

# NKG2D+CD4+ T Cells with Immune Suppressive Property Increase in Patients with Colorectal Cancer

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**Some studies suggest that small populations of CD4+ T cells with activation-independent, constitutive, NKG2D expression are found in normal peripheral blood and have immune suppressive properties. The present study was designed to investigate NKG2D expression on CD4+ T lymphocytes and its relationship to immune evasion in colorectal cancer patients. We examined NKG2D expression on both circulating and tumor-infiltrating CD4+ and CD8+ T cells or NK cells and evaluated it by multicolor flow cytometry. Furthermore, intracellular cytokine staining was carried out to determine the cytokine profile of NKG2D+CD4+ T cells in colorectal cancer patients. As a result, NKG2D expression on circulating and tumor-infiltrating CD8+ T cells and NK cells was downregulated in colorectal cancer patients. On the other hand, circulating and tumor-infiltrating NKG2D+CD4+ T cells increased in colorectal cancer patients. NKG2D+CD4+ T cells produced more immune suppressive cytokines, such as interleukin-10 and transforming growth factor- $\beta$ 1, than did NKG2D-CD4+ T cells. Increased NKG2D+CD4+ T cells as well as decreased NKG2D expression on CD8+ T cells and NK cells may be one of the key mechanisms responsible for immune evasion by tumors in colorectal cancer.**

**Key words:** CD4+ T lymphocyte; colorectal cancer; NKG2D

T cells play an essential role in the immunosurveillance and destruction of tumor cells. Accumulating evidence indicates that specific T-cell immune responses can be raised against many tumors (Boon et al., 1994; Sahin et al., 1997). Nonetheless, attempts to translate this knowledge into clinically effective immunotherapies have met with only limited success (Dunn et al., 2004; Rosenberg et al., 2004), because tumors develop mechanisms that allow them to escape host immune responses (Dunn et al., 2002; Whiteside, 2003). One of the major mechanisms is the activity of T cells with negative immune regulatory function at tumor sites, which can markedly suppress immune responses and induce immune tolerance (Woo et al., 2001; Liyanage

et al., 2002; Curiel et al., 2004; Wang et al., 2004, 2005). Negative immune regulatory activity at tumor sites has typically been attributed to regulatory T (Treg) cells. A high density of tumor-infiltrating Foxp3+ Tregs has been associated with poor outcomes in various solid tumors, including ovarian (Curiel et al., 2004; Sato et al., 2005), pancreatic (Hiraoka et al., 2006), and hepatocellular carcinomas (Gao et al., 2007; Kobayashi et al., 2007). Therefore, it is essential to understand the detailed mechanisms of immune cells with regulatory function in cancer patients to develop more effective immunotherapy.

Recent findings suggest that other T cell subsets can function as suppressors of antitumor

Abbreviations: NK, natural killer; PBMC, peripheral blood mononuclear cell; TIL, tumor infiltrating lymphocyte; Treg, regulatory T

immune responses (Shevach, 2002; Wang, 2006). One such T cell might be the NKG2D+CD4+ T cell. NKG2D is a type II C-lectin-like protein encoded by a gene located next to the NKG2A, NKG2C and NKG2E genes within the natural killer (NK) gene complex on human chromosome 12p12-p13 and mouse chromosome 6 (Glienke et al., 1998). NKG2D is an activating cell surface receptor expressed by NK cells, gamma-delta T cells, some cytolytic CD8+ alpha-beta T cells and NKT cells (Bauer et al., 1999; Vivier et al., 2002; Raulet, 2003; Watzl, 2003). In cancer patients, tumor infiltrating and systemic NK cells and CD8+ T cells often express little NKG2D and are functionally compromised (Groh et al., 2002). On the other hand, small populations of CD4+ T cells with activation-independent, constitutive, NKG2D expression occur in normal peripheral blood (Bauer et al., 1999; Groh et al., 2006; Allez et al., 2007; Sundstrom et al., 2007). Groh et al. (2006) recently demonstrated that increased populations of CD4+ T cells among tumor infiltrating lymphocytes (TILs) and in peripheral blood were positive for NKG2D in cancer patients with breast, lung, colon and ovarian carcinomas and melanomas. They also showed that NKG2D+CD4+ T cells observed in cancer patients appeared biased toward an IL-10- and TGF-dominated cytokine profile, indicating that NKG2D+CD4+ T cells had immune suppressive properties. However, little is known about NKG2D+CD4+ T cells in cancer patients thus far.

