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Molecular Immunology 128 (2020) 205–218

Contents lists available at ScienceDirect

Molecular Immunology

journal homepage: www.elsevier.com/locate/molimm

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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ARTICLE INFO

Keywords:
Colorectal cancer
NK cells
NKT cells
NK cell receptors
NK cell receptor ligands

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.

Abbreviations: ADCC, Antibody-dependent Cell-mediated Cytotoxicity; BAG6, BCL2-associated Athanogene 6; CC, Colon Cancer; CFP, Complement Factor P; CI, Confidence Interval; CLEC2A, C-type Lectin Domain Family 2 Member A; CLEC2D, C-type Lectin Domain Family 2 Member D; COAD, Colon Adenocarcinoma; CRC, Colorectal Cancer; DFS, Disease-free Survival; DNAM-1, DNAX Accessory Molecule-1; ECM, Extracellular Matrix; EMT, Epithelial-mesenchymal Transition; FFPE, Formalin-fixed Paraffin-embedded; H&E, Hematoxylin and Eosin; HLA, Human Leukocyte Antigen; HR, Hazard Ratio; IFN, Interferon; IHC, Immunohistochemistry; ILT2, Ig-like Transcript 2; KIR, Killer Cell Immunoglobulin-like Receptor; LGALS3, Galectin-3; LUMC, Leiden University Medical Center; MFI, Median Fluorescence Intensity; MIC, MHC Class I-related Chain; MSI, Multispectral Imaging; NCR, Natural Cytotoxicity Receptor; NID1, Nidogen-1; NK, Natural Killer; NKG2A, Natural Killer Group 2-A; NKG2C, Natural Killer Group 2-C; NKG2D, Natural Killer Group 2-D; NKT, Natural Killer T; OS, Overall Survival; PBMC, Peripheral Blood Mononuclear Cell; PCNA, Proliferating Cell Nuclear Antigen; PDGFD, Platelet-derived Growth Factor D; PVR, Poliovirus Receptor; SLAM, Signaling Lymphocytic Activation Molecule; TCGA, The Cancer Genome Atlas (TCGA); TCR, T Cell Receptor; TME, Tumor Microenvironment; TNM, Tumor Node Metastasis; ULBP, UL-16 Binding Protein; VIM, Vimentin.

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https://doi.org/10.1016/j.molimm.2020.10.012
Received 14 August 2020; Received in revised form 1 October 2020; Accepted 14 October 2020
Available online 1 November 2020

1. 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 (Campbell and Hasegawa, 2013; Pazina et al., 2017). Different inhibitory and activating receptors play a role in this process to dynamically regulate the activation state of NK cells (Biassoni et al., 2001), and probably NKT cells as well (Krijgsman et al., 2018). 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) CD158 h/j/l/g/e (Ivarsson et al., 2014). CD16 (FcγRIII) on NK cells mediates antibody-dependent cell--mediated cytotoxicity (ADCC) (Dutertre et al., 2008). Additionally, NKT cells express an invariant Vα24 T cell receptor (TCR) which functions as an activating receptor on these cells (Krijgsman et al., 2018). 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 (Ivarsson et al., 2014; Shiroishi et al., 2003). 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 (Ivarsson et al., 2014; Claus et al., 2019). Important roles have been implicated for NK- and NKT cells in tumor development and progression in different cancer types, including colorectal cancer (CRC) (Krijgsman et al., 2018; Coppola et al., 2015; McEwen-Smith et al., 2015).

We (Krijgsman et al., 2019a) and others (Peng et al., 2013; Rocca et al., 2013) 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 (Cooper et al., 2001; Jonges et al., 2001). Our study showed that circulating $CD56^{dim}$ NK cells were phenotypically altered in CRC patients, whereas $CD56^{bright}$ NK cells were not (Krijgsman et al., 2019a). 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 (Rocca et al., 2013). 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 (Krijgsman et al., 2020). This implies 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 (Renukaradhya et al., 2006; Chong et al., 2015).

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 (Krijgsman et al., 2019a; Peng et al., 2013; Rocca et al., 2013). We focused on the ligands galectin-3 and Proliferating Cell Nuclear Antigen (PCNA) as these ligands showed clinical relevance in cancer based on literature (Wang et al., 2014; Pogge von Strandmann et al., 2015; Rosental et al., 2012). Galectin-3 may be expressed by tumor cells on their cell surface which promotes epithelial-mesenchymal transition (EMT) of tumor cells (Liu and Rabinovich, 2005). Furthermore, tumor cells can upregulate expression of PCNA which facilitates and controls DNA replication via DNA polymerases (Stoimenov and Helleday, 2009; Rosental et al., 2011; Qasim et al., 2012). Upon binding, galectin-3 and PCNA inhibit the function of the receptors NKp30 (Wang et al., 2014) and NKp44 (Rosental et al., 2011), respectively. Upregulation of galectin-3 and PCNA have been shown to result in decreased NK cell-mediated lysis of tumor cells (Wang et al., 2014; Pogge von Strandmann et al., 2015; Rosental et al., 2012). 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 (Krijgsman et al., 2018; Long et al., 2013), and therefore have clinical importance in mediating immunosurveillance (Concha-Benavente et al., 2016; King et al., 2018). The inhibitory KIRs CD158a and CD158b that were investigated in this study both recognize HLA-C molecules and therefore compete for binding (Ivarsson et al., 2014). The TCR on NKT cells recognizes different glycolipids in the context of the HLA-like molecule CD1d (Bendelac et al., 2007). Co-stimulation of the CD161 receptor is vital in TCR/CD1d interactions, since its presence is necessary for TCR activation (Exley et al., 1998). Several studies have now provided evidence that NK cell receptor-ligand interactions are involved in tumor surveillance (Barrow et al., 2019) and have therefore suggested them as new targets for immune checkpoint inhibitors (Yang et al., 2020). 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.

