Correlation of Blood T-Cells to Intratumoural Density and Location of CD3⁺ and CD8⁺ T-Cells in Colorectal Cancer

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Abstract. *Aim: To test the feasibility of conducting parallel analyses of circulating T-cells in blood and intratumoural T-cells in colorectal cancer. A pre-operative 'liquid biopsy' to determine immune status would facilitate clinical decision-making. Materials and Methods: A total of 18 patients with stage I-III colorectal cancer (CRC) were included. Blood was analyzed for T-cell type (CD3⁺, CD4⁺ and CD8⁺) and count using flow cytometry. Intratumoural T-cells were stained using immunohistochemistry and quantified by digital pathology. Tumour location was defined as invasive front (IF) or tumour center (TC). Results: The number of CD3⁺ and CD4⁺ T-cells in pre-surgical blood samples correlated with the number of CD3⁺ T-cells found in the IF (Spearman $\rho=0.558$, $p<0.05$ and 0.598 , $p<0.01$ respectively) and CD3⁺ in the TC ($\rho=0.496$, $p<0.05$, and $\rho=0.637$, $p<0.01$, respectively). A strong correlation was found between CD4⁺ cells in blood and CD8⁺ T-cells found in the TC and IF ($\rho=0.602$ and $\rho=0.591$, $p<0.01$). Conclusion: There is a correlation between blood CD3⁺ and CD4⁺ T-cells and the T-cells found at the TC and IF.*

The immune system is being increasingly recognized as an integral component in carcinogenesis, cancer biology and

patient prognosis in colorectal cancer (CRC) (1, 2). Furthermore, escape from immune surveillance is regarded as a cancer hallmark (3). Therefore, the understanding of how cancer cells avoid detection by the immune system is a research field of great interest, with new promising immunotherapy cancer treatments having been developed and under development (4, 5).

Solid tumours often contain a range of different immune cells. In CRC, the presence of tumour-infiltrating lymphocytes, as expressed by the number of CD8⁺ T-cells at the tumour centre (TC) and invasive front (IF) is strongly related to clinical outcome (1, 6-8). Several other associations between increased immune response and disease outcome have been reported, e.g. using analysis of lymphocyte to neutrophil score (9), total lymphocytic score (10), and immunoresponse *in situ* using the immunoscore (11). In particular, the immunoscore shows that the number of T-cells (CD3⁺) and cytotoxic T-cells (CD8⁺) at both the tumour center (TC) and at the tumour invasive front (IF) relate to a favourable outcome (12). This becomes important as the current Tumour-Node-Metastasis (TNM) staging relies heavily on nodal status (with pN0 or pN+ for no or presence of node metastasis, respectively). Notably, TNM staging, although in widespread use, is imprecise with risk of both over- and undertreatment (13). Therefore, including information that may enhance staging precision beyond the lymph node status is needed. Immunoprofiling has proven to be of prognostic relevance (14), and recently claimed to be superior to previous prognostic markers such as microsatellite instability (MSI) in CRC (15). However, there is a link between MSI and immune response in that previous studies have found that patients with MSI have a higher percentage of CD8⁺ T-cells, but a lower percentage of CD4⁺ T-cells in tumour compared to those with microsatellite stable (MSS) disease (16). Another form of MSI includes elevated microsatellite instability at selected tetranucleotide repeats (EMAST) (17), which has been associated with a higher density of CD8⁺ at tumour nests (18) and was included together with MSI in this study for comparison.

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How the *in situ* tumour immunoprofile relates to circulating T-cells is less well-known. The possibility of doing a 'liquid biopsy' in order to immune-stage a patient in advance of surgery may have both prognostic and predictive abilities as novel immunotherapeutic treatments become available.

Thus, we designed a feasibility study to assess the potential for measuring circulating T-cells and correlating this with the T-cell-profile in tumour tissue after resection of stage I-III CRC. We set out to investigate patterns of CD3⁺ (total T-cells), CD4⁺ (helper T-cells) and CD8⁺ (cytotoxic T-cells) T-cells in blood samples taken before surgery compared with the CD3⁺ and CD8⁺ immunoprofile at the tumour site (Figure 1), hypothesising that the immunoprofile at the tumour could be reflected in the blood sample of the same patient. A preoperative "liquid biopsy" to determine immune status would greatly facilitate decision-making if proven feasible and accurate.

