

## Original article:

### Reduced NK-Cell Activity in Patients with Metastatic Colon Cancer

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#### ABSTRACT

Natural killer cells (NK-cells) are believed to play an essential role in the immune surveillance against tumors and infectious diseases. The role of NK-cells in colon cancer remains obscure, since increased as well as decreased percentages and/or activity of NK-cells in comparison to control patients have been reported. Percentage and cytolytic activity of NK-cells in the peripheral blood were analyzed in 42 patients with colon cancer before surgery and one year thereafter in comparison to control patients with non-malignant diseases. Patients without distant metastasis at the time of diagnosis displayed a significantly increased percentage of NK-cells as well as sustained NK-cell activity in the peripheral blood prior to surgery when compared to control patients. In contrast, patients with metastatic disease at the time of diagnosis displayed significantly decreased NK-cell activity in the peripheral blood when compared to control patients. One year after surgery, patients who remained free of metastasis still displayed sustained NK-cell activity, whereas patients who developed metastasis presented with profoundly decreased levels of NK-cell activity. Further analysis of these patients revealed that patients who developed metastasis within the first year after surgery already displayed reduced NK-cell activity prior to curative colorectal surgery. These observations indicate that metastatic spread of colorectal cancer is associated with decreased NK-cell activity. It remains speculative whether decreased NK-cell activity precedes the development of metastasis and thus may help to identify patients with a high risk of rapid tumor progression following curative colorectal surgery.

**Keywords:** Natural killer cells, cytolytic activity, colon cancer, metastasis

#### INTRODUCTION

Natural killer (NK) cells are CD3<sup>+</sup> lymphocytes, expressing CD16 and/or CD56 and account for up to 15% of human peripheral blood lymphocytes. In addition to their distinct phenotype, NK-cells can be distinguished from naive T and B lymphocytes by their capability to kill a variety of target cells including solid tumor cells, leukemia cells and virus-infected target cells as well as cells without MHC expression (Brittenden et al., 1996; Sandel et al., 2005). Due to the broad range of

potential target cells and the capability of lysing target cells “spontaneously” without prior sensitization, NK-cells are believed to play an essential role in the immune surveillance against tumors and infectious diseases (Brittenden et al., 1996; Hanna et al., 1982).

In malignancy, NK-cells appear to represent a first line of defense against the metastatic spread of tumor cells (Gorelik et al., 1982). Evidence for this hypothesis came from animal studies revealing rapid metastatic spread of cancer in the absence of NK-cells

(Gorelik et al., 1982). In accordance with these observations decreased activity or low numbers of circulating NK-cells in comparison to control patients have been reported to be associated with progression of cancer in humans (Chuang et al., 1990; Gonzalez et al., 1998; Lahat et al., 1989; Kondo et al., 2003). However, there are also reports about unchanged numbers and sustained activity of NK-cells in patients with malignant disease (Aparicio-Pages et al., 1989; Bonilla et al., 1990).

Conflicting results have also been obtained in patients with colon cancer. Decreased NK-cell activity in comparison to control patients has been observed irrespective of disease activity or in patients with advanced disease only. Other studies reported unchanged or even increased NK-cell activity in patients with colorectal cancer (Kondo et al., 2003; Aparicio-Pages et al., 1989; Koda et al., 2003; Tartter et al., 1987; Liljefors et al., 2003). Due to these discrepancies, the prognostic significance of NK-cell numbers and NK-cell activity in patients with colon cancer remains to be fully elucidated.

In the present study NK-cell number and activity in the peripheral blood were analyzed in 42 patients with colon cancer with or without distant metastasis in comparison to control patients with non-malignant diseases. In order to evaluate alterations of the percentage and activity of NK-cells during the course of the disease, all patients with colon cancer were examined prior to surgery and one year thereafter with particular emphasis on metastatic spread of the tumor.

Patients with primary non-metastatic cancer, who remained free of metastasis during the observation period displayed levels of NK-cell activity comparable to control patients prior to surgery and one year thereafter. In contrast, patients who developed metastasis after curative surgery displayed decreased NK-cell activity one year after surgery, thereby resembling patients who presented with metastasis at the time of diagnosis.