2. Materials and methods

2.1. 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 (Krijgsman et al., 2019a; Krijgsman et al., 2020). Hence, pre- and postoperative PBMC samples were collected and analyzed for 24 patients (Krijgsman et al., 2020). 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.

2.2. Public data

RNASeq data from The Cancer Genome Atlas (TCGA) colon adenocarcinoma (COAD) cohort was downloaded using TCGABiolinks (Colaprico et al., 2016). 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.

2.3. 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.

2.4. 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 (Krijgsman et al., 2019b). 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 immunohistochemistry (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).

2.5. 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 increased 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)].

2.6. Flow cytometry

PBMC samples were isolated and immunophenotyped using multiparameter flow cytometry as previously published (Krijgsman et al., 2019a; Krijgsman et al., 2020). 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 prior to surgery (Krijgsman et al., 2019a), and after surgery (Krijgsman et al., 2020). 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.

2.7. 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)*.

2.8. 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 cell 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 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 cell 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.

3. Results

3.1. 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 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 (Krijgsman et al., 2019a). 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

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 (Krijgsman et al., 2019a). 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	64 (82.1%)						
Colon	14 (17.9%)	61 (82.4%)	ns	24 (100%)	**0.025**	59 (83.1%)	ns
Rectum		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							
No	45 (57.7%)	43 (58.1%)	ns	13 (54.2%)	ns	40 (42.3%)	ns
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	68 (87.2%)						
No	10 (12.8%)	65 (87.8%)	ns	21 (87.5%)	ns	61 (85.9%)	ns
Yes		9 (12.2%)		3 (12.5%)		10 (14.1%)	
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%)	