Materials and Methods

Patients with non-metastatic primary CRC were recruited from an ongoing prospective, clinical and molecular biomarker outcomes project for translational cancer research in resectable primary CRC and liver metastasis (19). All included patients provided written informed consent. The Regional Ethics Committee (REK Helse Vest, #2012/742) approved the project and the project is registered at www.ClinicalTrials.gov (NCT#01762813). Clinicopathological demographics were recorded. Pathology staging was carried out as per the TNM system (20) using a standardized gross pathology and microscopic histopathology template for reporting. For the purpose of the current study design, each patient was his/her own control, using the pre-surgical blood sample profile in comparison to the intratumoural tissue immunoprofile for each case.

Blood sample preparation and flow cytometry. A registered nurse acquired pre-surgical blood samples the week immediately before surgery, and at follow-up at about 1 month post-surgery.

Blood was collected in EDTA-coated vacuum containers for determining the percentages of CD3⁺, CD4⁺ and CD8⁺ T-cells in erythrocyte-lysed whole blood. A kit was acquired from BD Biosciences (NJ, USA) with CD3 fluorescein isothiocyanate (FITC)/CD8 phycoerythrin (PE)/CD45 peridinin chlorophyll (PerCP)/CD4 allophycocyanin (APC)-conjugated antibodies for staining. Cells were prepared according to protocol using 100 µl full blood. BD Pharm Lyse was used at appropriate dilution to lyse erythrocytes, according to protocol. Collected blood samples were prepared for flow cytometry analysis on the same day or, alternatively, fixed in 2% paraformaldehyde solution after antibody staining for analysis one day later. Total leukocytes were marked using CD45⁺ and the lymphocyte population was identified on a side scatter vs. CD45⁺ scatter diagram. The number of CD3⁺ (T-cells), CD4⁺ (T-helper) and CD8⁺ (cytotoxic) T-cells was calculated from the lymphocyte population. To avoid double-positive cells, the number of cells that had CD4⁺ marking and was also CD8⁺ was deducted from the total number of cytotoxic cells.

The samples were run on a BD Accuri™ C6 (BD Biosciences) or a Cytotflex (Beckman-Coulter Inc., Indianapolis, USA) flow cytometer

both systems equipped with a blue and red laser, two light scatter detectors, and four fluorescence detectors with optical filters optimized for the detection of FITC, PE, PerCP, and APC. For analysis, the corresponding software (BD Biosciences Accuri C6 analysis software and CytExpert Beckman-Coulter) was used to calculate absolute numbers of the different T-cell populations per millilitre of blood.

Immunohistochemistry. Antigen retrieval and antibody dilution were optimised prior to the study onset. To ensure uniform handling of samples, all sections were processed simultaneously. Paraffin sections adjacent to the haematoxylin-eosin (H&E) sections used for histology were mounted onto Superfrost Plus slides and dried overnight at 37°C followed by one hour at 60°C.

Sections were deparaffinized in xylene and rehydrated in decreasing concentrations of ethanol. Antigen were retrieved with a highly stabilized retrieval system (ImmunoPrep, Instrumec, Oslo, Norway) using 10 mM TRIS/1 mM EDTA (pH 9.0) as the retrieval buffer. Sections were heated for 3 min at 110°C followed by 10 min at 95°C and cooled to 20°C. CD3 (Dako Clone F7.2.38; Dako, Glostrup, Denmark), was used at dilution 1:75 and CD8⁺ (Dako Clone C8/144B) was used at dilution 1:50. Dako Antibody Diluent (S0809) was used as diluent.

