Detailed phenotypic characterization of tumor-infiltrating lymphocytes in colorectal cancer patients and correlations with systemic P53 T cell reactivity
INTRODUCTION

Over the past decade, new research insights substantiate the theory that the immune system plays an important role in the pathophysiology of malignancies. This so-called tumor immuno-surveillance is reflected in the presence and composition of an inflammatory infiltrate, often seen in solid tumors. Tumor-infiltrating lymphocytes (TIL) play a key role in the host reaction against the tumor. The presence and phenotypic composition of TIL have shown to correlate with clinical prognostic parameters in different types of solid malignancies. Moreover, in several epithelial malignancies, like colorectal cancer and ovarian cancer, the exact localization of subgroups of infiltrating cells within the tumor, i.e. direct contact between immune cells and tumor cells, is of significance. In a majority of cases, most TIL reside in the tumor-associated stroma compartment, without direct contact with the malignant epithelial cells. Menon et al suggested that the interaction between immune cells and tumor cells is prevented by the presence of a basal membrane-like structure around the tumor cells. But also other inhibitory factors, i.e. chemokines or expression of immuno-modulating cell surface antigens, are likely to play a role.

Recently, we demonstrated that the presence of P53 reactive lymphocytes in the peripheral blood of pre-operative colorectal cancer patients correlated with an increased infiltration of leukocytes in the primary tumor. To investigate this, we made a detailed study of TIL in a group of colorectal cancer patients in relation to the presence of systemic anti-P53 T cell reactivity. We studied primary tumors of 16 patients using a multi-color immuno-fluorescence silver-gold staining method. TIL were identified using antibodies against CD3, CD8 and CD57 with different immunofluorescent labels, which enabled us to detect seven different lymphocyte phenotypes. Phenotypes were quantified, separate for tumor stroma and tumor epithelium. Intra-tumor epithelial lymphocytes were detected in all samples. Of all detectable intra-epithelial lymphocytes, CD3+CD8−CD57− cells were the dominant cell phenotype (mean 44%; SD 25%; range 5-89%), followed by CD3−CD8+CD57− cells (29%; SD 24%; range 2-85%). In the stromal compartment CD3+CD8−CD57− cells (44%; SD 19%; range 16-76%) were the most common found phenotype, followed by CD3+CD8−CD57− cells (32%; SD 18%; range 6-70%) and CD3−CD8+CD57− cells (8%; SD 7%; range 0-21%). Systemic P53 reactivity was previously studied in the same patient group by determination of P53-peptide specific proliferation/cytokine release in peripheral blood lymphocytes for each patient. Patients with a P53-specific INF-γ response before surgery showed significantly higher total numbers of CD3+CD8−CD57− lymphocytes, i.e. cytotoxic T cells, compared to patients without such a response. Our results suggest that the presence of a systemic T cell type 1 anti-tumor response in colorectal cancer patients is related to a more effective recruitment of effector T-cells in the primary tumor.

ABSTRACT

The efficacy of an anti-tumor response may be reflected by the number and phenotype of tumor-infiltrating lymphocytes (TIL). To investigate this, we made a detailed study of TIL in a group of colorectal cancer patients in relation to the presence of systemic anti-P53 T cell reactivity. We studied primary tumors of 16 patients using a multi-color immuno-fluorescence silver-gold staining method. TIL were identified using antibodies against CD3, CD8 and CD57 with different immunofluorescent labels, which enabled us to detect seven different lymphocyte phenotypes. Phenotypes were quantified, separate for tumor stroma and tumor epithelium. Intra-tumor epithelial lymphocytes were detected in all samples. Of all detectable intra-epithelial lymphocytes, CD3+CD8−CD57− cells were the dominant cell phenotype (mean 44%; SD 25%; range 5-89%), followed by CD3−CD8+CD57− cells (29%; SD 24%; range 2-85%). In the stromal compartment CD3+CD8−CD57− cells (44%; SD 19%; range 16-76%) were the most common found phenotype, followed by CD3+CD8−CD57− cells (32%; SD 18%; range 6-70%) and CD3−CD8+CD57− cells (8%; SD 7%; range 0-21%). Systemic P53 reactivity was previously studied in the same patient group by determination of P53-peptide specific proliferation/cytokine release in peripheral blood lymphocytes for each patient. Patients with a P53-specific INF-γ response before surgery showed significantly higher total numbers of CD3+CD8−CD57− lymphocytes, i.e. cytotoxic T cells, compared to patients without such a response. Our results suggest that the presence of a systemic T cell type 1 anti-tumor response in colorectal cancer patients is related to a more effective recruitment of effector T-cells in the primary tumor.

