of canonical natural killer T cells in mucosal immunity and inflammation. *Am J Physiol Gastrointest Liver Physiol* **2008**, *294*, G1-8, doi:10.1152/ajpgi.00437.2007.
37. Galante, N.Z.; Ozaki, K.S.; Cenedeze, M.A.; Kallas, E.G.; Salomao, R.; Pacheco-Silva, A.; Camara, N.O. Frequency of Valpha24+Vbeta11+ NKT cells in peripheral blood of human kidney transplantation recipients. *Int Immunopharmacol* **2005**, *5*, 53-58, doi:10.1016/j.intimp.2004.09.013.
38. Lynch, L.; O'Shea, D.; Winter, D.C.; Geoghegan, J.; Doherty, D.G.; O'Farrelly, C. Invariant NKT cells and CD1d(+) cells amass in human omentum and are depleted in patients with cancer and obesity. *Eur J Immunol* **2009**, *39*, 1893-1901, doi:10.1002/eji.200939349.
39. Chan, A.C.; Serwecinska, L.; Cochrane, A.; Harrison, L.C.; Godfrey, D.I.; Berzins, S.P. Immune characterization of an individual with an exceptionally high natural killer T cell frequency and her immediate family. *Clin Exp Immunol* **2009**, *156*, 238-245, doi:10.1111/j.1365-2249.2009.03888.x.
40. Woo, S.Y.; Jung, Y.J.; Ryu, K.H.; Park, H.Y.; Kie, J.H.; Im, S.A.; Chung, W.S.; Han, H.S.; Seoh, J.Y. In vitro differentiation of natural killer T cells from human cord blood CD34+ cells. *Br J Haematol* **2003**, *121*, 148-156.
41. Gao, B.; Radaeva, S.; Park, O. Liver natural killer and natural killer T cells: immunobiology and emerging roles in liver diseases. *J Leukoc Biol* **2009**, *86*, 513-528, doi:10.1189/JLB.0309135.
42. Timonen, T.; Ortaldo, J.R.; Herberman, R.B. Characteristics of human large granular lymphocytes and relationship to natural killer and K cells. *J Exp Med* **1981**, *153*, 569-582.
43. Nakatani, K.; Kaneda, K.; Seki, S.; Nakajima, Y. Pit cells as liver-associated natural killer cells: morphology and function. *Med Electron Microsc* **2004**, *37*, 29-36, doi:10.1007/s00795-003-0229-9.
44. Daibata, M.; Matsuo, Y.; Machida, H.; Taguchi, T.; Ohtsuki, Y.; Taguchi, H. Differential gene-expression profiling in the leukemia cell lines derived from indolent and aggressive phases of CD56+ T-cell large granular lymphocyte leukemia. *Int J Cancer* **2004**, *108*, 845-851, doi:10.1002/ijc.11647.
45. van der Vliet, H.J.; Pinedo, H.M.; von Blomberg, B.M.; van den Eertwegh, A.J.; Scheper, R.J.; Giaccone, G. Natural Killer T cells. *Lancet Oncol* **2002**, *3*, 574.
46. Gumperz, J.E.; Miyake, S.; Yamamura, T.; Brenner, M.B. Functionally distinct subsets of CD1d-restricted natural killer T cells revealed by CD1d tetramer staining. *J Exp Med* **2002**, *195*, 625-636.
47. Hammond, K.J.; Poulton, L.D.; Palmisano, L.J.; Silveira, P.A.; Godfrey, D.I.; Baxter, A.G. alpha/beta-T cell receptor (TCR)+CD4-CD8- (NKT) thymocytes prevent insulin-dependent diabetes mellitus in nonobese diabetic (NOD)/Lt mice by the influence of interleukin (IL)-4 and/or IL-10. *J Exp Med* **1998**, *187*, 1047-1056.
48. Lehuen, A.; Lantz, O.; Beaudoin, L.; Laloux, V.; Carnaud, C.; Bendelac, A.; Bach, J.F.; Monteiro, R.C. Overexpression of natural killer T cells protects Valpha14- Jalpha281 transgenic nonobese diabetic mice against diabetes. *J Exp Med* **1998**, *188*, 1831-1839.
49. Terabe, M.; Matsui, S.; Noben-Trauth, N.; Chen, H.; Watson, C.; Donaldson, D.D.; Carbone, D.P.; Paul, W.E.; Berzofsky, J.A. NKT cell-mediated repression of tumor immunosurveillance by IL-13 and the IL-4R-STAT6 pathway. *Nat Immunol* **2000**, *1*, 515-520, doi:10.1038/82771.
50. Moreira-Teixeira, L.; Resende, M.; Coffre, M.; Devergne, O.; Herbeuval, J.P.; Hermine, O.; Schneider, E.; Rogge, L.; Ruemmele, F.M.; Dy, M., et al. Proinflammatory environment dictates the IL-17-producing capacity of human invariant NKT cells. *J Immunol* **2011**, *186*, 5758-5765, doi:10.4049/jimmunol.1003043.
51. Snyder-Cappione, J.E.; Tincati, C.; Eccles-James, I.G.; Cappione, A.J.; Ndhlovu, L.C.; Koth, L.L.; Nixon, D.F. A comprehensive ex vivo functional analysis of human NKT cells reveals production of MIP1-alpha and MIP1-beta, a lack of IL-17, and a Th1-bias in males. *PLoS One* **2010**, *5*, e15412, doi:10.1371/journal.pone.0015412.
52. Moreira-Teixeira, L.; Resende, M.; Devergne, O.; Herbeuval, J.P.; Hermine, O.; Schneider, E.; Dy, M.; Cordeiro-da-Silva, A.; Leite-de-Moraes, M.C. Rapamycin combined with TGF-beta converts human

invariant NKT cells into suppressive Foxp3+ regulatory cells. *J Immunol* **2012**, *188*, 624-631, doi:10.4049/jimmunol.1102281.

53. Chang, P.P.; Barral, P.; Fitch, J.; Pratama, A.; Ma, C.S.; Kallies, A.; Hogan, J.J.; Cerundolo, V.; Tangye, S.G.; Bittman, R., et al. Identification of Bcl-6-dependent follicular helper NKT cells that provide cognate help for B cell responses. *Nat Immunol* **2012**, *13*, 35-43, doi:10.1038/ni.2166.
54. Gapin, L. Development of invariant natural killer T cells. *Curr Opin Immunol* **2016**, *39*, 68-74, doi:10.1016/j.coi.2016.01.001.
55. Sullivan, B.A.; Nagarajan, N.A.; Wingender, G.; Wang, J.; Scott, I.; Tsuji, M.; Franck, R.W.; Porcelli, S.A.; Zajonc, D.M.; Kronenberg, M. Mechanisms for glycolipid antigen-driven cytokine polarization by Valpha14i NKT cells. *J Immunol* **2010**, *184*, 141-153, doi:10.4049/jimmunol.0902880.
56. DuPage, M.; Bluestone, J.A. Harnessing the plasticity of CD4(+) T cells to treat immune-mediated disease. *Nat Rev Immunol* **2016**, *16*, 149-163, doi:10.1038/nri.2015.18.
57. van der Vliet, H.J.; Wang, R.; Yue, S.C.; Koon, H.B.; Balk, S.P.; Exley, M.A. Circulating myeloid dendritic cells of advanced cancer patients result in reduced activation and a biased cytokine profile in invariant NKT cells. *J Immunol* **2008**, *180*, 7287-7293.
58. Heczey, A.; Liu, D.; Tian, G.; Courtney, A.N.; Wei, J.; Marinova, E.; Gao, X.; Guo, L.; Yvon, E.; Hicks, J., et al. Invariant NKT cells with chimeric antigen receptor provide a novel platform for safe and effective cancer immunotherapy. *Blood* **2014**, *124*, 2824-2833, doi:10.1182/blood-2013-11-541235.
59. Metelitsa, L.S. Flow cytometry for natural killer T cells: multi-parameter methods for multifunctional cells. *Clin Immunol* **2004**, *110*, 267-276, doi:10.1016/j.clim.2003.11.005.
60. Porcelli, S.; Gerdes, D.; Fertig, A.M.; Balk, S.P. Human T cells expressing an invariant V alpha 24-J alpha Q TCR alpha are CD4- and heterogeneous with respect to TCR beta expression. *Hum Immunol* **1996**, *48*, 63-67.
61. Exley, M.; Garcia, J.; Balk, S.P.; Porcelli, S. Requirements for CD1d recognition by human invariant Valpha24+ CD4-CD8- T cells. *J Exp Med* **1997**, *186*, 109-120.
62. Kent, S.C.; Hafler, D.A.; Strominger, J.L.; Wilson, S.B. Noncanonical Valpha24JalphaQ T cells with conservative alpha chain CDR3 region amino acid substitutions are restricted by CD1d. *Hum Immunol* **1999**, *60*, 1080-1089.
63. Lee, P.T.; Putnam, A.; Benlagha, K.; Teyton, L.; Gottlieb, P.A.; Bendelac, A. Testing the NKT cell hypothesis of human IDDM pathogenesis. *J Clin Invest* **2002**, *110*, 793-800, doi:10.1172/JCI15832.
64. Schumann, J.; Voyle, R.B.; Wei, B.Y.; MacDonald, H.R. Cutting edge: influence of the TCR V beta domain on the avidity of CD1d:alpha-galactosylceramide binding by invariant V alpha 14 NKT cells. *J Immunol* **2003**, *170*, 5815-5819.
65. Benlagha, K.; Weiss, A.; Beavis, A.; Teyton, L.; Bendelac, A. In vivo identification of glycolipid antigen-specific T cells using fluorescent CD1d tetramers. *J Exp Med* **2000**, *191*, 1895-1903.
66. Matsuda, J.L.; Naidenko, O.V.; Gapin, L.; Nakayama, T.; Taniguchi, M.; Wang, C.R.; Koezuka, Y.; Kronenberg, M. Tracking the response of natural killer T cells to a glycolipid antigen using CD1d tetramers. *J Exp Med* **2000**, *192*, 741-754.
67. Sidobre, S.; Kronenberg, M. CD1 tetramers: a powerful tool for the analysis of glycolipid-reactive T cells. *J Immunol Methods* **2002**, *268*, 107-121.
68. Sag, D.; Ozkan, M.; Kronenberg, M.; Wingender, G. Improved Detection of Cytokines Produced by Invariant NKT Cells. *Sci Rep* **2017**, *7*, 16607, doi:10.1038/s41598-017-16832-1.
69. van Der Vliet, H.J.; Nishi, N.; de Gruijl, T.D.; von Blomberg, B.M.; van den Eertwegh, A.J.; Pinedo, H.M.; Giaccone, G.; Scheper, R.J. Human natural killer T cells acquire a memory-activated phenotype before birth. *Blood* **2000**, *95*, 2440-2442.
70. Tian, G.; Courtney, A.N.; Jena, B.; Heczey, A.; Liu, D.; Marinova, E.; Guo, L.; Xu, X.; Torikai, H.; Mo, Q., et al. CD62L+ NKT cells have prolonged persistence and antitumor activity in vivo. *J Clin Invest* **2016**, *126*, 2341-2355, doi:10.1172/JCI83476.

71. Kitayama, S.; Zhang, R.; Liu, T.Y.; Ueda, N.; Iriguchi, S.; Yasui, Y.; Kawai, Y.; Tatsumi, M.; Hirai, N.; Mizoro, Y., et al. Cellular Adjuvant Properties, Direct Cytotoxicity of Re-differentiated Valpha24 Invariant NKT-like Cells from Human Induced Pluripotent Stem Cells. *Stem Cell Reports* **2016**, *6*, 213-227, doi:10.1016/j.stemcr.2016.01.005.
72. Hintzen, R.Q.; de Jong, R.; Lens, S.M.; Brouwer, M.; Baars, P.; van Lier, R.A. Regulation of CD27 expression on subsets of mature T-lymphocytes. *J Immunol* **1993**, *151*, 2426-2435.
73. Hendriks, J.; Gravestein, L.A.; Tesselaar, K.; van Lier, R.A.; Schumacher, T.N.; Borst, J. CD27 is required for generation and long-term maintenance of T cell immunity. *Nat Immunol* **2000**, *1*, 433-440, doi:10.1038/80877.
74. Kim, S.; Lalani, S.; Parekh, V.V.; Vincent, T.L.; Wu, L.; Van Kaer, L. Impact of bacteria on the phenotype, functions, and therapeutic activities of invariant NKT cells in mice. *J Clin Invest* **2008**, *118*, 2301-2315, doi:10.1172/JCI33071.
75. Hudspeth, K.; Silva-Santos, B.; Mavilio, D. Natural cytotoxicity receptors: broader expression patterns and functions in innate and adaptive immune cells. *Front Immunol* **2013**, *4*, 69, doi:10.3389/fimmu.2013.00069.
76. Harada, M.; Seino, K.; Wakao, H.; Sakata, S.; Ishizuka, Y.; Ito, T.; Kojo, S.; Nakayama, T.; Taniguchi, M. Down-regulation of the invariant Valpha14 antigen receptor in NKT cells upon activation. *Int Immunol* **2004**, *16*, 241-247.
77. Ikarashi, Y.; Iizuka, A.; Koshidaka, Y.; Heike, Y.; Takaue, Y.; Yoshida, M.; Kronenberg, M.; Wakasugi, H. Phenotypical and functional alterations during the expansion phase of invariant Valpha14 natural killer T (Valpha14i NKT) cells in mice primed with alpha-galactosylceramide. *Immunology* **2005**, *116*, 30-37, doi:10.1111/j.1365-2567.2005.02193.x.
78. Baginska, J.; Viry, E.; Paggetti, J.; Medves, S.; Berchem, G.; Moussay, E.; Janji, B. The critical role of the tumor microenvironment in shaping natural killer cell-mediated anti-tumor immunity. *Front Immunol* **2013**, *4*, 490, doi:10.3389/fimmu.2013.00490.
79. Kim, C.H.; Johnston, B.; Butcher, E.C. Trafficking machinery of NKT cells: shared and differential chemokine receptor expression among V alpha 24(+)V beta 11(+) NKT cell subsets with distinct cytokine-producing capacity. *Blood* **2002**, *100*, 11-16, doi:10.1182/blood-2001-12-0196.
80. Thomas, S.Y.; Hou, R.; Boyson, J.E.; Means, T.K.; Hess, C.; Olson, D.P.; Strominger, J.L.; Brenner, M.B.; Gumperz, J.E.; Wilson, S.B., et al. CD1d-restricted NKT cells express a chemokine receptor profile indicative of Th1-type inflammatory homing cells. *J Immunol* **2003**, *171*, 2571-2580.
81. Campbell, J.J.; Qin, S.; Unutmaz, D.; Soler, D.; Murphy, K.E.; Hodge, M.R.; Wu, L.; Butcher, E.C. Unique subpopulations of CD56+ NK and NK-T peripheral blood lymphocytes identified by chemokine receptor expression repertoire. *J Immunol* **2001**, *166*, 6477-6482.
82. Liu, D.; Song, L.; Brawley, V.S.; Robison, N.; Wei, J.; Gao, X.; Tian, G.; Margol, A.; Ahmed, N.; Asgharzadeh, S., et al. Medulloblastoma expresses CD1d and can be targeted for immunotherapy with NKT cells. *Clin Immunol* **2013**, *149*, 55-64, doi:10.1016/j.clim.2013.06.005.
83. Zhao, J.; Weng, X.; Bagchi, S.; Wang, C.R. Polyclonal type II natural killer T cells require PLZF and SAP for their development and contribute to CpG-mediated antitumor response. *Proc Natl Acad Sci U S A* **2014**, *111*, 2674-2679, doi:10.1073/pnas.1323845111.
84. Fuss, I.J.; Joshi, B.; Yang, Z.; Degheidy, H.; Fichtner-Feigl, S.; de Souza, H.; Rieder, F.; Scaldaferri, F.; Schirbel, A.; Scarpa, M., et al. IL-13Ralpha2-bearing, type II NKT cells reactive to sulfatide self-antigen populate the mucosa of ulcerative colitis. *Gut* **2014**, *63*, 1728-1736, doi:10.1136/gutjnl-2013-305671.
85. Marrero, I.; Ware, R.; Kumar, V. Type II NKT Cells in Inflammation, Autoimmunity, Microbial Immunity, and Cancer. *Front Immunol* **2015**, *6*, 316, doi:10.3389/fimmu.2015.00316.
86. Terabe, M.; Swann, J.; Ambrosino, E.; Sinha, P.; Takaku, S.; Hayakawa, Y.; Godfrey, D.I.; Ostrand-Rosenberg, S.; Smyth, M.J.; Berzofsky, J.A. A nonclassical non-Valpha14Jalpha18 CD1d-restricted (type

II) NKT cell is sufficient for down-regulation of tumor immunosurveillance. *J Exp Med* **2005**, *202*, 1627-1633, doi:10.1084/jem.20051381.

87. Renukaradhya, G.J.; Khan, M.A.; Vieira, M.; Du, W.; Gervay-Hague, J.; Brutkiewicz, R.R. Type I NKT cells protect (and type II NKT cells suppress) the host's innate antitumor immune response to a B-cell lymphoma. *Blood* **2008**, *111*, 5637-5645, doi:10.1182/blood-2007-05-092866.
88. Terabe, M.; Berzofsky, J.A. The immunoregulatory role of type I and type II NKT cells in cancer and other diseases. *Cancer Immunol Immunother* **2014**, *63*, 199-213, doi:10.1007/s00262-013-1509-4.
89. Arrenberg, P.; Halder, R.; Dai, Y.; Maricic, I.; Kumar, V. Oligoclonality and innate-like features in the TCR repertoire of type II NKT cells reactive to a beta-linked self-glycolipid. *Proc Natl Acad Sci U S A* **2010**, *107*, 10984-10989, doi:10.1073/pnas.1000576107.
90. Parish, S.; Izhak, L.; Xia, Z.; Suzuki, M.; Terabe, M.; Berzofsky, J. Identification of sulfatide reactive type II NKT cells using CD1d dimers. *Journal of Immunology* **2013**, *190*.
91. Peng, L.S.; Mao, F.Y.; Zhao, Y.L.; Wang, T.T.; Chen, N.; Zhang, J.Y.; Cheng, P.; Li, W.H.; Lv, Y.P.; Teng, Y.S., et al. Altered phenotypic and functional characteristics of CD3+CD56+ NKT-like cells in human gastric cancer. *Oncotarget* **2016**, *7*, 55222-55230, doi:10.18632/oncotarget.10484.
92. Li, X.F.; Dai, D.; Song, X.Y.; Liu, J.J.; Zhu, L.; Zhu, X.; Ma, W.; Xu, W. A different representation of natural T cells and natural killer cells between tumor-infiltrating and periphery lymphocytes in human hepatocellular carcinoma. *Oncol Lett* **2017**, *13*, 3291-3298, doi:10.3892/ol.2017.5808.
93. Bondzio, I.; Arendt, A.; Schmitz, J.; Huppert, V. KIR positive subsets of human CD56+CD3+NKT cells are different in frequency from equivalant KIR positive CD56+CD3-NK cell subsets. *Blood* **2007**, *110*, 37b-37b.
94. van den Heuvel, M.J.; Peralta, C.G.; Hatta, K.; Han, V.K.; Clark, D.A. Decline in number of elevated blood CD3(+) CD56(+) NKT cells in response to intravenous immunoglobulin treatment correlates with successful pregnancy. *Am J Reprod Immunol* **2007**, *58*, 447-459, doi:10.1111/j.1600-0897.2007.00529.x.
95. Jiang, Y.; Cui, X.; Cui, C.; Zhang, J.; Zhou, F.; Zhang, Z.; Fu, Y.; Xu, J.; Chu, Z.; Liu, J., et al. The function of CD3+CD56+ NKT-like cells in HIV-infected individuals. *Biomed Res Int* **2014**, *2014*, 863625, doi:10.1155/2014/863625.
96. Bai, Y.; Zhang, Y.; Yang, Q.; Hou, Y.; Hu, N.; Wang, D.; Sun, H. The aberrant expression of stimulatory and inhibitory killer immunoglobulin-like receptors in NK- and NKT-cells contributes to lupus. *Clinical Laboratory Journal For Clinical Laboratories And Laboratories Related* **2014**, *60*, 717-727.
97. Mingari, M.C.; Schiavetti, F.; Ponte, M.; Vitale, C.; Maggi, E.; Romagnani, S.; Demarest, J.; Pantaleo, G.; Fauci, A.S.; Moretta, L. Human CD8+ T lymphocyte subsets that express HLA class I-specific inhibitory receptors represent oligoclonally or monoclonally expanded cell populations. *Proc Natl Acad Sci U S A* **1996**, *93*, 12433-12438.
98. Bendelac, A.; Lantz, O.; Quimby, M.E.; Yewdell, J.W.; Bennink, J.R.; Brutkiewicz, R.R. CD1 recognition by mouse NK1+ T lymphocytes. *Science* **1995**, *268*, 863-865.
99. Blumberg, R.S.; Terhorst, C.; Bleicher, P.; McDermott, F.V.; Allan, C.H.; Landau, S.B.; Trier, J.S.; Balk, S.P. Expression of a nonpolymorphic MHC class I-like molecule, CD1D, by human intestinal epithelial cells. *J Immunol* **1991**, *147*, 2518-2524.
100. Canchis, P.W.; Bhan, A.K.; Landau, S.B.; Yang, L.; Balk, S.P.; Blumberg, R.S. Tissue distribution of the non-polymorphic major histocompatibility complex class I-like molecule, CD1d. *Immunology* **1993**, *80*, 561-565.
101. Bendelac, A.; Savage, P.B.; Teyton, L. The biology of NKT cells. *Annu Rev Immunol* **2007**, *25*, 297-336, doi:10.1146/annurev.immunol.25.022106.141711.
102. Barral, D.C.; Brenner, M.B. CD1 antigen presentation: how it works. *Nat Rev Immunol* **2007**, *7*, 929-941, doi:10.1038/nri2191.

103. Jayawardena-Wolf, J.; Benlagha, K.; Chiu, Y.H.; Mehr, R.; Bendelac, A. CD1d endosomal trafficking is independently regulated by an intrinsic CD1d-encoded tyrosine motif and by the invariant chain. *Immunity* **2001**, *15*, 897-908.

104. Salio, M.; Ghadbane, H.; Dushek, O.; Shepherd, D.; Cypen, J.; Gileadi, U.; Aichinger, M.C.; Napolitani, G.; Qi, X.; van der Merwe, P.A., et al. Saposins modulate human invariant Natural Killer T cells self-reactivity and facilitate lipid exchange with CD1d molecules during antigen presentation. *Proc Natl Acad Sci U S A* **2013**, *110*, E4753-4761, doi:10.1073/pnas.1310050110.

105. Selvanantham, T.; Escalante, N.K.; Cruz Tleugabulova, M.; Fieve, S.; Girardin, S.E.; Philpott, D.J.; Mallevaey, T. Nod1 and Nod2 enhance TLR-mediated invariant NKT cell activation during bacterial infection. *J Immunol* **2013**, *191*, 5646-5654, doi:10.4049/jimmunol.1301412.

106. De Santo, C.; Arscott, R.; Booth, S.; Karydis, I.; Jones, M.; Asher, R.; Salio, M.; Middleton, M.; Cerundolo, V. Invariant NKT cells modulate the suppressive activity of IL-10-secreting neutrophils differentiated with serum amyloid A. *Nat Immunol* **2010**, *11*, 1039-1046, doi:10.1038/ni.1942.

107. Brigl, M.; Bry, L.; Kent, S.C.; Gumperz, J.E.; Brenner, M.B. Mechanism of CD1d-restricted natural killer T cell activation during microbial infection. *Nat Immunol* **2003**, *4*, 1230-1237, doi:10.1038/ni1002.

108. Kinjo, Y.; Tupin, E.; Wu, D.; Fujio, M.; Garcia-Navarro, R.; Benhnia, M.R.; Zajonc, D.M.; Ben-Menachem, G.; Ainge, G.D.; Painter, G.F., et al. Natural killer T cells recognize diacylglycerol antigens from pathogenic bacteria. *Nat Immunol* **2006**, *7*, 978-986, doi:10.1038/ni1380.

109. Fischer, K.; Scotet, E.; Niemeyer, M.; Koebernick, H.; Zerrahn, J.; Maillet, S.; Hurwitz, R.; Kursar, M.; Bonneville, M.; Kaufmann, S.H., et al. Mycobacterial phosphatidylinositol mannoside is a natural antigen for CD1d-restricted T cells. *Proc Natl Acad Sci U S A* **2004**, *101*, 10685-10690, doi:10.1073/pnas.0403787101.

110. Kinjo, Y.; Wu, D.; Kim, G.; Xing, G.W.; Poles, M.A.; Ho, D.D.; Tsuji, M.; Kawahara, K.; Wong, C.H.; Kronenberg, M. Recognition of bacterial glycosphingolipids by natural killer T cells. *Nature* **2005**, *434*, 520-525, doi:10.1038/nature03407.

111. Mattner, J.; Debord, K.L.; Ismail, N.; Goff, R.D.; Cantu, C., 3rd; Zhou, D.; Saint-Mezard, P.; Wang, V.; Gao, Y.; Yin, N., et al. Exogenous and endogenous glycolipid antigens activate NKT cells during microbial infections. *Nature* **2005**, *434*, 525-529, doi:10.1038/nature03408.

112. Sriram, V.; Du, W.; Gervay-Hague, J.; Brutkiewicz, R.R. Cell wall glycosphingolipids of Sphingomonas paucimobilis are CD1d-specific ligands for NKT cells. *Eur J Immunol* **2005**, *35*, 1692-1701, doi:10.1002/eji.200526157.

113. Brennan, P.J.; Tatituri, R.V.; Brigl, M.; Kim, E.Y.; Tuli, A.; Sanderson, J.P.; Gadola, S.D.; Hsu, F.F.; Besra, G.S.; Brenner, M.B. Invariant natural killer T cells recognize lipid self antigen induced by microbial danger signals. *Nat Immunol* **2011**, *12*, 1202-1211, doi:10.1038/ni.2143.

114. Chang, D.H.; Deng, H.; Matthews, P.; Krasovsky, J.; Ragupathi, G.; Spisek, R.; Mazumder, A.; Vesole, D.H.; Jagannath, S.; Dhodapkar, M.V. Inflammation-associated lysophospholipids as ligands for CD1d-restricted T cells in human cancer. *Blood* **2008**, *112*, 1308-1316, doi:10.1182/blood-2008-04-149831.

115. Tatituri, R.V.; Watts, G.F.; Bhowruth, V.; Barton, N.; Rothchild, A.; Hsu, F.F.; Almeida, C.F.; Cox, L.R.; Eggeling, L.; Cardell, S., et al. Recognition of microbial and mammalian phospholipid antigens by NKT cells with diverse TCRs. *Proc Natl Acad Sci U S A* **2013**, *110*, 1827-1832, doi:10.1073/pnas.1220601110.

116. Wingender, G.; Krebs, P.; Beutler, B.; Kronenberg, M. Antigen-specific cytotoxicity by invariant NKT cells in vivo is CD95/CD178-dependent and is correlated with antigenic potency. *J Immunol* **2010**, *185*, 2721-2729, doi:10.4049/jimmunol.1001018.

117. Crowe, N.Y.; Uldrich, A.P.; Kyparissoudis, K.; Hammond, K.J.; Hayakawa, Y.; Sidobre, S.; Keating, R.; Kronenberg, M.; Smyth, M.J.; Godfrey, D.I. Glycolipid antigen drives rapid expansion and sustained cytokine production by NK T cells. *J Immunol* **2003**, *171*, 4020-4027.

118. Nagaleekar, V.K.; Sabio, G.; Aktan, I.; Chant, A.; Howe, I.W.; Thornton, T.M.; Benoit, P.J.; Davis, R.J.; Rincon, M.; Boyson, J.E. Translational control of NKT cell cytokine production by p38 MAPK. *J Immunol* **2011**, *186*, 4140-4146, doi:10.4049/jimmunol.1002614.
119. Coquet, J.M.; Chakravarti, S.; Kyparissoudis, K.; McNab, F.W.; Pitt, L.A.; McKenzie, B.S.; Berzins, S.P.; Smyth, M.J.; Godfrey, D.I. Diverse cytokine production by NKT cell subsets and identification of an IL-17-producing CD4-NK1.1- NKT cell population. *Proc Natl Acad Sci U S A* **2008**, *105*, 11287-11292, doi:10.1073/pnas.0801631105.
120. Nakagawa, R.; Nagafune, I.; Tazunoki, Y.; Ehara, H.; Tomura, H.; Iijima, R.; Motoki, K.; Kamishohara, M.; Seki, S. Mechanisms of the antimetastatic effect in the liver and of the hepatocyte injury induced by alpha-galactosylceramide in mice. *J Immunol* **2001**, *166*, 6578-6584.
121. Bernin, H.; Fehling, H.; Marggraff, C.; Tannich, E.; Lotter, H. The cytokine profile of human NKT cells and PBMCs is dependent on donor sex and stimulus. *Med Microbiol Immunol* **2016**, *205*, 321-332, doi:10.1007/s00430-016-0449-y.
122. Long, E.O.; Kim, H.S.; Liu, D.; Peterson, M.E.; Rajagopalan, S. Controlling natural killer cell responses: integration of signals for activation and inhibition. *Annu Rev Immunol* **2013**, *31*, 227-258, doi:10.1146/annurev-immunol-020711-075005.
123. Pegram, H.J.; Andrews, D.M.; Smyth, M.J.; Darcy, P.K.; Kershaw, M.H. Activating and inhibitory receptors of natural killer cells. *Immunol Cell Biol* **2011**, *89*, 216-224, doi:10.1038/icb.2010.78.
124. Gonzalez, S.; Lopez-Soto, A.; Suarez-Alvarez, B.; Lopez-Vazquez, A.; Lopez-Larrea, C. NKG2D ligands: key targets of the immune response. *Trends Immunol* **2008**, *29*, 397-403, doi:10.1016/j.it.2008.04.007.
125. Bottino, C.; Castriconi, R.; Pende, D.; Rivera, P.; Nanni, M.; Carnemolla, B.; Cantoni, C.; Grassi, J.; Marcenaro, S.; Reymond, N., et al. Identification of PVR (CD155) and Nectin-2 (CD112) as cell surface ligands for the human DNAM-1 (CD226) activating molecule. *J Exp Med* **2003**, *198*, 557-567, doi:10.1084/jem.20030788.
126. Moretta, A.; Pende, D.; Locatelli, F.; Moretta, L. Activating and inhibitory killer immunoglobulin-like receptors (KIR) in haploidentical haemopoietic stem cell transplantation to cure high-risk leukaemias. *Clin Exp Immunol* **2009**, *157*, 325-331, doi:10.1111/j.1365-2249.2009.03983.x.
127. Mandelboim, O.; Davis, D.M.; Reyburn, H.T.; Vales-Gomez, M.; Sheu, E.G.; Pazmany, L.; Strominger, J.L. Enhancement of class II-restricted T cell responses by costimulatory NK receptors for class I MHC proteins. *Science* **1996**, *274*, 2097-2100.
128. Mandelboim, O.; Kent, S.; Davis, D.M.; Wilson, S.B.; Okazaki, T.; Jackson, R.; Hafler, D.; Strominger, J.L. Natural killer activating receptors trigger interferon gamma secretion from T cells and natural killer cells. *Proc Natl Acad Sci U S A* **1998**, *95*, 3798-3803.
129. Martin, M.P.; Gao, X.; Lee, J.H.; Nelson, G.W.; Detels, R.; Goedert, J.J.; Buchbinder, S.; Hoots, K.; Vlahov, D.; Trowsdale, J., et al. Epistatic interaction between KIR3DS1 and HLA-B delays the progression to AIDS. *Nat Genet* **2002**, *31*, 429-434, doi:10.1038/ng934.
130. Vilches, C.; Parham, P. KIR: diverse, rapidly evolving receptors of innate and adaptive immunity. *Annu Rev Immunol* **2002**, *20*, 217-251, doi:10.1146/annurev.immunol.20.092501.134942.
131. Guerra, N.; Michel, F.; Gati, A.; Gaudin, C.; Mishal, Z.; Escudier, B.; Acuto, O.; Chouaib, S.; Caignard, A. Engagement of the inhibitory receptor CD158a interrupts TCR signaling, preventing dynamic membrane reorganization in CTL/tumor cell interaction. *Blood* **2002**, *100*, 2874-2881, doi:10.1182/blood-2002-02-0643.
132. De Maria, A.; Ferraris, A.; Guastella, M.; Pilia, S.; Cantoni, C.; Polero, L.; Mingari, M.C.; Bassetti, D.; Fauci, A.S.; Moretta, L. Expression of HLA class I-specific inhibitory natural killer cell receptors in HIV-specific cytolytic T lymphocytes: impairment of specific cytolytic functions. *Proc Natl Acad Sci U S A* **1997**, *94*, 10285-10288.
133. D'Andrea, A.; Chang, C.; Phillips, J.H.; Lanier, L.L. Regulation of T cell lymphokine production by killer cell inhibitory receptor recognition of self HLA class I alleles. *J Exp Med* **1996**, *184*, 789-794.

134. Phillips, J.H.; Gumperz, J.E.; Parham, P.; Lanier, L.L. Superantigen-dependent, cell-mediated cytotoxicity inhibited by MHC class I receptors on T lymphocytes. *Science* **1995**, *268*, 403-405.
135. Kawamura, T.; Takeda, K.; Kaneda, H.; Matsumoto, H.; Hayakawa, Y.; Raulet, D.H.; Ikarashi, Y.; Kronenberg, M.; Yagita, H.; Kinoshita, K., et al. NKG2A inhibits invariant NKT cell activation in hepatic injury. *J Immunol* **2009**, *182*, 250-258.
136. Dutertre, C.A.; Bonnin-Gelize, E.; Pulford, K.; Bourel, D.; Fridman, W.H.; Teillaud, J.L. A novel subset of NK cells expressing high levels of inhibitory FcgammaRIIB modulating antibody-dependent function. *J Leukoc Biol* **2008**, *84*, 1511-1520, doi:10.1189/jlb.0608343.
137. Nagarajan, N.A.; Kronenberg, M. Invariant NKT cells amplify the innate immune response to lipopolysaccharide. *J Immunol* **2007**, *178*, 2706-2713.
138. Leite-De-Moraes, M.C.; Hameg, A.; Arnould, A.; Machavoine, F.; Koezuka, Y.; Schneider, E.; Herbelin, A.; Dy, M. A distinct IL-18-induced pathway to fully activate NK T lymphocytes independently from TCR engagement. *J Immunol* **1999**, *163*, 5871-5876.
139. Fehniger, T.A.; Shah, M.H.; Turner, M.J.; VanDeusen, J.B.; Whitman, S.P.; Cooper, M.A.; Suzuki, K.; Wechser, M.; Goodsaid, F.; Caligiuri, M.A. Differential cytokine and chemokine gene expression by human NK cells following activation with IL-18 or IL-15 in combination with IL-12: implications for the innate immune response. *J Immunol* **1999**, *162*, 4511-4520.
140. Fujii, S.; Shimizu, K.; Smith, C.; Bonifaz, L.; Steinman, R.M. Activation of natural killer T cells by alpha-galactosylceramide rapidly induces the full maturation of dendritic cells in vivo and thereby acts as an adjuvant for combined CD4 and CD8 T cell immunity to a coadministered protein. *J Exp Med* **2003**, *198*, 267-279, doi:10.1084/jem.20030324.
141. Fujii, S.; Liu, K.; Smith, C.; Bonito, A.J.; Steinman, R.M. The linkage of innate to adaptive immunity via maturing dendritic cells in vivo requires CD40 ligation in addition to antigen presentation and CD80/86 costimulation. *J Exp Med* **2004**, *199*, 1607-1618, doi:10.1084/jem.20040317.
142. Okamura, H.; Tsutsi, H.; Komatsu, T.; Yutsudo, M.; Hakura, A.; Tanimoto, T.; Torigoe, K.; Okura, T.; Nukada, Y.; Hattori, K., et al. Cloning of a new cytokine that induces IFN-gamma production by T cells. *Nature* **1995**, *378*, 88-91, doi:10.1038/378088a0.
143. Bonish, B.; Jullien, D.; Dutronc, Y.; Huang, B.B.; Modlin, R.; Spada, F.M.; Porcelli, S.A.; Nickoloff, B.J. Overexpression of CD1d by keratinocytes in psoriasis and CD1d-dependent IFN-gamma production by NK-T cells. *J Immunol* **2000**, *165*, 4076-4085.
144. Singh, A.K.; Gaur, P.; Das, S.N. Natural killer T cell anergy, co-stimulatory molecules and immunotherapeutic interventions. *Hum Immunol* **2014**, *75*, 250-260, doi:10.1016/j.humimm.2013.12.004.
145. Parekh, V.V.; Wilson, M.T.; Olivares-Villagomez, D.; Singh, A.K.; Wu, L.; Wang, C.R.; Joyce, S.; Van Kaer, L. Glycolipid antigen induces long-term natural killer T cell anergy in mice. *J Clin Invest* **2005**, *115*, 2572-2583, doi:10.1172/JCI24762.
146. Sullivan, B.A.; Kronenberg, M. Activation or anergy: NKT cells are stunned by alpha-galactosylceramide. *J Clin Invest* **2005**, *115*, 2328-2329, doi:10.1172/JCI26297.
147. Iyoda, T.; Ushida, M.; Kimura, Y.; Minamino, K.; Hayuka, A.; Yokohata, S.; Ehara, H.; Inaba, K. Invariant NKT cell anergy is induced by a strong TCR-mediated signal plus co-stimulation. *Int Immunol* **2010**, *22*, 905-913, doi:10.1093/intimm/dxq444.
148. Sidobre, S.; Hammond, K.J.; Benazet-Sidobre, L.; Maltsev, S.D.; Richardson, S.K.; Ndonye, R.M.; Howell, A.R.; Sakai, T.; Besra, G.S.; Porcelli, S.A., et al. The T cell antigen receptor expressed by Valpha14i NKT cells has a unique mode of glycosphingolipid antigen recognition. *Proc Natl Acad Sci U S A* **2004**, *101*, 12254-12259, doi:10.1073/pnas.0404632101.
149. Kuklina, E.M. Molecular mechanisms of T-cell anergy. *Biochemistry (Mosc)* **2013**, *78*, 144-156, doi:10.1134/S000629791302003X.

150. Crespo, J.; Sun, H.; Welling, T.H.; Tian, Z.; Zou, W. T cell anergy, exhaustion, senescence, and stemness in the tumor microenvironment. *Curr Opin Immunol* **2013**, *25*, 214-221, doi:10.1016/j.coi.2012.12.003.
151. Parekh, V.V.; Lalani, S.; Van Kaer, L. The in vivo response of invariant natural killer T cells to glycolipid antigens. *Int Rev Immunol* **2007**, *26*, 31-48, doi:10.1080/08830180601070179.
152. Leite-de-Moraes, M.C.; Herbelin, A.; Gouarin, C.; Koezuka, Y.; Schneider, E.; Dy, M. Fas/Fas ligand interactions promote activation-induced cell death of NK T lymphocytes. *J Immunol* **2000**, *165*, 4367-4371.
153. Yang, J.Q.; Saxena, V.; Xu, H.; Van Kaer, L.; Wang, C.R.; Singh, R.R. Repeated alpha-galactosylceramide administration results in expansion of NK T cells and alleviates inflammatory dermatitis in MRL-lpr/lpr mice. *J Immunol* **2003**, *171*, 4439-4446.
154. Sag, D.; Krause, P.; Hedrick, C.C.; Kronenberg, M.; Wingender, G. IL-10-producing NKT10 cells are a distinct regulatory invariant NKT cell subset. *J Clin Invest* **2014**, *124*, 3725-3740, doi:10.1172/JCI72308.
155. Maldonado, R.A.; von Andrian, U.H. How tolerogenic dendritic cells induce regulatory T cells. *Adv Immunol* **2010**, *108*, 111-165, doi:10.1016/B978-0-12-380995-7.00004-5.
156. Mantovani, A.; Sozzani, S.; Locati, M.; Allavena, P.; Sica, A. Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. *Trends Immunol* **2002**, *23*, 549-555.
157. Burdin, N.; Brossay, L.; Kronenberg, M. Immunization with alpha-galactosylceramide polarizes CD1-reactive NK T cells towards Th2 cytokine synthesis. *Eur J Immunol* **1999**, *29*, 2014-2025.
158. Gottschalk, C.; Mettke, E.; Kurts, C. The Role of Invariant Natural Killer T Cells in Dendritic Cell Licensing, Cross-Priming, and Memory CD8(+) T Cell Generation. *Front Immunol* **2015**, *6*, 379, doi:10.3389/fimmu.2015.00379.
159. Terabe, M.; Matsui, S.; Park, J.M.; Mamura, M.; Noben-Trauth, N.; Donaldson, D.D.; Chen, W.; Wahl, S.M.; Ledbetter, S.; Pratt, B., et al. Transforming growth factor-beta production and myeloid cells are an effector mechanism through which CD1d-restricted T cells block cytotoxic T lymphocyte-mediated tumor immunosurveillance: abrogation prevents tumor recurrence. *J Exp Med* **2003**, *198*, 1741-1752, doi:10.1084/jem.20022227.
160. Park, J.M.; Terabe, M.; van den Broeke, L.T.; Donaldson, D.D.; Berzofsky, J.A. Unmasking immunosurveillance against a syngeneic colon cancer by elimination of CD4+ NKT regulatory cells and IL-13. *Int J Cancer* **2005**, *114*, 80-87, doi:10.1002/ijc.20669.
161. O'Garra, A. Cytokines induce the development of functionally heterogeneous T helper cell subsets. *Immunity* **1998**, *8*, 275-283.
162. Levings, M.K.; Sangregorio, R.; Galbiati, F.; Squadrone, S.; de Waal Malefyt, R.; Roncarolo, M.G. IFN-alpha and IL-10 induce the differentiation of human type 1 T regulatory cells. *J Immunol* **2001**, *166*, 5530-5539.
163. Galli, G.; Nuti, S.; Tavarini, S.; Galli-Stampino, L.; De Lalla, C.; Casorati, G.; Dellabona, P.; Abrignani, S. CD1d-restricted help to B cells by human invariant natural killer T lymphocytes. *J Exp Med* **2003**, *197*, 1051-1057, doi:10.1084/jem.20021616.
164. Tonti, E.; Fedeli, M.; Napolitano, A.; Iannacone, M.; von Andrian, U.H.; Guidotti, L.G.; Abrignani, S.; Casorati, G.; Dellabona, P. Follicular helper NKT cells induce limited B cell responses and germinal center formation in the absence of CD4(+) T cell help. *J Immunol* **2012**, *188*, 3217-3222, doi:10.4049/jimmunol.1103501.
165. Barral, P.; Eckl-Dorna, J.; Harwood, N.E.; De Santo, C.; Salio, M.; Illarionov, P.; Besra, G.S.; Cerundolo, V.; Batista, F.D. B cell receptor-mediated uptake of CD1d-restricted antigen augments antibody responses by recruiting invariant NKT cell help in vivo. *Proc Natl Acad Sci U S A* **2008**, *105*, 8345-8350, doi:10.1073/pnas.0802968105.

166. Leadbetter, E.A.; Brigl, M.; Illarionov, P.; Cohen, N.; Luteran, M.C.; Pillai, S.; Besra, G.S.; Brenner, M.B. NK T cells provide lipid antigen-specific cognate help for B cells. *Proc Natl Acad Sci U S A* **2008**, *105*, 8339-8344, doi:10.1073/pnas.0801375105.

167. Chang, Y.J.; Huang, J.R.; Tsai, Y.C.; Hung, J.T.; Wu, D.; Fujio, M.; Wong, C.H.; Yu, A.L. Potent immune-modulating and anticancer effects of NKT cell stimulatory glycolipids. *Proc Natl Acad Sci U S A* **2007**, *104*, 10299-10304, doi:10.1073/pnas.0703824104.

168. Brigl, M.; Brenner, M.B. CD1: antigen presentation and T cell function. *Annu Rev Immunol* **2004**, *22*, 817-890, doi:10.1146/annurev.immunol.22.012703.104608.

169. Nieuwenhuis, E.E.; Matsumoto, T.; Exley, M.; Schleipman, R.A.; Glickman, J.; Bailey, D.T.; Corazza, N.; Colgan, S.P.; Onderdonk, A.B.; Blumberg, R.S. CD1d-dependent macrophage-mediated clearance of Pseudomonas aeruginosa from lung. *Nat Med* **2002**, *8*, 588-593, doi:10.1038/nm0602-588.

170. Sada-Ovalle, I.; Chiba, A.; Gonzales, A.; Brenner, M.B.; Behar, S.M. Innate invariant NKT cells recognize Mycobacterium tuberculosis-infected macrophages, produce interferon-gamma, and kill intracellular bacteria. *PLoS Pathog* **2008**, *4*, e1000239, doi:10.1371/journal.ppat.1000239.

171. Hegde, S.; Chen, X.; Keaton, J.M.; Reddington, F.; Besra, G.S.; Gumperz, J.E. NKT cells direct monocytes into a DC differentiation pathway. *J Leukoc Biol* **2007**, *81*, 1224-1235, doi:10.1189/jlb.1206718.

172. Xu, H.; Kramer, M.; Spengler, H.P.; Peters, J.H. Dendritic cells differentiated from human monocytes through a combination of IL-4, GM-CSF and IFN-gamma exhibit phenotype and function of blood dendritic cells. *Adv Exp Med Biol* **1995**, *378*, 75-78.

173. Kobayashi, E.; Motoki, K.; Uchida, T.; Fukushima, H.; Koezuka, Y. KRN7000, a novel immunomodulator, and its antitumor activities. *Oncol Res* **1995**, *7*, 529-534.

174. Morita, M.; Motoki, K.; Akimoto, K.; Natori, T.; Sakai, T.; Sawa, E.; Yamaji, K.; Koezuka, Y.; Kobayashi, E.; Fukushima, H. Structure-activity relationship of alpha-galactosylceramides against B16-bearing mice. *J Med Chem* **1995**, *38*, 2176-2187.

175. Motoki, K.; Morita, M.; Kobayashi, E.; Uchida, T.; Akimoto, K.; Fukushima, H.; Koezuka, Y. Immunostimulatory and antitumor activities of monoglycosylceramides having various sugar moieties. *Biol Pharm Bull* **1995**, *18*, 1487-1491.

176. Kawano, T.; Cui, J.; Koezuka, Y.; Toura, I.; Kaneko, Y.; Motoki, K.; Ueno, H.; Nakagawa, R.; Sato, H.; Kondo, E., et al. CD1d-restricted and TCR-mediated activation of valpha14 NKT cells by glycosylceramides. *Science* **1997**, *278*, 1626-1629.

177. Nakui, M.; Ohta, A.; Sekimoto, M.; Sato, M.; Iwakabe, K.; Yahata, T.; Kitamura, H.; Koda, T.; Kawano, T.; Makuuchi, H., et al. Potentiation of antitumor effect of NKT cell ligand, alpha-galactosylceramide by combination with IL-12 on lung metastasis of malignant melanoma cells. *Clin Exp Metastasis* **2000**, *18*, 147-153.

178. Smyth, M.J.; Crowe, N.Y.; Pellicci, D.G.; Kyparissoudis, K.; Kelly, J.M.; Takeda, K.; Yagita, H.; Godfrey, D.I. Sequential production of interferon-gamma by NK1.1(+) T cells and natural killer cells is essential for the antimetastatic effect of alpha-galactosylceramide. *Blood* **2002**, *99*, 1259-1266.

179. Toura, I.; Kawano, T.; Akutsu, Y.; Nakayama, T.; Ochiai, T.; Taniguchi, M. Cutting edge: inhibition of experimental tumor metastasis by dendritic cells pulsed with alpha-galactosylceramide. *J Immunol* **1999**, *163*, 2387-2391.

180. Hayakawa, Y.; Rovero, S.; Forni, G.; Smyth, M.J. Alpha-galactosylceramide (KRN7000) suppression of chemical- and oncogene-dependent carcinogenesis. *Proc Natl Acad Sci U S A* **2003**, *100*, 9464-9469, doi:10.1073/pnas.1630663100.

181. Smyth, M.J.; Thia, K.Y.; Street, S.E.; Cretney, E.; Trapani, J.A.; Taniguchi, M.; Kawano, T.; Pelikan, S.B.; Crowe, N.Y.; Godfrey, D.I. Differential tumor surveillance by natural killer (NK) and NKT cells. *J Exp Med* **2000**, *191*, 661-668.

182. Nishikawa, H.; Kato, T.; Tawara, I.; Takemitsu, T.; Saito, K.; Wang, L.; Ikarashi, Y.; Wakasugi, H.; Nakayama, T.; Taniguchi, M., et al. Accelerated chemically induced tumor development mediated by

CD4+CD25+ regulatory T cells in wild-type hosts. *Proc Natl Acad Sci U S A* **2005**, *102*, 9253-9257, doi:10.1073/pnas.0503852102.

183. Swann, J.B.; Uldrich, A.P.; van Dommelen, S.; Sharkey, J.; Murray, W.K.; Godfrey, D.I.; Smyth, M.J. Type I natural killer T cells suppress tumors caused by p53 loss in mice. *Blood* **2009**, *113*, 6382-6385, doi:10.1182/blood-2009-01-198564.
184. Gebremeskel, S.; Clattenburg, D.R.; Slauenwhite, D.; Lobert, L.; Johnston, B. Natural killer T cell activation overcomes immunosuppression to enhance clearance of postsurgical breast cancer metastasis in mice. *Oncoimmunology* **2015**, *4*, e995562, doi:10.1080/2162402X.2014.995562.
185. Gebremeskel, S.; Lobert, L.; Tanner, K.; Walker, B.; Oliphant, T.; Clarke, L.E.; Dellaire, G.; Johnston, B. Natural Killer T-cell Immunotherapy in Combination with Chemotherapy-Induced Immunogenic Cell Death Targets Metastatic Breast Cancer. *Cancer Immunol Res* **2017**, *5*, 1086-1097, doi:10.1158/2326-6066.CIR-17-0229.
186. Smyth, M.J.; Cretney, E.; Takeda, K.; Wiltrout, R.H.; Sedger, L.M.; Kayagaki, N.; Yagita, H.; Okumura, K. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) contributes to interferon gamma-dependent natural killer cell protection from tumor metastasis. *J Exp Med* **2001**, *193*, 661-670.
187. Hayakawa, Y.; Takeda, K.; Yagita, H.; Kakuta, S.; Iwakura, Y.; Van Kaer, L.; Saiki, I.; Okumura, K. Critical contribution of IFN-gamma and NK cells, but not perforin-mediated cytotoxicity, to anti-metastatic effect of alpha-galactosylceramide. *Eur J Immunol* **2001**, *31*, 1720-1727.
188. O'Konek, J.J.; Illarionov, P.; Khursigara, D.S.; Ambrosino, E.; Izhak, L.; Castillo, B.F., 2nd; Raju, R.; Khalili, M.; Kim, H.Y.; Howell, A.R., et al. Mouse and human iNKT cell agonist beta-mannosylceramide reveals a distinct mechanism of tumor immunity. *J Clin Invest* **2011**, *121*, 683-694, doi:10.1172/JCI42314.
189. Schmieg, J.; Yang, G.; Franck, R.W.; Tsuji, M. Superior protection against malaria and melanoma metastases by a C-glycoside analogue of the natural killer T cell ligand alpha-Galactosylceramide. *J Exp Med* **2003**, *198*, 1631-1641, doi:10.1084/jem.20031192.
190. Aspeslagh, S.; Li, Y.; Yu, E.D.; Pauwels, N.; Trappeniers, M.; Girardi, E.; Decruy, T.; Van Beneden, K.; Venken, K.; Drennan, M., et al. Galactose-modified iNKT cell agonists stabilized by an induced fit of CD1d prevent tumour metastasis. *EMBO J* **2011**, *30*, 2294-2305, doi:10.1038/emboj.2011.145.
191. Dashtsoodol, N.; Shigeura, T.; Tashiro, T.; Aihara, M.; Chikanishi, T.; Okada, H.; Hanada, K.; Sano, H.; Kurogi, A.; Taniguchi, M. Natural Killer T Cell-Targeted Immunotherapy Mediating Long-term Memory Responses and Strong Antitumor Activity. *Front Immunol* **2017**, *8*, 1206, doi:10.3389/fimmu.2017.01206.
192. Metelitsa, L.S.; Weinberg, K.I.; Emanuel, P.D.; Seeger, R.C. Expression of CD1d by myelomonocytic leukemias provides a target for cytotoxic NKT cells. *Leukemia* **2003**, *17*, 1068-1077, doi:10.1038/sj.leu.2402943.
193. Takahashi, T.; Haraguchi, K.; Chiba, S.; Yasukawa, M.; Shibata, Y.; Hirai, H. Valpha24+ natural killer T-cell responses against T-acute lymphoblastic leukaemia cells: implications for immunotherapy. *Br J Haematol* **2003**, *122*, 231-239.
194. Dhodapkar, M.V.; Geller, M.D.; Chang, D.H.; Shimizu, K.; Fujii, S.; Dhodapkar, K.M.; Krasovsky, J. A reversible defect in natural killer T cell function characterizes the progression of premalignant to malignant multiple myeloma. *J Exp Med* **2003**, *197*, 1667-1676, doi:10.1084/jem.20021650.
195. Chong, T.W.; Goh, F.Y.; Sim, M.Y.; Huang, H.H.; Thike, A.A.; Lim, W.K.; Teh, B.T.; Tan, P.H. CD1d expression in renal cell carcinoma is associated with higher relapse rates, poorer cancer-specific and overall survival. *J Clin Pathol* **2015**, *68*, 200-205, doi:10.1136/jclinpath-2014-202735.
196. Ni, C.; Wu, P.; Wu, X.; Zhang, T.; Zhang, T.; Wang, Z.; Zhang, S.; Qiu, F.; Huang, J. Thymosin alpha1 enhanced cytotoxicity of iNKT cells against colon cancer via upregulating CD1d expression. *Cancer Lett* **2015**, *356*, 579-588, doi:10.1016/j.canlet.2014.10.002.

197. Gumperz, J.E.; Roy, C.; Makowska, A.; Lum, D.; Sugita, M.; Podrebarac, T.; Koezuka, Y.; Porcelli, S.A.; Cardell, S.; Brenner, M.B., et al. Murine CD1d-restricted T cell recognition of cellular lipids. *Immunity* **2000**, *12*, 211-221.
198. Song, L.; Asgharzadeh, S.; Salo, J.; Engell, K.; Wu, H.W.; Sposto, R.; Ara, T.; Silverman, A.M.; DeClerck, Y.A.; Seeger, R.C., et al. Valpha24-invariant NKT cells mediate antitumor activity via killing of tumor-associated macrophages. *J Clin Invest* **2009**, *119*, 1524-1536, doi:10.1172/JCI37869.
199. Wu, D.Y.; Segal, N.H.; Sidobre, S.; Kronenberg, M.; Chapman, P.B. Cross-presentation of disialoganglioside GD3 to natural killer T cells. *J Exp Med* **2003**, *198*, 173-181, doi:10.1084/jem.20030446.
200. Olshefski, R.; Ladisch, S. Intercellular transfer of shed tumor cell gangliosides. *FEBS Lett* **1996**, *386*, 11-14.
201. Nicol, A.; Nieda, M.; Koezuka, Y.; Porcelli, S.; Suzuki, K.; Tadokoro, K.; Durrant, S.; Juji, T. Human invariant valpha24+ natural killer T cells activated by alpha-galactosylceramide (KRN7000) have cytotoxic anti-tumour activity through mechanisms distinct from T cells and natural killer cells. *Immunology* **2000**, *99*, 229-234.
202. Gansuvd, B.; Hagihara, M.; Yu, Y.; Inoue, H.; Ueda, Y.; Tsuchiya, T.; Masui, A.; Ando, K.; Nakamura, Y.; Munkhtuvshin, N., et al. Human umbilical cord blood NK T cells kill tumors by multiple cytotoxic mechanisms. *Hum Immunol* **2002**, *63*, 164-175.
203. Nishimura, T.; Kitamura, H.; Iwakabe, K.; Yahata, T.; Ohta, A.; Sato, M.; Takeda, K.; Okumura, K.; Van Kaer, L.; Kawano, T., et al. The interface between innate and acquired immunity: glycolipid antigen presentation by CD1d-expressing dendritic cells to NKT cells induces the differentiation of antigen-specific cytotoxic T lymphocytes. *Int Immunol* **2000**, *12*, 987-994.
204. Ishihara, S.; Nieda, M.; Kitayama, J.; Osada, T.; Yabe, T.; Kikuchi, A.; Koezuka, Y.; Porcelli, S.A.; Tadokoro, K.; Nagawa, H., et al. Alpha-glycosylceramides enhance the antitumor cytotoxicity of hepatic lymphocytes obtained from cancer patients by activating CD3-CD56+ NK cells in vitro. *J Immunol* **2000**, *165*, 1659-1664.
205. Escriba-Garcia, L.; Alvarez-Fernandez, C.; Tellez-Gabriel, M.; Sierra, J.; Briones, J. Dendritic cells combined with tumor cells and alpha-galactosylceramide induce a potent, therapeutic and NK-cell dependent antitumor immunity in B cell lymphoma. *J Transl Med* **2017**, *15*, 115, doi:10.1186/s12967-017-1219-3.
206. Ambrosino, E.; Terabe, M.; Halder, R.C.; Peng, J.; Takaku, S.; Miyake, S.; Yamamura, T.; Kumar, V.; Berzofsky, J.A. Cross-regulation between type I and type II NKT cells in regulating tumor immunity: a new immunoregulatory axis. *J Immunol* **2007**, *179*, 5126-5136.
207. Fichtner-Feigl, S.; Terabe, M.; Kitani, A.; Young, C.A.; Fuss, I.; Geissler, E.K.; Schlitt, H.J.; Berzofsky, J.A.; Strober, W. Restoration of tumor immunosurveillance via targeting of interleukin-13 receptor-alpha 2. *Cancer Res* **2008**, *68*, 3467-3475, doi:10.1158/0008-5472.CAN-07-5301.
208. Zhao, J.; Bagchi, S.; Wang, C.R. Type II natural killer T cells foster the antitumor activity of CpG-oligodeoxynucleotides. *Oncoimmunology* **2014**, *3*, e28977, doi:10.4161/onci.28977.
209. Bricard, G.; Cesson, V.; Devevre, E.; Bouzourene, H.; Barbey, C.; Rufer, N.; Im, J.S.; Alves, P.M.; Martinet, O.; Halkic, N., et al. Enrichment of human CD4+ V(alpha)24/Vbeta11 invariant NKT cells in intrahepatic malignant tumors. *J Immunol* **2009**, *182*, 5140-5151, doi:10.4049/jimmunol.0711086.
210. Tachibana, T.; Onodera, H.; Tsuruyama, T.; Mori, A.; Nagayama, S.; Hiai, H.; Imamura, M. Increased intratumor Valpha24-positive natural killer T cells: a prognostic factor for primary colorectal carcinomas. *Clin Cancer Res* **2005**, *11*, 7322-7327, doi:10.1158/1078-0432.CCR-05-0877.
211. Hishiki, T.; Mise, N.; Harada, K.; Ihara, F.; Takami, M.; Saito, T.; Terui, K.; Nakata, M.; Komatsu, S.; Yoshida, H., et al. Invariant natural killer T infiltration in neuroblastoma with favorable outcome. *Pediatr Surg Int* **2017**, 10.1007/s00383-017-4189-x, doi:10.1007/s00383-017-4189-x.

212. Lundgren, S.; Warfvinge, C.F.; Elebro, J.; Heby, M.; Nodin, B.; Krzyzanowska, A.; Bjartell, A.; Leandersson, K.; Eberhard, J.; Jirstrom, K. The Prognostic Impact of NK/NKT Cell Density in Periampullary Adenocarcinoma Differs by Morphological Type and Adjuvant Treatment. *PLoS One* **2016**, *11*, e0156497, doi:10.1371/journal.pone.0156497.
213. Metelitsa, L.S.; Wu, H.W.; Wang, H.; Yang, Y.; Warsi, Z.; Asgharzadeh, S.; Groshen, S.; Wilson, S.B.; Seeger, R.C. Natural killer T cells infiltrate neuroblastomas expressing the chemokine CCL2. *J Exp Med* **2004**, *199*, 1213-1221, doi:10.1084/jem.20031462.
214. Molling, J.W.; Kolgen, W.; van der Vliet, H.J.; Boomsma, M.F.; Kruizenga, H.; Smorenburg, C.H.; Molenkamp, B.G.; Langendijk, J.A.; Leemans, C.R.; von Blomberg, B.M., et al. Peripheral blood IFN-gamma-secreting Valpha24+Vbeta11+ NKT cell numbers are decreased in cancer patients independent of tumor type or tumor load. *Int J Cancer* **2005**, *116*, 87-93, doi:10.1002/ijc.20998.
215. Schneiders, F.L.; de Bruin, R.C.; van den Eertwegh, A.J.; Scheper, R.J.; Leemans, C.R.; Brakenhoff, R.H.; Langendijk, J.A.; Verheul, H.M.; de Gruijl, T.D.; Molling, J.W., et al. Circulating invariant natural killer T-cell numbers predict outcome in head and neck squamous cell carcinoma: updated analysis with 10-year follow-up. *J Clin Oncol* **2012**, *30*, 567-570, doi:10.1200/JCO.2011.38.8819.
216. Molling, J.W.; Langius, J.A.; Langendijk, J.A.; Leemans, C.R.; Bontkes, H.J.; van der Vliet, H.J.; von Blomberg, B.M.; Scheper, R.J.; van den Eertwegh, A.J. Low levels of circulating invariant natural killer T cells predict poor clinical outcome in patients with head and neck squamous cell carcinoma. *J Clin Oncol* **2007**, *25*, 862-868, doi:10.1200/JCO.2006.08.5787.
217. Crough, T.; Purdie, D.M.; Okai, M.; Maksoud, A.; Nieda, M.; Nicol, A.J. Modulation of human Valpha24(+)Vbeta11(+) NKT cells by age, malignancy and conventional anticancer therapies. *Br J Cancer* **2004**, *91*, 1880-1886, doi:10.1038/sj.bjc.6602218.
218. Giaccone, G.; Punt, C.J.; Ando, Y.; Ruijter, R.; Nishi, N.; Peters, M.; von Blomberg, B.M.; Scheper, R.J.; van der Vliet, H.J.; van den Eertwegh, A.J., et al. A phase I study of the natural killer T-cell ligand alpha-galactosylceramide (KRN7000) in patients with solid tumors. *Clin Cancer Res* **2002**, *8*, 3702-3709.
219. Singh, A.K.; Shukla, N.K.; Das, S.N. Altered invariant natural killer T cell subsets and its functions in patients with oral squamous cell carcinoma. *Scand J Immunol* **2013**, *78*, 468-477, doi:10.1111/sji.12104.
220. Tahir, S.M.; Cheng, O.; Shaulov, A.; Koezuka, Y.; Bubley, G.J.; Wilson, S.B.; Balk, S.P.; Exley, M.A. Loss of IFN-gamma production by invariant NK T cells in advanced cancer. *J Immunol* **2001**, *167*, 4046-4050.
221. Klatka, J.; Grywalska, E.; Hymos, A.; Krasowska, E.; Mielnik, M.; Siwicka-Gieroba, D.; Markowicz, J.; Trojanowski, P.; Olszanski, W.; Rolinski, J. Subpopulations of natural killer-T-like cells before and after surgical treatment of laryngeal cancer. *Cent Eur J Immunol* **2017**, *42*, 252-258, doi:10.5114/ceji.2017.70967.
222. Klatka, J.; Grywalska, E.; Wasiak, M.; Markowicz, J.; Trojanowski, P.; Olszanski, W.; Rolinski, J. The percentage of iNKT cells among other immune cells at various clinical stages of laryngeal cancer. *Postepy Hig Med Dosw (Online)* **2016**, *70*, 392-399.
223. Yanagisawa, K.; Seino, K.; Ishikawa, Y.; Nozue, M.; Todoroki, T.; Fukao, K. Impaired proliferative response of V alpha 24 NKT cells from cancer patients against alpha-galactosylceramide. *J Immunol* **2002**, *168*, 6494-6499.
224. Motohashi, S.; Okamoto, Y.; Yoshino, I.; Nakayama, T. Anti-tumor immune responses induced by iNKT cell-based immunotherapy for lung cancer and head and neck cancer. *Clin Immunol* **2011**, *140*, 167-176, doi:10.1016/j.clim.2011.01.009.
225. Exley, M.A.; Lynch, L.; Varghese, B.; Nowak, M.; Alatrakchi, N.; Balk, S.P. Developing understanding of the roles of CD1d-restricted T cell subsets in cancer: reversing tumor-induced defects. *Clin Immunol* **2011**, *140*, 184-195, doi:10.1016/j.clim.2011.04.017.
226. Xie, D.; Zhu, S.; Bai, L. Lactic acid in tumor microenvironments causes dysfunction of NKT cells by interfering with mTOR signaling. *Sci China Life Sci* **2016**, *59*, 1290-1296, doi:10.1007/s11427-016-0348-7.

227. Wang, H.; Yang, D.; Xu, W.; Wang, Y.; Ruan, Z.; Zhao, T.; Han, J.; Wu, Y. Tumor-derived soluble MICs impair CD3(+)CD56(+) NKT-like cell cytotoxicity in cancer patients. *Immunol Lett* **2008**, *120*, 65-71, doi:10.1016/j.imlet.2008.07.001.
228. Renukaradhya, G.J.; Sriram, V.; Du, W.; Gervay-Hague, J.; Van Kaer, L.; Brutkiewicz, R.R. Inhibition of antitumor immunity by invariant natural killer T cells in a T-cell lymphoma model in vivo. *Int J Cancer* **2006**, *118*, 3045-3053, doi:10.1002/ijc.21764.
229. Quatromoni, J.G.; Eruslanov, E. Tumor-associated macrophages: function, phenotype, and link to prognosis in human lung cancer. *Am J Transl Res* **2012**, *4*, 376-389.
230. Metelitsa, L.S. Anti-tumor potential of type-I NKT cells against CD1d-positive and CD1d-negative tumors in humans. *Clin Immunol* **2011**, *140*, 119-129, doi:10.1016/j.clim.2010.10.005.
231. Hayakawa, Y.; Takeda, K.; Yagita, H.; Smyth, M.J.; Van Kaer, L.; Okumura, K.; Saiki, I. IFN-gamma-mediated inhibition of tumor angiogenesis by natural killer T-cell ligand, alpha-galactosylceramide. *Blood* **2002**, *100*, 1728-1733.
232. Sun, T.; Yang, Y.; Luo, X.; Cheng, Y.; Zhang, M.; Wang, K.; Ge, C. Inhibition of tumor angiogenesis by interferon-gamma by suppression of tumor-associated macrophage differentiation. *Oncol Res* **2014**, *21*, 227-235, doi:10.3727/096504014X13890370410285.
233. Courtney, A.N.; Tian, G.W.; Liu, D.F.; Marinova, E.; Heczey, A.; Xu, X.; Guo, L.J.; Gao, X.H.; Metelitsa, L.S. Cross-talk between NKT cells and tumor associated macrophages in the tumor microenvironment. *Journal of Immunology* **2016**, *196*.
234. Raker, V.K.; Domogalla, M.P.; Steinbrink, K. Tolerogenic Dendritic Cells for Regulatory T Cell Induction in Man. *Front Immunol* **2015**, *6*, 569, doi:10.3389/fimmu.2015.00569.
235. Taniguchi, M.; Harada, M.; Dashtsoodol, N.; Kojo, S. Discovery of NKT cells and development of NKT cell-targeted anti-tumor immunotherapy. *Proc Jpn Acad Ser B Phys Biol Sci* **2015**, *91*, 292-304, doi:10.2183/pjab.91.292.
236. Lippitz, B.E. Cytokine patterns in patients with cancer: a systematic review. *Lancet Oncol* **2013**, *14*, e218-228, doi:10.1016/S1470-2045(12)70582-X.
237. Whiteside, T.L. The role of regulatory T cells in cancer immunology. *Immunotargets Ther* **2015**, *4*, 159-171, doi:10.2147/ITT.S55415.
238. Zhang, Z.; Su, T.; He, L.; Wang, H.; Ji, G.; Liu, X.; Zhang, Y.; Dong, G. Identification and functional analysis of ligands for natural killer cell activating receptors in colon carcinoma. *Tohoku J Exp Med* **2012**, *226*, 59-68, doi:10.6120/tjem.226.59.
239. de Kruijf, E.M.; Sajet, A.; van Nes, J.G.; Putter, H.; Smit, V.T.; Eagle, R.A.; Jafferji, I.; Trowsdale, J.; Liefers, G.J.; van de Velde, C.J., et al. NKG2D ligand tumor expression and association with clinical outcome in early breast cancer patients: an observational study. *BMC Cancer* **2012**, *12*, 24, doi:10.1186/1471-2407-12-24.
240. Garrido, F.; Cabrera, T.; Concha, A.; Glew, S.; Ruiz-Cabello, F.; Stern, P.L. Natural history of HLA expression during tumour development. *Immunol Today* **1993**, *14*, 491-499, doi:10.1016/0167-5699(93)90264-L.
241. Garrido, F.; Ruiz-Cabello, F.; Cabrera, T.; Perez-Villar, J.J.; Lopez-Botet, M.; Duggan-Keen, M.; Stern, P.L. Implications for immunosurveillance of altered HLA class I phenotypes in human tumours. *Immunol Today* **1997**, *18*, 89-95.
242. Marincola, F.M.; Jaffee, E.M.; Hicklin, D.J.; Ferrone, S. Escape of human solid tumors from T-cell recognition: molecular mechanisms and functional significance. *Adv Immunol* **2000**, *74*, 181-273.
243. Seliger, B.; Cabrera, T.; Garrido, F.; Ferrone, S. HLA class I antigen abnormalities and immune escape by malignant cells. *Semin Cancer Biol* **2002**, *12*, 3-13, doi:10.1006/scbi.2001.0404.
244. Chen, M.L.; Pittet, M.J.; Gorelik, L.; Flavell, R.A.; Weissleder, R.; von Boehmer, H.; Khazaie, K. Regulatory T cells suppress tumor-specific CD8 T cell cytotoxicity through TGF-beta signals in vivo. *Proc Natl Acad Sci U S A* **2005**, *102*, 419-424, doi:10.1073/pnas.0408197102.

245. Strauss, L.; Bergmann, C.; Szczepanski, M.; Gooding, W.; Johnson, J.T.; Whiteside, T.L. A unique subset of CD4+CD25highFoxp3+ T cells secreting interleukin-10 and transforming growth factor-beta1 mediates suppression in the tumor microenvironment. *Clin Cancer Res* **2007**, *13*, 4345-4354, doi:10.1158/1078-0432.CCR-07-0472.

246. Nair, S.; Dhodapkar, M.V. Natural Killer T Cells in Cancer Immunotherapy. *Front Immunol* **2017**, *8*, 1178, doi:10.3389/fimmu.2017.01178.

247. Ishikawa, A.; Motohashi, S.; Ishikawa, E.; Fuchida, H.; Higashino, K.; Otsuji, M.; Iizasa, T.; Nakayama, T.; Taniguchi, M.; Fujisawa, T. A phase I study of alpha-galactosylceramide (KRN7000)-pulsed dendritic cells in patients with advanced and recurrent non-small cell lung cancer. *Clin Cancer Res* **2005**, *11*, 1910-1917, doi:10.1158/1078-0432.CCR-04-1453.

248. Chang, D.H.; Osman, K.; Connolly, J.; Kukreja, A.; Krasovsky, J.; Pack, M.; Hutchinson, A.; Geller, M.; Liu, N.; Annable, R., et al. Sustained expansion of NKT cells and antigen-specific T cells after injection of alpha-galactosyl-ceramide loaded mature dendritic cells in cancer patients. *J Exp Med* **2005**, *201*, 1503-1517, doi:10.1084/jem.20042592.

