Natural Killer Cells are Scarce in Colorectal Carcinoma Tissue Despite High Levels of Chemokines and Cytokines

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Abstract

Purpose: Tumor infiltrating T lymphocytes in colorectal cancer (CRC) have prognostic impact, but the role of natural killer (NK) cells in CRC tissue is unclear. The contribution of intratumoral cytokines and chemokines in shaping the composition of the inflammatory lymphocytic infiltrate is also unclear.

Experimental Design: In this study, localization and densities of NK and T cells within primary CRC, CRC liver metastases, adenomas, and normal tissues were analyzed on whole tissue sections from 112 patients. In a subset of these patients, the most important 50 cytokines and chemokines were quantified in paired serum, primary CRC and adjacent mucosa samples and in CRC liver metastases and correlated with NK and T-cell infiltration, respectively.

Results: The various compartments displayed characteristic differences like significantly higher chemokine concentrations in CRC tissue. Most importantly, despite high local chemokine levels, NK cells were generally scarce within CRC tumor tissues, independent of human leukocyte antigen (HLA) class I expression. Adjacent normal mucosa contained normal levels of NK cells. In contrast, corresponding T-cell numbers varied substantially and were positively correlated with higher chemokine levels.

Conclusions: Our findings indicate a distinct regulation of NK cells versus T cells in the CRC tumor microenvironment. NK-cell migration into CRC tumor tissue is obviously impaired early during tumor development by mechanisms that do not affect T-cell infiltration.

Introduction

Colorectal carcinoma (CRC) is one of the most common malignancies and a major cause of cancer death worldwide (1). The development and course of CRC is influenced through factors like microsatellite instability or inflammatory mediators (2). Inflammation can promote tumor progression and metastasis formation. The innate and adaptive immune system also can protect the host against tumor development through mechanisms of immunosurveillance (3). Natural killer (NK) cells are part of the innate immune system and have diverse biological functions including the ability to recognize and kill a variety of tumor cells (4). Cytolysis is performed via the release of cytotoxic granula containing perforin and granzyme B (GrmB) or by the induction of death receptor-mediated apoptosis. NK cells are also able to produce a broad spectrum of chemokines and cytokines. The involvement of NK cells and the related cytokines and chemokines in the tumor microenvironment and their clinical impact in CRC remains not well understood.

NK cells and their cytokines and chemokines play a pivotal role in normal homeostasis and tissue differentiation of the gut (5, 6). To assess the direct relationship between chemokines and infiltration of T and NK cells into CRC, we combined complete tissue section immune-cell quantification (virtual microscopy), utilizing an antibody against the NK-cell-specific receptor NKP46, and multiplex-based chemokine and cytokine quantification (4, 7). This novel methodology allowed for the first time a detailed quantification of infiltrating NK and T cells and...
NKp46 nonmalignant colon tissue. Because CD56 that contains identical amounts of NK cells compared to chemokine levels compared to adjacent normal mucosa stages on, despite the presence of significantly higher patients were females, age range 40 to 78 years (mean cancer) staging system (8). Thirty-three percent of the (UICC)-TNM (tumor node metastasis, system for staging analysis. Histopathologic and clinical findings were scored according to the International Union Against Cancer medical ethics committee of the University of Heidelberg, liver”). All material was obtained after approval by the colon mucosa”) from patients without malignant disease and liver metastases and (ii) nonmalignant colon (“normal mucosa”) were evaluated (Supplementary centers of the primary CRC tumor but also adjacent and subsequent objective evaluation (10–12). Not only metastatic disease (stage IV) had liver metastases. Twelve noncorresponding liver metastases (“liver metastases”) paraffin-embedded specimens were analyzed. Nonmalignant liver tissue that was surgically removed during resection of hemangiomas was included (“normal liver”). Adenomas were obtained from surgically removed specimens and consisted of 10 samples from 10 different patients. Six cryo-conserved samples consisting of 3 primary tumors and 3 liver metastases were used for protein quantification. Two additional primary tumors could only be used for cytokine and chemokine measurements. Pathological reports were available for all tissues, no samples from patients with inflammatory bowel disease or chronic inflammation (e.g., hepatitis, diverticulitis, etc.) were included in this analysis. In CRC samples, the whole primary tumor (“tumor”) and the adjacent morphologically normal mucosa (“adjacent normal mucosa”) were analyzed separately after dissection. For the evaluation of HLA class I expression and NKp46+ NK-cell density, 20 independent primary CRC samples from stage I to IV (5 each) patients were analyzed.