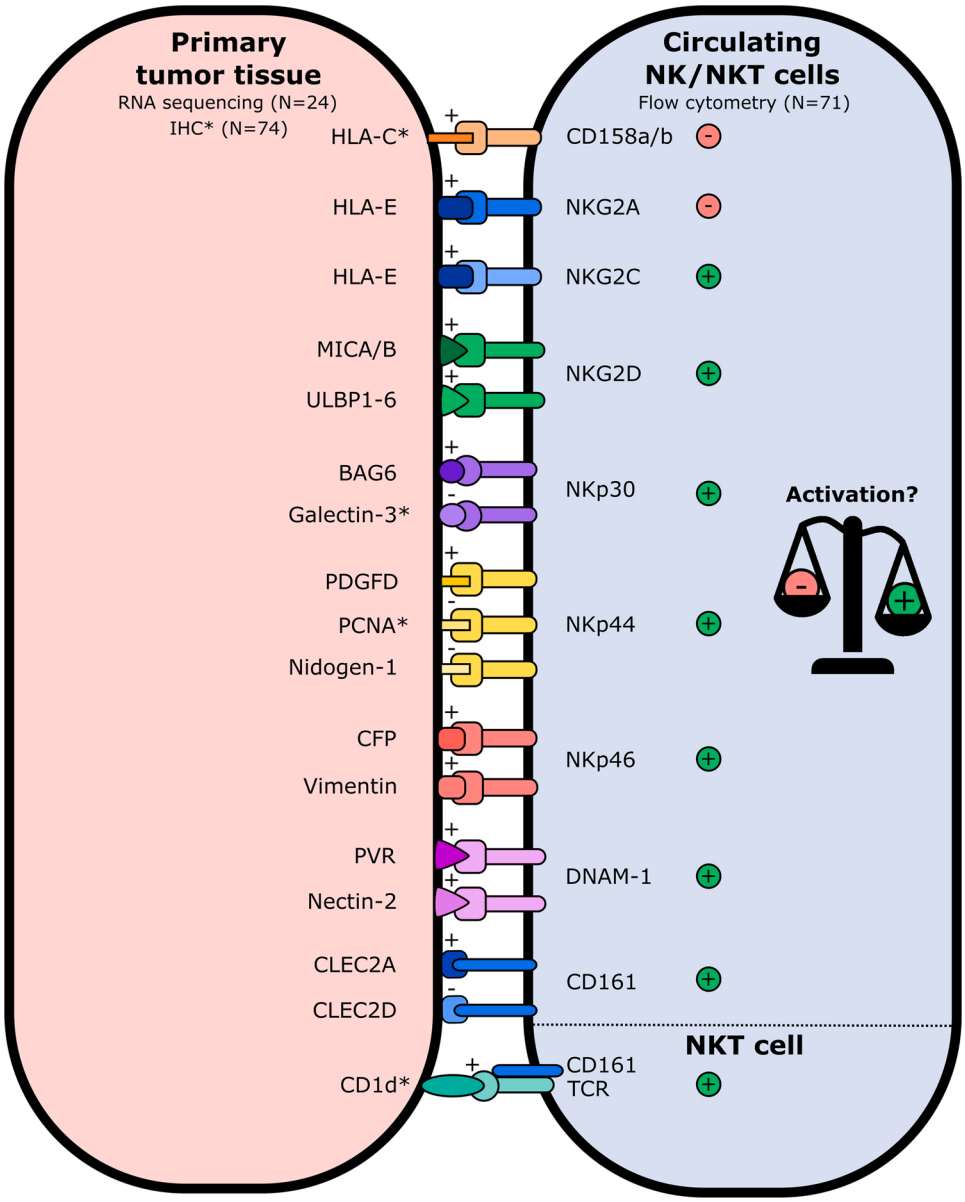

Fig. 1. Overview of the studied NK cell receptor ligands in primary tumor tissue, and NK cell receptors on circulating NK- and NKT cells in CRC patients.
Overview of the studied NK cell receptor ligands in primary tumor tissue, and the receptors they bind to on circulating NK- and NKT cells in CRC patients. 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 (Krijgsman et al., 2019a). 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).

sample availability. Fig. 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.

3.2. 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 (Fig. 2). These stainings were then used for automated image analysis to score the expression of 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. Fig. 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 Fig. 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.