249. Uchida, T.; Horiguchi, S.; Tanaka, Y.; Yamamoto, H.; Kunii, N.; Motohashi, S.; Taniguchi, M.; Nakayama, T.; Okamoto, Y. Phase I study of alpha-galactosylceramide-pulsed antigen presenting cells administration to the nasal submucosa in unresectable or recurrent head and neck cancer. *Cancer Immunol Immunother* **2008**, *57*, 337-345, doi:10.1007/s00262-007-0373-5.

250. Nicol, A.J.; Tazbirkova, A.; Nieda, M. Comparison of clinical and immunological effects of intravenous and intradermal administration of alpha-galactosylceramide (KRN7000)-pulsed dendritic cells. *Clin Cancer Res* **2011**, *17*, 5140-5151, doi:10.1158/1078-0432.CCR-10-3105.

251. Nieda, M.; Okai, M.; Tazbirkova, A.; Lin, H.; Yamaura, A.; Ide, K.; Abraham, R.; Juji, T.; Macfarlane, D.J.; Nicol, A.J. Therapeutic activation of Valpha24+Vbeta11+ NKT cells in human subjects results in highly coordinated secondary activation of acquired and innate immunity. *Blood* **2004**, *103*, 383-389, doi:10.1182/blood-2003-04-1155.

252. Motohashi, S.; Nagato, K.; Kunii, N.; Yamamoto, H.; Yamasaki, K.; Okita, K.; Hanaoka, H.; Shimizu, N.; Suzuki, M.; Yoshino, I., et al. A phase I-II study of alpha-galactosylceramide-pulsed IL-2/GM-CSF-cultured peripheral blood mononuclear cells in patients with advanced and recurrent non-small cell lung cancer. *J Immunol* **2009**, *182*, 2492-2501, doi:10.4049/jimmunol.0800126.

253. Nagato, K.; Motohashi, S.; Ishibashi, F.; Okita, K.; Yamasaki, K.; Moriya, Y.; Hoshino, H.; Yoshida, S.; Hanaoka, H.; Fujii, S., et al. Accumulation of activated invariant natural killer T cells in the tumor microenvironment after alpha-galactosylceramide-pulsed antigen presenting cells. *J Clin Immunol* **2012**, *32*, 1071-1081, doi:10.1007/s10875-012-9697-9.

254. Motohashi, S.; Ishikawa, A.; Ishikawa, E.; Otsuji, M.; Iizasa, T.; Hanaoka, H.; Shimizu, N.; Horiguchi, S.; Okamoto, Y.; Fujii, S., et al. A phase I study of in vitro expanded natural killer T cells in patients with advanced and recurrent non-small cell lung cancer. *Clin Cancer Res* **2006**, *12*, 6079-6086, doi:10.1158/1078-0432.CCR-06-0114.

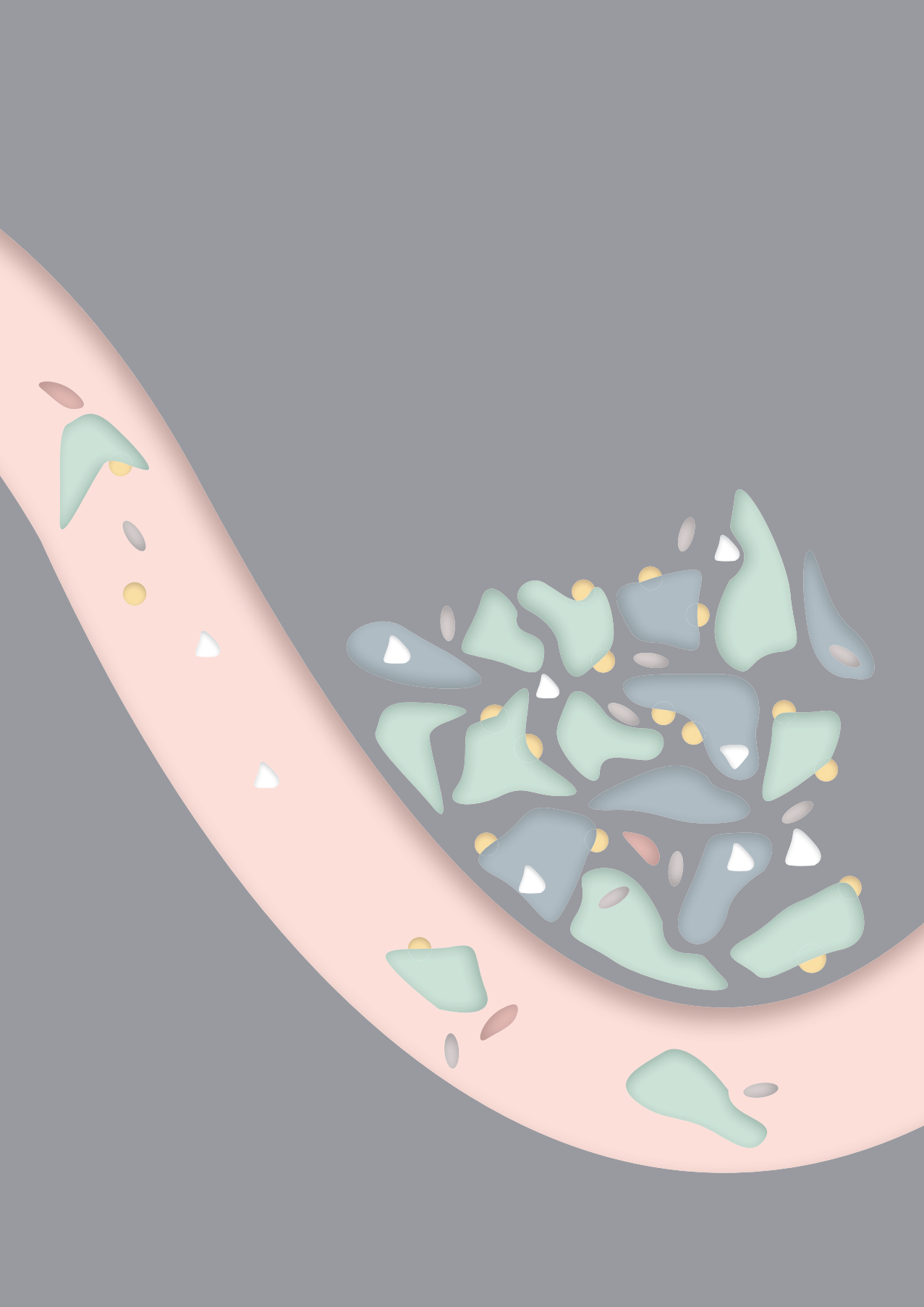
255. Exley, M.A.; Friedlander, P.; Alatrakchi, N.; Vriend, L.; Yue, S.; Sasada, T.; Zeng, W.; Mizukami, Y.; Clark, J.; Nemer, D., et al. Adoptive Transfer of Invariant NKT Cells as Immunotherapy for Advanced Melanoma: A Phase I Clinical Trial. *Clin Cancer Res* **2017**, *23*, 3510-3519, doi:10.1158/1078-0432.CCR-16-0600.

256. Yamasaki, K.; Horiguchi, S.; Kurosaki, M.; Kunii, N.; Nagato, K.; Hanaoka, H.; Shimizu, N.; Ueno, N.; Yamamoto, S.; Taniguchi, M., et al. Induction of NKT cell-specific immune responses in cancer tissues after NKT cell-targeted adoptive immunotherapy. *Clin Immunol* **2011**, *138*, 255-265, doi:10.1016/j.clim.2010.11.014.

257. Kunii, N.; Horiguchi, S.; Motohashi, S.; Yamamoto, H.; Ueno, N.; Yamamoto, S.; Sakurai, D.; Taniguchi, M.; Nakayama, T.; Okamoto, Y. Combination therapy of in vitro-expanded natural killer T cells and

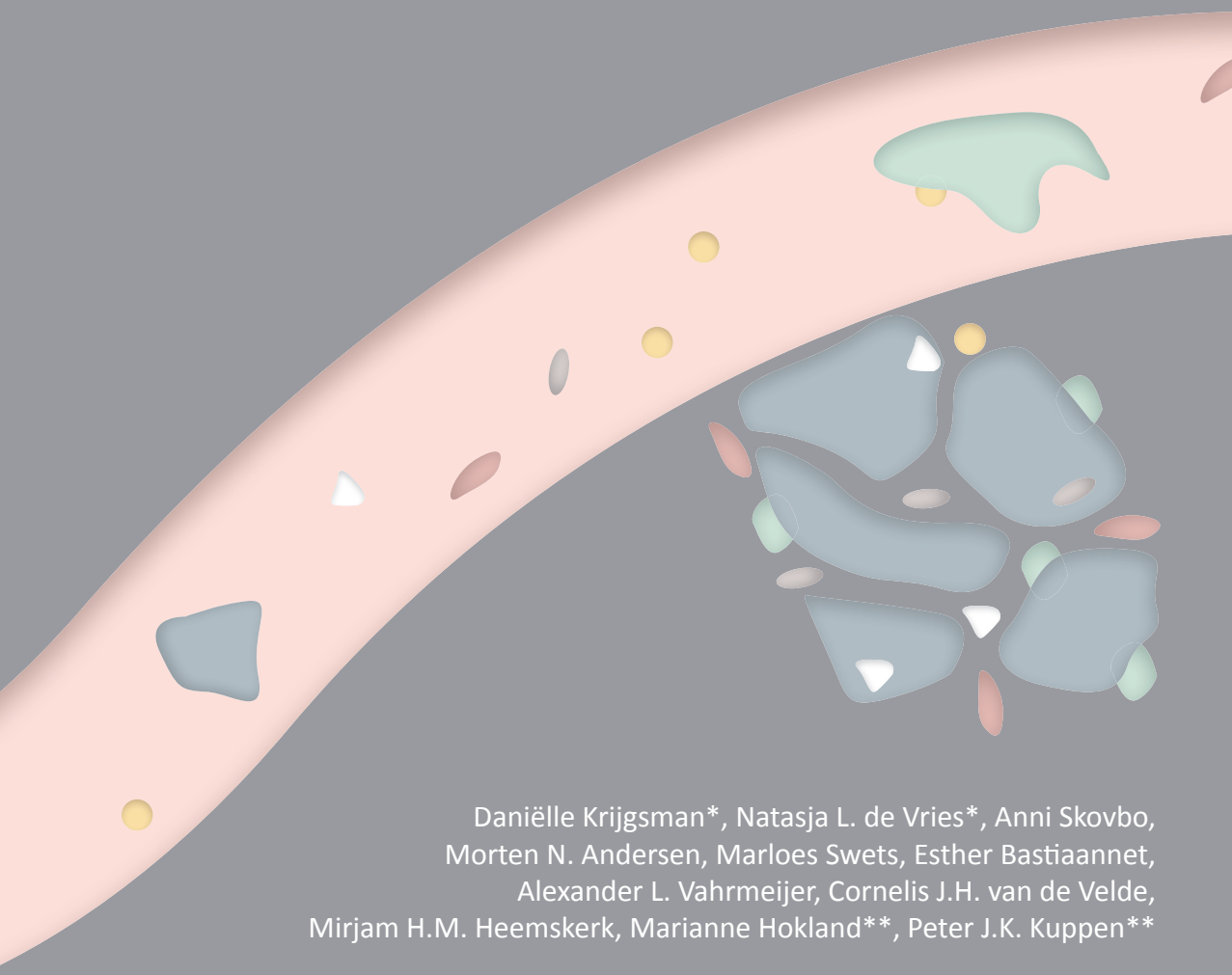
alpha-galactosylceramide-pulsed antigen-presenting cells in patients with recurrent head and neck carcinoma. *Cancer Sci* **2009**, *100*, 1092-1098, doi:10.1111/j.1349-7006.2009.01135.x.

258. Bollino, D.; Webb, T.J. Chimeric antigen receptor-engineered natural killer and natural killer T cells for cancer immunotherapy. *Transl Res* **2017**, *187*, 32-43, doi:10.1016/j.trsl.2017.06.003.
259. Stirnemann, K.; Romero, J.F.; Baldi, L.; Robert, B.; Cesson, V.; Besra, G.S.; Zauderer, M.; Wurm, F.; Corradin, G.; Mach, J.P., et al. Sustained activation and tumor targeting of NKT cells using a CD1d-anti-HER2-scFv fusion protein induce antitumor effects in mice. *J Clin Invest* **2008**, *118*, 994-1005, doi:10.1172/JCI33249.
260. Corgnac, S.; Perret, R.; Derre, L.; Zhang, L.; Stirnemann, K.; Zauderer, M.; Speiser, D.E.; Mach, J.P.; Romero, P.; Donda, A. CD1d-antibody fusion proteins target iNKT cells to the tumor and trigger long-term therapeutic responses. *Cancer Immunol Immunother* **2013**, *62*, 747-760, doi:10.1007/s00262-012-1381-7.
261. Horn, L.A.; Ciavattone, N.G.; Atkinson, R.; Woldergerima, N.; Wolf, J.; Clements, V.K.; Sinha, P.; Poudel, M.; Ostrand-Rosenberg, S. CD3xPDL1 bi-specific T cell engager (BiTE) simultaneously activates T cells and NKT cells, kills PDL1(+) tumor cells, and extends the survival of tumor-bearing humanized mice. *Oncotarget* **2017**, *8*, 57964-57980, doi:10.18632/oncotarget.19865.
262. Gasser, O.; Sharples, K.J.; Barrow, C.; Williams, G.M.; Bauer, E.; Wood, C.E.; Mester, B.; Dzhelali, M.; Caygill, G.; Jones, J., et al. A phase I vaccination study with dendritic cells loaded with NY-ESO-1 and alpha-galactosylceramide: induction of polyfunctional T cells in high-risk melanoma patients. *Cancer Immunol Immunother* **2017**, 10.1007/s00262-017-2085-9, doi:10.1007/s00262-017-2085-9.
263. Watarai, H.; Yamada, D.; Fujii, S.; Taniguchi, M.; Koseki, H. Induced pluripotency as a potential path towards iNKT cell-mediated cancer immunotherapy. *Int J Hematol* **2012**, *95*, 624-631, doi:10.1007/s12185-012-1091-0.
264. Watarai, H.; Fujii, S.; Yamada, D.; Rybouchkin, A.; Sakata, S.; Nagata, Y.; Iida-Kobayashi, M.; Sekine-Kondo, E.; Shimizu, K.; Shozaki, Y., et al. Murine induced pluripotent stem cells can be derived from and differentiate into natural killer T cells. *J Clin Invest* **2010**, *120*, 2610-2618, doi:10.1172/JCI42027.
265. Yamada, D.; Iyoda, T.; Vizcardo, R.; Shimizu, K.; Sato, Y.; Endo, T.A.; Kitahara, G.; Okoshi, M.; Kobayashi, M.; Sakurai, M., et al. Efficient Regeneration of Human Valpha24(+) Invariant Natural Killer T Cells and Their Anti-Tumor Activity In Vivo. *Stem Cells* **2016**, *34*, 2852-2860, doi:10.1002/stem.2465.
266. Parekh, V.V.; Lalani, S.; Kim, S.; Halder, R.; Azuma, M.; Yagita, H.; Kumar, V.; Wu, L.; Kaer, L.V. PD-1/PD-L blockade prevents anergy induction and enhances the anti-tumor activities of glycolipid-activated invariant NKT cells. *J Immunol* **2009**, *182*, 2816-2826, doi:10.4049/jimmunol.0803648.
267. Kamata, T.; Suzuki, A.; Mise, N.; Ihara, F.; Takami, M.; Makita, Y.; Horinaka, A.; Harada, K.; Kunii, N.; Yoshida, S., et al. Blockade of programmed death-1/programmed death ligand pathway enhances the antitumor immunity of human invariant natural killer T cells. *Cancer Immunol Immunother* **2016**, *65*, 1477-1489, doi:10.1007/s00262-016-1901-y.
268. Klatka, J.; Grywalska, E.; Hymos, A.; Guz, M.; Polberg, K.; Rolinski, J.; Stepulak, A. Cyclooxygenase-2 Inhibition Enhances Proliferation of NKT Cells Derived from Patients with Laryngeal Cancer. *Anticancer Res* **2017**, *37*, 4059-4066, doi:10.21873/anticanres.11791.
269. Laurent, X.; Bertin, B.; Renault, N.; Farce, A.; Speca, S.; Milhomme, O.; Millet, R.; Desreumaux, P.; Henon, E.; Chavatte, P. Switching invariant natural killer T (iNKT) cell response from anticancerous to anti-inflammatory effect: molecular bases. *J Med Chem* **2014**, *57*, 5489-5508, doi:10.1021/jm4010863.
270. Liu, Z.; Guo, J. NKT-cell glycolipid agonist as adjuvant in synthetic vaccine. *Carbohydr Res* **2017**, *452*, 78-90, doi:10.1016/j.carres.2017.10.006.
271. Carreno, L.J.; Saavedra-Avila, N.A.; Porcelli, S.A. Synthetic glycolipid activators of natural killer T cells as immunotherapeutic agents. *Clin Transl Immunology* **2016**, *5*, e69, doi:10.1038/cti.2016.14



CHAPTER 3

Characterization of circulating T-, NK-, and NKT cell subsets in patients with colorectal cancer: the peripheral blood immune cell profile



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Abstract

Objective: As the development and progression of colorectal cancer (CRC) are known to be affected by the immune system, cell subsets such as T cells, natural killer (NK) cells, and natural killer T (NKT) cells are considered interesting targets for immunotherapy- and clinical biomarker research. Until now, the role of systemic immune profiles in tumor progression remains unclear. In this study, we aimed to characterize the immunophenotype of circulating T cells, NK cells, and NKT-like cells in patients with CRC, and to subsequently correlate these immunophenotypes to clinical follow-up data.

Methods: Using multiparameter flow cytometry, the subset distribution and immunophenotype of T cells ($CD3^+CD56^-$), $CD56^{dim}$ NK cells ($CD3^-CD56^{dim}$), $CD56^{bright}$ NK cells ($CD3^-CD56^{bright}$), and NKT-like ($CD3^+CD56^+$) cells were investigated in peripheral blood mononuclear cell (PBMC) samples from 71 CRC patients and 19 healthy donors.

Results: CRC patients showed profound differences in immune cell subset distribution and their immunophenotype compared to healthy donors, as characterized by increased percentage of regulatory T cells, and reduced expression level of the natural cytotoxicity receptors NKp44 and NKp46 on both $CD56^{dim}$ NK cells and NKT-like cells. Finally, we showed in a multivariate analysis that above median percentage of $CD16^+$ NKT-like cells was independently associated with shorter disease-free survival in CRC patients.

Conclusion: The altered phenotype of circulating immune cell subsets in CRC and its association with clinical outcome highlight the potential use of PBMC subsets as prognostic biomarkers in CRC, thereby contributing to better insight into the role of systemic immune profiles in tumor progression.

Introduction

Colorectal cancer (CRC) is a major contributor to cancer-related morbidity and mortality [1]. Approximately one million new cases of CRC arise per year throughout the world, with more than half a million deaths annually [1]. For colorectal tumors that have not spread to distant sites, surgery is the primary treatment [2]. However, around 25% of the patients present with unresectable metastatic disease at the time of diagnosis, and up to 50% percent of early stage patients develop recurrence or dissemination of the disease following surgery [3]. In order to reduce the risk of relapse and to prolong the survival of patients with metastatic disease, treatment strategies need to be optimized. Therefore, biomarker application is important since it provides information for therapeutic decision making. As the development and progression of CRC are known to be affected by the immune system, cell subsets such as T cells, natural killer (NK) cells, and natural killer T (NKT) cells are considered interesting targets for immunotherapy- and clinical biomarker research. Importantly, the phenotype of circulating lymphocyte subsets may reflect the local immune response in the tumor microenvironment (TME), thereby providing potentially important information regarding disease progression in CRC [4-6].

Different T cell subsets have been described over the years, each with distinct functions that promote, or inhibit, anti-tumor immune responses. For instance, cytotoxic ($CD8^+$) T cells recognize tumor-associated antigens presented by classical HLA class I molecules. Additionally, $CD4^+$ T cells play an important regulatory role via secretion of cytokines and activation of cytotoxic T cells and B cells [7]. Regulatory T cells (T_{reg}), a subpopulation of $CD4^+$ T cells, have an immunosuppressive function [8]. In order to escape immune recognition by cytotoxic T cells, tumor cells may down-regulate classical HLA class I expression [9]. Studies showed that aberrant HLA class I expression commonly occurs in colorectal tumors [10-14]. These tumors represent potential targets for NK cells [15], which are known to be able to recognize and kill tumor cells with down-regulated HLA class I molecules [16,17].

NK cells can be subdivided based on their CD56 expression: $CD56^{bright}$ NK cells are generally associated with immunoregulatory properties and production of pro-inflammatory cytokines, while $CD56^{dim}$ NK cells primarily exert cytotoxic functions [18,19]. The NK cell activity is dependent on a delicate balance between activating and inhibitory signals from cell surface receptors [20]. 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. Additionally, CD16 (FcγRIII) on NK cells mediates antibody-dependent cell-mediated cytotoxicity (ADCC) [21]. Furthermore, CD8 is considered a stimulatory cell surface receptor since it enhances the cytolytic activity of NK cells [22]. NK cells also

express a range of receptors that provide inhibitory signals upon stimulation. NK cell inhibitory receptors include natural killer group 2-A (NKG2A), and killer cell immunoglobulin (Ig)-like receptors CD158a and CD158b.

Apart from NK- and T cells, peripheral blood comprises other leucocyte subsets with the ability to induce anti-tumor effects. For instance, NKT cells constitute a unique subset of T cells that lie at the interface between innate and adaptive immunity. Unlike conventional T cells, NKT cells express a T cell receptor that recognizes glycolipids presented by the HLA-like molecule CD1d [23]. Besides, NKT cells express various markers typically associated with NK cells (activating- and inhibitory cell surface receptors) [24]. NKT cells possess cytotoxic capabilities, but are primarily considered to have an important regulatory function via the secretion of large amounts of pro- or anti-inflammatory cytokines upon activation, thereby resulting in amplification or dampening of the immune response [25]. Due to a lack of specific markers, it is not possible as yet to identify the entire NKT cell population using flow cytometry or immunohistochemistry [25]. In many studies, co-expression of CD3 and CD56 is used to identify NKT cells [26-31]. Although it is likely that the $CD3^+CD56^+$ cell population includes "true" CD1d-restricted NKT cells, it has to be taken into account that conventional T cells have also been reported to express CD56 [32,33]. Thus, since it is unclear whether all $CD3^+CD56^+$ cells are CD1d restricted, this population is often referred to as "NKT-like".

In this study, we aimed to characterize the immunophenotype of circulating T cells, NK cells, and NKT-like cells in CRC patients using multiparameter flow cytometry, and to subsequently correlate these immunophenotypes to clinical follow-up data.

Materials and methods

Study population

The study population comprised patients diagnosed with CRC who underwent surgical resection of their colorectal tumor at the Leiden University Medical Center (LUMC, the Netherlands) between 2001 and 2007. From these patients, peripheral blood samples were collected within a month prior to surgery. In the present study, patients with histologically proven primary colorectal tumors, Tumor-Node-Metastasis (TNM) stage 0-IV, surgical R0 resection, and a minimal amount of 5 million cryopreserved peripheral blood mononuclear cells (PBMCs) were included (N=87). Patients with multiple colorectal tumors at the time of diagnosis or resection were excluded. Patient follow-up was performed until August 2017, with a median follow-up time of 12 years (range 0.1-15.8 years).

Peripheral blood samples obtained from 19 healthy donors (Dept. of Hematology, LUMC) served as controls.

Isolation of peripheral blood mononuclear cells

PBMCs were isolated by Ficoll-Paque (density 1.077g/ml, provided by the apothecary LUMC) density-gradient centrifugation. All PBMC samples were cryopreserved in liquid nitrogen until time of analysis in freezing medium containing 20% heat-inactivated FCS (Thermo Fisher Scientific, Waltham, MA, USA) and 10% DMSO (Avantor, Center Valley, PA, USA). Additionally, PBMCs were isolated from the buffy coat of a healthy donor obtained from the Blood Bank at Aarhus University Hospital (Dept. Clinical Immunology, Aarhus University Hospital, Skejby, Denmark) which was used as an internal control in the flow cytometry experiments. After isolation using Histopaque (Sigma-Aldrich, St. Louis, MO, USA) density-gradient centrifugation, these PBMCs were cryopreserved at -150°C until time of use in RPMI-1640 medium (Lonza, Basel, Switzerland) containing 20% heat-inactivated FCS (GE Healthcare, Little Chalfont, United Kingdom), and 10% DMSO (Sigma-Aldrich).

Flow cytometry antibody staining

Extensive immunophenotyping of circulating T cells, NK cells, and NKT-like cells was performed using multiparameter flow cytometry with directly fluorochrome-conjugated mouse mAb (Supplementary Table 1). Briefly, PBMCs were thawed at 37°C, washed in PBS (Lonza)/10% FCS (GE Healthcare), and cell count was determined using a NucleoCounter®NC-250™ (ChemoMetec, Allerod, Denmark) according to the manufacturer's instructions. PBMCs were adjusted to a concentration of $10x10^6$ cells/ml and blocked for non-specific Ab binding in PBS/0.5% BSA (Merck Millipore, Billerica, MA, USA)/0.09% sodium azide buffer (Ampliqon, Odense, Denmark) with 5% human AB serum (from a batch of healthy donors obtained from the Blood Bank at Aarhus University Hospital) or 50 µg/ml human Ig (Human Ig, CSL Behring, Bern, Switzerland) for 15 minutes. Three multicolor flow cytometry panels were designed for the identification of circulating T cells, NK cells, and NKT-like cells (Supplementary Table 1). For each flow panel, a cocktail of Ab was prepared in BD brilliant stain buffer (BD Biosciences) in order to prevent aggregation of brilliant violet fluorochromes. PBMCs ($0.5x10^6$ cells) were incubated with the antibody cocktails in the dark for 30 min at room temperature (flow panel 1 and 2) or at 4°C (flow panel 3), as these conditions provided optimal binding for the included Ab in each panel. To exclude dead cells from the analysis, a live/dead fixable near-infrared dead cell stain kit (Life Technologies, Carlsbad, CA, USA) was included in each staining. After incubation with

the Ab cocktails, the cells were washed twice in PBS/0.5% BSA/0.09% sodium azide buffer. Next, PBMCs were fixed in PBS/0.9% formaldehyde (Sigma-Aldrich) before analysis.

Flow cytometry data analysis

Samples were analyzed immediately after staining on the LSR Fortessa (BD Biosciences) flow cytometer running FACSDiva™ software version 8.0 (BD Biosciences). The data set was analyzed using FlowJo software version 10.1 (Tree Star Inc., Ashland, OR, USA). In each experiment, PBMCs derived from the same buffy coat (described above) were included as an internal control. Instrument performance was verified daily using the Cytometer Setup & Tracking (CS&T) system (BD Biosciences), applying CS&T application settings to ensure comparable flow cytometry results over time. Compensation was carried out with CompBeads (BD Biosciences), OneComp eBeads (eBioscience, Inc., San Diego, CA), CompBeads Plus (BD Biosciences), and ArC reactive beads (Life Technologies) according to the manufacturer's protocol. The threshold for positive staining was determined using unstained- or fluorescence minus one (FMO) controls when necessary [34,35]. FMO controls were used for CD56, CD158a, and NKG2A in flow panel 1. Since there was no spectral overlap between the fluorochromes brilliant violet 711 (CD158a) and allophycocyanin (NKG2A), FMO controls for CD158a and NKG2A were combined in a fluorescence minus two (FM2) control following proper verification. Furthermore, an FMO control for CD56 in flow panel 2 was included. To identify circulating T cells, NK cells, and NKT-like cells, a sequential gating strategy for each flow panel was created based on the buffy coat internal control sample (Supplementary Figure 1, 2, and 3). The expression of phenotypic markers on circulating T cells, NK cells and NKT-like cells was then evaluated by the median fluorescence intensity (MFI) and/or the percentage of positive cells. The markers CD3, CD14 and CD56 were strictly used for gating and not studied in further detail.

Corrections and statistical analyses

The NKG2A antibody was changed regarding isotype and clone during the flow cytometry experiments due to low quality of a newly ordered Ab batch. The internal control buffy coat was used to correct for differences in the MFI and percentage of positive lymphocytes regarding NKG2A between the two used Ab clones. Statistical analyses were conducted using SPSS statistical software (IBM SPSS Statistics 23, Chicago, USA). The Mann-Whitney U test was used to compare the age of the CRC patients with the healthy donors. The sex of CRC patients was compared with healthy donors using a Pearson Chi-square test. Additionally, independent samples T tests and Mann-Whitney U tests, where appropriate,

were used in order to compare patients with healthy donors and to evaluate differences in patient and tumor characteristics. Furthermore, the Spearman correlation test was used to study the relation of phenotypic markers on different immune cell subsets. Throughout the text, the median percentages or MFI are reported including standard deviations (SD). Kaplan-Meier analyses and Log-rank tests were used to investigate and compare survival within patient subgroups. Our primary clinical endpoint was disease-free survival (DFS), which was defined as the time from surgery until recurrence of disease or death, whichever came first, or end of follow-up (censored). Cox regression analysis was used for univariate and multivariate analyses for DFS. We corrected for multiple testing using the Benjamini-Hochberg method, by which adjusted *P*-values were calculated (indicated by *P**) [36]. *P*- and *P**-values ≤0.05 were considered statistically significant.

Results

Patient characteristics

In total, flow cytometry data of 71/87 (81.6%) CRC patients could be included in the analysis. Eight samples were excluded due to low viability of the PBMCs (<50% viable cells). Additionally, samples from two patients were obtained prior to resection of liver metastases instead of the primary colorectal tumor and, therefore, also excluded from further analysis. Furthermore, four patients were excluded from this study due to a confirmed diagnosis of Lynch syndrome. Finally, two patients were excluded due to pre-surgical chemotherapy before sample collection. Eleven patients included in the study had undergone local radiotherapy prior to the collection of the blood sample. Since local radiotherapy is unlikely to affect the immune system systemically, these patients were not excluded from this study. Table 1 summarizes the clinico-pathological characteristics of the 71 CRC patients included in the analyses, together with 19 healthy donors. When comparing the CRC patients with the healthy donors, no significant difference was observed in relation to sex (Table 1). A trend was observed towards a higher age in the CRC patients compared to the healthy donors, which was not statistically significant (Table 1). Flow cytometry panels 1 and 2 (NK cells and NKT-like cells) were investigated in all included 71 CRC patients and 19 healthy donors. Flow cytometry panel 3 (T cells) was investigated in 47 CRC patients and 10 healthy donors.

Table 1. Patient demographics and tumor characteristics. In this study, the immunophenotype of peripheral blood immune cell profiles was investigated in 71 CRC patients and 19 healthy donors.

	CRC patients (N=71)	**Healthy donors** (N=19)	*P*-value
Age*			0.059
Mean (years)	65.6	55.6	
Range (years)	25-85	22-83	
Sex			0.557
Female	32 (45.1%)	10 (52.6%)	
Male	39 (54.9%)	9 (47.4%)	
Tumor location			
Colon	59 (83.1%)		
Rectum	12 (16.9%)		
TNM classification			
Stage 0	4 (5.6%)		
Stage I	10 (14.1%)		
Stage II	22 (35.2%)		
Stage III	22 (33.8%)		
Stage IV	8 (11.3%)		
Tumor differentiation			
Well/moderate	55 (77.5%)		
Poor	13 (18.3%)		
Unknown	3 (4.2%)		
Tumor-positive lymph nodes			
Yes	30 (56.3%)		
No	40 (42.3%)		
Unknown	1 (1.4%)		
Neoadjuvant radiotherapy			
Yes	10 (14.1%)		
No	61 (85.9%)		
Adjuvant chemotherapy			
Yes	27 (38.0%)		
No	44 (62.0%)		

*Age at time of surgery

Increased percentage of circulating regulatory T cells in colorectal cancer patients compared to healthy donors

In this study, we compared the immunophenotype of circulating T cells ($CD3^+CD56^-$), NK cells ($CD3^-CD56^+$), and NKT-like cells ($CD3^+CD56^+$) between CRC patients and healthy donors. First of all, the distribution of the different immune cell subsets was studied (Table 2). We hypothesized that CRC patients would present with a general immunocompromised state of circulating immune cells,

reflected by a shift in the balance between cytotoxic and regulatory cell subsets. Similar proportions of circulating T cells (*P**=0.883), NK cells (*P**=0.768), and NKT-like cells (*P**=0.904) (% of total lymphocytes) were observed in CRC patients and healthy donors (Figure 1A;1E;1H respectively). Furthermore, no differences were observed in the distribution of circulating $CD8^+$ (*P**=0.670) and $CD4^+$ (*P**=0.689) T cells (% of T cells) or distribution of circulating $CD56^{dim}$ (*P**=0.197) and $CD56^{bright}$ (*P**=0.196) NK cell populations (% of NK cells) (Figure 1B;1C;1F;1G respectively). In contrast, CRC patients presented with an increased percentage of circulating $CD127^{low}CD25^+$ T_{reg} (*P**<0.001) (% of $CD3^+CD4^+$ T cells) (Figure 1D) compared to healthy donors.

Table 2. Comparison between peripheral blood immune cell profiles in CRC patients and healthy donors. The distribution of circulating immune cell subsets and their immunophenotype was compared between CRC patients (N=71) and healthy donors (N=19). *P*-values were corrected for multiple testing using the Benjamini-Hochberg method, by which adjusted *P*-values were calculated (indicated by *P**). *P**-values ≤0.05 were considered statistically significant and are indicated in bold.

	Healthy controls (N=19)		**CRC patients (N=71)**			
	Mean	SD	Mean	SD	*P*-value	Corrected *P**-value
Subset distribution						
*T cells (%)**	59.4	8.2	57.5	13.2	0.669^T	0.883
*$CD8^+$ T cells (%)**	20.7	8.3	18.7	10.4	0.378^U	0.670
*$CD4^+$ T cells (%)**	42.1	6.1	44.3	12.2	0.400^T	0.689
*$CD127^{low}CD25^+$ T_{reg} (%)**	5.5	0.5	7.7	2.4	0.000^U	**0.000**
NK cells (%)	12.8	5.4	15.4	8.5	0.508^U	0.768
$CD56^{dim}$ NK cells (%)	93.4	4.2	95.0	4.2	0.054^U	0.197
$CD56^{bright}$ NK cells (%)	6.6	4.2	5.1	4.2	0.057^U	0.196
NKT-like cells (%)	4.3	2.8	6.1	6.4	0.656^U	0.904
$CD56^{dim}$ NK cells						
$CD16^+$ (%)	85.3	6.1	83.7	10.2	0.909^U	0.989
$CD158a^+$ (%)	35.2	17.1	31.9	16.9	0.477^U	0.739
$CD158b^+$ (%)	37.0	11.4	37.5	14.7	0.786^U	0.937
$NKG2A^+$ (%)	45.6	19.3	43.4	19.0	0.656^T	0.884
$NKG2A^+$ (MFI)	6398	2138	7368	2459	0.275^U	0.533
$NKG2C^+$ (%)	13.7	13.0	16.2	17.9	0.909^U	0.972
$NKG2C^+$ (MFI)	2506	2797	2965	3094	0.213^U	0.440
CD161 (MFI)	3111	804	3057	1130	0.816^T	0.903
$CD8^+$ (%)	34.3	14.3	31.5	14.4	0.467^U	0.742
$CD8^+$ (MFI)	3481	774	3067	1159	0.067^U	0.208
DNAM-1 (MFI)	560	106	548	146	0.741^T	0.919
$NKG2D^+$ (%)	89.9	4.4	91.4	4.9	0.134^U	0.346
$NKG2D^+$ (MFI)	3600	1108	3429	1113	0.554^T	0.818
NKp30 (MFI)	1960	752	1630	900	0.052^U	0.202
$NKp44^+$ (%)	0.7	0.6	0.6	0.6	0.081^U	0.239

Table 1. continued

	Healthy controls (N=19)		CRC patients (N=71)			
	Mean	SD	Mean	SD	*P*-value	Corrected *P**-value
NKp44 (MFI)	131	13	115	26	0.002[U]	**0.041**
NKp46$^+$ (%)	48.7	18.0	34.7	16.9	0.002[T]	**0.031**
NKp46 (MFI)	732	345	478	271	0.003[U]	**0.031**
CD56^{bright} NK cells						
CD16$^+$ (%)	3.0	2.8	2.5	1.8	0.415[U]	0.695
CD158a$^+$ (%)	6.6	4.2	7.0	4.8	0.793[U]	0.928
CD158b$^+$ (%)	6.4	3.0	8.6	5.5	0.097[U]	0.273
NKG2A$^+$ (%)	6.6	4.6	4.5	3.3	0.044[U]	0.210
NKG2A$^+$ (MFI)	17020	7986	19052	5829	0.165 [U]	0.365
NKG2C$^+$ (%)	1.9	2.2	1.2	1.1	0.145[U]	0.333
NKG2C$^+$ (MFI)	1760	967	3038	3831	0.017[U]	0.105
CD161 (MFI)	2128	568	1767	578	0.012[U]	0.093
CD8$^+$ (%)	2.4	1.6	1.8	1.7	0.044[U]	0.195
CD8$^+$ (MFI)	2951	886	3098	1318	0.945[U]	0.977
DNAM-1 (MFI)	765	130	782	161	0.675[T]	0.872
NKG2D$^+$ (%)	6.2	4.1	4.7	3.7	0.062[U]	0.202
NKG2D$^+$ (MFI)	5321	1354	6210	1837	0.037[U]	0.191
NKp30 (MFI)	2145	418	2413	769	0.048[T]	0.198
NKp44$^+$ (%)	2.0	1.5	1.3	1.2	0.024[U]	0.135
NKp44 (MFI)	252	49	236	78	0.099[U]	0.267
NKp46$^+$ (%)	86.1	8.1	80.7	14.3	0.135[U]	0.322
NKp46 (MFI)	2343	613	2090	812	0.211[T]	0.451
NKT-like cells						
CD16$^+$ (%)	35.6	17.6	24.6	13.7	0.013[U]	0.090
CD158a$^+$ (%)	12.0	12.9	9.4	9.8	0.957[U]	0.973
CD158b$^+$ (%)	18.8	19.2	19.2	19.2	0.793[U]	0.910
NKG2A$^+$ (%)	31.9	25.4	28.3	21.0	0.692[U]	0.876
NKG2A$^+$ (MFI)	4529	4416	3444	3757	0.604[U]	0.851
NKG2C$^+$ (%)	15.0	15.6	14.2	13.3	0.909[U]	0.955
NKG2C$^+$ (MFI)	2308	2256	3205	3300	0.283[U]	0.532
CD161 (MFI)	4509	4407	4445	5434	0.452[U]	0.737
CD8$^+$ (%)	64.1	16.9	70.4	17.9	0.134[U]	0.332
CD8$^+$ (MFI)	11870	6011	11673	5556	0.968[U]	0.968
DNAM-1 (MFI)	847	211	766	279	0.238[T]	0.476
NKG2D$^+$ (%)	85.4	14.5	88.6	10.7	0.345[U]	0.629
NKG2D$^+$ (MFI)	4730	1589	4995	2041	0.602[T]	0.868
NKp30 (MFI)	217	70.1	250	136	0.797[U]	0.898
NKp44$^+$ (%)	1.4	1.0	1.4	1.2	0.774[U]	0.941
NKp44 (MFI)	134	17	120	33	0.002[U]	**0.025**
NKp46$^+$ (%)	5.4	1.9	4.6	2.8	0.011[U]	0.097
NKp46 (MFI)	112	53	61	47	0.000[U]	**0.000**

[*] T cells were investigated in 10 healthy donors and 47 CRC patients

[T] Independent samples T test

[U] Mann-Whitney U test

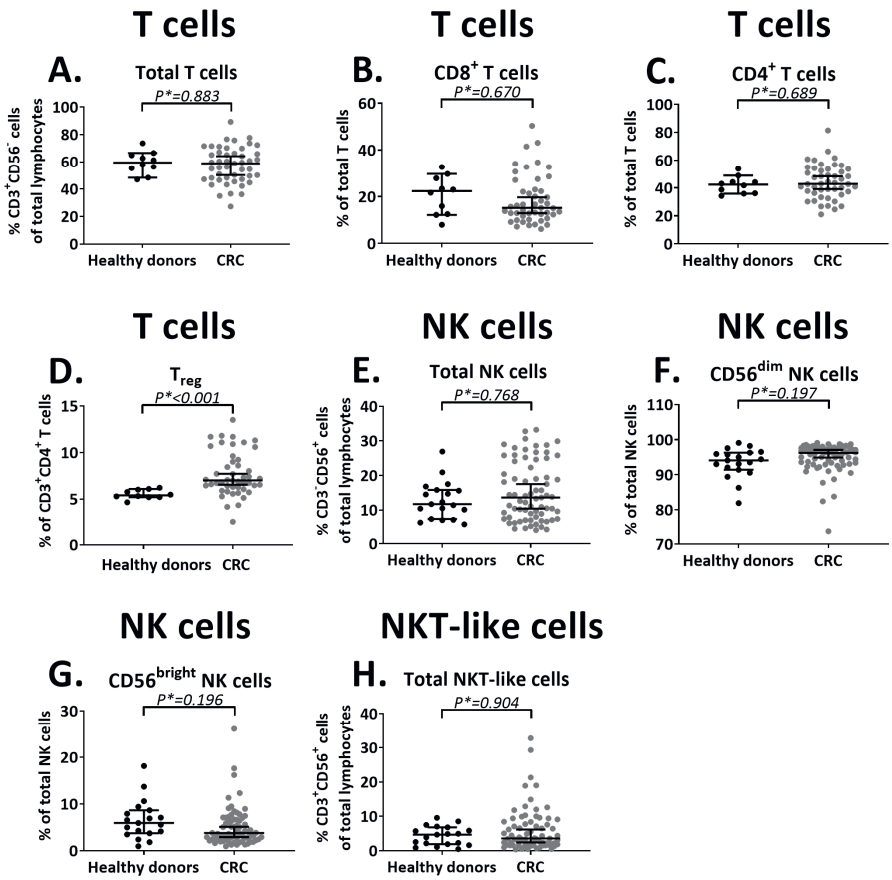


Figure 1. The peripheral blood immune cell subset distribution in CRC patients compared to healthy donors. The distribution of circulating immune cell subsets was compared between CRC patients (grey dots) and healthy donors (black dots). **A.** The total percentage of T cells (% $CD3^+CD56^-$ cells of total lymphocytes). **B.** Percentage of $CD8^+$ T cells (% of total T cells). **C.** Percentage of $CD4^+$ T cells (% of total T cells). **D.** Percentage of T_{reg} (% of $CD3^+CD4^+$ T cells). **E.** The total percentage of NK cells (% $CD3^-CD56^+$ cells of total lymphocytes). **F.** Percentage of $CD56^{dim}$ NK cells (% of total NK cells). **G.** Percentage of $CD56^{bright}$ NK cells (% of total NK cells). **H.** The total percentage of NKT-like cells (% $CD3^+CD56^+$ cells of total lymphocytes). The bars show median percentage of the respective immune cell subset including 95% CI.

Reduced expression of natural cytotoxicity receptors on circulating $CD56^{dim}$ NK cells and NKT-like cells from colorectal cancer patients compared to healthy donors

We subsequently evaluated the immunophenotype of circulating NK cells and NKT-like cells in CRC patients compared to healthy donors (Table 2). The percentage of $NKp44^+$ cells within the $CD56^{dim}$ NK

cell subset and NKT-like subset were comparable between CRC patients and healthy donors (*P**=0.239 and *P**=0.941, respectively) (Figure 2A;2B). In contrast, the expression level (MFI) of NKp44 was decreased in CRC patients on both $CD56^{dim}$ NK cells (*P**=0.041) and NKT-like cells (*P**=0.025) compared to healthy donors (Figure 2D;2E). Interestingly, the presence (*P*=0.013) and expression (*P*<0.001) of NKp44 on $CD56^{dim}$ NK cells and NKT-like cells strongly correlated (Figure 2C;2F). Hence, patients with a low percentage positive cells or expression level of NKp44 for $CD56^{dim}$ NK cells also had a low percentage positive cells or expression level of NKp44 for NKT-like cells and the other way around. Additionally, we observed a decreased percentage of $NKp46^{+}$ cells within the $CD56^{dim}$ NK cell subset compared to healthy donors (*P**=0.031) (Figure 2G), whereas in the NKT-like cell population, no statistically significant difference was found, only a trend (*P**=0.097) (Figure 2H). In contrast to NKp44, the percentage $NKp46^{+}$ cells did not correlate (only a trend) between $CD56^{dim}$ NK cells and NKT-like cells (*P*=0.098) (Fig. 2i). Furthermore, the expression level of NKp46 was decreased on both $CD56^{dim}$ NK cells (*P**=0.031) and NKT-like cells (*P**<0.001) in CRC patients compared to healthy donors (Figure 2J;2K). Expression level of NKp46 strongly correlated on $CD56^{dim}$ NK cells and NKT-like cells (*P*=0.004) (Figure 2L). In contrast to the $CD56^{dim}$ NK cell and NKT-like cell populations, the percentage positive cells and expression level of NKp44 and NKp46 were not decreased in the $CD56^{bright}$ NK cell population (Table 2). No differences in other phenotypic markers were observed related to NK cells or NKT-like cells between CRC patients and healthy donors (Table 2).

No correlation between distribution of immune cell subsets and immunophenotype of circulating lymphocytes and tumor characteristics

After discovering different peripheral blood immune cell profiles in CRC patients compared to healthy donors, the relationship with disease stage was investigated. The course of tumor progression entails a series of stages that ultimately leads to the formation of metastases in distant organs. It is hypothesized that the systemic immune system is activated during tumor progression in CRC upon disruption of the basal membrane located underneath the intestinal epithelium which allows tumor cells to grow into surrounding tissue and to enter blood vessels. As such, CRC patients were subdivided according to their TNM at the time of diagnosis into patients without disruption of the basal membrane (stage 0/I, N=13), and patients with disruption of the basal membrane (stage II/III/IV, N=58). No differences were observed in the distribution of immune cell subsets or their immunophenotype between CRC patients with or without disruption of the basal membrane. Furthermore, we did not observe any correlation between the immune cell subset distribution or

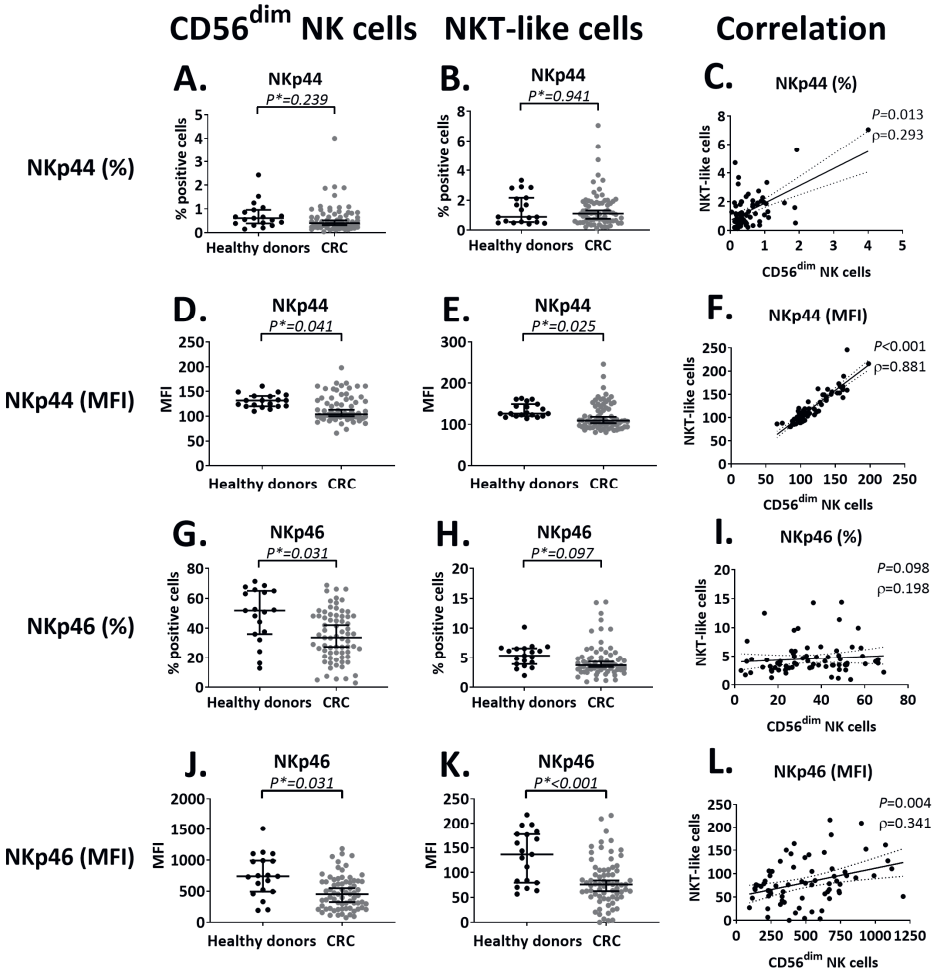


Figure 2. The peripheral blood immunophenotype of CD56^{dim} NK cells and NKT-like cells in CRC patients compared to healthy donors. The peripheral blood immune cell profile was compared between CRC patients (grey dots) and healthy donors (black dots). **A.** The percentage of NKp44^{+} CD56^{dim} NK cells. **B.** Percentage of NKp44^{+} NKT-like cells. **C.** Correlation between the percentage of NKp44^{+} CD56^{dim} NK cells and NKT-like cells. **D.** Expression level of NKp44 on CD56^{dim} NK cells. **E.** Expression level of NKp44 on NKT-like cells. **F.** Correlation between expression level of NKp44 on CD56^{dim} NK cells and NKT-like cells. **G.** Percentage of NKp46^{+} CD56^{dim} NK cells. **H.** Percentage of NKp46^{+} NKT-like cells. **I.** Correlation between the percentage of NKp46^{+} CD56^{dim} NK cells and NKT-like cells. **J.** Expression level of NKp46 on CD56^{dim} NK cells **K.** Expression level of NKp46 on NKT-like cells. **L.** Correlation between expression level of NKp46 on CD56^{dim} NK cells and NKT-like cells. The bars show median percentage or expression level of the respective immunophenotypic marker including 95% CI.

immunophenotype of circulating lymphocytes and tumor location, lymph node invasion or tumor differentiation grade (data not shown).

Association between the peripheral blood immune cell profile and disease-free survival of colorectal cancer patients

We subsequently wondered whether peripheral blood immune cell profiles were associated with clinical outcome in CRC patients. Therefore, survival plots as well as univariate and multivariate analyses were generated for stage II and III CRC patients at risk of developing metastases. The median percentage of positive cells or expression level of the immunophenotypic marker was used as cutoff. Kaplan-Meier plots and Log-rank tests revealed that CRC patients with above-median (>6.9%, N=12)

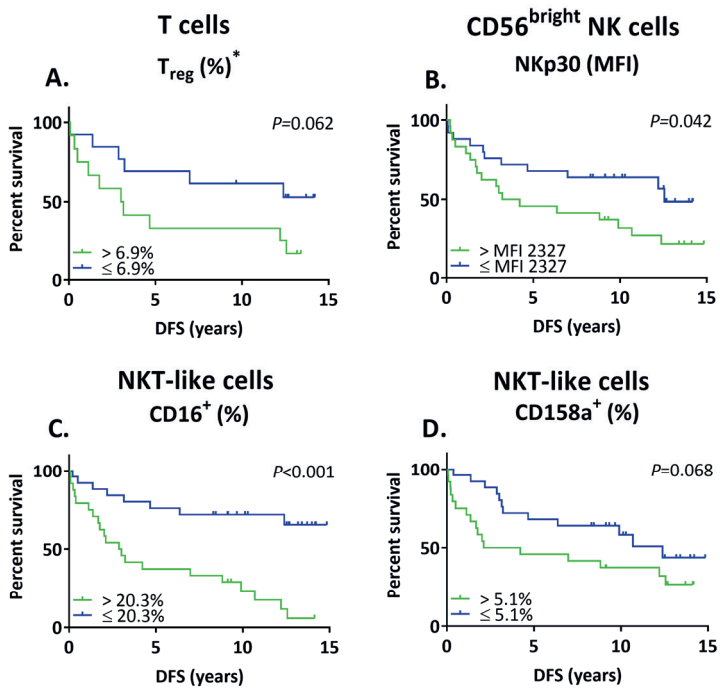


Figure 3. Relationship between the immune subset distribution and immunophenotype of circulating lymphocyte subsets with DFS of CRC patients. DFS curves are shown for stage II and III CRC patients (N=49) at risk for development of metastases. Stratifications were based on the median percentage of positive cells or expression level of the respective immunophenotypic marker. **A.** The percentage of T_{reg} (% $CD3^+CD4^+$ T cells). **B.** NKp30 expression level on $CD56^{bright}$ NK cells. **C.** Percentage of $CD16^+$ circulating NKT-like cells and, **D.** Percentage of $CD158a^+$ NKT-like cells. * This marker was only studied in 25 CRC patients at risk for development of metastasis (stage II and III).

percentage of circulating T_{reg} showed a trend towards shorter DFS compared to CRC patients with below-median (N=13) percentage (*P*=0.062) (Figure 3A), with a hazard ratio (HR) of 2.551 (95% confidence interval (CI) 0.921-7.069, *P*=0.072). Furthermore, survival analyses revealed that above-median (MFI >2327, N=24) expression of NKp30 on $CD56^{bright}$ NK cells (*P*=0.042) and above-median percentage (>20.3%, N=24) of $CD16^{+}$ NKT-like cells (*P*<0.001) were associated with short DFS in CRC patients, with a HR of 2.143 (95% CI 1.009-4.551, *P*=0.047) and 4.697 (95% CI 2.046-10.783, *P*<0.001), respectively (Figure 3B;3C). In contrast, CRC patients with above-median (>5.1%, N=24) percentage of inhibitory receptor $CD158a^{+}$ NKT-like cells showed a trend towards shorter DFS compared to patients with below-median (N=25) percentage (*P*=0.068) (Figure 3D) with a HR of 1.969 (95% CI 0.937-4.136, *P*=0.074). A multivariate analysis was performed for DFS in CRC patients which revealed that above-median percentage of activating receptor $CD16^{+}$ NKT-like cells (HR 4.192, 95% CI 1.578-11.137, *P*=0.004) remained significantly associated with shorter DFS in CRC patients when corrected for age, sex, tumor stage and lymph node invasion (Table 3). Thus, the presence of CD16 on circulating NKT-like cells may be a novel prognostic biomarker candidate for clinical outcome of CRC patients. No other correlations between immunophenotype and DFS were observed (Supplementary table 2).

Discussion

Recently, a new scoring system based on the immune profile in the tumor area, the 'immunoscore', has shown to be a strong prognostic biomarker in CRC [37-41]. High density of $CD3^{+}$ and $CD8^{+}$ tumor-infiltrating lymphocytes was reported to correlate with good clinical outcome in CRC [37,39-41]. We hypothesized that the distribution and phenotype of circulating immune cell subsets may reflect the local immune response in the TME, thereby providing potentially important information regarding disease progression and therapeutic decision making in CRC. In order to elucidate the role of the peripheral blood immune system in tumor progression and metastasis in CRC, this study aimed to characterize the distribution and immunophenotype of circulating T cells, NK cells, and NKT-like cells in CRC patients. These peripheral blood immune cell profiles were subsequently correlated to clinical follow-up data.

We first demonstrated that CRC patients present with alterations in peripheral blood immune subset distribution. We observed a significant increase in the percentage of circulating T_{reg} in CRC patients compared to healthy donors. This observation is in line with other studies [6,42,43], and suggests tumor escape in CRC by increasing T_{reg} which are known to suppress the immune system and, thereby, may support tumor progression using a plethora of different mechanisms [44,45].

Table 3. Univariate and multivariate analyses for DFS of CRC patients. Univariate and multivariate analyses for DFS were generated for stage II and III CRC patients (N=49) at risk for development of metastases. The median percentage of positive cells or median expression level was used as a cutoff (≤ median (N=25) and > median (N=24)). *P*-values, HRs, and their CIs were estimated from a Cox proportional hazard regression model, in which the first category was used as reference group. Statistically significant *P*-values are indicated in bold. Abbreviations: Ref. (reference group).

	Univariate analysis			**Multivariate analysis****		
Parameters	HR	95% CI	*P*-value	HR	95% CI	*P*-value
Age*	1.0	1.0-1.1	0.053			
Sex						
Female	Ref.					
Male	1.9	0.9-4.1	0.085			
Tumor type						
Colon	Ref.					
Rectum	1.3	0.5-3.2	0.571			
Tumor stage						
Stage II	Ref.					
Stage III	2.9	1.4-6.4	**0.006**			
Tumor differentiation grade						
Well/moderate	Ref.					
Poor	0.9	0.3-2.7	0.885			
Lymph node invasion						
No	Ref.					
Yes	2.8	1.3-6.2	**0.010**			
T_{reg} (%)***						
≤ 6.9%	Ref.			Ref.		
> 6.9%	2.6	0.9-7.1	0.072	3.1	0.7-13.3	0.119
NKp30 $CD56^{bright}$ NK cells (MFI)						
≤ MFI 2327	Ref.			Ref.		
> MFI 2327	2.1	1.0-4.6	**0.047**	1.8	0.8-3.9	0.169
$CD16^{+}$ NKT-like cells (%)						
≤ 20.3%	Ref.			Ref.		
> 20.3%	4.7	2.0-10.8	**<0.001**	4.2	1.6-11.1	**0.004**
$CD158a^{+}$ NKT-like cells (%)						
≤ 5.1%	Ref.			Ref.		
> 5.1%	2.0	0.9-4.1	0.074	1.8	0.8-3.9	0.134

* Age at time of surgery

** Corrected for age, sex, tumor stage and lymph node invasion

*** This marker was only studied in 25 CRC patients at risk for development of metastasis (stage II and III)

Additionally, we demonstrated that CRC patients present with an altered immunophenotype of circulating $CD56^{dim}$ NK cells compared to healthy donors, characterized by reduced expression of the NCRs NKp44 and NKp46. These findings are consistent with previous studies that showed a reduced number of activating receptor (e.g. NKG2D, NKp30, NKp46, and DNAM-1)-positive NK cells in peripheral blood of patients with CRC [4,5]. Additionally, NK cells with downregulated activating receptors showed impaired IFN-γ secretion and degranulation upon

activation, thereby implying impaired function [4]. These studies did, however, not discriminate between the two major subpopulations of NK cells, namely $CD56^{dim}$ and $CD56^{bright}$ NK cells, which are known to differ in phenotype as well as function [18,19]. Whereas $CD56^{dim}$ NK cells have the potential to kill tumor cells, $CD56^{bright}$ NK cells primarily produce large amounts of cytokines [18,19]. We showed that circulating $CD56^{dim}$ NK cells were phenotypically altered in CRC patients, whereas $CD56^{bright}$ NK cells were not, suggesting that this NK cell subset, involved in cytokine production, is phenotypically, and thereby probably also functionally, not altered in CRC patients. This implicates different roles of $CD56^{dim}$ and $CD56^{bright}$ NK cells in cancer progression, thereby emphasizing the need to discriminate between these NK cell subsets in future studies. Furthermore, we also showed downregulation of the NCRs NKp44 and NKp46 on circulating NKT-like cells in CRC patients compared to healthy donors. In contrast to NK cells, the immunophenotype of circulating NKT cells (in both healthy donors and cancer patients) is not well defined due to the lack of specific markers to identify this immune cell subset. To our knowledge, only one study reported on the immunophenotype of circulating NKT-like cells in CRC. This study showed significantly lower numbers of circulating $NKG2D^{+}$ NKT-like cells in CRC patients with metastatic disease compared to healthy donors [46]. Our results suggest that presence of a tumor affects expression levels of NKp44 and NKp46 receptors on both $CD56^{dim}$ NK cells and NKT-like cells. Over the years, many studies have focused on the effects of a tumor on NK cell function. For instance, NK cells in the TME adapt to survive hypoxic stress by upregulating hypoxia-inducible factor (HIF)-1α which is associated with impaired upregulation of NKp44 and NKp46 in response to activating cytokines such as IL-2, IL-12, IL-15 and IL-21 [47]. Furthermore, immune cells and fibroblasts in the TME may produce immunosuppressive cytokines and signal molecules that downregulate [48-50] or prevent IL-2-induced upregulation [51,52] of NCRs on NK cells. Furthermore, tumor cells may induce downregulation of NCRs on NK cells as a response to hypoxic stress via prostaglandin E2 production or lactate release [53,54]. Finally, tumor cells may alter the function of NK cells via overexpression of NK cell ligands. For instance, overexpression of proliferating cell nuclear antigen (PCNA) by tumor cells impaired NKp44-mediated NK cell attack in an *in vitro* model [55,56]. Whether PCNA also actively downregulates the NKp44 receptor on NK cells is not clear and requires additional research. In conclusion, we observed reduced expression of the NCRs NKp44 and NKp46 on circulating $CD56^{dim}$ NK cells and NKT-like cells in CRC patients, which may be caused by the presence of the tumor via various mechanisms, resulting in functional impairment in these subsets, thereby promoting tumor escape.

Furthermore, we addressed the question whether the peripheral blood immune cell profile in CRC patients was related to tumor stage. Although the peripheral blood immune cell profile was clearly altered in CRC patients as compared to healthy controls, no major shift in the distribution of

PBMC subsets was observed in relation with tumor stage of CRC patients. Hence, differences in the peripheral blood immune cell profile were mainly related to the fact that these patients had a colorectal tumor rather than to tumor stage. We did, however, observe a relationship between the immune subset distribution and phenotype of circulating PBMCs and clinical outcome. This suggests that circulating immune cell profiles have prognostic value (i.e. biomarker candidates) for the identification of patients with low tumor grades with a high risk of developing metastatic disease. In general, it is hypothesized that decreased percentages of cytotoxic immune cell subsets or expression levels of activating receptors and increased percentages of regulatory immune cell subsets or expression levels of inhibitory receptors represent an immunocompromised state of the immune system and would therefore be correlated with shorter DFS in CRC patients [4,57,58]. In line with this hypothesis, our results revealed that CRC patients with high numbers of circulating T_{reg} showed a trend towards shorter DFS compared to patients with low numbers of circulating T_{reg}, an association also observed by others. For instance, an association was reported between T_{reg}-mediated suppression of tumor-specific $CD4^+$ T cells prior to surgery and tumor recurrence in CRC patients at 12 months [59], whereas FOLFIRI (levo-leucovorin, 5-fluorouracil, irinotexan)- bevacizumab therapy of metastatic CRC patients was associated with better clinical outcome, but also with reduced numbers of circulating T_{reg} [60,61]. Additionally, in line with our hypothesis, patients with a high percentage of inhibitory receptor $CD158a^+$ circulating NKT-like cells showed a trend towards shorter DFS. Since the CD158a receptor recognizes HLA class I molecules, it would be interesting to study HLA class I tumor cell expression in primary colorectal tumors to investigate whether there is an association with the presence of circulating $CD158a^+$ NKT-like immune cells. In contrast with the hypothesis, we observed a statistically significant association between a high expression level of activating receptor NKp30 on circulating $CD56^{bright}$ NK cells and a high percentage of $CD16^+$ circulating NKT-like cells and short DFS in CRC patients. A multivariate analysis revealed that the latter, high percentage of $CD16^+$ NKT-like cells, was even independently associated with shorter DFS in CRC patients. From these observations, it might be deduced that the NKp30 and CD16 receptors are activated during the course of tumor cell dissemination. The exact role of the NKp30 and CD16 receptors in the process of dissemination in CRC patients has yet to be elucidated. Possibly, the status of these receptors in patients with poor clinical outcome might affect the tumor immune response, thus acting as an active selection mechanism by which tumor cells escape immune-mediated destruction. In that case, it would for instance be likely that ligands for these receptors on tumor cells are downregulated.

In conclusion, we demonstrated profound differences in subset distribution and immunophenotype of PBMCs in CRC patients compared to healthy donors, characterized by an

increased percentage of T_{reg}, and reduced expression of NCRs NKp44 and NKp46 on both $CD56^{dim}$ NK cells and NKT-like cells. Additionally, a multivariate analysis revealed that above median percentage of $CD16^{+}$ NKT-like cells was independently associated with shorter DFS in CRC patients. These findings highlight the potential use of circulating lymphocyte subsets as prognostic biomarkers in CRC and contribute to better insight into the role of systemic immune profiles in tumor progression.

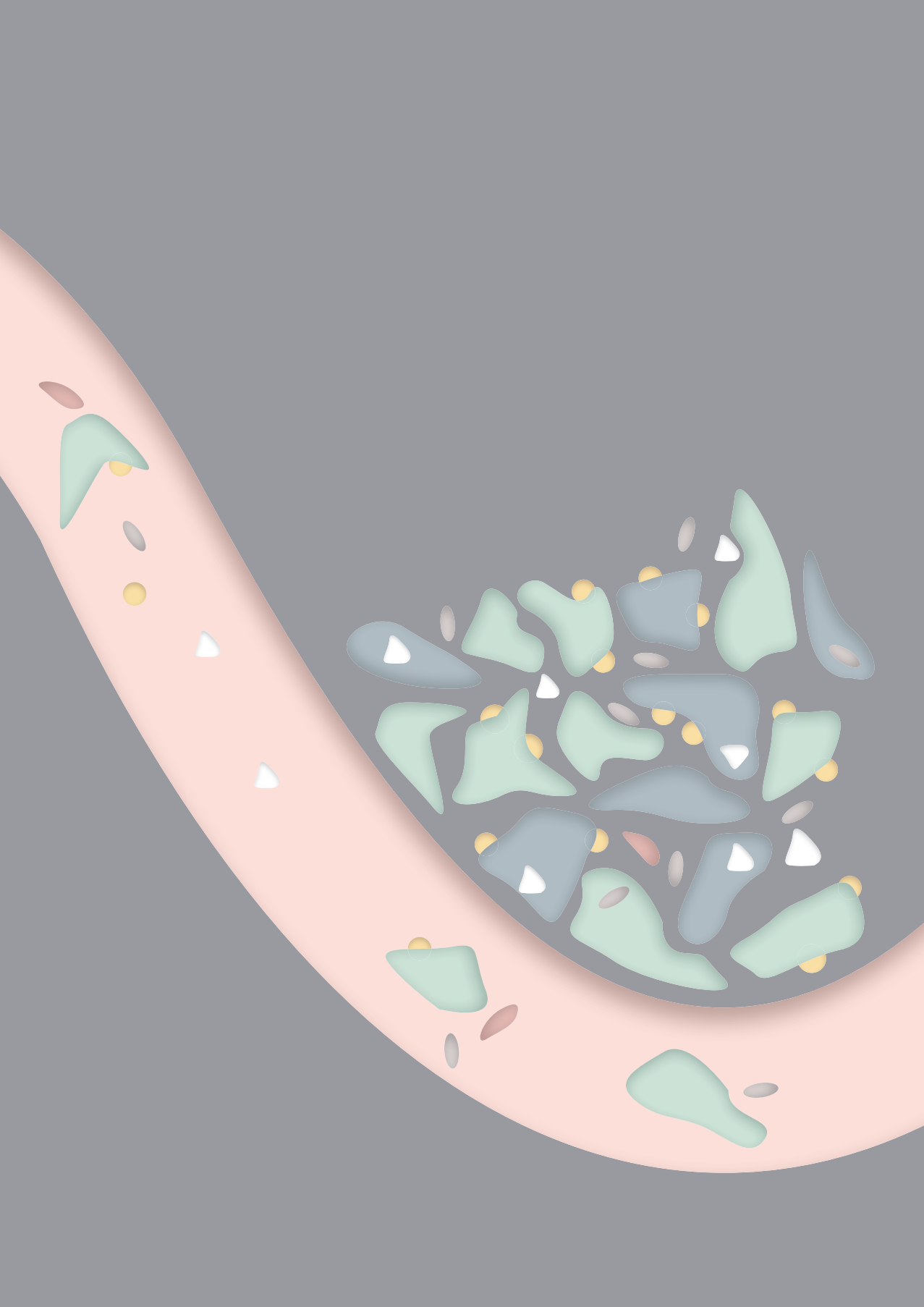
References

1. Torre, L.A., Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, Jemal A. Global cancer statistics, 2012. *CA Cancer J Clin* **2015**, *65*, 87-108.
2. Schmoll, H.J., Van Cutsem E, Stein A, Valentini V, Glimelius B, Haustermans K, et al. ESMO Consensus Guidelines for management of patients with colon and rectal cancer. a personalized approach to clinical decision making. *Ann Oncol* **2012**, *23*, 2479-2516, doi:mds236 [pii];10.1093/annonc/mds236 [doi].
3. Van Cutsem, E., Cervantes A, Nordlinger B, Arnold D; ESMO Guidelines Working Group. Metastatic colorectal cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. *Ann Oncol* **2014**, *Suppl 3*, iii1-9.
4. Rocca, Y.S., Roberti MP, Arriaga JM, Amat M, Bruno L, Pampena MB, et al. Altered phenotype in peripheral blood and tumor-associated NK cells from colorectal cancer patients. *Innate Immun* **2013**, *19*, 76-85, doi:1753425912453187 [pii];10.1177/1753425912453187 [doi].
5. Peng, Y.P., Zhu Y, Zhang JJ, Xu ZK, Qian ZY, Dai CC, et al. Comprehensive analysis of the percentage of surface receptors and cytotoxic granules positive natural killer cells in patients with pancreatic cancer, gastric cancer, and colorectal cancer. *J Transl Med* **2013**, *11*, 262, doi:1479-5876-11-262 [pii];10.1186/1479-5876-11-262 [doi].
6. Ling, K.L., Pratap SE, Bates GJ, Singh B, Mortensen NJ, George BD, et al. Increased frequency of regulatory T cells in peripheral blood and tumour infiltrating lymphocytes in colorectal cancer patients. *Cancer Immun* **2007**, *7*, 7.
7. Luckheeram, R.V.; Zhou, R.; Verma, A.D.; Xia, B. CD4(+)T cells: differentiation and functions. *Clin Dev Immunol* **2012**, *2012*, 925135, doi:10.1155/2012/925135.
8. Zhang, X., Kelaria S, Kerstetter J, Wang J. The functional and prognostic implications of regulatory T cells in colorectal carcinoma. *J Gastrointest Oncol* **2015**, *6*, 307-313, doi:10.3978/j.issn.2078-6891.2015.017 [doi];jgo-06-03-307 [pii].
9. Aptsiauri, N., Cabrera T, Mendez R, Garcia-Lora A, Ruiz-Cabello F, Garrido F. Role of altered expression of HLA class I molecules in cancer progression. *Adv Exp Med Biol* **2007**, *601*, 123-131.
10. Menon, A.G., Morreau H, Tollenaar RA, Alphenaar E, Van Puijenbroek M, Putter H, et al. Down-regulation of HLA-A expression correlates with a better prognosis in colorectal cancer patients. *Lab Invest* **2002**, *82*, 1725-1733.
11. Watson, N.F., Ramage JM, Madjd Z, Spendlove I, Ellis IO, Scholefield JH, et al. Immunosurveillance is active in colorectal cancer as downregulation but not complete loss of MHC class I expression correlates with a poor prognosis. *Int J Cancer* **2006**, *118*, 6-10, doi:10.1002/ijc.21303 [doi].
12. Zeestraten, E.C.; Reimers, M.S.; Saadatmand, S.; Goossens-Beumer, I.J.; Dekker, J.W.; Liefers, G.J.; van den Elsen, P.J.; van de Velde, C.J.; Kuppen, P.J. Combined analysis of HLA class I, HLA-E and HLA-G predicts prognosis in colon cancer patients. *Br.J.Cancer* **2014**, *110*, 459-468, doi:bjc2013696 [pii];10.1038/bjc.2013.696 [doi].
13. Sandel, M.H., Speetjens FM, Menon AG, Albertsson PA, Basse PH, Hokland M, et al. Natural killer cells infiltrating colorectal cancer and MHC class I expression. *Mol Immunol* **2005**, *42*, 541-546, doi:S0161-5890(04)00314-1 [pii];10.1016/j.molimm.2004.07.039 [doi].
14. Swets, M., König MH, Zaalberg A, Dekker-Ensink NG, Gelderblom H, van de Velde CJ, et al. HLA-G and classical HLA class I expression in primary colorectal cancer and associated liver metastases. *Hum Immunol* **2016**, *doi: 10.1016/j.humimm.2016.03.001*, doi:10.1016/j.humimm.2016.03.001.
15. Chang, C.C., Campoli M, Ferrone S. Classical and nonclassical HLA class I antigen and NK Cell-activating ligand changes in malignant cells: current challenges and future directions. *Adv Cancer Res* **2005**, *93*, 189-234, doi:S0065230X05930066 [pii];10.1016/S0065-230X(05)93006-6 [doi].