Immunohistochemistry for NK and T cells and evaluation of HLA class I expression

Tissue specimens were immunohistochemically analyzed for their infiltration with NK cells (defined as NKp46+), T cells (CD3ε+ cells), and CD56+ NK and T cells. HLA class I expression was also evaluated as shown in Supplementary Figure S1. Mouse monoclonal antibodies recognizing human CD3ε (clone PS1, Acris), CD56 (clone IB6, Novocastra), and NKp46 (clone 195314, R&D Systems) were used. For HLA class I expression, EMR8–5, a monoclonal antipan HLA-class I heavy chain antibody (clone EMR8–5, MBL) was used (9). All processing steps were performed with a BOND-II autostainer (Leica) according to manufacturers recommendations. Antigen detection was performed by a color reaction with 3,3-diaminobenzidine (DAB+ chromogen, Menarini).

Evaluation of immunostaining in tissue samples

Manual or semi-manual evaluations of stained cell densities on immunohistological slides are influenced by observer bias and usually limited to small regions of tissue (especially for Tissue Microarrays). Therefore, we have developed a novel system to automate the process of whole slide tissue preparation and staining and subsequent objective evaluation (10–12). Not only centers of the primary CRC tumor but also adjacent normal mucosal tissues were evaluated (Supplementary Fig. S2). High-precision quantification of cell densities was performed as described previously (11, 12) using the quantification of the corresponding chemokine/cytokine composition in primary CRC, adjacent normal mucosa, and liver metastases. Our studies demonstrate that NKp46+ NK cells are scarce in CRC tissue from early stages on, despite the presence of significantly higher chemokine levels compared to adjacent normal mucosa that contains identical amounts of NK cells compared to nonmalignant colon tissue. Because CD56+ and NKp46− cell numbers are generally not equivalent, CD56 alone cannot be used as a surrogate marker for NK cells in immunohistochemistry. The amount of effector molecules like TRAIL, GrMβ, and others rather correlated with T-cell than with NK-cell infiltration. The here presented approach aimed at clarifying the differential role of NK cells in the tumor microenvironment of CRC, giving a detailed account of the parameters involved in “immunosurveillance” and immunotherapeutic strategies.

Materials and Methods

Patients and tissue samples

A set of 112 samples was analyzed, consisting of (i) surgically resected CRC primary tumors from patients with liver metastases and (ii) nonmalignant colon (“normal colon mucosa”) from patients without malignant disease and liver specimens from noncancer patients (“normal liver”). All material was obtained after approval by the medical ethics committee of the University of Heidelberg, written consent was obtained from all patients prior to analysis. Histopathologic and clinical findings were scored according to the International Union Against Cancer (UICC)-TNM (tumor node metastasis, system for staging cancer) staging system (8). Thirty-three percent of the patients were females, age range 40 to 78 years (mean 63.7). Ten samples were UICC stage I, 12 samples stage II, 12 samples stage III, and 14 samples were stage IV. Ten percent of the tumors were well differentiated (G1). 70% of tumors were of intermediate differentiation (G2), and poor differentiation (G3) in 20% of tumors. Tumor samples were typed for MSI using BAT25, BAT26, and CAT25 (2), no microsatellite instability was found. Patients with metastatic disease (stage IV) had liver metastases. Twelve noncorresponding liver metastases (“liver metastases”) were included in this analysis. In CRC samples, the whole primary tumor (“tumor”) and the adjacent morphologically normal mucosa (“adjacent normal mucosa”) were analyzed separately after dissection. For the evaluation of HLA class I expression and NKp46+ NK-cell density, 20 independent primary CRC samples from stage I to IV (5 each) patients were analyzed.