3.3. 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

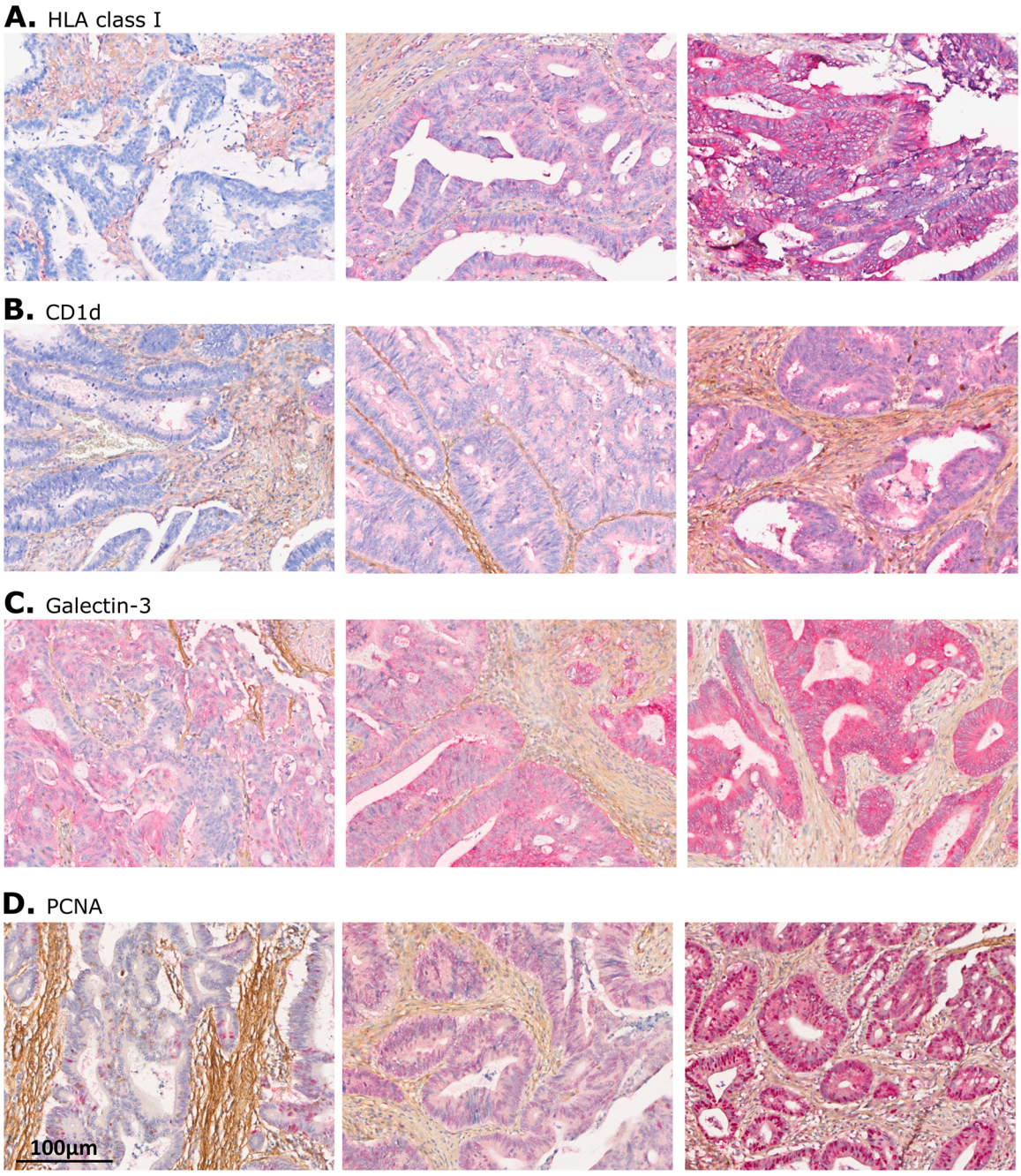

Fig. 2. IHC of NK cell ligands in primary colorectal tumors.
An IHC double staining was set up to analyze NK cell ligand expression in primary colorectal tumors. Stromal tissue, blood vessels, and immune cells were stained brown whereas NK cell ligand expression was stained pink. Cell nuclei were stained 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).

Supplementary Fig. 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 Fig. 1B. Fig. 4A 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 (Fig. 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

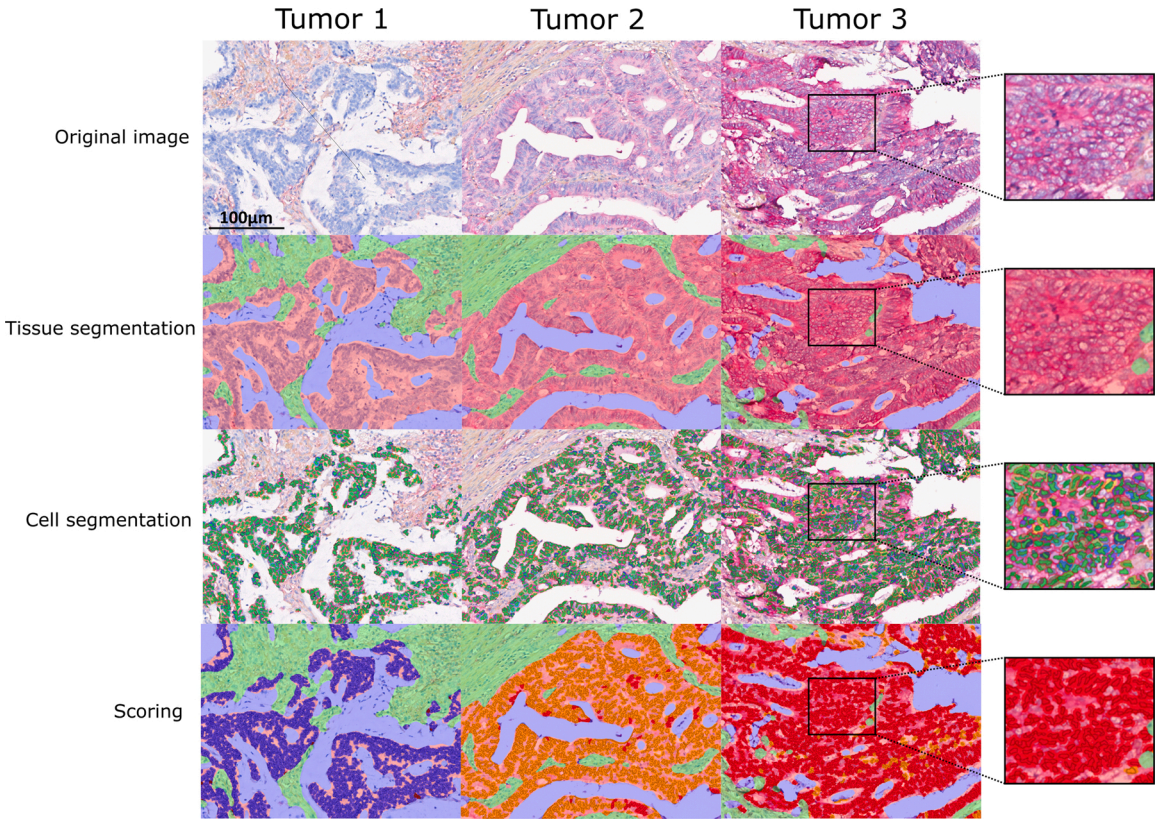

Fig. 3. IHC double staining with subsequent automated image analysis of HLA class I expression in three primary colorectal tumors.
An IHC double staining was set up to analyze HLA class I expression in primary colorectal tumors. Stromal tissue, blood vessels, and immune cells were stained brown whereas 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).

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 Fig. 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

into adjacent tissue.