16. Caligiuri, M.A. Human natural killer cells. *Blood* **2008**, *112*, 461-469, doi:112/3/461 [pii];10.1182/blood-2007-09-077438 [doi].
17. Vivier, E., Tomasello E, Baratin M, Walzer T, Ugolini S. Functions of natural killer cells. *Nat Immunol* **2008**, *9*, 503-510, doi:ni1582 [pii];10.1038/ni1582 [doi].
18. Cooper, M.A.; Fehniger, T.A.; Caligiuri, M.A. The biology of human natural killer-cell subsets. *Trends Immunol* **2001**, *22*, 633-640.
19. Jonges, L.E.; Albertsson, P.; van Vlierberghe, R.L.; Ensink, N.G.; Johansson, B.R.; van de Velde, C.J.; Fleuren, G.J.; Nannmark, U.; Kuppen, P.J. The phenotypic heterogeneity of human natural killer cells: presence of at least 48 different subsets in the peripheral blood. *Scand J Immunol* **2001**, *53*, 103-110.
20. Biassoni, R., Cantoni C, Pende D, Sivori S, Parolini S, Vitale M, et al. Human natural killer cell receptors and co-receptors. *Immunol Rev* **2001**, *181*, 203-214.
21. Dutertre, C.A., Bonnin-Gelize E, Pulford K, Bourel D, Fridman WH, Teillaud JL. A novel subset of NK cells expressing high levels of inhibitory FcgammaRIIB modulating antibody-dependent function. *J Leukoc Biol* **2008**, *84*, 1511-1520, doi:jlb.0608343 [pii];10.1189/jlb.0608343 [doi].
22. Addison, E.G.; North, J.; Bakhsh, I.; Marden, C.; Haq, S.; Al-Sarraj, S.; Malayeri, R.; Wickremasinghe, R.G.; Davies, J.K.; Lowdell, M.W. Ligation of CD8alpha on human natural killer cells prevents activation-induced apoptosis and enhances cytolytic activity. *Immunology* **2005**, *116*, 354-361, doi:10.1111/j.1365-2567.2005.02235.x.
23. Bendelac, A.; Savage, P.B.; Teyton, L. The biology of NKT cells. *Annu Rev Immunol* **2007**, *25*, 297-336, doi:10.1146/annurev.immunol.25.022106.141711.
24. Makino, Y., Kanno R, Ito T, Higashino K, Taniguchi M. Predominant expression of invariant V alpha 14+ TCR alpha chain in NK1.1+ T cell populations. *Int Immunol* **1995**, *7*, 1157-1161.
25. Krijgsman, D.; Hokland, M.; Kuppen, P.J.K. The Role of Natural Killer T Cells in Cancer-A Phenotypical and Functional Approach. *Front Immunol* **2018**, *9*, 367, doi:10.3389/fimmu.2018.00367.
26. Campbell, J.J.; Qin, S.; Unutmaz, D.; Soler, D.; Murphy, K.E.; Hodge, M.R.; Wu, L.; Butcher, E.C. Unique subpopulations of CD56+ NK and NK-T peripheral blood lymphocytes identified by chemokine receptor expression repertoire. *J Immunol* **2001**, *166*, 6477-6482.
27. van den Heuvel, M.J.; Peralta, C.G.; Hatta, K.; Han, V.K.; Clark, D.A. Decline in number of elevated blood CD3(+) CD56(+) NKT cells in response to intravenous immunoglobulin treatment correlates with successful pregnancy. *Am J Reprod Immunol* **2007**, *58*, 447-459, doi:10.1111/j.1600-0897.2007.00529.x.
28. Chan, W.K.; Rujkijyanont, P.; Neale, G.; Yang, J.; Bari, R.; Das Gupta, N.; Holladay, M.; Rooney, B.; Leung, W. Multiplex and genome-wide analyses reveal distinctive properties of KIR+ and CD56+ T cells in human blood. *J Immunol* **2013**, *191*, 1625-1636, doi:10.4049/jimmunol.1300111.
29. Jiang, Y.; Cui, X.; Cui, C.; Zhang, J.; Zhou, F.; Zhang, Z.; Fu, Y.; Xu, J.; Chu, Z.; Liu, J., et al. The function of CD3+CD56+ NKT-like cells in HIV-infected individuals. *Biomed Res Int* **2014**, *2014*, 863625, doi:10.1155/2014/863625.
30. Peng, L.S.; Mao, F.Y.; Zhao, Y.L.; Wang, T.T.; Chen, N.; Zhang, J.Y.; Cheng, P.; Li, W.H.; Lv, Y.P.; Teng, Y.S., et al. Altered phenotypic and functional characteristics of CD3+CD56+ NKT-like cells in human gastric cancer. *Oncotarget* **2016**, *7*, 55222-55230, doi:10.18632/oncotarget.10484.
31. Li, X.F.; Dai, D.; Song, X.Y.; Liu, J.J.; Zhu, L.; Zhu, X.; Ma, W.; Xu, W. A different representation of natural T cells and natural killer cells between tumor-infiltrating and periphery lymphocytes in human hepatocellular carcinoma. *Oncol Lett* **2017**, *13*, 3291-3298, doi:10.3892/ol.2017.5808.
32. Kim, C.H.; Johnston, B.; Butcher, E.C. Trafficking machinery of NKT cells: shared and differential chemokine receptor expression among V alpha 24(+)V beta 11(+) NKT cell subsets with distinct cytokine-producing capacity. *Blood* **2002**, *100*, 11-16, doi:10.1182/blood-2001-12-0196.
33. Godfrey, D.I.; MacDonald, H.R.; Kronenberg, M.; Smyth, M.J.; Van Kaer, L. NKT cells: what's in a name? *Nat Rev Immunol* **2004**, *4*, 231-237, doi:10.1038/nri1309.

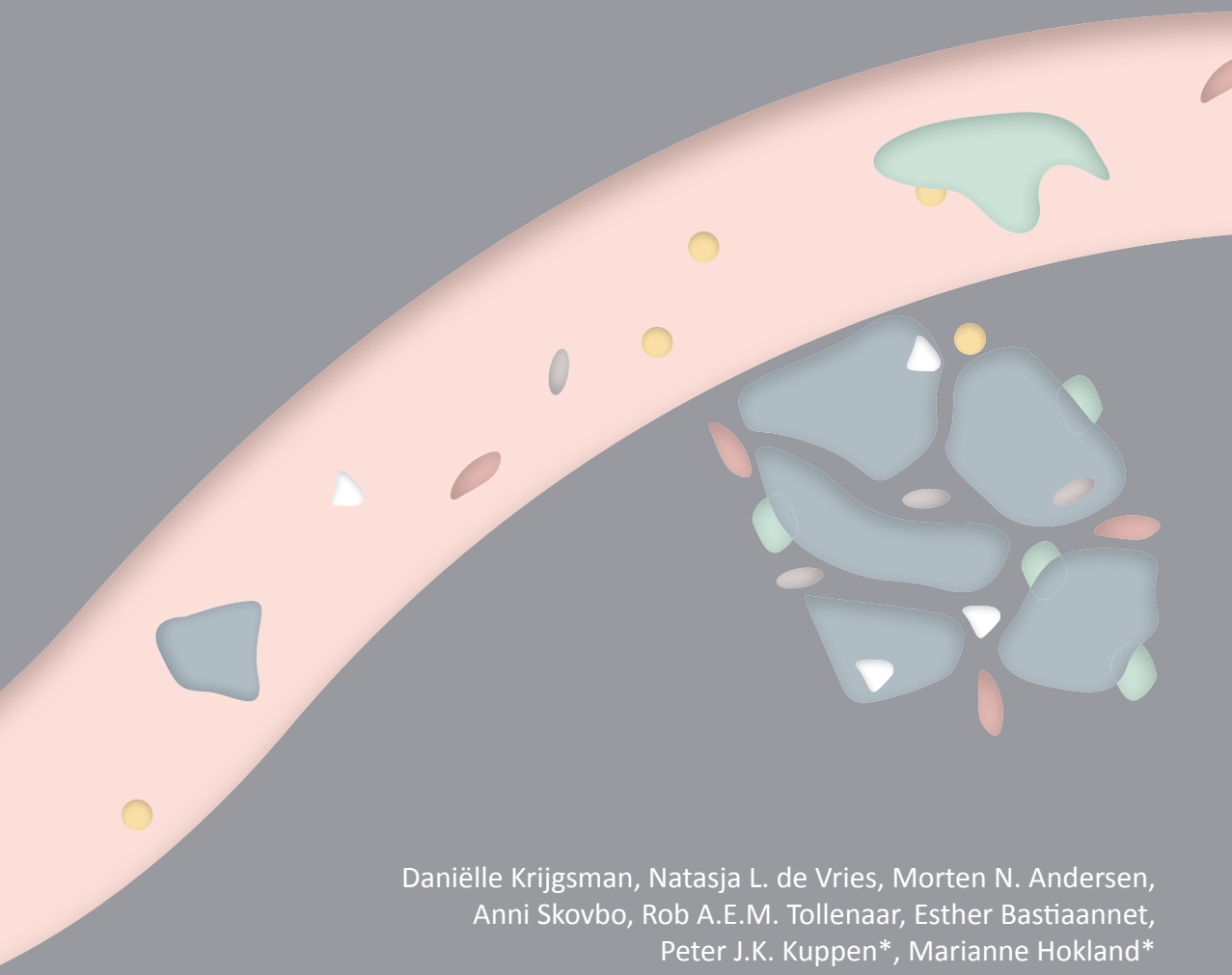
34. Maecker, H.T., Trotter J. Flow cytometry controls, instrument setup, and the determination of positivity. *Cytometry A* **2006**, *69*, 1037-1042.
35. Roederer, M. Spectral compensation for flow cytometry: visualization artifacts, limitations, and caveats. *Cytometry* **2001**, *45*, 194-205, doi:10.1002/1097-0320(20011101)45:3<194::AID-CYTO1163>3.0.CO;2-C [pii].
36. Benjamini, Y.; Hochberg, Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *Journal of the Royal Statistical Society. Series B.* **1995**, *57*, 289-300.
37. Galon, J.; Mlecnik, B.; Bindea, G.; Angell, H.K.; Berger, A.; Lagorce, C.; Lugli, A.; Zlobec, I.; Hartmann, A.; Bifulco, C., et al. Towards the introduction of the 'Immunoscore' in the classification of malignant tumours. *J. Pathol.* **2014**, *232*, 199-209.
38. Pagès, F.; Kirilovsky, A.; Mlecnik, B.; Asslaber, M.; Tosolini, M.; Bindea, G.; Lagorce, C.; Wind, P.; Marliot, F.; Bruneval, P., et al. In situ cytotoxic and memory T cells predict outcome in patients with early-stage colorectal cancer. *J. Clin. Oncol.* **2009**, *27*, 5944-5951.
39. Galon, J.; Costes, A.; Sanchez-Cabo, F.; Kirilovsky, A.; Mlecnik, B.; Lagorce-Pages, C.; Tosolini, M.; Camus, M.; Berger, A.; Wind, P., et al. Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. *Science* **2006**, *313*, 1960-1964, doi:10.1126/science.1129139.
40. Mlecnik, B.; Tosolini, M.; Kirilovsky, A.; Berger, A.; Bindea, G.; Meatchi, T.; Bruneval, P.; Trajanoski, Z.; Fridman, W.H.; Pages, F., et al. Histopathologic-based prognostic factors of colorectal cancers are associated with the state of the local immune reaction. *J Clin Oncol* **2011**, *29*, 610-618, doi:10.1200/JCO.2010.30.5425.
41. Pages, F.; Mlecnik, B.; Marliot, F.; Bindea, G.; Ou, F.S.; Bifulco, C.; Lugli, A.; Zlobec, I.; Rau, T.T.; Berger, M.D., et al. International validation of the consensus Immunoscore for the classification of colon cancer: a prognostic and accuracy study. *Lancet* **2018**, *391*, 2128-2139, doi:10.1016/S0140-6736(18)30789-X.
42. Liu, Z., Huang Q, Liu G, Dang L, Chu D, Tao K, et al. Presence of FOXP3(+)Treg cells is correlated with colorectal cancer progression. *Int J Clin Exp Med* **2014**, *7*, 1781-1785.
43. Wolf, A.M., Wolf D, Steurer M, Gastl G, Gunsilius E, Grubeck-Loebenstein B. Increase of regulatory T cells in the peripheral blood of cancer patients. *Clin Cancer Res* **2003**, *9*, 606-612.
44. Campbell, D.J., Koch MA. Phenotypical and functional specialization of FOXP3+ regulatory T cells. *Nat Rev Immunol* **2011**, *11*, 119-130.
45. Lin, Y.C.; Mahalingam, J.; Chiang, J.M.; Su, P.J.; Chu, Y.Y.; Lai, H.Y.; Fang, J.H.; Huang, C.T.; Chiu, C.T.; Lin, C.Y. Activated but not resting regulatory T cells accumulated in tumor microenvironment and correlated with tumor progression in patients with colorectal cancer. *Int J Cancer* **2013**, *132*, 1341-1350, doi:10.1002/ijc.27784.
46. Gharagozloo, M.; Rezaei, A.; Kalantari, H.; Bahador, A.; Hassannejad, N.; Maracy, M.; Nouri, N.; Sedghi, M.; Ghazanfari, H.; Bayat, B. Decline in peripheral blood NKG2D+CD3+CD56+ NKT cells in metastatic colorectal cancer patients. *Bratisl Lek Listy* **2018**, *119*, 6-11, doi:10.4149/BLL_2018_002.
47. Balsamo, M.; Manzini, C.; Pietra, G.; Raggi, F.; Blengio, F.; Mingari, M.C.; Varesio, L.; Moretta, L.; Bosco, M.C.; Vitale, M. Hypoxia downregulates the expression of activating receptors involved in NK-cell-mediated target cell killing without affecting ADCC. *Eur J Immunol* **2013**, *43*, 2756-2764, doi:10.1002/eji.201343448.
48. Vitale, M.; Cantoni, C.; Pietra, G.; Mingari, M.C.; Moretta, L. Effect of tumor cells and tumor microenvironment on NK-cell function. *Eur J Immunol* **2014**, *44*, 1582-1592, doi:10.1002/eji.201344272.
49. Han, B.; Mao, F.Y.; Zhao, Y.L.; Lv, Y.P.; Teng, Y.S.; Duan, M.; Chen, W.; Cheng, P.; Wang, T.T.; Liang, Z.Y., et al. Altered NKp30, NKp46, NKG2D, and DNAM-1 Expression on Circulating NK Cells Is Associated with Tumor Progression in Human Gastric Cancer. *J Immunol Res* **2018**, *2018*, 6248590, doi:10.1155/2018/6248590.

50. Valayer, A.; Brea, D.; Lajoie, L.; Avezard, L.; Combes-Soia, L.; Labas, V.; Korkmaz, B.; Thibault, G.; Baranek, T.; Si-Tahar, M. Neutrophils can disarm NK cell response through cleavage of NKp46. *J Leukoc Biol* **2017**, *101*, 253-259, doi:10.1189/jlb.3AB0316-140RR.
51. Balsamo, M.; Scordamaglia, F.; Pietra, G.; Manzini, C.; Cantoni, C.; Boitano, M.; Queirolo, P.; Vermi, W.; Facchetti, F.; Moretta, A., et al. Melanoma-associated fibroblasts modulate NK cell phenotype and antitumor cytotoxicity. *Proc Natl Acad Sci U S A* **2009**, *106*, 20847-20852, doi:10.1073/pnas.0906481106.
52. Li, T.; Yi, S.; Liu, W.; Jia, C.; Wang, G.; Hua, X.; Tai, Y.; Zhang, Q.; Chen, G. Colorectal carcinoma-derived fibroblasts modulate natural killer cell phenotype and antitumor cytotoxicity. *Med Oncol* **2013**, *30*, 663, doi:10.1007/s12032-013-0663-z.
53. Pietra, G.; Manzini, C.; Rivara, S.; Vitale, M.; Cantoni, C.; Petretto, A.; Balsamo, M.; Conte, R.; Benelli, R.; Minghelli, S., et al. Melanoma cells inhibit natural killer cell function by modulating the expression of activating receptors and cytolytic activity. *Cancer Res* **2012**, *72*, 1407-1415, doi:10.1158/0008-5472.CAN-11-2544.
54. Husain, Z.; Huang, Y.; Seth, P.; Sukhatme, V.P. Tumor-derived lactate modifies antitumor immune response: effect on myeloid-derived suppressor cells and NK cells. *J Immunol* **2013**, *191*, 1486-1495, doi:10.4049/jimmunol.1202702.
55. Rosental, B.; Brusilovsky, M.; Hadad, U.; Oz, D.; Appel, M.Y.; Afergan, F.; Yossef, R.; Rosenberg, L.A.; Aharoni, A.; Cerwenka, A., et al. Proliferating cell nuclear antigen is a novel inhibitory ligand for the natural cytotoxicity receptor NKp44. *J Immunol* **2011**, *187*, 5693-5702, doi:10.4049/jimmunol.1102267.
56. Horton, N.C.; Mathew, S.O.; Mathew, P.A. Novel interaction between proliferating cell nuclear antigen and HLA I on the surface of tumor cells inhibits NK cell function through NKp44. *PLoS One* **2013**, *8*, e59552, doi:10.1371/journal.pone.0059552.
57. Scurr, M.; Gallimore, A.; Godkin, A. T cell subsets and colorectal cancer: discerning the good from the bad. *Cell Immunol* **2012**, *279*, 21-24, doi:10.1016/j.cellimm.2012.08.004.
58. Choi, J.; Maeng, H.G.; Lee, S.J.; Kim, Y.J.; Kim, D.W.; Lee, H.N.; Namgung, J.H.; Oh, H.M.; Kim, T.J.; Jeong, J.E., et al. Diagnostic value of peripheral blood immune profiling in colorectal cancer. *Ann Surg Treat Res* **2018**, *94*, 312-321, doi:10.4174/astr.2018.94.6.312.
59. Betts, G.; Jones, E.; Junaid, S.; El-Shanawany, T.; Scurr, M.; Mizen, P.; Kumar, M.; Jones, S.; Rees, B.; Williams, G., et al. Suppression of tumour-specific CD4(+) T cells by regulatory T cells is associated with progression of human colorectal cancer. *Gut* **2012**, *61*, 1163-1171, doi:10.1136/gutjnl-2011-300970.
60. Roselli, M.; Formica, V.; Cereda, V.; Jochems, C.; Richards, J.; Grenga, I.; Orlandi, A.; Ferroni, P.; Guadagni, F.; Schlom, J. The association of clinical outcome and peripheral T-cell subsets in metastatic colorectal cancer patients receiving first-line FOLFIRI plus bevacizumab therapy. *Oncoimmunology* **2016**, *5*, e1188243, doi:10.1080/2162402X.2016.1188243.
61. Xu, T.; Lu, J.; An, H. The relative change in regulatory T cells / T helper lymphocytes ratio as parameter for prediction of therapy efficacy in metastatic colorectal cancer patients. *Oncotarget* **2017**, *8*, 109079-109093, doi:10.18632/oncotarget.22606.



CHAPTER 4

The effects of tumor resection and adjuvant therapy On the peripheral blood immune cell profile In patients with colon carcinoma



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Abstract

Objective: The subset distribution and immunophenotype of circulating immune cells ("peripheral blood immune cell profile") may reflect tumor development and response to cancer treatment. In order to use the peripheral blood immune cell profile as biomarker to monitor patients over time, it is crucial to know how immune cell subsets respond to therapeutic interventions. In this study, we investigated the effects of tumor resection and adjuvant therapy on the peripheral blood immune cell profile in patients with colon carcinoma (CC).

Methods: The subset distribution and immunophenotype of T cells ($CD3^+CD56^-$), $CD56^{dim}$ NK cells ($CD3^-CD56^{dim}$), $CD56^{bright}$ NK cells ($CD3^-CD56^{bright}$), and NKT-like cells ($CD3^+CD56^+$) were studied in preoperative and postoperative peripheral blood mononuclear cell (PBMC) samples of 24 patients with CC by multiparameter flow cytometry. Changes in immunophenotype of circulating immune cells after tumor resection were studied in patients treated with and without (capecitabine-based) adjuvant therapy.

Results: The NKT-like cell (% of total PBMCs) and $CD8^+$ T cell (% of total T cells) populations expanded in the peripheral blood of non-adjuvant treated CC patients after surgery. NK- and NKT-like cells showed upregulation of activating receptors and downregulation of inhibitory receptors in non-adjuvant treated CC patients after surgery. These changes were not observed in the peripheral blood of adjuvant treated CC patients.

Conclusions: Our results suggest tumor-induced suppression of NK- and NKT-like cells in CC patients, an effect that could not be detected after tumor resection. In contrast, adjuvant therapy maintained tumor-induced immunosuppression of NK- and NKT-like cells in CC patients.

Introduction

Colon carcinoma (CC) is a tumor type with a high incidence and, combined with rectal cancer, globally accounting for more than 1 million new cases and around 600.000 deaths annually [1-3]. Despite improved surgical procedures and adjuvant treatment strategies, around 30% of the patients, without metastatic disease at time of diagnosis, develop recurrence or dissemination of the disease following successful resection of the primary tumor [4]. Currently, different biomarkers are evaluated for their prognostic value that may be used to identify cancer patients at risk of disease progression. Hence, biomarker application in cancer patients is crucial since it may provide information for therapeutic decision making, including decisions on the frequency of clinical follow-up, thereby possibly prolonging survival and reducing the risk of recurrence in cancer patients. Over the past decades, it has become clear that immunosurveillance plays a pivotal role in colon cancer by protecting the host against tumor development and progression. For instance, the immunoscore, representing $CD3^+/CD8^+$ T lymphocyte density in tumor tissue, is a strong prognostic factor in colorectal cancer (CRC) [5-9]. Low density of cytotoxic ($CD8^+$) tumor-infiltrating T cells was reported to correlate with poor clinical outcome in CRC [5,6,10,11]. However, it must be realized that the immunoscore can only be determined once at time of surgery because it is performed on resection material. Therefore, it does not provide information regarding treatment response of patients over time. In general, immune cells are present in relatively low numbers in the tissue of primary tumors, whereas they are abundantly present in peripheral blood. Interestingly, the subset distribution and immunophenotype of circulating immune cells ("peripheral blood immune cell profile") are associated with clinical outcome of CRC patients [12]. In contrast to the immunoscore, the peripheral blood immune cell profile can be monitored in patients over time since it only requires blood sampling.

The peripheral blood comprises different immune cell subsets, among which are natural killer (NK) cells that can be subdivided based on their CD56 expression. $CD56^{dim}$ NK cells have an important cytotoxic function while $CD56^{bright}$ NK cells are generally associated with production of pro-inflammatory cytokines and immunoregulatory properties [13,14]. Furthermore, peripheral blood contains a unique natural killer T (NKT) cell population with characteristics of both NK cells and NKT cells. Co-expression of CD3 and CD56 can be used to identify this immune subset in the circulation using flow cytometry, which is often referred to as "NKT-like" [15]. Like other immune cell subsets, NK and NKT-like cell activity is dependent on a delicate balance between activating and inhibitory signals from cell surface receptors [16]. 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 NKp30, NKp44, and NKp46, that recognize a variety of stress-induced molecules that may be present on tumor cells. Additionally, the CD16 (FcγRIII) receptor mediates antibody-dependent cell-mediated cytotoxicity and CD8 enhances the cytolytic activity of NK cells [17,18]. NK cell inhibitory receptors include natural killer group 2-A (NKG2A), and killer cell immunoglobulin (Ig)-like receptors CD158a and CD158b that recognize human leukocyte antigen (HLA) class I molecules. Recently, we [12] and others [19-21] showed that the peripheral blood immune cell profile is altered in CRC patients compared to healthy donors, characterized by downregulation of activating receptors on circulating $CD56^{dim}$ NK cells and NKT-like cells.

Several studies reported a critical role for the tumor microenvironment (TME) in shaping NK- and NKT-like receptor-mediated anti-tumor immunity [15,22,23]. For instance, immune-modulating effects of NK cells have been attributed to hypoxic conditions [24], and immunosuppressive cytokines and signaling molecules [23,25,26] present in the TME. As a result of these immune modulations, NK- and NKT-like cells may downregulate activating receptors and, as a result, may become functionally impaired, thereby promoting tumor escape. Up till now, it is unclear whether TME-induced immune-modulations of circulating NK- and NKT-like cells are reversible. Furthermore, chemotherapy, often used for adjuvant treatment of CRC patients, has been reported to have an immunosuppressive effect on the immune system, including overall impairment of NK cell responses [27]. In order to use the peripheral blood immune cell profile as biomarker to monitor cancer patients over time, it is crucial to know how immune cell subsets respond to therapeutic interventions. In a recent paper [12], we studied the preoperative peripheral blood immune cell profile in patients with CRC. In this study, we investigated the peripheral blood immune cell profile in patients with available postoperative blood samples in order to study the effects of tumor resection and adjuvant therapy. It was not possible to study the effects of tumor resection and adjuvant therapy in patients with rectum tumors due to limited sample availability. Therefore, we focused on patients with colon tumors. Additionally, we selected patients with Tumor-Node-Metastasis (TNM) stage II and III who are at risk for development of metastases after surgery and are, therefore, the patients that would qualify for biomarker monitoring over time. In summary, our collection of peripheral blood mononuclear cells (PBMCs) before and after tumor resection enabled, for the first time, to study the effects of tumor resection as well as adjuvant therapy on the subset distribution and immunophenotype of circulating T-, NK- and NKT-like cells in CC patients.

Materials & Methods

Study population

The study population comprised patients diagnosed with CC who underwent surgical resection of their primary tumor at the Leiden University Medical Center (LUMC, the Netherlands) between 2001 and 2007. Patients were included in this study based on availability of PBMC samples. PBMC samples were collected within a month prior to surgery and ≥2 months after surgery (mean 8.2 months after surgery, range 2.2-17.7). When the patient started adjuvant therapy directly after surgery, postoperative PBMC samples were obtained after therapy completion. These samples were obtained ≥5 months after the final therapy date (mean 8.4 months after surgery, range 6.7-13.7). Most adjuvant therapy cycles consisted of 3 weeks of therapy, therefore, the included postoperative PBMC samples were obtained ≥4 months after therapy completion. In the present study, patients with histologically proven primary CC, TNM stage II and III, surgical R0 resection, and a minimal amount of 5 million cryopreserved preoperative as well as postoperative PBMCs were included (N=26). Clinicopathological data of all patients were available. All blood samples were obtained after approval by the Medical Ethical Committee of the LUMC (protocol number P000.193). All procedures performed in this study were in accordance with the ethical standards of the Dutch law ("WMO", medical research involving human subjects act), and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. All CC patients included in this study agreed to our use of their PBMCs and data for research purposes prior to blood sampling via written informed consent and they moreover agreed to anonymous publication of the resulting data.

Isolation of peripheral blood mononuclear cells

PBMCs from CC patients were isolated and cryopreserved as previously described [12]. PBMCs were also isolated from the buffy coat of a random blood donor obtained from the Blood Bank at Aarhus University Hospital (Dept. Clinical Immunology, Aarhus University Hospital, Skejby, Denmark), which was used as an internal control in the flow cytometry experiments [12].

Flow cytometry antibody staining and data analysis

All preoperative PBMC samples from CC patients were previously immunophenotyped and reported on as part of a CRC study [12]. All postoperative PBMC samples selected for the current study were immunophenotyped following the same protocol. Briefly, PBMC samples were thawed following a

standard protocol and the cell concentration was adjusted to $10x10^6$ cells/ml. PBMCs were then blocked for 15-30 minutes at room temperature with 50 µg/ml human IgG (CSL Behring, Bern, Switzerland) and subsequently stained with two distinct antibody panels containing selected NK- and NKT cell markers as previously described [12]. After the staining procedure, samples were fixed in PBS supplemented with 0.9% formaldehyde (Sigma-Aldrich, St. Louis, MO) and analyzed the same day by flow cytometry on a 4-laser equipped LSRFortessa (BD Biosciences, San Diego, CA) using Diva 7.0 software (BD Biosciences). Results were analyzed with FlowJo software v10.1 (Tree Star Inc., Ashland, OR). The buffy coat was used as an internal control in order to check for any inter-experimental variation. Expression of phenotypic markers on peripheral blood T-, NK- and NKT-like cells was evaluated by the median fluorescence intensity (MFI) and/or the percentage of positive cells. A compensation matrix was calculated to compensate for spillover of signal into other channels in the multicolor flow cytometry experiments. This matrix was set up using single stains on CompBeads (BD Biosciences,) OneComp eBeads (eBioscience, San Diego, CA), CompBeads Plus (BD Biosciences), and ArC reactive beads (Life Technologies). A standardized gating strategy based on a healthy donor buffy coat was used in order to study the distribution of markers on specific lymphocyte subsets as described previously [12].

Statistical analyses

Statistical analyses were conducted using SPSS statistical software (IBM SPSS Statistics 23, Chicago, USA). The age of patients treated with adjuvant therapy was compared with the age of patients treated without adjuvant therapy using the Mann Whitney-U test. Preoperative and postoperative PBMC samples from patients were compared using the paired samples T test and Wilcoxon signed-rank test, for normally distributed and not normally distributed variables respectively. We corrected for multiple testing using the Benjamini-Hochberg method, by which adjusted *P*-values were calculated (indicated by *P**) [28]. *P**-values ≤0.05 were considered statistically significant.

Results

Study population

The subset distribution and immunophenotype of circulating T-, NK-, and NKT-like cells was studied pre- and postoperatively in patients diagnosed with CC TNM stage II and III (N=26). One postoperative sample was excluded due to low viability of the PBMCs (<50% viable cells). Additionally, one patient was diagnosed with Lynch syndrome. Since tumors from patients with Lynch syndrome are

immunologically different compared to tumors from patients with sporadic cancer [29], this patient was excluded from analyses, resulting in a total study population of 24 patients. One patient had undergone local radiotherapy prior to the collection of the preoperative PBMC sample. In total, 15 patients underwent surgical resection of their tumor only (non-adjuvant treated group) and 9 patients received additional therapy after surgery (adjuvant treated group). Two of these 9 patients were treated with capecitabine monotherapy, six patients were treated with a combination of capecitabine and oxaliplatin, and one patient was treated with a combination of capecitabine, oxaliplatin and bevacizumab. Table 1 summarizes the clinicopathological characteristics of the 24 patients included in the analyses. Patients undergoing adjuvant therapy were younger (median 56 years) compared to patients that did not receive adjuvant therapy (median 73 years, *P*=0.002). The low number of patients did not allow for analyzing the peripheral blood immune cell profile according to age.

Table 1. Patient demographics and tumor characteristics. Clinicopathological data of the 24 CC patients in this study. Abbreviations: CC (colon carcinoma), TNM (Tumor-Node-Metastasis).

	CC patients Total population	**CC patients No adjuvant therapy**	**CC patients Adjuvant therapy***
	(N=24)	(N=15)	(N=9)
Age at time of surgery			
Median (years)	65	73	56
Range (years)	25-85	56-85	25-71
Sex			
Female	11 (45.8%)	7 (46.7%)	4 (44.4%)
Male	13 (54.2%)	8 (53.3%)	5 (55.6%)
TNM classification			
Stage II	12 (50.0%)	10 (66.7%)	2 (22.2%)
Stage III	12 (50.0%)	5 (33.3%)	7 (77.8%)
Tumor differentiation			
Well/moderate	21 (87.5%)	12 (80.0%)	9 (100%)
Poor	3 (12.5%)	3 (20.0%)	0 (0%)
Neoadjuvant radiotherapy			
Yes	1 (4.2%)	1 (6.7%)	0 (0%)
No	23 (95.8%)	14 (93.3%)	9 (100%)

* Capecitabine monotherapy, capecitabine with oxaliplatin, or a combination of capecitabine, oxaliplatin and bevacizumab

Table 2. Effects of tumor resection and adjuvant therapy on the peripheral blood immune cell profile in CC patients. The peripheral blood immune profile was studied in preoperative and postoperative PBMC samples from CC patients treated without (N=15) and with (N=9) adjuvant therapy using multiparameter flow cytometry. Statistically significant *P**-values are indicated in bold. Abbreviations: CC (colon carcinoma), MFI (median fluorescence intensity), NK (natural killer), NKT (natural killer T), SD (standard deviation).

	No adjuvant therapy Preoperative CC patients (N=15)		**No adjuvant therapy Postoperative CC patients (N=15)**			**Adjuvant therapy Preoperative CC patients (N=9)**		**Adjuvant therapy Postoperative CC patients (N=9)**		
	Mean	SD	Mean	SD	*P**-value	Mean	SD	Mean	SD	*P**-value
Subset distribution										
T cells (%)	60.3	16.7	89.8	10.4	0.586^{W}	56.5	9.8	61.5	8.4	0.369^{W}
CD8^{+} T cells (%)	28.4	11.7	35.8	14.4	**0.034^{T}**	32.6	13.4	36.6	15.7	0.455^{T}
NK cells (%)	9.9	4.8	12.2	5.4	0.148^{W}	16.0	8.1	14.9	6.0	0.657^{W}
CD56^{dim} NK cells (%)	92.2	6.1	95.4	3.0	0.083^{W}	96.5	1.9	95.0	4.1	0.535^{W}
CD56^{bright} NK cells (%)	6.8	6.1	4.6	3.0	0.090^{W}	3.5	1.9	4.9	4.1	0.514^{W}
NKT-like cells (%)	5.8	3.8	7.6	5.0	**0.049^{W}**	5.8	6.7	6.6	8.0	0.494^{W}
CD56^{dim} NK cells										
CD16^{+} (%)	80.0	12.4	82.7	6.0	0.988^{W}	81.0	11.8	84.8	6.2	0.651^{W}
CD158a^{+} (%)	28.5	14.9	27.0	14.2	0.085^{T}	37.1	20.9	34.6	20.2	0.436^{T}
CD158b^{+} (%)	37.8	13.6	38.2	13.5	0.993^{W}	34.4	14.3	32.9	15.3	1.003^{W}
NKG2A^{+} (%)	48.9	18.6	43.8	16.6	0.133^{T}	38.6	13.8	40.3	13.3	0.551^{T}
NKG2A^{+} (MFI)	9042	3049	7929	3827	0.086^{W}	5726	1932	6935	2156	0.253^{W}
NKG2C^{+} (%)	21.8	15.2	22.7	16.5	0.972^{W}	17.5	20.0	19.3	20.0	0.615^{W}
NKG2C^{+} (MFI)	4070	3242	4614	3829	0.085^{W}	4238	5774	4338	5496	0.503^{W}
CD161 (MFI)	2923	1263	2820	1124	0.557^{T}	2848	1463	3025	1369	0.638^{T}
CD8^{+} (%)	23.8	8.8	24.0	9.2	0.862^{W}	30.7	20.7	37.6	17.9	0.240^{W}
CD8^{+} (MFI)	2899	1333	3853	1120	0.060^{W}	3850	1343	4370	1582	0.561^{W}
DNAM-1 (MFI)	517	155	540	148	0.562^{T}	516	140	572	131	0.305^{T}
NKG2D^{+} (%)	89.6	6.5	91.2	3.8	0.439^{W}	90.5	5.0	91.0	4.0	0.986^{W}
NKG2D^{+} (MFI)	3717	1344	4475	1037	**0.045^{T}**	3452	1247	4015	778	0.483^{T}
NKp30 (MFI)	1482	963	1423	933	0.807^{T}	1442	570	1755	802	0.327^{T}
NKp44^{+} (%)	0.5	0.3	0.7	0.4	0.132^{W}	0.7	0.5	0.7	0.3	0.488^{W}
NKp44 (MFI)	118	28	145	10	**0.036^{T}**	133	25	145	15	0.420^{T}

Table 2. continued

	No adjuvant therapy Preoperative CC patients (N=15)		No adjuvant therapy Postoperative CC patients (N=15)			Adjuvant therapy Preoperative CC patients (N=9)		Adjuvant therapy Postoperative CC patients (N=9)		
	Mean	SD	Mean	SD	*P**-value	Mean	SD	Mean	SD	*P**-value
NKp46$^+$ (%)	36.2	19.9	34.6	17.0	0.803^T	36.2	17.7	45.5	18.6	0.482^T
NKp46 (MFI)	529	313	500	295	0.846^W	510	239	671	354	0.496^W
CD56^{bright} NK cells										
CD16$^+$ (%)	2.8	1.8	1.9	1.4	**0.040^W**	1.6	1.1	2.9	2.8	0.344^W
CD158a$^+$ (%)	5.0	3.1	5.8	3.1	0.519^W	9.7	6.8	7.8	5.9	0.753^W
CD158b$^+$ (%)	9.0	8.7	10.0	7.2	0.596^W	8.8	5.1	7.9	7.3	0.969^W
NKG2A$^+$ (%)	5.5	3.1	4.3	3.2	0.224^W	3.1	2.0	4.6	4.1	0.494^W
NKG2A$^+$ (MFI)	21772	6987	18008	5705	0.045^W	17143	4384	19518	5110	0.473^W
NKG2C$^+$ (%)	1.5	1.4	2.1	2.2	0.829^W	1.1	0.7	1.7	1.1	0.476^W
NKG2C$^+$ (MFI)	2956	2358	3007	2901	0.928^W	2414	1675	2515	1882	0.740^W
CD161 (MFI)	1709	752	1852	550	0.295^T	2105	572	2177	553	0.686^T
CD8$^+$ (%)	1.9	1.3	1.5	1.1	0.505^W	1.2	0.7	2.4	2.1	0.348^W
CD8$^+$ (MFI)	3085	1629	3677	1365	0.051^W	3379	715	3720	1551	0.644^W
DNAM-1 (MFI)	790	183	806	154	0.775^T	781	139	877	116	0.240^T
NKG2D$^+$ (%)	6.2	5.4	4.0	2.4	0.103^W	3.4	1.9	4.8	3.9	0.459^W
NKG2D$^+$ (MFI)	7064	2766	8248	1954	0.051^T	6379	1973	7096	1501	0.501^T
NKp30 (MFI)	2134	572	2271	509	0.434^T	2231	507	2524	719	0.309^T
NKp44$^+$ (%)	1.3	0.8	1.4	1.2	1.000^W	1.2	1.1	1.7	1.1	0.472^W
NKp44 (MFI)	222	61	270	47	0.081^W	295	141	306	63	0.451^W
NKp46$^+$ (%)	79.7	16.5	78.2	15.5	0.725^W	75.6	20.9	89.0	5.7	0.225^W
NKp46 (MFI)	2237	1030	2184	482	0.813^W	1893	762	2753	818	0.330^W
NKT-like cells										
CD16$^+$ (%)	21.1	13.4	20.2	15.1	0.263^W	16.7	9.6	26.5	14.6	0.210^W
CD158a$^+$ (%)	8.6	11.1	7.4	10.1	**0.048^W**	10.4	12.5	12.6	12.9	0.601^W
CD158b$^+$ (%)	23.8	26.3	21.1	24.2	0.161^W	8.7	4.7	11.0	5.2	0.417^W
NKG2A$^+$ (%)	21.1	18.1	15.3	14.2	**0.030^W**	33.2	17.7	30.6	14.5	0.822^W
NKG2A$^+$ (MFI)	3334	3813	6250	5937	0.081^W	7398	4106	10845	4848	0.220^W

Table 2. continued

	No adjuvant therapy Preoperative CC patients (N=15)		**No adjuvant therapy Postoperative CC patients (N=15)**			**Adjuvant therapy Preoperative CC patients (N=9)**		**Adjuvant therapy Postoperative CC patients (N=9)**		
	Mean	SD	Mean	SD	*P**-value	Mean	SD	Mean	SD	*P**-value
NKG2C⁺ (%)	15.1	14.0	13.3	12.2	0.895[W]	14.4	13.0	14.1	13.1	0.547[W]
NKG2C⁺ (MFI)	2657	2160	3029	3185	0.971[W]	1694	2488	1685	2403	0.631[W]
CD161 (MFI)	2126	1796	2055	1689	0.975[W]	7309	9278	4287	3896	0.459[W]
CD8⁺ (%)	76.4	16.4	77.8	15.5	0.255[W]	75.5	16.4	76.7	17.3	0.618[W]
CD8⁺ (MFI)	11727	5260	15077	6501	**0.030[T]**	15142	6583	18619	9320	0.342[T]
DNAM-1 (MFI)	726	329	730	381	0.974[T]	712	191	876	211	0.210[T]
NKG2D⁺ (%)	92.0	9.0	92.5	8.5	0.098[W]	91.8	7.4	92.5	8.3	0.663[W]
NKG2D⁺ (MFI)	6176	2776	7155	2225	**0.048[T]**	4836	2465	6413	2180	0.192[T]
NKp30 (MFI)	235	159	157	26	0.468[W]	183	65	182	60	0.953[W]
NKp44⁺ (%)	1.1	0.6	2.1	0.7	**0.040[W]**	2.2	1.5	2.5	1.1	0.588[W]
NKp44 (MFI)	122	34	156	12	**0.038[W]**	147	45.8	152	13.3	0.490[W]
NKp46⁺ (%)	3.8	1.0	3.1	1.0	0.081[W]	3.0	1.3	4.3	2.1	0.349[W]
NKp46 (MFI)	94	41	84	31	0.570[T]	56	25	69.0	31	0.506[T]

[T] Paired samples T test

[W] Wilxoxon signed-rank test

Expansion of NKT-like and CD8⁺ T cell populations in peripheral blood after surgery in non-adjuvant treated CC patients

First, the effects of tumor resection and adjuvant therapy on the distribution of peripheral blood immune subsets were studied in non-adjuvant treated (N=15) and adjuvant treated (N=9) CC patients (Table 2). The percentages of total NK cells (% $CD3^-CD56^+$ cells of total PBMCs), $CD56^{dim}$ NK cells (% of total NK cells), and $CD56^{bright}$ NK cells (% of total NK cells) did neither change in non-adjuvant treated

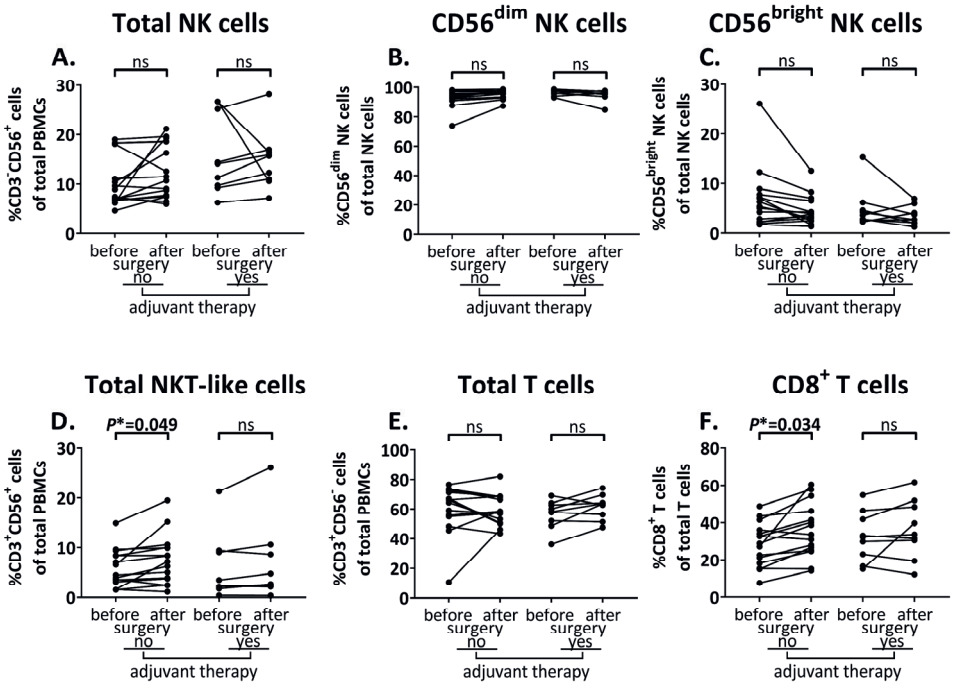


Figure 1. Effects of tumor resection and adjuvant therapy on the peripheral blood immune cell subset distribution in CC patients. Multiparameter flow cytometry was used to study the peripheral blood immune profile in PBMC samples from CC patients. The distribution of circulating immune cell subsets was compared before and after surgery in CC patients that did (N=9) or did not (N=15) receive adjuvant therapy. **A.** Total percentage of NK cells (% $CD3^-CD56^+$ cells of total PBMCs). **B.** Percentage of $CD56^{dim}$ NK cells (% of total NK cells). **C.** Percentage of $CD56^{bright}$ NK cells (% of total NK cells). **D.** Total percentage of NKT-like cells (% $CD3^+CD56^+$ cells of total PBMCs). **E.** Total percentage of T cells (% $CD3^+CD56^-$ cells of total PBMCs). **F.** Percentage of $CD8^+$ T cells (% of total T cells). Pre- and postoperative samples from each patient are connected with a line. *P**-values ≤0.05 were considered statistically significant. Abbreviations: CC (colon carcinoma), PBMCs (peripheral blood mononuclear cells), NK (natural killer), NKT (natural killer T), ns (not significant).

patients nor in adjuvant treated patients after resection of the primary tumor (Figure 1A;1B;1C, respectively). The percentage of NKT-like cells (% $CD3^+CD56^+$ cells of total PBMCs) increased (*P**=0.049) in non-adjuvant treated patients after surgery (Figure 1D). Furthermore, although the percentage of total T cells (% $CD3^+CD56^-$ cells of total PBMCs) was not altered in non-adjuvant treated patients after resection of the primary tumor (Figure 1E), the percentage of $CD8^+$ T cells (% of total T cells) was significantly increased (*P**=0.034, Figure 1F). No change was observed in the percentage of NKT-like cells or $CD8^+$ T cells in adjuvant treated patients after surgery.

Increased expression of activating receptors and downregulation of inhibitory receptors on NK cells in peripheral blood after surgery in non-adjuvant treated CC patients

After analyzing the distribution of immune cell subsets, we compared the immunophenotype of NK- and NKT-like cells before and after tumor resection in non-adjuvant treated and adjuvant treated CC patients (Table 2). Within the $CD56^{dim}$ NK cell population, the expression levels of activating receptors NKp44 (*P**=0.036) and NKG2D (*P**=0.045) increased after surgery in non-adjuvant treated patients (Figure 2A;2B, respectively). Additionally, a trend was observed towards a higher expression level of the co-stimulatory molecule CD8 in non-adjuvant treated patients after surgery (*P**=0.060) (Figure 2C).

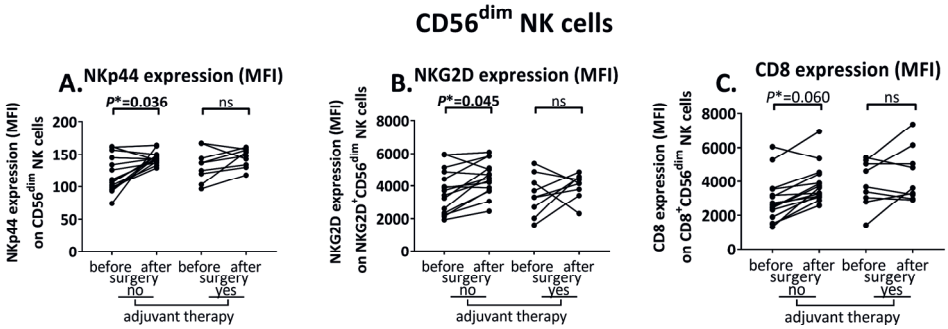


Figure 2. Effects of tumor resection and adjuvant therapy on the immunophenotype of $CD56^{dim}$ NK cells in CC patients. Multiparameter flow cytometry was used to study the peripheral blood immune profile in PBMC samples from CC patients. The immunophenotype of $CD56^{dim}$ NK cells was compared before and after surgery in CC patients that did (N=9) or did not (N=15) receive adjuvant therapy. **A.** Expression level of NKp44 on $CD56^{dim}$ NK cells. **B.** Expression level of NKG2D on $NKG2D^+CD56^{dim}$ NK cells. **C.** Expression level of CD8 on $CD8^+CD56^{dim}$ NK cells. Pre- and postoperative samples from each patient are connected with a line. Statistically significant *P**-values are indicated in bold. Abbreviations: CC (colon carcinoma), MFI (median fluorescence intensity), NK (natural killer), ns (not significant).

The expression levels of NKp44, NKG2D and CD8 did not change on CD56^{dim} NK cells after tumor resection in adjuvant treated patients (Figure 2A;2B;2C, respectively). In line with these results, a trend towards increased expression levels of NKG2D (*P**=0.051) and CD8 (*P**=0.051) was observed on CD56^{bright} NK cells in non-adjuvant treated patients (Figure 3A;3B, respectively). No change in expression levels of NKG2D and CD8 was observed on CD56^{bright} NK cells after tumor resection in adjuvant treated patients. The expression level of the inhibitory receptor NKG2A (*P**=0.045) on CD56^{bright} cells decreased in non-adjuvant treated patients after surgery (Figure 3C). In contrast, the percentage of stimulatory receptor CD16^{+} CD56^{bright} NK cells was also decreased (*P**=0.040) in non-adjuvant treated patients after tumor resection (Figure 3D). The expression level of NKG2A and the

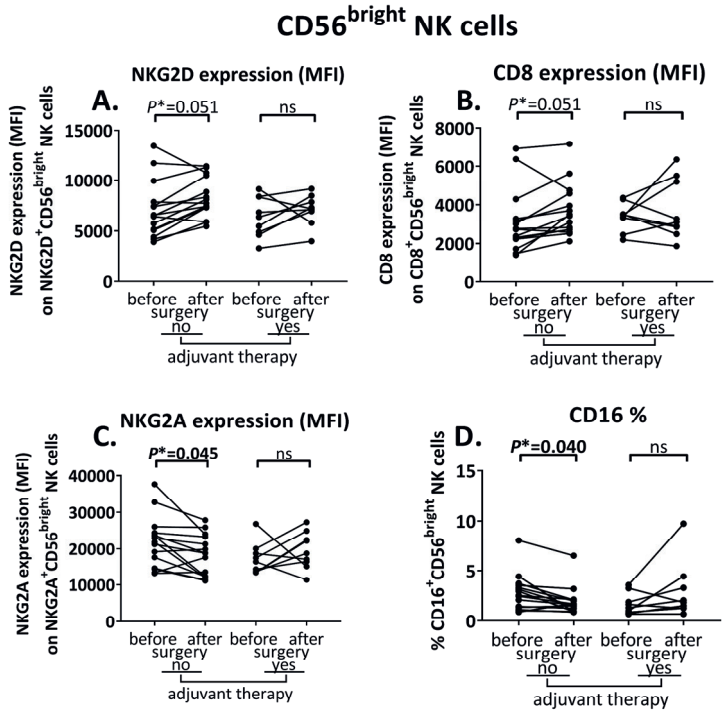


Figure 3. Effects of tumor resection and adjuvant therapy on the immunophenotype of CD56^{bright} NK cells in CC patients. Multiparameter flow cytometry was used to study the peripheral blood immune profile in PBMC samples from CC patients. The immunophenotype of CD56^{bright} NK cells was compared before and after surgery in CC patients that did (N=9) or did not (N=15) receive adjuvant therapy. **A.** Expression level of NKG2D on NKG2D^{+}CD56^{bright} NK cells. **B.** Expression level of CD8 on CD8^{+}CD56^{bright} cells. **C.** Expression level of NKG2A on NKG2A^{+}CD56^{bright} NK cells. **D.** Percentage of CD16^{+}CD56^{bright} NK cells. Pre- and postoperative samples from each patient are connected with a line. Statistically significant *P**-values are indicated in bold. Abbreviations: CC (colon carcinoma), MFI (median fluorescence intensity), NK (natural killer), ns (not significant).

percentage of CD16$^+$ cells did not change after surgery in adjuvant treated patients. In summary, circulating NK cells acquired expression of cell surface markers associated with functional activity after surgery in non-adjuvant treated CC patients as compared to before surgery.

Increased expression of activating receptors and downregulation of inhibitory receptors on NKT-like cells in peripheral blood after surgery in non-adjuvant treated CC patients

Within the NKT-like cell subset, the percentages of NKT-like cells expressing the inhibitory receptors CD158a or NKG2A decreased in non-adjuvant treated patients after surgery (*P**=0.048 and *P**=0.030, respectively), whereas no change was observed in adjuvant treated patients (Figure 4A;4B). In contrast, the expression levels of activating receptors NKG2D (*P**=0.048) and co-stimulatory receptor CD8 (*P**=0.030) on NKT-like cells increased in non-adjuvant treated patients after surgery (Figure 4C;4D). No change in NKT-like cell expression levels of NKG2D and CD8 were observed after surgery in adjuvant treated patients (Figure 4C;4D). Additionally, an increased percentage of NKp44$^+$ NKT-like cells was observed in non-adjuvant treated patients after surgery (*P**=0.040, Figure 4E). The expression level of NKp44 on NKT-like cells showed a similar pattern as the percentage of NKp44$^+$ NKT-like cells and was increased in non-adjuvant treated patients after surgery (*P**=0.038, Figure 4F). The percentage of NKp44$^+$ NKT-like cells, as well as the expression level of NKp44 on NKT-like cells, did not change in adjuvant treated patients after surgery (Figure 4E;4F). In summary, as also observed for the immunophenotype of NK cells, NKT-like cells acquired expression of cell surface markers associated with functional activity after surgery in non-adjuvant treated CC patients.

Discussion

As shown in our recent study [12], the peripheral blood immune cell profile comprises a potential pool of biomarkers in CRC. We hypothesized that this profile might change upon therapeutic interventions. Therefore, we studied the peripheral blood immune profile in available postoperative PBMC samples (N=24) from TNM stage II and III CC patients included in our previous study [12]. Our collection of PBMCs before and after tumor resection enabled, for the first time, to study the effects of tumor resection as well as adjuvant therapy on the subset distribution and immunophenotype of circulating immune cells in CC. Here, we focused on NK- and NKT-like cell subsets as several studies have reported an altered phenotype of these cells in peripheral blood of CRC patients compared to healthy donors, characterized by downregulation of activating receptors, which suggests impaired function of these immune cell subsets in cancer patients [12,19,20]. A critical role of the TME was implied in shaping

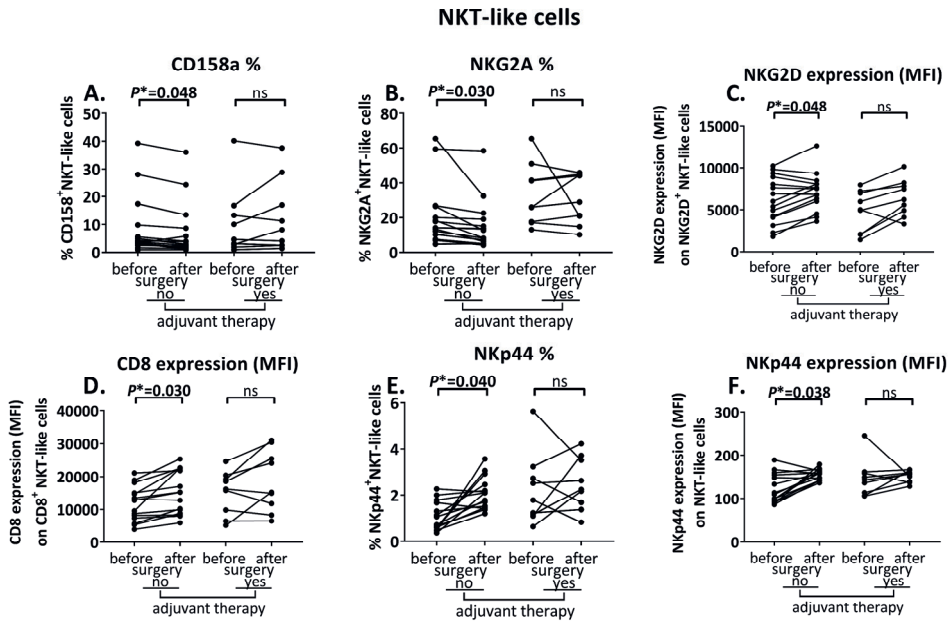


Figure 4. Effects of tumor resection and adjuvant therapy on the immunophenotype of NKT-like cells in CC patients. Multiparameter flow cytometry was used to study the peripheral blood immune profile in PBMC samples from CC patients. The immunophenotype of NKT-like cells was compared before and after surgery in CC patients that did (N=9) or did not (N=15) receive adjuvant therapy. **A.** Percentage of CD158a$^+$NKT-like cells. **B.** Percentage of NKG2A$^+$NKT-like cells. **C.** Expression level of NKG2D on NKG2D$^+$NKT-like cells. **D.** Expression level of CD8 on CD8$^+$NKT-like cells. **E.** Percentage of NKp44$^+$NKT-like cells. **F.** Expression level of NKp44 on NKT-like cells. Pre- and postoperative samples from each patient are connected with a line. Statistically significant *P**-values are indicated in bold. Abbreviations: CC (colon carcinoma), CI (confidence interval), MFI (median fluorescence intensity), NKT (natural killer T), ns (not significant).

NK- and NKT-like receptor-mediated anti-tumor immunity [15,22,23], which could be a result of hypoxic conditions, immunosuppressive cytokines, or fibroblasts present in the TME [23-26].

We observed changes in peripheral blood immune cell profile after resection of the primary tumor in non-adjuvant treated patients, including expansion of NKT-like (% of total PBMCs) and CD8$^+$ T (% of total T cells) cell populations. An increase in percentage of these cell types was also observed after surgery in non-adjuvant treated patients with laryngeal cancer [30]. Importantly, the reported expansion of the NKT-like and CD8$^+$ T cell populations in the present study and by Klatka *et al.* [30] is relative, and does not provide information about a change in absolute numbers of these immune subsets. It was not possible to assess cells/ml counts in the present study since our samples were

frozen PBMCs, not whole blood. Thus, trying to make measurements of cells/ml would be highly affected by the Ficoll procedure and sample preparation. This is a limitation of our study and should be considered when drawing conclusions. Grimm *et al.* reported no absolute increase of $CD8^+$ T cells in patients with oral squamous cell carcinoma (OSCC) after surgery [31], suggesting no effect on absolute immune cell numbers after surgery. However, it must be realized that this study was focused on OSCC patients that might have a different preoperative peripheral blood immune profile compared to CC patients which might also respond different to tumor resection. Furthermore, OSCC patients are often treated with neoadjuvant radiotherapy which might influence the immune system over a long period of time. The study by Grimm *et al.* did not specify neoadjuvant and/or adjuvant treatment of the included OSCC patients in their study. Up till now, studies reported on the numbers of circulating immune cells and subset distribution in cancer patients after surgery [30-32], but not on the immunophenotype of specific immune cell subsets. It is important that this is considered since the immunophenotype of immune cells is closely related to their function. Hence, expansion of the NKT-like cell population in CC patients after surgery does not necessarily mean that more effector cells are present. In this study, we showed that expression of activating receptors including NKG2D, NKp44, and CD8, was upregulated on NKT-like cells after surgery in non-adjuvant treated patients. Additionally, we observed a decrease in the percentage of inhibitory receptor $CD158a^+$ and $NKG2A^+$ NKT-like cells, suggesting that the NKT-like cell population expands and acquires expression of cell surface markers associated with functional activity in CC patients after tumor resection. In contrast to studies on laryngeal cancer [30] and oral squamous cell carcinoma [31], we did not observe expansion of the NK cell population after tumor resection. We did, however, observe a change in immunophenotype of NK cells resembling the pattern observed in NKT-like cells. Hence, we observed upregulation of activating receptors NKG2D, NKp44 and CD8 on $CD56^{dim}$ NK cells in non-adjuvant treated CC patients after tumor resection. In line with this, upregulation of activating receptors NKG2D and CD8 was observed on $CD56^{bright}$ NK cells as well as downregulation of the inhibitory receptor NKG2A after tumor resection in non-adjuvant treated CC patients, suggesting that both $CD56^{dim}$ NK and $CD56^{bright}$ NK cells acquired a more active phenotype after tumor resection and, therefore, possibly recovered from TME-induced immunosuppression. The only activating marker that was downregulated in CC patients after tumor resection was the percentage of $CD16^+$ cells within the $CD56^{bright}$ NK cell population, suggesting differences in TME-induced recovery of $CD56^{dim}$ and $CD56^{bright}$ NK cells. This is in line with a previous study in which we also reported differences in immunophenotype of $CD56^{dim}$ in comparison with $CD56^{bright}$ NK cells in preoperative CRC patients [12],

implicating different roles for NK cell subsets in patients, thereby emphasizing the need to analyze these subsets separately.

Several studies investigated short-term effects of surgical resection on the immune system in cancer patients (1-14 days after surgery) [32-36], whereas only a few studies focused on the long-term effects (≥6 weeks after surgery). Although these studies did not investigate the expression of different activating and inhibitory cell surface receptors, they all report expansion of cytotoxic immune cell subsets after surgery, including NK cells, NKT cells, and $CD8^+$ T cells [30-32], suggesting a more active immune system in patients after tumor resection, which is in line with our results. Importantly, the changes in immunophenotype of NK- and NKT-like cells observed in our study after surgery were restricted to patients that did not receive any adjuvant therapy, suggesting that adjuvant therapy delays or even prevents the recovery of TME-induced suppression of NK- and NKT-like cells. As the postoperative samples from adjuvant treated patients were obtained ≥5 months after the final therapy date, this implies long lasting immunosuppressive effects of adjuvant therapy. This is in line with a study in breast cancer patients that reported decreased numbers of $CD4^+$ T cells and B cells even 9 months after completion of chemotherapy [37].

The fact that therapeutic interventions influenced the peripheral blood immune cell profile in CC patients, has consequences regarding its biomarker potential. In an ideal situation, biomarkers such as circulating immune cells could be used to monitor treatment response and risk of recurrence in patients over time. Our data suggest that TME-induced immunosuppression is de-activated after resection of the primary tumor. In theory, patients without complete resection of the tumor or patients with micrometastases after tumor resection still have a TME present which could mean that TME-induced immunosuppression of NK- and NKT-like cells is not removed. Despite the small sample size of our cohort and statistical correction for multiple testing, we still found significant results in our study. Further research is required to investigate whether the peripheral blood immune cell profile can be used as biomarker to determine the presence of residual tumor cells in cancer patients.

In conclusion, we observed expansion of NKT-like cells and $CD8^+$ T cells, and activation of both NK- and NKT-like cells after tumor resection in CC patients. This suggests TME-induced suppression of NK cells and NKT-like cells, an effect that could not be detected after surgical resection of the primary tumor. The changes in peripheral blood immune cell profile after tumor resection were restricted to patients that did not receive any adjuvant therapy, suggesting that adjuvant therapy delays or even prevents the recovery of TME-induced suppressed NK- and NKT-like cells in CC patients after surgery. These observations are of importance for using the peripheral blood immune cell profile

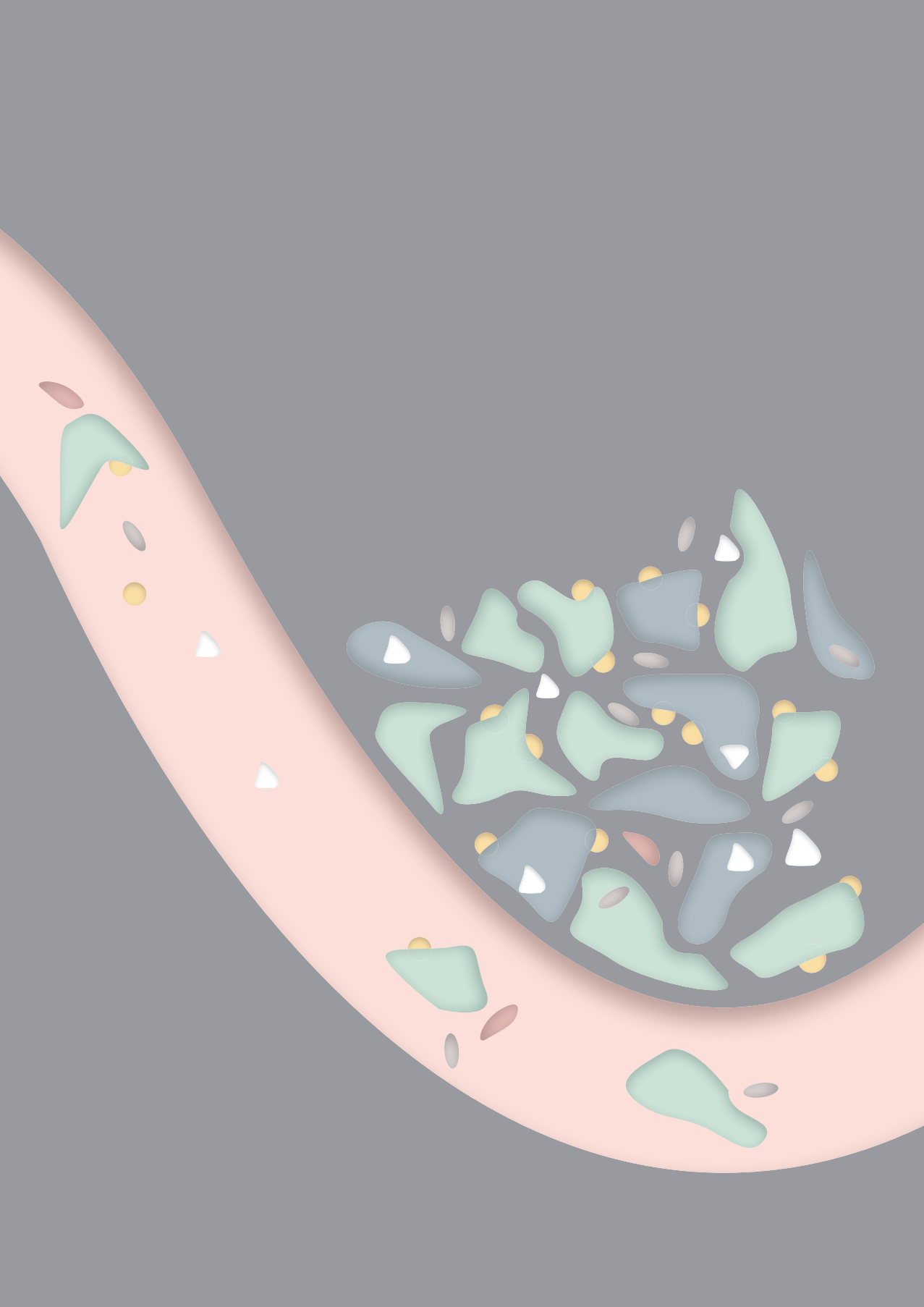
as a biomarker in CC, but should also be considered when developing cancer immunotherapy for CC patients.

References

1. Ferlay, J.; Parkin, D.M.; Steliarova-Foucher, E. Estimates of cancer incidence and mortality in Europe in 2008. *Eur J Cancer* **2010**, *46*, 765-781, doi:10.1016/j.ejca.2009.12.014.
2. Ferlay, J.; Steliarova-Foucher, E.; Lortet-Tieulent, J.; Rosso, S.; Coebergh, J.W.; Comber, H.; Forman, D.; Bray, F. Cancer incidence and mortality patterns in Europe: estimates for 40 countries in 2012. *Eur J Cancer* **2013**, *49*, 1374-1403, doi:10.1016/j.ejca.2012.12.027.
3. Ferlay, J.; Soerjomataram, I.; Dikshit, R.; Eser, S.; Mathers, C.; Rebelo, M.; Parkin, D.M.; Forman, D.; Bray, F. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. *Int J Cancer* **2015**, *136*, E359-386, doi:10.1002/ijc.29210.
4. Van Cutsem, E., Cervantes A, Nordlinger B, Arnold D; ESMO Guidelines Working Group. Metastatic colorectal cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. *Ann Oncol* **2014**, *Suppl 3*, iii1-9.
5. Galon, J.; Costes, A.; Sanchez-Cabo, F.; Kirilovsky, A.; Mlecnik, B.; Lagorce-Pages, C.; Tosolini, M.; Camus, M.; Berger, A.; Wind, P., et al. Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. *Science* **2006**, *313*, 1960-1964, doi:10.1126/science.1129139.
6. Mlecnik, B.; Tosolini, M.; Kirilovsky, A.; Berger, A.; Bindea, G.; Meatchi, T.; Bruneval, P.; Trajanoski, Z.; Fridman, W.H.; Pages, F., et al. Histopathologic-based prognostic factors of colorectal cancers are associated with the state of the local immune reaction. *J Clin Oncol* **2011**, *29*, 610-618, doi:10.1200/JCO.2010.30.5425.
7. Finn, O.J. Host response in tumor diagnosis and prognosis: importance of immunologists and pathologists alliance. *Exp Mol Pathol* **2012**, *93*, 315-318, doi:10.1016/j.yexmp.2012.10.013.
8. Galon, J.; Mlecnik, B.; Bindea, G.; Angell, H.K.; Berger, A.; Lagorce, C.; Lugli, A.; Zlobec, I.; Hartmann, A.; Bifulco, C., et al. Towards the introduction of the 'Immunoscore' in the classification of malignant tumours. *J Pathol* **2014**, *232*, 199-209, doi:10.1002/path.4287.
9. Mlecnik, B.; Bindea, G.; Angell, H.K.; Maby, P.; Angelova, M.; Tougeron, D.; Church, S.E.; Lafontaine, L.; Fischer, M.; Fredriksen, T., et al. Integrative Analyses of Colorectal Cancer Show Immunoscore Is a Stronger Predictor of Patient Survival Than Microsatellite Instability. *Immunity* **2016**, *44*, 698-711, doi:10.1016/j.immuni.2016.02.025.
10. Galon, J.; Mlecnik, B.; Bindea, G.; Angell, H.K.; Berger, A.; Lagorce, C.; Lugli, A.; Zlobec, I.; Hartmann, A.; Bifulco, C., et al. Towards the introduction of the 'Immunoscore' in the classification of malignant tumours. *J. Pathol.* **2014**, *232*, 199-209.
11. Pages, F.; Mlecnik, B.; Marliot, F.; Bindea, G.; Ou, F.S.; Bifulco, C.; Lugli, A.; Zlobec, I.; Rau, T.T.; Berger, M.D., et al. International validation of the consensus Immunoscore for the classification of colon cancer: a prognostic and accuracy study. *Lancet* **2018**, *391*, 2128-2139, doi:10.1016/S0140-6736(18)30789-X.
12. Krijgsman, D.; de Vries, N.L.; Skovbo, A.; Andersen, M.N.; Swets, M.; Bastiaannet, E.; Vahrmeijer, A.L.; van de Velde, C.J.H.; Heemskerk, M.H.M.; Hokland, M., et al. Characterization of circulating T-, NK-, and NKT cell subsets in patients with colorectal cancer: the peripheral blood immune cell profile. *Cancer Immunol Immunother* **2019**, 10.1007/s00262-019-02343-7, doi:10.1007/s00262-019-02343-7.
13. Cooper, M.A.; Fehniger, T.A.; Caligiuri, M.A. The biology of human natural killer-cell subsets. *Trends Immunol* **2001**, *22*, 633-640.
14. Jonges, L.E.; Albertsson, P.; van Vlierberghe, R.L.; Ensink, N.G.; Johansson, B.R.; van de Velde, C.J.; Fleuren, G.J.; Nannmark, U.; Kuppen, P.J. The phenotypic heterogeneity of human natural killer cells: presence of at least 48 different subsets in the peripheral blood. *Scand J Immunol* **2001**, *53*, 103-110.
15. Krijgsman, D.; Hokland, M.; Kuppen, P.J.K. The Role of Natural Killer T Cells in Cancer-A Phenotypical and Functional Approach. *Front Immunol* **2018**, *9*, 367, doi:10.3389/fimmu.2018.00367.