Translational Relevance

Prognosis of colorectal cancer (CRC) patients is dependent on the local immunological tumor microenvironment. Therefore, there is an urgent need to dissect the immunological mechanisms involved in the differences in the prognosis. Here we show that natural killer (NK) cells are generally absent in CRC of all stages. We provide evidence that this absence is not dependent on simple lack of one of the known factors: local cytokines, chemokines, or adhesion molecules. Furthermore, our data indicate a pivotal role for an escape from NK cells for CRC tumorigenesis, as already adenomas show this absence of NK cells. Differential and combined modification of the above mentioned factors to overcome this cancer-protective milieu therefore plays an important role for future clinical trials harnessing NK cells. Additionally we propose, that quantification of NK cells within CRC tissue can be used as an important parameter for the detection of response to therapy.
VIS software package (Visiopharm). For nonmalignant colorectal specimens the complete mucosal layer with adjacent stroma tissue was evaluated. For metastatic lesions, the complete intratumoral area was analyzed. In primary CRC samples, the complete tissue section was analyzed. Due to differences in tissue size the evaluated region ranges for each sample in size from 16 to 110 mm², a total of approximately 5,500 mm² was analyzed. Evaluation of HLA class I expression was performed using measurements of relative staining intensity across complete regions of 1 mm².

Chemokine and cytokine detection in tissue lysates and corresponding sera
Lysates were prepared according to the manufacturer’s instructions (BioRad Laboratories). A 2-laser reader simultaneously quantifies cytokines and chemokines. Standard curves and concentrations were calculated with Bio-Plex Manager 4.1.1 on the basis of the 5-parameter logistic plot regression formula. The detection sensitivity of all analytes ranged from 2 pg/mL to 30 ng/mL. For details see Supplementary Materials and Methods.

Statistical analysis
Statistical analyses were performed with SPSS 16.0 software (SPSS). For the comparison of individual variables, exact Mann–Whitney U-tests or Kruskal–Wallis tests were carried out as appropriate. For paired sample analysis the Wilcoxon signed-rank test was used. Results with 2-tailed $P$ values $<0.05$ were judged to be statistically significant (see Supplementary Materials and Methods).

Results

NKp46⁺ NK cells are detectable in normal mucosa but significantly reduced in primary CRC tissue and liver metastases
Substantial numbers of NKp46⁺ NK cells were found in healthy lamina propria, surrounding colonic crypts and also in the muscular layer (Fig. 1A and D), as well as in the subserosal fat tissue (NK cell numbers in normal colon tissue, see Supplementary Table S1). Direct comparison of NKp46⁺ cell densities in CRC patients between primary tumor and adjacent normal mucosa revealed a significant difference with lower numbers in tumor tissues (Fig. 1D and E, paired samples analysis, $P < 0.001$). NKp46⁺ NK-cell densities within primary tumors were markedly lower (see Supplementary Table S1) as compared to adjacent normal mucosa (see Supplementary Table S1). Comparing the densities of adjacent normal mucosa from all patients with cell densities from all primary tumors, the decrease in NKp46⁺ NK cells was statistically significant (Fig. 1E, $P < 0.001$). Adjacent normal mucosa contained significantly higher numbers of NKp46⁺ NK cells than primary CRC tissue. The slope of the gradient (adjacent NK-cell number : tumor NK-cell number) was not associated with age (Spearman’s rank correlation rho = −0.199, $P = 0.363$) or a specific age group. The difference between surrounding normal mucosa and primary CRC was also observed between adenomas and adjacent normal mucosa, whereby practically no NKp46⁺ NK infiltration was detectable in adenomas (median 3.95 cells/mm²; data not shown). The comparison of NK-cell numbers of the adjacent normal mucosa from tumor samples with normal mucosa from patients without malignancies showed no statistically significant difference (Fig. 1D, $P = 0.451$). NKp46⁺ NK cells were also analyzed in an independent cohort of liver metastases of colorectal cancers (Fig. 2A). These liver metastases did not contain any or only very low numbers of infiltrating NKp46⁺ NK cells, whereas substantial numbers of NKp46⁺ NK cells were found in normal liver tissue (Fig. 2B, $P < 0.01$).