Colorectal cancer is the fourth most commonly diagnosed malignancy, with an estimated 1,023,000 new cases and 529,000 deaths each year (Parkin et al., 2005). Considering the important function of immune cells with regulatory function, such as Tregs, in tumor progression and prognosis, it is extremely important to determine the presence of NKG2D+CD4+ T cells with immune suppressive function in colorectal cancer patients. Furthermore, NKG2D expression on CD8+ T cells and NK cells has not yet been determined in colorectal cancer. In the present study, we determined NKG2D expression on CD4+ and CD8+ T cells and NK cells obtained from colorectal cancer patients. We

also analyzed the function of NKG2D+CD4+ T cells to investigate one of the mechanisms responsible for immune evasion in patients with colorectal cancer.

## Materials and Methods

### *Patients and normal donors*

Forty-two patients (22 males and 20 females), treated at Tottori University Hospital and pathologically diagnosed with colorectal cancer, were enrolled in this study. None of the patients received radiotherapy, chemotherapy or any other medical intervention before donating blood. Informed consent for blood donation was obtained from all individuals. Patient characteristics are shown in Table 1. Healthy controls ( $n = 24$ ; 18 males and 6 females) were age-matched ( $62.5 \pm 8.9$  years for the controls versus  $65.3 \pm 9.1$  years for the patients), and each experiment was performed in parallel. The clinicopathological findings were determined according to the Japanese Classification of Colorectal Carcinoma (Japanese Society for Cancer of the Colon and Rectum, 2009).

### *Preparation of peripheral blood mononuclear cells (PBMCs)*

An amount (40 mL) of peripheral blood was drawn from each of the controls and patients before surgery or chemotherapy and PBMCs were separated by centrifugation over a Ficoll-Paque (Pharmacia, Uppsala, Sweden) gradient.

### *Isolation of TILs*

Freshly excised tumor tissues were minced and incubated in 1.5 mg/mL of collagenase D (Wako Pure Chemical Industries, Osaka, Japan). Cell suspensions were then filtered through a mesh filter (BD Falcon, Franklin Lakes, NJ).

**Flow cytometry analysis**

Flow cytometry analysis was performed on a FACSCalibur (Becton Dickinson, Franklin Lakes, NJ), using the following antibodies: anti-CD3-PE-Cy5 (Biolegend, San Diego, CA), anti-CD4-FITC, anti-CD4-PE-Cy5, anti-CD8-FITC, anti-CD56-FITC and anti-NKG2D-PE (BD Pharmingen, Franklin Lakes, NJ). For intracellular cytokine staining, PBMCs were cultured in the presence of either Leukocyte Activation Cocktail (BD Pharmingen) or lipopolysaccharide (Calbiochem, Darmstadt, Germany). Anti-cytokine antibodies were anti-IFN- $\gamma$ -FITC (BD Pharmingen), anti-IL-10-Alexa fluor 488 (eBioscience, San Diego, CA) and anti-LAP-TGF- $\beta$ 1-PerCP (R&D systems, Minneapolis, MN). For the staining of IFN- $\gamma$  and IL-10, cells were fixed and permeabilized with BD Cytotfix/Cytoperm solution.

**Media**

Culture medium consisted of RPMI 1640 (Cambrex Bio Science Walkersville, Walkersville, MD), 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA) and 10% heat-inactivated human serum AB (Gemini Bio-Products, Woodlands, CA).