Recently, we demonstrated that the presence of P53 reactive lymphocytes in the peripheral blood of pre-operative colorectal cancer patients correlated with an increased infiltration of leukocytes in the primary tumor. P53 is considered to be an important tumor-associated antigen for colorectal adenocarcinomas. In that study infiltrating leukocytes were detected with the pan-leukocyte marker CD45. In order to gain more insight in the natural immune response against colorectal tumors we sought to further characterize these tumor-infiltrating leukocytes. For this phenotypic study we used a combination of the markers CD3, CD8 and CD57, which all have shown to correlate with clinical prognosis. CD3 serves as a co-receptor for the T cell receptor and therefore represents T lymphocytes. CD8 serves as a co-receptor for the T cell receptor and is mainly expressed by cytotoxic T cells (CTL). CD57 is also called human natural killer-1 (HNK-1) or LEU7 and is considered an adhesion molecule. It is not exactly clear on what type of cell CD57 is expressed, but it is considered to represent NK cells. By making use of a combined triple-color immuno-fluorescence and immuno-gold-silver stain technique, we were able to characterize seven different phenotypes in a single staining, enabling us to differentiate between T cells, NK cells and CTL, and determine their exact intra-tumoral localization.
Combined bright-field and fluorescent confocal scans were made of 25 representative tumor fields (0.14 mm² per field) in each tissue section. Serially obtained parallel tissue sections were stained with standard haematoxilin-eosin to aid in the histological evaluation of the tissue samples. The confocal laser-scanning Microscope (Zeiss LSM510; Zeiss, Jena, Germany) was used in a multi-track setting. In every slide, Alexa-488 was excited at 488nm and detected using a 505-530 band-pass filter, Alexa-546 was excited at 543nm and detected using a 560-615nm band-pass filter, and Alexa-647 was excited at 633nm and detected using a 650nm long-pass filter. Using these settings, the three different fluorescent signals were detected separately without significant background in the other channels. For the detection of the IGSS we used bright field microscopy using the 633nm laser. The IGSS staining signal for cytokeratin expression was shown in grey tones. For detailed microscope settings, see Vlierberghe et al. (2005).25

Analysis of the scans was performed without knowledge of the clinical outcome and p53 reactivity of the patients. The large number of different cell types, in combination with the need to differentiate between two tumor compartments, made a manual analysis less feasible. This created a need for tools to aid in the analysis of these large amounts of data. Specifically for this purpose, our department of Molecular cell biology, developed a novel software application, resulting in a comprehensive semi-automated computerized scan analysis system. Quantification, localization and phenotypy of TIL were recorded directly into an Excel worksheet, after which they were recalculated into a format suitable for statistical analysis in SPSS.