CD56 staining detects NKp46⁺ NK cells as well as CD56⁺ CD3ε⁺ T cells
CD56 is routinely used in immunohistochemistry for the detection of NK cells. To address the question whether all CD56⁺ cells are NKp46⁺ NK cells, we compared the NKp46⁺-cell densities with CD56⁺ and CD3ε⁺ densities (see Fig. 1A–C) and we quantified CD56⁺ versus NKp46⁺ cells/mm² and calculated the ratios in normal mucosa and normal liver (Fig. 3). In normal colon mucosa, an almost 20-fold excess of CD56⁺ cells was found (see Supplementary Table 1). In normal liver samples CD56⁺ cells were also increased (Fig. 3A and B, Supplementary Table S1). To test whether this abundance of CD56⁺ cells in normal mucosa resulted primarily from staining of CD56⁺ T cells, T cells were identified by CD3ε (Fig. 3C and D). Direct comparison of NKp46 and CD3ε staining revealed higher numbers of infiltrating T cells compared to NK cells, especially in primary CRC, a strong CD3ε⁺ T-cell infiltration (Figs. 1B and 3C and D, see Supplementary Table S1). In adjacent normal mucosa (compared to tumor), this ratio was significantly lower (see Supplementary Table S1), while between adjacent mucosa and normal mucosa, no statistical difference was observed ($P = 0.313$, see Fig. 3C and D).

We conclude (i) that specific markers like NKp46 are required for a precise quantification and localization of tissue-infiltrating NK cells since CD56 staining does not discriminate between NK and T cells and (ii) that in primary CRC tissue and in liver metastases, significantly less NK cells than T cells were found. There was no dependency of NK cell density on HLA class I expression in the tumor (see Supplementary Fig. S3).

NKp46⁺ NK cell infiltration is independent from expression of selectins or adhesion molecules
Publicly available expression data (ArrayExpress, and BioGPS) of colorectal cancer and normal colon tissue revealed a general low expression of L-, P-, and E-selectin in both tumor and normal mucosa tissues without significant differences. In liver metastases and adjacent normal

http://www.ebi.ac.uk/microarray-as/ae/
http://biogps.gnf.org
Figure 1. Immunohistochemistry for NKp46⁺, CD3ε⁺, and CD56⁺-cell distributions. A. Immunohistochemical staining for NKp46 and representative magnification from different regions. Overview magnification ×1.5, higher magnifications ×12. Counterstained with haematoxylin. B. Immunohistochemical staining for CD3ε and representative magnification from different regions. C. Immunohistochemical staining for CD56 and representative magnification from different regions.
Figure 1. (Cont’d) (D) Distribution of NKp46+ cell densities [cells/mm²] in different regions (adjacent normal mucosa versus tumor, Mann–Whitney U-test, ***P < 0.001). (E) Paired depiction of corresponding cell densities in the tumor and in adjacent normal mucosa (P1040, P1080, P1085 labels indicate individual patients from which frozen material was available for protein quantification, Wilcoxon signed rank test, ***P < 0.001).

Figure 2. NKp46+ NK-cell distribution in liver and CRC liver metastases. (A) Microscopical analyses of normal liver and CRC liver metastases, comparing NKp46 staining and CD56 staining arrows indicate positive lymphocytes, asterisks mark CD56+ small bile ducts. (B) Distribution of NKp46+ NK-cell densities in normal liver and in colorectal cancer liver metastases (Mann–Whitney U-test, **P < 0.01).
liver, L-, P-, and E-selectin expression was very low in the invasive margin and absent in the other compartments, including normal liver (data not shown and (13)). With respect to adhesion molecules, substantial differences at the protein level could be detected for ICAM-1 and VCAM-1 with significantly higher concentrations in CRC tissue versus adjacent mucosa and serum (Supplementary Fig. S4).

Elevated chemokine levels in CRC tissue promote infiltration of T cells but not NK cells