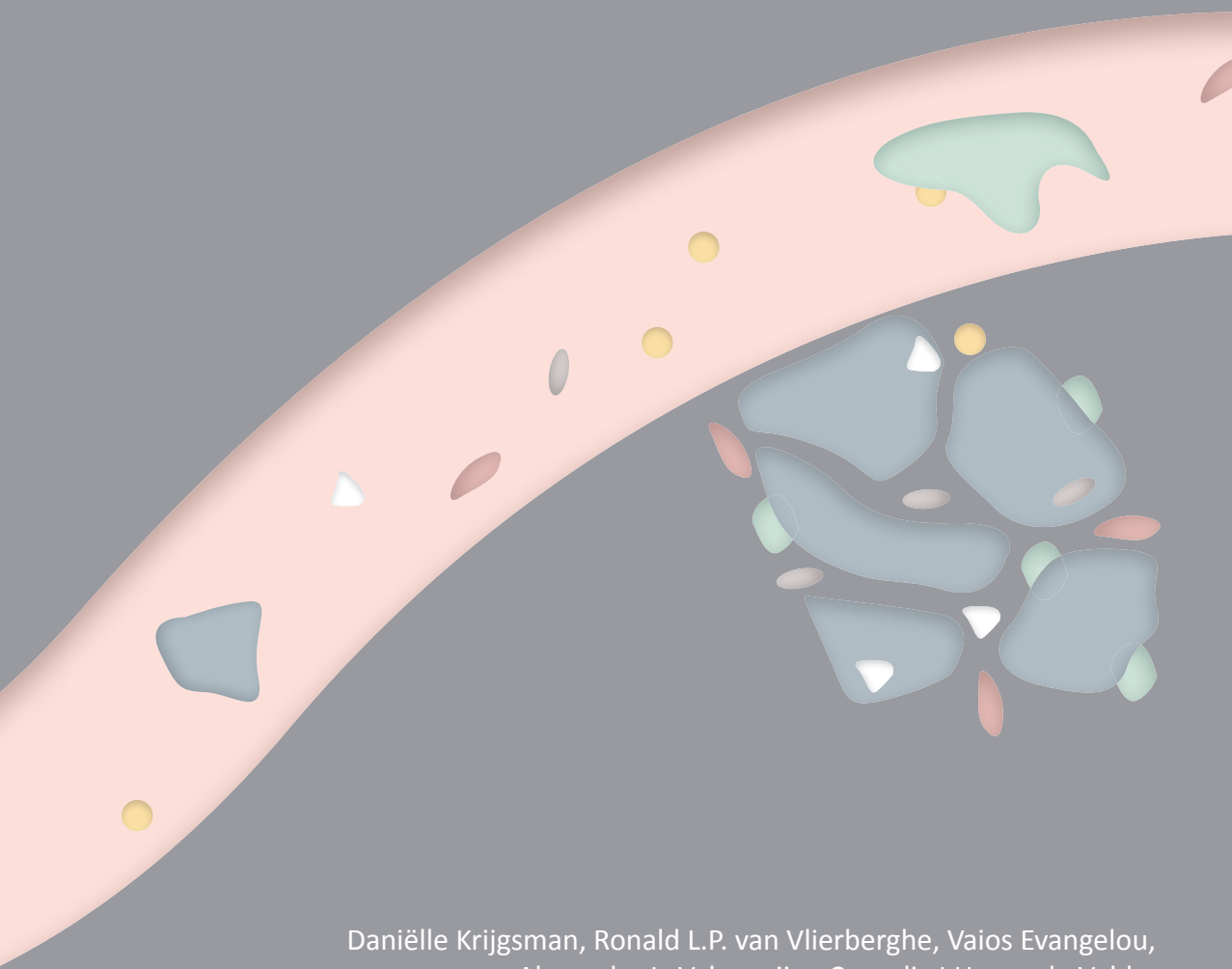
16. Biassoni, R., Cantoni C, Pende D, Sivori S, Parolini S, Vitale M, et al. Human natural killer cell receptors and co-receptors. *Immunol Rev* **2001**, *181*, 203-214.
17. Dutertre, C.A.; Bonnin-Gelize, E.; Pulford, K.; Bourel, D.; Fridman, W.H.; Teillaud, J.L. A novel subset of NK cells expressing high levels of inhibitory FcgammaRIIB modulating antibody-dependent function. *J Leukoc Biol* **2008**, *84*, 1511-1520, doi:10.1189/jlb.0608343.
18. Addison, E.G.; North, J.; Bakhsh, I.; Marden, C.; Haq, S.; Al-Sarraj, S.; Malayeri, R.; Wickremasinghe, R.G.; Davies, J.K.; Lowdell, M.W. Ligation of CD8alpha on human natural killer cells prevents activation-induced apoptosis and enhances cytolytic activity. *Immunology* **2005**, *116*, 354-361, doi:10.1111/j.1365-2567.2005.02235.x.
19. Peng, Y.P., Zhu Y, Zhang JJ, Xu ZK, Qian ZY, Dai CC, et al. Comprehensive analysis of the percentage of surface receptors and cytotoxic granules positive natural killer cells in patients with pancreatic cancer, gastric cancer, and colorectal cancer. *J Transl Med* **2013**, *11*, 262, doi:1479-5876-11-262 [pii];10.1186/1479-5876-11-262 [doi].
20. Rocca, Y.S., Roberti MP, Arriaga JM, Amat M, Bruno L, Pampena MB, et al. Altered phenotype in peripheral blood and tumor-associated NK cells from colorectal cancer patients. *Innate Immun* **2013**, *19*, 76-85, doi:1753425912453187 [pii];10.1177/1753425912453187 [doi].
21. Gharagozloo, M.; Rezaei, A.; Kalantari, H.; Bahador, A.; Hassannejad, N.; Maracy, M.; Nouri, N.; Sedghi, M.; Ghazanfari, H.; Bayat, B. Decline in peripheral blood NKG2D+CD3+CD56+ NKT cells in metastatic colorectal cancer patients. *Bratisl Lek Listy* **2018**, *119*, 6-11, doi:10.4149/BLL_2018_002.
22. Baginska, J.; Viry, E.; Paggetti, J.; Medves, S.; Berchem, G.; Moussay, E.; Janji, B. The critical role of the tumor microenvironment in shaping natural killer cell-mediated anti-tumor immunity. *Front Immunol* **2013**, *4*, 490, doi:10.3389/fimmu.2013.00490.
23. Vitale, M.; Cantoni, C.; Pietra, G.; Mingari, M.C.; Moretta, L. Effect of tumor cells and tumor microenvironment on NK-cell function. *Eur J Immunol* **2014**, *44*, 1582-1592, doi:10.1002/eji.201344272.
24. Balsamo, M.; Manzini, C.; Pietra, G.; Raggi, F.; Blengio, F.; Mingari, M.C.; Varesio, L.; Moretta, L.; Bosco, M.C.; Vitale, M. Hypoxia downregulates the expression of activating receptors involved in NK-cell-mediated target cell killing without affecting ADCC. *Eur J Immunol* **2013**, *43*, 2756-2764, doi:10.1002/eji.201343448.
25. Han, B.; Mao, F.Y.; Zhao, Y.L.; Lv, Y.P.; Teng, Y.S.; Duan, M.; Chen, W.; Cheng, P.; Wang, T.T.; Liang, Z.Y., et al. Altered NKp30, NKp46, NKG2D, and DNAM-1 Expression on Circulating NK Cells Is Associated with Tumor Progression in Human Gastric Cancer. *J Immunol Res* **2018**, *2018*, 6248590, doi:10.1155/2018/6248590.
26. Valayer, A.; Brea, D.; Lajoie, L.; Avezard, L.; Combes-Soia, L.; Labas, V.; Korkmaz, B.; Thibault, G.; Baranek, T.; Si-Tahar, M. Neutrophils can disarm NK cell response through cleavage of NKp46. *J Leukoc Biol* **2017**, *101*, 253-259, doi:10.1189/jlb.3AB0316-140RR.
27. Bracci, L.; Schiavoni, G.; Sistigu, A.; Belardelli, F. Immune-based mechanisms of cytotoxic chemotherapy: implications for the design of novel and rationale-based combined treatments against cancer. *Cell Death Differ* **2014**, *21*, 15-25, doi:10.1038/cdd.2013.67.
28. Benjamini, Y.; Hochberg, Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *Journal of the Royal Statistical Society. Series B.* **1995**, *57*, 289-300.
29. Lynch, H.T.; Drescher, K.M.; de la Chapelle, A. Immunology and the Lynch syndrome. *Gastroenterology* **2008**, *134*, 1246-1249, doi:10.1053/j.gastro.2008.02.008.
30. Klatka, J.; Grywalska, E.; Hymos, A.; Krasowska, E.; Mielnik, M.; Siwicka-Gieroba, D.; Markowicz, J.; Trojanowski, P.; Olszanski, W.; Rolinski, J. Subpopulations of natural killer-T-like cells before and after surgical treatment of laryngeal cancer. *Cent Eur J Immunol* **2017**, *42*, 252-258, doi:10.5114/ceji.2017.70967.

31. Grimm, M.; Feyen, O.; Hofmann, H.; Teriete, P.; Biegner, T.; Munz, A.; Reinert, S. Immunophenotyping of patients with oral squamous cell carcinoma in peripheral blood and associated tumor tissue. *Tumour Biol* **2016**, *37*, 3807-3816, doi:10.1007/s13277-015-4224-2.
32. Angka, L.; Martel, A.B.; Kilgour, M.; Jeong, A.; Sadiq, M.; de Souza, C.T.; Baker, L.; Kennedy, M.A.; Kekre, N.; Auer, R.C. Natural Killer Cell IFNgamma Secretion is Profoundly Suppressed Following Colorectal Cancer Surgery. *Ann Surg Oncol* **2018**, *25*, 3747-3754, doi:10.1245/s10434-018-6691-3.
33. Molling, J.W.; Kolgen, W.; van der Vliet, H.J.; Boomsma, M.F.; Kruizenga, H.; Smorenburg, C.H.; Molenkamp, B.G.; Langendijk, J.A.; Leemans, C.R.; von Blomberg, B.M., et al. Peripheral blood IFN-gamma-secreting Valpha24+Vbeta11+ NKT cell numbers are decreased in cancer patients independent of tumor type or tumor load. *Int J Cancer* **2005**, *116*, 87-93, doi:10.1002/ijc.20998.
34. Dovsak, T.; Ihan, A.; Didanovic, V.; Kansky, A.; Verdenik, M.; Hren, N.I. Effect of surgery and radiotherapy on complete blood count, lymphocyte subsets and inflammatory response in patients with advanced oral cancer. *BMC Cancer* **2018**, *18*, 235, doi:10.1186/s12885-018-4136-9.
35. Wang, W.H.; Xu, H.Y.; Zhao, Z.M.; Zhang, G.M.; Lin, F.W. Dynamic and significant changes of T-cell subgroups in breast cancer patients during surgery and chemotherapy. *Int Immunopharmacol* **2018**, *65*, 279-283, doi:10.1016/j.intimp.2018.09.039.
36. Lachmann, G.; von Haefen, C.; Kurth, J.; Yuerek, F.; Spies, C. Innate immunity recovers earlier than acquired immunity during severe postoperative immunosuppression. *Int J Med Sci* **2018**, *15*, 1-9, doi:10.7150/ijms.21433.
37. Verma, R.; Foster, R.E.; Horgan, K.; Mounsey, K.; Nixon, H.; Smalle, N.; Hughes, T.A.; Carter, C.R. Lymphocyte depletion and repopulation after chemotherapy for primary breast cancer. *Breast Cancer Res* **2016**, *18*, 10, doi:10.1186/s13058-015-0669-x.



CHAPTER 5

A method for semi-automated image analysis of HLA class I tumor epithelium expression in rectal cancer



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Abstract

Biomarkers may hold the key towards development and improvement of personalized cancer treatment. For instance, tumor expression of immune system-related proteins may reveal the tumor immune status and, accordingly, determine choice for type of immunotherapy. Therefore, objective evaluation of tumor biomarker expression is needed but often challenging. For instance, human leukocyte antigen (HLA) class I tumor epithelium expression is cumbersome to quantify by eye due to its presence on both tumor epithelial cells and tumor stromal cells, as well as tumor-infiltrating immune cells. In this study, we solved this problem by setting up an immunohistochemical (IHC) double staining using a tissue microarray (TMA) of rectal tumors wherein HLA class I expression was colored with a blue chromogen, whereas non-epithelial tissue was visualized with a brown chromogen. We subsequently developed a semi-automated image analysis method that identified tumor epithelium as well as the percentage of HLA class I-positive tumor epithelium. Using this technique, we compared HCA2/HC10 and EMR8-5 antibodies for the assessment of HLA class I tumor expression and concluded that EMR8-5 is the superior antibody for this purpose. This IHC double staining can in principle be used for scoring of any biomarker expressed by tumor epithelium.

Introduction

Tissue biomarkers have a variety of applications and their use in the field of oncology is widespread. Immunohistochemistry (IHC) is used worldwide regarding morphological and pathological evaluation of tumor biomarkers, but several limitations and difficulties have been reported [1,2]. The evaluation of IHC staining's of tumor tissue sections usually relies on visual microscopic inspection, manual annotation procedures, and inter-observer agreement. This method is prone to subjective criteria and will always be qualitative rather than quantitative. In our opinion, computer-assisted image analysis is crucial for determination of oncological biomarkers to acquire quantitative, objective and reproducible data, especially for large cohorts as used in tissue microarrays (TMAs).

In this study, human leukocyte antigen (HLA) class I was chosen as tissue biomarker of interest for semi-automated analysis on a TMA of rectal cancer. Via presentation of tumor-associated antigens by HLA class I molecules, tumor cells can be recognized and killed by cytotoxic T cells. HLA molecules, therefore, play an important role in anti-tumor immune responses. Several cancer types, including rectal cancer, have been reported to downregulate HLA class I expression [3-5], which might lead to tumor escape from T cells. Studies showed that the degree of HLA class I expression on tumor cells contains important information regarding clinical outcome of patients for various cancer types [3-11]. Therefore, tumor HLA class I expression evaluation may be important for clinical cancer prognosis, but may also be included in the choice of immunotherapy for specific cancer patients. Unfortunately, HLA class I expression is cumbersome to quantify by eye on tumor epithelium, specifically due to its high heterogeneity in expression pattern and its presence on both tumor epithelial cells and tumor stromal cells, as well as tumor-infiltrating immune cells. Additionally, the evaluation of HLA class I expression is complicated due to the widespread use of antibodies that only recognize a selection of HLA class I A, B, and C alleles, such as HCA2 and HC10 [12-14]. These two antibodies are often combined to study HLA class I expression in order to cover the detection of as many different HLA class I alleles as possible [4-6,15]. Unfortunately, HCA2 cross-reacts with non-classical HLA class I molecules HLA-E, HLA-F, and HLA-G [12,13], thereby possibly leading to overestimation of the total HLA class I tumor expression. The introduction of a novel monoclonal antibody, EMR8-5, recognizing, and only recognizing, HLA class I A, B, and C alleles [16], may circumvent undetected reactivity and unwanted cross reactivity. In conclusion, HLA class I is a difficult and therefore particularly suited tissue biomarker for setting up semi-automated analysis.

In order to solve the problem of discriminating between tumor epithelium and non-epithelial tissue, we developed a double staining wherein HLA class I was visualized with a blue chromogen,

whereas all non-epithelial tissue, i.e. stromal cells, blood vessels, and immune cells, was colored with a brown chromogen. Using a negative selection method, tumor epithelium could automatically be selected by excluding all brown-stained non-epithelial tissue. With this method we scored HLA class I expression in tumor epithelium in a TMA of primary tumors from rectal cancer patients. Next, we investigated whether EMR8-5 better detects HLA class I expression in tumor epithelium in rectal cancer than the combined HCA2/HC10 antibodies.

Materials and Methods

Study population

The study population consisted of 495 patients diagnosed with rectal cancer included in the Dutch total mesorectal excision (TME) trial (January 12th, 1996, DUT-KWF-CKVO-9504, EORTC-40971, EU-96020) who underwent TME surgery without pre-operative radiotherapy [7]. All patients included in the TME trial gave written informed consent for participation and retrospective use of samples gathered during the trial. A TMA was produced as described in the study by Reimers *et al.* [5], and used in this study. Tissue sections (4 µm) of the TMA blocks were cut following a standard procedure and transferred onto glass slides using tape.

Antibodies

The mouse monoclonal antibodies HCA2 and HC10 were used, which were kindly provided by Prof. Dr. J. Neefjes (LUMC, Leiden, The Netherlands). HCA2 recognizes the heavy chains of all HLA-A molecules except HLA-A24, as well as some HLA-B, HLA-C, HLA-E, HLA-F, and HLA-G heavy chains [12,13]. HC10 is known to react with HLA-B and HLA-C heavy chains and with some alleles of HLA-A heavy chains (HLA-A*10, A*28, A*29, A*30, A*31, A*32, and A*33) [14]. Furthermore, the mouse monoclonal antibody EMR8-5 (ab70328, AbCam, Cambridge, UK) was used, which is reported to be an anti-pan classical HLA class I antibody, recognizing HLA-A, -B, and -C alleles [16]. It has been shown to strongly react with the extracellular domains that were tested (i.e. HLA- A*2402, A*0101, A*1101, A*0201, A*0207, B*0702, B*0801, B*1501, B*3501, B*4001, B*4002, B*4006, B*4403, Cw*0102, Cw*0801, Cw*1202, and Cw*1502) [16]. Additionally, a mix of rabbit polyclonal antibodies targeting collagen I, collagen VI, and elastin (ab34710, ab6588, and ab23747 respectively, all from AbCam) was used in order to stain extracellular matrix (ECM), to stain stromal tissue and blood vessels in tumor tissue. A rabbit monoclonal anti-CD45 antibody (ab40763, AbCam) was included to target tumor-infiltrating immune cells. For each antibody, the dilution to obtain optimal staining was determined.

Immunohistochemistry

An IHC double staining was set up wherein HLA expression was visualized with blue chromogen, whereas tumor stromal tissue, blood vessels, and tumor-infiltrating immune cells were colored with brown chromogen. Briefly, 4 μm thick TMA sections were deparaffinized and rehydrated followed by heat-mediated antigen retrieval in Envision^{TM} FLEX target retrieval solution low pH (DAKO, Glostrup, Denmark) using a PT Link module (DAKO, Glostrup, Denmark). Endogenous peroxidase and phosphatase activity were blocked for 10 minutes with BloxAll solution (Vector Laboratories, Burlingame, USA). Two distinct antibody mixes were prepared in PBS/BSA 1% containing mouse (either HCA2/HC10 or EMR8-5) and rabbit antibodies (against collagen I, collagen VI, elastin and CD45) in the predetermined optimal dilutions. Tissue sections were then incubated overnight with either the HCA2/HC10-ECM-CD45 antibody mix or the EMR8-5-ECM-CD45 antibody mix. The following day, sections were incubated with AP-labelled secondary anti-mouse antibodies (MACH-2 Mouse AP-polymer, Biocare Medical, CA, USA) and developed with a Vector Blue Substrate kit (Vector Laboratories, Burlingame, USA). Sections were subsequently incubated with anti-rabbit HRP-labelled secondary antibodies (Rabbit Envision, DAKO, Glostrup, Denmark) and developed with a DAB substrate kit (DAKO, Glostrup, Denmark). Note that the TMA tissue sections were not counterstained with haematoxylin, like in a standard IHC staining procedure. Finally, the sections were dehydrated and mounted with Ecomount (Biocare Medical, CA, USA).

Semi-automated image analyses

Stained sections were scanned using an IntelliSite Digital pathology slide scanner (Philips, Eindhoven, The Netherlands). Images of the scanned sections were opened with Philips Digital Pathology Solution software (release 2.3.1.1, Philips Electronics). Single tumor TMA cores were then identified within the scanned sections and exported as JPEG images (20x magnification). Next, the JPEG images were imported into *AxioVision* digital image processing software (release 4.9.1, Zeiss) and HLA class I expression in the TMA tumor cores was assessed as percentage HLA class I-positive tumor epithelium from the tumor epithelium area using the following method (For detailed description, see supplementary data). In short, the tissue area of interest in the TMA core was annotated manually in *AxioVision* software, thereby excluding necrotic areas and artefacts as a result of the staining procedure to prevent interference with the analysis. The first step of the computer-assisted analysis was tissue selection based on a threshold in the RGB channels (determined for each staining) for automatic exclusion of areas within the annotated region that did not contain tissue. TMA cores

containing <350,000 pixel^2 in the tissue area (equivalent to <10% tissue area of the total core area) were excluded from analysis. In the second step, tumor epithelium was identified using hue luminance saturation (HLS) settings to discriminate between brown-stained stromal tissue, blood vessels, immune cells and HLA class I-positive or negative tumor epithelium. To correct for deposition of blue chromogen outside the tissue area, the settings for this step in the analysis were manually adapted for each TMA core. TMA cores with <5% tumor epithelium area (of the total tissue area) were excluded from analysis. Finally, HLA class I-positive tumor epithelium was identified within the total defined tumor epithelium area. This was accomplished by generation of a black and white image which was sorted into 256 levels of greyscale from black (0) to white (255). The two independent observers then determined the threshold for positive staining based on blinded assessment of five randomly selected TMA cores using the following method. The threshold for positive staining was decreased by 1 level at a time by the person responsible for the automated analysis until the independent observers indicated that the threshold resulted in optimal separation of HLA class I-positive and negative tumor epithelium. The mean of the 5 determined thresholds was used as cut-off value. Finally, to include the whole cell area of an HLA class I-positively stained tumor cell, the non-stained area within the cell (i.e. cytoplasm and nucleus) was included into the total tumor area considered as HLA class I-positive. The percentage of HLA class I-positive tumor epithelium among the total tumor epithelium area was semi-automatically scored in steps of 0.1%. In addition, HLA class I scores were categorized as follows: <60%, 60-80%, 80-95%, or 95-100% HLA class I-positive tumor epithelium.

Statistical analyses

The percentage of HLA class I-positive tumor epithelium expression was compared between assessment by HCA2/HC10 and EMR8-5 antibodies using the Spearman correlation test. Furthermore, a Chi square test was used to correlate HLA class I categorical scoring between assessment by HCA2/HC10 and EMR8-5 antibodies. *P*-values ≤0.05 were considered statistically significant.

Results

HLA class I double staining

HLA class I expression was evaluated in a TMA of 495 primary rectal tumors either by a combination of HCA2/HC10 antibodies or by EMR8-5. It was chosen to analyze one TMA core per patient in order to include a high variety of tissue cores in the analysis regarding morphology and HLA class I expression

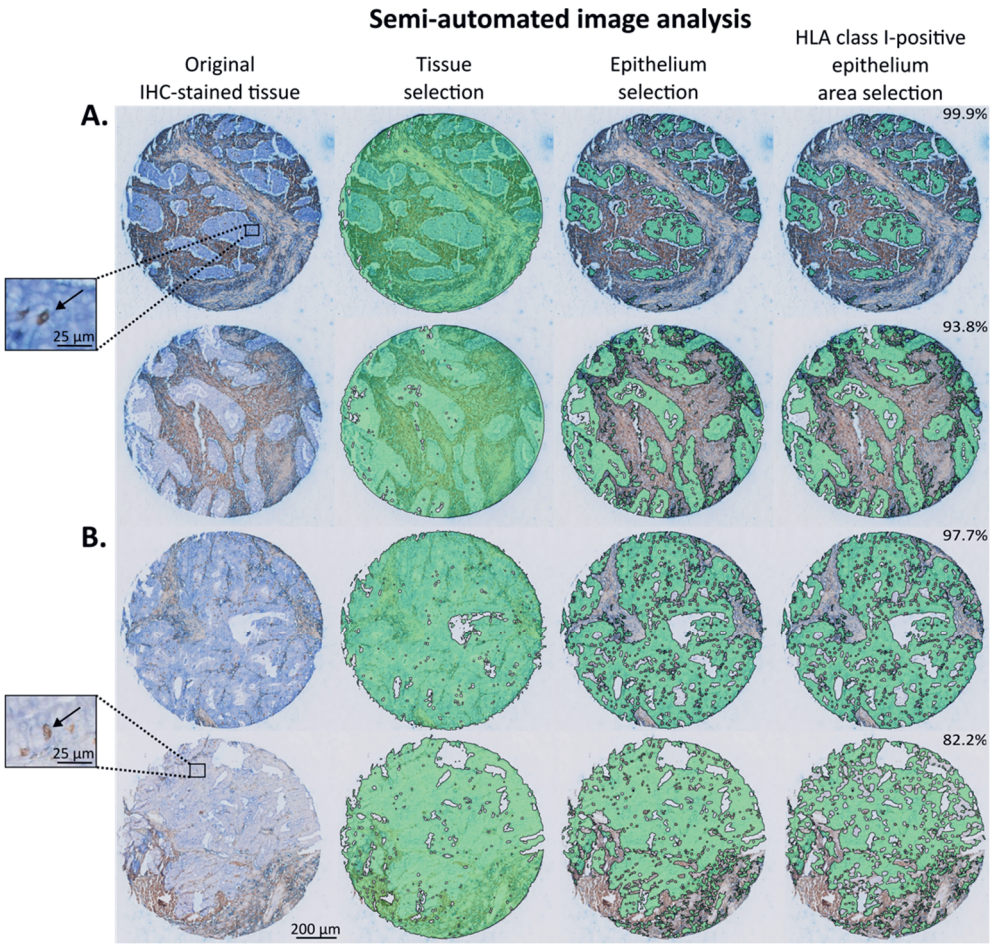


Figure 1. IHC double staining with subsequent semi-automated image analysis for HLA class I expression in four different TMA cores of rectal tumors with HCA2/HC10 or EMR8-5 antibodies. An IHC double staining was set up to analyze HLA class I expression in rectal cancer. Stromal tissue, blood vessels, and immune cells were stained brown whereas HLA class I expression (HCA2/HC10 antibody mix or EMR8-5) was stained blue. Representative images are presented of **A.** two TMA cores stained with the HCA2/HC10 antibody mix and **B.** two TMA cores stained with EMR8-5 antibodies. The arrows in the high magnification inserts indicate (brown-stained) tumor-infiltrating immune cells. The images illustrate the different steps of the semi-automated image analysis of HLA class I expression in rectal cancer. Tissue selected in each step of the analysis is indicated in green. First, all tissue in the core was selected. Second, tumor epithelium was identified within the tissue selection by subtraction of the brown stroma. Third, the percentage of HLA class I-positive epithelium area was scored within the epithelium selection as displayed in the upper right corner of each TMA core. Abbreviations: HLA (human leukocyte antigen), IHC (immunohistochemistry), TMA (tissue microarray).

as heterogeneity of HLA class I expression among tumors and not within the tumors was subject of this study. Due to staining artefacts and loss of tissue cores during the staining procedure, the HCA2/HC10 and EMR8-5 staining's could be evaluated in 284 (57.4%) and 298 tissue cores (60.2%) respectively. In total, 280 tissue cores were successfully evaluated for both IHC staining's. Figure 1 shows an example of HLA class I IHC staining in TMA cores of rectal tumors as assessed using HCA2/HC10 (Figure 1A) and EMR8-5 antibodies (Figure 1B). Please note that no overlap of blue and brown chromogens was present on tumor epithelium using this double staining, allowing for clear evaluation of HLA class I expression by tumor epithelium. Furthermore, this staining method enabled clear visualization of brown-stained tumor-infiltrating immune cells (inserts Figure 1).

Semi-automated scoring of HLA class I expression with HCA2/HC10 antibodies

HLA class I expression was semi-automatically scored in the tissue cores IHC-stained with HCA2/HC10 antibodies. Figure 1A shows the sequential steps in the semi-automated image analysis, starting with selection of the total tissue area using RGB color settings. Due to deposition of blue chromogen outside the tissue area, probably as a result of binding of the antibodies to remnants of the tape that was used to transfer the TMA sections onto glass slides, the total tissue area was overestimated in approximately 20% of the evaluated TMA cores. These non-tissue containing areas were excluded from the semi-automated analysis in the next step in which all non-epithelial tissue was excluded from the tissue selection (i.e. negative selection). Please note that brown-stained tumor-infiltrating immune cells, together with brown-stained stromal tissue, were excluded from the tumor epithelium area selection (Figure 1A). Due to different composition of stromal tissue between tumors, the brown staining intensity varied between the tissue cores but this did not affect the image analysis. Finally, the area of HLA class I-positive tumor epithelium was selected. Based on blinded manual assessment of five randomly selected TMA cores, the threshold for HLA class I-positive staining using semi-automated image analysis was determined as greyscale level 195. Thus, every pixel present in the tumor epithelium selection with a greyscale level between 0-195 was defined as HLA class I-positive, while all pixels with a greyscale level between 196-255 were defined as HLA class I negative. Figure 2 shows a representative area of the TMA stained for HLA class I expression, using the method we developed. In total, 9/19 (42.1%) of the TMA cores containing rectal tumor tissue in Figure 2 could not be analyzed due to loss of tissue cores during the staining procedure, a phenomenon that is often observed when evaluating stained TMA tissue sections. The output from the semi-automated analysis for the example cores shown in Figure 2 is summarized in Table 1 and expressed as the number of

pixels in the tissue area, tumor epithelium area and HLA class I-positive tumor epithelium area with the corresponding percentages. This information can be used to compensate for variation in the amount of tissue in different cores in case multiple tumor cores are evaluated per patient. In this study, we evaluated a single tumor core per patient and therefore, we did not compensate for variation in the amount of tissue in different cores.

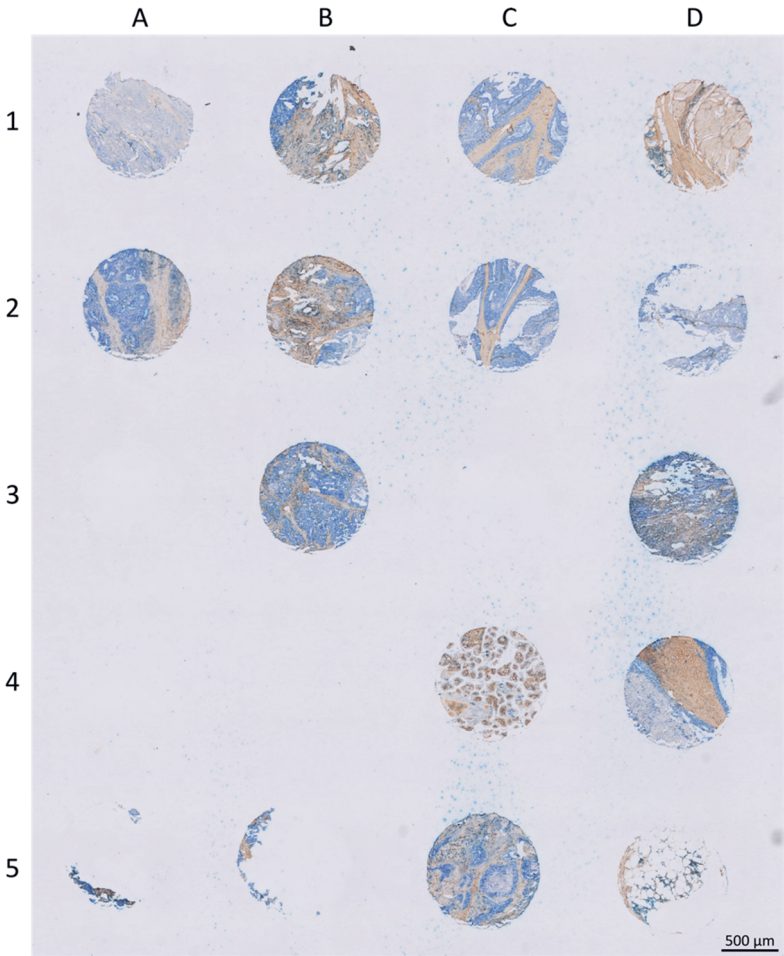


Figure 2. Example of TMA cores with rectal tumor tissue stained for HLA class I expression with EMR8-5 antibodies. An IHC double staining was set up to analyze HLA class I expression in rectal cancer. Stromal tissue, blood vessels, and immune cells were stained brown whereas HLA class I expression (EMR8-5) was stained blue. A representative area of a TMA stained for HLA class I expression, containing 20 TMA cores, is shown. Abbreviations: HLA (human leukocyte antigen), IHC (immunohistochemistry), TMA (tissue microarray).

Table 1. Example of the semi-automated image analysis output of TMA cores with rectal tumor tissue stained for HLA class I expression with EMR8-5 antibodies. The table summarizes the output of the semi-automated image analysis of the TMA cores stained for HLA class I expression shown in figure 2. TMA cores A3, A4, A5, B4, B5, C3 and D5 were excluded from analyses since <350,000 pixel^2 were present in the tissue area (equivalent to <10% tissue area of the total core area. C5 contained control placenta tissue and was therefore not analyzed. Finally, D1 could not be analyzed since the tissue that may be tumor epithelium stained brown. As a result, no discrimination could be made in D1 between tumor epithelium and non-epithelial tissue. Abbreviations: HLA (human leukocyte antigen), NA (not applicable), TMA (tissue microarray).

	Analyzed	**Tissue area**		**Tumor epithelium area**		**HLA class I-positive tumor area**	
TMA Core	**y/n**	**Pixel^2**	**% of total core area**	**Pixel^2**	**% of tissue area**	**Pixel^2**	**% of tumor epithelium area**
A1	Y	2493655	71.2%	1561920	62.6%	1107401	70.9%
A2	Y	2965056	84.7%	1107682	37.4%	1094390	98.8%
A3	N	NA	NA	NA	NA	NA	NA
A4	N	NA	NA	NA	NA	NA	NA
A5	N	152566	4.4%	NA	NA	NA	NA
B1	Y	2399358	68.6%	263289	11,0%	263026	99.9%
B2	Y	2552283	72.9%	301687	11.8%	299877	99.4%
B3	Y	2899882	82.9%	1578167	54.4%	1549760	98.2%
B4	N	NA	NA	NA	NA	NA	NA
B5	N	173445	5.0%	NA	NA	NA	NA
C1	Y	2803175	80.1%	1328377	47.4%	1253988	94.4%
C2	Y	2393691	68.4%	1567623	65.5%	1500215	95.7%
C3	N	NA	NA	NA	NA	NA	NA
C4	N	NA	NA	NA	NA	NA	NA
C5	Y	3230220	92.3%	1233284	38.1%	1209852	98.1%
D1	N	2370722	67.7%	NA	NA	NA	NA
D2	Y	1212719	34.6%	936256	77.2%	870718	93.0%
D3	Y	2810333	80.3%	690631	24.6%	556649	80.6%
D4	y	1965909	56.2%	590244	30.0%	526498	89.2%
D5	N	238838	6.8%	NA	NA	NA	NA

The mean percentage HLA class-I positive tumor epithelium area scored with the HCA2/HC10 antibody mix was 96.7%±5.3 (Table 2). In total, 220/284 (77.5%) of the TMA cores were scored with 95-100% HLA class I-positive tumor epithelium (Table 2). Additionally, 56/284 (19.7%) and 8/284 (2.8%) tissue cores were scored with 80-95% and 60-80% HLA class I-positive tumor epithelium respectively (Table 2). No TMA cores were scored with <60% HLA class I-positive tumor epithelium (Table 2). These results indicate that the double staining method and subsequent semi-automated image analysis can be used to score HLA class I expression in TMA cores of rectal cancer, enabling use of objective and consequent scoring criteria.

Semi-automated scoring of HLA class I expression using EMR8-5 antibodies

Next, we assessed HLA class I expression in TMA cores of rectal cancer with EMR8-5 antibodies using the same image analysis workflow as described above. The threshold for HLA class I-positive staining was used as determined for the HCA2/HC10 stained TMA cores. Figure 1B shows two representative TMA cores stained with EMR8-5 with the sequential steps in the semi-automated image analysis. More variation was observed in the percentages of HLA class I-positive tumor epithelium in TMA cores with EMR8-5 compared to HCA2/HC10 (Table 2). The mean percentage HLA class-I positive tumor epithelium area scored with the EMR8-5 antibodies was 92.9%±9.5 (Table 2). In contrast with the HCA2/HC10 antibody mix, almost no deposition of blue chromogen outside the tissue area was observed in the EMR8-5 staining, suggesting that EMR8-5 did not bind to remnants of the used tape. As a result, the total tissue area was not overestimated using EMR8-5. In conclusion, these results indicate that our IHC double staining technique and subsequent semi-automated image analysis can be used to score HLA class I expression on tumor epithelium using antibodies that recognize different HLA class I epitopes.

Table 2. Overview of semi-automated scoring of HLA class I expression in a TMA of rectal cancer using HCA2/HC10 or EMR8-5 antibodies. Tumor epithelium expression of HLA class I was scored in TMA tumor cores of 284 rectal cancer patients with HCA2/HC10 antibodies, and in 298 patients with EMR8-5 antibodies. The mean percentage of HLA class I-positive tumor epithelium area is shown in the table. In addition, HLA class I scores were categorized as follows: <60%, 60-80%, 80-95%, or 95-100% HLA class I-positive tumor epithelium. Abbreviation: HLA (human leukocyte antigen), SD (standard deviation).

	HCA2/HC10 N=284 (%)	**EMR8-5** N=298 (%)
HLA class I expression		
mean±SD	96.7±5.3	92.9±9.5
Range	69.1-100.0	18.9-100.0
HLA class I expression		
<60%	0 (0.0)	3 (1.0)
60-80%	8 (2.8)	16 (5.4)
80-95%	56 (19.7)	113 (37.9)
95-100%	220 (77.5)	166 (55.7)

Comparison of HCA2/HC10 and EMR8-5 antibodies for the assessment of HLA class I expression

Finally, we compared the percentage of HLA class I-positive tumor epithelium in TMA cores of rectal cancer as assessed by HCA2/HC10 and EMR8-5 antibodies, respectively. In total, 280 tissue cores were successfully analyzed for both IHC staining's. Scorings of HLA class I expression on tumor epithelium with HCA2/HC10 and EMR8-5 antibodies significantly correlated (ρ=0.136, *P*=0.022). Hence, tumor cores scored with a high area percentage of HLA class-I positive tumor epithelium assessed by HCA2/HC10 antibodies were also scored with a high area percentage of HLA class-I positive tumor

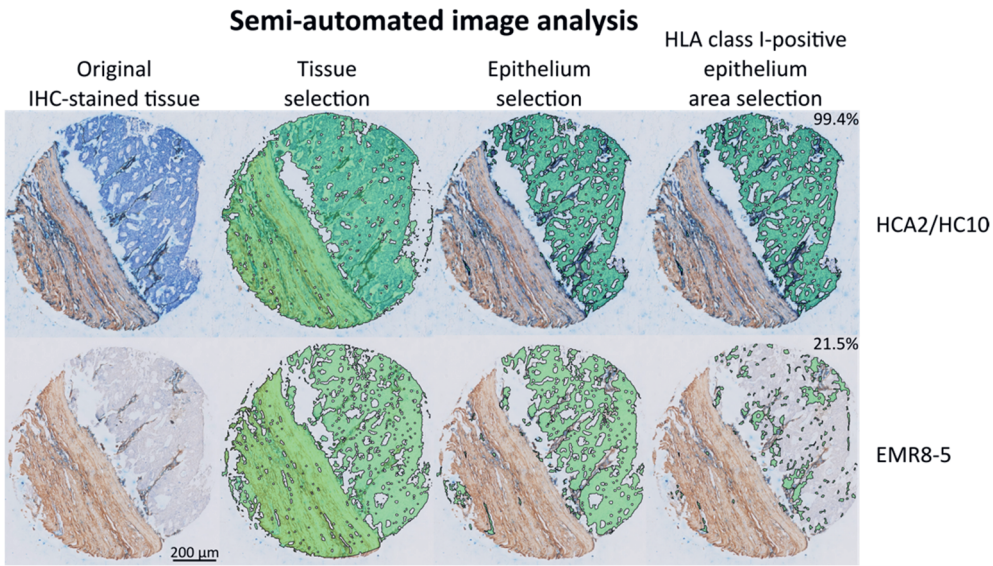


Figure 3. Comparison of HCA2/HC10 and EMR8-5 antibodies for the evaluation of HLA class I expression in a TMA core of rectal cancer. An IHC double staining was set up to analyze HLA class I expression with HCA2/HC10 and EMR8-5 antibodies in a TMA of rectal cancer. Stromal tissue, blood vessels, and immune cells were stained brown whereas HLA class I expression was stained blue. The percentage of HLA class I-positive tumor epithelium area (indicated in the figure) was evaluated using semi-automated image analysis. Tissue selected by the software in each step of the analysis is indicated in green. First, all tissue in the TMA cores was selected. Second, tumor epithelium was identified within the tissue selection. Third, the percentage of HLA class I-positive epithelium area was scored within the epithelium selection. The example shows a TMA core that was observed to be positively stained with the HCA2/HC10 antibody mix, while being mostly negative regarding EMR8-5**.** These tissue cores might express non-classical HLA class I molecules such as HLA-E, HLA-F and/or HLA-G that are recognized by the HCA2/HC10 antibody mix but not by EMR8-5. Abbreviations: HLA (human leukocyte antigen), IHC (immunohistochemistry), TMA (tissue microarray).

epithelium using EMR8.5 antibodies and the other way around. However, scoring of HLA class I expression in categories did not correlate when assessed with HCA2/HC10 and EMR8-5 antibodies (*P*=0.101). We hypothesized that tumor epithelium would be scored equal or higher for HLA class I expression with HCA2/HC10 compared to EMR8-5 antibodies due to known cross reactivity of HCA2 with non-classical HLA molecules [12,13]. In the majority of the TMA cores (82.5%), the percentage of HLA class I-positive tumor epithelium was scored equal (±10%) with HCA2/HC10 and EMR8-5 antibodies. In a fraction of the tissue cores (4.3%), the percentage of HLA class I-positive epithelium was scored >10% higher with EMR8-5 compared to HCA2/HC10. When examined in further detail, the scored differences in these tissue cores were due to inaccurate selection of the tumor epithelium as a result of tissue damage and a relatively high amount of blue chromogen deposition outside the tissue area in the HCA2/HC10 staining. In contrast, 4 times as many tissue cores (13.2%) were observed with >10% higher HLA class I-positive percentage tumor epithelium area when assessed by HCA2/HC10 antibodies compared to EMR8-5. A representative tumor that was scored >10% higher with HCA2/HC10 compared to EMR8-5 is shown in Figure 3. TMA cores with this staining pattern might express non-classical HLA class I molecules such as HLA-E, HLA-F and/or HLA-G that are recognized by HCA2/HC10 antibodies, but not by EMR8-5. In conclusion, the staining patterns of HCA2/HC10 and EMR8-5 antibodies are different, characterized by recognition of additional epitopes by HCA2/HC10, most likely non-classical HLA class I molecules.

Discussion

In order to study the expression of clinical predictive and prognostic biomarkers in tumor tissue using IHC, it is essential that scoring is standardized. Here, we presented an IHC double staining and subsequent semi-automated image analysis method that can be used to score the tumor epithelium expression of HLA class I in rectal cancer. Importantly, our developed technique has a major advantage by allowing discrimination between epithelium and non-epithelial tissue, which enabled simple semi-automated tissue selection based on color. This method therefore enabled quantification of a biomarker (i.e. HLA class I) on tumor epithelium, even when also expressed by stromal tissue and/or immune cells. Using our relatively simple IHC double staining technique, a straight forward slide scanner is sufficient in order to obtain images that can be analyzed at digital platforms that support JPEG files and that may be chosen based on availability and/or user experience. With the described image analysis method using *AxioVision* software, it was possible to calculate the percentage of HLA class I-positive tumor epithelium with a set threshold, thereby acquiring objective data. Since this

scoring method is relatively fast and simple, it is highly suitable for analysis of biomarker expression on tumor tissue in both research and clinical settings. To the best of our knowledge, we are the first to describe a double staining method for semi-automated image analysis of biomarkers expressed by cancer tissue using a TMA.

Many different techniques have been reported to evaluate biomarkers on tumor tissue [17-22]. Some studies, using *MATLAB* software, could give insights in the relocation of tumor biomarkers between a cell's membrane, cytoplasm and nucleus using RGB unmixing of images of conventional IHC-stained sections (DAB and haematoxylin signals) [20,21]. Additionally, *MATLAB* software (*MIAQuant* code) was recently reported as novel computational method for the quantification of IHC stained tissue sections [23,24]. This software is already used in clinical cancer research for determination of the expression of microRNAs in melanomas, which might have value in prediction of poor immunotherapy outcome [25]. As no discrimination could be made between tumor epithelium and non-epithelial tissue, this method is not suitable for quantification of tumor biomarkers that are also expressed by non-epithelial cells. Other studies reported that the software platform *ImageJ* can be used to evaluate the expression of biomarkers based on its immunohistochemical staining intensity on a TMA in a high throughput manner [17-19]. Using this method, the tumor-epithelium specific expression of biomarkers could be analyzed since the biomarker expression signal on epithelium was higher compared to non-epithelial tissue. Hence, the threshold for positive staining was set in a way that the relatively low signal in stromal tissue was scored as negative. Unfortunately, this method cannot be used for tumor epithelium-specific evaluation of biomarkers like HLA class I, that are also expressed at high levels on non-tumor cells. Recent studies using digital image analysis software *QuPath* or *ImageJ* showed that it is in principle possible to score biomarkers such as p53, Ki67, PR, HER2, and CD3 on tumor epithelium cells with standard IHC and haematoxylin counterstaining without a need for tumor or stromal identification markers [26-28]. However, it should be considered that the deep learning algorithms used in these analyses have disadvantages as a large number of training samples are required that need to be manually annotated by experts, thereby making it a time-consuming method to set up [29,30]. Additionally, deep learning algorithms often require the use of expensive microscopes and complicated software. For the future, it is likely that deep-learning algorithms in combination with the presented IHC double staining are even better for accurate scoring of biomarker expression on tumor epithelium. Importantly, deep learning algorithms might be less complicated to set up based on our IHC double staining since we used staining of non-epithelial cells instead of morphological features to distinguish tumor cells from other cells.

Although our semi-automated image analysis method has many advantages compared to other automated image analysis techniques, it also presents with some limitations. For instance, it provides information regarding the percentage of HLA class I-positive tumor epithelial area instead of percentage of tumor cells. Inclusion of a nuclear stain to the presented IHC staining would be required to estimate the latter. For this purpose, it is necessary to discriminate between more than two colors on one tissue slide for which the current software like we used is not suitable. Deep learning software, for instance in combination with spectral imaging, may solve this problem. Additionally, although infiltrating immune cells could be visualized and excluded from the tumor epithelium selection, it was not possible to quantify them in TMA tumor cores. This was due to the use of one chromogen for both immune cells and other non-epithelial cells, thereby complicating discrimination of these cell types. For detailed analyses of infiltrating immune cells, multiplex immunofluorescence is advised [31,32]. Furthermore, our IHC double staining does not discriminate between normal epithelium and tumor epithelium. This is not an issue when analyzing TMA tumor cores since the location of these tumor tissue cores has been annotated and checked by a pathologist. When analyzing whole tissue sections, however, it will be necessary to visually verify which areas contain tumor epithelium and should therefore be analyzed. Finally, as described in the Materials and Methods section, the presented IHC double staining uses species-specific antibodies, meaning that in combination with the rabbit-derived ECM/CD45 antibody mix, biomarkers can be evaluated using any non-rabbit-derived antibodies. In case only rabbit antibodies are available for the evaluation of a specific biomarker, a new ECM/CD45 mix has to be prepared with antibodies derived from another species.

HCA2 and HC10 antibodies are often used to study HLA class I expression in cancer [4-6,15]. Importantly, the combination of HCA2 and HC10 antibodies overestimates the total HLA class I expression due to the cross reaction of HCA2 with non-classical HLA molecules [12,13] that are known to be present in rectal tumors [5,15]. In this study, we showed that some TMA cores of rectal cancer positively stained with HCA2/HC10 antibodies while being negative regarding EMR8-5, thereby suggesting that EMR8-5 indeed does not cross react with additional non-classical HLA molecules [16]. Therefore, in our opinion, EMR8-5 is a better monoclonal antibody to assess the expression of HLA class I specifically compared to a mix of HCA2/HC10 antibodies.

In conclusion, the presented IHC double staining and subsequent semi-automated image analysis can be used for the scoring of HLA class I expression on tumor epithelium, thereby acquiring quantitative and objective data. This staining method and automated analysis can be expanded to any other tissue biomarker and also to different (epithelial) cancer types, and is therefore widely applicable for tumor biomarker analysis.

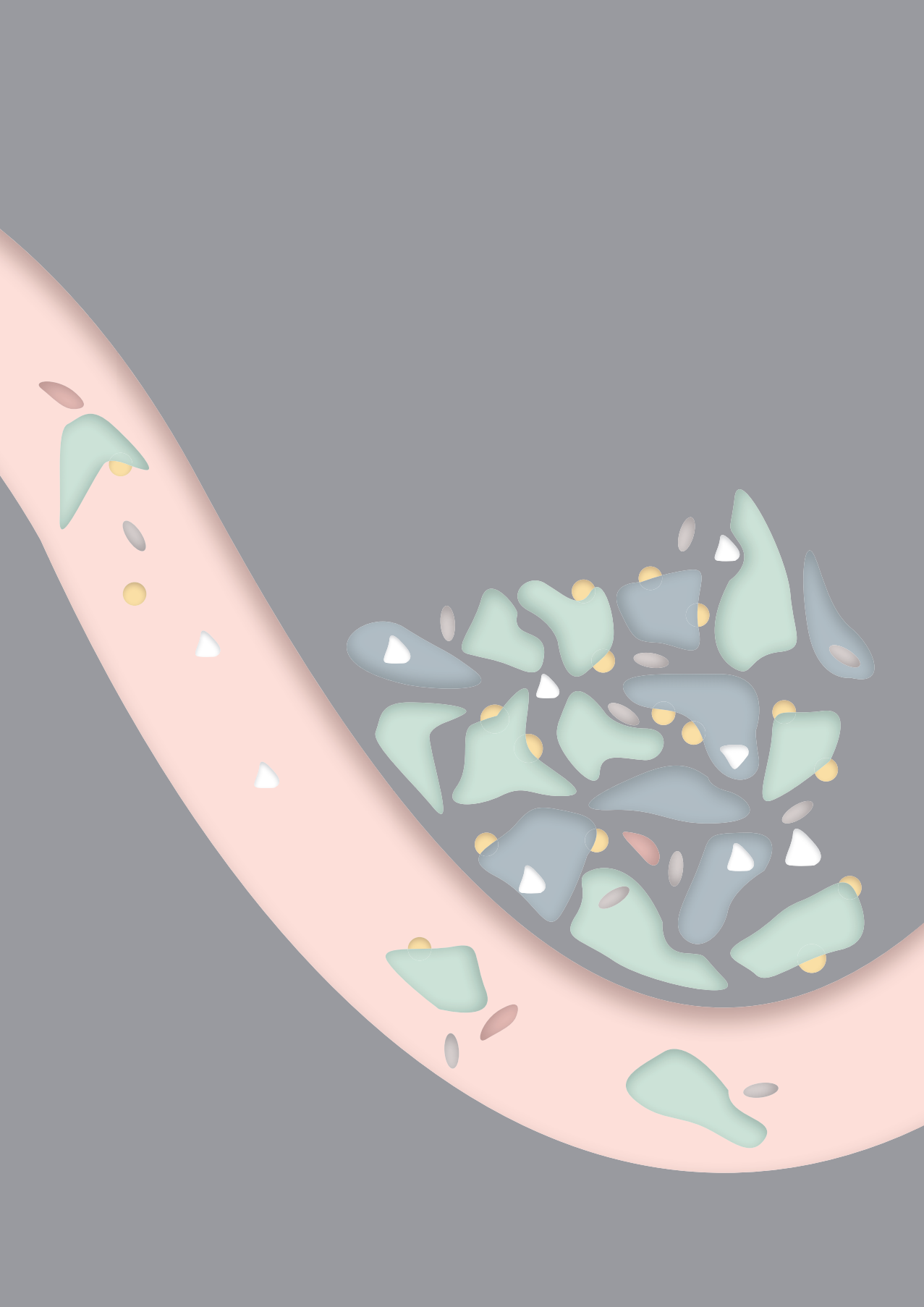
References

1. Yaziji, H.; Barry, T. Diagnostic Immunohistochemistry: what can go wrong? *Adv Anat Pathol* **2006**, *13*, 238-246, doi:10.1097/01.pap.0000213041.39070.2f.
2. Matos, L.L.; Trufelli, D.C.; de Matos, M.G.; da Silva Pinhal, M.A. Immunohistochemistry as an important tool in biomarkers detection and clinical practice. *Biomark Insights* **2010**, *5*, 9-20.
3. de Bruin, E.C.; van de Velde, C.J.; van Krieken, J.H.; Marijnen, C.A.; Medema, J.P. Epithelial human leukocyte antigen-DR expression predicts reduced recurrence rates and prolonged survival in rectal cancer patients. *Clin Cancer Res* **2008**, *14*, 1073-1079, doi:10.1158/1078-0432.CCR-07-1597.
4. Speetjens, F.M.; de Bruin, E.C.; Morreau, H.; Zeestraten, E.C.; Putter, H.; van Krieken, J.H.; van Buren, M.M.; van Velzen, M.; Dekker-Ensink, N.G.; van de Velde, C.J., et al. Clinical impact of HLA class I expression in rectal cancer. *Cancer Immunol Immunother* **2008**, *57*, 601-609, doi:10.1007/s00262-007-0396-y.
5. Reimers, M.S.; Engels, C.C.; Putter, H.; Morreau, H.; Liefers, G.J.; van de Velde, C.J.; Kuppen, P.J. Prognostic value of HLA class I, HLA-E, HLA-G and Tregs in rectal cancer: a retrospective cohort study. *BMC Cancer* **2014**, *14*, 486, doi:10.1186/1471-2407-14-486.
6. Zeestraten, E.C.; Reimers, M.S.; Saadatmand, S.; Goossens-Beumer, I.J.; Dekker, J.W.; Liefers, G.J.; van den Elsen, P.J.; van de Velde, C.J.; Kuppen, P.J. Combined analysis of HLA class I, HLA-E and HLA-G predicts prognosis in colon cancer patients. *Br J Cancer* **2014**, *110*, 459-468, doi:10.1038/bjc.2013.696.
7. Kapiteijn, E.; Kranenbarg, E.K.; Steup, W.H.; Taat, C.W.; Rutten, H.J.; Wiggers, T.; van Krieken, J.H.; Hermans, J.; Leer, J.W.; van de Velde, C.J. Total mesorectal excision (TME) with or without preoperative radiotherapy in the treatment of primary rectal cancer. Prospective randomised trial with standard operative and histopathological techniques. Dutch ColoRectal Cancer Group. *Eur J Surg* **1999**, *165*, 410-420, doi:10.1080/110241599750006613.
8. Watson, N.F.; Ramage, J.M.; Madjd, Z.; Spendlove, I.; Ellis, I.O.; Scholefield, J.H.; Durrant, L.G. Immunosurveillance is active in colorectal cancer as downregulation but not complete loss of MHC class I expression correlates with a poor prognosis. *Int J Cancer* **2006**, *118*, 6-10, doi:10.1002/ijc.21303.
9. Sandel, M.H.; Speetjens, F.M.; Menon, A.G.; Albertsson, P.A.; Basse, P.H.; Hokland, M.; Nagelkerke, J.F.; Tollenaar, R.A.; van de Velde, C.J.; Kuppen, P.J. Natural killer cells infiltrating colorectal cancer and MHC class I expression. *Mol Immunol* **2005**, *42*, 541-546, doi:10.1016/j.molimm.2004.07.039.
10. Mizukami, Y.; Kono, K.; Maruyama, T.; Watanabe, M.; Kawaguchi, Y.; Kamimura, K.; Fujii, H. Downregulation of HLA Class I molecules in the tumour is associated with a poor prognosis in patients with oesophageal squamous cell carcinoma. *Br J Cancer* **2008**, *99*, 1462-1467, doi:10.1038/sj.bjc.6604715.
11. Kaneko, K.; Ishigami, S.; Kijima, Y.; Funasako, Y.; Hirata, M.; Okumura, H.; Shinchi, H.; Koriyama, C.; Ueno, S.; Yoshinaka, H., et al. Clinical implication of HLA class I expression in breast cancer. *BMC Cancer* **2011**, *11*, 454, doi:10.1186/1471-2407-11-454.
12. Seitz, C.; Uchanska-Ziegler, B.; Zank, A.; Ziegler, A. The monoclonal antibody HCA2 recognises a broadly shared epitope on selected classical as well as several non-classical HLA class I molecules. *Mol Immunol* **1998**, *35*, 819-827.
13. Sernee, M.F.; Ploegh, H.L.; Schust, D.J. Why certain antibodies cross-react with HLA-A and HLA-G: epitope mapping of two common MHC class I reagents. *Mol Immunol* **1998**, *35*, 177-188.
14. Perosa, F.; Luccarelli, G.; Prete, M.; Favoino, E.; Ferrone, S.; Dammacco, F. Beta 2-microglobulin-free HLA class I heavy chain epitope mimicry by monoclonal antibody HC-10-specific peptide. *J Immunol* **2003**, *171*, 1918-1926.
15. Swets, M.; Konig, M.H.; Zaalberg, A.; Dekker-Ensink, N.G.; Gelderblom, H.; van de Velde, C.J.; van den Elsen, P.J.; Kuppen, P.J. HLA-G and classical HLA class I expression in primary colorectal cancer and associated liver metastases. *Hum Immunol* **2016**, *77*, 773-779, doi:10.1016/j.humimm.2016.03.001.

16. Torigoe, T.; Asanuma, H.; Nakazawa, E.; Tamura, Y.; Hirohashi, Y.; Yamamoto, E.; Kanaseki, T.; Hasegawa, T.; Sato, N. Establishment of a monoclonal anti-pan HLA class I antibody suitable for immunostaining of formalin-fixed tissue: unusually high frequency of down-regulation in breast cancer tissues. *Pathol Int* **2012**, *62*, 303-308, doi:10.1111/j.1440-1827.2012.02789.x.
17. Wang, Q.; Symes, A.J.; Kane, C.A.; Freeman, A.; Nariculam, J.; Munson, P.; Thrasivoulou, C.; Masters, J.R.; Ahmed, A. A novel role for Wnt/Ca2+ signaling in actin cytoskeleton remodeling and cell motility in prostate cancer. *PLoS One* **2010**, *5*, e10456, doi:10.1371/journal.pone.0010456.
18. Symes, A.J.; Eilertsen, M.; Millar, M.; Nariculam, J.; Freeman, A.; Notara, M.; Feneley, M.R.; Patel, H.R.; Masters, J.R.; Ahmed, A. Quantitative analysis of BTF3, HINT1, NDRG1 and ODC1 protein over-expression in human prostate cancer tissue. *PLoS One* **2013**, *8*, e84295, doi:10.1371/journal.pone.0084295.
19. Cao, K.; Arthurs, C.; Atta-Ul, A.; Millar, M.; Beltran, M.; Neuhaus, J.; Horn, L.C.; Henrique, R.; Ahmed, A.; Thrasivoulou, C. Quantitative Analysis of Seven New Prostate Cancer Biomarkers and the Potential Future of the 'Biomarker Laboratory'. *Diagnostics (Basel)* **2018**, *8*, doi:10.3390/diagnostics8030049.
20. Newberg, J.; Murphy, R.F. A framework for the automated analysis of subcellular patterns in human protein atlas images. *J Proteome Res* **2008**, *7*, 2300-2308, doi:10.1021/pr7007626.
21. Kumar, A.; Rao, A.; Bhavani, S.; Newberg, J.Y.; Murphy, R.F. Automated analysis of immunohistochemistry images identifies candidate location biomarkers for cancers. *Proc Natl Acad Sci U S A* **2014**, *111*, 18249-18254, doi:10.1073/pnas.1415120112.
22. Ali, H.R.; Irwin, M.; Morris, L.; Dawson, S.J.; Blows, F.M.; Provenzano, E.; Mahler-Araujo, B.; Pharoah, P.D.; Walton, N.A.; Brenton, J.D., et al. Astronomical algorithms for automated analysis of tissue protein expression in breast cancer. *Br J Cancer* **2013**, *108*, 602-612, doi:10.1038/bjc.2012.558.
23. Casiraghi, E.; Cossa, M.; Huber, V.; Rivoltini, L.; Tozzi, M.; Villa, A.; Vergani, B. MIAQuant, a novel system for automatic segmentation, measurement, and localization comparison of different biomarkers from serialized histological slices. *Eur J Histochem* **2017**, *61*, 2838, doi:10.4081/ejh.2017.2838.
24. Casiraghi, E.; Huber, V.; Frasca, M.; Cossa, M.; Tozzi, M.; Rivoltini, L.; Leone, B.E.; Villa, A.; Vergani, B. A novel computational method for automatic segmentation, quantification and comparative analysis of immunohistochemically labeled tissue sections. *BMC Bioinformatics* **2018**, *19*, 357, doi:10.1186/s12859-018-2302-3.
25. Huber, V.; Vallacchi, V.; Fleming, V.; Hu, X.; Cova, A.; Dugo, M.; Shahaj, E.; Sulsenti, R.; Vergani, E.; Filipazzi, P., et al. Tumor-derived microRNAs induce myeloid suppressor cells and predict immunotherapy resistance in melanoma. *J Clin Invest* **2018**, *128*, 5505-5516, doi:10.1172/JCI98060.
26. Bankhead, P.; Fernandez, J.A.; McArt, D.G.; Boyle, D.P.; Li, G.; Loughrey, M.B.; Irwin, G.W.; Harkin, D.P.; James, J.A.; McQuaid, S., et al. Integrated tumor identification and automated scoring minimizes pathologist involvement and provides new insights to key biomarkers in breast cancer. *Lab Invest* **2018**, *98*, 15-26, doi:10.1038/labinvest.2017.131.
27. Loughrey, M.B.; Bankhead, P.; Coleman, H.G.; Hagan, R.S.; Craig, S.; McCorry, A.M.B.; Gray, R.T.; McQuaid, S.; Dunne, P.D.; Hamilton, P.W., et al. Validation of the systematic scoring of immunohistochemically stained tumour tissue microarrays using QuPath digital image analysis. *Histopathology* **2018**, 10.1111/his.13516, doi:10.1111/his.13516.
28. Liu, J.; Xu, B.; Zheng, C.; Gong, Y.; Garibaldi, J.; Soria, D.; Green, A.; Ellis, I.O.; Zou, W.; Qiu, G. An End-to-End Deep Learning Histochemical Scoring System for Breast Cancer TMA. *IEEE Trans Med Imaging* **2019**, *38*, 617-628, doi:10.1109/TMI.2018.2868333.
29. Komura, D.; Ishikawa, S. Machine Learning Methods for Histopathological Image Analysis. *Comput Struct Biotechnol J* **2018**, *16*, 34-42, doi:10.1016/j.csbj.2018.01.001.
30. Sirinukunwattana, K.; Ahmed Raza, S.E.; Tsang, Y.W.; Snead, D.R.J.; Cree, I.A.; Rajpoot, N.M. A spatially constrained deep learning framework for detection of epithelial tumor nuclei in cancer histology

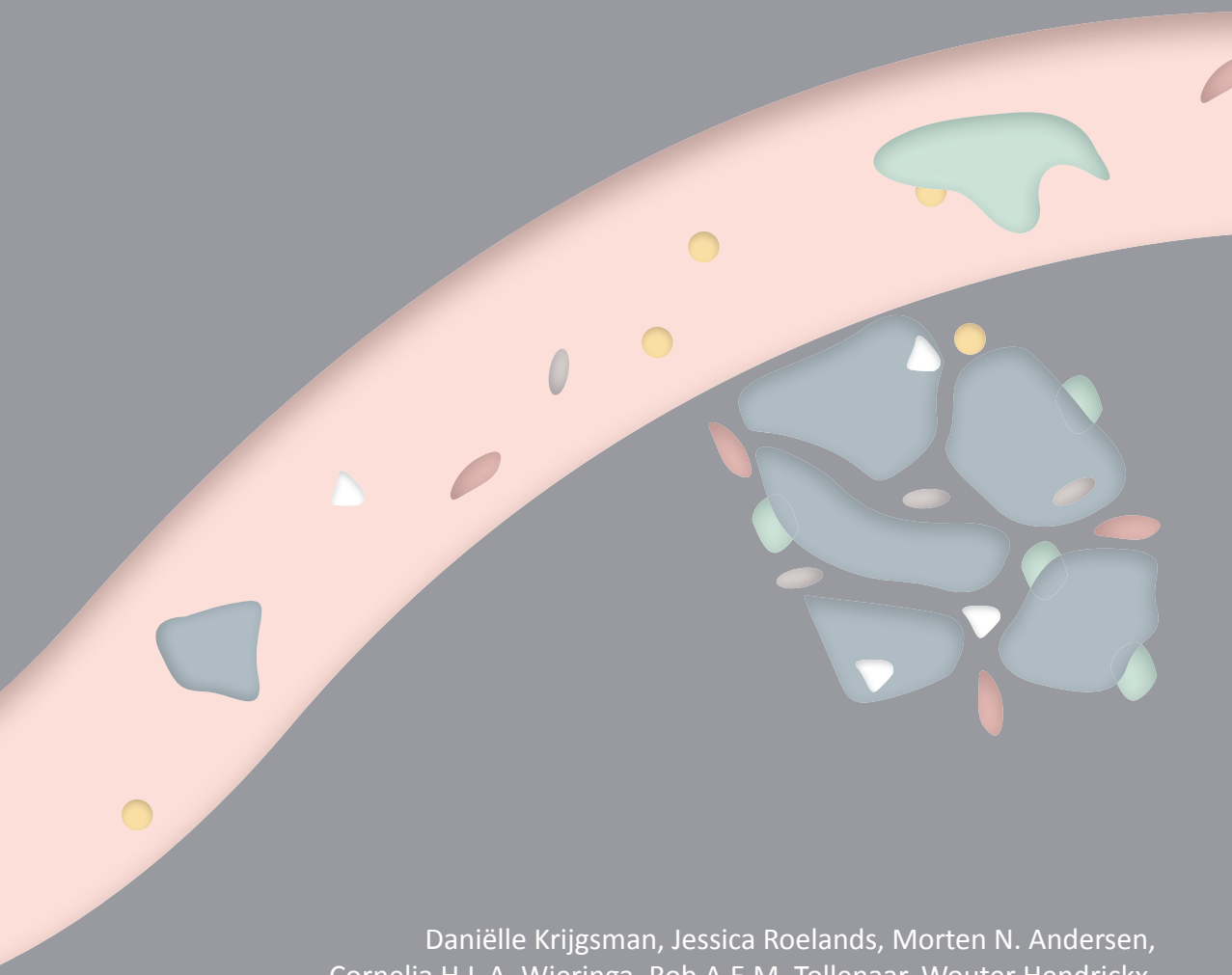
images. *Springer International Publishing Switzerland* **2015**, 10.1007/978-3-319-28194-0_19, 154-162, doi:10.1007/978-3-319-28194-0_19.

31. Parra, E.R.; Uraoka, N.; Jiang, M.; Cook, P.; Gibbons, D.; Forget, M.A.; Bernatchez, C.; Haymaker, C.; Wistuba, II; Rodriguez-Canales, J. Validation of multiplex immunofluorescence panels using multispectral microscopy for immune-profiling of formalin-fixed and paraffin-embedded human tumor tissues. *Sci Rep* **2017**, *7*, 13380, doi:10.1038/s41598-017-13942-8.
32. Park, I.J.; An, S.; Kim, S.Y.; Lim, H.M.; Hong, S.M.; Kim, M.J.; Kim, Y.J.; Yu, C.S. Prediction of radio-responsiveness with immune-profiling in patients with rectal cancer. *Oncotarget* **2017**, *8*, 79793-79802, doi:10.18632/oncotarget.19558.



CHAPTER 6

Expression of NK cell receptor ligands in primary colorectal cancer tissue in relation to the phenotype of circulating NK- and NKT cells, and clinical outcome



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Abstract

Introduction: Natural killer (NK) cells and natural killer T (NKT) cells are implicated in the development and progression of colorectal cancer (CRC). Tumor cells express NK cell receptor ligands that modulate their function. This study aimed to investigate the expression of such ligands in CRC in relation to the phenotype of circulating NK- and NKT cells, and clinical outcome.

Methods: Primary tumor tissues were analyzed for protein expression of NK cell ligands using immunohistochemistry with automated image analysis in a cohort of 78 CRC patients. For 24 of the 78 patients, RNA expression of NK cell ligands was analyzed in primary tumor tissue using RNA sequencing. Receptor expression on circulating NK- and NKT cells was previously measured by us in 71 of the 78 patients using flow cytometry.

Results: High Proliferating Cell Nuclear Antigen (PCNA) protein expression in the primary tumor associated with shorter disease-free survival (DFS) of CRC patients (P=0.026). A trend was observed towards shorter DFS in CRC patients with above-median galectin-3 protein expression in the primary tumor (P=0.055). High protein expression of galectin-3, CD1d, and human leukocyte antigen (HLA) class I, and high RNA expression of *UL16-binding protein (ULBP)-1, -2, and -5,* and *HLA-E* in the tumor tissue correlated with low expression of the corresponding receptors on circulating NK- or NKT cells (P<0.05).

Conclusions: Galectin-3 and PCNA expression in the primary tumor may be prognostic biomarkers in CRC patients. Furthermore, our results suggest that NK cell receptor ligands expressed by tumor cells may modulate the phenotype of circulating NK- and NKT cells, and facilitate immune escape of metastasizing cells.

Introduction

It has become increasingly clear that natural killer (NK) cells and natural killer T (NKT) cells use cell surface receptors to regulate their response to abnormal cells, including virus-infected cells and tumor cells [1,2]. Different inhibitory and activating receptors play a role in this process to dynamically regulate the activation state of NK cells [3], and probably NKT cells as well [4]. The activating receptors include natural killer group 2-C (NKG2C), natural killer group 2-D (NKG2D), DNAX accessory molecule-1 (DNAM-1), CD161, and the natural cytotoxicity receptors (NCRs) NKp30, NKp44, and NKp46. Other important activating receptors include the killer cell Immunoglobulin-like receptors (KIRs) CD158h/j/l/g/e [5]. CD16 (FcγRIII) on NK cells mediates antibody-dependent cell-mediated cytotoxicity (ADCC) [6]. Additionally, NKT cells express an invariant Vα24 T cell receptor (TCR) which functions as an activating receptor on these cells [4]. NK- and NKT cells also express a range of receptors that provide inhibitory signals upon stimulation, including natural killer group 2-A (NKG2A) and KIRs CD158a/b/f/e/k/z, as well as the Ig-like transcript 2 (ILT2) receptor [5,7]. Furthermore, NK cells express receptors with both inhibiting and activating functions, depending on the binding motifs they attract during downstream signaling. These receptors include signaling lymphocytic activation molecule (SLAM)F4, SLAMF6, and SLAMF7 receptors, and the KIR CD158d [5,8]. Important roles have been implicated for NK- and NKT cells in tumor development and progression in different cancer types, including colorectal cancer (CRC) [4,9,10].