The EnVision™ Flex detection system (Dako) was used for visualization. Sections were incubated for 5 min with peroxidase-blocking reagent (SM801), 30 min with the primary antibody, 20 min with the EnVision™ FLEX/HRP Detection Reagent (SM802), 10 min with EnVision™ FLEX DAB+ Chromogen (DM827) and Substrate Buffer (SM803) mix and 5 min with EnVision™ FLEX Hematoxylin (K8008). The slides were then dehydrated and mounted. All immunohistochemical stainings were performed using a Dako Autostainer Link 48 instrument and EnVision™ FLEX Wash Buffer.

Immune scoring using Image software. Tumour sections were scanned at ×40 magnification using a Leica SCN400 slide scanner (Leica Biosystems, Wetzlar, Germany) and uploaded to the image analysis software, Visiopharm® (Hoersholm, Denmark). We performed two separate analyses to score T-cell immune response: whole-section analyses of CD3⁺ and CD8⁺ cells in the TC and IF and measurement of intraepithelial and stromal T-cells in a 2 mm diameter circle in the TC and IF.

For whole-section analyses, the areas of TC and IF were marked manually in Visiopharm® on both the CD3⁺- and CD8⁺-stained sections. The same area was marked on both CD3⁺- and CD8⁺-stained section. The Visiopharm program identified the CD3⁺- and CD8⁺-positive T-cells (Figure 2a) in the marked areas. Visiopharm measured the area of CD3⁺ and CD8⁺ positivity and transformed this area into a number of positive T-cells based on an estimation of the mean area of a lymphocyte (60 µm²).

For measurement of tumour intraepithelial and stromal CD3⁺ and CD8⁺ T-cells, an area of 3.14 mm² was marked in both the TC and IF using a 2 mm diameter circle. The epithelium and stroma were separated inside the circle by programming in Visiopharm® (Figure 2b), followed by detection of CD3⁺- and CD8⁺-positive T-cells (Figure 2b). The same area was used for both CD3⁺- and CD8⁺-stained sections. For both analyses, the number of CD3⁺- and CD8⁺-positive T-cells was calculated per square millimetre. In addition, the CD8⁺/CD3⁺-ratio was calculated.

Analysis of MSI. Following slide review by an experienced pathologist, 20 µm tumour and tumour-free sections were cut from

Figure 1. Immune profiling of patients with colorectal cancer. Blood samples were acquired from patients 1 week before and 1 month after primary surgery and analyzed using flow cytometry. The identified lymphocyte population was further analyzed as described in the Materials and Methods. Tumours were sectioned and T-cells stained using antibodies for CD3⁺ and CD8⁺. Digital image analysis software was used to measure T-cell immune response at the tumour centre (TC) and invasive front (IF). The lower panel illustrates how colorectal tumour with dying necrotic cells releases neo-antigens, which are detected by antigen-presenting cells (APC) in close vicinity to the tumour. In response to antigen presentation on APCs, lymph nodes produce cytotoxic T-cells (CD8⁺) which attack the tumour and increase the numbers of T-cells circulate in the peripheral blood.

formalin-fixed paraffin-embedded blocks for DNA extraction, using the Tissue DNA E.Z.N.A. kit (Omega Bio-Tech®, Norcross, GA, USA) according to the manufacturer's instructions.

DNA extracted from tumour tissue and the corresponding normal tissue (from surgical resection margins) was then amplified by polymerase chain reaction (PCR)-amplified with five tetranucleotide microsatellite primer pairs (EMAST: D20S85, D20S82, D9S242, D8S321, MYCL1, 5'-terminus fluorescently labelled) and five mononucleotide microsatellite primer pairs (MSI: NR-27, NR-21, NR-24, BAT-25, BAT-26, 5'-terminus fluorescently labelled).

PCR conditions were as follows an initial denaturation step of 5 min at 95°C, followed by 37 cycles of denaturation (30 sec at 95°C), annealing (90 sec at 55°C) and extension (30 sec at 72°C), and concluded by a final elongation step (30 min at 60°C).