Retrospective analysis of the five patients who developed metastasis within one year after surgery revealed that these patients already presented with low levels of NK-cell activity prior to surgery. These observations indicate that metastatic spread of colorectal cancer surgery may be associated with decreased NK-cell activity in the peripheral blood. A possible prognostic significance of decreased NK-cell activity remains to be elucidated.

## MATERIAL AND METHODS

### *Patients studied*

50 patients with colon cancer were included in this study. 6 patients refused the follow-up analysis one year after surgery, 2 patients were lost in follow-up. These 8 patients were excluded from the analysis. The remaining 42 patients with colon cancer were retrospectively divided into three groups according to disease stage and tumor progression during follow-up:

A: patients with initially non-metastatic colon cancer who remained free of metastatic tumor growth within the first year after primary surgery (“without metastasis”, n= 15). This group consisted of 8 men and 7 women with a mean age of  $65\pm 17$  years (range 48-81 years).

B: patients with initially non-metastatic colon cancer who developed metastatic tumor growth within the first year after primary surgery (“metachronous metastasis”, n=5). This group consisted of 2 men and 3 women with a mean age of  $55\pm 18$  years (range 41-79 years).

C: patients with metastatic tumor growth at the time of diagnosis (“synchronous metastasis”, n=22). This group consisted of 12 men and 10 women with a mean age of  $63\pm 24$  years (range 41-88 years).

Patients undergoing surgery for non-malignant disease (cholecystolithiasis,

inguinal hernia or peripheral vascular disease) served as control group (n=20). This group consisted of 9 men and 11 women with a mean age of 59±28 years (range 45-88 years). Patients with benign inflammatory diseases (cholecystitis, colitis, diverticulitis) were excluded from the control group, in order to avoid effects of the inflammatory processes on NK-cell function.

Patients with initially non-metastatic tumor (group A and B) underwent elective colorectal cancer surgery. In all cases diagnosis and stage of disease were confirmed by histopathological examination of the resected specimens. TNM and UICC stages are depicted in Table 1.

**Table 1:** Tumor stages of 42 patients with colon cancer.

	Group A without metastasis, n=15 n (%)	Group B metachronous metastasis, n=5 n (%)	Group C synchronous metastasis, n=22 n (%)
T1	-	-	-
T2	5 (33.3)	1 (20.0)	5 (22.7)
T3	9 (60.0)	2 (40.0)	13 (59.1)
T4	1 (6.7)	2 (40.0)	4 (18.2)
UICC I	4 (26.7)	1 (20.0)	-
UICC II	6 (40.0)	1 (20.0)	-
UICC III	5 (33.3)	3 (80.0)	-
UICC IV	-	-	22 (100.0)

Patients with advanced metastatic colon cancer (group C) were admitted to the hospital either for surgery or chemotherapy. 16 of the 22 patients had liver metastases, three patients presented with pulmonary metastasis and three patients showed ubiquitous tumor growth.

The study was approved by the ethics committee of the Humboldt University and all patients gave written informed consent prior to enrollment in the study.

Venous blood samples were obtained from all patients one day prior to surgery or prior to chemotherapy. Patients of Group A and B were re-examined one year after primary surgery. At that time point patients underwent abdominal ultrasound, chest x-ray, analysis of tumor marker levels (CEA and CA19-9) in the peripheral blood as well as CT scan when indicated in order to screen for distant metastasis.

#### *Isolation of PBL*

Peripheral blood lymphocytes were isolated from heparinized venous blood samples by Ficoll hypaque density gradient centrifugation. All blood samples were

obtained before surgery or before chemotherapy. Isolated lymphocytes were cultured at 37°C and 5% CO<sub>2</sub> (v/v) in RPMI 1640 medium (Gibco BRL, USA) supplemented with 1mM sodium pyruvate, 0.1 mM non-essential amino acids, 2mM L-glutamine, 100U/ml penicillin, 0.1 mg/ml streptomycin, 0.05 mM 2-mercaptoethanol and 5% heat-inactivated fetal calf serum (FCS) (Gibco BRL, USA).