We [11] and others [12,13] reported phenotypic dysregulation of NK- and NKT cells in peripheral blood of CRC patients as compared to healthy donors, characterized by downregulation of the NCRs NKp30, NKp44, and NKp46, and NKG2D. NK cells can be subdivided based on their CD56 expression: $CD56^{dim}$ NK cells primarily exert cytotoxic functions, while $CD56^{bright}$ NK cells are generally associated with immunoregulatory properties and production of pro-inflammatory cytokines [14,15]. Our study showed that circulating $CD56^{dim}$ NK cells were phenotypically altered in CRC patients, whereas $CD56^{bright}$ NK cells were not [11]. This implicates different roles of $CD56^{dim}$ and $CD56^{bright}$ NK cells in cancer progression, thereby emphasizing the need to discriminate between these NK cell subsets in future studies. Furthermore, circulating NK cells from CRC patients were observed to show impaired interferon (IFN)-γ secretion and degranulation upon activation [13]. 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. Interestingly, we showed that expression of NKp44 and NKG2D on circulating NK- and NKT cells increased in CRC patients after curative tumor resection [16]. This suggests a suppressive influence of the primary

tumor and its tumor microenvironment (TME) on NK- and NKT cell phenotype, which is abolished after surgical resection of the tumor. Several studies suggested a role for expression of NK cell ligands by tumor cells in the modulation of NK- and NKT cell phenotype [17,18].

The activating receptors expressed by NK- and NKT cells recognize different cell stress-related ligands that may be expressed and/or secreted by tumor cells. The inhibitory receptors all recognize different human leukocyte antigen (HLA) class I molecules. In this study, we focused on ligands that bind to activating receptors (NCRs and NKG2D) that were shown to be downregulated on circulating NK- and NKT cells in CRC patients as discussed above [11-13]. We focused on the ligands galectin-3 and Proliferating Cell Nuclear Antigen (PCNA) as these ligands showed clinical relevance in cancer based on literature [19-21]. Galectin-3 may be expressed by tumor cells on their cell surface which promotes epithelial-mesenchymal transition (EMT) of tumor cells [22]. Furthermore, tumor cells can upregulate expression of PCNA which facilitates and controls DNA replication via DNA polymerases [23-25]. Upon binding, galectin-3 and PCNA inhibit the function of the receptors NKp30 [19] and NKp44 [24], respectively. Upregulation of galectin-3 and PCNA have been shown to result in decreased NK cell-mediated lysis of tumor cells [19-21]. Furthermore, we focused on KIR receptors in this study, and the TCR on NKT cells specifically, as these receptors play crucial roles in the inhibition and activation of NK- and NKT cells [4,26], and therefore have clinical importance in mediating immunosurveillance [27,28]. The inhibitory KIRs CD158a and CD158b that were investigated in this study both recognize HLA-C molecules and therefore compete for binding [5]. Furthermore, the TCR on NKT cells recognizes different glycolipids in the context of the HLA-like molecule CD1d [29]. Co-stimulation of the CD161 receptor is vital in TCR/CD1d interactions, since its presence is necessary for TCR activation [30]. Several studies have now provided evidence that NK receptor-ligand interactions are involved in tumor surveillance [31] and have therefore suggested them as new targets for immune checkpoint inhibitors [32]. However, the underlying biology of these receptor-ligand interactions and their role in immune evasion remains unclear. Therefore, the aim of the present study was to investigate protein and RNA expression of NK cell ligands in primary colorectal tumors, and relate this expression to the phenotype of circulating $CD56^{dim}$ NK cells, $CD56^{bright}$ NK cells, and NKT cells in the peripheral blood, and clinical outcome of the patients.

Materials and methods

Study population

Seventy-eight patients, diagnosed with Tumor Node Metastasis (TNM) stage 0-IV colorectal adenocarcinomas between 2001 and 2007 in the Leiden University Medical Center (LUMC, the Netherlands), were included in the present study. All patients underwent surgical tumor resection. None of the patients received pre-operative chemotherapy or were diagnosed with Lynch syndrome. Formalin-fixed paraffin-embedded (FFPE) tumor tissue was obtained from primary CRC tissues of all 78 included patients. Furthermore, frozen primary tumor tissue for RNA-sequencing was obtained from 24 of the 78 patients. Peripheral blood mononuclear cells (PBMCs) were previously collected and analyzed by us for 71 of the 78 patients prior to surgery, and for 24 of the 78 patients after surgery as described elsewhere [11,16]. Hence, pre- and postoperative PBMC samples were collected and analyzed for 24 patients [16]. Clinicopathological data of all patients were available. All blood samples were obtained after approval by the Medical Ethical Committee of the LUMC (protocol number P000.193). Frozen tumor samples for RNA-sequencing were used following the code of good conduct regarding secondary use of human tissue as described in "Human Tissue and Medical Research: Code of Conduct for responsible use (2011)" Drawn up by the FEDERA. All procedures performed in this study were in accordance with the ethical standards of the Dutch law ("WMO", medical research involving human subjects act), and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. All CRC patients included in this study agreed to our use of their PBMCs and data for research purposes prior to blood sampling by written informed consent, and agreed to anonymous publication of the resulting data.

Public data

RNASeq data from The Cancer Genome Atlas (TCGA) colon adenocarcinoma (COAD) cohort was downloaded using TCGABiolinks [33]. Normalization of RNA-seq data was performed within lanes, between lanes, and per quantile using the same package. Subsequently, samples were filtered from the dataset using the "ExtractTissueSpecificSamples" function from TCGA Assembler package (v2.03.) to select tissue type solid primary tumor ("TP", N=478) and solid normal tissue ("NT", N=41) samples.

Antibodies

Mouse monoclonal antibodies were used to stain HLA class I (EMR8.5, ab70328, Abcam, Cambridge,

UK), galectin-3 (A3A12, ab2785, Abcam), PCNA (PC10, 13-3900, Invitrogen, Leiden, The Netherlands), and CD1d (NOR3.2 (NOR3.2/13.17, ab11076, Abcam) in tumor tissue. Additionally, a mix of rabbit polyclonal antibodies targeting collagen I, collagen VI, and elastin (ab34710, ab6588, and ab23747 respectively, all from AbCam) was used in order to stain extracellular matrix (ECM) and blood vessels in tumor stromal tissue. A rabbit monoclonal anti-CD45 antibody (ab40763, AbCam) was included to target tumor-infiltrating immune cells. For each antibody, the dilution to obtain optimal staining was determined.

Immunohistochemistry

In order to automatically detect and score biomarkers expressed on CRC cells, it is essential that the software can discriminate between epithelium and other cell types in the tumor tissue. Therefore, we previously set up a double staining protocol wherein a biomarker (HLA class I) was visualized with blue chromogen, whereas all non-epithelial tissue, i.e. stromal tissue, blood vessels, and immune cells, was colored with brown chromogen [34]. In this previously described staining, no counterstaining was included, thereby disabling the possibility to identify cells. In the present study, a hematoxylin counterstaining was added to the previously developed staining, and the blue chromogen that was used to detect biomarker expression on tumor epithelium was changed to pink. Using this method, expression of NK cell ligands was studied on tumor epithelial cells of primary CRC tumors. Briefly, four sequential whole tumor tissue sections (4 µm thick) were obtained from the tumors of 78 CRC patients and used for HLA class I, galectin-3, PCNA, and CD1d IHC stainings, respectively. The tumor tissue sections were deparaffinized and rehydrated followed by heat-mediated antigen retrieval in Envision^{TM} FLEX target retrieval solution low pH (DAKO, Glostrup, Denmark) using a PT Link module (DAKO). Endogenous peroxidase and phosphatase activity were blocked with BloxAll solution (Vector Laboratories, Burlingame, CA, USA) for 10 minutes. An antibody mix was prepared in PBS/BSA 1% containing mouse (either anti-HLA class I, anti-galectin-3, anti-PCNA, or anti-CD1d) and rabbit antibodies (against collagen I, collagen VI, elastin and CD45) in the predetermined optimal dilutions. Tissue sections were then incubated overnight. The following day, sections were incubated with AP-labelled secondary anti-mouse antibodies (MACH-2 Mouse AP-polymer, Biocare Medical, Pacheco, CA, USA) and developed with a Vulcan Fast Red chromogen kit (Biocare Medical). Sections were subsequently incubated with anti-rabbit HRP-labelled secondary antibodies (Rabbit Envision, DAKO) and developed with a DAB substrate kit (DAKO). Tissue sections were then counterstained with

hematoxylin (Klinipath, Amsterdam, The Netherlands). Finally, the sections were dehydrated and mounted with Ecomount (Biocare Medical).

Automated image analyses

The VECTRA 3.0 automated quantitative pathology imaging system (Akoya Biosciences, Marlborough MA, USA) was used for imaging of the multiplexed-stained slides. The whole tissue sections were scanned at a 10x magnification. In consultation with a pathologist, it was assessed whether a tumor border could be identified in the tissue sections where tumor epithelium tissue was adjacent to normal epithelium and/or other normal tissue. The tumor border was defined as the band of 1 mm at the transition between tumor and normal tissue. The tumor center area was defined as the area containing tumor epithelium that was not adjacent to any normal tissue within a range of 1 mm. PhenoChart software (Akoya Biosciences, 1.0.4.) was used to randomly select 6 multispectral imaging (MSI) fields within the tumor center, which were then scanned at a higher resolution (20x). Since PCNA expression was expected to be higher at the tumor border due to its cell proliferative function, 5 MSI fields were also selected at the tumor border in the tissue sections stained for PCNA. InForm software (Akoya Biosciences, 2.2.1) was used to prepare a spectral library of every fluorophore. Spectral unmixing was then performed on the multiplexed-stained slides. Thereafter, in order to automatically define tumor epithelium, stroma, and areas without tissue, a tissue segmentation algorithm was trained using InForm software based on DAB (stroma) and hematoxylin (tumor epithelium and stroma) signals within the selected tumor areas. Then, a cell segmentation algorithm was set up based on detection of cell nuclei using the hematoxylin signal, followed by detection of the cytoplasm and cell membrane of the cells using signals from the pink staining. The cell membrane of tumor epithelial cells was then scored as negative, weak positive, or strong positive for either HLA class I, galectin-3, PCNA, or CD1d expression using set thresholds. Two independent observers determined the thresholds based on blinded assessment of 10 randomly selected MSI fields from PCNA-stained tumor tissue sections. The thresholds were slowly increased by 1 level at a time until the independent observers indicated that the thresholds resulted in optimal visual separation of negative (no staining), weak PCNA positive (weakly stained), and strong PCNA positive (strongly stained) tumor cells. The determined thresholds on the PCNA-stained tissue sections were also used to score HLA class I, galectin-3, and CD1d expression. Hence, all sections were quantified with image analysis software using the same criteria. The percentage of positive cells (the percentage of cells with weak and strong staining combined) was used for further analyses. Additionally, the H-score was used for further

analyses since it also takes the intensity of the staining into account. This H-score was calculated by the InForm software using the following formula: [(0 * % of negative cells) + (1 * % of weak positive cells) + (2 * % of strong positive cells)].

Flow cytometry

PBMC samples were isolated and immunophenotyped using multiparameter flow cytometry as previously published [11,16]. Briefly, expression of CD16, CD158a, CD158b, NKG2A, NKG2C, CD161, CD8, DNAM-1, NKG2D, NKp30, NKp44, and NKp46 were determined on circulating $CD56^{dim}$ NK cells, $CD56^{bright}$ NK cells, and NKT cells [11,16]. Where possible, the percentage of positive cells and median fluorescence intensity (MFI) of these receptors were determined since both the presence of the receptor as the expression level can be biologically relevant.

RNA sequencing

Fresh frozen tumor samples available from the biobank (Dept. of Surgery, LUMC) were sectioned for nucleic acid extraction using a cryostat. Tissue sections flanking the corresponding samples were hematoxylin and eosin (H&E) stained to confirm tissue morphology. RNA and DNA were isolated using the AllPrep DNA/RNA Mini Kit (Qiagen, Venlo, The Netherlands) automated in the QIAcube, according to manufactures protocol. RNA sequencing (HiSeq4000) data was aligned to the human reference genome (hg38) using HISAT2 (v2.1.0). Within lane, between lane, and per quantile normalization was performed on the raw counts (featureCounts, subreads v1.5.1) with R package EDASeq (v2.12.0). RNA expression of the following NK cell receptor ligands was investigated in primary tumor tissue: *HLA-C, HLA-E, CD1d, MIC-A, MIC-B, ULBP1-6, PCNA, platelet-derived growth factor D (PDGFD), nidogen-1 (NID1), complement factor P (CFP), vimentin (VIM), C-type lectin domain family 2 member (CLEC2)-A and -D, poliovirus receptor (PVR), NECTIN2, LGALS3 (galectin-3), and BCL2-associated athanogene 6 (BAG6).*

Statistical analyses

Statistical analyses were performed using SPSS software (IBM SPSS Statistics 24, Chicago, IL, USA). A paired t-test was used in order to compare PCNA expression in the tumor center and tumor border of primary colorectal tumors. Kaplan-Meier analyses and Log-rank tests were used to correlate NK receptor ligand expression with overall survival (OS) and disease-free survival (DFS). OS was defined as the time from surgery until death, or end of follow-up (censored). DFS was defined as the time from

Table 1. Patient demographics and tumor characteristics. Clinicopathological data of the 78 CRC patients in this study, and the subgroups of patients investigated using different techniques. The column describing the patient demographics and tumor characteristics of the 71 CRC patients analyzed with flow cytometry, is adapted from [7]. The patient and tumor characteristics of the patient subgroups were compared to the full cohort using Mann-Whitney *U*- and Chi-squared tests. Statistically significant *P*-values (≤0.05) are indicated in bold. Abbreviations: CRC (Colorectal Cancer), IHC (Immunohistochemistry), NK (Natural Killer), NKT (Natural Killer T), ns (not significant), RNA seq (RNA sequencing), TNM (Tumor-Node-Metastasis).

		NK cell receptor ligands (tumor tissue)				**NK cell receptors (circulating NK/NKT cells)**	
	Full CRC cohort (N=78)	**IHC (N=74)**	***P*-value**	**RNA seq (N=24)**	***P*-value**	**Flow cytometry (N=71)**	***P*-value**
Age at time of surgery							
Median (years)	67	67	ns	69	ns	67	ns
Range (years)	25-85	25-85		47-83		25-85	
Sex							
Female	35 (44.9%)	35 (47.3%)	ns	8 (33.3%)	ns	32 (45.1%)	ns
Male	43 (55.1%)	39 (52.7%)		16 (66.7%)		39 (54.9%)	
Tumor location			ns		**0.025**		ns
Colon	64 (82.1%)	61 (82.4%)		24 (100%)		59 (83.1%)	
Rectum	14 (17.9%)	13 (17.6%)		0 (0%)		12 (16.9%)	
TNM classification							
Stage 0/I	16 (20.5%)	14 (19.0%)	ns	5 (20.8%)	ns	14 (19.7%)	ns
Stage II	26 (33.3%)	26 (35.1%)		7 (29.2%)		22 (35.2%)	
Stage III	26 (33.3%)	24 (32.4%)		5 (20.8%)		22 (33.8%)	
Stage IV	10 (12.9%)	10 (13.5%)		7 (29.2%)		8 (11.3%)	
Tumor differentiation							
Well/moderate	62 (79.5%)	60 (81.1%)	ns	18 (75.0%)	ns	55 (77.5%)	ns
Poor	13 (16.7%)	13 (17.6%)		4 (16.7%)		13 (18.3%)	
Unknown	3 (3.8%)	1 (1.4%)		2 (8.3%)		3 (4.2%)	
Positive lymph nodes			ns		ns		ns
No	45 (57.7%)	43 (58.1%)		13 (54.2%)		40 (42.3%)	
Yes	32 (41.0%)	31 (41.9%)		11 (45.8%)		30 (56.3%)	
Unknown	1 (1.3%)	0 (0%)		0 (0%)		1 (1.4%)	
Neoadjuvant radiotherapy			ns		ns		ns
No	68 (87.2%)	65 (87.8%)		21 (87.5%)		61 (85.9%)	
Yes	10 (12.8%)	9 (12.2%)		3 (12.5%)		10 (14.1%)	

Table 1. Continued

		NK cell receptor ligands (tumor tissue)				NK cell receptors (circulating NK/NKT cells)	
	Full CRC cohort (N=78)	**IHC (N=74)**	***P*-value**	**RNA seq (N=24)**	***P*-value**	**Flow cytometry (N=71)**	***P*-value**
Adjuvant chemotherapy							
No	48 (61.5%)	46 (62.2%)	ns	15 (62.5%)	ns	44 (62.0%)	ns
Yes	30 (38.5%)	28 (37.8%)		9 (37.5%)		27 (38.0%)	

surgery until first evidence of disease recurrence or until death, whichever came first, or end of follow-up (censored). Cox regression analysis was used for univariate and multivariate analyses. R package "ggplot2" (v3.3.2) was used to plot expression of NK cell ligands in solid primary tumor and normal tissue samples from the TCGA-COAD cohort. An unpaired t-test was performed to test difference in expression between tissue types. The corresponding statistical significance was plotted in the graph using "ggubr" (v.0.2.3). The Pearson correlation test was used to correlate NK ligand protein expression in the primary tumor with receptor expression on circulating NK- and NKT cells. R package "stats" (R version 3.5.1) was used to calculate Pearson's r for correlations between log2-transformed, quantile-normalized gene expression values for NK cell receptor ligands (RNASeq) and receptor expression on circulating NK- and NKT cells. Correlation coefficients between all well-defined NK cell ligands and corresponding receptors were visualized in heatmaps using "ComplexHeatmap" (v2.1.2). *P*-values ≤0.05 were considered statistically significant.

Results

Study population

The study cohort consisted of 78 CRC patients diagnosed in the LUMC in the Netherlands. Patient and tumor characteristics are shown in Table 1. Due to staining artefacts, protein expression of NK cell receptor ligands could be evaluated in 74 of the 78 primary tumors using immunohistochemistry (IHC). Furthermore, due to limited sample availability, RNA expression of NK cell receptor ligands was studied in primary tumor tissue in a subgroup of the full cohort (N=24) using RNA sequencing. Receptor expression on circulating NK- and NKT cells was previously measured by us in a subgroup of patients with available PBMC samples (N=71) using flow cytometry [11]. The patient and tumor characteristics of these subgroups are summarized in Table 1 and show no significant differences compared to the full cohort, thereby indicating that no bias was introduced. The exception was the RNA subgroup which was restricted to a group of patients with colon tumors due to limited sample availability. Figure 1 shows an overview of the measured NK cell receptor ligands in primary tumor tissue in this study, and their associated NK cell receptors expressed on circulating NK- and NKT cells.

Immunohistochemistry and automated image analysis

Whole tissue sections from the tumors of 74 CRC patients were stained for HLA class I, CD1d, galectin-3, and PCNA expression using IHC. Varying expression patterns and staining intensities were observed (Figure 2). These stainings were then used for automated image analysis to score the expression of

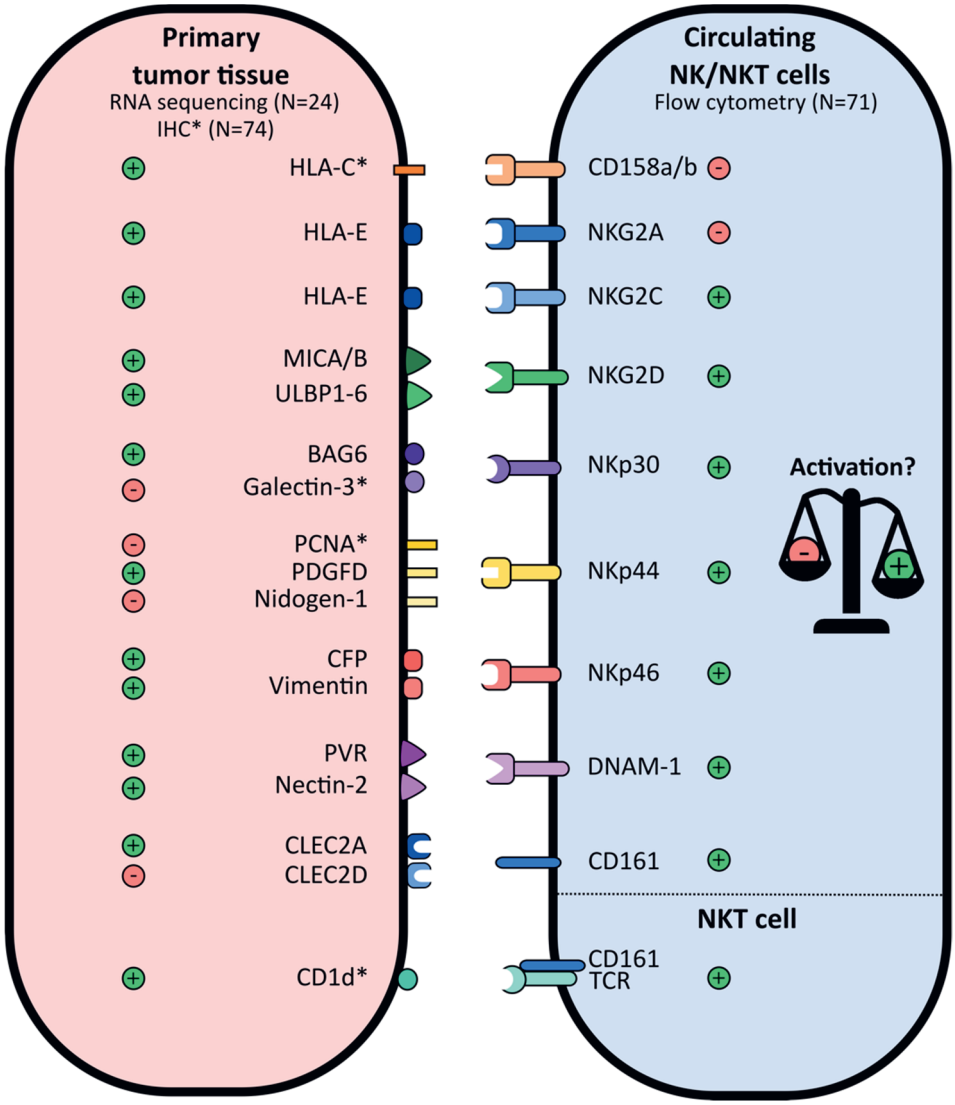


Figure 1. Overview of the studied NK cell receptor ligands in primary tumor tissue, and NK cell receptors on circulating NK- and NKT cells in CRC patients. Overview of the studied NK cell receptor ligands in primary tumor tissue, and the receptors they bind to on circulating NK- and NKT cells in CRC patients. The activating (+) and/or inhibiting (-) effects of the NK cell ligands upon interaction with their receptors is indicated, as well as the activating (+) or inhibiting (-) functions of the receptors on NK- and NKT cell activation. All indicated NK cell receptor ligands were studied on RNA level using RNA sequencing (N=24). The NK cell ligands indicated by the asterisk were also studied on protein level using IHC (N=74). In IHC, HLA-C was studied in combination with HLA-A and HLA-B (total HLA class I). On NKT cells, CD161 functions as co-stimulatory factor for TCR activation via CD1d. Expression of NK- and NKT cell receptors was studied by us on circulating NK- and NKT cells in 71 CRC patients using multiparameter flow cytometry as described elsewhere [11]. Abbreviations: BAG6 (BCL2-associated Athanogene 6), CRC (Colorectal Cancer), CFP (Complement Factor P), CLEC2 (C-type Lectin Domain

Family Member 2), DNAM-1 (DNAX Accessory Molecule-1), HLA (Human Leukocyte Antigen), IHC (Immunohistochemistry), MIC (MHC class I-related Chain), NK (Natural Killer), NKG2 (Natural Killer Group 2), NKT (Natural Killer T), PCNA (Proliferating Cell Nuclear Antigen), PDGFD (Platelet-Derived Growth Factor D), PVR (Poliovirus Receptor), TCR (T Cell Receptor), ULBP (UL-16 Binding Protein).

HLA class I, CD1d, galectin-3, and PCNA (pink staining) per tumor cell. The expression of these NK cell ligands was quantified on the cell membrane of tumor epithelial cells specifically. Hence, only surface expression of these proteins was assessed on tumor cells. Figure 3 shows examples of the automated image analysis of HLA class I expression on primary colorectal tumors from 3 different patients. The output of the image analysis of these 3 tumors is summarized in Table 2 and includes the percentage of HLA class I negative, weak positive, and strong positive cells, and the calculated H-score. The image analysis pipeline as presented in Figure 3 was applied for all stained tissue sections in this study. Protein expression of HLA class I, CD1d, galectin-3, and PCNA was therefore scored in a standardized and reproducible way using set thresholds as explained in the Materials and Methods section. For further analyses, weak and strong expression of HLA class I, CD1d, galectin-3, and PCNA by tumor cells was combined in order to analyse the effect of expression versus no expression of these receptor ligands.

Higher expression of PCNA protein in the tumor border compared to the tumor center

The majority of the studied colorectal tumor cells expressed HLA class I (median 96%, range 54-100), and galectin-3 (median 96%, range 55-100). In contrast, expression of CD1d was much more heterogeneous (median 82%, range 1-100). Additionally, the staining intensity of HLA class I, galectin-3, and CD1d was highly heterogeneous, as reflected in a wide range of the H-score (median 106, range 53-190; median 157, range 76-191; median 85, range 1-165, respectively). A summary of the expression data, including the relation between the percentage of positive cells and H-score of HLA class I, CD1d, and galectin-3 is presented in Supplementary Figure 1A. Interestingly, the majority of the studied tumors expressed low levels of PCNA (median 6.4%, range 0.0-96.0). Since PCNA is involved in cell division, we expected to find high expression in tumor cells. When evaluating the stained tumor tissue sections, we noted that the staining pattern throughout the tumor (i.e. tumor center) was different as compared to the tumor border. Therefore, we decided to compare PCNA protein expression in the tumor center and border in 46 CRC patients for which we could evaluate both. A summary of the expression data and the relation between the percentage of $PCNA^+$ cells and H-score in the tumor center and tumor border is presented in Supplementary Figure 1B. Figure 4A

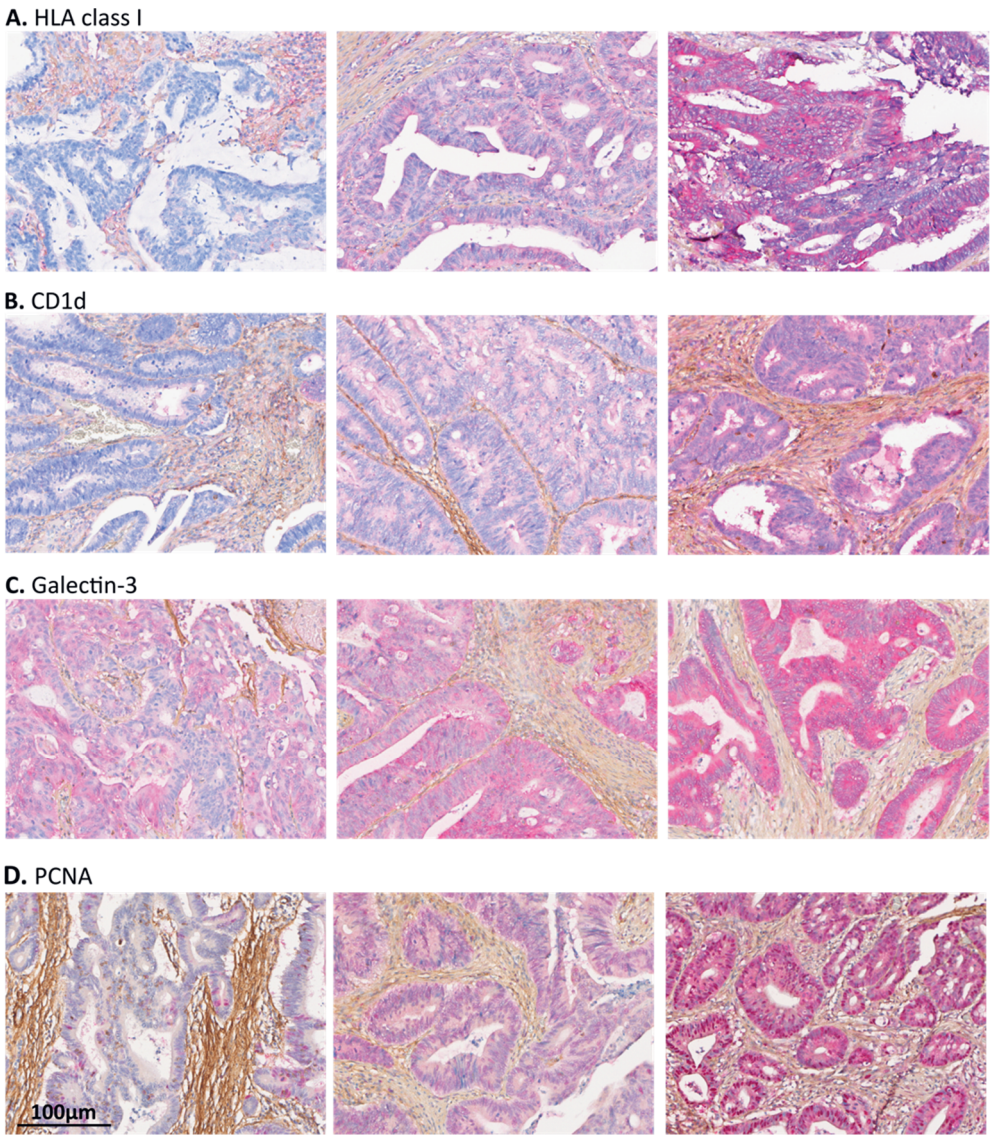


Figure 2. IHC of NK cell ligands in primary colorectal tumors. An IHC double staining was set up to analyze NK cell ligand expression in primary colorectal tumors. Stromal tissue, blood vessels, and immune cells were stained brown whereas NK cell ligand expression was stained pink. Cell nuclei were stained blue. For each staining, representative images are presented of three primary CRC tumors with negative cell membrane staining (left), strong cell membrane staining (right), and moderate cell membrane staining (middle) of **A.** HLA class I, **B.** CD1d, **C.** Galectin-3, and **D.** PCNA. All studied tumors were positive regarding galectin-3 cell membrane expression. The left figure therefore represents the weakest staining observed regarding galectin-3. Abbreviations: HLA (Human Leukocyte Antigen), IHC (Immunohistochemistry), NK (Natural Killer), PCNA (Proliferating Cell Nuclear Antigen).

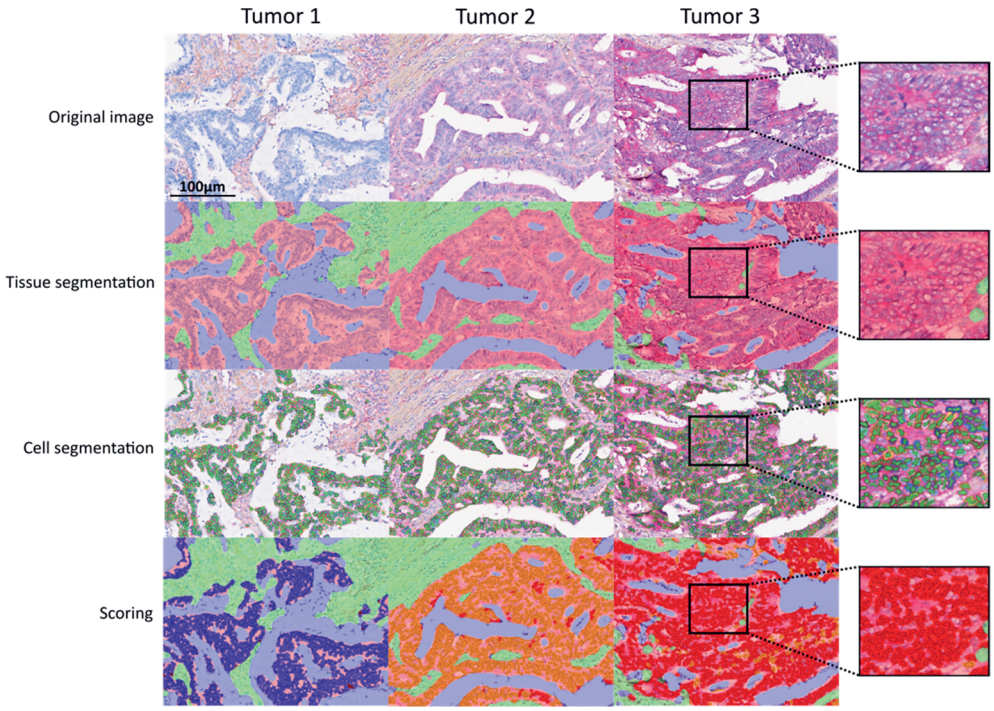


Figure 3. IHC double staining with subsequent automated image analysis of HLA class I expression in three primary colorectal tumors. An IHC double staining was set up to analyze HLA class I expression in primary colorectal tumors. Stromal tissue, blood vessels, and immune cells were stained brown whereas HLA class I expression was stained pink. Cell nuclei were stained blue. Representative images are presented of three primary colorectal tumors with variable HLA class I expression. The images illustrate the different steps of the automated image analysis of HLA class I expression. First, different tissue types were discriminated using a tissue segmentation algorithm (red: tumor epithelium; green: stromal tissue; blue: area without tissue). Cell nuclei, cytoplasm, and cell membranes were then identified within the tumor epithelium area using a cell segmentation algorithm based on hematoxylin staining. Finally, HLA class I expression was scored on the cell membrane of tumor epithelial cells as the percentage of HLA class I^+ cells. (blue: HLA class I negative; orange: weak HLA class I expression; red: strong HLA class I expression). Abbreviations: IHC (Immunohistochemistry), HLA (Human Leukocyte Antigen).

illustrates different staining patterns between the tumor center and tumor border in 2 distinct colorectal tumors. The percentage of $PCNA^+$ cells (P<0.001) and PCNA H-score (P<0.001) were significantly higher in the tumor border compared to the tumor center (Figure 4B). This indicates that tumor cells in the center of the tumor only multiply occasionally, as reflected by low expression of PCNA, possibly showing limited availability of oxygen and nutrients in the tumor center. In contrast, the tumor cells at the tumor border divide much more often, as reflected by high expression of PCNA, thereby facilitating invasive growth into adjacent tissue.

Table 2. Scoring of HLA class I expression in three colorectal tumors. An IHC double staining was set up to analyze HLA class I expression in primary colorectal tumors using automated image analysis. In this table, the output of the scoring algorithm is presented of the three tumors from Figure 3 including the percentage of negative, weakly positive, and strongly positive HLA class I tumor epithelial cells. Additionally, the H-score (based on the percentage of positive tumor epithelial cells and the staining intensity) was calculated as described in the Materials and Methods section. Abbreviations: IHC (Immunohistochemistry), HLA (Human Leukocyte Antigen).

Automated image analysis HLA class I	Tumor 1	Tumor 2	Tumor 3
Total number of tumor epithelial cells	1029	1366	1512
Total number of negative cells	987	2	2
Negative cells (% of total cells)	95.9%	0.2%	0.1%
Total number of weak positive cells	42	1291	136
Weak positive cells (% of total cells)	4.1%	94.5%	9.0%
Total number of strong positive cells	0	73	1374
Strong positive cells (% of total cells)	0.0%	5.3%	90.9%
Average H-score	4	105	191

High expression of PCNA and Galectin-3 protein is associated with short DFS

Next, we investigated the association between the cell surface expression of HLA class I, CD1d, galectin-3, and PCNA, as detected by IHC, and tumor characteristics. No associations were observed with TNM classification, tumor location, tumor differentiation grade, or tumor-lymph node invasion (data not shown). Additionally, the association between HLA class I, CD1d, galectin-3, and PCNA expression in the tumor center and clinical outcome was investigated in CRC patients. The patient population (N=74) was divided into two groups using the median expression level as cut-off. No associations were observed with OS (data not shown). Patients with TNM stage IV tumors were excluded from the DFS analyses (N=10) since they already presented with metastatic disease at time of diagnosis. No associations were observed between expression of HLA class I and CD1d in the primary tumor and DFS (data not shown). Patients with above-median percentage of $PCNA^+$ tumor cells in the tumor center showed significantly shorter DFS (P=0.026) compared to patients with below-median percentage of $PCNA^+$ tumor cells in the tumor center, with a hazard ratio (HR) of 2.3 (95% confidence interval (CI) 1.1-4.7, P=0.031) (Figure 5A). Based on these results, we wondered whether the percentage of $PCNA^+$ tumor cells in the tumor border was also associated with DFS. In contrast to the tumor center, the percentage of $PCNA^+$ tumor cells in the tumor border did not correlate with DFS

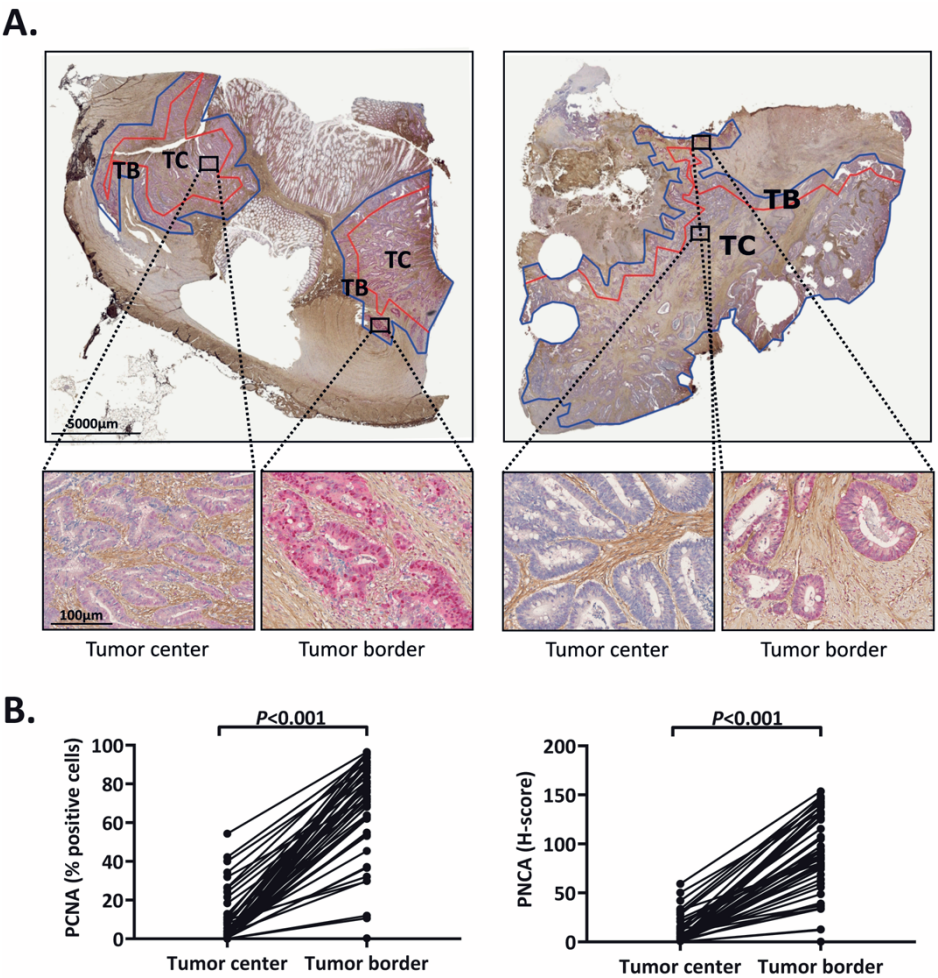


Figure 4. PCNA expression in the tumor center versus tumor border in primary colorectal tumors. An IHC double staining was set up to analyze PCNA expression in primary colorectal tumors. Stromal tissue, blood vessels, and immune cells were stained brown whereas PCNA expression was stained pink. Cell nuclei were stained blue. PCNA expression was then automatically scored in 46 primary colorectal tumors in the tumor center and in the tumor border regarding percentage of positive cells and H-score as described in the Materials and Methods section. **A.** Two representative images of higher PCNA expression in the tumor border compared to the tumor center. **B.** Higher percentage of $PCNA^{+}$ cells and PCNA H-score in the tumor border compared to the tumor center (N=46). Statistically significant *P*-values (≤0.05) are indicated in bold. Abbreviations: CRC (Colorectal Cancer), IHC (Immunohistochemistry), PCNA (Proliferating Cell Nuclear Antigen), TB (tumor border), TC (tumor center).

(N=40, *P*=0.512) as visualized in Figure 5B. Furthermore, patients with above-median galectin-3 H-score showed a trend towards shorter DFS (*P*=0.055) compared to patients with below-median galectin-3 H-score with a HR of 2.0 (95% CI 1.0-4.2, *P*=0.060) (Figure 5C). A multivariate analysis was performed for DFS in CRC patients which revealed that above-median percentage of PCNA$^+$ tumor cells in the tumor center (HR 1.4, 95% CI 0.6-3.3, *P*=0.388) and above-median galectin-3 H-score (HR 1.5, 95% CI 0.7-3.1, *P*=0.310) were not independently associated with DFS when corrected for age and TNM classification.

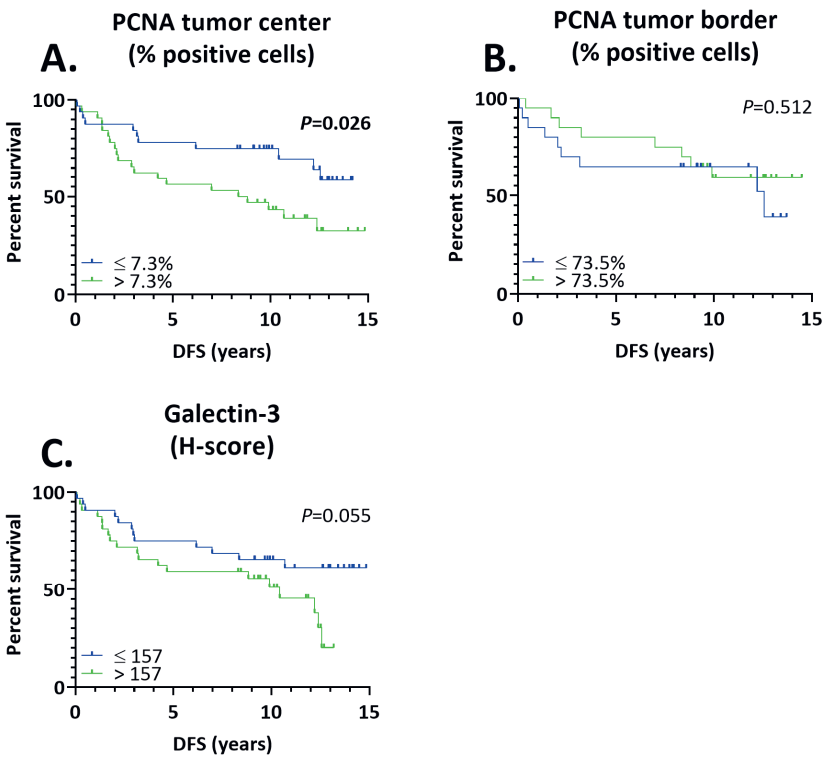


Figure 5. Relationship between PCNA and galectin-3 expression in primary tumors with DFS of CRC patients. DFS curves are shown for TNM stage 0-III CRC patients at risk for development of metastases (N=64). Stratifications were based on the median percentage of PCNA$^+$ cells and galectin-3 H-score, respectively. **A.** Relationship between PCNA expression in the tumor center (percentage of PCNA$^+$ cells) and clinical outcome of 64 CRC patients. **B.** Relationship between PCNA expression in the tumor border (percentage of PCNA$^+$ cells) and clinical outcome of 40 CRC patients. **C.** Relationship between galectin-3 expression (galectin-3 H-score) and clinical outcome of 64 CRC patients. Statistically significant *P*-values (≤0.05) are indicated in bold. Abbreviations: CRC (Colorectal Cancer), DFS (Disease-Free Survival), PCNA (Proliferating Cell Nuclear Antigen), TNM (Tumor-Node-Metastasis).

Association between protein expression of galectin-3, CD1d, and HLA class I in the primary tumor and receptor expression on circulating NK- and NKT cells

Importantly, the immunologic effect of the presence of NK cell receptor ligands on tumor cells is dependent on the interaction with their receptors on NK- and NKT cells. Therefore, we studied the correlation between protein expression of NK cell ligands in the primary tumor in relation to receptor expression on circulating $CD56^{dim}$ NK cells, $CD56^{bright}$ NK cells, and NKT cells in CRC patients. In total, protein expression data in the primary tumors and data on receptor expression on circulating NK- and NKT cells was available for 67 CRC patients included in our study. The results from the correlation analyses are summarized in Supplementary Table 1. The results revealed a significant negative correlation between the percentage of galectin-3^+ cells (P=0.002) as well as galectin H-score (P=0.026) and NKp30 expression level on NKT cells (Figure 6A). Thus, high expression of negative regulator galectin-3 was associated with low NKp30 expression on circulating NKT cells. Additionally, the percentage of $CD1d^+$ tumor cells (P=0.007) and CD1d H-score (P=0.015) negatively correlated with the expression level of TCR co-stimulatory receptor CD161 on circulating NKT cells (Figure 6B). Furthermore, the percentage of HLA class I^+ cells (P=0.023) but not HLA class I H-score (P=0.128) negatively correlated with the percentage of $CD158b^+$ $CD56^{bright}$ NK cells in the circulation of CRC patients (Figure 6C). In total, 24 CRC patients in our study had available preoperative as well as postoperative PBMC samples. Interestingly, a positive correlation was observed between the percentage of galectin-3^+ tumor cells and the change in expression level of NKp30 on circulating NKT cells after tumor resection (P<0.001, Supplementary Figure 2). Hence, CRC patients with high galectin-3 tumor expression showed a slight upregulation of the NKp30 receptor on circulating NKT cells after surgery whereas CRC patients with relatively low galectin-3 tumor expression showed downregulation of the NKp30 receptor after surgery. This suggests a direct modulatory effect of galectin-3 on NKp30 expression on NKT cells. In summary, expression of some of the studied NK cell ligands in the primary tumor associated with expression of receptors on circulating NK- and NKT cells in CRC patients. This implies that NK cell ligands expressed by primary tumor cells influence the expression levels of the receptors they bind to, thereby possibly influencing the activation of circulating NK- and NKT cells, which might result in immune escape and outgrowth of distant metastases.

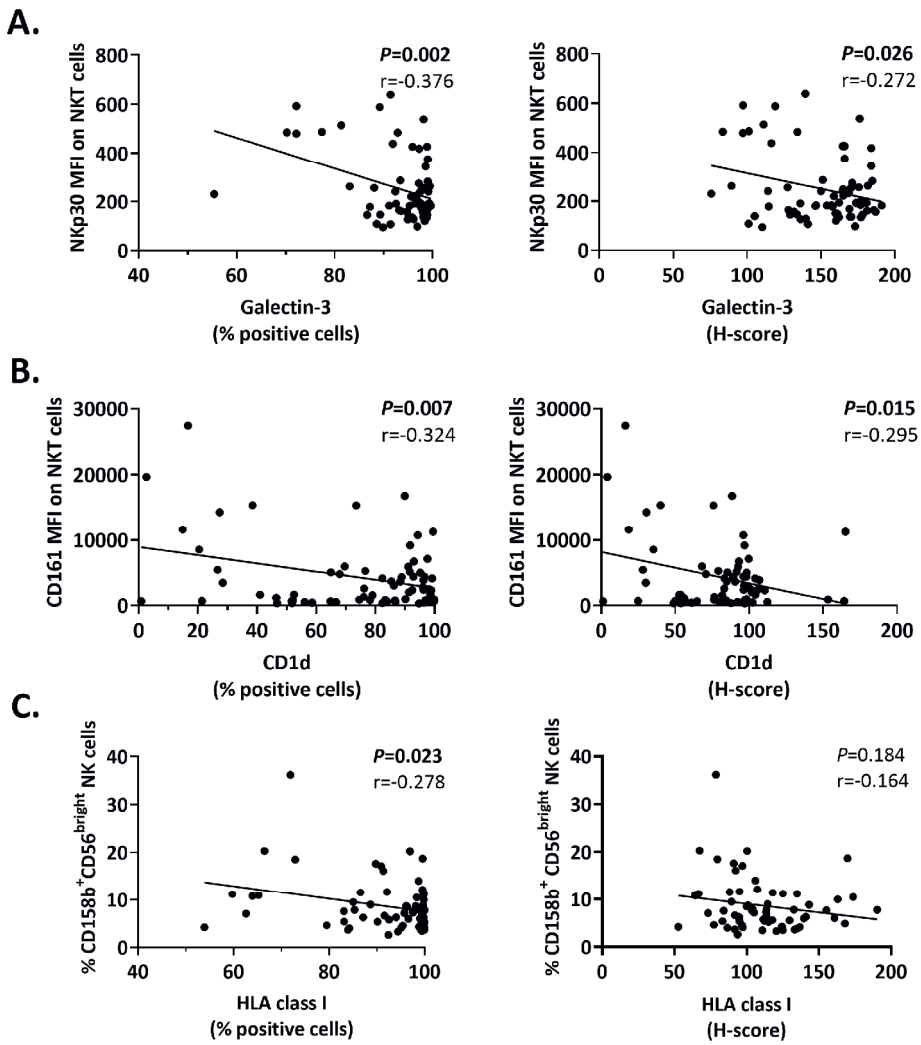


Figure 6. Correlation between NK cell receptor ligand expression in tumor tissue (protein) and their associated receptors on circulating NK and NKT cells (protein) in CRC patients. Protein expression of NK cell receptor ligands was quantified in the primary tumor of CRC patients using IHC and subsequent automated image analysis (x-axis), whereas expression of receptors on circulating NK- and NKT cells in peripheral blood of CRC patients was measured using multiparameter flow cytometry (y-axis, N=67). **A.** Correlation between galectin-3 expression in the primary tumor and NKp30 expression on circulating NKT cells. **B.** Correlation between CD1d expression in the primary tumor and CD161 expression on circulating NKT cells. **C.** Correlation between HLA class I expression in the primary tumor and the percentage of circulating CD158b^{+} CD56^{bright} NK cells. Pearson's r, and corresponding *P*-values are indicated. Statistically significant *P*-values (≤0.05) are indicated in bold. Abbreviations: CRC (Colorectal Cancer), HLA (Human Leukocyte Antigen), IHC (Immunohistochemistry), NK (Natural Killer), NKT (Natural Killer T).

Association between RNA expression of NKG2D ligands in the primary tumor and NKG2D expression on circulating NKT cells

Importantly, the analyses described above are focused on a single ligand and the receptor it binds to. However, the activation of NK- and NKT cells is much more complicated than that. Some receptors are known to recognize and bind multiple ligands which might all have different effects (either activating or inhibiting) as illustrated in Figure 1. Therefore, we further investigated the correlation between RNA expression of NK cell ligands in primary tumor tissue in relation to receptor expression on circulating $CD56^{dim}$ NK cells, $CD56^{bright}$ NK cells, and NKT cells. Using RNA expression data, it was possible to study all well-defined NK cell ligands described in literature. First, to verify expression of NK cell ligands in colon cancer (CC) samples as compared to normal tissue, we utilized the publicly available data from the TCGA-COAD cohort. Indeed, the expression of NK cell ligands was significantly different in primary tumors compared to normal solid tissue for most of the analyzed genes (Supplementary Figure 3), suggesting deregulation in CC. We then proceeded to evaluate the relation between NK cell ligands and corresponding receptor expression in our cohort. In total, RNA expression data in the primary tumors and data on receptor expression on circulating NK- and NKT cells was available for 24 CC patients included in this study. For 23 of the 24 CC patients, protein expression data of HLA class I, CD1d, galectin-3, and PCNA in the primary tumor was available as described above. For the ligands we studied on protein level using IHC, no significant correlation was observed between level of protein and corresponding RNA expression (data not shown). We studied the correlation between RNA expression of NK cell ligands and receptor expression in peripheral blood as summarized in the heatmaps in Supplementary Figure 4. Strong negative correlations were observed between RNA expression of the ligands *ULBP1* (P=0.017), *ULBP2* (P=0.028), and *ULBP5* (P=0.011) and the percentage of $NKG2D^{+}$ circulating NKT cells (Figure 7A and 7B). No correlations were observed between expression of NK cell ligands and the phenotype of circulating NK cells (Supplementary Figure 4). This suggest that for some NK cell ligands, the transcript abundance associated with NKT cell phenotype, suggesting modulation of circulating NKT cells by tumor cells. This might be an important mechanism though which colon (and rectum) tumors can escape from the immune system, resulting in the formation of distant metastases.

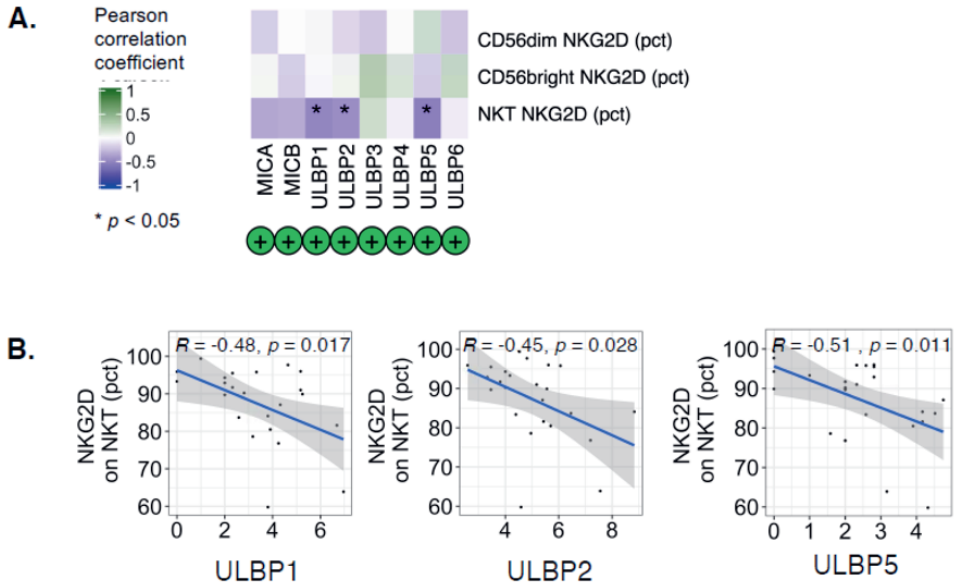


Figure 7. Correlation between expression of NK cell receptor ligands in tumor tissue (RNA) and their associated receptors on circulating NK- and NKT cells (protein) in CC patients. RNA expression of NK cell receptor ligands was analyzed in the primary tumor of CC patients using RNA sequencing whereas expression of receptors on circulating NK- and NKT cells in peripheral blood of CC patients was measured using multiparameter flow cytometry (N=24). **A.** Heatmaps of correlations between RNA NK ligand expression in primary colon tumors and receptor expression on circulating NK- and NKT cells. **B.** Scatterplots of log2 transformed, normalized gene expression of NK receptor ligands in primary colon tumors (x-axis) and receptor expression on circulating NK- and NKT cells (y-axis). Pearson's r, and corresponding *P*-values are indicated. Statistically significant *P*-values (≤0.05) are indicated in **A.** by the asterisk. Abbreviations: CC (Colon Cancer), MIC (MHC class I-related Chain), NK (Natural Killer), NKT (Natural Killer T), pct (percentage), ULBP (UL-16 Binding Protein).

Discussion

In this study, we investigated protein and RNA expression of NK cell receptor ligands in primary colorectal tumors in relation to clinical outcome, and the expression of receptors on circulating NK- and NKT cells in the peripheral blood.

We showed that higher PCNA tumor expression in the tumor center was associated with shorter DFS in the included CRC patients. In literature, controversial results have been reported regarding the potential prognostic implications of PCNA expression. High PCNA expression was considered an unfavorable prognostic marker in several CRC studies [35-37], but other studies suggested that it had no significance, and even reported an association with favorable outcome

[38,39]. A recent meta-analysis concluded that high PCNA expression was associated with poor clinical outcome in CRC patients, and suggested that PCNA might be a prognostic biomarker candidate to predict clinical outcome [40]. The discrepancies between studies may be explained by the fact that PCNA expression shows different staining patterns throughout the tumor. In the present study, we showed that PCNA expression in the tumor border was significantly higher compared to the tumor center. To our knowledge, this has not been considered previously. Whereas PCNA expression in the tumor center was associated with DFS, PCNA expression in the tumor border was not. Thus, the location of scoring PCNA in the tumor may influence the results. Furthermore, discrepancies in studies could also be explained by the fact that PCNA has different functions in tumor progression depending on the cell compartment it resides. When located in the nucleus, PCNA is involved in cell proliferation. However, when cancer cells overexpress PCNA and/or interact with NKp44^{+} NK cells, PCNA relocates from the nucleus towards the cell membrane where it may function as a ligand for NK- and NKT cells [21,24]. In the present study, we strictly quantified PCNA expression on the cell membrane of tumor cells by automated image analysis since we aimed to investigate the immune-modulating functions of this protein, whereas other studies investigated combined nucleus, cytoplasmic, and membrane expression, thereby studying the different functions of PCNA in tumor progression combined [35,37]. A uniform way of scoring IHC images could diminish the discrepancies between studies. Here, automated image analysis is crucial for obtaining standardized, robust, objective, and reproducible scoring data.

We observed a trend in this study towards short DFS in patients with high galectin-3 tumor expression which is in line with other studies [41-44]. This suggests galectin-3 may have value as a prognostic biomarker in CRC. The fact that we did not observe a statistically significant association was likely due to the limited sample size of our study cohort.

We also studied the correlation between protein and RNA expression of NK cell ligands in the primary tumor, and expression of their receptors on circulating NK- and NKT cells. Remarkably, no significant correlation was observed between level of protein and corresponding RNA expression. This may reflect differences in dynamics and kinetics of RNA and protein. However, among RNA profiles of NK cell ligands and expression of receptors on circulating NKT cells we did find correlations. Significant negative correlations were observed between RNA expression of *ULBP1*, *-2*, and *-5* and NKG2D receptor expression on NKT cells in CRC patients. This may suggest that for some NK cell ligands, the level of RNA expression reflects modulation of NKT cell phenotype by tumor cells and/or their soluble products. Many studies demonstrated that the NKG2D receptor plays an important role in cancer development and progression. Hence, surface expression of NKG2D ligands on tumor cells induced an

effective anti-tumor immune response in early CRC cancer stages [45-47]. However, sustained NKG2D ligand expression, as well as shedding of soluble NKG2D ligands into the circulation, counteracted NKG2D-dependent NK cell activity in later stages of CRC via downregulation of the NKG2D receptor [47-50], which is in line with our results. Therefore, overexpression of NKG2D ligands by tumor cells, and/or shedding of NKG2D ligands in the circulation might be important immune escape strategies for tumor cells. Schmiedel *et al.* argued that shedding of NKG2D ligands has several major advantages for cancer cells in terms of immune evasion (even more than NKG2D overexpression by tumor cells) [51]. This is because soluble NKG2D ligands are unable to activate NKG2D receptor-bearing immune cells while still being capable of binding the NKG2D receptor, thereby causing internalization of the receptor [51]. Therefore, antibodies neutralizing soluble NK cell ligands, as well as soluble NKG2D receptors to catch away soluble NKG2D ligands from the circulation, are worth pursuing in future clinical development.

In the present study, we also observed a similar pattern regarding protein expression of inhibitory ligand galectin-3 by tumor cells and expression of the NKp30 receptor on circulating NKT cells. Hence, high expression of galectin-3 by tumor cells correlated with low expression of the NKp30 receptor on NKT cells. Due to the inhibitory functions of galectin-3, it was proposed that expression and secretion of galectin-3 represents a novel pathway to escape NKp30-mediated NK cell immunosurveillance [19]. To our knowledge, we are the first to describe that high expression of the inhibitory ligand galectin-3 by tumor cells may ultimately result in downregulation of the NKp30 receptor on NKT cells in peripheral blood, thereby dysregulating NKp30-mediated activation of NKT cells. In addition to NKp30, galectin-3 has also been reported to bind the NKG2D binding site of MICA, thereby shielding this binding site and severely impairing NKG2D-mediated NK cell activation [52]. Besides its suppressive function on NK- and NKT cells, membrane-bound galectin-3 also promotes EMT of tumor cells, thereby increasing their invasiveness [22]. Additionally, soluble galectin-3 in the TME has been shown to suppress the expansion of tumor-reactive T cells, and polarize macrophages toward the immunosuppressive M2 phenotype [53]. Galectin-3 is therefore an interesting target for cancer immunotherapy. An important question is whether the immune modulating functions of galectin-3 are mediated via its membrane-bound or soluble form. Since direct interaction of galectin-3 and circulating immune cells is necessary in order for immune modulation to take place, it is most likely that soluble galectin-3 in the circulation is responsible for this process. Importantly, different NKp30 splice variants have been identified recently with different functions [54]. Whereas the NKp30A and NKp30B splice variants are the activating isoforms, NKp30C has been associated with immunosuppressive effects on NK cells [20,54]. Although speculative, the immunosuppressive effects

of tumor-expressed and/or secreted galectin-3 may be the result of a shift in NKp30A/B splice variants towards NKp30C in cancer patients [20]. In our study, it was not possible to distinguish between expression of different NKp30 isoforms on circulating NK- and NKT cells (protein level) due to lack of specific antibodies. Galectin-3 and its complex immune-modulating effects are an interesting topic of research and should be explored further in future studies.

We observed a negative correlation between CD1d protein expression on tumor cells and expression of the co-stimulatory molecule CD161 on circulating NKT cells in CRC patients. CD161 is crucial for activation of the TCR on NKT cells in a CD1d-dependent manner [30]. To our knowledge, no previous study has investigated the relation between CD1d protein expression in the primary tumor and CD161 expression on circulating NKT cells in cancer patients. The observed negative correlation in our study may implicate impairment of TCR-mediated activation of NKT cells in CD1d-expressing tumors, and therefore immune escape from NKT cells. Future studies should focus on the underlying biology of impairment of TCR-mediated activation of NKT cells and whether this can be restored. Furthermore, we observed a negative correlation between the percentage HLA class I^+ tumor cells on protein level and the percentage of $CD158b^+$ $CD56^{bright}$ NK cells in the circulation of CRC patients. Interaction between HLA class I and CD158b results in inhibition of NK cells. Our results indicate that especially patients with a high percentage of HLA class I^+ tumor cells show relatively low expression of the CD158b receptor on NK cells. In these patients, HLA class I might induce less inhibitory signals towards NK cells compared to patients with moderate HLA class I expression and relatively higher CD158b expression. In line with this hypothesis, a study by Ewen *et al.* reported that human NK cells are unleashed from KIR/HLA class I inhibition via downregulation of KIRs, resulting in enhanced killing of tumor cells [55]. The fact that the CD158b receptor is downregulated in patients with HLA class I^+ tumors suggests enhanced killing of tumor cells instead of immune escape. Further research is needed to explain this.

Our results suggest that NK cell ligands galectin-3, CD1d, HLA class I, and ULBPs modulate the expression of their receptors (NKp30, CD1d, CD158b, and NKG2D, respectively) on circulating NK- and NKT cells. This may influence their function, including their ability to directly kill tumor cells, as well as their ability to produce and secrete large amounts of cytokines in order to activate other immune cells. As a result, circulating tumor cells might escape immune recognition in the peripheral blood and form distant metastases. These observations show that the primary tumor, including its expression and secretion of immune-modulating NK cell ligands, highly influences the tumor-immune response in the circulation of patients. This emphasizes the importance of a system-based biology approach in future studies to further investigate tumor-immune interactions on different levels in a

single patient, including the primary tumor, the circulation, and, for instance, the metastatic site as well [56]. This enables us to acquire an overall picture of the immune system in relation to disease progression which will help us to better understand the underlying biology of tumor-immune interactions.

Importantly, the correlations between expression of NK cell ligands in primary tumors and receptor expression on circulating immune cells that we observed in the present study were largely restricted to NKT cells. This emphasizes the important role for NKT cells in tumor progression and implicates the involvement of these cell types in immune escape. Furthermore, in line with our recently published review on the role of NKT cells in cancer [4], our results suggest that chronic stimulation of NKT cells via ligand-receptor interactions is associated with downregulation of their corresponding receptors, irrespective of the fact whether the ligand-receptor interaction activates or inhibits their cellular function. Although speculative, this might imply the presence of a highly complex protective feedback mechanism in NK- and NKT cells that prevents both overstimulation and overinhibition of these cell types. Interestingly, we showed that downregulation of the NKp30 expression on circulating NKT cells in CRC patients was not permanent as this receptor was upregulated after removing the putative source of galectin-3 expression (i.e. the primary tumor) via tumor resection.

The strength of our study is the fact that we investigated the tumor-immune response in CRC patients on different levels. The design of these types of studies is difficult since unique and consequent sample collection is crucial, including concomitant collection of FFPE and frozen tumor tissue, as well as PBMCs from the same patients. As a result of these difficulties, the sample size of our study is relatively small which could be considered a limitation. Despite this small sample size, we observed significant correlations, suggesting that NK cell receptor ligands are involved in modulation of the phenotype of circulating NK- and NKT cells and are therefore interesting to study further. In the future, it is relevant to perform functional assays to study the consequences of the phenotypical modulations we observed, as well as studying other NK cell receptor ligands.

In conclusion, we have shown that galectin-3 and PCNA protein expression in the primary tumor are potential prognostic biomarkers in CRC patients. Furthermore, we have shown associations between expression of different NK cell receptor ligands in the primary tumor and receptor expression on circulating NK- and NKT cells. This suggests that NK cell ligands expressed by tumor cells modulate the phenotype of circulating NK- and NKT cells, thereby possibly influencing their function. As a result, circulating tumor cells might escape immune recognition in peripheral blood, resulting in the formation of distant metastases. With this study, we gained more knowledge concerning tumor-

immune interactions, not only involving the primary tumor, but also the circulation of CRC patients. In the future, this new knowledge may lead to the discovery of new immune-related biomarkers, and new targets for immunotherapies aimed at enhancing the function of NK- and NKT cells in the context of cancer.

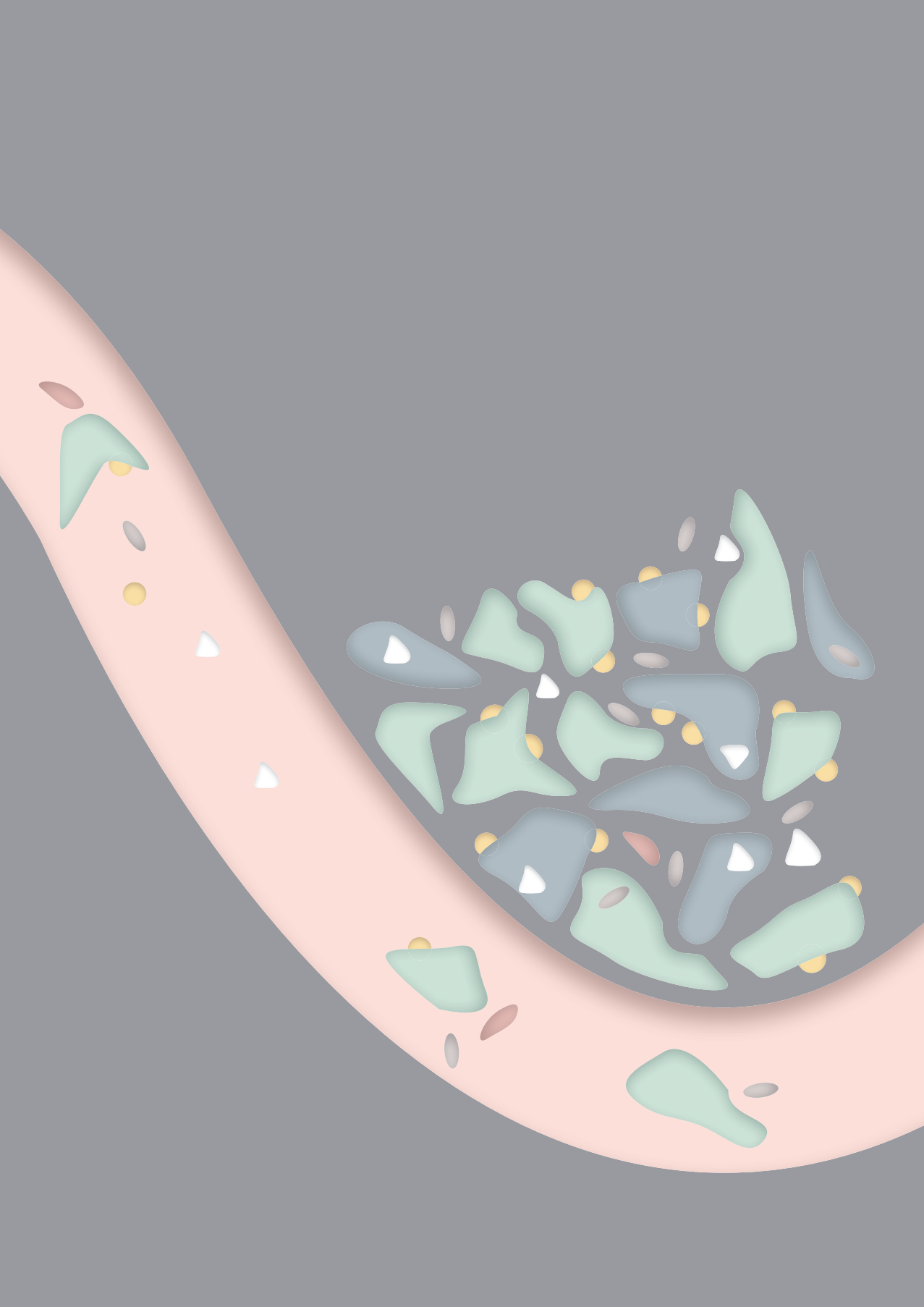
References

1. Campbell, K.S.; Hasegawa, J. Natural killer cell biology: an update and future directions. *J Allergy Clin Immunol* **2013**, *132*, 536-544, doi:10.1016/j.jaci.2013.07.006.
2. Pazina, T.; Shemesh, A.; Brusilovsky, M.; Porgador, A.; Campbell, K.S. Regulation of the Functions of Natural Cytotoxicity Receptors by Interactions with Diverse Ligands and Alterations in Splice Variant Expression. *Front Immunol* **2017**, *8*, 369, doi:10.3389/fimmu.2017.00369.
3. Biassoni, R.; Cantoni, C.; Pende, D.; Sivori, S.; Parolini, S.; Vitale, M.; Bottino, C.; Moretta, A. Human natural killer cell receptors and co-receptors. *Immunol Rev* **2001**, *181*, 203-214, doi:10.1034/j.1600-065x.2001.1810117.x.
4. Krijgsman, D.; Hokland, M.; Kuppen, P.J.K. The Role of Natural Killer T Cells in Cancer-A Phenotypical and Functional Approach. *Front Immunol* **2018**, *9*, 367, doi:10.3389/fimmu.2018.00367.
5. Ivarsson, M.A.; Michaelsson, J.; Fauriat, C. Activating killer cell Ig-like receptors in health and disease. *Front Immunol* **2014**, *5*, 184, doi:10.3389/fimmu.2014.00184.
6. Dutertre, C.A.; Bonnin-Gelize, E.; Pulford, K.; Bourel, D.; Fridman, W.H.; Teillaud, J.L. A novel subset of NK cells expressing high levels of inhibitory FcgammaRIIB modulating antibody-dependent function. *J Leukoc Biol* **2008**, *84*, 1511-1520, doi:10.1189/jlb.0608343.
7. Shiroishi, M.; Tsumoto, K.; Amano, K.; Shirakihara, Y.; Colonna, M.; Braud, V.M.; Allan, D.S.; Makadzange, A.; Rowland-Jones, S.; Willcox, B., et al. Human inhibitory receptors Ig-like transcript 2 (ILT2) and ILT4 compete with CD8 for MHC class I binding and bind preferentially to HLA-G. *Proc Natl Acad Sci U S A* **2003**, *100*, 8856-8861, doi:10.1073/pnas.1431057100.
8. Claus, M.; Urlaub, D.; Fasbender, F.; Watzl, C. SLAM family receptors in natural killer cells - Mediators of adhesion, activation and inhibition via cis and trans interactions. *Clin Immunol* **2019**, *204*, 37-42, doi:10.1016/j.clim.2018.10.011.
9. Coppola, A.; Arriga, R.; Lauro, D.; Del Principe, M.I.; Buccisano, F.; Maurillo, L.; Palomba, P.; Venditti, A.; Sconocchia, G. NK Cell Inflammation in the Clinical Outcome of Colorectal Carcinoma. *Front Med (Lausanne)* **2015**, *2*, 33, doi:10.3389/fmed.2015.00033.
10. McEwen-Smith, R.M.; Salio, M.; Cerundolo, V. The regulatory role of invariant NKT cells in tumor immunity. *Cancer Immunol Res* **2015**, *3*, 425-435, doi:10.1158/2326-6066.CIR-15-0062.
11. Krijgsman, D.; de Vries, N.L.; Skovbo, A.; Andersen, M.N.; Swets, M.; Bastiaannet, E.; Vahrmeijer, A.L.; van de Velde, C.J.H.; Heemskerk, M.H.M.; Hokland, M., et al. Characterization of circulating T-, NK-, and NKT cell subsets in patients with colorectal cancer: the peripheral blood immune cell profile. *Cancer Immunol Immunother* **2019**, *68*, 1011-1024, doi:10.1007/s00262-019-02343-7.
12. Peng, Y.P.; Zhu, Y.; Zhang, J.J.; Xu, Z.K.; Qian, Z.Y.; Dai, C.C.; Jiang, K.R.; Wu, J.L.; Gao, W.T.; Li, Q., et al. Comprehensive analysis of the percentage of surface receptors and cytotoxic granules positive natural killer cells in patients with pancreatic cancer, gastric cancer, and colorectal cancer. *J Transl Med* **2013**, *11*, 262, doi:10.1186/1479-5876-11-262.
13. Rocca, Y.S.; Roberti, M.P.; Arriaga, J.M.; Amat, M.; Bruno, L.; Pampena, M.B.; Huertas, E.; Loria, F.S.; Pairola, A.; Bianchini, M., et al. Altered phenotype in peripheral blood and tumor-associated NK cells from colorectal cancer patients. *Innate Immun* **2013**, *19*, 76-85, doi:10.1177/1753425912453187.
14. Cooper, M.A.; Fehniger, T.A.; Caligiuri, M.A. The biology of human natural killer-cell subsets. *Trends Immunol* **2001**, *22*, 633-640, doi:10.1016/s1471-4906(01)02060-9.
15. Jonges, L.E.; Albertsson, P.; van Vlierberghe, R.L.; Ensink, N.G.; Johansson, B.R.; van de Velde, C.J.; Fleuren, G.J.; Nannmark, U.; Kuppen, P.J. The phenotypic heterogeneity of human natural killer cells: presence of at least 48 different subsets in the peripheral blood. *Scand J Immunol* **2001**, *53*, 103-110, doi:10.1046/j.1365-3083.2001.00838.x.