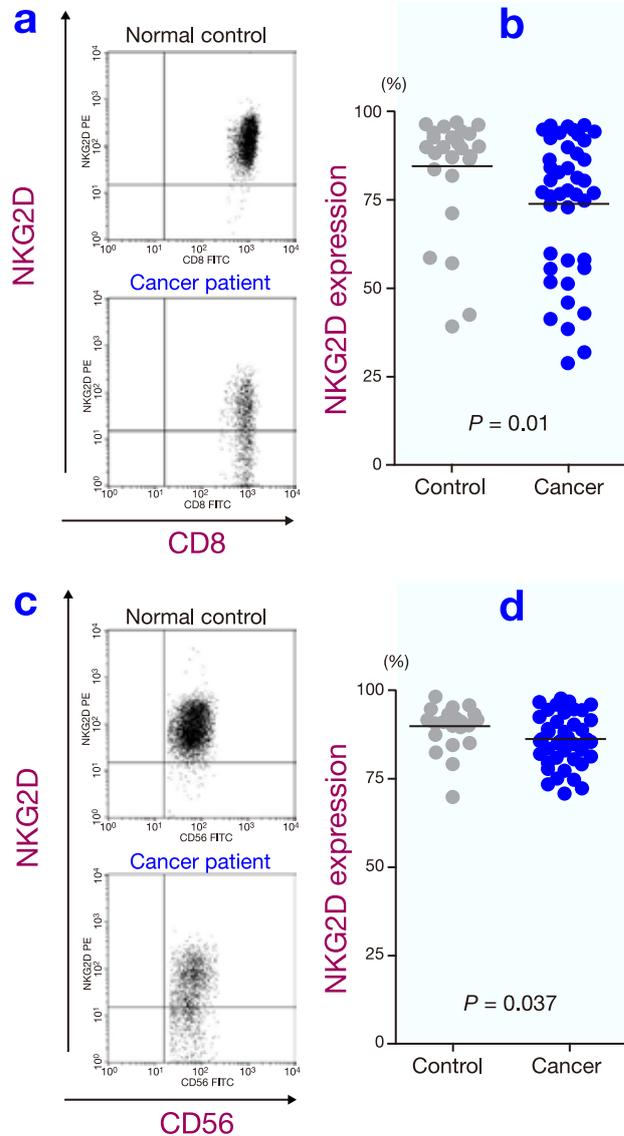
**Statistical analysis**

To determine statistical differences between the 2 groups, either paired *t*-tests or Mann-Whitney *U*-tests were used. The accepted level of significance was  $P < 0.05$ . GraphPad Prism software (GraphPad Software, La Jolla, CA) was used for all statistical analyses.

**Results**

**NKG2D expression on CD8+ T lymphocytes and NK cells in patients with colorectal cancer**

NKG2D expression on circulating CD8+ T lymphocytes and NK cells is downregulated in various



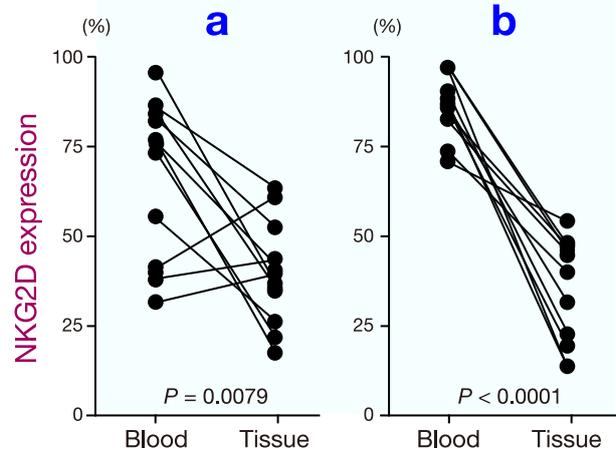
**Fig. 1.** NKG2D expression on circulating CD8+ T cells or NK cells.

- a:** Representative result of NKG2D expression on circulating CD8+ T cells in either normal controls or colorectal cancer patients by FACS.
- b:** NKG2D expression on circulating CD8+ T cells in colorectal cancer patients was significantly lower than in normal controls ( $P = 0.01$ ).
- c:** Representative result of NKG2D expression on circulating NK cells in either normal controls or colorectal cancer patients by FACS.
- d:** NKG2D expression on circulating NK cells in colorectal cancer patients was significantly lower than in normal controls ( $P = 0.037$ ).

types of cancer patients, but this has not yet been determined in colorectal cancer. Therefore, we first determined NKG2D expression on circulating CD8+ T lymphocytes and NK cells in both normal controls and colorectal cancer patients. NKG2D expression of circulating CD8+ T lymphocytes in colorectal cancer patients ( $73.6 \pm 19.6\%$ ) was significantly lower than that in normal controls ( $84.5 \pm 16.0\%$ ;  $P = 0.01$ ; Figs. 1a and b). Furthermore, NKG2D expression of circulating NK cells in colorectal cancer patients ( $86.3 \pm 7.3\%$ ) was significantly lower than those in normal controls ( $90.0 \pm 6.2\%$ ;  $P = 0.037$ ; Figs. 1c and d).