3.4. 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, $P = 0.031$) (Fig. 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 (N = 40, $P = 0.512$) as visualized in Fig. 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, $P = 0.060$) (Fig. 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

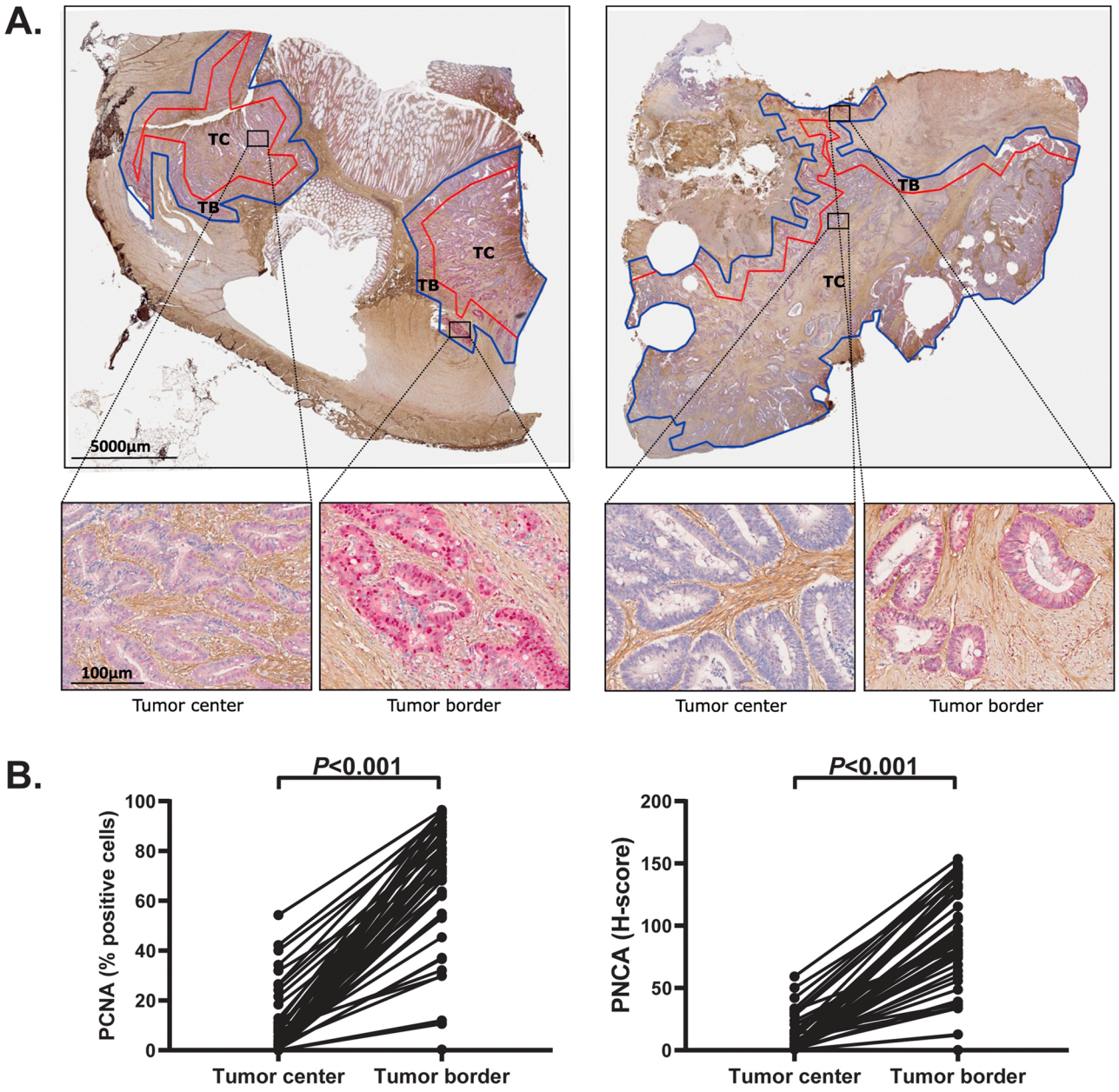

Fig. 4. PCNA expression in the tumor center versus tumor border in primary colorectal tumors.
An IHC double staining was set up to analyze PCNA expression in primary colorectal tumors. Stromal tissue, blood vessels, and immune cells were stained brown whereas PCNA expression was stained pink. Cell nuclei 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).

independently associated with DFS when corrected for age and TNM classification.