The PCR products were then analysed for fragment lengths on a 3130xl GeneticAnalyser (Applied Biosystems, Waltham, MA, USA), with GeneMapper v3.7 software (Applied Biosystems). Tumour samples were compared to their corresponding normal samples. Those showing any number of extra peaks at $\pm 4n$ ($n \neq 0$) (tetranucleotide markers, EMAST), or $\pm 1n$ or $2n$ ($n \neq 0$) (mono- and dinucleotide markers respectively, MSI) were scored as unstable for that marker.

Definition of EMAST and MSI. To detect EMAST, either direct sequencing or fragment analysis are generally used, with most laboratories adopting a panel of five tetranucleotide polymorphic markers (at least two unstable markers to score EMAST positivity). In CRC, up to seven microsatellite markers have been used, with EMAST considered present (EMAST⁺) when at least

Figure 2. Digital programming for detection of CD3⁺ and CD8⁺ T-cells. Pre-scanned colorectal tumour sections were used for analysis. a: Visiopharm® digital programming was used to mark out the tumour invasive front (IF; blue line) and tumour centre (TC; green line) in all tumour samples. The program then measured the full area (red) and then the area positive for CD3⁺ and CD8⁺ cells, as described in the Materials and Methods. b: Representative image of the marking process using 2 mm diameter circles for identifying immune infiltrative CD3⁺ and CD8⁺ cells in cancer epithelia and cancer stroma at the TC (green) and IF (red). This method was used for the analysis presented in Tables II and III.

Figure 3. Flow cytometric analysis of T-cell populations in blood samples acquired before and after colorectal surgery. Blood samples were gated using CD45⁺ leukocyte marker to distinguish the lymphocyte population in erythrocyte-lysed full blood. *a*: The marked area shows the lymphocyte population. These cells were subsequently analysed for expression of CD3⁺ for total T-cell numbers, CD8⁺ for cytotoxic and CD4⁺ for T-helper cells. *b*: Graph showing the median number of T-cell populations in blood acquired prior to (1 week or less) and post (4 weeks) surgery in the same patients, grouped by node-negative (■) and node-positive (□) tumours. N=18.

one marker was found unstable. Here, the most used definitions of at least two out of five unstable tetranucleotide markers was used to confirm EMAST. Samples with instability in at least two out of five markers (40%) were recorded as EMAST-positive with/without MSI. If two out of five markers were positive in both panels they were listed as EMAST-positive and MSI-H. Instability in one out of five markers was scored as EMAST-negative and

MSI-L when using the MSI panel, respectively. If no unstable marker was found, the specimens were considered as microsatellite stable (MSS). MSI analysis was carried out as previously described (21, 22). Two investigators completed the scoring process independently, blinded to each other's results. Discordance between scoring was addressed with re-running of the samples from the PCR step onwards, and re-scoring.

Table I. Patient clinicopathological characteristics.

	N (%)
Median age (range), years	76.5 (56-86)
<70 Years	6 (33.3)
≥70 Years	12 (66.6)
Gender	
Male	6 (33.3)
Female	12 (66.6)
TNM stage	
I (pN0)	5 (27.7)
II (pN0)	8 (44.4)
III (pN+)	5 (27.7)
Location of tumour	
Colon	12 (66.6)
Rectum	6 (33.3)
MSI status	
MSS	16 (88.8)
MSI	2 (11.1)

MSI: Microsatellite instability. N=18.

Statistics. All statistical analyses were performed using IBM® SPSS® v. 23 for Mac and Windows, version 23 (IBM Corp., Armonk, NY, USA). Assuming a non-Gaussian distribution of variables, all tests used were non-parametric. Correlation analyses were performed using Spearman's rank correlation coefficient (ρ). All tests were two-tailed and statistical significance was set at $p < 0.050$.

Results

The study recruited 21 patients, of whom 18 had invasive adenocarcinomas and were included. Three patients were excluded due to having adenoma and not carcinoma. Patients clinicopathological characteristics are listed in Table I. The test for MSI at mono- and tetranucleotides (EMAST) found that two out of 18 patients met the criteria for EMAST, whereas 16 were Microsatellite stable (MSS). The two EMAST+ tumours demonstrated no increased immune response (lymphocyte count) compared to non-EMAST and MSS tumours. The low number of MSI/EMAST cases (N=2) in this series precluded further statistical testing.