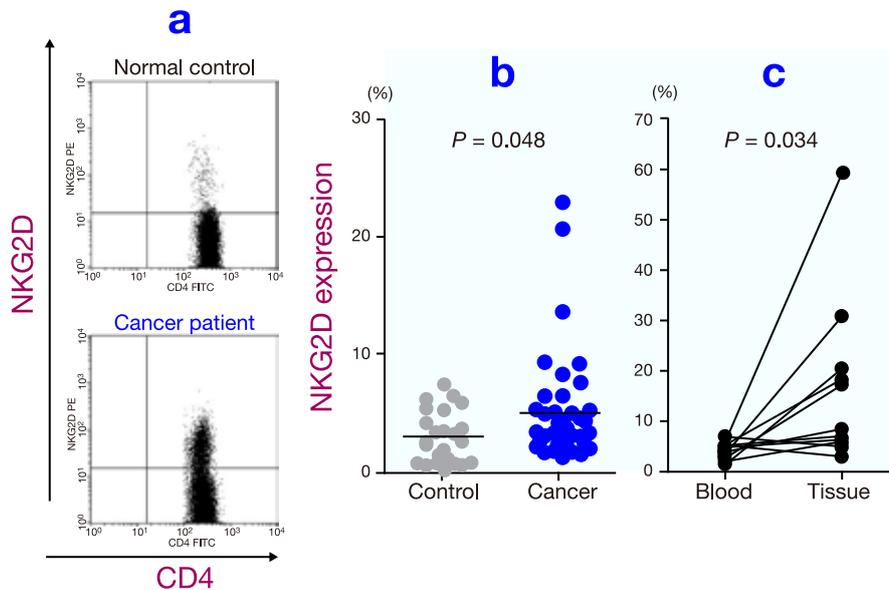
**NKG2D expression on CD8+ T cells and NK cells in the tissue of colorectal cancer**

We then determined NKG2D expression on CD8+ T cells and NK cells obtained from colorectal cancer tissue. NKG2D expression on CD8+ T cells in the tissue of colorectal cancer ( $40.0 \pm 14.9\%$ ) was significantly lower than that of circulating CD8+ T



**Fig. 2.** NKG2D expression on tumor infiltrating CD8+ T cells or NK cells.

- a: NKG2D expression on CD8+ T cells in tissue of colorectal cancer was significantly lower than that on circulating CD8+ T cells ( $P = 0.0079$ ).
- b: NKG2D expression on NK cells in tissue of colorectal cancer was significantly lower than on circulating NK cells ( $P < 0.0001$ ).



**Fig. 3.** NKG2D expression on CD4+ T cells.

- a: Representative result of NKG2D expression on circulating CD4+ T cells in either normal controls or colorectal cancer patients by FACS.
- b: The frequency of NKG2D+CD4+ T cells in colorectal cancer patients was significantly higher than in normal controls ( $P = 0.048$ ).
- c: The frequency of NKG2D+CD4+ T cells in tissue of colorectal cancer was significantly higher than in circulating CD4+ T cells ( $P = 0.034$ ).

cells ( $67.7 \pm 22.0\%$ ;  $P = 0.0079$ ; Fig. 2a). NKG2D expression on NK cells in the tissue of colorectal cancers ( $34.8 \pm 15.1\%$ ) was also significantly lower than that of circulating NK cells ( $86.6 \pm 8.9\%$ ;  $P < 0.0001$ ; Fig. 2b).

**Increased NKG2D+CD4+ T cells among TILs and in peripheral blood in colorectal patients**

CD4+ T cells in peripheral blood were significantly more positive for NKG2D in colorectal cancer patients ( $5.0 \pm 4.6\%$ ) than that in normal controls ( $3.0 \pm 2.2\%$ ;  $P = 0.048$ ; Figs. 3a and b). There were significantly more NKG2D+CD4+ T cells in the tissue of colorectal cancers ( $16.5 \pm 16.6\%$ ) than those in peripheral blood ( $4.1 \pm 1.7\%$ ;  $P = 0.034$ ; Fig. 3c).

Table 1 shows the correlation between the frequency of NKG2D+CD4+ T cells and various clinicopathological factors. No significant differences in the frequency of NKG2D+CD4+ T cells were observed in terms of depth of invasion, lymph node metastasis, liver metastasis and stage of disease.