#### *Phenotype analysis of PBL*

Phenotype analysis of peripheral blood lymphocytes was performed by two-color flow cytometry. The following monoclonal antibodies were used: FITC mouse anti-human CD3 (clone UCHT1), PE and FITC mouse anti-human CD16 (3G8) and PE mouse anti-human CD56 (B159) all purchased from Pharmingen (San Diego, CA, USA).

For staining, 100 µl heparinized blood was incubated with FITC and PE-labeled antibodies for 30 min. at 4°C. Red blood cells were lysed by addition of FACS Lysing Solution (Becton Dickinson & Co., Mountain View, CA, USA) for 10 minutes at room

temperature. Cells were washed three times in FACS medium and fixed in 1% paraformaldehyde solution. Two-color cytofluorometric analysis was performed on a FACScan (Becton Dickinson, Mountain View, CA ). A total of 20.000 events were counted, after dead cells and epithelial cells had been excluded by gating on forward and side light scatter. Percentages reported are based on cells in the gated region only. Data were analyzed using Cell Quest software (Becton Dickinson & Co., Mountain View, CA).

#### *Cytotoxicity assay*

Cytotoxicity was measured in a [<sup>51</sup>-Cr] release assay against the human colonic adenocarcinoma cell line DLD-1 and the NK-sensitive cell line K-562. Target cells were labeled with <sup>51</sup>NaCr<sub>2</sub>O<sub>3</sub> (200μCi/5x10<sup>6</sup> cells) (ICN, Eschwege, Germany) for one hour at 37°C and were washed three times in RPMI 1640. Target cells were then plated in triplicates at 2x10<sup>3</sup> cells/well in 96-well microtiter plates. Effector cells were plated into the wells in various numbers in order to give effector:target ratios ranging from 10:1 to 200:1 in a total volume of 200μl. After a 16-h-incubation period at 37°C, cells were pelleted by centrifugation. Supernatants were collected for determination of radioactivity using a γ-counter (1470 Wizard, Wallac, Albertville, USA). Cytotoxicity was calculated as : % target cell lysis = (sample release - spontaneous release) / (maximal release - spontaneous release) × 100. Spontaneous release was < 10%.

#### *Statistics*

For each set of data, the mean and standard error of the mean were calculated. Student's *t*-test was performed to determine differences between the study groups. Significance was reached at p<0.05.

## RESULTS

### *Increased percentage of NK-cells in the peripheral blood of patients with primary non-metastatic colon cancer.*

The first experiments were conducted in order to analyze the percentage of NK-cells in the peripheral blood of patients with primary non-metastatic colorectal cancer in comparison to patients with metastatic disease and control patients (Table 2). In control patients CD3<sup>+</sup>CD16<sup>+</sup> NK-cells accounted for 11.9% of isolated PBL. All patients without metastasis at the time of diagnosis (group A and B) displayed a significantly increased percentage of NK-cells in the peripheral blood when compared to control patients (Table 2). This increased percentage of NK-cells could be detected prior to surgery in patients who remained free of metastasis after surgery (group A) as well as in patients who experienced metastatic tumor spread within the first postoperative year (group B). The latter presented with the highest percentage of NK-cells (21.4%) of all groups before surgery. Patients without metastasis before surgery also showed a slight increase of CD3<sup>+</sup>CD56<sup>+</sup> NK-cells in the peripheral blood when compared to control patients, but this difference did not reach statistic significance. In contrast, patients with already established metastasis (group C) did not display changes in the number of CD3<sup>+</sup>CD16<sup>+</sup> or CD3<sup>+</sup>CD56<sup>+</sup> NK-cells when compared to control patients (Table 2).