3.5. 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 (Fig. 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 (Fig. 6B). Furthermore, the percentage of HLA class I^{+} cells (P = 0.023) but not HLA class I H-score (P = 0.128) negatively correlated

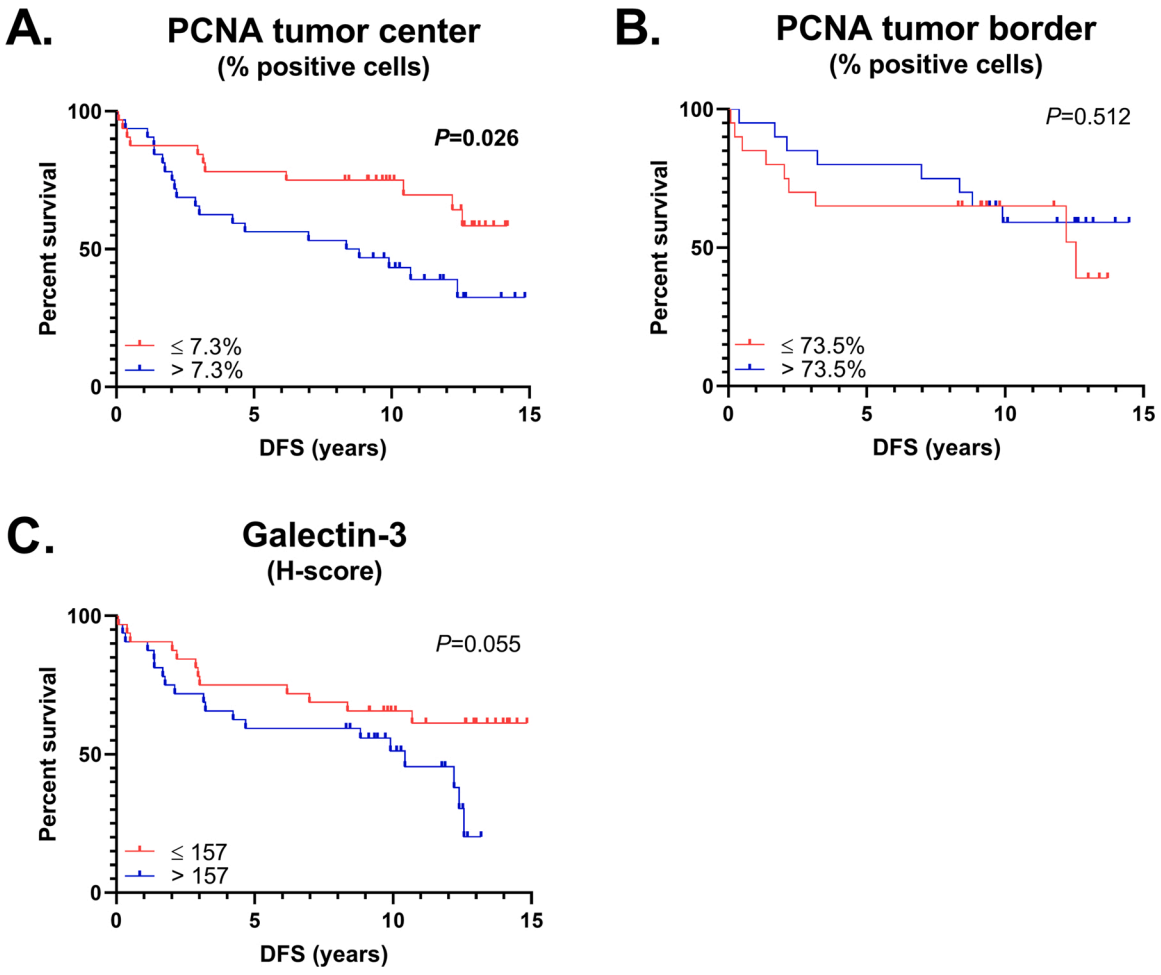

Fig. 5. Relationship between PCNA and galectin-3 expression in primary tumors with DFS of CRC patients.
DFS curves are shown for TNM stage 0-III CRC patients at risk for development of metastases (N = 64). Stratifications were based on the median percentage of $PCNA^+$ cells and galectin-3 H-score, respectively. **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).

with the percentage of $CD158b^+$ $CD56^{bright}$ NK cells in the circulation of CRC patients (Fig. 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 Fig. 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.

3.6. 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 Fig. 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 Fig. 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