Immune profiling of whole blood before and after surgery. To test whether there was a significant change in T-cells in blood samples acquired before and after primary surgery, we analyzed the T-cell population by flow cytometry and gated for high CD45+/low side-scatter to identify the lymphocyte population (Figure 3a). The paired pre- and postoperative blood samples showed no significant change in T-cells when comparing CD3+, CD4+ and CD8+ frequencies in the 18 patients. To further test if the clinical staging of the tumour

Table II. Correlation coefficients for associations between blood T-lymphocyte populations (CD3+, CD4+ and CD8+) and T-lymphocytes (CD3+, CD8+) at the tumour invasive front and centre.

Blood	Tumour invasive front		Tumour centre	
	CD3+	CD8+	CD3+	CD8+
CD3+	0.558*	0.541*	0.496*	0.515*
CD8+	0.129	0.083	-0.067	0.061
CD4+	0.598**	0.602**	0.637**	0.591**

Significant at the *0.05 level, **0.01 level (2-tailed). N=18.

would influence these scores, we grouped the patients into node-negative and node-positive groups. Although not statistically significant, the median numbers of CD3+ and CD4+ T-cells were higher in patients with node-positive disease before surgery, but the CD8+ T-cell frequencies were lower (Figure 3b). After surgery, the CD3+ T-cell count dropped in patients with node-positive disease, however, it increased in patients with node-negative disease (Figure 3b). The median number of CD8+ T-cells increased in patients with node-negative disease after surgery, while in those with node-positive disease, there seemed to be little change (Figure 3b). Furthermore, CD4+ T- helper cells increased after surgery in those with node-negative tumours, whilst the median decreased to less than half in those with node-positive tumours. None of these changes were statistically significant according to Mann-Whitney U-test grouping the patients into node-negative and node-positive groups.

Comparison of blood T-cell frequencies vs. T-cell infiltration at the tumour. There were significant positive correlation between a high number of CD3+ T-cells in blood before surgery and CD3+ cells found at both the TC, and at the IF ($p < 0.05$) (Table II). The strongest correlation was between CD4+ cells in blood and CD3+ and CD8+ T-cells both at the TC and IF ($p < 0.01$). There was no significant correlation between CD8+ T-cells in blood and the tumour, neither at TC nor at IF.

CD8+ T-cells over CD3+ T- cells at IF and TC compared to blood T- cells. When analyzing the ratio of CD8+/CD3+ T-cells in whole-section tumour slides compared with blood T-cells, we found a significant correlation between the number of CD3+ T-cells in blood and the CD8+/CD3+ ratio at the TC ($p = 0.037$) (Table III). There was no significant correlation between the other T-cell populations and that found at the TC or IF.

Comparing TNM stage to CD3+ and CD8+ T-cells using area of TC and IF. When comparing the number of CD3+ and CD8+ T-cells at the TC and IF by T-stage, we found that

Table III. Correlation coefficient for associations of total (CD3⁺), cytotoxic (CD8⁺) and T-helper (CD4⁺) lymphocytes found in blood with the ratio of cytotoxic to total lymphocyte count at the invasive front and tumour centre in tissue.

	Invasive front CD8 ⁺ /CD3 ⁺	Tumour centre CD8 ⁺ /CD3 ⁺
Blood		
CD3 ⁺	0.051	0.495*
CD8 ⁺	0.030	0.344
CD4 ⁺	0.036	0.439

*Significant at the 0.05 level (2-sided); N=18.

the median number of CD3⁺ and CD8⁺ T-cells decreased in the IF and TC in tumour with higher T-stage (Figure 4). None of the data reached statistical significance.