One year after surgery, patients who remained free of metastatic tumor growth (group A) still displayed an increased percentage of NK-cells in the peripheral blood (Table 2). In contrast, patients who had developed metastasis (group B) presented with a percentage of NK-cells undistinguishable from control patients, but profoundly decreased in comparison to their preoperative values. This observations show, that metastatic spread of colorectal cancer is not associated with significant alterations of the percentage of NK-cells in the peripheral blood when compared to control patients. However, a decrease of preoperatively

increased percentages of NK-cells may initially non-metastatic colorectal cancer. indicate metastatic spread in patients with

**Table 2:** Percentage of NK-cells in the peripheral blood of patients with colon cancer<sup>a</sup>

	Control n=20	Group A without metastasis n=15	Group B metachronous metastasis n=5	Group C synchronous metastasis n=22
<b>preop</b>				
CD3 <sup>+</sup> CD16 <sup>+</sup>	11.9 ± 6.5	16.4 ± 5.1*	21.4 ± 5.6*	13.6 ± 6.7
CD3 <sup>+</sup> CD56 <sup>+</sup>	12.2 ± 7.0	12.8 ± 7.5	15.2 ± 5.8	11.7 ± 4.8
<b>postop</b>				
CD3 <sup>+</sup> CD16 <sup>+</sup>		17.9 ± 7.3*	13.0 ± 1.8	
CD3 <sup>+</sup> CD56 <sup>+</sup>		18.3 ± 6.5*	11.5 ± 2.0	

<sup>a</sup>Phenotypic analysis of peripheral blood lymphocytes was performed in patients with colon cancer with and without metastasis and control patients prior to surgery and one year thereafter. Utilizing two-color FACS analysis, the percentages of the various lymphocyte subsets were determined. Values are depicted as mean ± SEM of all patients in each group.

\*p<0.05 vs. control patients.

*Cytolytic NK-cell activity is reduced in patients with metastatic colon cancer.*

A characteristic function of NK-cells is their spontaneous cytotoxicity which can be

analyzed in a <sup>51</sup>[Cr]-release assay against the human NK-sensitive K-562 cells (Table 3, Figure 1).

**Table 3:** NK-cell activity of isolated PBL in relation to tumor stage<sup>a</sup>

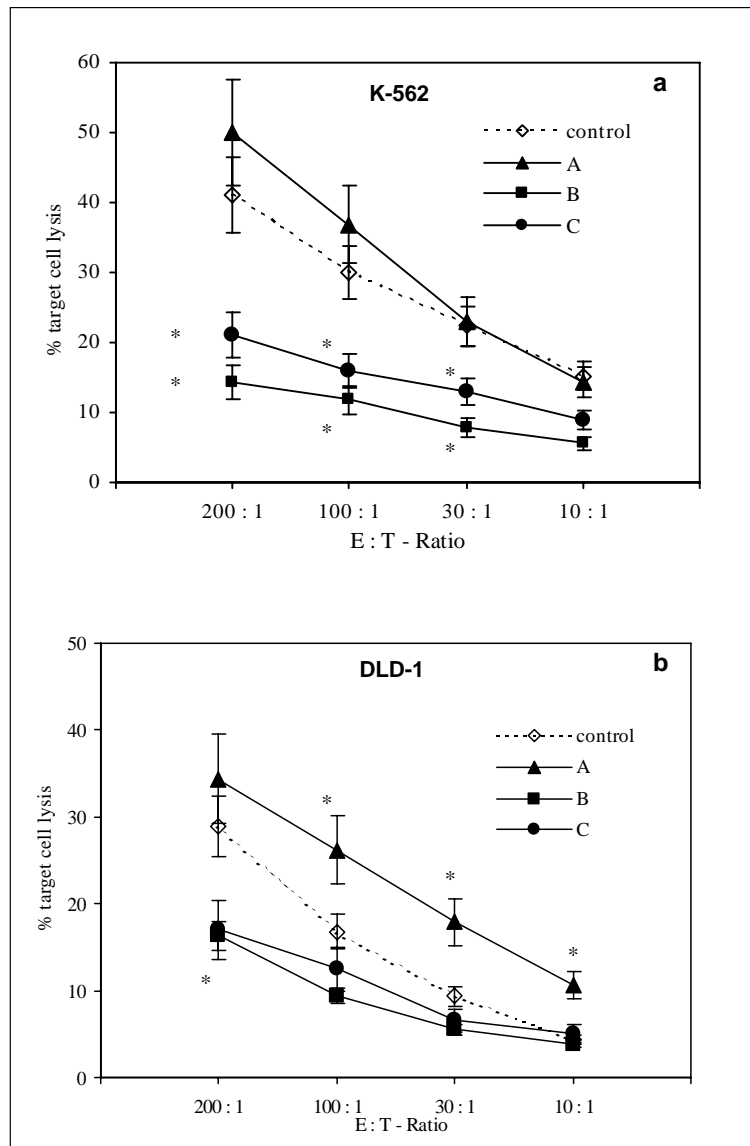
tumor stage	Control n=20	Group A without metastasis n=15	Group B metachronous metastasis n=5	Group C synchronous metastasis n=22
<b>preop</b>				
<b>mean</b>	<b>41.1±5.8</b>	<b>50.0±7.4</b>	<b>11.1±2.5*</b>	<b>21.1±3.6*</b>
UICC I		45.6±6.8	12.5 <sup>#</sup>	
UICC II		56.2±7.9	7.9 <sup>#</sup>	
UICC III		39.4±7.3	11.8±2.7 <sup>†</sup>	
UICC IV				21.1±3.6*
<b>postop</b>				
<b>mean</b>	<b>41.1±5.8</b>	<b>41.6±6.9</b>	<b>14.2±4.8*</b>	<b>21.1±3.6*</b>