As shown in Figure 1, colon carcinoma tissues were preferentially infiltrated by T cells, despite the fact that NK and T cells were found in almost equal numbers in the surrounding mucosa. Therefore, we determined the chemokine pattern in the different tissue compartments using frozen material from serial sections of 5 patients. NK cells consist of 2 distinct subgroups: CD56<sup>bright</sup> and CD56<sup>dim</sup> cells (14, 15). The analysis of the CD56<sup>bright</sup> NK-cell-relevant chemokines CXCL9, CXCL10, CCL3, CCL4 in serum, corresponding adjacent normal mucosa, and primary CRC tissue revealed significantly elevated levels in CRC (P < 0.05, Fig. 4A). A similar gradient was detected for the CD56<sup>dim</sup> NK-cell-relevant chemokines CXCL8, CXCL1, CXCL5, and CXCL12 (Fig. 4B and Supplementary Fig. S5) showing significantly higher concentrations in tumor tissue. These data indicated that the chemokine composition in primary CRC tissue was able to support CD56<sup>bright</sup> and CD56<sup>dim</sup> NK-cell infiltration. CCL5 was the only chemokine that showed higher concentrations in serum than in tissues. Thus, elevated concentrations in CRC tissue were not a general feature of all chemokines. Despite this gradient of CD56<sup>bright</sup> as well as CD56<sup>dim</sup> NK-cell-attracting chemokines, the number of NKp46<sup>+</sup> NK cells, was significantly decreased in CRC (Fig. 1).

Chemokine levels show better correlation with T-cell than NK-cell infiltration

To directly compare the impact of chemokines for NK- and T-cell infiltration, we chose 3 representative CRC tissues with different numbers of infiltrating T and NK cells: P1040 with high infiltration of T cells (607/mm²) and NK cells (36/mm²), P1085 with high T cells (234/mm²) but low NK-cell numbers (3/mm²) and P1080 with virtually no T cells (0.5/mm²) and low NK cells (6/mm²). T cells exceeded NK-cell numbers by a factor of approximately 10. We observed a clear positive correlation since the number of T cells increased with elevated concentrations of CXCL10, CXCL8, CXCL5, CXCL1, and CX3CL1. In contrast, only small numbers of NK cells infiltrated the tumor tissue samples despite their high local chemokine levels.

In liver metastases of 3 independent colon carcinoma patients (LM62, LM63, LM65; Fig. 5), lower concentrations...
Figure 4. Elevated chemokine levels in primary CRC tissue. Relevant chemokines for the recruitment of NK cells were analyzed in paired serum, adjacent normal mucosa and CRC primary tumor tissue lysates of 5 representative CRC patients. Additional data is shown in Supplementary Figure S4, including a table that summarizes the NK subset-specific chemokine receptors and the corresponding ligands. (A) Relevant chemokines for CD56bright NK cells. (B) Relevant chemokines for CD56dim NK cells. Each data point indicates one patient and the median values of the chemokine concentrations show significantly elevated levels (Kruskal–Wallis test, \( P < 0.05 \)) in CRC tissues compared to adjacent mucosa, with the exceptions of CCL5 (RANTES) and CX3CL1 (Fractalkine).
Figure 5. Comparison of T- and NK-cell infiltration and corresponding chemokine concentrations. Primary CRC tissues of 3 representative patients P1080, P1085, and P1040 (panel A) and CRC liver metastatic tissues of 3 different patients, LM62, LM63, and LM65 (panel B) were analyzed for T- and NK-cell infiltration (cell numbers/mm²) and the corresponding protein concentrations of the NK-relevant chemokines. The numbers of infiltrating T and NK cells are shown for each patient in the upper part of the panels (for primary CRCs and CRC liver metastases). For each patient sample, the chemokine concentrations are displayed below for primary CRC and CRC liver metastases. For additional data see Supplementary Figure S5.
of chemokines were detected compared to primary CRC tissue. Remarkably, NK cells are almost absent within the metastases (with 0.2 NK cells/mm² for LM62, 0.28 for LM63 and 1.3 for LM65) and no general correlation between high chemokine concentrations and high T- or NK-cell numbers was observed in liver metastases.

The absence of tumor-infiltrating T and NK cells is reflected by low levels of effector molecules