16. Krijgsman, D.; De Vries, N.L.; Andersen, M.N.; Skovbo, A.; Tollenaar, R.; Bastiaannet, E.; Kuppen, P.J.K.; Hokland, M. The effects of tumor resection and adjuvant therapy on the peripheral blood immune cell profile in patients with colon carcinoma. *Cancer Immunol Immunother* **2020**, 10.1007/s00262-020-02590-z, doi:10.1007/s00262-020-02590-z.
17. Renukaradhya, G.J.; Sriram, V.; Du, W.; Gervay-Hague, J.; Van Kaer, L.; Brutkiewicz, R.R. Inhibition of antitumor immunity by invariant natural killer T cells in a T-cell lymphoma model in vivo. *Int J Cancer* **2006**, *118*, 3045-3053, doi:10.1002/ijc.21764.
18. Chong, T.W.; Goh, F.Y.; Sim, M.Y.; Huang, H.H.; Thike, A.A.; Lim, W.K.; Teh, B.T.; Tan, P.H. CD1d expression in renal cell carcinoma is associated with higher relapse rates, poorer cancer-specific and overall survival. *J Clin Pathol* **2015**, *68*, 200-205, doi:10.1136/jclinpath-2014-202735.
19. Wang, W.; Guo, H.; Geng, J.; Zheng, X.; Wei, H.; Sun, R.; Tian, Z. Tumor-released Galectin-3, a soluble inhibitory ligand of human NKp30, plays an important role in tumor escape from NK cell attack. *J Biol Chem* **2014**, *289*, 33311-33319, doi:10.1074/jbc.M114.603464.
20. Pogge von Strandmann, E.; Shatnyeva, O.; Hansen, H.P. NKp30 and its ligands: emerging players in tumor immune evasion from natural killer cells. *Ann Transl Med* **2015**, *3*, 314, doi:10.3978/j.issn.2305-5839.2015.09.08.
21. Rosental, B.; Hadad, U.; Brusilovsky, M.; Campbell, K.S.; Porgador, A. A novel mechanism for cancer cells to evade immune attack by NK cells: The interaction between NKp44 and proliferating cell nuclear antigen. *Oncoimmunology* **2012**, *1*, 572-574.
22. Liu, F.T.; Rabinovich, G.A. Galectins as modulators of tumour progression. *Nat Rev Cancer* **2005**, *5*, 29-41, doi:10.1038/nrc1527.
23. Stoimenov, I.; Helleday, T. PCNA on the crossroad of cancer. *Biochemical Society transactions* **2009**, *37*, 605-613, doi:10.1042/bst0370605.
24. Rosental, B.; Brusilovsky, M.; Hadad, U.; Oz, D.; Appel, M.Y.; Afergan, F.; Yossef, R.; Rosenberg, L.A.; Aharoni, A.; Cerwenka, A., et al. Proliferating cell nuclear antigen is a novel inhibitory ligand for the natural cytotoxicity receptor NKp44. *J Immunol* **2011**, *187*, 5693-5702, doi:10.4049/jimmunol.1102267.
25. Qasim, B.J.; Ali, H.H.; Hussein, A.G. Immunohistochemical expression of PCNA and CD34 in colorectal adenomas and carcinomas using specified automated cellular image analysis system: a clinicopathologic study. *Saudi journal of gastroenterology : official journal of the Saudi Gastroenterology Association* **2012**, *18*, 268-276, doi:10.4103/1319-3767.98435.
26. Long, E.O.; Kim, H.S.; Liu, D.; Peterson, M.E.; Rajagopalan, S. Controlling natural killer cell responses: integration of signals for activation and inhibition. *Annu Rev Immunol* **2013**, *31*, 227-258, doi:10.1146/annurev-immunol-020711-075005.
27. Concha-Benavente, F.; Srivastava, R.; Ferrone, S.; Ferris, R.L. Immunological and clinical significance of HLA class I antigen processing machinery component defects in malignant cells. *Oral Oncol* **2016**, *58*, 52-58, doi:10.1016/j.oraloncology.2016.05.008.
28. King, L.A.; Lameris, R.; de Gruijl, T.D.; van der Vliet, H.J. CD1d-Invariant Natural Killer T Cell-Based Cancer Immunotherapy: alpha-Galactosylceramide and Beyond. *Front Immunol* **2018**, *9*, 1519, doi:10.3389/fimmu.2018.01519.
29. Bendelac, A.; Savage, P.B.; Teyton, L. The biology of NKT cells. *Annu Rev Immunol* **2007**, *25*, 297-336, doi:10.1146/annurev.immunol.25.022106.141711.
30. Exley, M.; Porcelli, S.; Furman, M.; Garcia, J.; Balk, S. CD161 (NKR-P1A) costimulation of CD1d-dependent activation of human T cells expressing invariant V alpha 24 J alpha Q T cell receptor alpha chains. *J Exp Med* **1998**, *188*, 867-876, doi:10.1084/jem.188.5.867.
31. Barrow, A.D.; Martin, C.J.; Colonna, M. The Natural Cytotoxicity Receptors in Health and Disease. *Front Immunol* **2019**, *10*, 909, doi:10.3389/fimmu.2019.00909.
32. Yang, C.; Li, Y.; Yang, Y.; Chen, Z. Overview of Strategies to Improve Therapy against Tumors Using Natural Killer Cell. *J Immunol Res* **2020**, *2020*, 8459496, doi:10.1155/2020/8459496.

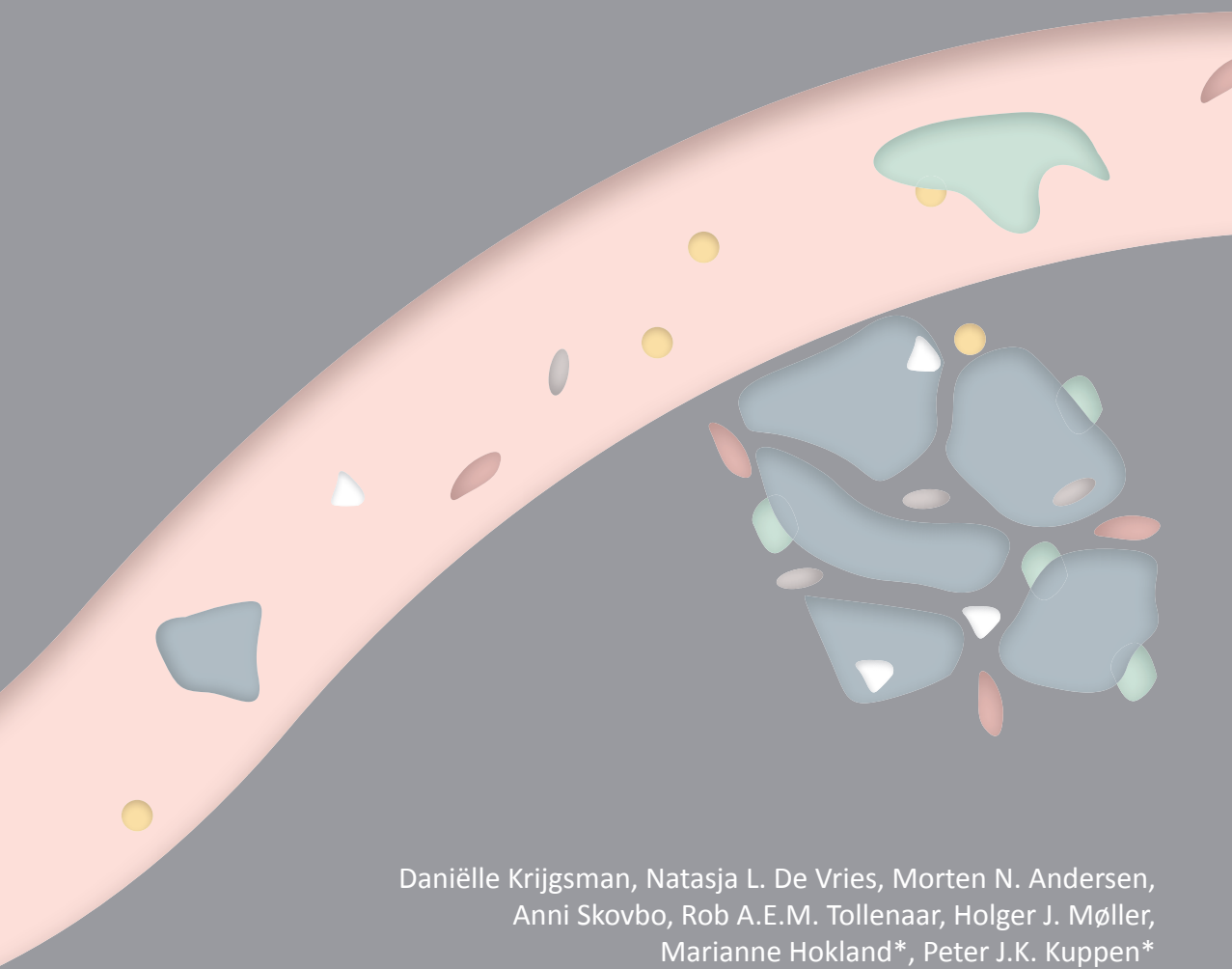
33. Colaprico, A.; Silva, T.C.; Olsen, C.; Garofano, L.; Cava, C.; Garolini, D.; Sabedot, T.S.; Malta, T.M.; Pagnotta, S.M.; Castiglioni, I., et al. TCGAbiolinks: an R/Bioconductor package for integrative analysis of TCGA data. *Nucleic Acids Res* **2016**, *44*, e71, doi:10.1093/nar/gkv1507.
34. Krijgsman, D.; Van Vlierberghe, R.L.P.; Evangelou, V.; Vahrmeijer, A.L.; Van de Velde, C.J.H.; Sier, C.F.M.; Kuppen, P.J.K. A method for semi-automated image analysis of HLA class I tumour epithelium expression in rectal cancer. *Eur J Histochem* **2019**, *63*, doi:10.4081/ejh.2019.3028.
35. Huh, J.W.; Kim, H.R.; Kim, Y.J. Proliferating cell nuclear antigen as a prognostic factor after total mesorectal excision of stage II-III rectal cancer. *Ann Surg Oncol* **2009**, *16*, 1494-1500, doi:10.1245/s10434-009-0424-6.
36. Li, X.F.; Jiang, Z.; Gao, Y.; Li, C.X.; Shen, B.Z. Combination of three-gene immunohistochemical panel and magnetic resonance imaging-detected extramural vascular invasion to assess prognosis in non-advanced rectal cancer patients. *World J Gastroenterol* **2016**, *22*, 8576-8583, doi:10.3748/wjg.v22.i38.8576.
37. Jin, L.J.; Chen, W.B.; Zhang, X.Y.; Bai, J.; Zhao, H.C.; Wang, Z.Y. Analysis of factors potentially predicting prognosis of colorectal cancer. *World J Gastrointest Oncol* **2019**, *11*, 1206-1217, doi:10.4251/wjgo.v11.i12.1206.
38. Neoptolemos, J.P.; Oates, G.D.; Newbold, K.M.; Robson, A.M.; McConkey, C.; Powell, J. Cyclin/proliferation cell nuclear antigen immunohistochemistry does not improve the prognostic power of Dukes' or Jass' classifications for colorectal cancer. *Br J Surg* **1995**, *82*, 184-187, doi:10.1002/bjs.1800820214.
39. Paradiso, A.; Rabinovich, M.; Vallejo, C.; Machiavelli, M.; Romero, A.; Perez, J.; Lacava, J.; Cuevas, M.A.; Rodriquez, R.; Leone, B., et al. p53 and PCNA expression in advanced colorectal cancer: response to chemotherapy and long-term prognosis. *Int J Cancer* **1996**, *69*, 437-441, doi:10.1002/(SICI)1097-0215(19961220)69:6<437::AID-IJC2>3.0.CO;2-5.
40. Zhou, H.; Huang, T.; Xiong, Y.; Peng, L.; Wang, R.; Zhang, G.J. The prognostic value of proliferating cell nuclear antigen expression in colorectal cancer: A meta-analysis. *Medicine (Baltimore)* **2018**, *97*, e13752, doi:10.1097/MD.0000000000013752.
41. Endo, K.; Kohnoe, S.; Tsujita, E.; Watanabe, A.; Nakashima, H.; Baba, H.; Maehara, Y. Galectin-3 expression is a potent prognostic marker in colorectal cancer. *Anticancer research* **2005**, *25*, 3117-3121.
42. Dong, R.; Zhang, M.; Hu, Q.; Zheng, S.; Soh, A.; Zheng, Y.; Yuan, H. Galectin-3 as a novel biomarker for disease diagnosis and a target for therapy (Review). *International journal of molecular medicine* **2018**, *41*, 599-614, doi:10.3892/ijmm.2017.3311.
43. Tao, L.; Jin, L.; Dechun, L.; Hongqiang, Y.; Changhua, K.; Guijun, L. Galectin-3 Expression in Colorectal Cancer and its Correlation with Clinical Pathological Characteristics and Prognosis. *Open medicine (Warsaw, Poland)* **2017**, *12*, 226-230, doi:10.1515/med-2017-0032.
44. Nakamura, M.; Inufusa, H.; Adachi, T.; Aga, M.; Kurimoto, M.; Nakatani, Y.; Wakano, T.; Nakajima, A.; Hida, J.I.; Miyake, M., et al. Involvement of galectin-3 expression in colorectal cancer progression and metastasis. *International journal of oncology* **1999**, *15*, 143-148.
45. Watson, N.F.; Spendlove, I.; Madjd, Z.; McGilvray, R.; Green, A.R.; Ellis, I.O.; Scholefield, J.H.; Durrant, L.G. Expression of the stress-related MHC class I chain-related protein MICA is an indicator of good prognosis in colorectal cancer patients. *Int J Cancer* **2006**, *118*, 1445-1452, doi:10.1002/ijc.21510.
46. McGilvray, R.W.; Eagle, R.A.; Watson, N.F.; Al-Attar, A.; Ball, G.; Jafferji, I.; Trowsdale, J.; Durrant, L.G. NKG2D ligand expression in human colorectal cancer reveals associations with prognosis and evidence for immunoediting. *Clin Cancer Res* **2009**, *15*, 6993-7002, doi:10.1158/1078-0432.CCR-09-0991.
47. Liu, H.; Wang, S.; Xin, J.; Wang, J.; Yao, C.; Zhang, Z. Role of NKG2D and its ligands in cancer immunotherapy. *Am J Cancer Res* **2019**, *9*, 2064-2078.

48. Coudert, J.D.; Zimmer, J.; Tomasello, E.; Cebecauer, M.; Colonna, M.; Vivier, E.; Held, W. Altered NKG2D function in NK cells induced by chronic exposure to NKG2D ligand-expressing tumor cells. *Blood* **2005**, *106*, 1711-1717, doi:10.1182/blood-2005-03-0918.
49. Fernandez-Messina, L.; Ashiru, O.; Boutet, P.; Aguera-Gonzalez, S.; Skepper, J.N.; Reyburn, H.T.; Vales-Gomez, M. Differential mechanisms of shedding of the glycosylphosphatidylinositol (GPI)-anchored NKG2D ligands. *J Biol Chem* **2010**, *285*, 8543-8551, doi:10.1074/jbc.M109.045906.
50. Doubrovina, E.S.; Doubrovin, M.M.; Vider, E.; Sisson, R.B.; O'Reilly, R.J.; Dupont, B.; Vyas, Y.M. Evasion from NK cell immunity by MHC class I chain-related molecules expressing colon adenocarcinoma. *J Immunol* **2003**, *171*, 6891-6899, doi:10.4049/jimmunol.171.12.6891.
51. Schmiedel, D.; Mandelboim, O. NKG2D Ligands-Critical Targets for Cancer Immune Escape and Therapy. *Front Immunol* **2018**, *9*, 2040, doi:10.3389/fimmu.2018.02040.
52. Tsuboi, S.; Sutoh, M.; Hatakeyama, S.; Hiraoka, N.; Habuchi, T.; Horikawa, Y.; Hashimoto, Y.; Yoneyama, T.; Mori, K.; Koie, T., et al. A novel strategy for evasion of NK cell immunity by tumours expressing core2 O-glycans. *EMBO J* **2011**, *30*, 3173-3185, doi:10.1038/emboj.2011.215.
53. Farhad, M.; Rolig, A.S.; Redmond, W.L. The role of Galectin-3 in modulating tumor growth and immunosuppression within the tumor microenvironment. *Oncoimmunology* **2018**, *7*, e1434467, doi:10.1080/2162402X.2018.1434467.
54. Delahaye, N.F.; Rusakiewicz, S.; Martins, I.; Menard, C.; Roux, S.; Lyonnet, L.; Paul, P.; Sarabi, M.; Chaput, N.; Semeraro, M., et al. Alternatively spliced NKp30 isoforms affect the prognosis of gastrointestinal stromal tumors. *Nat Med* **2011**, *17*, 700-707, doi:10.1038/nm.2366.
55. Ewen, E.M.; Pahl, J.H.W.; Miller, M.; Watzl, C.; Cerwenka, A. KIR downregulation by IL-12/15/18 unleashes human NK cells from KIR/HLA-I inhibition and enhances killing of tumor cells. *Eur J Immunol* **2018**, *48*, 355-365, doi:10.1002/eji.201747128.
56. de Vries, N.L.; Swets, M.; Vahrmeijer, A.L.; Hokland, M.; Kuppen, P.J. The Immunogenicity of Colorectal Cancer in Relation to Tumor Development and Treatment. *Int J Mol Sci* **2016**, *17*, doi:10.3390/ijms17071030.



CHAPTER 7

CD163 as a biomarker in colorectal cancer: The expression on circulating monocytes and tumor associated macrophages, and the soluble form in the blood



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Abstract

The macrophage-associated molecule CD163 has been reported as prognostic biomarker in different cancer types, but its role in colorectal cancer (CRC) is unclear. We studied CD163 in the tumor microenvironment and circulation of patients with CRC in relation to clinicopathological parameters. An enzyme-linked immunosorbent assay (ELISA) was used to measure the serum sCD163 levels and multiparameter flow cytometry was used to study the peripheral blood monocytes and their CD163 expression in CRC patients (N=78) and healthy donors (N=50). The distribution of tumor-associated macrophages (TAMs) was studied in primary colorectal tumors with multiplex immunofluorescence. We showed that CRC patients with above-median sCD163 level had a shorter overall survival (OS, *P*=0.035) as well as disease-free survival (DFS, *P*=0.005). The above-median sCD163 remained significantly associated with a shorter DFS in the multivariate analysis (*P*=0.049). Moreover, a shorter OS was observed in CRC patients with an above-median total monocyte percentage (*P*=0.007). The number and phenotype of the stromal and intraepithelial TAMs in colorectal tumors were not associated with clinical outcome. In conclusion, sCD163 and monocytes in the circulation may be potential prognostic biomarkers in CRC patients, whereas TAMs in the tumor showed no association with clinical outcome. Thus, our results emphasize the importance of the innate systemic immune response in CRC disease progression.

Introduction

Colorectal cancer (CRC) remains one of the leading causes of cancer-related deaths worldwide [1]. Approximately 25% of CRC patients have distant metastases at diagnosis [2]. Additionally, up to 25% of the patients diagnosed in the early stages eventually relapse or develop distant metastases following radical surgery and adjuvant chemotherapy [2,3]. In order to optimize treatment strategies, it is crucial that biomarkers are identified that associate with clinical outcome. Due to its critical role in combating tumor development and progression, the immune system has become an important focus in biomarker research. Studies have indicated important roles for monocytes and macrophages in CRC development and progression [4].

Monocytes can be divided into subsets based on their CD14 and CD16 expression levels. Classical monocytes ($CD14^{++}CD16^{-}$) develop in the bone marrow from myeloid progenitor cells and enter the circulation where they may differentiate into intermediate monocytes ($CD14^{++}CD16^{+}$) and, subsequently, to nonclassical monocytes ($CD14^{+}CD16^{++}$) [5]. Classical monocytes are the most prevalent subset in peripheral blood and are important phagocytes [6]. Intermediate monocytes are potent producers of pro-inflammatory cytokines, whereas nonclassical monocytes produce anti-inflammatory cytokines [6]. Recent meta-analyses have shown that, considering peripheral blood leukocytes, a high lymphocyte-to-monocyte ratio was a significant predictor of better overall survival (OS), disease-free survival (DFS) and cancer-specific survival in CRC patients [7,8]. However, circulating monocyte subsets and the monocyte–macrophage marker CD163 have not been widely investigated in CRC patients.

CD163 is a 130-kDa transmembrane scavenger receptor solely expressed by monocytes and macrophages mediating the endocytic uptake of haptoglobin–hemoglobin (Hp-Hb) complexes that form upon intravascular hemolysis [9]. Upon internalization, Hp-Hb complexes are degraded in lysosomes thereby producing anti-inflammatory heme metabolites [9] that dampen the inflammatory response of monocytes and macrophages [10]. CD163 can be cleaved from the cell membrane of monocytes and macrophages by the protease ADAM17/TACE upon activation by pro-inflammatory stimuli [11]. Soluble CD163 (sCD163) is an important biomarker in various inflammatory diseases including sepsis, liver disease, and macrophage activation syndrome [12]. In addition, high sCD163 levels have been associated with disease progression and clinical outcome in different cancer types [13–17]. When monocytes leave the circulation and migrate into tissue, they differentiate into macrophages. Uncommitted M0 macrophages have been described to polarize into pro-inflammatory macrophages (the so-called M1 phenotype) with a high inducible nitric oxide synthase (iNOS)

expression, or into macrophages associated with wound healing and anti-inflammatory functions (the so-called M2 phenotype) with a high CD163 expression [18,19]. Tumor-associated macrophages (TAMs) have been reported to express high levels of CD163 (i.e., M2 phenotype) and the density of these TAMs is associated with unfavorable clinical outcome in numerous human cancers [20–22]. Additionally, M3 TAMs have also been described with an M1/M2 or M2/M1 switch phenotype, both in mice [23] and humans [24,25].

Although CD163 has been reported a prognostic biomarker in different cancer types, its role in CRC is still unclear and requires further investigation. For instance, a high CD163+ TAM density has been reported to associate with both unfavorable [26–28] and favorable clinical outcome [24,25,29–31] in CRC. Therefore, we decided to study CD163 in a broader context, comprising both the tumor microenvironment and circulation of CRC patients. We investigated CD163 expressed by circulating monocytes and TAMs, and the sCD163 in the blood in relation to clinicopathological parameters in CRC.

Materials and methods

Study Population and Patient-Derived Material

Seventy-eight patients diagnosed with tumor node metastasis (TNM) stage 0-IV CRC between 2001 and 2007 at Leiden University Medical Center (LUMC, the Netherlands) were included in the present study, and all underwent surgical resection. None of the patients received pre-operative chemotherapy nor were they diagnosed with Lynch syndrome. The pre-operative sera and peripheral blood mononuclear cells (PBMCs) were collected within a month prior to surgery. The post-operative serum samples were collected during routine checks in the outpatient clinic (mean 6.2 months after surgery, range 2-14). The post-operative samples obtained ≤2 months after surgery, or ≤5 months after the final therapy date in case a patient started adjuvant chemotherapy, were excluded as treatment may have influenced the peripheral blood immune system. Forty serum samples from healthy spouses of cancer patients and 10 PBMC samples from healthy blood donors were included as controls in this study. For the collection of serum samples, the peripheral blood of CRC patients was obtained (Dept. of Surgery, LUMC, The Netherlands) in BD Vacutainer serum separation transport tubes (BD Biosciences, Breda, The Netherlands). The tubes were centrifuged for 12 min at 1000xg after which the serum (supernatant) was frozen at -80°C. The PBMCs were isolated and cryopreserved as described previously [39]. Formalin-fixed paraffin-embedded (FFPE) tumor tissue was obtained from primary CRC tissues (Dept. of Pathology, LUMC, The Netherlands). The clinicopathological data

of all patients and healthy donors were available. All materials were obtained after approval by the Medical Ethical Committee of LUMC (protocol number P000.193). Written informed consent was obtained from all CRC patients and healthy donors included in the study.

Enzyme-Linked Immunosorbent Assay for the Detection of the sCD163 Levels in Serum

Serum samples were thawed and the sCD163 concentrations in serum were measured by an enzyme-linked immunosorbent assay (ELISA) using a BEP-2000 ELISA-analyzer (Dade Behring, Siemens, Erlangen, Germany) essentially as previously described [53]. Briefly, 96-wells plates were coated with polyclonal rabbit anti-CD163 IgG [9] diluted in a carbonate buffer (20 mM, pH 9.6). The wells were then washed three times in PBS, and 100 µL serum (diluted 1:101 in PBS/0.2% bovine serum albumin (BSA, Sigma-Aldrich, St. Louis, MO, USA)) supplemented with 0.25% Tween20 (Merck, Søborg, Denmark) was added and incubated for 90 min. After washing the wells, monoclonal anti-CD163 (clone GHI/61, BD Biosciences, Franklin Lakes, NJ, USA) was added and incubated for 60 min. After washing, peroxidase-labelled antibodies (goat anti-mouse immunoglobulins, DAKO, Glostrup, Denmark) were added and incubated for 60 min. The wells were washed and TMB ONE (Kem-En-Tec Nordic, Taastrup, Denmark) was added and incubated for 3 min. Finally, H3PO4 (1 M in water) was added to the wells and the plate was read on a BEP-2000 ELISA-analyzer. The internal control samples and serum standards were included in each run.

Multiparameter Flow Cytometry for the Detection of CD163 on Circulating Monocyte Subsets

The PBMC samples were thawed and cells were counted using a NucleoCounter NC-250 (Chemometec, Allerod, Denmark). The cell concentration was adjusted to 10 million/mL and the PBMCs were blocked for 15-30 min at room temperature (RT) with 50 µg/mL human IgG (CSL Behring, Bern, Switzerland) to prevent nonspecific antibody binding [54]. The PBMCs were then incubated with mouse anti-human antibodies against T cell and monocyte markers including CD3, CD4, CD8, CD14, CD16, CD25, CD45, CD127 and CD163 (for details see Table S2) as described previously [39]. Only one batch of each antibody type was used. Immediately after staining, the samples were analyzed on the LSRFortessa (BD Biosciences) flow cytometer running FACSDivaTM software version 8.0 (BD Biosciences). FlowJo software version 10.1 (Tree Star Inc., Ashland, OR, USA) was used to analyze the data. In order to identify any inter-experimental variation, a buffy coat from a healthy donor obtained from Aarhus University Hospital, Denmark, was used as an internal control (PBMC reference sample).

The threshold for positive staining was determined using unstained or fluorescence minus one (FMO) controls. In the present study, we used an FMO control for CD16. A standardized gating strategy based on the measurements of the PBMC reference sample was used to identify monocyte subpopulations (Figure S1). The expression of CD163 was then determined by the median fluorescence intensity (MFI) of the total monocyte population, as well as for the classical ($CD14^{++}CD16^{-}$), intermediate ($CD14^{++}CD16^{+}$), and nonclassical ($CD14^{+}CD16^{++}$) monocyte subsets separately. Additionally, regulatory T cells (Tregs, $CD127^{low}CD25^{+}$) were identified as described previously [39].

Multiplex Immunofluorescence for the Detection of TAMs

In total, 4 µm FFPE whole tumor tissue sections were cut and stained with macrophage-related markers using the Akoya Biosciences tyrosine amplification (TSA) method for multiplex immunofluorescence. Briefly, FFPE tissue sections were deparaffinized and rehydrated, and fixed with PBS/1% formaldehyde (Klinipath, Breda, The Netherlands) for 5 min at RT. Thereafter, the endogenous peroxidase activity was blocked by an incubation with 0.3% H2O2 (Millipore BV, The Netherlands) followed by a heat-induced antigen retrieval using a PT link module (DAKO). The tissue sections then underwent four staining cycles. Briefly, during every staining cycle, the sections were incubated with one type of primary antibody, anti-CD68 (KP1, DAKO), anti-iNOS (ab3523, AbCam, Cambridge, UK), anti-CD163 (NCL-L-CD163, DAKO), and finally anti-cytokeratin (EA1/EA3, DAKO). After each incubation round with primary antibodies, sections were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (anti-mouse Envision, DAKO or anti-rabbit Envision, DAKO, depending on the species of which the primary antibodies were derived). The sections were then developed using Opal 570, Opal 690, Opal 520, or Opal 620 fluorophores (all from Akoya Biosciences) dissolved in 1x amplification buffer (Akoya Biosciences). After this visualization step, the sections were microwaved in AR6 buffer (Akoya Biosciences) to strip the antibody complexes from the sections and to perform antigen retrieval for the next staining cycle. The staining procedure described above was repeated three more times until all the epitopes of interest were targeted. Next, all sections were counterstained with DAPI (Sigma-Aldrich) and mounted with ProLong Gold Antifade Mountant (Thermo Fisher Scientific, Bleiswijk, The Netherlands).

Automated Image Analyses

The VECTRA 3.0 automated quantitative pathology imaging system (Akoya Biosciences) was used for imaging of the multiplexed-stained slides. The whole tissue sections were scanned at a 10x

magnification. PhenoChart software (Akoya Biosciences, 1.0.4.) was used to randomly select 6 multispectral imaging (MSI) fields within the tumor regions, defined as areas containing at least 30% tumor epithelium based on the anti-cytokeratin staining and DAPI signal, which were then scanned at a higher resolution (20x). InForm software (Akoya Biosciences, 2.2.1) was used to prepare a spectral library of every fluorophore. Spectral unmixing was then performed on the multiplexed-stained slides and the background signals were extracted using InForm software. Thereafter, a tissue segmentation algorithm was trained using InForm software in order to automatically define tumor epithelium, stroma, and areas without tissue based on anti-cytokeratin antibodies and DAPI signals. A cell segmentation algorithm was set up based on the detection of cell nuclei using the DAPI signal. A phenotyping algorithm was trained to distinguish macrophages ($CD68^+$) from non-macrophages ($CD68^-$) within the tumor epithelium and stromal compartments separately. Finally, the iNOS and CD163 expression were scored on the identified TAMs using a set threshold to identify M0 ($iNOS^-CD163^-$), M1 ($iNOS^+CD163^-$), M2 ($iNOS^-CD163^+$), and M3 ($iNOS^+CD163^+$) TAMs. Subsequently, the cell density (cells/mm^2) and subset distribution (percentage of the total stromal or intraepithelial TAMs) were calculated.

Statistical Analyses

Statistical analyses were performed using SPSS software (IBM SPSS Statistics 22, Chicago, IL, USA). Independent samples T tests and Mann-Whitney U tests were used in order to compare the markers between CRC patients and healthy donors. Dependent samples T tests were used to study the change in sCD163 concentrations between pre-operative and post-operative serum samples. Independent samples T tests, Mann-Whitney U tests, Kruskal–Wallis tests, ANOVA, and the Spearman's rho correlation test were used to relate monocytes, sCD163, and macrophages with tumor characteristics. The Spearman's rho test was used to study the correlation between the serum sCD163 levels and the CD163 expression on monocytes and TAMs. In addition, Kaplan-Meier analyses and log-rank tests were used to correlate monocytes, sCD163, and macrophages with patients' OS and DFS. The OS was defined as the time from surgery until death, or the end of follow-up (censored). The DFS was defined as the time from surgery until the first sign of disease recurrence or until death, whichever came first, or the end of the follow-up (censored). A Cox regression analysis was used for the univariate and multivariate analyses. *P*-values ≤0.05 were considered statistically significant.

Results

Study Population

We investigated CD163 expressed by circulating monocytes and TAMs, and its soluble circulating form (sCD163) in relation to the clinicopathological parameters in CRC. In total, 78 CRC patients were included in the study. Due to a limited sample availability, sCD163, monocytes, and TAMs were studied in subgroups of this cohort, as visualized in Figure 1. As controls, sCD163 was studied in the serum of 40 healthy donors. Additionally, the CD163 expression on circulating monocytes was studied in 10 healthy donors. The clinicopathological characteristics of the 78 CRC patients and healthy donors are summarized in Table 1. No differences were observed between the distribution of age or sex between the 78 CRC patients and the 40 healthy serum donors. The age of the healthy PBMC donors was significantly lower than the CRC patients (*P*=0.028). This was due to the limited PBMC sample availability from elderly healthy donors. No differences were found regarding the distribution of sex between patients and healthy PBMC donors.

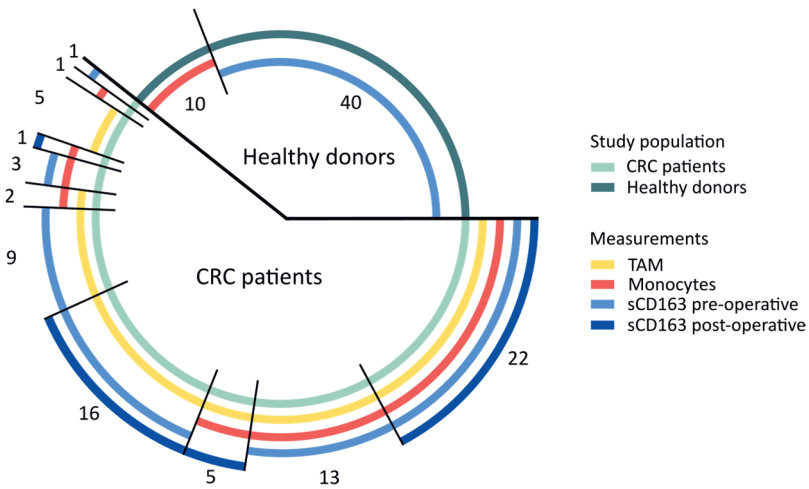


Figure 1. Sample availability for the measurement of monocytes, sCD163 and TAMs in CRC patients and healthy donors. Monocytes, sCD163 and TAMs were studied in 78 CRC patients. TAMs and monocytes were studied in 72 and 47 CRC patients, respectively. Additionally, sCD163 levels were studied in 64 pre-operative and 44 post-operative patients. Finally, monocytes were studied in 10 healthy donors whereas sCD163 levels were studied in 40 healthy donors. The numbers in the figure indicate the number of patients in each subgroup with overlapping samples. Abbreviations: CRC (colorectal cancer), ELISA (enzyme-linked immunosorbent assay), PBMC (peripheral blood mononuclear cells), sCD163 (soluble CD163), TAM (tumor-associated macrophages).

Table 1. Clinicopathological characteristics of patients with CRC and healthy donors in the study. Statistically significant *P*-values (≤0.05) are indicated in bold. Abbreviations: CRC (colorectal cancer), PBMC (peripheral blood mononuclear cells), TNM (Tumor Node Metastasis).

	CRC patients	**Healthy serum donors**		**Healthy PBMC donors**	
	(N=78)	(N=40)	*P*-value	(N=10)	*P*-value
Age*			0.392		**0.028**
Mean (years)	65.9	63.8		48.8	
Range (years)	25-85	26-82		22-78	
Sex			0.597		0.951
Female	35 (44.9%)	20 (50.0%)		5 (50.0%)	
Male	34 (55.1%)	20 (50.0%)		5 (50.0%)	
Tumor location					
Colon	64 (82.1%)				
Rectum	14 (17.9%)				
TNM classification					
Stage 0	4 (5.1%)				
Stage I	12 (15.4%)				
Stage II	26 (33.3%)				
Stage III	26 (33.3%)				
Stage IV	10 (12.8%)				
Tumor differentiation					
Well/moderate	62 (79.5%)				
Poor	13 (16.7%)				
Unknown	3 (3.8%)				
Tumor-positive lymph nodes					
No	45 (57.7%)				
Yes	32 (41.0%)				
Unknown	1 (1.3%)				
Neoadjuvant radiotherapy					
No	69 (88.5%)				
Yes	9 (11.5%)				
Adjuvant chemotherapy					
No	49 (62.8%)				
Yes	29 (37.2%)				

*Age at time of surgery was used for patients and time of serum/ PBMC donation for healthy donors

Trend towards Increased sCD163 Levels in CRC Patients with a Higher TNM Classification

We studied the levels of sCD163 in the pre-operative (N=64) and post-operative serum samples (N=44) derived from CRC patients and in 40 healthy donors. The majority of the measured sCD163 levels from healthy donors and CRC patients were within the reference range (0.7-3.9 mg/L) with no difference in the sCD163 levels between the two groups (Figure 2A, *P*=0.267). In the 39 patients with pre-operative and post-operative serum samples available, we observed that sCD163 levels did not change after

resection of the tumor (*P*=0.723, Figure 2A). We also investigated the association between sCD163 levels and tumor characteristics (Table S1A). Although no correlation was observed between the sCD163 levels and TNM stage in a Spearman's rho correlation test (*P*=0.141), an intergroup analysis revealed that patients with TNM stage IV tumors showed a trend towards higher sCD163 levels compared to TNM stage 0/I patients (*P*=0.052, Figure 2B). No association was observed between the sCD163 levels in CRC patients and tumor location, differentiation grade or tumor-lymph node invasion (Table S1A).

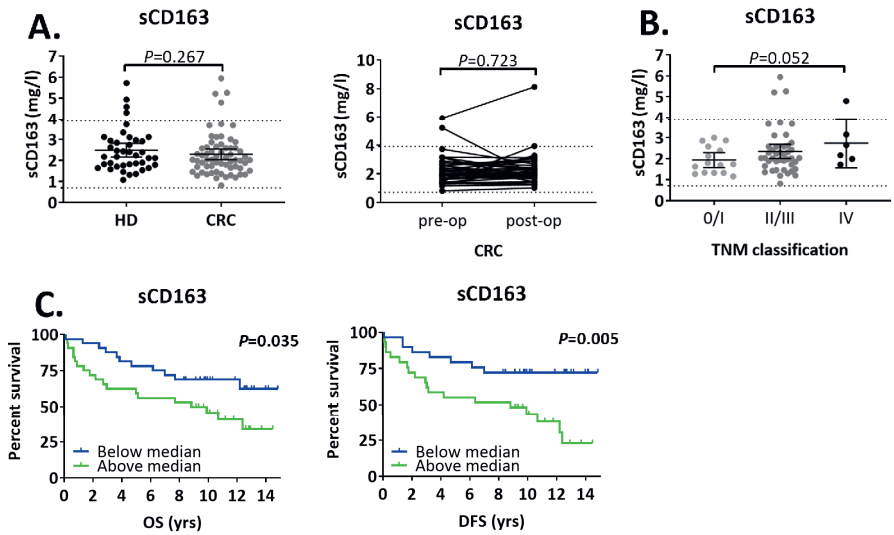


Figure 2. sCD163 levels in serum of CRC patients and healthy donors as measured by ELISA in relation to clinicopathological parameters. **A.** Comparison of sCD163 serum levels in healthy donors (N=40) and pre-operative CRC patients (N=64), and the change in sCD163 levels in CRC patients after surgery (N=39). **B.** Association between the sCD163 levels in CRC patients and TNM stage (stage 0/I, N=15; stage II/III, N=43; stage IV, N=6). **C.** Association between the sCD163 levels and clinical outcome in CRC patients. Kaplan-Meier curves for the OS are shown for TNM stage 0-IV CRC patients (N=64) and Kaplan-Meier curves for DFS are shown for the TNM stage 0-III CRC patients (N=58). Stratifications were based on the median sCD163 level (2.0 mg/l). The bars (**A**, left figure; **B**) show the median sCD163 level with a 95% CI whereas the dotted lines show the reference sCD163 levels (0.7-3.9 mg/l). Statistically significant *P*-values (≤0.05) are indicated in bold. Abbreviations: CI (confidence interval), CRC (colorectal cancer), DFS (disease-free survival), HD (healthy donor), OS (overall survival), post-op (post-operative), pre-op (pre-operative), sCD163 (soluble CD163), TNM (Tumor, Node, Metastasis), yrs (years).

High sCD163 Levels Are Associated with a Shorter OS and DFS in CRC Patients

Next, the association between the sCD163 levels and clinical outcome was investigated in CRC patients. The patient population (N=64) was divided into two groups using the median concentration of sCD163 (2.0 mg/L) as a cutoff. We observed that above-median sCD163 levels in CRC patients were associated with a shorter OS (*P*=0.035), with a hazard ratio (HR) of 2.2 (95% confidence interval (CI) 1.0-4.6, *P*=0.040). Patients with TNM stage IV tumors were excluded from the DFS analyses (N=6) since they already presented metastatic disease at the time of blood sampling. Patients with above-median sCD163 levels showed a significantly shorter DFS (*P*=0.005) compared to patients with below-median sCD163 levels, with a hazard ratio (HR) of 3.1 (CI 1.4-7.1, *P*=0.007) (Figure 2C). A multivariate analysis was performed for DFS and OS in CRC patients which revealed that above-median sCD163 levels (HR 2.4, 95% CI 1.0–5.7, *P*=0.049) remained significantly associated with a shorter DFS when corrected for age (category ≤70 or >70 years) and TNM classification (Table 2), but not with the OS (HR 1.5, 95% CI 0.7-3.3, *P*=0.291, Table 3).

Table 2. Univariate and multivariate analyses of sCD163 serum levels for the DFS of CRC patients. Univariate and multivariate analyses for DFS were generated for stage 0-III CRC patients (N=58). The median sCD163 level (2.0 mg/l) was used as a cutoff. Statistically significant *P*-values (≤0.05) are indicated in bold. Abbreviations: CI (confidence interval), CRC (colorectal cancer), DFS (disease-free survival), HR (hazard ratio), sCD163 (soluble CD163), TNM (Tumor Node Metastasis).

	Univariate analysis for DFS			**Multivariate analysis* for DFS**		
Parameter	HR	95% CI	*P*-value	HR	95% CI	*P*-value
Age (continuous)	1.0	1.0-1.1	0.340			
Age						
≤70 years	1.0					
>70 years	2.0	0.9-4.3	0.072			
Sex						
Female	1.0					
Male	1.4	0.7-3.1	0.358			
TNM classification						
Stage 0/I	1.0					
Stage II	2.0	0.5-7.6	0.299			
Stage III	5.9	1.7-20.4	**0.005**			
Tumor location						
Colon	1.0					
Rectum	1.9	0.8-4.5	0.117			
Tumor differentiation grade						
Well/moderate	1.0					
Poor	0.9	0.3-2.5	0.775			

Table 2. Continued

	Univariate analysis for DFS			Multivariate analysis* for DFS		
Parameter	HR	95% CI	*P*-value	HR	95% CI	*P*-value
sCD163 (continuous)	1.1	0.8-1.6	0.446	1.0	0.7-1.4	0.903
sCD163						
Below median	1.0			1.0		
Above median	3.1	1.4-7.1	**0.007**	2.4	1.0-5.7	**0.049**

* Corrected for age (categorized as ≤70 or >70 years age) and TNM classification

Table 3. Univariate and multivariate analyses of sCD163 serum levels for the OS of CRC patients. Univariate and multivariate analyses for OS were generated for stage 0-IV CRC patients (N=64). The median sCD163 level (2.0 mg/l) was used as a cutoff. Statistically significant *P*-values (≤0.05) are indicated in bold. Abbreviations: CI (confidence interval), CRC (colorectal cancer), HR (hazard ratio), OS (overall survival), sCD163 (soluble CD163), TNM (Tumor Node Metastasis).

	Univariate analysis for OS			Multivariate analysis* for OS		
Parameter	HR	95% CI	*P*-value	HR	95% CI	*P*-value
Age (continuous)	1.0	1.0-1.1	**0.039**			
Age						
≤70 years	1.0					
>70 years	2.9	1.4-6.1	**0.005**			
Sex						
Female	1.0					
Male	1.9	0.9-4.1	0.101			
TNM classification						
Stage 0/I	1.0					
Stage II	1.7	0.4-6.4	0.459			
Stage III	4.5	1.3-15.8	**0.018**			
Stage IV	30.7	6.6-143.0	**<0.001**			
Tumor location						
Colon	1.0					
Rectum	1.5	0.7-3.4	0.314			
Tumor differentiation grade						
Well/moderate	1.0					
Poor	1.5	0.6-3.4	0.393			
sCD163 (continuous)	1.2	0.9-1.6	0.303	1.0	0.7-1.4	0.960
sCD163						
Below median	1.0			1.0		
Above median	2.2	1.0-4.6	**0.040**	1.5	0.7-3.3	0.291

* Corrected for age (categorized as ≤70 or >70 years age) and TNM classification.

Expression of Membrane-Bound CD163 on Circulating Classical Monocytes Is Decreased in CRC Patients Compared to Healthy Donors

We studied the presence of circulating CD14+ and/or CD163+ monocytes in pre-operative PBMC samples from CRC patients (N=47) and healthy donors (N=10) with multiparameter flow cytometry using a standardized gating strategy (Figure S1). The total monocyte percentage (% of $CD45^+$ PBMCs) was comparable between CRC patients and healthy donors (*P*=0.425, Figure 3A). The monocyte population was further divided into classical ($CD14^{++}CD16^-$), intermediate ($CD14^{++}CD16^+$) and nonclassical ($CD14^+CD16^{++}$) monocyte subsets. No statistically significant differences were observed in the percentage (of total monocytes) of classical (*P*=0.975), intermediate (*P*=0.536), or nonclassical (*P*=0.116) monocytes when CRC patients were compared to healthy donors (Figure 3A). Interestingly, CD163 was expressed to a lower extent in the total monocyte population in CRC patients compared to healthy donors (*P*=0.007, Figure 3A). The decreased expression of CD163 was observed only in classical monocytes (*P*=0.006), and not in intermediate (*P*=0.522) or nonclassical monocytes (*P*=0.193, Figure 3A). Interestingly, the percentage of total monocytes positively correlated with the percentage of total Tregs (*P*=0.019, Figure S2). No association was observed between the CD163 expression on monocytes and the percentage of Tregs in the peripheral blood of CRC patients (*P*=0.745). Additionally, no significant correlation was observed between the CD163 expression on monocytes and serum sCD163 levels in CRC patients (*P*=0.482, Figure S3).

Increased Monocyte Percentage in More Advanced Tumors

Next, we examined the association between the total monocyte percentage and monocyte subsets (Table S1B) and their level of CD163 expression (Table S1C) with tumor characteristics. A positive correlation was observed between the total percentage of monocytes and TNM stage in CRC patients (*P*=0.004). An intergroup analysis revealed that patients with TNM stage IV tumors (N=8) showed a significantly higher total monocyte percentage compared to patients with TNM stage 0/I tumors (N=14, *P*=0.016, Figure 3B). Additionally, patients with poorly differentiated tumors (N=11) showed a trend towards a higher percentage of circulating monocytes compared to patients with well or moderately differentiated tumors (N=34, *P*=0.055, Figure 3B). This was restricted to the classical monocytes (*P*=0.039, Figure 3C). Furthermore, the percentage of total monocytes was higher in patients with tumor-positive lymph nodes (N=17) compared to patients without tumor-positive lymph nodes (N=30, *P*=0.011, Figure 3B). No significant associations were observed between tumor

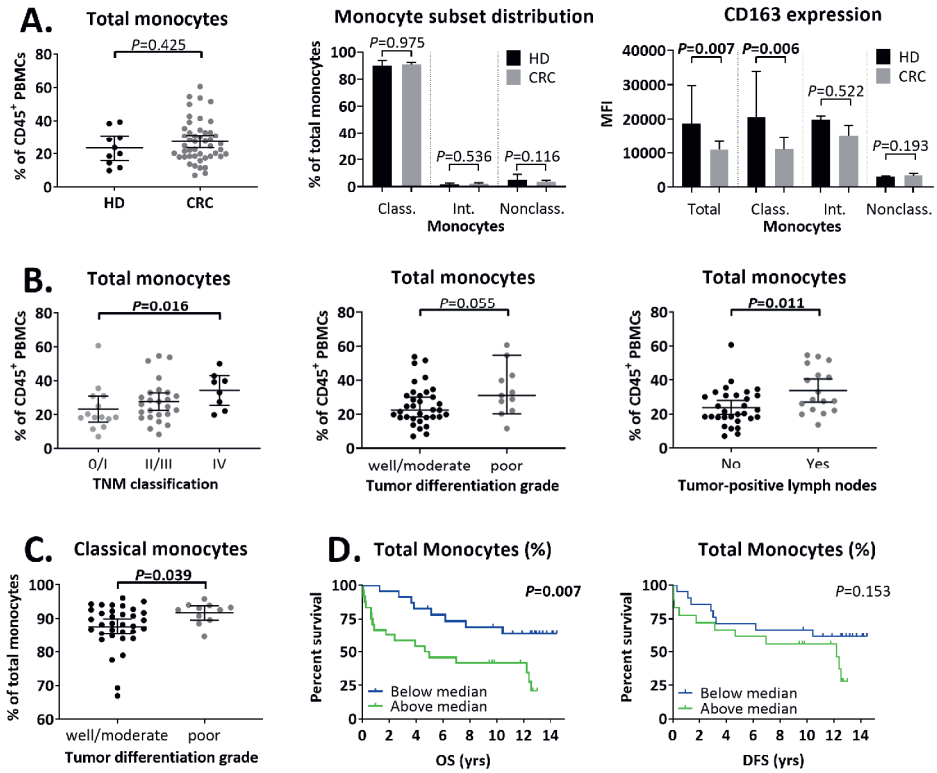


Figure 3. Distribution of monocyte subsets and their level of CD163 expression in the peripheral blood of CRC patients and healthy donors as measured by flow cytometry in relation to clinicopathological parameters. **A.** Comparison of the total monocyte percentage, monocyte subset distribution ($CD14^{++}CD16^{-}$ classical, $CD14^{++}CD16^{+}$ intermediate and $CD14^{+}CD16^{++}$ nonclassical monocytes), and CD163 expression level on these monocyte subsets between healthy donors (N=10) and CRC patients (N=47). **B.** Association between the total monocyte percentage and TNM stage (stage 0/I, N=14; stage II/III, N=25; stage IV, N=8), differentiation grade (well/moderate, N=34; poor, N=11) and tumor-positive lymph nodes (no, N=30; yes, N=17) in CRC patients. **C.** Association between the percentage of classical monocytes and differentiation grade (well/moderate, N=34; poor, N=11) in CRC patients. **D.** Association between the total monocyte percentage and clinical outcome in CRC patients. Kaplan-Meier curves for the OS are shown for TNM stage 0-IV CRC patients (N=47) and Kaplan-Meier curves for the DFS are shown for stage 0-III CRC patients (N=39). Stratifications were based on the median total monocyte percentage (24.9%). The bars in figure (**A-C**) show the median with a 95% CI. Statistically significant *P*-values (≤0.05) are indicated in bold. Abbreviations: CI (confidence interval), Class. (classical), CRC (colorectal cancer), DFS (disease-free survival), HD (healthy donor), Int. (Intermediate), OS (overall survival), MFI (median fluorescence intensity), Nonclass. (Nonclassical), PBMCs (peripheral blood mononuclear cells), TNM (Tumor, Node, Metastasis), yrs (years).

characteristics, the percentage of intermediate or nonclassical monocytes, or CD163 expression (Table S2B,C).

Association between Total Monocyte Percentage and Clinical Outcome in CRC Patients

We subsequently investigated if the total monocyte percentage in CRC patients was associated with clinical outcome. Kaplan-Meier plots and log-rank tests revealed a shorter OS in CRC patients with an above-median (≥24.9%) total monocyte percentage (N=24) compared to CRC patients with a below-median percentage (N=23, *P*=0.007, Figure 3D) with a HR of 3.0 (95% CI 3.1-7.1, *P*=0.010). Patients with TNM stage IV tumors were excluded from the DFS analyses (N=8). No association was observed between the percentage of total monocytes and DFS (*P*=0.153, Figure 3D) with a HR of 1.9 (95% CI 0.8-4.8, *P*=0.161). A multivariate analysis showed that the above-median percentage of circulating monocytes was not independently associated with a shorter OS when corrected for age and TNM classification. The percentage of classical, intermediate, and nonclassical monocytes, and the CD163 expression level on monocytes, were not associated with clinical outcome (data not shown).

TAMs in the Stromal Compartment of Primary Colorectal Tumors Have an M2-Polarized ($iNOS^-CD163^+$) Phenotype whereas in the Epithelium M0- ($iNOS^-CD163^-$) and M1-Polarized ($iNOS^+CD163^-$) Phenotypes Are Predominant

Multiplex immunofluorescent imaging was used to identify the presence of stromal TAM (sTAM) and intraepithelial TAM (ieTAM) subsets in the primary tumors of CRC patients (N=72). The sTAMs were successfully quantified in all 72 included primary colorectal tumors. Due to the occasional expression of CD68 in tumor epithelium cells, the total number of ieTAMs was sometimes overestimated. In total, four patients were identified as outliers with an overestimated cell density of the total ieTAMs and were therefore excluded from the analyses. M0 ($iNOS^-CD163^-$), M1 ($iNOS^+CD163^-$), M2 ($iNOS^-CD163^+$), and M3 ($iNOS^+CD163^+$) TAMs could be identified in primary colorectal tumors as illustrated in Figure 4A. Although the majority of TAMs were identified in the stromal compartment, TAMs were also observed to infiltrate the tumor epithelium compartment of some tumors. Figure 4B shows representative examples of colorectal tumors with high numbers of sTAMs and ieTAMs, respectively. In the majority of the studied tumors, the cell density of sTAMs was higher compared to ieTAMs (Figure 5A). In both stromal and epithelial tissue compartments, the TAM subsets were not equally distributed (*P*<0.001, Figure 5A). The majority of sTAMs showed a M2-polarized phenotype (63%±17), whereas smaller numbers of M0 (21%±14), M1 (7%±8) and M3 (9%±9) sTAMs were found (Figure 5A).

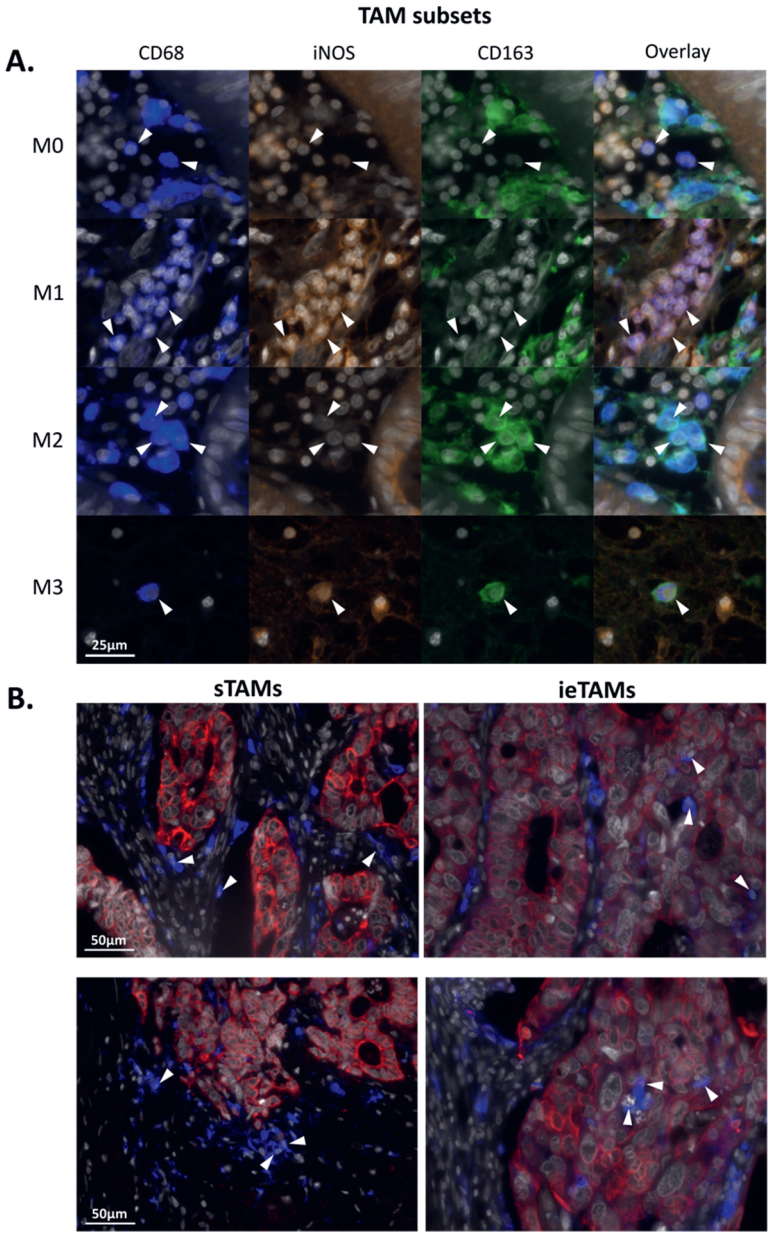


Figure 4: *TAM subsets in primary colorectal tumors as visualized by multiplex immunofluorescence.* **A.** Image of M0 ($CD68^+iNOS^-CD163^-$), M1 ($CD68^+iNOS^+CD163^-$), M2 ($CD68^+iNOS^-CD163^+$) and M3 ($CD68^+iNOS^+CD163^+$) TAMs. **B.** Representative images of colorectal tumors with high numbers of sTAMs and ieTAMs (white: DAPI; red: cytokeratin$^+$ tumor epithelium; blue: $CD68^+$ TAMs). The white arrows indicate examples of TAMs with indicated phenotypes (**A**) or localization (**B**). Abbreviations: CRC (colorectal cancer), iNOS (inducible nitric oxide synthase), ieTAM (intraepithelial TAM), sTAM (stromal TAM), TAM (tumor-associated macrophage).

Interestingly, the distribution of ieTAMs showed a different pattern with relatively high numbers of M0 (37%±20) and M1 (36%±19) ieTAMs compared to low numbers of M2 (15%±10) and M3 (12%±12) ieTAMs (Figure 5A). Hence, sTAMs primarily showed an immunosuppressive (M2) phenotype whereas the majority of ieTAMs showed a naïve (M0) or inflammatory (M1) phenotype.

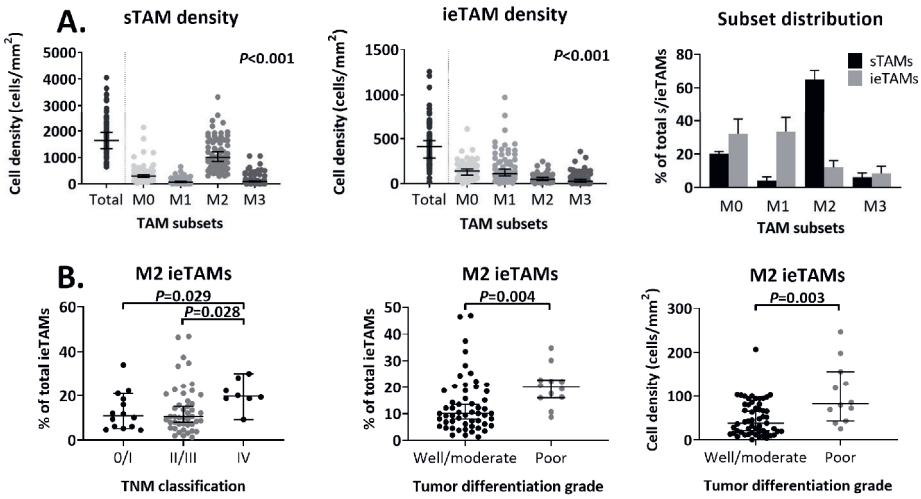


Figure 5. Distribution of the TAM subsets in the stromal and intraepithelial compartments of primary colorectal tumors as measured by multiplex immunofluorescence in relation to clinicopathological parameters. **A.** Distribution of sTAM and ieTAM subsets ($CD68^+iNOS^-CD163^-$ M0, $CD68^+iNOS^+CD163^-$ M1, $CD68^+iNOS^-CD163^+$ M2 and $CD68^+iNOS^+CD163^+$ M3 TAMs) in primary colorectal tumors (N=72 and N=68, respectively). **B.** Associations between the percentage of M2 ieTAMs and TNM stage (stage 0/I, N=14; stage II/III, N=46; stage IV, N=8), and between the percentage of M2 ieTAMs and M2 ieTAM density and tumor differentiation grade (well/moderate, N=55; poor, N=12) in primary colorectal tumors. The bars show the median with a 95% CI. Statistically significant *P*-values (≤0.05) are indicated in bold. Abbreviations: CI (confidence interval), CRC (colorectal cancer), iNOS (inducible nitric oxide synthase), ieTAM (intraepithelial TAM), sTAM (stromal TAM), TAM (tumor-associated macrophage), TNM (Tumor, Node, Metastasis).

Increased M2 TAM Percentage in the Epithelial Compartment of Advanced Tumors

Next, the association between the cell density and distribution of different TAM subsets in the stromal (Table S1D,E) and epithelial (Table S1F,G) compartments of colorectal tumors and tumor characteristics was studied. The cell density and subset distribution of sTAMs were not associated with tumor characteristics (Table S1D and S1E, respectively). Although no correlation was observed between the percentage of M2 ieTAMs and the TNM stage (*P*=0.205), an intergroup analysis revealed

that patients with TNM stage IV tumors showed a higher percentage of M2 ieTAMs compared to TNM stage 0/I patients (*P*=0.029) and TNM stage II/III patients (*P*=0.028, Figure 5B). Furthermore, the percentage of M2 ieTAMs as well as the M2 ieTAM density were observed to be higher in patients with poorly differentiated tumors (N=12) compared to patients with well or moderately differentiated tumors (N=55, *P*=0.004 and *P*=0.003, respectively, Figure 5B). The cell density and subset distribution of the sTAMs and ieTAMs were not correlated with clinical outcome (data not shown). Interestingly, a trend was observed towards a positive correlation between the cell density of M2 sTAMs and the total percentage of circulating Tregs (*P*=0.077), as well as for the cell density of M2 ieTAMs and Tregs (*P*=0.103, Figure S4). No significant correlation was observed between the M2 sTAM or ieTAM density and serum sCD163 levels in CRC patients (*P*=0.989 and *P*=0.498, respectively, Figure S5).

Discussion

Innate immune cells in the circulation and tumor microenvironment participate in several stages of cancer progression [32]. The macrophage-associated molecule CD163 has been reported to be a prognostic biomarker in different cancer types [13-17,20-22], but its role in CRC is still unclear. We decided to study CD163 in a broader context, comprising both the tumor microenvironment and circulation of CRC patients. We investigated the CD163 expressed by circulating monocytes and TAMs, and its soluble circulating form (sCD163) in relation to clinicopathological parameters in CRC.

We evaluated the levels of sCD163 in serum and observed no differences between healthy donors and CRC patients, which is in agreement with studies on ovarian cancer [13], melanoma [14], and multiple myeloma [17], but in contrast with a study on hepatocellular carcinoma [15] and another study in CRC patients by Ding et al. [16]. The latter showed increased sCD163 levels in the total CRC patient population compared to healthy donors [16]. However, subset analyses revealed that early stage CRC patients showed comparable sCD163 levels with healthy donors [16]. This is in line with our results that showed increased sCD163 levels in CRC patients with advanced cancer stages. The total study population by Ding et al. contained relatively more patients with advanced cancer stages compared to our cohort, which may explain the discrepancy. Additionally, although the majority of our healthy donors had sCD163 levels within the reference range (0.7-3.9 mg/L), four healthy donors showed elevated levels. The healthy serum donors included in our study were spouses from cancer patients which may have been less healthy (i.e., relatively higher sCD163 levels) compared to the healthy donors included in the study by Ding et al. Excluding the healthy donor outliers from analysis

did not affect the results of our analyses. In the present study, we observed no change in the sCD163 level of CRC patients after tumor resection. The post-operative serum samples were obtained within a range of 2-14 months after surgery, suggesting that resection of the primary tumor did not influence sCD163 levels or, alternatively, that it takes longer than the studied interval to detect a change in sCD163 levels after surgical resection of the tumor. In line with studies on other cancers [13-15,17] and a study on CRC specifically [16], a high sCD163 level was an independent predictor of DFS in our CRC cohort. Therefore, sCD163 levels may be used in a clinical setting as a prognostic biomarker. Additional research in a prospective setting is required to further investigate the potential use of sCD163 as prognostic biomarker in CRC.

We also observed comparable monocyte percentages and distribution of circulating monocyte subsets between CRC patients and healthy donors. Within the patient population, patients with the highest total monocyte frequency had more advanced tumors, represented by a high TNM classification, poor differentiation grade, and tumor-positive lymph nodes. This is in agreement with the meta-analysis by Tan et al. that reported an association between decreased lymphocyte-to-monocyte ratios and high tumor invasion depths and larger tumor sizes [7]. Additionally, in line with other studies [7,8], we observed a trend towards a shorter OS in CRC patients with a high percentage of total circulating monocytes. Such an association was not observed in our study for DFS. This may be due to the limited number of patients in our study cohort, but may also suggest that the monocyte percentages are associated with comorbidities instead of cancer-specific deaths. This has also been suggested by other studies that showed an association between high monocyte percentages and cardiovascular disease [33] and infectious diseases [34]. Furthermore, we showed in this study that the percentage of circulating monocytes positively correlated with the percentage of circulating Tregs. This is in line with other studies that suggested a positive feedback loop between these cell types [35,36]. Among others, monocytes can produce CCL5 and express the TIE2 receptor that recruits and expands immunosuppressive Tregs, respectively [37,38]. Additionally, Tregs produce IL-4, IL-10, and IL-13 that direct the differentiation of classical monocytes into M2 TAMs with further immunosuppressive functions [35,36]. In line with these studies, we observed a trend towards a positive correlation between the cell density of M2 TAMs and total percentage of circulating Tregs in CRC patients. Furthermore, we [39] and others [40-42] reported an association between a high percentage of circulating Tregs and poor clinical outcome in CRC patients. This suggests that monocytes/TAMs and Tregs closely interact and, additionally, play important roles in cancer development and progression.

The CD163 expression level was significantly lower on monocytes from CRC patients compared to healthy donors, suggesting that colorectal tumors influence the phenotype of monocytes. The mechanism behind the phenotypic regulation of monocytes, and other circulating innate immune cells, such as natural killer and natural killer T cells, are not fully understood, but immunosuppressive cytokines are thought to play a major role, along with the hypoxic conditions generated in the tumor microenvironment [39,43,44]. In our study, a decreased expression of CD163 on monocytes in CRC patients, compared to healthy donors, was not accompanied by elevated sCD163 levels. Additionally, there was no correlation between the CD163 expression on monocytes and sCD163 levels. This is in contrast with other studies that reported an inverse relation between the sCD163 and monocytic membrane expression of CD163 [11,45]. However, it is also argued that most sCD163 does not come from monocytes but from tissue macrophages [12,46], and thus, an increased shedding of CD163 from monocytes might not significantly increase the total sCD163. This hypothesis was further supported by Sugaya et al. who reported an association between the high infiltration of CD163+ cells in the skin and high sCD163 levels in patients with cutaneous T cell lymphoma [47]. These observations were not confirmed in our study. Importantly, other factors should also be considered when studying the shedding of the CD163 receptor from macrophages and monocytes, such as the presence and function of the ADAM17/TACE protease which mediates the cleavage of the CD163 receptor [11]. In the present study, the CD163 expression on circulating monocytes was not associated with tumor characteristics and clinical outcome.

We also characterized TAM subsets in primary colorectal tumors with an automated image analysis. Although the markers used in this study are often used to identify total (CD68), M1 (iNOS) and M2 (CD163) TAMs, we have to consider that TAMs show a remarkable functional plasticity and often express markers characteristic of both activation states [48]. Hence, the vast majority of macrophages have a functional phenotype on a scale in which M1 and M2 macrophages represent the extremes [49]. This makes subdividing TAMs into different subsets based on any type of cell surface markers with immunofluorescence difficult. In the present study, we observed that M2 TAMs were predominant in stromal tissue of colorectal tumors as also reported by others [27,50]. Additionally, Edin et al. reported more M2 compared to M1 TAMs at the invasive front of colorectal tumors [25]. This suggests that most sTAMs have an M2-polarized phenotype associated with immunosuppression. In contrast, Koelzer et al. reported that 40% of the total TAM population in colorectal tumors showed a M2 phenotype, whereas 60% presented an M1 phenotype [31]. A case-by-case analysis showed a positive correlation between the stromal CD68 counts and total tumor CD163 counts, but not with the total tumor iNOS counts [31]. Although not further studied, these

results suggested that the majority of sTAMs had an M2-polarized phenotype. Since the total TAM population showed higher percentages of M1 TAMs compared to M2 TAMs, this might suggest that many ieTAMs with M1-polarized phenotypes were present in the colorectal tumors included in the study of Koelzer et al. In agreement with this observation, we reported an M0- and M1-biased TAM phenotype in the epithelial compartment of colorectal tumors in the present study. This observation is also supported by Kim et al. who reported low densities of M2 ieTAMs compared to total ieTAMs, suggesting that most ieTAMs had a phenotype other than M2, most likely M1 [50]. In summary, we and others showed that, in contrast to sTAMs, primary ieTAMs have an M1 phenotype that are associated with pro-inflammatory functions. As the function of TAMs might be dependent on their spatial distribution, it seems crucial to characterize TAMs in tumor epithelium and stroma separately in future studies.