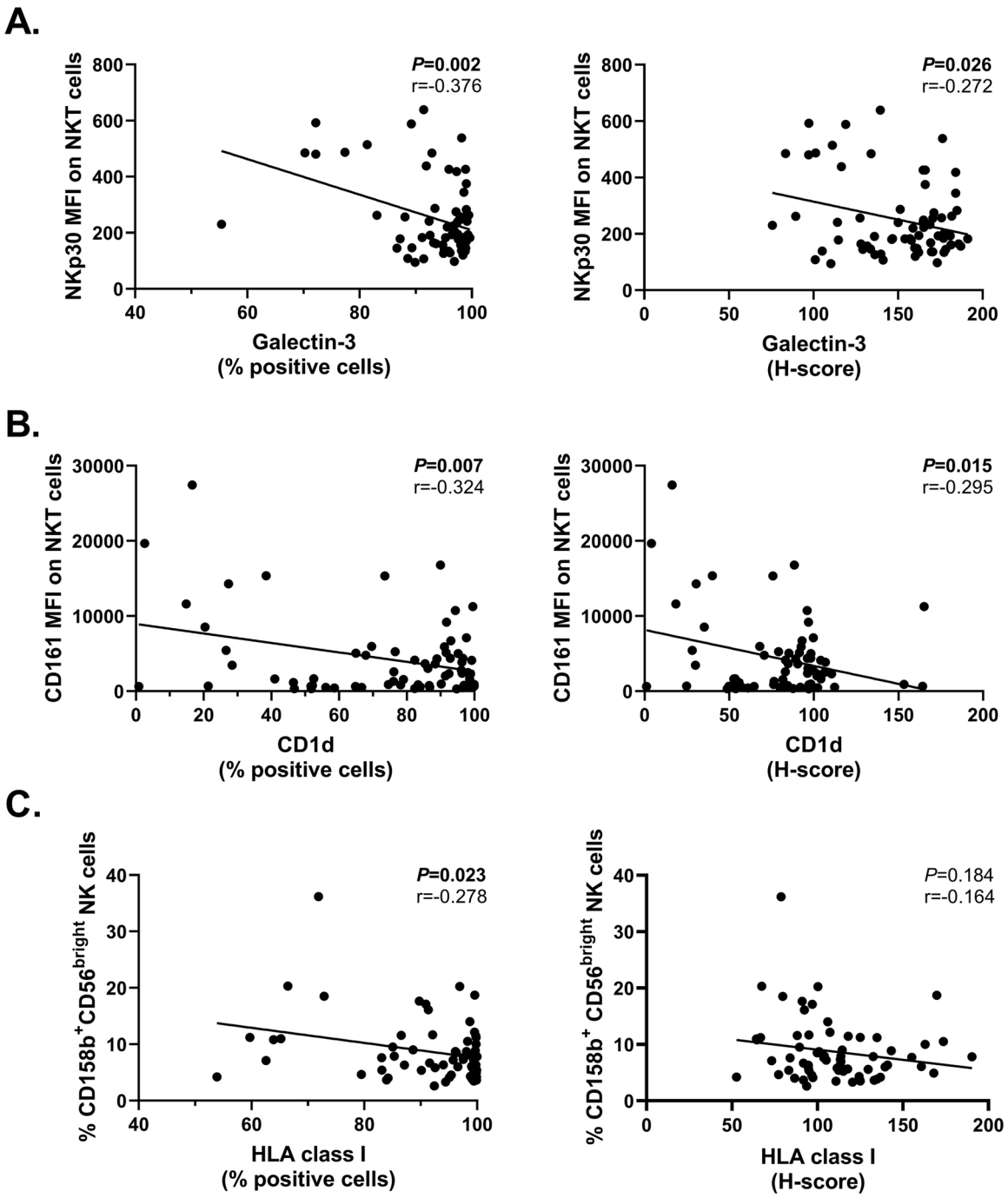

Fig. 6. Correlation between NK cell receptor ligand expression in tumor tissue (protein) and their associated receptors on circulating NK and NKT cells (protein) in CRC patients.

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 ($\leq$0.05) are indicated in bold. Abbreviations: CRC (Colorectal Cancer), HLA (Human Leukocyte Antigen), IHC (Immunohistochemistry), NK (Natural Killer), NKT (Natural Killer T).

Supplementary Fig. 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 (Fig. 7A and 7B). No correlations were observed between expression of NK cell ligands and the phenotype of circulating NK cells (Supplementary Fig. 4). In summary, 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.