The activation status of effector functions of immune cells (in the same tissue samples as shown in Figure 5) was analyzed via measurement of effector molecules like the soluble death receptor ligands CD95L (FasL) and TRAIL, the cytotoxin GrmB, and the proinflammatory cytokines IFN-γ and TNF-α (Fig. 6). The general activation status of T and NK cells was determined by the amount of soluble CD25. In direct comparison adjacent normal mucosa and tumor tissue, P1040 (□) and P1085 (△) showed increased effector molecule concentrations, that is, sCD95L, GrmB, and TNF-α in tumor tissue whereas concentrations were decreased in P1080 (○). Therefore, the concentrations of effector molecules positively correlated to T-cell numbers in the corresponding tissue compartments. P1080 virtually lacked tumor-infiltration by T cells, whereas the T-cell numbers in the adjacent mucosa were comparable to P1085 and P1040. The slightly higher number of NK cells in the tumor tissue of P1080 compared to P1085 was not reflected by the amounts of effector molecules, indicating that mainly T cells serve as source for these molecules. Similar to the chemokines, these effector molecules can be divided into 2 groups according to their highest concentrations in tumor tissue or their lowest concentrations in adjacent mucosa, respectively. The correlation between high T-cell infiltration and high levels of sCD95L and GrmB, indicates a localized immune response that is, however, insufficient to control tumor growth. In contrast, TRAIL and IFN-γ seem to be distributed rather systemically and less characteristic for the tumor microenvironment. Importantly, the liver metastases showed less than 10% of the effector molecule concentrations compared to primary tumor tissues, which also correlated with lower T- and NK-cell infiltration. This remarkable decrease in effector molecules in metastatic tissue reflects the postulated stepwise-impaired immune recognition from primary tumor tissue to metastases.

Discussion

The role of NK cells in solid tumors and especially in CRC is not well understood, specifically whether NK cells also contribute to an immune surveillance (16). For CRC, high densities of infiltrating lymphocytes, high levels of CD3, GrmB, CXCL8, an IFN-γ/IRF1-driven Th1 response and low angiogenesis were shown to have a strong prognostic impact (17, 18). Since most of the above parameters are also expressed or produced by NK cells, we aimed to delineate the contribution of NK cells to these observations. Our results, therefore, could be interpreted in different aspects: (a) the infiltration of NK cells following treatment is a possible predictive marker; (b) migration of NK cells into tumor needs to be an aim of clinical trials, regardless of the stage of the disease; (c) simple administration of a single cytokine/chemokine is unlikely to yield NK migration and clinical benefit; and (d) cytokines/chemokine levels are lower in metastatic
lesions (and cannot be compared to the primary tumor) and this has to be taken into account when designing interventional trials. In detail, the comparison of the NK-specific marker Nkp46 with CD56 revealed that NK-cell quantities cannot be estimated from staining with CD56 alone (19–21). For other gastrointestinal cancers, the available data is ambiguous due to the use of CD56 in stainings. We have shown in our previous work (22), that renal cell cancer tissue contains large numbers of NK cells, contrasting with our findings in CRC. But apparently, the tumor biology for different cancer entities goes along with different patterns of NK-cell presence, pointing to a fundamental difference in the immunological microarchitecture. These differences might be an explanation for the clinical differences and different prognosis of different tumor entities.

We further show that reduced or lost HLA class I expression on tumor cells is not sufficient to recruit NK cells (Supplementary Fig. S4; 23). A gradient of NK cells from normal levels in adjacent normal mucosa down to very few cells in CRC tissue was found across stage UICC I–IV and in adenomas, suggesting that the underlying mechanism is already present in early stages of tumor development. While for the NK cells an early exclusion seems to be decisive for CRC, the other immune cell subsets—and especially T cells—seem to have a not so uniform presence during the different stages of the diseases. The latter may very well reflect the developments along the adenoma–carcinoma–metastasis sequence, the NK-cell exclusion in contrast seems like a pivotal early step. Our data clearly demonstrate that the prognostic impact of CD3, IL-8, and GrmB in CRC is provided by infiltrating T cells and not NK cells.

Trafficking and migration of NK cells into nonlymphoid organs is determined by 3 systems, that is, selectins, adhesion molecules and chemokines. In our expression profiles studies, no significant difference in E-1, L-, and P-selectin expression was found between CRC and normal mucosa tissues suggesting that in the colon, selectins are less important for NK-cell transmigration. In contrast, significantly higher concentrations of ICAM-1 and VCAM-1 were detected in CRC versus adjacent mucosa tissue. VCAM-1/alpha4 integrin and/or ICAM-1/LFA-1 interactions were shown to be important for T-cell binding and transmigration through HEV (24). While these high ICAM-1 and VCAM-1 levels may contribute to the increased T-cell infiltration into CRC tissue, they are obviously insufficient to mediate transmigration of NK cells.