Discussion

This study showed that there was a significant correlation of the number of circulating CD3⁺ and CD4⁺ T-cells with high CD3⁺ and CD8⁺ numbers at the IF and TC. Previous studies have shown that subsets of regulatory CD4⁺ T-cells (CD4⁺Forkhead box P3⁺) showed a significant increase in peripheral blood from patients with CRC compared to healthy individuals (23). In this feasibility study, we did not assess the different sub-populations of CD4⁺ T-cells but rather the number of cells per millilitre of blood comparatively to T-cells at tumour site. The use of CD4⁺ as a prognostic marker at the tumour is unclear due to the complexity of T-helper subsets. Because of the suggested use of an immunoscore for CRC prognostic evaluation (CD3⁺ and CD8⁺), we excluded CD4⁺ as a marker in our intratumoural analysis. Although not significant, we did see a trend for a higher number of T-cells (CD3⁺ and CD4⁺) in those with node-negative *vs.* node-positive disease in pre-surgical blood analysis. Previous reports have shown that patients with a low neutrophil to lymphocyte ratio, which could be due to an increase in the number of lymphocytes, were significantly associated with higher TNM staging and shorter overall survival (24).

The beneficial effect of a cancer-specific immune reaction is now well-established as being related to better outcome, whereas a lower immune response is associated with a worse prognosis (2, 25, 26). The current study did not claim to investigate prognostic outcomes, but rather test the association between specific immune cells found in circulating blood compared to those found in the intratumoural tissue. The number of CD8⁺ and CD3⁺ T-cells as biomarkers at the TC and IF in CRC tumours to differentiate which patients have a higher risk of relapse has

Figure 4. Comparing CD3⁺ and CD8⁺ T-cells at the tumour centre (TC) and invasive front (IF) by T-stage. The graph illustrates the median number of CD3⁺ and CD8⁺ T-cells found at the IF and at TC at different T-stages in our patient cohort. T1: N=1, T2: N=6, T3: N=11.

been proposed by an Immunoscore® (15, 27). However, whether corresponding T-cells in blood can identify patients with higher risk of relapse needs further investigation.

It is interesting that the most significant association between T-cells in blood and that at the tumour site was found for CD4⁺ T-cells, which are a class of T-cells especially important in the adaptive immune system for enhancing or suppressing an immune response (28). However, there have been conflicting reports regarding the prognostic value of CD4⁺ T-cells evaluated in cancer tissues (12), where recently it was suggested that CD4⁺ Th17 cells together with macrophages maintained a positive feedback loop in patients with CRC inducing inflammation which could be initiating rather than preventing tumourigenesis (29). CD4⁺ T-cells include a range of different sub-classes, which would have to be evaluated and scored for prognostic purposes in a larger cohort. This was, however, beyond the scope of our initial study, and we chose a standard T-cell panel for blood analysis and the better described method for CD3⁺ and CD8⁺ detection at the tumour site for our analysis.

Although our cohort is too small to establish firm conclusions at this stage, there is a strong association between the circulating blood T-cell profile with the intratumoural immune response. We, thus, believe the current findings warrant further pursuit both in larger cohorts, and

more specifically, with regard to subgroups of known molecular traits, such as MSI type tumours.

The limitations of our study includes, a low number of patients and lack of a validation cohort. Validation would be necessary to demonstrate any predictive or prognostic impact. However, the current study was merely testing the hypothesis that there was an association between blood and tumour T-cell count using two methods, as described in the “cancer-immunity cycle” (4), which we were able to confirm. Thus, the next step will be to verify the results using a larger, prospective cohort of patients as we have optimized the techniques and efficiency of sample analysis.

The inclusion of scoring CD3⁺ and CD8⁺ T-cells by means of digital pathology has its strength in objectively assessing the score. Our patient samples are from a cohort of consecutive patients and thus should not have an inherent referral bias. Whether the results apply more specifically to subgroups (*e.g.* CRC with/without MSI; rectal cancer with/without pre-operative radiation therapy; presence or no presence of node metastases etc.) remains to be determined in larger patient cohorts.

Ethics

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. The project and research biobank was approved by the Regional Ethics Committee of the Western Health Authority (REK Helse Vest, #2012/742) and by the Institutional review board (Helse Stavanger HF Protocol Record #29034/2012), and registered at www.ClinicalTrials.gov NCT#01762813.

Conflicts of Interest

The Authors declare that they have no conflict of interest in regard to this study.

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