<sup>a</sup> PBL were isolated from heparinized venous blood samples and cytolytic activity of the isolated cells was tested against the NK-sensitive cell line K-562 in a [<sup>51</sup>-Cr] release assay. Results are depicted as % target cell lysis at an effector:target ratio of 200:1 (mean±SEM of all patients in each group). <sup>#</sup> group consisted of only one patient. \* p<0.05 versus control

Specific cytotoxicity of PBL was tested against the human adenocarcinoma cells DLD-1. Cytolytic activity was tested prior to surgery and one year thereafter.

PBL from control patients displayed cytolytic activity against K-562 cells (Figure 1a) and to a lesser degree against the human colon carcinoma cells DLD-1 (Figure 1b).

**Figure 1:** Preoperative cytotoxicity of isolated PBL from patients with colon cancer and control patients.



PBL were isolated from heparinized venous blood samples of control patients (open diamonds), patients without metastasis (A, closed triangles), patients with metachronous metastasis (B, closed squares) or patients with synchronous metastasis (C, closed circles) prior to surgery. Cytolytic activity of the isolated cells was tested against the NK-sensitive cell line K-562 (upper panel, a) and the colon carcinoma cell line DLD-1 (lower panel, b) in a  $^{51}\text{-Cr}$  release assay. Results are depicted as % target cell lysis at various effector : target ratios (mean  $\pm$  SEM of all patients in each time group).

\*  $p < 0.05$  versus control patients.

In comparison, PBL from patients with established metastatic tumor growth (Group C) displayed significantly decreased cytolytic activity against both, the NK-sensitive target K-562, and the colon cancer cells DLD-1 (Figure 1a and 1b). This reduced NK-activity in these patients was not

due to a reduced number of peripheral NK-cells, since the percentage of NK-cells in patients with metastatic tumor growth was similar to the percentage of NK-cells in control patients at that time point (Table 2).

Patients with initially non-metastatic colon cancer who remained free of metastasis during the observation period (Group A) were capable of mounting a cytolytic response against K-562 cells preoperatively comparable to control patients (Figure 1a). The cytolytic activity against DLD-1 cells was significantly increased in these patients (Figure 1b). It has to be noted, that patients with non-metastatic tumor growth displayed an increased percentage of NK-cells at that time point (Table 2) suggesting an impaired function of the cytolytic cells. Similar results were obtained one year after surgery. At that time point, the level of NK-cell activity of patients without distant metastasis was again comparable to the level of NK-cell activity of control patients (Table 3).

Different results were obtained from the five patients who experienced metastatic spread after curative surgery (Group B) (Table 3 and Figure 1). They showed a significant reduction of the cytolytic activity of peripheral blood lymphocytes against both targets before surgery, i.e. before the development of metastasis when compared to control patients (Figure 1). This decreased NK-activity was observed despite the significantly increased percentage of NK-cells in the peripheral blood (Table 2) pointing towards a profound disturbance of NK-cell function. One year after surgery, when metastasis had been detected in these five patients, their level of NK-cell activity was again undistinguishable from the NK-cell activity of patients who had presented with metastasis at the time of diagnosis (group C) (Table 3).