Regarding chemokines, the most information is available from animal models that posulated chemokines to be one of the most potent driving forces for lymphocyte recruitment into tumor tissue (25). But here, higher concentrations of chemokines are found in the tumor without significant NK-cell infiltration in CRC tissue. Unexpectedly, the significantly highest concentrations of attracting chemokines for both CD56bright and CD56dim NK subsets were observed in tumor tissue compared to adjacent mucosa. Thus, the increasing chemokine gradients from adjacent mucosa towards tumor tissue should favor an infiltration of both NK subsets and of T cells due to their overlapping chemokine receptor repertoire (26, 27). Since NK cells are scarce in CRC tissue, chemokines alone seem to be insufficient to recruit or retain NK cells in the tumor. In other studies, the chemokine composition of human CRC and liver metastasis samples was addressed using an ex vivo culture system (28) or at the genome wide expression level (29). Interestingly, similar chemokine concentrations for IL-8 (CXCL8) and IP-10 (CXCL10) were observed in ex vivo cultured metastatic CRC tissue. Although expression levels can only give estimates for protein levels, previous work (29) shows relative changes between tumor and metastasis in accordance with the here reported protein levels.

Remarkably, in nonmalignant tissue like placenta, these chemokines, CXCL12, CX3CL1, and CXCL10 in particular, were shown to mediate recruitment of peripheral NK cells into the decidua (30). Hence, tumor tissue containing high concentrations of these chemokines appears to possess mechanisms that prohibit this chemokine-driven recruitment and other mechanisms than a simple lack of chemokines must be responsible for the exclusion of NK cells from CRC tissue. One explanation could be an unresponsiveness of NK cells to chemokines in the tumor vicinity. Another possibility may be a “chemorepulsion” of NK cells, an effect that has been also proposed as “fugetaxis” in murine melanoma models where high CXCL12 concentrations led to a repulsion of antigen-specific CXCR4+ T cells (31). A similar counterproductive effect has been demonstrated for high concentrations of CXCL8 regarding migration of CD8+ T cells (32). The novel concept of “immune contexture” incorporates several intratumoral parameters like high T-cell levels, high CX3CL1, CXCL10, and CXCL19 expression and low VEGF expression defining the major features associated with an optimal immune reaction that further translates into a prognostic impact (33). However, colon carcinoma cells can produce CXCL8, VEGF, and other factors (34) that may rather support invasion, metastasis formation, and autologous chemotaxis (29, 34). Therefore the chemokine milieu may represent a double-edged sword by simultaneously recruiting immune cells and promoting tumor growth (29). Although only low NK-cell numbers were detectable in CRC, it was of interest to analyze molecules involved in cytotoxicity. High amounts of GrmB and sCD95L were detected in some CRC tissues compared to adjacent mucosa, which may be predominantly derived from infiltrating T cells. This observation indicates a specific activation of effector cells in the tumor microenvironment, also supporting the “immune contexture” concept (33). The concentrations of TRAIL, IFN-γ, TNF-α, and sCD25 were higher in tumor tissue than in adjacent mucosa but did not exceed serum levels suggesting their rather systemic distribution and a limited T- and NK-cell activation.
was no clear Th1, Th2, or Th17 pattern, which would indicate a coordinated immune reaction, instead we found an immunosuppressive microenvironment especially in CRC metastases (Supplementary Fig. S5). Furthermore, the most important NK activation cytokines were very low or absent (Supplementary Fig. S5; ref. 35). IL-18, the most important NK activation cytokine, was very low or absent (Supplementary Fig. S5; ref. 35). IL-18 is very important for NK cell activation, and its absence suggests that NK cells are not activated in CRC.

In conclusion, using the novel approach of in situ quantification of immune cells in combination with quantification of a comprehensive set of immune modulators allowed us to identify distribution patterns between primary tumor, adjacent normal mucosa, metastasis, and serum. The modification or reversal of the exclusion of NK cells from tumor tissue should be addressed in future clinical trials, for instance by an elegant combination of immune activating and promigratory strategies to see whether the abrogation of this NK deprivation would initiate an antitumor immune response in CRC.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed. The study sponsors have no influence in the study design, in the collection, analysis, and interpretation of data.

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