**Cytokine profile of NKG2D+CD4+ T cells in patients with colorectal cancer**

Finally, we determined the cytokine profile of NKG2D+CD4+ T cells to show their immune suppressive function. NKG2D+CD4+ T cells produced significantly less IFN- $\gamma$  than NKG2D-CD4+ T cells (Fig. 4a). On the other hand, expression of the immune suppressive cytokines, IL-10 and

**Table 1. The frequency of NKG2D+CD4+ T cells and clinicopathological characteristics in colorectal cancer patients**

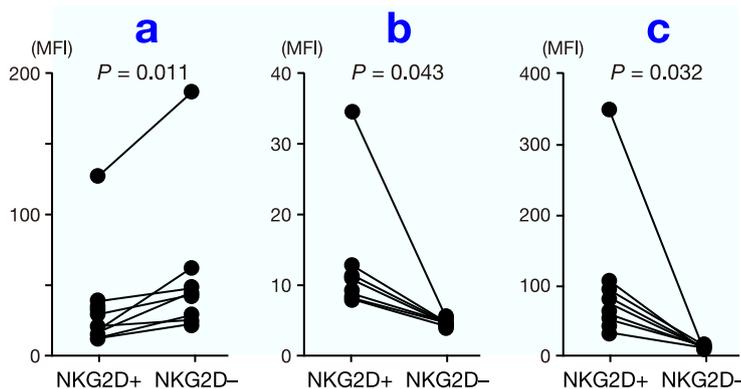
	Number of patients	NKG2D+CD4+ T cells (%)	P value
<b>Depth of invasion*</b>			
M/SM/MP	6	$7.4 \pm 6.9$	0.22
SS/SE/SI (A/AI)	25	$3.9 \pm 1.9$	
<b>Lymph node metastasis</b>			
Absent	17	$5.0 \pm 4.4$	0.71
Present	14	$4.1 \pm 2.3$	
<b>Lymphatic invasion†</b>			
ly0/1	14	$5.7 \pm 4.9$	0.24
ly2/3	17	$3.7 \pm 1.7$	
<b>Vascular invasion‡</b>			
v0/1	14	$5.6 \pm 4.8$	0.21
v2/3	17	$3.7 \pm 2.0$	
<b>Liver metastasis</b>			
Absent	28	$4.6 \pm 3.7$	0.53
Present	3	$5.0 \pm 2.6$	
<b>Stage of disease</b>			
Stage I/II	17	$5.0 \pm 4.4$	0.71
Stage III/IV	14	$4.1 \pm 2.3$	

\* M, tumor invasion of mucosa; SM, tumor invasion of sub-mucosa; MP, tumor invasion of muscularis propria; SS, tumor invasion of subserosa; SE, tumor invasion of serosal; SI, direct tumor invasion of other organs or structures; A, tumor invasion through muscularis propria; AI, direct tumor invasion of other organs or structures.

† Lymphatic invasion: ly0–ly3, grade of lymphatic vessel invasion.

‡ Vascular invasion: v0–v3, grade of vascular invasion.

TGF- $\beta$ 1, by NKG2D+CD4+ T cells was significantly increased compared to NKG2D-CD4+ T cells (Figs. 4b and c).



**Fig. 4.** Cytokine profile of CD4+ T cells.

- a:** IFN-gamma.
- b:** IL-10.
- c:** TGF-beta 1.

MFI, mean fluorescence intensity.

## Discussion

CD8+ T cells and NK cells are thought to play an important role in the control of tumors as a result of their cytotoxic activity and by releasing soluble factors. It has been reported that the functions of CD8+ T cells and NK cells are impaired in cancer patients, which is related to immune evasion by cancer. Although the detailed mechanisms responsible for impaired function of CD8+ T cells and NK cells remain unclear, recent studies demonstrated that decreased NKG2D expression on CD8+ T cells and NK cells was closely related to this phenomenon (Groh et al., 2002; Wu et al., 2004). In the present study, we demonstrated that the NKG2D expression of circulating CD8+ T cells and NK cells in colorectal cancer patients was significantly lower than that in normal controls. Furthermore, the reduction in NKG2D expression on CD8+ T cells and NK cells in the tissue of colorectal cancer was more striking than in peripheral blood. We have previously shown that NKG2D expression significantly correlated with IFN- $\gamma$  production in CD8+ T cells in patients with gastric cancer, indicating that downregulated NKG2D expression is closely related to the low responsiveness of CD8+ T cells to cancers (Osaki et al., 2007). Moreover, Groh et al. (2002) reported that NKG2D-low MART-1 specific CD8+ T cells isolated from TILs from a MIC-positive melanoma showed no or little induction of IFN- $\gamma$  after stimulation with MART-1 peptide, while a substantial proportion of identically treated NKG2D high MART-1-specific T cells from a MIC-negative melanoma produced a strong IFN- $\gamma$  response. With regard to the function of NK cells, it has been demonstrated that downregulated NKG2D expression correlated with a reduction in cytotoxic activity by NK cells (Wu et al., 2004). Therefore, the downregulated NKG2D expression of CD8+ T cells and NK cells observed in cancer tissue and peripheral blood in the present study might be one of the key mechanisms by which colorectal cancer impairs the function of immune cells such as CD8+ T cells and NK cells.