To our knowledge, we are the first to study the presence of TAMs with M3 characteristics in human tissue. M3 TAMs have already been described in mice, also known as TAMs with an M1/M2 or M2/M1 switch phenotype, and thus are positive for both M1 (Ly6C) and M2 (CXCR1) markers [23]. Additionally, TAMs have also been reported to express both M1 and M2 markers in humans [24,25], although they have never been quantified and related to clinical parameters until now. In our study, we used a combination of the human M1 (iNOS) and M2 (CD163) markers to identify M3 macrophages ($iNOS^+CD163^+$). M3 TAMs are reported to have anti-tumor activities in an Ehrlich ascites [51] and a prostate cancer mouse model [52]. In our cohort, we did not observe an association between M3 TAM densities in the stroma or epithelium from primary colorectal tumors and tumor characteristics or clinical outcome. Therefore, the function of M3 TAMs in CRC remains unclear. In the present study, the percentage of M2 ieTAMs positively correlated with the TNM stage and differentiation grade. Hence, M2 ieTAMs were more present in colorectal tumors with advanced stages. Two other studies reported the correlation between ieTAMs and tumor characteristics [31,50]. Whereas Koelzer et al. did not show any correlation between the total ieTAM counts and TNM stage or differentiation grade [31], Kim et al. showed higher total ieTAM counts in tumors with higher TNM stages [50]. In contrast, the M2 ieTAM counts were not associated with the TNM stage [50]. Additionally, we did not observe any correlations between the sTAM or ieTAM densities or subset distributions and clinical outcome of CRC patients in the present study. Koelzer et al. reported that, independent from TAM localization, high CD68 counts were associated with a longer OS [31]. In contrast, Kim et al. reported an association between a high intraepithelial CD68 density and worse outcome, but not for a high stromal CD68 density [50]. In summary, many contradictory findings have been reported on the association of TAMs in colorectal tumors with tumor characteristics and clinical outcome. Therefore, the role of TAMs in

CRC remains elusive. Major issues in TAM research include the differences in used markers, techniques, and analyzing methods, which makes it difficult to compare studies, calling for more standardized assays. Based on our findings and others, it seems crucial to take the spatial distribution of TAMs in CRC into account. This should be investigated and validated in future studies.

In conclusion, we have shown that monocytes and sCD163 in the circulation are potential prognostic biomarkers to predict disease progression in CRC patients, whereas the TAM densities and phenotypes in the primary tumor are not, thereby emphasizing the importance of the innate systemic immune system in CRC disease progression.

References

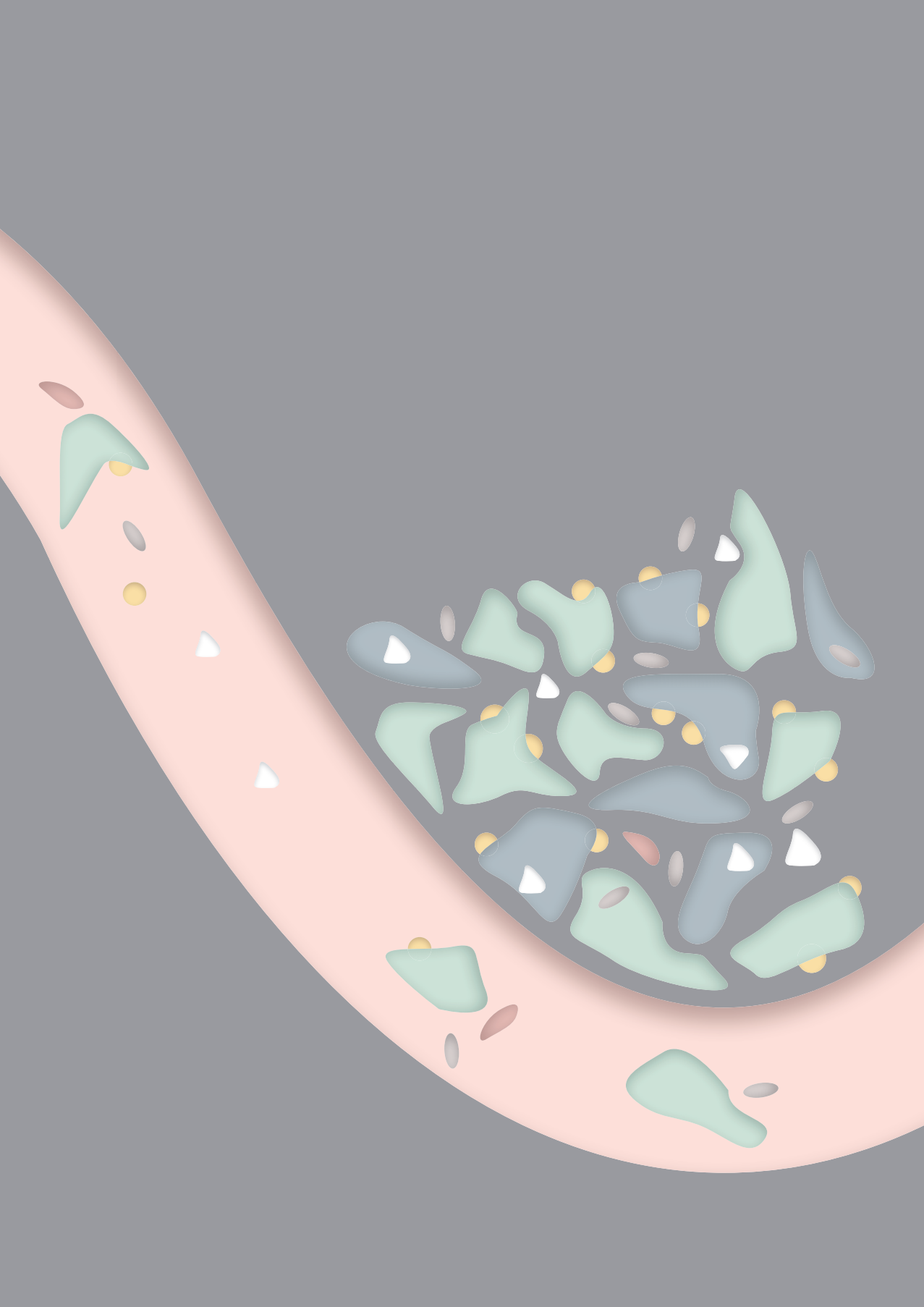
1. Ferlay, J.; Soerjomataram, I.; Dikshit, R.; Eser, S.; Mathers, C.; Rebelo, M.; Parkin, D.M.; Forman, D.; Bray, F. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. *Int J Cancer* **2015**, *136*, E359-386, doi:10.1002/ijc.29210.
2. Van Cutsem, E.; Cervantes, A.; Nordlinger, B.; Arnold, D.; Group, E.G.W. Metastatic colorectal cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. *Ann Oncol* **2014**, *25 Suppl 3*, iii1-9, doi:10.1093/annonc/mdu260.
3. Edge, S.; Byrd, D.; Compton, C.; Fritz, A.; Greene, F.; Trotti, A. *AJCC Cancer Staging Manual. 7th.*; Springer; New York, 2010.
4. Richards, D.M.; Hettinger, J.; Feuerer, M. Monocytes and macrophages in cancer: development and functions. *Cancer Microenviron* **2013**, *6*, 179-191, doi:10.1007/s12307-012-0123-x.
5. Ziegler-Heitbrock, L.; Ancuta, P.; Crowe, S.; Dalod, M.; Grau, V.; Hart, D.N.; Leenen, P.J.; Liu, Y.J.; MacPherson, G.; Randolph, G.J., et al. Nomenclature of monocytes and dendritic cells in blood. *Blood* **2010**, *116*, e74-80, doi:10.1182/blood-2010-02-258558.
6. Yang, J.; Zhang, L.; Yu, C.; Yang, X.F.; Wang, H. Monocyte and macrophage differentiation: circulation inflammatory monocyte as biomarker for inflammatory diseases. *Biomark Res* **2014**, *2*, 1, doi:10.1186/2050-7771-2-1.
7. Tan, D.; Fu, Y.; Tong, W.; Li, F. Prognostic significance of lymphocyte to monocyte ratio in colorectal cancer: A meta-analysis. *Int J Surg* **2018**, *55*, 128-138, doi:10.1016/j.ijsu.2018.05.030.
8. Song, W.; Wang, K.; Zhang, R.J.; Zou, S.B. Prognostic value of the lymphocyte monocyte ratio in patients with colorectal cancer: A meta-analysis. *Medicine (Baltimore)* **2016**, *95*, e5540, doi:10.1097/MD.0000000000005540.
9. Kristiansen, M.; Graversen, J.H.; Jacobsen, C.; Sonne, O.; Hoffman, H.J.; Law, S.K.; Moestrup, S.K. Identification of the haemoglobin scavenger receptor. *Nature* **2001**, *409*, 198-201, doi:10.1038/35051594.
10. Otterbein, L.E.; Soares, M.P.; Yamashita, K.; Bach, F.H. Heme oxygenase-1: unleashing the protective properties of heme. *Trends Immunol* **2003**, *24*, 449-455.
11. Etzerodt, A.; Maniecki, M.B.; Moller, K.; Moller, H.J.; Moestrup, S.K. Tumor necrosis factor alpha-converting enzyme (TACE/ADAM17) mediates ectodomain shedding of the scavenger receptor CD163. *J Leukoc Biol* **2010**, *88*, 1201-1205, doi:10.1189/jlb.0410235.
12. Moller, H.J. Soluble CD163. *Scand J Clin Lab Invest* **2012**, *72*, 1-13, doi:10.3109/00365513.2011.626868.
13. No, J.H.; Moon, J.M.; Kim, K.; Kim, Y.B. Prognostic significance of serum soluble CD163 level in patients with epithelial ovarian cancer. *Gynecol Obstet Invest* **2013**, *75*, 263-267, doi:10.1159/000349892.
14. Jensen, T.O.; Schmidt, H.; Moller, H.J.; Hoyer, M.; Maniecki, M.B.; Sjoegren, P.; Christensen, I.J.; Steiniche, T. Macrophage markers in serum and tumor have prognostic impact in American Joint Committee on Cancer stage I/II melanoma. *J Clin Oncol* **2009**, *27*, 3330-3337, doi:10.1200/JCO.2008.19.9919.
15. Kazankov, K.; Rode, A.; Simonsen, K.; Villadsen, G.E.; Nicoll, A.; Moller, H.J.; Lim, L.; Angus, P.; Kronborg, I.; Arachchi, N., et al. Macrophage activation marker soluble CD163 may predict disease progression in hepatocellular carcinoma. *Scand J Clin Lab Invest* **2016**, *76*, 64-73, doi:10.3109/00365513.2015.1099722.
16. Ding, D.; Yao, Y.; Yang, C.; Zhang, S. Identification of mannose receptor and CD163 as novel biomarkers for colorectal cancer. *Cancer Biomark* **2018**, *21*, 689-700, doi:10.3233/CBM-170796.
17. Andersen, M.N.; Abildgaard, N.; Maniecki, M.B.; Moller, H.J.; Andersen, N.F. Monocyte/macrophage-derived soluble CD163: a novel biomarker in multiple myeloma. *Eur J Haematol* **2014**, *93*, 41-47, doi:10.1111/ejh.12296.

18. Mantovani, A.; Sica, A.; Sozzani, S.; Allavena, P.; Vecchi, A.; Locati, M. The chemokine system in diverse forms of macrophage activation and polarization. *Trends Immunol* **2004**, *25*, 677-686, doi:10.1016/j.it.2004.09.015.
19. Porcheray, F.; Viaud, S.; Rimaniol, A.C.; Leone, C.; Samah, B.; Dereuddre-Bosquet, N.; Dormont, D.; Gras, G. Macrophage activation switching: an asset for the resolution of inflammation. *Clin Exp Immunol* **2005**, *142*, 481-489, doi:10.1111/j.1365-2249.2005.02934.x.
20. Lissbrant, I.F.; Stattin, P.; Wikstrom, P.; Damber, J.E.; Egevad, L.; Bergh, A. Tumor associated macrophages in human prostate cancer: relation to clinicopathological variables and survival. *Int J Oncol* **2000**, *17*, 445-451.
21. Koide, N.; Nishio, A.; Sato, T.; Sugiyama, A.; Miyagawa, S. Significance of macrophage chemoattractant protein-1 expression and macrophage infiltration in squamous cell carcinoma of the esophagus. *Am J Gastroenterol* **2004**, *99*, 1667-1674, doi:10.1111/j.1572-0241.2004.30733.x.
22. Tsutsui, S.; Yasuda, K.; Suzuki, K.; Tahara, K.; Higashi, H.; Era, S. Macrophage infiltration and its prognostic implications in breast cancer: the relationship with VEGF expression and microvessel density. *Oncol Rep* **2005**, *14*, 425-431.
23. Jackaman, C.; Yeoh, T.L.; Acuil, M.L.; Gardner, J.K.; Nelson, D.J. Murine mesothelioma induces locally-proliferating IL-10(+)TNF-alpha(+)CD206(-)CX3CR1(+) M3 macrophages that can be selectively depleted by chemotherapy or immunotherapy. *Oncoimmunology* **2016**, *5*, e1173299, doi:10.1080/2162402X.2016.1173299.
24. Zhou, Q.; Peng, R.Q.; Wu, X.J.; Xia, Q.; Hou, J.H.; Ding, Y.; Zhou, Q.M.; Zhang, X.; Pang, Z.Z.; Wan, D.S., et al. The density of macrophages in the invasive front is inversely correlated to liver metastasis in colon cancer. *J Transl Med* **2010**, *8*, 13, doi:10.1186/1479-5876-8-13.
25. Edin, S.; Wikberg, M.L.; Dahlin, A.M.; Rutegard, J.; Oberg, A.; Oldenborg, P.A.; Palmqvist, R. The distribution of macrophages with a M1 or M2 phenotype in relation to prognosis and the molecular characteristics of colorectal cancer. *PLoS One* **2012**, *7*, e47045, doi:10.1371/journal.pone.0047045.
26. Shabo, I.; Olsson, H.; Elkarim, R.; Sun, X.F.; Svanvik, J. Macrophage Infiltration in Tumor Stroma is Related to Tumor Cell Expression of CD163 in Colorectal Cancer. *Cancer Microenviron* **2014**, *7*, 61-69, doi:10.1007/s12307-014-0145-7.
27. Waniczek, D.; Lorenc, Z.; Snietura, M.; Wesecki, M.; Kopec, A.; Muc-Wierzgon, M. Tumor-Associated Macrophages and Regulatory T Cells Infiltration and the Clinical Outcome in Colorectal Cancer. *Arch Immunol Ther Exp (Warsz)* **2017**, 10.1007/s00005-017-0463-9, doi:10.1007/s00005-017-0463-9.
28. Shibutani, M.; Maeda, K.; Nagahara, H.; Fukuoka, T.; Nakao, S.; Matsutani, S.; Hirakawa, K.; Ohira, M. The peripheral monocyte count is associated with the density of tumor-associated macrophages in the tumor microenvironment of colorectal cancer: a retrospective study. *BMC Cancer* **2017**, *17*, 404, doi:10.1186/s12885-017-3395-1.
29. Forssell, J.; Oberg, A.; Henriksson, M.L.; Stenling, R.; Jung, A.; Palmqvist, R. High macrophage infiltration along the tumor front correlates with improved survival in colon cancer. *Clin Cancer Res* **2007**, *13*, 1472-1479, doi:10.1158/1078-0432.CCR-06-2073.
30. Gulubova, M.; Ananiev, J.; Yovchev, Y.; Julianov, A.; Karashmalakov, A.; Vlaykova, T. The density of macrophages in colorectal cancer is inversely correlated to TGF-beta1 expression and patients' survival. *J Mol Histol* **2013**, *44*, 679-692, doi:10.1007/s10735-013-9520-9.
31. Koelzer, V.H.; Canonica, K.; Dawson, H.; Sokol, L.; Karamitopoulou-Diamantis, E.; Lugli, A.; Zlobec, I. Phenotyping of tumor-associated macrophages in colorectal cancer: Impact on single cell invasion (tumor budding) and clinicopathological outcome. *Oncoimmunology* **2016**, *5*, e1106677, doi:10.1080/2162402X.2015.1106677.
32. Krijgsman, D.; de Vries, N.L.; Skovbo, A.; Andersen, M.N.; Swets, M.; Bastiaannet, E.; Vahrmeijer, A.L.; van de Velde, C.J.H.; Heemskerk, M.H.M.; Hokland, M., et al. Characterization of circulating T-, NK-, and

NKT cell subsets in patients with colorectal cancer: the peripheral blood immune cell profile. *Cancer Immunol Immunother* **2019**, *68*, 1011-1024, doi:10.1007/s00262-019-02343-7.

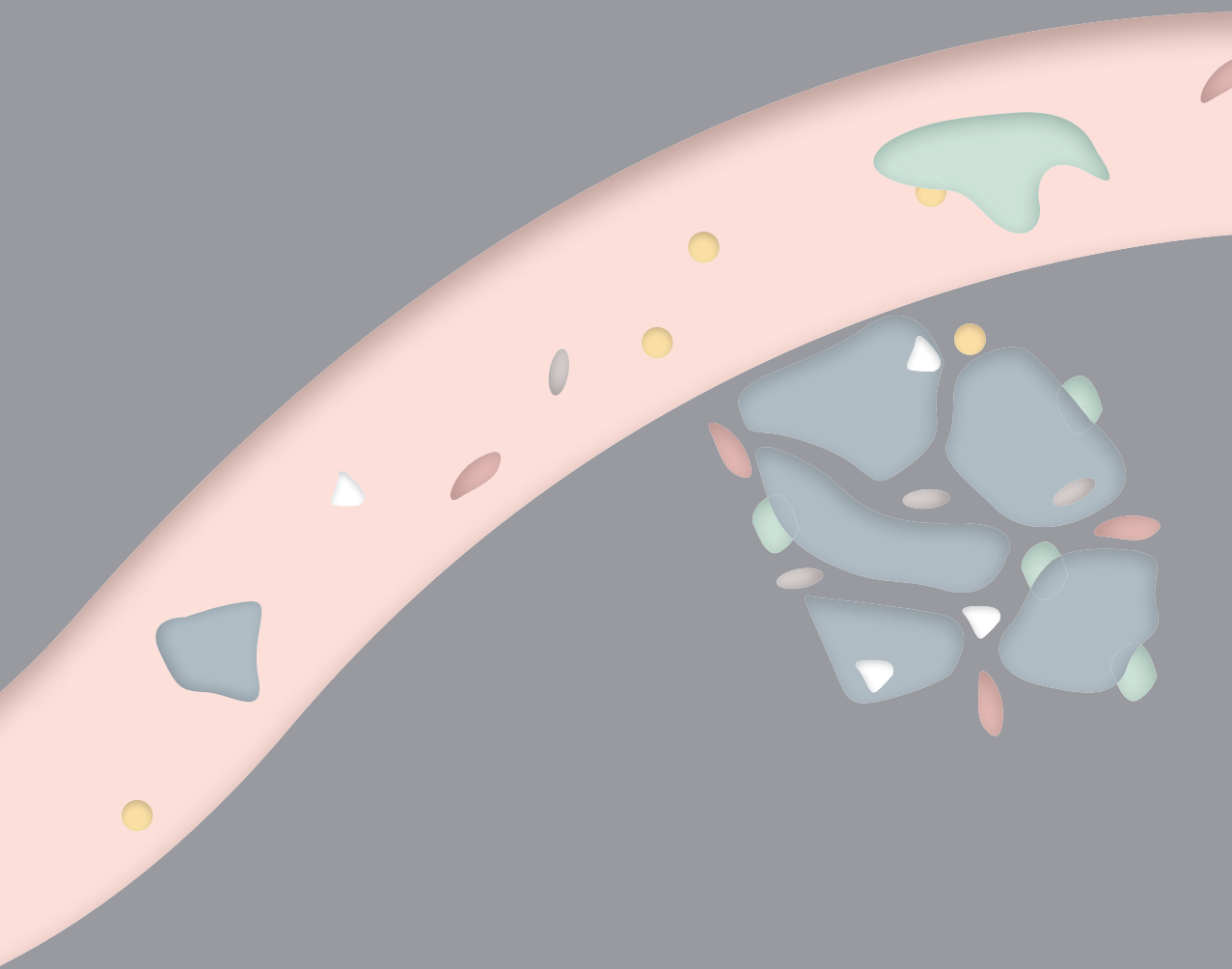
33. Moller, H.J.; Hald, K.; Moestrup, S.K. Characterization of an enzyme-linked immunosorbent assay for soluble CD163. *Scand J Clin Lab Invest* **2002**, *62*, 293-299.
34. Andersen, M.N.; Al-Karradi, S.N.; Kragstrup, T.W.; Hokland, M. Elimination of erroneous results in flow cytometry caused by antibody binding to Fc receptors on human monocytes and macrophages. *Cytometry A* **2016**, *89*, 1001-1009, doi:10.1002/cyto.a.22995.
35. Mantovani, A.; Allavena, P.; Sica, A.; Balkwill, F. Cancer-related inflammation. *Nature* **2008**, *454*, 436-444, doi:10.1038/nature07205.
36. Shahid, F.; Lip, G.Y.H.; Shantsila, E. Role of Monocytes in Heart Failure and Atrial Fibrillation. *J Am Heart Assoc* **2018**, *7*, doi:10.1161/JAHA.117.007849.
37. Shi, C.; Pamer, E.G. Monocyte recruitment during infection and inflammation. *Nat Rev Immunol* **2011**, *11*, 762-774, doi:10.1038/nri3070.
38. Pommier, A.; Audemard, A.; Durand, A.; Lengagne, R.; Delpoux, A.; Martin, B.; Douguet, L.; Le Campion, A.; Kato, M.; Avril, M.F., et al. Inflammatory monocytes are potent antitumor effectors controlled by regulatory CD4+ T cells. *Proc Natl Acad Sci U S A* **2013**, *110*, 13085-13090, doi:10.1073/pnas.1300314110.
39. Tiemessen, M.M.; Jagger, A.L.; Evans, H.G.; van Herwijnen, M.J.; John, S.; Taams, L.S. CD4+CD25+Foxp3+ regulatory T cells induce alternative activation of human monocytes/macrophages. *Proc Natl Acad Sci U S A* **2007**, *104*, 19446-19451, doi:10.1073/pnas.0706832104.
40. Schlecker, E.; Stojanovic, A.; Eisen, C.; Quack, C.; Falk, C.S.; Umansky, V.; Cerwenka, A. Tumor-infiltrating monocytic myeloid-derived suppressor cells mediate CCR5-dependent recruitment of regulatory T cells favoring tumor growth. *J Immunol* **2012**, *189*, 5602-5611, doi:10.4049/jimmunol.1201018.
41. Coffelt, S.B.; Chen, Y.Y.; Muthana, M.; Welford, A.F.; Tal, A.O.; Scholz, A.; Plate, K.H.; Reiss, Y.; Murdoch, C.; De Palma, M., et al. Angiopoietin 2 stimulates TIE2-expressing monocytes to suppress T cell activation and to promote regulatory T cell expansion. *J Immunol* **2011**, *186*, 4183-4190, doi:10.4049/jimmunol.1002802.
42. Betts, G.; Jones, E.; Junaid, S.; El-Shanawany, T.; Scurr, M.; Mizen, P.; Kumar, M.; Jones, S.; Rees, B.; Williams, G., et al. Suppression of tumour-specific CD4(+) T cells by regulatory T cells is associated with progression of human colorectal cancer. *Gut* **2012**, *61*, 1163-1171, doi:10.1136/gutjnl-2011-300970.
43. Roselli, M.; Formica, V.; Cereda, V.; Jochems, C.; Richards, J.; Grenga, I.; Orlandi, A.; Ferroni, P.; Guadagni, F.; Schlom, J. The association of clinical outcome and peripheral T-cell subsets in metastatic colorectal cancer patients receiving first-line FOLFIRI plus bevacizumab therapy. *Oncoimmunology* **2016**, *5*, e1188243, doi:10.1080/2162402X.2016.1188243.
44. Xu, T.; Lu, J.; An, H. The relative change in regulatory T cells / T helper lymphocytes ratio as parameter for prediction of therapy efficacy in metastatic colorectal cancer patients. *Oncotarget* **2017**, *8*, 109079-109093, doi:10.18632/oncotarget.22606.
45. Li, C.; Luo, X.; Lin, Y.; Tang, X.; Ling, L.; Wang, L.; Jiang, Y. A Higher Frequency of CD14+ CD169+ Monocytes/Macrophages in Patients with Colorectal Cancer. *PLoS One* **2015**, *10*, e0141817, doi:10.1371/journal.pone.0141817.
46. Maciejewski, R.; Radej, S.; Furmaga, J.; Chroscicki, A.; Rudzki, S.; Rolinski, J.; Wallner, G. Evaluation of immature monocyte-derived dendritic cells generated from patients with colorectal cancer. *Pol Przegl Chir* **2013**, *85*, 714-720, doi:10.2478/pjs-2013-0109.
47. Davis, B.H.; Zarev, P.V. Human monocyte CD163 expression inversely correlates with soluble CD163 plasma levels. *Cytometry B Clin Cytom* **2005**, *63*, 16-22, doi:10.1002/cyto.b.20031.
48. Moestrup, S.K.; Moller, H.J. CD163: a regulated hemoglobin scavenger receptor with a role in the anti-inflammatory response. *Ann Med* **2004**, *36*, 347-354.

49. Sugaya, M.; Miyagaki, T.; Ohmatsu, H.; Suga, H.; Kai, H.; Kamata, M.; Fujita, H.; Asano, Y.; Tada, Y.; Kadono, T., et al. Association of the numbers of CD163(+) cells in lesional skin and serum levels of soluble CD163 with disease progression of cutaneous T cell lymphoma. *J Dermatol Sci* **2012**, *68*, 45-51, doi:10.1016/j.jdermsci.2012.07.007.
50. Yang, M.; McKay, D.; Pollard, J.W.; Lewis, C.E. Diverse Functions of Macrophages in Different Tumor Microenvironments. *Cancer Res* **2018**, *78*, 5492-5503, doi:10.1158/0008-5472.CAN-18-1367.
51. Braster, R.; Bogels, M.; Beelen, R.H.; van Egmond, M. The delicate balance of macrophages in colorectal cancer; their role in tumour development and therapeutic potential. *Immunobiology* **2017**, *222*, 21-30, doi:10.1016/j.imbio.2015.08.011.
52. Kim, Y.; Wen, X.; Bae, J.M.; Kim, J.H.; Cho, N.Y.; Kang, G.H. The distribution of intratumoral macrophages correlates with molecular phenotypes and impacts prognosis in colorectal carcinoma. *Histopathology* **2018**, *73*, 663-671, doi:10.1111/his.13674.
53. Kalish, S.; Lyamina, S.; Manukhina, E.; Malyshev, Y.; Raetskaya, A.; Malyshev, I. M3 Macrophages Stop Division of Tumor Cells In Vitro and Extend Survival of Mice with Ehrlich Ascites Carcinoma. *Med Sci Monit Basic Res* **2017**, *23*, 8-19.
54. Tsai, C.S.; Chen, F.H.; Wang, C.C.; Huang, H.L.; Jung, S.M.; Wu, C.J.; Lee, C.C.; McBride, W.H.; Chiang, C.S.; Hong, J.H. Macrophages from irradiated tumors express higher levels of iNOS, arginase-I and COX-2, and promote tumor growth. *Int J Radiat Oncol Biol Phys* **2007**, *68*, 499-507, doi:10.1016/j.ijrobp.2007.01.041.



CHAPTER 8

General discussion and future perspectives



Summary and discussion

Over the past years, major progress has been made in the treatment of colorectal cancer (CRC) patients including introduction of new (neo)-adjuvant treatment strategies and surgical techniques. These developments have resulted in improvement of survival of CRC patients [1], but there is still considerable potential. Due to the crucial role of tumor-immune interactions in development and progression of CRC, immune-related biomarkers have become an important focus of research. Research aims at better identification of CRC patients with a high risk for disease progression, and selection of patients who would benefit most from immunotherapeutic strategies. This thesis focused on systemic immune-related biomarkers in CRC with as ultimate goal to better understand the underlying biology of tumor-immune interactions in the circulation of CRC patients. The primary focus was on natural killer (NK) cells and natural killer T (NKT) cells since these cell types are able to recognize and kill tumor cells with downregulated human leukocyte antigen (HLA) class I expression, a feature that is often observed in CRC.

Circulating NK- and NKT cells

NKT cells constitute a unique subset of T cells at the interface between the innate and adaptive immune system displaying features of both NK- and T cells. NKT cells possess cytotoxic capabilities, but are primarily considered to have a regulatory function via the secretion of large amounts of pro- or anti-inflammatory cytokines upon activation, thereby resulting in amplification or dampening of the immune response [2]. In **Chapter 2**, we argued that, because of their major modulating effects on immune responses, NKT cells might be important mediators of tumor immunosurveillance. Therefore, NKT cells are interesting targets for immunotherapy. Over the past years, multiple clinical trials have been performed aimed at activation of NKT cells in cancer patients using the synthetic compound α-GalCer. However, up till now, the beneficial clinical responses in patients are limited [3-5]. In **Chapter 2** we argued that this is because the contribution of functionally altered NKT cells to the failure of NKT cell-based immunotherapies has been largely ignored. During tumor progression, NKT cells may become overstimulated which results in deletion of a part of the NKT cell population in cancer patients via activation-induced cell death. Additionally, the remaining NKT cells may become hyporesponsive (i.e. anergic), or start secreting immunosuppressive cytokines, thereby facilitating tumor progression and immune escape. Therefore, we concluded that there should be three important focuses of NKT cell research in cancer patients: (1) expansion of the NKT cell population, (2) prevention and/or

breaking of NKT cell anergy, and (3) skewing of NKT cells towards subsets with potent antitumor activity.

In order to gain more insights in the role and function of circulating immune cell subsets in tumor progression, including NKT cells, we studied the peripheral blood immune profile of CRC patients before and after tumor resection as described in **Chapter 3 and 4**, respectively. In these studies, we observed downregulation of activating receptors on circulating NK- and NKT cells in CRC patients before tumor resection, thereby implying impaired function. CRC patients therefore seem to have an immunocompromised state of their circulating NK- and NKT cells, which might contribute to immune escape and disease progression. These findings are consistent with previous studies that showed low numbers of peripheral blood NK cells expressing activating receptors in preoperative CRC patients [6,7]. Interestingly, we showed in **Chapter 4** that expression of several activating receptors was upregulated on circulating NK- and NKT cells in CRC patients after surgical removal of the primary tumor, whereas inhibitory receptors were downregulated. Hence, the immunosuppressive state of NK- and NKT cells in preoperative CRC patients was not observed anymore after surgery. In line with our results, others [8-10] also reported higher numbers of cytotoxic immune cell subsets in cancer patients after surgery, including NK cells, NKT cells, and $CD8^+$ T cells. These observations imply that the altered phenotype, and possibly function, of circulating immune cells might be induced by the primary tumor. Thus, tumors may have a suppressive effect on the systemic immune system which possibly leads to escape of tumor cells from recognition and elimination in the circulation, thereby resulting in the formation of distant metastasis.

We showed in **Chapter 3** that a high percentage of $CD16^+$ circulating NKT cells was associated with short disease-free survival (DFS) in CRC patients, independent from TNM classification and age. Thus, it seems that the CD16 receptor, which is involved in antibody-dependent cell-mediated cytotoxicity, is upregulated on NKT cells during the course of tumor cell dissemination in CRC patients. The biological function of CD16 on NKT cells is, however, still unclear. Of course, we have to keep in mind that the immune system is highly complex and expression of one specific receptor on one distinct immune cell subset might not be causally related to disease progression and, therefore, clinical outcome. It is essential to understand the underlying biology of tumor-immune responses if we want to use systemic immune-related biomarkers for clinical purposes in the future. This should be an important focus of future studies.

We showed in **Chapter 4** that the immunosuppressive state of NK- and NKT cell receptors was not altered after tumor resection in CRC patients that received adjuvant therapy, even months after therapy completion, which is in line with results from a study on breast cancer [11]. Although adjuvant therapy is administered to patients in order to kill residual tumor cells and to decrease the risk of recurrence or distant metastasis, it has to be considered that at the same time, adjuvant therapy compromises the immune system for a long period of time. In this period, the immune system is probably not able to induce an effective tumor-immune response against any residual tumor cells. In the future, patients might be treated with a combination of adjuvant chemotherapy and immunotherapy in order to counteract the immunosuppressive effects of chemotherapy, thereby diminishing the risk to develop metastases. Rocca *et al.* reported that a combination of IL-2 and IL-15 could upregulate the expression of activating receptors on CRC patient-derived dysregulated circulating NK cells *ex vivo* [12]. Although these results seem promising, this approach to activate patient-derived NK cells into potent tumor-killing cells closely resembles the lymphokine-activated killer (LAK) cell therapy strategy introduced over 30 years ago [13]. Patient-derived lymphocytes were obtained using lymphocytapheresis, and cultured in the presence of IL-2 in order to activate immune cells (mostly NK cells) to heighten their cytotoxicity towards malignant cells [14]. These activated immune cells, also called LAK cells, were then re-administered to the patient in conjunction with high-dose IL-2. Although some patients with metastatic renal cancer and melanoma showed prolonged remission [15], the effects were limited, while the procedure was highly time-consuming and logistically complex. In addition, many toxic effects were reported, mainly related to increased vascular permeability induced by IL-2. This strongly suggests that upregulation of activating receptors on patient-derived NK cells *ex vivo* using cytokines is not useful in the clinic as cancer immunotherapy. Instead, it might be more promising to develop immunotherapies that target specific receptors on NK- and NKT cells in order to enhance the function of these cells in cancer patients. In this way, major toxic side effects (as observed after systemic IL-2 treatment) may be prevented. Additionally, targeted immunotherapy does not require *ex vivo* culturing of patient NK- and NKT cells, thereby making it a feasible treatment strategy for daily use in the clinic. In order to design these types of immunotherapies, it is crucial to understand the underlying biology of tumor-induced immunosuppression of NK cells, since we need to know which receptors to target, what the effect of this would be, and how to target these receptors.

NK- and NKT cell ligands in primary tumors

In **Chapter 5 and 6**, we studied the underlying biology of tumor-induced immunosuppression of circulating NK- and NKT cells in CRC patients. Several studies suggested a role for expression of NK- and NKT cell ligands by tumor cells [16,17], as well as production of soluble NK- and NKT cell ligands by the tumor [18]. The activating receptors expressed by NK- and NKT cells recognize different stress-related soluble and membrane-bound ligands that may be secreted and/or expressed by tumor cells whereas the inhibitory receptors all recognize different HLA class I molecules. In **Chapter 5**, a semi-automated image analysis method was demonstrated which enabled scoring of biomarkers on tumor epithelium specifically, even when also expressed by stromal tissue and/or immune cells, as is the case for NK- and NKT cell ligands. Using this method, HLA class I expression could be quantified in tumor epithelium in a robust, reproducible, and objective manner. This method was further developed using deep learning software and used in **Chapter 6** to quantify expression of different NK- and NKT cell ligands in primary CRC tumors. In this chapter, we showed correlations between ligand expression in the primary tumor (both on protein and on RNA level) and receptor expression on circulating NK- and NKT cells. For instance, we observed a negative correlation between expression of the natural killer group 2D (NKG2D) ligands UL-16 binding protein (ULBP)-1, -2, and -5 in the primary tumor, and expression of the NKG2D receptor on circulating NKT cells. This implies that NK cell ligands expressed by the primary tumor indeed have immune-modulating effects on the peripheral blood immune profile which might result in immune escape. This hypothesis is supported by the study of Gharagozloo *et al.* which reported a decrease in circulating NKG2D^{+} NK cells in CRC patients with metastatic disease as compared to patients with early stage cancer [19]. Furthermore, studies showed that sustained NKG2D ligand expression, as well as shedding of soluble NKG2D ligands into the circulation, counteracted NKG2D-dependent NK cell activity in later stages of CRC via downregulation of the NKG2D receptor [20-23], which is in line with our results. Therefore, overexpression of NKG2D ligands by tumor cells, and/or shedding of NKG2D ligands in the circulation might be important immune escape strategies for tumor cells. Schmiedel *et al.* argued that shedding of NKG2D ligands has several major advantages for cancer cells in terms of immune evasion (even more than NKG2D overexpression by tumor cells) since soluble NKG2D ligands are unable to activate NKG2D receptor-bearing immune cells while still being capable of binding the NKG2D receptor, thereby causing internalization of the receptor [24]. In line with this hypothesis, multiple studies reported poor clinical outcome in cancer patients with high levels of soluble NKG2D ligands [25,26]. As also mentioned in **Chapter 2**, overstimulation of NKT cells (and NK cells as well) via their receptors might induce an

anergic and hyporesponsive state of these immune cell subsets, thereby rendering them useless in the defense against tumor growth. By preventing overstimulation of NK- and NKT cell receptors, the antitumor function of NK- and NKT cells might be restored. Ligand-receptor interactions on NK- and NKT cells can therefore be considered as immune checkpoints in tumor-immune interactions, thereby making them important new targets for inhibitors.

At the moment, NK cell-based clinical trials are mostly based on blocking the interaction between NK cell inhibitory receptors and their ligands using monoclonal antibodies [27,28]. This includes blocking the NKG2A/HLA-E axis using monalizumab, and the KIR ligand-receptor axis with lirilumab. Currently, several monalizumab-based clinical trials are ongoing [29], including a basket trial with advanced solid malignancies (NCT02671435). Interestingly, this trial reported durable partial responses in microsatellite-stable CRC patients treated with combinations of FOLFOX chemotherapy and VEGF, PD-L1, and NKG2A blocking antibodies [30]. Based on these promising results, a larger phase II trial is currently set up for high-risk microsatellite-stable CRC patients that underwent surgical resection of their primary tumor (NCT04145193). Different adjuvant treatment strategies will be tested in this trial based on FOLFOX chemotherapy in combination with NKG2A and PD-L1 blocking antibodies (monalizumab and durvalumab, respectively). Lirilumab therapy in patients with different cancer types has been proven to be safe, however, limited anti-tumor efficacy was observed [31,32]. Furthermore, pre-clinical studies also focus on blocking the ILT/HLA-G signaling pathway, as well as the interaction between TIGIT and its ligands CD155 and nectin-2. As our data clearly demonstrated, activating NK cell receptors are also potential targets for immune checkpoint inhibitors. Importantly, it is the integration of multiple activating and inhibitory signals that determines whether NK- and NKT cells become activated or not. Therefore, it seems crucial to target these cell types at multiple levels using immunotherapy, for instance by combining multiple immune checkpoint inhibitors that target inhibitory as well as activating receptors in order to skew NK- and NKT cells towards activation. Additionally, studies suggested that CD16-mediated antibody-dependent cellular cytotoxicity (ADCC) plays an important role in mediating anti-tumor effects of monoclonal antibody therapies by binding the Fc part of the therapeutic antibody, which in turn is bound to tumor-associated antigens on the tumor surface [33]. In line with this hypothesis, cetuximab was shown to restore the lytic capacity of dysregulated NK cells from CRC patients [12]. Furthermore, studies in different cancer types, including CRC, demonstrated clinical benefit of treatment with rituximab, trastuzumab, and cetuximab via enhancement of CD16-mediated ADCC by NK cells [34-36]. With this in mind, it seems beneficial to develop antibody-based immune checkpoint inhibitors that block receptor-ligand interactions on the

tumor cell-site, in order to benefit from CD16-mediated ADCC by NK cells. Up till now, it is unclear whether the CD16 receptor expressed by NKT cells has a similar role in mediating anti-tumor effects of monoclonal antibody therapies as compared to NK cells. As addressed in **Chapter 2**, NKT cells in cancer patients seem to be hyporesponsive (i.e. anergic), or start secreting immunosuppressive cytokines upon stimulation. Therefore, stimulating the CD16 receptor on NKT cells might, in contrast to NK cells, facilitate tumor progression and immune escape. This hypothesis is further supported by the results from **Chapter 3** in which we showed that CRC patients with high numbers of $CD16^+$ circulating NKT cells have poor clinical outcome. As thoroughly discussed in **Chapter 2**, it is crucial for future research to focus on prevention and/or breaking of NKT cell anergy and to skew these cells towards proinflammatory cytokine producing subsets with potent antitumor activity. Finally, antibodies neutralizing soluble NK cell ligands in the circulation are worth pursuing in future clinical development.

Monocytes and macrophages

Finally, we also studied CD163 as biomarker in CRC in **Chapter 7**, comprising both the circulation and tumor microenvironment. CD163 is expressed by circulating monocytes and M2-polarized tissue macrophages, that dampens the inflammatory response of these cell types [37]. CD163 is also present as a soluble molecule (sCD163) in the circulation when cleaved from the cell membrane of monocytes and macrophages by the protease ADAM17 [38]. In **Chapter 7**, it was shown that a high sCD163 level was an independent predictor of DFS in our CRC cohort, which is in line with studies in other cancers [39-42] and a study in CRC specifically [43]. Thus, it seems that patients with the highest immunosuppressive state, based on sCD163 levels, had the worst clinical outcome in our CRC cohort. This is in contradiction to what we showed in **Chapter 3** where patients with a relatively active immune system, based on the presence of high expression of activating receptors on NK- and NKT cells, were observed to have poor clinical outcome in the same study population. This emphasizes the fact that different cell types have distinct functions during tumor progression that are yet poorly understood.

Additionally, **Chapter 7** showed shorter overall survival (OS) in CRC patients with a high percentage of circulating monocytes (% of total $CD45^+$ cells). This was also reported by two meta-analyses that, in addition, also showed a correlation between a high percentage of circulating monocytes and DFS [44,45]. In contrast to sCD163 and monocytes in the circulation, ($CD163^+$) M2 tumor-associated macrophages (TAMs) in the primary tumor were not associated with clinical outcome in CRC patients

in our study. Other studies reported contradictory findings, in which high M2 TAM density has been reported to associate with both unfavorable [46-48], and favorable clinical outcome [49-53] in CRC. Importantly, the majority of these studies did not take the spatial distribution of TAMs in the primary tumor into account. In **Chapter 7**, we showed that the percentage of intraepithelial M2 TAMs positively correlated with TNM stage and differentiation grade whereas stromal M2 TAMs did not. In line with our results, Kim *et al.* showed higher intraepithelial TAM counts, but not stromal TAMs in colorectal tumors with higher TNM stages [54]. Furthermore, this study reported an association between high intraepithelial TAM counts and worse clinical outcome, but not for high stromal TAM counts [54]. These findings suggest a different role for stromal TAMs and intraepithelial TAMs in CRC progression. Therefore, based on our findings and others, it seems crucial to keep the spatial distribution of TAMs in CRC into account. This should be further investigated and validated in future studies.

In summary, **Chapter 7** showed that circulating monocyte markers may have prognostic value in CRC patients whereas TAMs in the primary tumor have not. Furthermore, we showed in **Chapter 3** that a high percentage of $CD16^+$ circulating NKT cells was associated with short disease-free survival (DFS) in CRC patients, independent from TNM classification and age. This illustrates that the peripheral blood immune profile is an important pool of potential biomarkers that may provide insight in the dynamic process of metastases development since they can be monitored over time. Most biomarker studies primarily focus on primary tumor-associated biomarkers, including the immunoscore as defined by Galon *et al.* [55]. This scoring system is based on quantification of infiltrated T cells in primary CRC tumors and showed that high density of $CD3^+$ and $CD8^+$ tumor-infiltrating T cells is strongly correlated with better clinical outcome [55-57]. As we have shown in **Chapter 3 and 4**, tumor-immune interactions are much more complex than the interaction between primary tumor cells and tumor-infiltrated immune cells only. The tumor-immune response consists of multiple different levels that are all intertwined. In order to estimate the state of the tumor-immune response in cancer patients, it is important to not only study tumor-immune interactions in the primary tumor, but in the circulation, and maybe the (pre)-metastatic niche [58] as well. Based on our findings, it might be interesting to expand the immunoscore with circulating biomarkers such as NK- and/or NKT cell markers, and/or monocyte-related markers in order to estimate the state of the immune system in the entire patient and thus, better predict clinical outcome of CRC patients.

Link between the primary tumor and circulating immune cells

Based on the data presented in this thesis, there are two important questions that require further attention. First of all, the question remains whether the primary tumor actively suppresses the peripheral blood immune profile in cancer patients in order to stimulate immune escape of circulating tumor cells in peripheral blood. In other words: is active immunoediting going on in the primary tumor that results in outgrowth of tumor cell clones that are most effective in suppressing the systemic immune system for survival benefit during dissemination of the disease? The answer to this question seems to be: "no". Since there is no continuous interaction between the primary tumor and circulating immune cells, it seems highly unlikely for immunoediting to have taken place due to lack of direct feedback. Primary tumor cells that are able to suppress the systemic immune system may have survival benefit when they enter the circulation. However, these cells do not necessarily have any survival benefit as long as they are located in the primary tumor, and, therefore, do not selectively grow out in the primary tumor. Instead, suppression of the peripheral blood immune system might merely be a bystander effect of tumor development, for instance via cytokine release, tumor metabolites, exosomes, and soluble NK cell receptor ligands that are produced by the tumor and released into the circulation. Based on our results, it seems this effect is induced during early tumor progression since the peripheral blood immune profile was equally suppressed in CRC patients with early and late cancer stages.

The next logical question is whether immunoediting takes place in the circulation of cancer patients. When tumor cells enter the circulation, they interact with circulating immune cells. The immune insensitive tumor cells are not recognized and, therefore, escape immune recognition in the peripheral blood. Although these tumor cell clones do not selectively grow out in peripheral blood, they do selectively survive, thereby resembling immunoediting. Whether a specific circulating tumor cell is able to successfully grow out into metastasis may depend on the immunoediting process taking place at the metastatic site. As the immune composition differs between organs, different immunoediting processes will take place, resulting in outgrowth of different tumor clones in different organs. In summary, immunoediting takes place in the primary tumor, next in the circulation, and finally at the metastatic site. This is a dynamic complex process in which different conditions apply for each compartment. Future research should focus on further unraveling the complexity of immunoediting, including the link between tumor-immune interactions in the primary tumor, the circulation, and the metastatic site.

Future perspectives

The immune system plays a pivotal role during tumor development and progression and has become an important pool of biomarkers in cancer. Up till now, most studies have primarily focused on tumor-immune interactions in the primary tumor, the most well-known being the immunoscore as defined by Galon *et al.* [55]. In this thesis, we illustrated that the tumor-immune response is much more complex and consists of multiple different levels that are all intertwined. In particular, we showed that the systemic tumor-immune response plays an important role during tumor progression and is therefore an interesting pool of biomarkers in cancer in addition to primary tumor-derived biomarkers. As is clear from this thesis, and from other studies as well [58-60], the immune system is a closely connected complex network. Therefore, in our opinion, it should be studied as one process in a patient, instead of as single stand-alone components as has been the standard in the past years. Hence, future research should focus on a system-based biology approach wherein the patient is studied *in toto*. Technical innovation is essential to establish this shift in research. Developments in multiparameter flow cytometry, mass cytometry, multiplex immunofluorescence, and software based on artificial intelligence have already accelerated research over the past decade. Whereas only a limited number of biomarkers could simultaneously be used to study different immune cell subsets and their phenotypes in tissues and peripheral blood in the past, nowadays this has already increased to simultaneous imaging and data processing of over 40 biomarkers by mass cytometry and multiplex immunofluorescence [61,62]. These new techniques enable us to acquire an overall picture of the immune system in relation to disease progression which will help us to better understand the underlying biology of tumor-immune interactions. In the future, this new knowledge may lead to the discovery of new immune-related biomarkers and new targets for immunotherapies. Early diagnosis of distant metastases, in combination with development of new treatment strategies, will get us closer in developing personalized medicine which will further improve the clinical prognosis of CRC patients in the future.

References

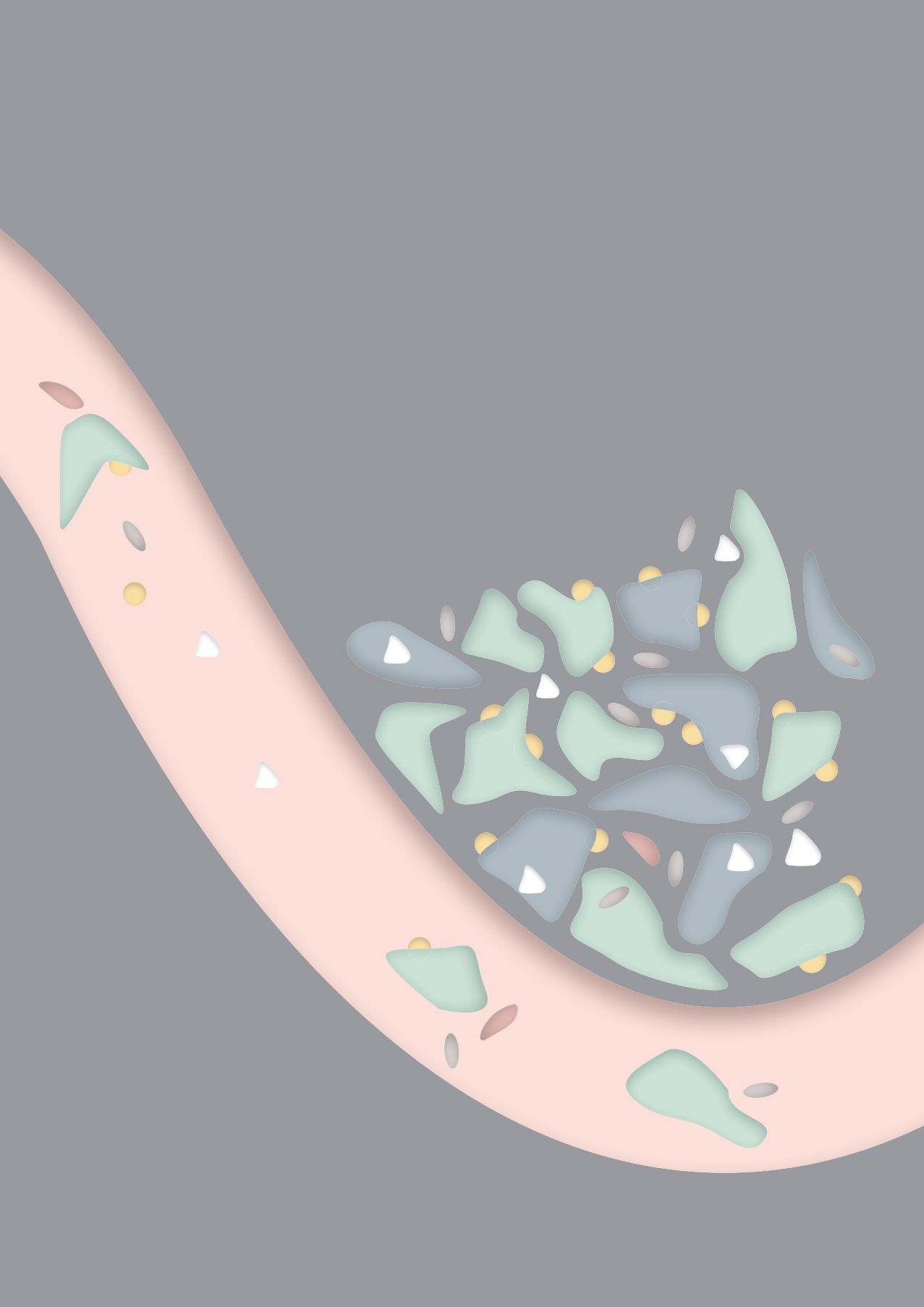
1. Brouwer, N.P.M.; Bos, A.; Lemmens, V.; Tanis, P.J.; Hugen, N.; Nagtegaal, I.D.; de Wilt, J.H.W.; Verhoeven, R.H.A. An overview of 25 years of incidence, treatment and outcome of colorectal cancer patients. *Int J Cancer* 2018, *143*, 2758-2766, doi:10.1002/ijc.31785.
2. Krijgsman, D.; Hokland, M.; Kuppen, P.J.K. The Role of Natural Killer T Cells in Cancer-A Phenotypical and Functional Approach. *Front Immunol* **2018**, *9*, 367, doi:10.3389/fimmu.2018.00367.
3. Giaccone, G.; Punt, C.J.; Ando, Y.; Ruijter, R.; Nishi, N.; Peters, M.; von Blomberg, B.M.; Scheper, R.J.; van der Vliet, H.J.; van den Eertwegh, A.J., et al. A phase I study of the natural killer T-cell ligand alpha-galactosylceramide (KRN7000) in patients with solid tumors. *Clin Cancer Res* **2002**, *8*, 3702-3709.
4. Motohashi, S.; Nagato, K.; Kunii, N.; Yamamoto, H.; Yamasaki, K.; Okita, K.; Hanaoka, H.; Shimizu, N.; Suzuki, M.; Yoshino, I., et al. A phase I-II study of alpha-galactosylceramide-pulsed IL-2/GM-CSF-cultured peripheral blood mononuclear cells in patients with advanced and recurrent non-small cell lung cancer. *J Immunol* **2009**, *182*, 2492-2501, doi:10.4049/jimmunol.0800126.
5. Exley, M.A.; Friedlander, P.; Alatrakchi, N.; Vriend, L.; Yue, S.; Sasada, T.; Zeng, W.; Mizukami, Y.; Clark, J.; Nemer, D., et al. Adoptive Transfer of Invariant NKT Cells as Immunotherapy for Advanced Melanoma: A Phase I Clinical Trial. *Clin Cancer Res* **2017**, *23*, 3510-3519, doi:10.1158/1078-0432.CCR-16-0600.
6. Rocca, Y.S.; Roberti, M.P.; Arriaga, J.M.; Amat, M.; Bruno, L.; Pampena, M.B.; Huertas, E.; Loria, F.S.; Pairola, A.; Bianchini, M., et al. Altered phenotype in peripheral blood and tumor-associated NK cells from colorectal cancer patients. *Innate Immun* **2013**, *19*, 76-85, doi:10.1177/1753425912453187.
7. Peng, Y.P.; Zhu, Y.; Zhang, J.J.; Xu, Z.K.; Qian, Z.Y.; Dai, C.C.; Jiang, K.R.; Wu, J.L.; Gao, W.T.; Li, Q., et al. Comprehensive analysis of the percentage of surface receptors and cytotoxic granules positive natural killer cells in patients with pancreatic cancer, gastric cancer, and colorectal cancer. *J Transl Med* **2013**, *11*, 262, doi:10.1186/1479-5876-11-262.
8. Grimm, M.; Feyen, O.; Hofmann, H.; Teriete, P.; Biegner, T.; Munz, A.; Reinert, S. Immunophenotyping of patients with oral squamous cell carcinoma in peripheral blood and associated tumor tissue. *Tumour Biol* **2016**, *37*, 3807-3816, doi:10.1007/s13277-015-4224-2.
9. Klatka, J.; Grywalska, E.; Hymos, A.; Krasowska, E.; Mielnik, M.; Siwicka-Gieroba, D.; Markowicz, J.; Trojanowski, P.; Olszanski, W.; Rolinski, J. Subpopulations of natural killer-T-like cells before and after surgical treatment of laryngeal cancer. *Cent Eur J Immunol* **2017**, *42*, 252-258, doi:10.5114/ceji.2017.70967.
10. Angka, L.; Martel, A.B.; Kilgour, M.; Jeong, A.; Sadiq, M.; de Souza, C.T.; Baker, L.; Kennedy, M.A.; Kekre, N.; Auer, R.C. Natural Killer Cell IFNgamma Secretion is Profoundly Suppressed Following Colorectal Cancer Surgery. *Ann Surg Oncol* **2018**, *25*, 3747-3754, doi:10.1245/s10434-018-6691-3.
11. Verma, R.; Foster, R.E.; Horgan, K.; Mounsey, K.; Nixon, H.; Smalle, N.; Hughes, T.A.; Carter, C.R. Lymphocyte depletion and repopulation after chemotherapy for primary breast cancer. *Breast Cancer Res* **2016**, *18*, 10, doi:10.1186/s13058-015-0669-x.
12. Rocca, Y.S.; Roberti, M.P.; Julia, E.P.; Pampena, M.B.; Bruno, L.; Rivero, S.; Huertas, E.; Sanchez Loria, F.; Pairola, A.; Caignard, A., et al. Phenotypic and Functional Dysregulated Blood NK Cells in Colorectal Cancer Patients Can Be Activated by Cetuximab Plus IL-2 or IL-15. *Front Immunol* **2016**, *7*, 413, doi:10.3389/fimmu.2016.00413.
13. Rosenberg, S.A.; Lotze, M.T.; Muul, L.M.; Leitman, S.; Chang, A.E.; Ettinghausen, S.E.; Matory, Y.L.; Skibber, J.M.; Shiloni, E.; Vetto, J.T., et al. Observations on the systemic administration of autologous lymphokine-activated killer cells and recombinant interleukin-2 to patients with metastatic cancer. *N Engl J Med* **1985**, *313*, 1485-1492, doi:10.1056/NEJM198512053132327.

14. Muul, L.M.; Nason-Burchenal, K.; Carter, C.S.; Cullis, H.; Slavin, D.; Hyatt, C.; Director, E.P.; Leitman, S.F.; Klein, H.G.; Rosenberg, S.A. Development of an automated closed system for generation of human lymphokine-activated killer (LAK) cells for use in adoptive immunotherapy. *J Immunol Methods* **1987**, *101*, 171-181, doi:10.1016/0022-1759(87)90148-7.
15. Rosenberg, S.A.; Lotze, M.T.; Yang, J.C.; Topalian, S.L.; Chang, A.E.; Schwartzentruber, D.J.; Aebersold, P.; Leitman, S.; Linehan, W.M.; Seipp, C.A., et al. Prospective randomized trial of high-dose interleukin-2 alone or in conjunction with lymphokine-activated killer cells for the treatment of patients with advanced cancer. *J Natl Cancer Inst* **1993**, *85*, 622-632, doi:10.1093/jnci/85.8.622.
16. Renukaradhya, G.J.; Sriram, V.; Du, W.; Gervay-Hague, J.; Van Kaer, L.; Brutkiewicz, R.R. Inhibition of antitumor immunity by invariant natural killer T cells in a T-cell lymphoma model in vivo. *Int J Cancer* **2006**, *118*, 3045-3053, doi:10.1002/ijc.21764.
17. Chong, T.W.; Goh, F.Y.; Sim, M.Y.; Huang, H.H.; Thike, A.A.; Lim, W.K.; Teh, B.T.; Tan, P.H. CD1d expression in renal cell carcinoma is associated with higher relapse rates, poorer cancer-specific and overall survival. *J Clin Pathol* **2015**, *68*, 200-205, doi:10.1136/jclinpath-2014-202735.
18. Wang, H.; Yang, D.; Xu, W.; Wang, Y.; Ruan, Z.; Zhao, T.; Han, J.; Wu, Y. Tumor-derived soluble MICs impair CD3(+)CD56(+) NKT-like cell cytotoxicity in cancer patients. *Immunol Lett* **2008**, *120*, 65-71, doi:10.1016/j.imlet.2008.07.001.
19. Gharagozloo, M.; Kalantari, H.; Rezaei, A.; Maracy, M.R.; Salehi, M.; Bahador, A.; Hassannejad, N.; Narimani, M.; Sanei, M.H.; Bayat, B., et al. The decrease in NKG2D+ Natural Killer cells in peripheral blood of patients with metastatic colorectal cancer. *Bratisl Lek Listy* **2015**, *116*, 296-301, doi:10.4149/bll_2015_056.
20. Coudert, J.D.; Zimmer, J.; Tomasello, E.; Cebecauer, M.; Colonna, M.; Vivier, E.; Held, W. Altered NKG2D function in NK cells induced by chronic exposure to NKG2D ligand-expressing tumor cells. *Blood* **2005**, *106*, 1711-1717, doi:10.1182/blood-2005-03-0918.
21. Fernandez-Messina, L.; Ashiru, O.; Boutet, P.; Aguera-Gonzalez, S.; Skepper, J.N.; Reyburn, H.T.; Vales-Gomez, M. Differential mechanisms of shedding of the glycosylphosphatidylinositol (GPI)-anchored NKG2D ligands. *J Biol Chem* **2010**, *285*, 8543-8551, doi:10.1074/jbc.M109.045906.
22. Liu, H.; Wang, S.; Xin, J.; Wang, J.; Yao, C.; Zhang, Z. Role of NKG2D and its ligands in cancer immunotherapy. *Am J Cancer Res* **2019**, *9*, 2064-2078.
23. Doubrovina, E.S.; Doubrovin, M.M.; Vider, E.; Sisson, R.B.; O'Reilly, R.J.; Dupont, B.; Vyas, Y.M. Evasion from NK cell immunity by MHC class I chain-related molecules expressing colon adenocarcinoma. *J Immunol* **2003**, *171*, 6891-6899, doi:10.4049/jimmunol.171.12.6891.
24. Schmiedel, D.; Mandelboim, O. NKG2D Ligands-Critical Targets for Cancer Immune Escape and Therapy. *Front Immunol* **2018**, *9*, 2040, doi:10.3389/fimmu.2018.02040.
25. Holdenrieder, S.; Stieber, P.; Peterfi, A.; Nagel, D.; Steinle, A.; Salih, H.R. Soluble MICB in malignant diseases: analysis of diagnostic significance and correlation with soluble MICA. *Cancer Immunol Immunother* **2006**, *55*, 1584-1589, doi:10.1007/s00262-006-0167-1.
26. Zhao, Y.; Chen, N.; Yu, Y.; Zhou, L.; Niu, C.; Liu, Y.; Tian, H.; Lv, Z.; Han, F.; Cui, J. Prognostic value of MICA/B in cancers: a systematic review and meta-analysis. *Oncotarget* **2017**, *8*, 96384-96395, doi:10.18632/oncotarget.21466.
27. Mariotti, F.R.; Quatrini, L.; Munari, E.; Vacca, P.; Tumino, N.; Pietra, G.; Mingari, M.C.; Moretta, L. Inhibitory checkpoints in human natural killer cells: IUPHAR Review 28. *Br J Pharmacol* **2020**, 10.1111/bph.15081, doi:10.1111/bph.15081.
28. Yang, C.; Li, Y.; Yang, Y.; Chen, Z. Overview of Strategies to Improve Therapy against Tumors Using Natural Killer Cell. *J Immunol Res* **2020**, *2020*, 8459496, doi:10.1155/2020/8459496.
29. Borst, L.; van der Burg, S.H.; van Hall, T. The NKG2A - HLA-E axis as a novel checkpoint in the tumor microenvironment. *Clin Cancer Res* **2020**, 10.1158/1078-0432.CCR-19-2095, doi:10.1158/1078-0432.CCR-19-2095.

30. Cho, M.; Bendell, J.C.; Han, S.W.; Naidoo, J.; Lieu, C.; Carneiro, B.A. Durvalumab plus monalizumab, mFOLFOX6, and bevacizumab in patients (pts) with metastatic micosatellite-stabel colorectal cancer (MSS-CRC). *Ann Oncol* **2019**, *30*.
31. Carlsten, M.; Korde, N.; Kotecha, R.; Reger, R.; Bor, S.; Kazandjian, D.; Landgren, O.; Childs, R.W. Checkpoint Inhibition of KIR2D with the Monoclonal Antibody IPH2101 Induces Contraction and Hyporesponsiveness of NK Cells in Patients with Myeloma. *Clin Cancer Res* **2016**, *22*, 5211-5222, doi:10.1158/1078-0432.CCR-16-1108.
32. Vey, N.; Karlin, L.; Sadot-Lebouvier, S.; Broussais, F.; Berton-Rigaud, D.; Rey, J.; Charbonnier, A.; Marie, D.; Andre, P.; Paturel, C., et al. A phase 1 study of lirilumab (antibody against killer immunoglobulin-like receptor antibody KIR2D; IPH2102) in patients with solid tumors and hematologic malignancies. *Oncotarget* **2018**, *9*, 17675-17688, doi:10.18632/oncotarget.24832.
33. Chester, C.; Fritsch, K.; Kohrt, H.E. Natural Killer Cell Immunomodulation: Targeting Activating, Inhibitory, and Co-stimulatory Receptor Signaling for Cancer Immunotherapy. *Front Immunol* **2015**, *6*, 601, doi:10.3389/fimmu.2015.00601.
34. Cartron, G.; Dacheux, L.; Salles, G.; Solal-Celigny, P.; Bardos, P.; Colombat, P.; Watier, H. Therapeutic activity of humanized anti-CD20 monoclonal antibody and polymorphism in IgG Fc receptor FcgammaRIIIa gene. *Blood* **2002**, *99*, 754-758, doi:10.1182/blood.v99.3.754.
35. Musolino, A.; Naldi, N.; Bortesi, B.; Pezzuolo, D.; Capelletti, M.; Missale, G.; Laccabue, D.; Zerbini, A.; Camisa, R.; Bisagni, G., et al. Immunoglobulin G fragment C receptor polymorphisms and clinical efficacy of trastuzumab-based therapy in patients with HER-2/neu-positive metastatic breast cancer. *J Clin Oncol* **2008**, *26*, 1789-1796, doi:10.1200/JCO.2007.14.8957.
36. Rodriguez, J.; Zarate, R.; Bandres, E.; Boni, V.; Hernandez, A.; Sola, J.J.; Honorato, B.; Bitarte, N.; Garcia-Foncillas, J. Fc gamma receptor polymorphisms as predictive markers of Cetuximab efficacy in epidermal growth factor receptor downstream-mutated metastatic colorectal cancer. *Eur J Cancer* **2012**, *48*, 1774-1780, doi:10.1016/j.ejca.2012.01.007.
37. Otterbein, L.E.; Soares, M.P.; Yamashita, K.; Bach, F.H. Heme oxygenase-1: unleashing the protective properties of heme. *Trends Immunol* **2003**, *24*, 449-455.
38. Etzerodt, A.; Maniecki, M.B.; Moller, K.; Moller, H.J.; Moestrup, S.K. Tumor necrosis factor alpha-converting enzyme (TACE/ADAM17) mediates ectodomain shedding of the scavenger receptor CD163. *J Leukoc Biol* **2010**, *88*, 1201-1205, doi:10.1189/jlb.0410235.
39. No, J.H.; Moon, J.M.; Kim, K.; Kim, Y.B. Prognostic significance of serum soluble CD163 level in patients with epithelial ovarian cancer. *Gynecol Obstet Invest* **2013**, *75*, 263-267, doi:10.1159/000349892.
40. Jensen, T.O.; Schmidt, H.; Moller, H.J.; Hoyer, M.; Maniecki, M.B.; Sjoegren, P.; Christensen, I.J.; Steiniche, T. Macrophage markers in serum and tumor have prognostic impact in American Joint Committee on Cancer stage I/II melanoma. *J Clin Oncol* **2009**, *27*, 3330-3337, doi:10.1200/JCO.2008.19.9919.
41. Kazankov, K.; Rode, A.; Simonsen, K.; Villadsen, G.E.; Nicoll, A.; Moller, H.J.; Lim, L.; Angus, P.; Kronborg, I.; Arachchi, N., et al. Macrophage activation marker soluble CD163 may predict disease progression in hepatocellular carcinoma. *Scand J Clin Lab Invest* **2016**, *76*, 64-73, doi:10.3109/00365513.2015.1099722.
42. Andersen, M.N.; Abildgaard, N.; Maniecki, M.B.; Moller, H.J.; Andersen, N.F. Monocyte/macrophage-derived soluble CD163: a novel biomarker in multiple myeloma. *Eur J Haematol* **2014**, *93*, 41-47, doi:10.1111/ejh.12296.
43. Ding, D.; Yao, Y.; Yang, C.; Zhang, S. Identification of mannose receptor and CD163 as novel biomarkers for colorectal cancer. *Cancer Biomark* **2018**, *21*, 689-700, doi:10.3233/CBM-170796.
44. Tan, D.; Fu, Y.; Tong, W.; Li, F. Prognostic significance of lymphocyte to monocyte ratio in colorectal cancer: A meta-analysis. *Int J Surg* **2018**, *55*, 128-138, doi:10.1016/j.ijsu.2018.05.030.

45. Song, W.; Wang, K.; Zhang, R.J.; Zou, S.B. Prognostic value of the lymphocyte monocyte ratio in patients with colorectal cancer: A meta-analysis. *Medicine (Baltimore)* **2016**, *95*, e5540, doi:10.1097/MD.0000000000005540.
46. Shabo, I.; Olsson, H.; Elkarim, R.; Sun, X.F.; Svanvik, J. Macrophage Infiltration in Tumor Stroma is Related to Tumor Cell Expression of CD163 in Colorectal Cancer. *Cancer Microenviron* **2014**, *7*, 61-69, doi:10.1007/s12307-014-0145-7.
47. Waniczek, D.; Lorenc, Z.; Snietura, M.; Wesecki, M.; Kopec, A.; Muc-Wierzgon, M. Tumor-Associated Macrophages and Regulatory T Cells Infiltration and the Clinical Outcome in Colorectal Cancer. *Arch Immunol Ther Exp (Warsz)* **2017**, 10.1007/s00005-017-0463-9, doi:10.1007/s00005-017-0463-9.
48. Shibutani, M.; Maeda, K.; Nagahara, H.; Fukuoka, T.; Nakao, S.; Matsutani, S.; Hirakawa, K.; Ohira, M. The peripheral monocyte count is associated with the density of tumor-associated macrophages in the tumor microenvironment of colorectal cancer: a retrospective study. *BMC Cancer* **2017**, *17*, 404, doi:10.1186/s12885-017-3395-1.
49. Forssell, J.; Oberg, A.; Henriksson, M.L.; Stenling, R.; Jung, A.; Palmqvist, R. High macrophage infiltration along the tumor front correlates with improved survival in colon cancer. *Clin Cancer Res* **2007**, *13*, 1472-1479, doi:10.1158/1078-0432.CCR-06-2073.
50. Zhou, Q.; Peng, R.Q.; Wu, X.J.; Xia, Q.; Hou, J.H.; Ding, Y.; Zhou, Q.M.; Zhang, X.; Pang, Z.Z.; Wan, D.S., et al. The density of macrophages in the invasive front is inversely correlated to liver metastasis in colon cancer. *J Transl Med* **2010**, *8*, 13, doi:10.1186/1479-5876-8-13.
51. Gulubova, M.; Ananiev, J.; Yovchev, Y.; Julianov, A.; Karashmalakov, A.; Vlaykova, T. The density of macrophages in colorectal cancer is inversely correlated to TGF-beta1 expression and patients' survival. *J Mol Histol* **2013**, *44*, 679-692, doi:10.1007/s10735-013-9520-9.
52. Koelzer, V.H.; Canonica, K.; Dawson, H.; Sokol, L.; Karamitopoulou-Diamantis, E.; Lugli, A.; Zlobec, I. Phenotyping of tumor-associated macrophages in colorectal cancer: Impact on single cell invasion (tumor budding) and clinicopathological outcome. *Oncoimmunology* **2016**, *5*, e1106677, doi:10.1080/2162402X.2015.1106677.
53. Edin, S.; Wikberg, M.L.; Dahlin, A.M.; Rutegard, J.; Oberg, A.; Oldenborg, P.A.; Palmqvist, R. The distribution of macrophages with a M1 or M2 phenotype in relation to prognosis and the molecular characteristics of colorectal cancer. *PLoS One* **2012**, *7*, e47045, doi:10.1371/journal.pone.0047045.
54. Kim, Y.; Wen, X.; Bae, J.M.; Kim, J.H.; Cho, N.Y.; Kang, G.H. The distribution of intratumoral macrophages correlates with molecular phenotypes and impacts prognosis in colorectal carcinoma. *Histopathology* **2018**, *73*, 663-671, doi:10.1111/his.13674.
55. Galon, J.; Costes, A.; Sanchez-Cabo, F.; Kirilovsky, A.; Mlecnik, B.; Lagorce-Pages, C.; Tosolini, M.; Camus, M.; Berger, A.; Wind, P., et al. Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. *Science* **2006**, *313*, 1960-1964, doi:10.1126/science.1129139.
56. Mlecnik, B.; Tosolini, M.; Kirilovsky, A.; Berger, A.; Bindea, G.; Meatchi, T.; Bruneval, P.; Trajanoski, Z.; Fridman, W.H.; Pages, F., et al. Histopathologic-based prognostic factors of colorectal cancers are associated with the state of the local immune reaction. *J Clin Oncol* **2011**, *29*, 610-618, doi:10.1200/JCO.2010.30.5425.
57. Galon, J.; Mlecnik, B.; Bindea, G.; Angell, H.K.; Berger, A.; Lagorce, C.; Lugli, A.; Zlobec, I.; Hartmann, A.; Bifulco, C., et al. Towards the introduction of the 'Immunoscore' in the classification of malignant tumours. *J Pathol* **2014**, *232*, 199-209, doi:10.1002/path.4287.
58. Doglioni, G.; Parik, S.; Fendt, S.M. Interactions in the (Pre)metastatic Niche Support Metastasis Formation. *Front Oncol* **2019**, *9*, 219, doi:10.3389/fonc.2019.00219.
59. Dumeaux, V.; Fjukstad, B.; Fjosne, H.E.; Frantzen, J.O.; Holmen, M.M.; Rodegerdts, E.; Schlichting, E.; Borresen-Dale, A.L.; Bongo, L.A.; Lund, E., et al. Interactions between the tumor and the blood systemic response of breast cancer patients. *PLoS Comput Biol* **2017**, *13*, e1005680, doi:10.1371/journal.pcbi.1005680.