The reliability of the automated analysis was validated by manual analysis of samples. The manual analysis consisted of the visual recognition, localization and quantification of cells. Cells were phenotyped according to their respective fluorescent signal; i.e. red (CD3⁺CD8⁻CD57⁻), green (CD3⁺CD8⁻CD57⁺), blue (CD3⁻CD8⁺CD57⁻), yellow (CD3⁻CD8⁺CD57⁺), magenta (CD3⁺CD8⁺CD57⁺), cyan (CD3⁻CD8⁺CD57⁺), white (CD3⁺CD8⁺CD57⁺). The automated image analysis software program recognized discrete objects in the scan pictures and returned their individual size and percentage of fluorescent signal. On basis of visual interpreta-
To validate our semi-automated image analysis method, we analyzed a tumor sample both fully manual and semi-automated, as described in the materials and methods section. Overall distribution and quantification of different types of cells showed a close correlation ($R^2 = 0.968$, $p < 0.01$), as shown in figure 2. In a few cases the scan pictures could not be analyzed with the semi-automated image analysis software. These pictures showed large and abundant clustering of cells in close vicinity and the analysis program software could not detect separate cells. Instead, these pictures were manually quantified. Only objects between 5 and 35 μm were used for the analysis.

**Statistical analysis**

All statistical analysis was done using the SPSS software package. The χ² test, Wilcoxon-Mann-Whitney rank-test and Spearman’s correlation were used appropriately. A $p$-value of less than 0.05 was considered statistically significant.

**Tumor Immunology**

**IGSS-IF staining**

The type of lymphocytes, based on three markers, and intra-tumoral localization. Figure 1 shows an example of such a staining and control slide of a human colon carcinoma. The staining method resulted in highly reproducible images with an overall clear insight in the tumor histology and infiltrating cells. All seven different phenotypes that could be found on basis of our primary antibody mix were detected (figure 2). In a few tissue samples we encountered some auto-fluorescent signal. This was mainly due to the presence of erythrocytes in vessels. These structures were morphologically well distinguishable from lymphocytes, as they appeared as black dots in the bright-field setting and often showed their biconcave shape. Background fluorescence from other tumor structures was generally also morphologically clearly distinguishable from specific (lymphocyte) fluorescent signal. We detected some inter- and intra-tumor variations in the expression of cytokeratin. Low or absent expression of cytokeratin was often accompanied with a low tumor differentiation grade.

**Quantification of TIL**

To validate our semi-automated image analysis method, we analyzed a tumor sample both fully manual and semi-automated, as described in the materials and methods section. Overall distribution and quantification of different types of cells showed a close correlation ($R^2 = 0.968$, $p < 0.01$), as shown in figure 2. In a few cases the scan pictures could not be analyzed with the semi-automated image analysis software. These pictures showed large and abundant clustering of cells in close vicinity and the analysis program software could not detect separate cells. Instead, these pictures were manually quantified. Only objects between 5 and 35 μm were used for the analysis.
There was a relative paucity of intra-epithelial CD3-CD8-CD57+ cells (mean 0.9%; SD 1.2%; range 0-3.1%).

In the stromal compartment CD3+CD8+CD57- cells (44%; SD 19%; range 16-76%) were the most common found phenotype, followed by CD3+CD8+CD57+ cells (32%; SD 18%; range 6-70%) and CD3+CD8-CD57- cells (8%; SD 7%; range 0-21%). As the overall intensity of TIL increased, the number of stromal CD3+CD8+CD57- cells decreased ($R^2 = -0.565; p = 0.02$). Compared to tumor stroma, the relative proportions of CD3+CD8-CD57- and CD3+CD8-CD57+ cells in the tumor epithelium were significantly increased ($p = 0.02$ for both; see figure 4). Compared to tumor epithelium on the other hand, numbers of CD3+CD8-CD57- and CD3+CD8+CD57+ cells were significantly increased in the tumor stroma ($p = 0.01$ and 0.004 respectively).

The majority of CD57 positive cells (84%) found in our set of tumors co-expressed CD3, suggesting that CD57+ TIL are mainly T cells. Only a small fraction of the remaining CD57+ TIL without T cell receptor (CD3) expression, co-expressed CD8. The CD3+CD57+ cell types hardly infiltrated in the tumor-epithelium (< 7%) and were mainly found in the tumor stroma.
In our former study we acquired data on p53 specific T helper immunity of peripheral blood lymphocytes before and after surgical excision of the primary tumor in the presently described patient cohort. T helper immunity was determined on basis of p53 specific production of key cytokines INF-γ, IL-4 and/or lymphocyte proliferation. Data on p53 reactivity was available for 15 of our 16 patients.