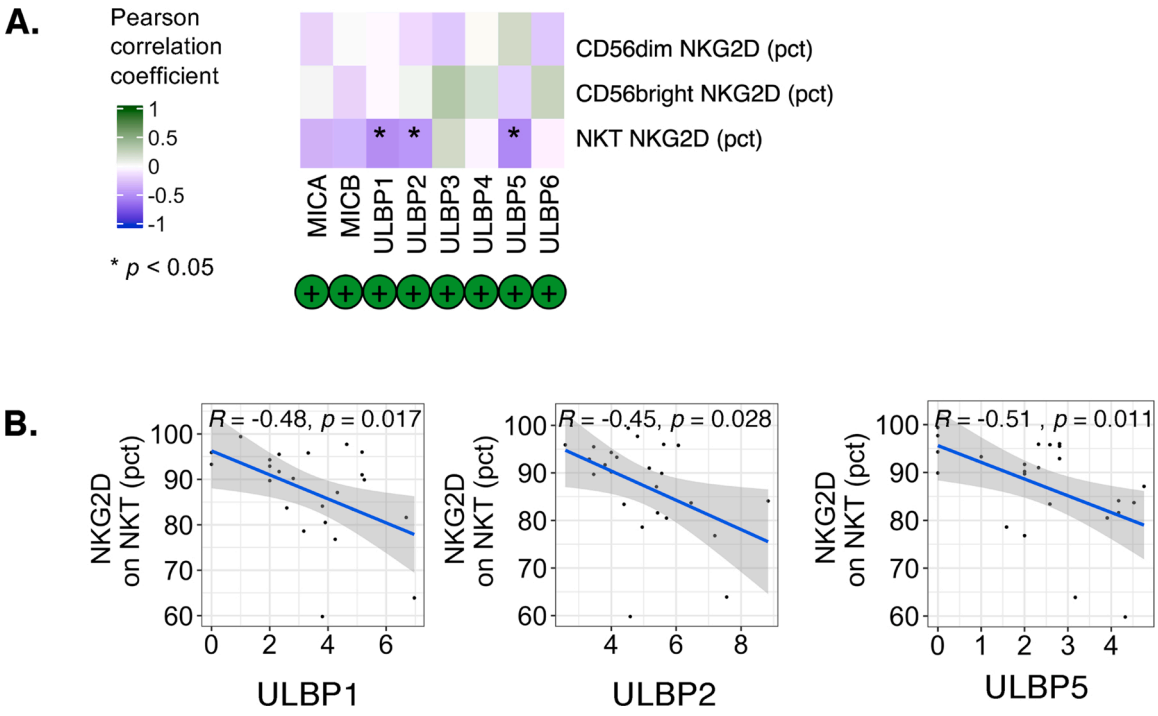

Fig. 7. Correlation between expression of NK cell receptor ligands in tumor tissue (RNA) and their associated receptors on circulating NK- and NKT cells (protein) in CC patients.

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 cell 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).

4. 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 (Huh et al., 2009; Li et al., 2016; Jin et al., 2019), but other studies suggested that it had no significance, and even reported an association with favorable outcome (Neoptolemos et al., 1995; Paradiso et al., 1996). 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 (Zhou et al., 2018). 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 taken into account 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 (Rosental et al., 2012; Rosental et al., 2011). 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 (Huh et al., 2009; Jin et al., 2019). 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 (Endo et al., 2005; Dong et al., 2018; Tao et al., 2017; Nakamura et al., 1999). 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 (Watson et al., 2006; McGilvray et al., 2009; Liu et al., 2019). 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 (Liu et al., 2019; Coudert et al., 2005; Fernandez-Messina et al., 2010; Doubrovina et al., 2003), 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) (Schmiedel and Mandelboim, 2018). 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 (Schmiedel and Mandelboim, 2018). 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 (Wang et al., 2014). 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 (Tsuboi et al., 2011). Besides its suppressive function on NK- and NKT cells, membrane-bound galectin-3 also promotes EMT of tumor cells, thereby increasing their invasiveness (Liu and Rabinovich, 2005). 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 (Farhad et al., 2018). 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 (Delahaye et al., 2011). Whereas the NKp30A and NKp30B splice variants are the activating isoforms, NKp30C has been associated with immunosuppressive effects on NK cells (Pogge von Strandmann et al., 2015; Delahaye et al., 2011). 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 (Pogge von Strandmann et al., 2015). 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 (Exley et al., 1998). 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 (Ewen et al., 2018). 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 (de Vries et al., 2016). 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 (Krijgsman et al., 2018), 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.

Declaration of Competing Interest

The authors declare no conflict of interest

Funding

DK received support from the European Commission (Erasmus Plus Programme, ET 2020), and Leiden University Fund (LUF International Study Fund (LISF)). JR received support from Qatar National Research Fund (grant number: JSREP07-010-3-005).

CRediT authorship contribution statement

Daniëlle Krijgsman: Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Data curation, Writing - original draft, Visualization, Project administration, Funding acquisition. **Jessica Roelands:** Methodology, Software, Formal analysis, Investigation, Data curation, Writing - original draft, Visualization, Funding acquisition. **Morten N. Andersen:** Writing - review & editing. **Cornelia H.L.A. Wieringa:** Methodology, Software, Formal analysis, Investigation, Writing - review & editing. **Rob A.E.M. Tollenaar:** Resources, Writing - review & editing, Supervision. **Wouter Hendrickx:** Methodology, Validation, Resources, Writing - review & editing, Funding acquisition. **Davide Bedognetti:** Methodology, Validation, Resources, Writing - review & editing, Funding acquisition. **Marianne Hokland:** Conceptualization, Resources, Writing - review & editing. **Peter J.K. Kuppen:** Conceptualization, Validation, Resources, Writing - review & editing, Supervision, Project administration, Funding acquisition.

Acknowledgements

We thank Ronald van Vlierberghe and Rob Keijzer (Department of Surgery, LUMC) for technical support, and dr. Arantza F. Sarasqueta (Department of Pathology, LUMC) for annotating the tumor border in several tumor tissue sections. Furthermore, we thank prof. dr. Hein Putter and dr. Nan van Geloven (Department of Biomedical Data Sciences, LUMC) for statistical advice. The flow cytometry data was acquired using the LSRFortessa flow cytometer at the FACS Core Facility (Aarhus University, Denmark).

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.molimm.2020.10.012.

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