NKG2D can be induced on human CD4+ T cells by TCR-CD3 complex stimulation (Groh et al., 2003). A previous study demonstrated that increased populations of CD4+ T cells among TILs and in peripheral blood were positive for NKG2D in cancer patients with breast, lung, colon and ovarian carcinomas and melanomas (Groh et al., 2006). In the present study, we have also demonstrated that the frequency of NKG2D+CD4+ T cells in patients with colorectal cancer is significantly more than in normal controls. Furthermore, the increased population of NKG2D+CD4+ T cells in the tissue of colorectal cancer was more striking than that in peripheral blood. Those increased NKG2D+CD4+ T cells produced less IFN- $\gamma$  than NKG2D-CD4+ T cells. On the other hand, NKG2D+CD4+ T cells produced more immunosuppressive cytokines, such as IL-10 and TGF- $\beta$ 1 than NKG2D-CD4+ T cells, indicating that the increased number of NKG2D+CD4+ T cells we observed in colorectal cancer patients in the present study had immunosuppressive properties and might correlate with immune evasion in colorectal cancer patients. To the best of our knowledge, this study is the first to demonstrate decreased NKG2D expression on CD8+ and NK cells and an increased population of NKG2D+CD4+ T cell with immune suppressive function in colorectal cancer.

The role of increased NKG2D+CD4+ T cells we observed in the present study in clinical settings remains unclear. In fact, the increased frequency of NKG2D+CD4+ T cells in peripheral blood was not correlated with disease progression, indicating that an increase in NKG2D+CD4+ T cells in peripheral blood might be an early event in the progression of colorectal cancer. On the other hand, some paper demonstrated that the presence of immune cells with immunosuppressive properties, such as Regulatory T cells which were characterized by CD4+Foxp3+ T cells, in the tissue of carcinoma was associated with poor outcome in various solid tumors, including ovarian (Curiel et al., 2004; Sato et al., 2005), pancreatic (Hiraoka et al., 2006), and hepatocellular carcinoma (Kobayashi et al., 2007; Gao et al., 2007). In fact, the increased

population of NKG2D+CD4+ T cells in the tissue of colorectal cancer was more striking than that in peripheral blood in the present study. Although we were not able to determine the prognostic significance of tumor-infiltrating NKG2D+CD4+ T cells due to small number of data, it is likely that tumor-infiltrating NKG2D+CD4+ T cells is associated with poor prognosis in colorectal cancer. Further investigation to show the prognostic significance of increased tumor-infiltrating NKG2D+CD4+ T cells is imperative.

The mechanisms by which NKG2D+CD4+ T cells increase remains unclear in the present study. In this regard, Groh et al. (2006) demonstrated that expansion of the NKG2D+CD4+ T cell population was dependent on the presence of tumor-associated MICA and correlates with serum concentrations of sMICA in cancer patients. In the present study, however, there was no difference in the concentration of sMICA between normal controls and colorectal cancer patients (data not shown). We are currently planning to perform some experiments to investigate the detailed mechanisms that induce expansion of NKG2D+CD4+ T cells in colorectal cancer patients.

In conclusion, our data demonstrate decreased NKG2D expression on both CD8+ T cells and NK cells and an increase in the NKG2D+CD4+ T cell population in colorectal cancer patients. NKG2D+CD4+ T cells exhibit immune suppressive function by producing cytokines, such as IL-10 and TGF- $\beta$ 1. Therefore, the MIC-NKG2D system might more exclusively contribute to immunosuppression by tumors in colorectal cancer. On the other hand, the mechanisms by which NKG2D+CD4+ T cells increase are still unknown. Further investigation to show the mechanisms leading to increased NKG2D+CD4+ T cells is urgently required.

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