Seven of these 15 patients showed p53-specific INF-γ production in their pre-surgery PMBC samples. We compared the intensity and phenotype of TIL between patients with and without this p53 specific T helper 1 response before surgery. The mean number of CD3+CD8+CD57+ lymphocytes in both stromal and epithelial compartment was significantly higher in the group of patients with a p53-specific T helper 1 response than in the patients that lacked this response before surgery (Figure 5; mean number $458 \pm 181$ cells/mm² versus $259 \pm 171$ cells/mm²; $p = 0.05$). There was no difference when considering the intra-epithelial or stromal compartment individually.

Analysis of phenotypes in the two different tumor compartments showed that CD3+CD8+CD57+ positive lymphocytes dominated in the tumor epithelium. Moreover, increased numbers of infiltration were mainly due to a higher quota of these cells. This finding seems to confirm the theory that TIL with a cytotoxic T-cell (CTL) phenotype, play an important role in the natural immunity against tumors. It is presumed that these CTL are primed against tumor-associated antigens (TAA) in secondary lymphatic tissue and then recruited into the tumor epithelium. The dominant cell phenotype in the tumor stroma was CD3+CD8-CD57-. Although we did not stain for CD4, presumably the majority of these cells were CD4+ T-helper cells.

P53 is generally found to be an important TAA in colorectal cancer. P53 specific reactivity can be measured through T-cell proliferation and/or cytokine production in the presence of P53 protein or P53-derived peptides. In a previous study we found a correlation between systemic P53 specific INF-γ production and number of infiltrating CD45 positive leukocytes before surgery. This suggested that a T helper 1 directed response led to a higher tumor infiltration of lymphocytes and would, therefore, be beneficial in an anti-tumor immune response. This theory is supported by recent findings in colorectal cancer and ovarian cancer. Using multi-color immunofluorescence-histochemistry analysis, we found a significantly higher number of CD3+CD8+CD57+ TIL in the group of patients with a p53-specific T helper response, compared to patients that lacked this response before surgery. This is consistent with the theory that the main anti-tumor effector cell phenotype is the cytotoxic T cell.
Increased infiltration intensity of these CD3+CD8+CD57- TIL may reflect the expansion and recruitment of these cells after the development of a systemic anti-tumor immune response. We expected to find high numbers of CD3+CD8+CD57- TIL specifically in the tumor epithelium of patients with systemic p53 specific immunity. This was indeed the case, but it did not reach statistical significance (data not shown), probably due to the small number of our study group. Other cell phenotypes that have shown to be of importance for anti-tumor immune response are NK cells and T helper cells. Some studies have put the emphasis on the role of NK cells in tumor immunity. CD57-expression has been considered as a natural killer cell marker and may also represent a characteristic of oligoclonal expansion of immune cells. A recent study showed that CD57+ T cells may act as special regulatory cells, suppressing the activity of conventional T helper cells, particularly T helper 1 cells. Patients with colorectal cancer have shown to display elevated levels of CD57+ T cells in the peripheral blood and at tumor sites. In our study we found CD57+ TIL mostly residing in the tumor stroma. Moreover, the vast majority of CD57+ TIL co-expressed CD3 without CD8, suggesting they are CD4+ T helper cells. The exact role of these cells remains to be investigated.

In summary, we made a detailed analysis of lymphocyte phenotype and localization in primary tumors of a group of colorectal cancer patients. CD3+CD8+CD57- lymphocytes were the dominant cell population in the tumor epithelium. CD3+CD8+CD57- lymphocyte infiltration was correlated to P53 specific T-helper immunity, suggesting that these patients had developed a natural systemic type 1 anti-tumor response, resulting in a more effective recruitment of effector T-cells to the primary tumor.

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REFERENCE LIST


Immunophenotype and activation status of the lymphocytic infiltrate in colorectal tumors with normal and down-regulated HLA class I

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