These observations suggest that reduced NK-cell activity is associated with metastatic tumor growth in patients with colon carcinoma. A possible prognostic significance of preoperatively reduced NK-cell activity in patients with colorectal cancer could be hypothesized.

## DISCUSSION

Soon after the discovery of NK-cells in 1975 and the description of their spontaneous, so called "natural" cytotoxicity, it was realized that these cells may play an important role in the host defense against tumors (Eremin et al., 1978). Various studies have been carried out since then, trying to establish a connection between tumor spread and percentage or activity of NK-cells. Until now contradictory observations have been made. Many investigators have found either decreased percentages of circulating NK-cells or decreased activity of isolated NK-cells in patients with advanced malignancies when compared to control patients (Brittenden et al., 1996; Chuang et al., 1990; Lahat et al., 1989; Tartter et al., 1987). In contrast, other studies revealed no changes in the cytolytic activity of NK-cells in patients with cancer (Aparicio-Pages et al., 1989; Bonilla et al., 1990; Gati et al., 2001) or even observed an increased activity of NK-cells in tumor patients in comparison to control patients (Liljefors et al., 2003; Nio et al., 1991).

In this study, decreased NK-cell activity in comparison to control patients was observed in patients with metastatic colon cancer and in patients with primary non-metastatic colon cancer who developed distant metastasis within one year after surgery. Both, patients with established metastasis as well as patients who developed metastasis following curative surgery for initially non-metastatic disease presented severely impaired NK-cell activity when compared to patients who remained free of metastatic spread or control patients. Similar observations have been made by Kondo et al. who reported about an increased risk of metachronous colorectal metastasis in patients with decreased NK-cell activity (Kondo et al., 2003). The importance of NK-cells in the host defense against malignant tumors had also been pointed out in other studies which showed that sustained NK-activity was associated with better survival in patients with laryngeal or colon cancer and decreased NK-activity predicted

poor prognosis in patients with metastatic colon cancer (Gonzalez et al., 1998; Liljefors et al., 2003).

Interestingly, the preoperative level of cytolytic activity of NK-cells in patients with colon cancer in this study did not correlate with the tumor stage. Among patients who remained free of metastasis, the level of NK-cell activity was not different between UICC I, II or III patients. Similarly all five patients who later developed metastasis displayed decreased levels of NK-cell activity prior to surgery, irrespective of their UICC stage. And although the significance of this observation is somewhat limited due to the small number of patients in this group, it could be hypothesized that a preoperatively reduced level of NK-cell activity compared to control patients may point towards an increased risk of metastatic tumor growth following curative colorectal surgery. Certainly, this hypothesis needs to be validated in larger studies.

The observation that both, patients with metachronous or synchronous metastasis displayed decreased NK-activity despite normal or even increased percentages of circulating NK-cells when compared to control patients further supports the hypothesis of profound disturbances of NK-cell function in these patients. However, impaired NK-cell function must also be assumed in patients who remained free of metastasis after colorectal surgery. In these patients the sustained level of NK-cell activity was achieved by increased percentages of NK-cells in the peripheral blood, suggesting a reduction of NK-cell activity compensated by the higher number of circulating NK-cells. Similar observations have been made by Berghella et al. (Berghella et al., 1994), who have shown an increased percentage of circulating NK-cells, but decreased activity of the isolated NK-cells in patients with advanced cancer. Suppressing factors, such as soluble MHC class I chain related molecules (MIC) which are downmodulating receptors on NK-cells have been hypothesized to be responsible for

this impairment of NK-cell function in malignant disease (Brittenden et al., 1996; Doubrovina et al., 2003). Furthermore, stress and surgical interventions as well as chemotherapy may account for disturbances of NK-cells in cancer (Koda et al., 2003; Ben-Eliayahu et al., 1990).

In summary, NK-cell activity seems to be an important host defense mechanism against metastatic spread of colon cancer. Our data suggest that decreased NK-cell activity may be associated with metastatic disease. The prognostic significance of decreased NK-cell activity in patients with initially non-metastatic colon cancer needs to be elucidated.

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