60. Ferguson Bennit, H.R.; Gonda, A.; McMullen, J.R.W.; Kabagwira, J.; Wall, N.R. Peripheral Blood Cell Interactions of Cancer-Derived Exosomes Affect Immune Function. *Cancer Microenviron* **2019**, *12*, 29-35, doi:10.1007/s12307-018-0209-1.
61. Ijsselsteijn, M.E.; van der Breggen, R.; Farina Sarasqueta, A.; Koning, F.; de Miranda, N. A 40-Marker Panel for High Dimensional Characterization of Cancer Immune Microenvironments by Imaging Mass Cytometry. *Front Immunol* **2019**, *10*, 2534, doi:10.3389/fimmu.2019.02534.
62. Tan, W.C.C.; Nerurkar, S.N.; Cai, H.Y.; Ng, H.H.M.; Wu, D.; Wee, Y.T.F.; Lim, J.C.T.; Yeong, J.; Lim, T.K.H. Overview of multiplex immunohistochemistry/immunofluorescence techniques in the era of cancer immunotherapy. *Cancer Commun (Lond)* **2020**, *40*, 135-153, doi:10.1002/cac2.12023.



APPENDICES

Nederlandse samenvatting
List of publications
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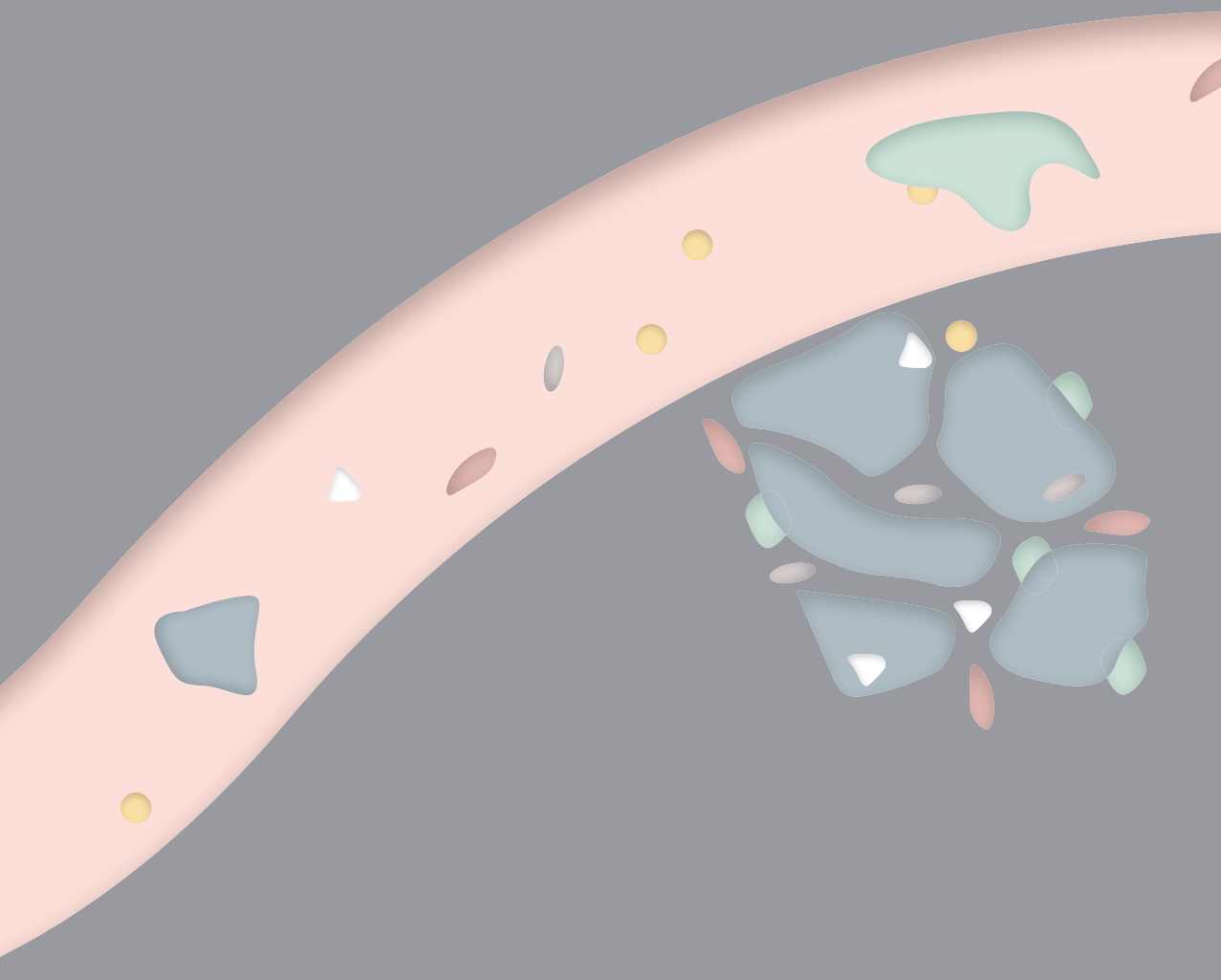


Dikkedarmkanker

In de afgelopen jaren is de duur van de overleving van patiënten met dikkedarmkanker, en het aantal patiënten dat overleeft, aanzienlijk verbeterd. Dit wordt verklaard door de effectiviteit van nieuwe behandelingen, die zowel voor (neo-adjuvant) als na (adjuvant) het operatief verwijderen van de primaire (oorspronkelijke) tumor toegepast worden [1]. Hoewel de overleving van patiënten is verbeterd, is er echter nog veel winst te behalen. Per jaar overlijden er namelijk wereldwijd nog altijd een half miljoen patiënten aan de gevolgen van dikkedarmkanker [2]. Ongeveer 25% van deze patiënten heeft op het moment dat de diagnose gesteld wordt al uitzaaiingen in andere organen, vaak in de lever, wat resulteert in een relatief kortere overleving van deze patiënten. Daarnaast ontwikkelt ongeveer 30% van de patiënten in een vroeg stadium alsnog uitzaaiingen na een succesvolle operatie [3]. De ontwikkeling van een “gepersonaliseerde behandeling”, waarbij aan iedere patiënt een therapie op maat aangeboden kan worden, is veelbelovend voor het vergroten van de overlevingskansen van patiënten met dikkedarmkanker. Het is hiervoor belangrijk dat de patiënten met een hoog risico op uitzaaiingen kunnen worden geïdentificeerd, aangezien deze patiënten in potentie het meeste baat zullen hebben bij adjuvante therapie. Daarnaast is het van belang dat er wordt onderzocht hoe patiënten die het best zullen reageren op een therapie geïdentificeerd kunnen worden. Ziekteprogressie (voortgang van de ziekte) en het aanslaan van de behandeling kunnen worden voorspeld door middel van zogenoemde “biomarkers”. Biomarkers zijn bijvoorbeeld specifieke moleculen of cellen die gemeten kunnen worden in de tumor of het bloed van patiënten. Recente ontwikkelingen in het kankeronderzoek hebben laten zien dat het immuunsysteem een belangrijke rol speelt in de ontwikkeling en groei van tumoren. Daarom zijn immuun-gerelateerde biomarkers een belangrijk onderwerp van onderzoek geworden.

De rol van het immuunsysteem in de primaire tumor

Tumorcellen ontstaan als gevolg van ontregeling van o.a. de normale controlemechanismen voor celgroei en celdeling. Dit vaak in samenhang met beschadigingen van het erfelijke materiaal van de cel (het DNA). Deze beschadigingen worden ook wel mutaties genoemd en kunnen verschillende oorzaken hebben. Ze kunnen bijvoorbeeld het resultaat zijn uv-straling, sigarettenrook, en asbest, die daarom ‘kankerverwekkend’ worden genoemd, maar ook van spontane fouten die optreden tijdens de celdeling. Tot slot zijn er ook erfelijke mutaties die van ouder op kind kunnen worden overgedragen en ervoor zorgen dat iemand een sterk verhoogd risico heeft op het ontwikkelen van bepaalde types

kanker. Op het moment dat er verschillende mutaties optreden die gerelateerd zijn aan processen die belangrijk zijn voor bijvoorbeeld de celdeling, kan een cel ongecontroleerd gaan delen en zich dus ontwikkelen tot een tumorcel. Tijdens de ongecontroleerde celdeling van de tumorcel zullen er nóg meer mutaties ontstaan waardoor de tumorcel er anders uit gaat zien dan normale cellen. Het immuunsysteem controleert voortdurend of lichaamscellen nog normaal functioneren. Daarvoor laten lichaamscellen aan cellen van het immuunsysteem op ieder moment een soort “paspoort” zien. Op hun celoppervlak steken bepaalde eiwitten uit (HLA-moleculen) die als presenteerblaadjes kleine fragmenten van willekeurige eiwitten uit de cel laten zien. Cellen van ons immuunsysteem (T-cellen) zijn voortdurend bezig om deze fragmenten te controleren en zodra ze iets onregelmatigs bespeuren, dus de aanwezigheid van een fragment afkomstig van een abnormaal eiwit als gevolg van een mutatie in het DNA, slaan ze alarm en wordt de betreffende cel vernietigd. Helaas kunnen tumorcellen ook DNA-mutaties verkrijgen waardoor ze eiwitten gaan maken die ervoor zorgen dat het immuunsysteem ze niet herkent en opruimt. Deze tumorcellen, die niet opgeruimd worden door het immuunsysteem, kunnen vervolgens verder groeien terwijl de tumorcellen die deze eiwitten niet hebben wél worden opgeruimd. Door de selectie van welke tumorcellen worden opgeruimd en welke blijven leven zal uiteindelijk een groot deel van de tumor bestaan uit cellen die niet herkend worden door het immuunsysteem. Dit proces noemen we “immuno editing” en leidt er uiteindelijk toe dat de tumor ontsnapt aan het immuunsysteem waardoor deze ongeremd verder kan groeien. De aanwezigheid van eiwitten die de reactie van het immuunsysteem (‘de immuunrespons’) tegen de tumorcellen onderdrukken, evenals de aanwezigheid van verschillende typen immuuncellen die de tumorcellen kunnen herkennen, zijn interessante biomarkers om de ziekteprogressie van patiënten te voorspellen. De “immunoscore”, zoals gedefinieerd door de onderzoeksgroep van de Franse onderzoeker Galon [4-7], laat bijvoorbeeld zien dat patiënten met darmkanker een betere overleving hebben als er veel T-cellen in hun tumor zitten. Er was echter ook een deel van de patiënten met relatief veel T-cellen in de tumor dat juist een slechte prognose had [4,5,7]. Deze tumoren hebben waarschijnlijk de HLA-moleculen uitgeschakeld waardoor de fragmenten van abnormale eiwitten in de tumorcellen niet meer gecontroleerd kunnen worden door T-cellen. Tumorcellen zonder HLA-moleculen ontsnappen dus aan de herkenning van T-cellen. Precies deze tumoren zijn potentiële aangrijpingspunten voor NK-cellen (Natural Killer) en NKT-cellen (Natural Killer T) die tumorcellen kunnen herkennen en opruimen zonder HLA-moleculen [8-10]. Echter zijn deze celtypes erg schaars in darmtumoren en lijken daarom geen grote rol te spelen in de immuunrespons gericht tegen de primaire tumor [11-14]. Samenvattend speelt het immuunsysteem dus een belangrijke rol in het opruimen van tumorcellen. Met name T-cellen spelen een grote rol in de immuunrespons gericht tegen de primaire tumor door het herkennen

en opruimen van tumorcellen via HLA-moleculen. Ontsnapping aan T-cellen is daarom een belangrijk proces voor tumorcellen in de primaire tumor van darmkankerpatiënten.

De rol van het immuunsysteem in de bloedbaan

Op een gegeven moment, gedurende ziekteprogressie, kunnen tumorcellen loslaten van de primaire tumor waarna deze in de bloedbaan (circulatie) terecht komen. Via deze weg kunnen tumorcellen uitzaaien naar lymfeklieren en andere organen. Of een circulerende tumorcel uiteindelijk leidt tot uitzaaiingen in lymfeklieren en/of andere organen hangt af van het feit of de betreffende tumorcel kan ontsnappen aan immuuncellen in de bloedbaan. NK-cellen en NKT-cellen zijn in relatief grote aantallen aanwezig in de bloedbaan, in tegenstelling tot in de primaire tumor [9], en zijn heel goed in staat om tumorcellen te doden. Waar ontsnapping aan T-cellen dus belangrijk is voor tumorcellen in de primaire tumor, is ontsnapping aan NK-cellen en NKT-cellen een belangrijk proces voor tumorcellen in de bloedbaan. De mate van aanwezigheid en activiteit van deze celtypes in het bloed worden daarom genoemd als potentiële biomarkers voor het voorspellen van het risico op uitzaaiingen in patiënten met darmkanker. In tegenstelling tot biomarkers ín de primaire tumor, die op één gefixeerd tijdstip bepaald worden (namelijk in het tumorweefsel dat verwijderd wordt tijdens de operatie), kunnen biomarkers in het bloed op meerdere tijdstippen worden bepaald door een simpele bloedafname. Hierdoor kan eventuele ziekteprogressie van patiënten worden gemonitord gedurende een lange periode.

Doel van dit proefschrift

Samenvattend kunnen verschillende interacties tussen tumorcellen en het immuunsysteem (tumor-immuun interacties) worden geïdentificeerd in patiënten met dikkedarmkanker, onder andere in de primaire tumor en in de bloedbaan, welke ieder een unieke rol spelen in ziekteprogressie [15]. Met name tumor-immuun interacties in de bloedbaan zijn een interessante focus van onderzoek aangezien de uitkomst hiervan bepaalt of tumorcellen in het bloed worden herkend en opgeruimd, of juist ontsnappen aan het immuunsysteem en vervolgens uitzaaien naar andere organen. Het doel van dit proefschrift was om meer te weten te komen over de onderliggende biologie van tumor-immuun interacties in het bloed van patiënten met dikkedarmkanker, en om een link te leggen tussen tumor-immuun interacties in de primaire tumor en in de bloedbaan.

NK-cellen en NKT-cellen in de bloedbaan

Hoofdstuk 2 behandelt alle huidige kennis over de recent ontdekte NKT-cellen en hun mogelijke rol in de ziekteprogressie van kankerpatiënten. NKT-cellen kunnen tumorcellen herkennen en opruimen, maar zijn vooral belangrijk voor het reguleren van de immuunrespons [16]. Na activatie produceren NKT-cellen grote hoeveelheden signaalmoleculen waardoor andere immuuncellen geactiveerd ofwel geremd worden. Mede door deze regulerende eigenschappen zijn NKT-cellen interessante aangrijpingspunten voor immuuntherapie. De laatste jaren zijn er verschillende klinische studies uitgevoerd in kankerpatiënten gericht op de activatie van NKT-cellen. Helaas hebben tot nu toe maar weinig patiënten baat bij deze behandeling [17-19]. In **Hoofdstuk 2** beargumenteren we dat dit mogelijk komt door de veranderde functie van NKT-cellen in kankerpatiënten. NKT-cellen kunnen overprikkeld worden in kankerpatiënten door te veel stimulatie door de tumor waardoor deze NKT-cellen doodgaan, niet meer reageren op activatie, of signaalmoleculen produceren die juist zorgen voor onderdrukking van het immuunsysteem. Hierdoor kunnen tumorcellen ontsnappen aan het immuunsysteem waardoor ziekteprogressie bevorderd wordt. Op basis van de besproken literatuur concludeerden we dat er drie belangrijke punten opgelost moeten worden voordat immuuntherapie op basis van NKT-cellen effectief kan zijn in de behandeling van kankerpatiënten: 1) het aantal NKT-cellen moet worden verhoogd in patiënten, 2) activatie van NKT-cellen moet worden hersteld, en 3) er moet gezorgd worden dat NKT-cellen signaalmoleculen produceren die zorgen voor activatie van andere immuuncellen, waardoor de immuunrespons tegen de tumorcellen een *boost* krijgt.

Om meer inzicht te krijgen in de rol van immuuncellen in het bloed in ziekteprogressie, inclusief NKT-cellen, hebben we het immuunprofiel in de bloedbaan (perifeer bloed immuunprofiel) nader bestudeerd in patiënten met dikkedarmkanker, zowel voor als na operatieve verwijdering van de tumor. Dit immuunprofiel bevat onder andere informatie over de aanwezigheid van verschillende subtypes immuuncellen in het bloed, maar ook informatie over de aanwezigheid van receptoren op deze cellen. Een receptor is een eiwit dat signalen doorgeeft aan de cel als er een specifiek molecuul aan bindt en kan ofwel zorgen voor remming of activatie van de cel. De aanwezigheid van remmende en activerende receptoren op immuuncellen geeft dus indirect informatie over hun activiteit en functie [20]. In **Hoofdstuk 3** laten we zien dat NK-cellen en NKT-cellen in het bloed van patiënten met dikkedarmkanker, voordat ze geopereerd worden, minder activerende receptoren hebben dan gezonde individuen. Dit suggereert dat NK-cellen en NKT-cellen minder functioneel zijn in deze patiënten, wat kan bijdragen aan de ontsnapping van tumorcellen aan het immuunsysteem en

daarmee ziekteprogressie. In **Hoofdstuk 4** laten we zien dat de aanwezigheid van activerende receptoren op NK-cellen en NKT-cellen toeneemt in darmkankerpatiënten na de operatie wanneer zij géén adjuvante therapie (therapie na de operatie) hebben gekregen, terwijl de aanwezigheid van remmende receptoren op deze cellen afneemt. Dit suggereert dat de onderdrukte functie van NK-cellen en NKT-cellen hersteld wordt in patiënten na het weghalen van de primaire tumor. Gebaseerd op deze bevindingen concludeerden we dat darmtumoren in staat zijn om immuuncellen in het bloed te onderdrukken, wat zou kunnen leiden tot ontsnapping van tumorcellen in de bloedbaan aan het immuunsysteem en vervolgens tot de vorming van uitzaaiingen in andere organen.

Daarnaast laten we in **Hoofdstuk 4** zien dat de aanwezigheid van activerende receptoren op NK-cellen en NKT-cellen in het bloed nog steeds verlaagd was na de operatie in darmkankerpatiënten die wél behandeld werden met adjuvante therapie. Dit effect was zelfs nog aantoonbaar maanden nadat de therapie was afgerond. Adjuvante therapie, zoals chemotherapie, wordt vaak aan patiënten gegeven om overgebleven tumorcellen na de operatie op te ruimen, en daarmee het risico op het terugkomen van de tumor en het risico op het ontwikkelen van uitzaaiingen te verkleinen. Echter, chemotherapie heeft langdurige onderdrukkende effecten op het immuunsysteem waardoor het immuunsysteem in deze periode waarschijnlijk ook geen effectieve immuunrespons opbouwt tegen overgebleven tumorcellen na de operatie. In de toekomst is het wellicht een optie om patiënten te behandelen met een combinatie van adjuvante chemotherapie en immuuntherapie om de immuun-onderdrukkende effecten van chemotherapie te neutraliseren, waardoor de kans op uitzaaiingen na de operatie kleiner wordt.

NK-cel liganden in de primaire tumor

In **Hoofdstuk 5 en 6** bestuderen we hoe een darmtumor NK-cellen en NKT-cellen in het bloed zou kunnen beïnvloeden. De receptoren op NK-cellen en NKT-cellen, zoals beschreven in voorgaande hoofdstukken, zorgen voor remming of activatie van deze celtypes op het moment dat ze binden aan specifieke eiwitten. De *activerende* receptoren op NK-cellen en NKT-cellen herkennen verschillende celstress-gerelateerde eiwitten, ook wel liganden genoemd, die door tumorcellen gemaakt kunnen worden. De *remmende* receptoren herkennen verschillende HLA-moleculen. De activatie van NK-cellen en NKT-cellen is dus afhankelijk van de interactie tussen de receptoren op deze cellen, en liganden die aanwezig kunnen zijn op tumorcellen. Verschillende studies hebben daarom

gesuggereerd dat de aanwezigheid van liganden op tumorcellen de activatie van NK-cellen en NKT-cellen zou kunnen beïnvloeden [21,22]. Dit hebben we daarom nader onderzocht.

Hoofdstuk 5 demonstreert een methode waarin we met behulp van een semiautomatisch algoritme de aanwezigheid van biomarkers, zoals liganden voor NK-cellen en NKT-cellen, kunnen bepalen in tumorweefsel. Hiervoor worden dunne plakjes tumorweefsel gesneden waarop specifieke eiwitten worden aangekleurd. Deze labtechniek noemen we "immunohistochemie". De hoeveelheid aankleuring is een maatstaf voor de hoeveelheid eiwit dat aanwezig is in de tumor. Deze aankleuring op tumorcellen wordt meestal met het oog (door middel van een microscoop) beoordeeld op aan- of afwezigheid, maar ook op intensiteit van de kleuring. Deze methode is echter erg gevoelig voor misinterpretatie aangezien het lastig is om op het oog tumorweefsels met dezelfde criteria te scoren. Dit is met name omdat er grote verschillen tussen patiënten kunnen zijn hoe het tumorweefsel er uit ziet. Daarnaast is het soms lastig om de aanwezigheid van biomarkers specifiek op tumorcellen te beoordelen aangezien deze ook regelmatig voorkomen op andere type cellen in de tumor, zoals bindweefsel, immuuncellen, zenuwen, en bloedvaten. Bovendien is het op het oog bijvoorbeeld niet mogelijk om voldoende nauwkeurig een percentage positieve tumorcellen te scoren. Deze limitaties hebben we opgelost in de methode zoals gepresenteerd in **Hoofdstuk 5**. Allereerst hebben we een immunohistochemiemethode met twee kleuren opgezet waarin we al het weefsel, behalve de tumorcellen, bruin hebben aangekleurd, en de biomarker van interesse op de tumorcel (in dit geval HLA-moleculen) blauw. In plaats van beoordeling met het oog door een microscoop hebben we de aangekleurde tumorweefsels gescand en vervolgens digitaal, met specifieke software, op een semiautomatische manier gescoord. Met semiautomatisch bedoelen we dat het grootste gedeelte van het algoritme automatisch werkte, maar nog enige handmatige instellingen en controlestappen vereiste. Door het excluderen van al het bruin aangekleurde weefsel was het mogelijk om de tumorcellen te selecteren waarop we vervolgens de aanwezigheid van HLA-moleculen door de software konden laten scoren volgens standaardcriteria. Met deze methode hebben we laten zien dat de aanwezigheid van HLA-moleculen in de tumor bepaald kon worden op een reproductieve, robuuste, en objectieve manier.

In **Hoofstuk 6** hebben we vervolgens deze analysemethode verder geoptimaliseerd met behulp van kunstmatige intelligentie en zogenaamde "deep-learning" (zelflerende) software. Vervolgens hebben we deze techniek gebruikt om de aanwezigheid van verschillende NK-cel en NKT-cel liganden te bepalen in darmtumoren. In deze studie laten we onder andere zien dat er een sterke negatieve

correlatie is tussen de mate van aanwezigheid van het ligand galectin-3 in de tumor, en mate van aanwezigheid van de bijbehorende receptor NKp30 op NKT-cellen in het bloed. Met andere woorden: hoe hoger de aanwezigheid van galectin-3 in de tumor, des te lager de aanwezigheid van de NKp30 receptor op NKT-cellen waardoor activatie in verminderde mate plaats kan vinden. Dit impliceert dat NK-cel en NKT-cel liganden het immuunprofiel in de bloedbaan kunnen beïnvloeden/onderdrukken wat ertoe kan leiden dat tumorcellen in de bloedbaan ontsnappen aan het immuunsysteem. Daarnaast ondersteunt dit resultaat de hypothese zoals gepresenteerd in **Hoofdstuk 2**: overprikkeling van NKT-cellen gedurende ziekteprogressie leidt tot verminderde functie waardoor ze waarschijnlijk tumorcellen niet meer effectief kunnen herkennen en opruimen. Door het voorkomen van overprikkeling van NK-cellen en NKT-cellen, bijvoorbeeld door het blokkeren van de interacties tussen specifieke receptoren en hun liganden, kan de anti-tumor functie van NK-cellen en NKT-cellen worden hersteld. Receptor-ligand interacties op NK-cellen en NKT-cellen zijn daarom potentiële nieuwe aangrijpingspunten voor immuuntherapie.

Biomarkers in de bloedbaan van darmkankerpatiënten

Tot slot beschrijven we in **Hoofdstuk 7** het onderzoek waarin we ons hebben gericht op monocyten en macrofagen. Deze immuuncellen spelen, net als NK-cellen en NKT-cellen, een grote rol in de immuunrespons gericht tegen de tumor. Monocyten en macrofagen breken dode cellen af, en produceren signaalmoleculen waardoor andere immuuncellen ofwel geactiveerd of geremd worden. Monocyten bevinden zich in het bloed en ontwikkelen zich tot macrofagen op het moment dat ze een weefsel binnendringen. CD163 is een molecuul dat aanwezig kan zijn op monocyten en macrofagen. Het zorgt ervoor dat deze celtypes signaalmoleculen produceren die een immuunrespons onderdrukken [23]. CD163 kan daarnaast ook van monocyten en macrofagen geknipt worden, waarna het als oplosbare vorm (sCD163) in de bloedbaan terecht komt [24]. In het bloed kan sCD163 de functie van diverse immuuncellen onderdrukken. In **Hoofdstuk 7** laten we zien dat hoge sCD163 concentraties in de circulatie van darmkankerpatiënten geassocieerd is met een kortere ziektevrije overleving (de tijd gedurende welke de patiënt overleeft zonder tekenen van kanker). Dit is in overeenstemming met andere studies in verschillende kankertypes en suggereert dat sCD163 concentraties gebruikt zouden kunnen worden als biomarker voor het voorspellen van ziektevrije overleving in patiënten [25-27]. Grote aantallen monocyten in het bloed waren geassocieerd met slechte overleving van patiënten. Aantallen en subtypes macrofagen in de tumor waren daarentegen niet geassocieerd met klinische uitkomst in darmkankerpatiënten. Samenvattend hebben we in deze

studie laten zien dat monocyten en sCD163 in het bloed wél biomarkerpotentie hebben voor het voorspellen van ziektevrije overleving van patiënten met darmkanker, en macrofagen ín de tumor niet. Daarnaast hebben we in **Hoofdstuk 3** laten zien dat aanwezigheid van specifieke receptoren op NKT-cellen in het bloed van invloed was op de overleving van patiënten met darmkanker: een hoog percentage $CD16^+$ NKT-cellen in het bloed was geassocieerd met slechte ziektevrije overleving. Over de biologische functie van de CD16 receptor op NKT-cellen is echter nog weinig bekend en moet nader bestudeerd worden. De bevindingen uit **Hoofdstuk 3 en 7** illustreren dat het immuunprofiel in de bloedbaan een belangrijke bron is van potentiële biomarkers om inzicht te geven in welke patiënten een hoog risico hebben op het ontwikkelen van uitzaaiingen na de operatie. Het is dus van belang om in gedachte te houden dat de immuunrespons tegen de tumor ingewikkelder is dan alleen de interactie tussen tumorcellen en immuuncellen in de primaire tumor. Om preciezer te zijn, de immuunrespons tegen de tumor bestaat uit verschillende niveaus welke allemaal samenhangen. Om een overzicht te krijgen hoe de staat is van een immuunrespons tegen de tumor moet daarom niet alleen naar de primaire tumor gekeken worden, maar ook naar wat er in de bloedbaan van deze patiënten aan de hand is. Gebaseerd op deze resultaten is het interessant om biomarkers afkomstig uit de bloedbaan, zoals NK-cellen, NKT-cellen, en/of monocyten, toe te voegen aan de “immunoscore” om zijn voorspellende waarde te vergroten.

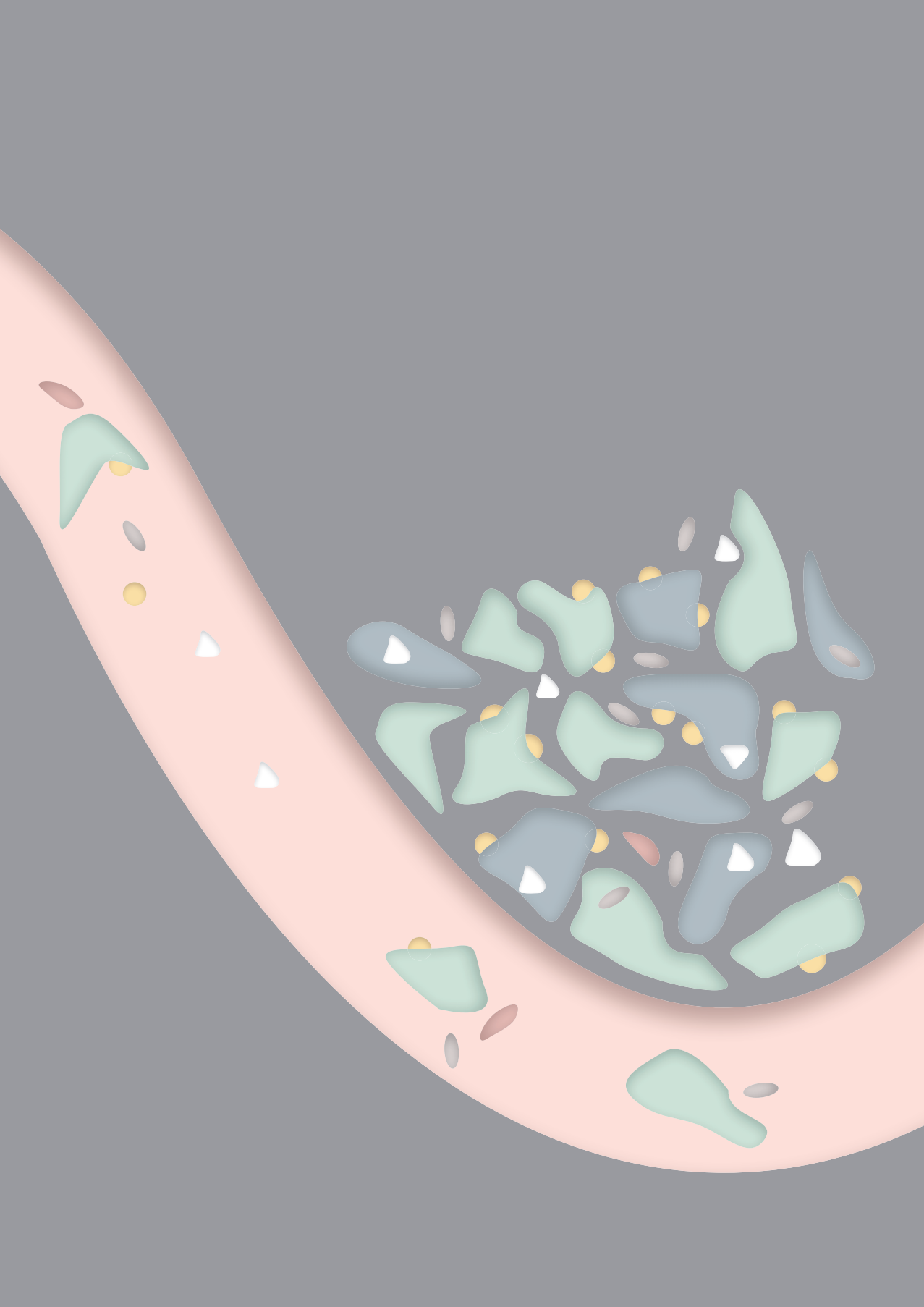
Toekomstperspectief

Het immuunsysteem speelt een cruciale rol in ziekteprogressie van darmkankerpatiënten en is daarom een belangrijke bron van mogelijke biomarkers om het risico op uitzaaiingen te kunnen voorspellen. Tot nu toe richtten de meeste studies zich op tumor-immuun interacties ín de primaire tumor, waarvan de meest bekende de “immunoscore” is zoals gedefinieerd door de onderzoeksgroep van Galon [6]. In dit proefschrift hebben we geïllustreerd dat de tumor-immuunrespons veel complexer is dan alleen de interactie tussen tumorcellen en immuuncellen in de primaire tumor: ook interacties elders in het lichaam zijn bepalend voor het ziekteverloop. De immuunrespons tegen de tumor is een complex netwerk dat bestaat uit verschillende niveaus die allemaal nauw samenhangen. Naar onze mening zou het daarom als één geheel bestudeerd moeten worden in een patiënt in plaats van in losse op zichzelf staande onderdelen. Toekomstig onderzoek moet zich daarom focussen op een zogenoemde “system-based biology” benadering waarin de patiënt als geheel wordt bestudeerd en er zowel naar de primaire tumor als naar het bloed van patiënten gekeken wordt en wellicht in de toekomst ook naar uitzaaiingen. Technische innovatie is essentieel voor focusverschuiving binnen kankeronderzoek. Onder andere ontwikkelingen in laboratoriumtechnieken en artificiële intelligentie hebben ertoe geleid dat we al meer dan 40 biomarkers tegelijkertijd in patiënten kunnen meten en verwerken [28,29]. Deze nieuwe technieken zorgen ervoor dat we overzicht krijgen hoe de staat is van de totale tumor-immuunrespons in een patiënt in relatie tot ziekteprogressie. Dit helpt ons om de interactie tussen de tumor en het immuunsysteem beter te begrijpen. In de toekomst kan deze kennis leiden tot de ontdekking van nieuwe biomarkers en nieuwe aangrijpingspunten voor immunotherapieën. Het beter voorspellen van het risico op het krijgen van uitzaaiingen, in combinatie met het ontwikkelen van nieuwe therapieën, brengt ons een stap dichterbij gepersonaliseerde behandeling. Deze vooruitgang zal de overleving van darmkankerpatiënten aanzienlijk verbeteren in de toekomst.

Referenties

1. Brouwer, N.P.M.; Bos, A.; Lemmens, V.; Tanis, P.J.; Hugen, N.; Nagtegaal, I.D.; de Wilt, J.H.W.; Verhoeven, R.H.A. An overview of 25 years of incidence, treatment and outcome of colorectal cancer patients. *Int J Cancer* **2018**, *143*, 2758-2766, doi:10.1002/ijc.31785.
2. Torre, L.A.; Bray, F.; Siegel, R.L.; Ferlay, J.; Lortet-Tieulent, J.; Jemal, A. Global cancer statistics, 2012. *CA Cancer J Clin* **2015**, *65*, 87-108, doi:10.3322/caac.21262.
3. Van Cutsem, E.; Cervantes, A.; Nordlinger, B.; Arnold, D.; Group, E.G.W. Metastatic colorectal cancer: ESMO Clinical Practice Guidelines for diagnosis, treatment and follow-up. *Ann Oncol* **2014**, *25 Suppl 3*, iii1-9, doi:10.1093/annonc/mdu260.
4. Galon, J.; Costes, A.; Sanchez-Cabo, F.; Kirilovsky, A.; Mlecnik, B.; Lagorce-Pages, C.; Tosolini, M.; Camus, M.; Berger, A.; Wind, P., et al. Type, density, and location of immune cells within human colorectal tumors predict clinical outcome. *Science* **2006**, *313*, 1960-1964, doi:10.1126/science.1129139.
5. Mlecnik, B.; Tosolini, M.; Kirilovsky, A.; Berger, A.; Bindea, G.; Meatchi, T.; Bruneval, P.; Trajanoski, Z.; Fridman, W.H.; Pages, F., et al. Histopathologic-based prognostic factors of colorectal cancers are associated with the state of the local immune reaction. *J Clin Oncol* **2011**, *29*, 610-618, doi:10.1200/JCO.2010.30.5425.
6. Galon, J.; Mlecnik, B.; Bindea, G.; Angell, H.K.; Berger, A.; Lagorce, C.; Lugli, A.; Zlobec, I.; Hartmann, A.; Bifulco, C., et al. Towards the introduction of the 'Immunoscore' in the classification of malignant tumours. *J Pathol* **2014**, *232*, 199-209, doi:10.1002/path.4287.
7. Pages, F.; Mlecnik, B.; Marliot, F.; Bindea, G.; Ou, F.S.; Bifulco, C.; Lugli, A.; Zlobec, I.; Rau, T.T.; Berger, M.D., et al. International validation of the consensus Immunoscore for the classification of colon cancer: a prognostic and accuracy study. *Lancet* **2018**, *391*, 2128-2139, doi:10.1016/S0140-6736(18)30789-X.
8. Chang, C.C.; Campoli, M.; Ferrone, S. Classical and nonclassical HLA class I antigen and NK Cell-activating ligand changes in malignant cells: current challenges and future directions. *Adv Cancer Res* **2005**, *93*, 189-234, doi:10.1016/S0065-230X(05)93006-6.
9. Caligiuri, M.A. Human natural killer cells. *Blood* **2008**, *112*, 461-469, doi:10.1182/blood-2007-09-077438.
10. Vivier, E.; Tomasello, E.; Baratin, M.; Walzer, T.; Ugolini, S. Functions of natural killer cells. *Nat Immunol* **2008**, *9*, 503-510, doi:10.1038/ni1582.
11. Sandel, M.H.; Speetjens, F.M.; Menon, A.G.; Albertsson, P.A.; Basse, P.H.; Hokland, M.; Nagelkerke, J.F.; Tollenaar, R.A.; van de Velde, C.J.; Kuppen, P.J. Natural killer cells infiltrating colorectal cancer and MHC class I expression. *Mol Immunol* **2005**, *42*, 541-546, doi:10.1016/j.molimm.2004.07.039.
12. Tachibana, T.; Onodera, H.; Tsuruyama, T.; Mori, A.; Nagayama, S.; Hiai, H.; Imamura, M. Increased intratumor Valpha24-positive natural killer T cells: a prognostic factor for primary colorectal carcinomas. *Clin Cancer Res* **2005**, *11*, 7322-7327, doi:10.1158/1078-0432.CCR-05-0877.
13. Menon, A.G.; Janssen-van Rhijn, C.M.; Morreau, H.; Putter, H.; Tollenaar, R.A.; van de Velde, C.J.; Fleuren, G.J.; Kuppen, P.J. Immune system and prognosis in colorectal cancer: a detailed immunohistochemical analysis. *Lab Invest* **2004**, *84*, 493-501, doi:10.1038/labinvest.3700055.
14. Sconocchia, G.; Eppenberger, S.; Spagnoli, G.C.; Tornillo, L.; Droeser, R.; Caratelli, S.; Ferrelli, F.; Coppola, A.; Arriga, R.; Lauro, D., et al. NK cells and T cells cooperate during the clinical course of colorectal cancer. *Oncoimmunology* **2014**, *3*, e952197, doi:10.4161/21624011.2014.952197.
15. de Vries, N.L.; Swets, M.; Vahrmeijer, A.L.; Hokland, M.; Kuppen, P.J. The Immunogenicity of Colorectal Cancer in Relation to Tumor Development and Treatment. *Int J Mol Sci* **2016**, *17*, doi:10.3390/ijms17071030.
16. Krijgsman, D.; Hokland, M.; Kuppen, P.J.K. The Role of Natural Killer T Cells in Cancer-A Phenotypical and Functional Approach. *Front Immunol* **2018**, *9*, 367, doi:10.3389/fimmu.2018.00367.

17. Giaccone, G.; Punt, C.J.; Ando, Y.; Ruijter, R.; Nishi, N.; Peters, M.; von Blomberg, B.M.; Scheper, R.J.; van der Vliet, H.J.; van den Eertwegh, A.J., et al. A phase I study of the natural killer T-cell ligand alpha-galactosylceramide (KRN7000) in patients with solid tumors. *Clin Cancer Res* **2002**, *8*, 3702-3709.
18. Motohashi, S.; Nagato, K.; Kunii, N.; Yamamoto, H.; Yamasaki, K.; Okita, K.; Hanaoka, H.; Shimizu, N.; Suzuki, M.; Yoshino, I., et al. A phase I-II study of alpha-galactosylceramide-pulsed IL-2/GM-CSF-cultured peripheral blood mononuclear cells in patients with advanced and recurrent non-small cell lung cancer. *J Immunol* **2009**, *182*, 2492-2501, doi:10.4049/jimmunol.0800126.
19. Exley, M.A.; Friedlander, P.; Alatrakchi, N.; Vriend, L.; Yue, S.; Sasada, T.; Zeng, W.; Mizukami, Y.; Clark, J.; Nemer, D., et al. Adoptive Transfer of Invariant NKT Cells as Immunotherapy for Advanced Melanoma: A Phase I Clinical Trial. *Clin Cancer Res* **2017**, *23*, 3510-3519, doi:10.1158/1078-0432.CCR-16-0600.
20. Biassoni, R.; Cantoni, C.; Pende, D.; Sivori, S.; Parolini, S.; Vitale, M.; Bottino, C.; Moretta, A. Human natural killer cell receptors and co-receptors. *Immunol Rev* **2001**, *181*, 203-214, doi:10.1034/j.1600-065x.2001.1810117.x.
21. Renukaradhya, G.J.; Sriram, V.; Du, W.; Gervay-Hague, J.; Van Kaer, L.; Brutkiewicz, R.R. Inhibition of antitumor immunity by invariant natural killer T cells in a T-cell lymphoma model in vivo. *Int J Cancer* **2006**, *118*, 3045-3053, doi:10.1002/ijc.21764.
22. Chong, T.W.; Goh, F.Y.; Sim, M.Y.; Huang, H.H.; Thike, A.A.; Lim, W.K.; Teh, B.T.; Tan, P.H. CD1d expression in renal cell carcinoma is associated with higher relapse rates, poorer cancer-specific and overall survival. *J Clin Pathol* **2015**, *68*, 200-205, doi:10.1136/jclinpath-2014-202735.
23. Otterbein, L.E.; Soares, M.P.; Yamashita, K.; Bach, F.H. Heme oxygenase-1: unleashing the protective properties of heme. *Trends Immunol* **2003**, *24*, 449-455, doi:10.1016/s1471-4906(03)00181-9.
24. Etzerodt, A.; Maniecki, M.B.; Moller, K.; Moller, H.J.; Moestrup, S.K. Tumor necrosis factor alpha-converting enzyme (TACE/ADAM17) mediates ectodomain shedding of the scavenger receptor CD163. *J Leukoc Biol* **2010**, *88*, 1201-1205, doi:10.1189/jlb.0410235.
25. No, J.H.; Moon, J.M.; Kim, K.; Kim, Y.B. Prognostic significance of serum soluble CD163 level in patients with epithelial ovarian cancer. *Gynecol Obstet Invest* **2013**, *75*, 263-267, doi:10.1159/000349892.
26. Kazankov, K.; Rode, A.; Simonsen, K.; Villadsen, G.E.; Nicoll, A.; Moller, H.J.; Lim, L.; Angus, P.; Kronborg, I.; Arachchi, N., et al. Macrophage activation marker soluble CD163 may predict disease progression in hepatocellular carcinoma. *Scand J Clin Lab Invest* **2016**, *76*, 64-73, doi:10.3109/00365513.2015.1099722.
27. Ding, D.; Yao, Y.; Yang, C.; Zhang, S. Identification of mannose receptor and CD163 as novel biomarkers for colorectal cancer. *Cancer Biomark* **2018**, *21*, 689-700, doi:10.3233/CBM-170796.
28. Ijsselsteijn, M.E.; van der Breggen, R.; Farina Sarasqueta, A.; Koning, F.; de Miranda, N. A 40-Marker Panel for High Dimensional Characterization of Cancer Immune Microenvironments by Imaging Mass Cytometry. *Front Immunol* **2019**, *10*, 2534, doi:10.3389/fimmu.2019.02534.
29. Tan, W.C.C.; Nerurkar, S.N.; Cai, H.Y.; Ng, H.H.M.; Wu, D.; Wee, Y.T.F.; Lim, J.C.T.; Yeong, J.; Lim, T.K.H. Overview of multiplex immunohistochemistry/immunofluorescence techniques in the era of cancer immunotherapy. *Cancer Commun (Lond)* **2020**, *40*, 135-153, doi:10.1002/cac2.12023.



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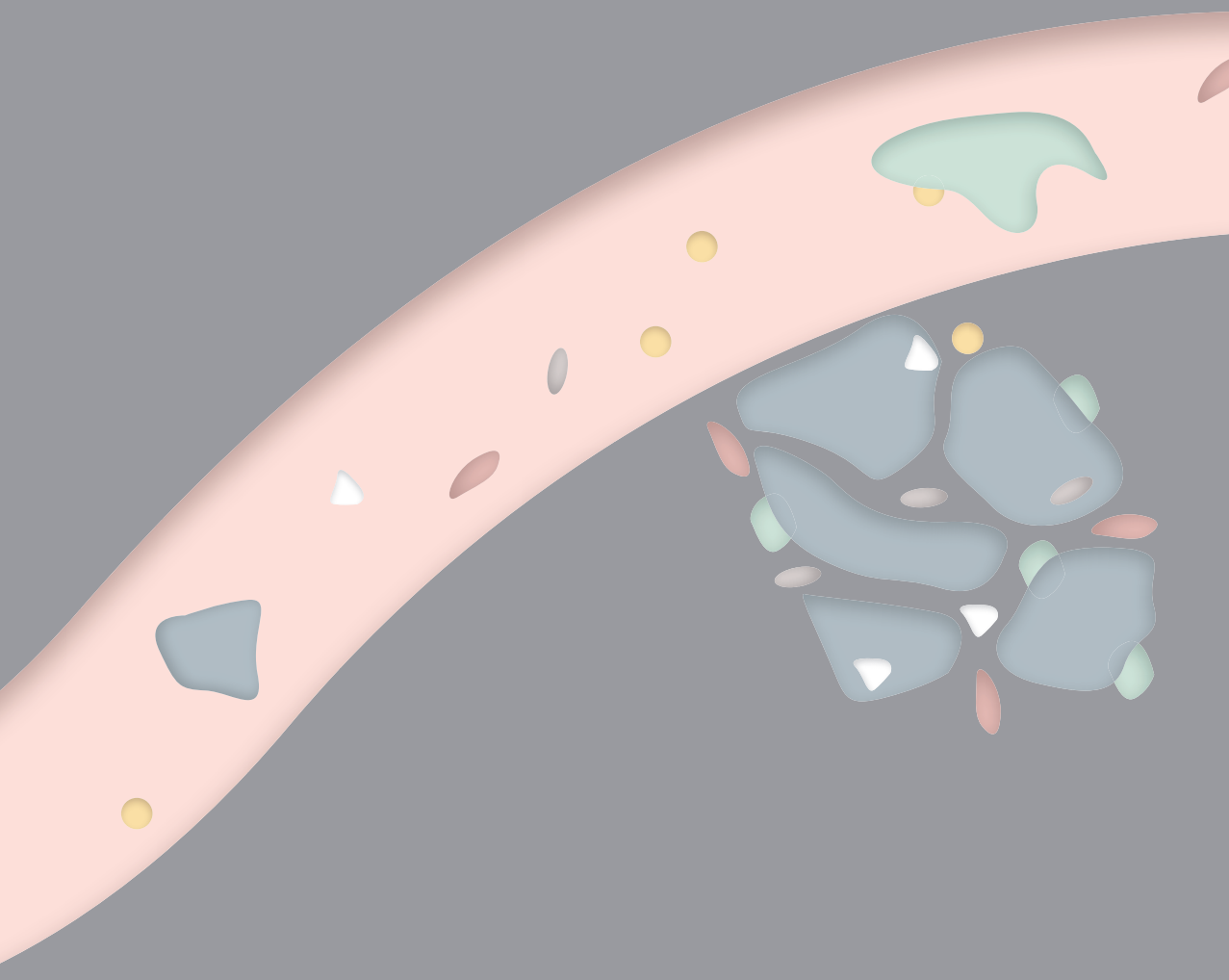


List of publications

J. Attia, C. Dessens, R. van de Water, R. Houvast, P.J.K. Kuppen, **D. Krijgsman**, The molecular and functional characteristics of HLA-G and the interaction with its receptors: where to intervene for cancer immunotherapy? *Int J Mol Sci.* 2020

D. Krijgsman, J. Roelands, M.N. Andersen, C.H.L.A. Wieringa, R.A.E.M. Tollenaar, W. Hendrickx, D. Bedognetti, M. Hokland, P.J.K. Kuppen, Expression of NK cell receptor ligands in primary colorectal cancer tissue in relation to the phenotype of circulating NK- and NKT cells, and clinical outcome, *Mol Immunol.* 2020

D. Krijgsman, N.L. De Vries, M.N. Andersen, A. Skovbo, R.A.E.M. Tollenaar, H.J. Møller, M. Hokland, P.J.K. Kuppen, CD163 as a biomarker in colorectal cancer: The expression on circulating monocytes and tumor-associated macrophages, and the soluble form in the blood, *Int J Mol Sci.* 2020

D. Krijgsman, J. Roelands, W. Hendrickx, D. Bedognetti, P.J.K. Kuppen, HLA-G: A New Immune Checkpoint in Cancer? *Int J Mol* Sci. 2020

D. Krijgsman, M.B. van Leeuwen, J. van der Ven, V. Almeida, R. Vlutters, D. Halter, P.J.K. Kuppen, C.J.H. van de Velde, R. Wimberger-Friedl, Quantitative whole slide assessment of tumor infiltrating CD8-positive lymphocytes in ER-positive breast cancer in relation to clinical outcome*, J Biom Health Inf.* 2020

D. Krijgsman, N.L. de Vries, M.N. Andersen, A. Skovbo, R.A.E.M. Tollenaar, E. Bastiaannet, P.J.K. Kuppen, M. Hokland, The effects of tumor resection and adjuvant therapy on the peripheral blood immune cell profile in patients with colon carcinoma, *Cancer Immunol Immunother.* 2020

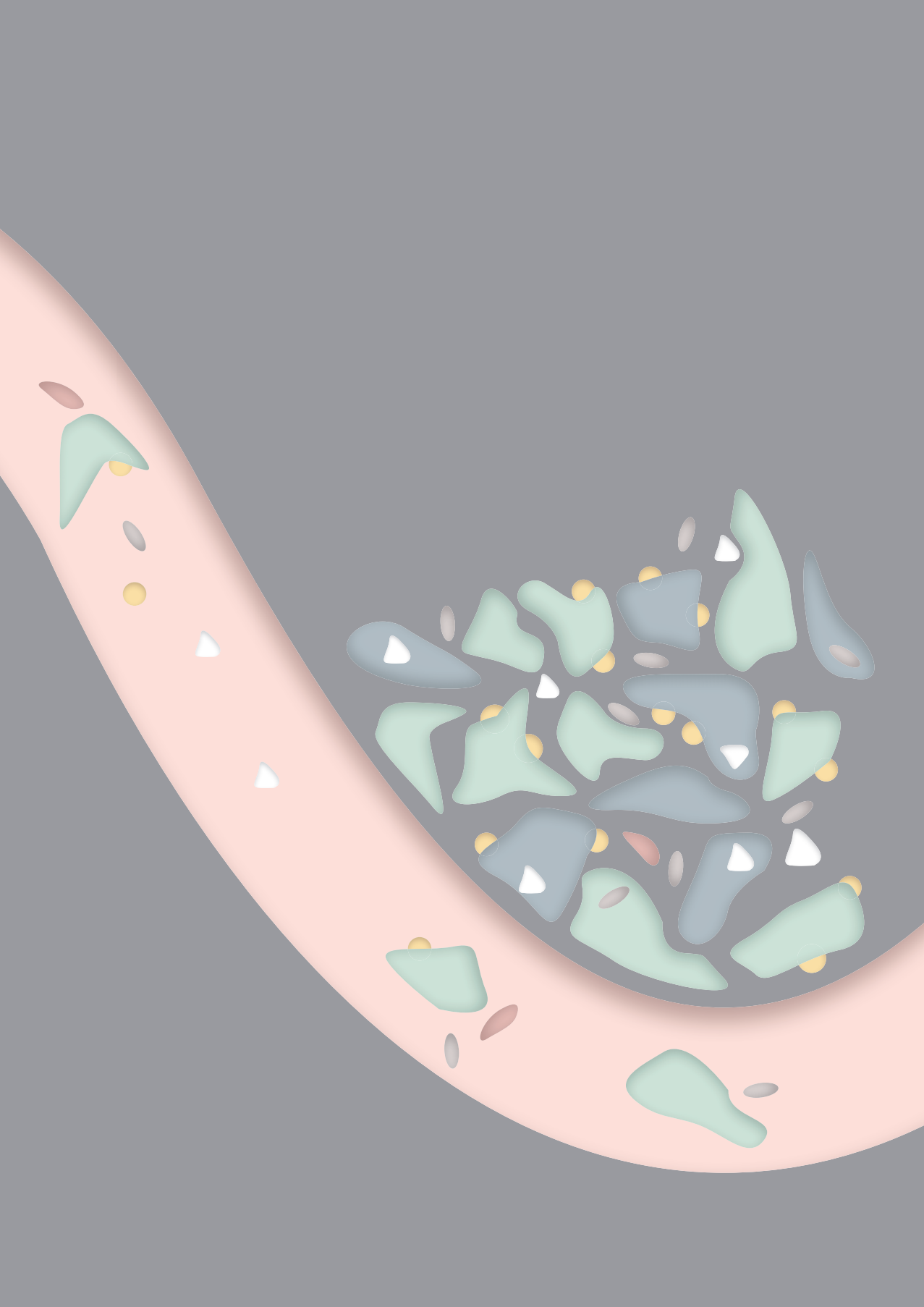
D. Krijgsman, R.L.P. van Vlierberghe, V. Evangelou, A.L. Vahrmeijer, C.J.H. van de Velde, C.F.M. Sier, P.J.K. Kuppen, A method for semi-automated image analysis of HLA class I tumour epithelium expression in rectal cancer, *Eur J Histochem.* 2019

D. Krijgsman, N.L. de Vries, A. Skovbo, M.N. Andersen, M. Swets, E. Bastiaannet, A.L. Vahrmeijer, C.J.H. van de Velde, M.H.M. Heemskerk, M. Hokland, P.J.K. Kuppen, Characterization of circulating T-, NK-, and NKT cell subsets in patients with colorectal cancer: the peripheral blood immune cell profile, *Cancer Immunol Immunother*. 2019

M. Swets, A. Wouters, **D. Krijgsman**, R.L.P. van Vlierberghe, A. Boot, J.D. van Eendenburg, T. van Wezel, H. Gelderblom, C.J.H. van de Velde, P.J. van den Elsen, P.J.K. Kuppen, HLA-G protein expression in colorectal cancer evaluated by immunohistochemistry and western blot analysis: Its expression characteristics remain enigmatic, *Clin Immunol.* 2018

D. Krijgsman, M. Hokland, P.J.K. Kuppen, The role of natural killer T cells in cancer – A phenotypical and functional approach, *Front Immunol*. 2018

G. Gezgin, S.J. Luk, J. Cao, M. Dogrusöz, D.M. van der Steen, R.S. Hagedoorn, **D. Krijgsman**, P.A. van der Velden, G.P.M. Luyten, K. Szuhai, J.W. Harbour, E.S. Jordanova, M.J. Jager, M.H.M. Heemskerk, PRAME: a potential target for immunotherapy in uveal melanoma*, JAMA ophtalmol.* 2017



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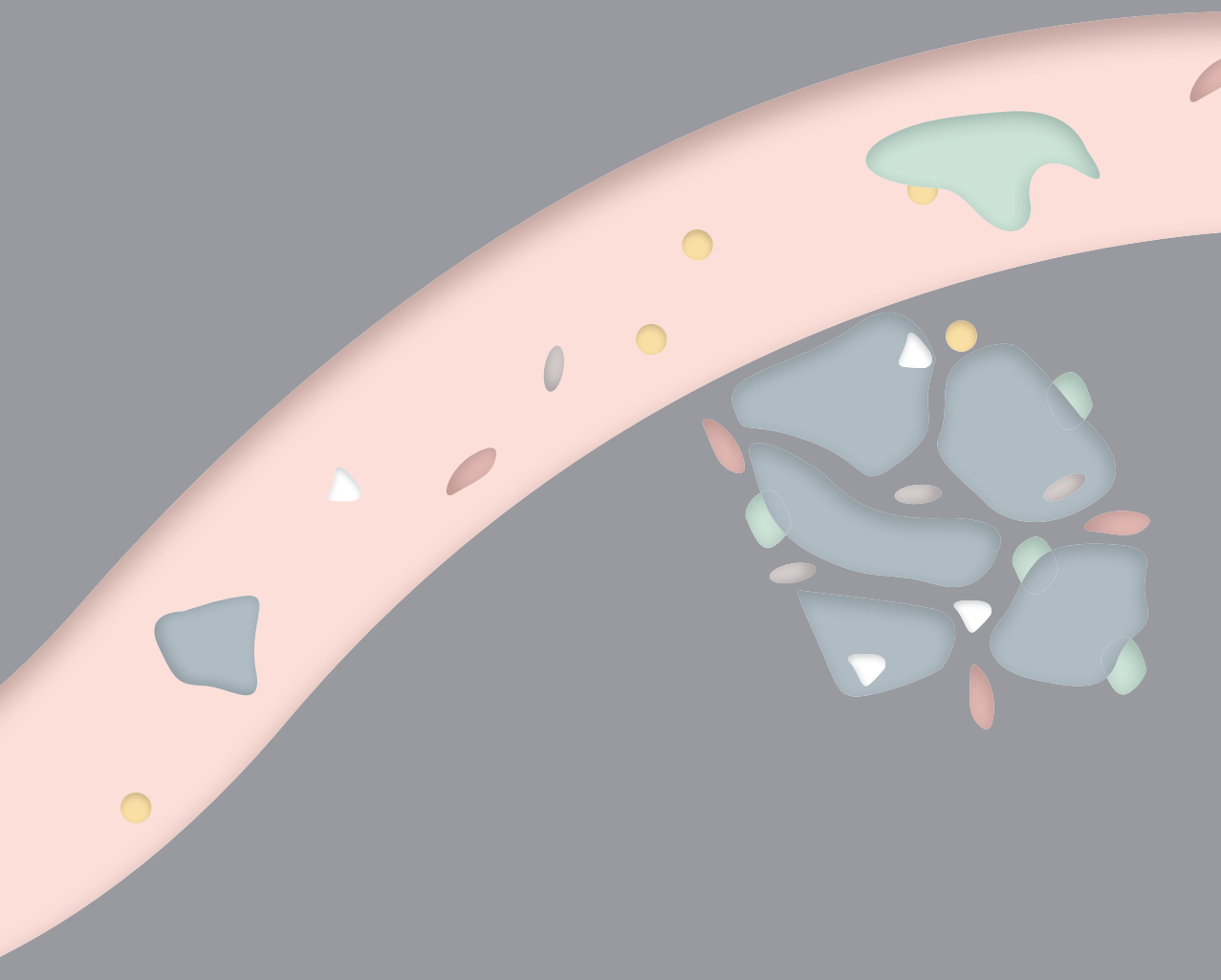
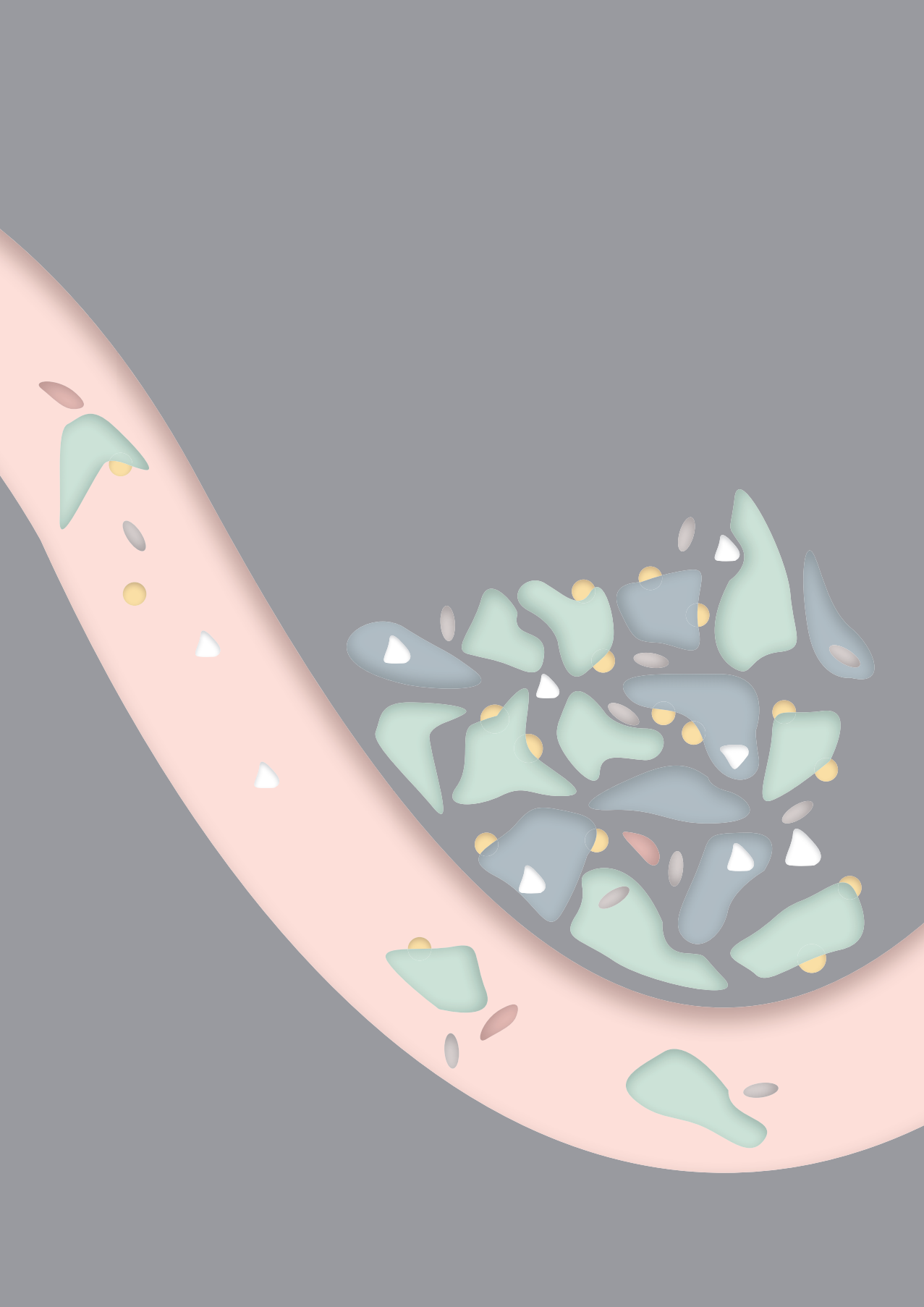


Curriculum Vitae

Daniëlle Krijgsman was born on April 21st 1993 in Spijkenisse, where she grew up with her parents and younger sister Mariska. After graduating from the Blaise Pascal in Spijkenisse in 2011, she started her study Biomedical Sciences at the Leiden University.

After obtaining her Bachelor's degree in Biomedical Sciences in 2014, she started the Research Master program that same year. During the Master program, she focused on tumor immunology subjects, resulting in obtainment of the "Cancer pathogenesis and therapy" certificate. Her first experience with tumor immunology research was during her first Biomedical Sciences Master's internship at the department of Ophthalmology at the Leiden University Medical Center (LUMC). This internship was performed under supervision of prof. dr. Martine J. Jager, and resulted in her first scientific publication on the use of PRAME as target for immunotherapy in uveal melanoma patients.

In 2016, she visited the lab of prof. dr. Marianne Hokland at the University of Aarhus in Denmark. There, she performed multiplex flow cytometry experiments to characterize the phenotype of circulating immune cell subsets in colorectal cancer patients. This project was carried out in collaboration with the department of Surgery at the LUMC, and was the starting point for her PhD and the current thesis. The subject of her PhD thesis was on tumor-immune interactions in colorectal cancer patients, supervised by dr. Peter J.K. Kuppen, dr. Alexander L. Vahrmeijer, and prof. dr. Rob A.E.M. Tollenaar. In October 2020, she started working as Postdoc in the lab of dr. Yvonne Vercoulen at the University Medical Center Utrecht, working on myeloid derived suppressor cells and IgA immunotherapy in cancer.



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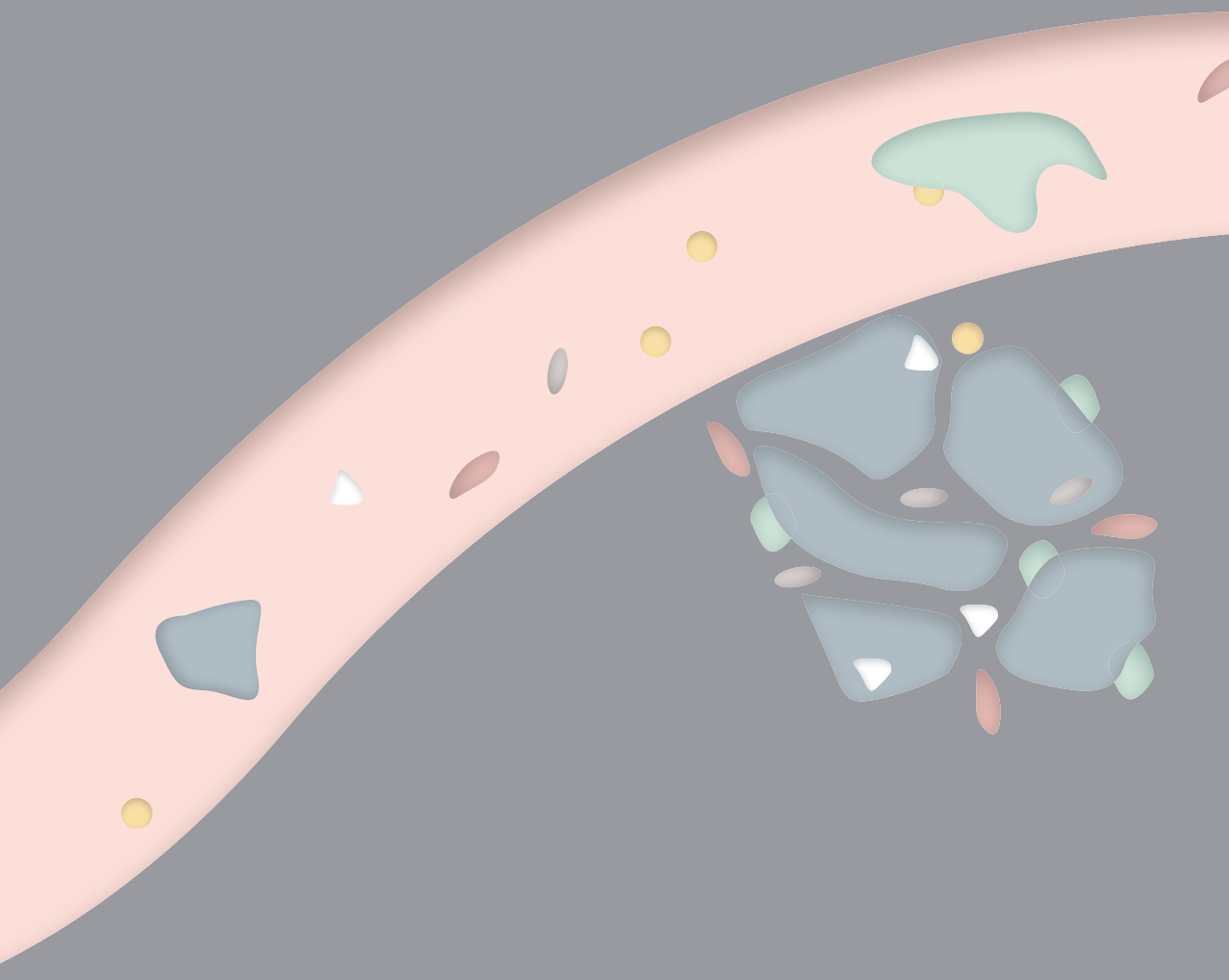

Dankwoord

Onderzoek doen en een proefschrift schrijven doe je niet alleen. Daarom wil ik graag iedereen bedanken voor zijn/haar bijdrage aan het tot stand komen van dit mooie boekje, waarvan ik er een aantal in het bijzonder wil noemen.

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Dear colleagues from Denmark, in particular prof. dr. Marianne Hokland. Dear Marianne, thank you so much for giving me the opportunity to be part of your department. Your view on research and life in general is remarkable and I hope we will continue working together in the future. Furthermore, I would like to thank Anni, Morten, Solveig, Line, Anne-Mette, and Kasper for many fruitful discussions, and, importantly, cake-sharing and movie nights. Thanks to you all, the time I spend in Denmark was one of the best things that ever happened to me.

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Daarnaast wil ik mijn collega's van het Heelkunde laboratorium bedanken voor de gezellige periode en de ondersteuning bij vele experimenten. Kees, jou ben ik bijzondere dank verschuldigd voor je betrokkenheid bij het schrijven van de technical note en je input voor het HLA-G-project. Je altijd kritische blik op onderzoek heeft ervoor gezorgd dat ik een betere onderzoeker ben geworden. Marloes, Tessa, en Jessica, wat heb ik door jullie een leuke tijd gehad als onderzoeker bij de Heelkunde. Ook al verliepen onze projecten niet altijd even soepel, we wisten elkaar altijd weer te motiveren om verder te gaan. Mede door onze vele wetenschappelijke koffie uurtjes kwam ik iedere dag met veel plezier naar het LUMC.

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Tot slot: promoveren is een unieke ervaring. Hoewel het een mooie en leuke tijd is geweest, was het ook hard werken. Soms is het dan makkelijk om te vergeten dat er buiten je promotie nog zoveel moois te zien en te doen is. Pap, zoals je in je eigen proefschrift schreef: “it is necessary to give priority to real life, as life is lived only once”. Hoewel je mijn proefschrift niet mee hebt mogen maken weet ik dat je trots op me bent.