Peripheral Foxp3+ regulatory T cells and natural killer group 2, member D expression levels in natural killer cells of patients with colorectal cancer

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Abstract. Foxp3+ regulatory T cells (Tregs) and natural killer group 2, member D (NKG2D)-positive natural killer (NK) cells are considered to be important in the immune escape of colorectal cancer (CRC). However, the association between these two variables remains obscure. Therefore, in the present study, the levels of peripheral Tregs and NKG2D expression in NK cells and the associations in CRC patients were investigated. A total of 35 CRC patients and 16 healthy controls were enrolled in this study. Flow cytometry was performed to assay Treg numbers and NKG2D expression levels in NK cells in peripheral blood samples. Serum carcino-embryonic antigen (CEA) protein was assayed by electrochemiluminescence. Peripheral Treg numbers were significantly increased ($P<0.05$), while NKG2D expression levels in NK cells were significantly reduced ($P<0.01$) in CRC patients compared with healthy controls. However, no significant differences were identified in Treg numbers between CRC patients with and without lymph node metastases and between CRC patients with different clinical stages of CRC. Similarly, no significant differences were detected in NKG2D expression levels in NK cells between the different patient groups. Statistical analysis revealed that increased Treg numbers were not correlated with reduced NKG2D expression levels in NK cells from CRC patients. In addition, no statistical correlation was observed between Treg numbers and serum CEA protein in CRC patients. In conclusion, the upregulation of Tregs was not significantly correlated with the downregulation of NKG2D expression in NK cells in peripheral blood from CRC patients.

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Introduction

$CD4^+CD25^+$ regulatory T cells (Tregs) are a specific subset of $CD4^+$ T cells, and it has been suggested that Tregs are important in maintaining immune self-tolerance and in tumor immune escape (1). Forkhead box protein 3 (Foxp3) has been identified as a $CD4^+CD25^+$ T cell-specific transcription factor and has been shown to be widely expressed in this cell type. Notably, Foxp3 regulates the development and function of Tregs. A number of studies have observed that Tregs may suppress the functions of $CD8^+$ T cells, natural killer (NK) cells, natural killer T (NKT) cells and dendritic cells by cell contact and transforming growth factor-β-dependent mechanisms (1-8) and that depletion of Tregs may result in effective antitumor immune responses (9-12). Patients with different types of cancer, including colorectal, ovarian, prostate and hepatocellular cancer, exhibit increased numbers of Tregs in the peripheral blood and tumor microenvironment; notably, the number of Tregs may have significant prognostic importance (13-18). Thus, Treg-targeting agents are currently being investigated in clinical trials and are a promising approach for cancer treatment (19-21).

NK cells are important innate effectors that defend the host against viruses, bacteria, parasites and tumor cells. The NK cells respond to immune stimuli by killing or ignoring target cells, depending on the balance between activating and inhibitory signals. These signals are produced by the interactions of activating and inhibitory receptors expressed on NK cells with the corresponding ligands expressed on target cells. Natural killer group 2, member D (NKG2D) is a crucial activating receptor located on NK cells and possibly on the surface of $CD8^+$ T cells, γδ T cells, NKT cells and certain activated $CD4^+$ T cells. NKG2D recognizes and binds corresponding ligands, including major histocompatibility complex class I-related antigens A and B and UL16-binding proteins expressed on tumor cells, and subsequently generates activating signals, triggering NK cells to kill the target cells.

A number of studies have demonstrated that NKG2D is critical for tumor surveillance (22-26). In a previous study, NKG2D expression levels were found to be significantly downregulated in NK cells collected from the peripheral blood of patients with colorectal cancer (CRC) and inhibition of the NKG2D signaling pathway with anti-NKG2D antibodies resulted in notably reduced cytotoxicity as well as CD107a degranulation in *ex vivo* experiments. Thus, reduced NKG2D expression levels may be associated with the suppression of NK cell activity in CRC (27).

Although the key role of Tregs as an immunosuppressive cell population is widely accepted, the association between Tregs and NKG2D expression levels in NK cells in CRC requires evaluation. In the present study, peripheral Treg numbers and NKG2D expression levels in NK cells were assayed in parallel using flow cytometry and the association between these variables in CRC patients was determined.

Materials and methods

Patients and controls. A total of 35 patients (18 males and 17 females) with primary CRC and 16 healthy donors (8 males and 8 females) were enrolled in this study. Patients were hospitalized in the gastrointestinal surgery ward of Shandong Provincial Hospital Affiliated to Shandong University (Jinan, China). The CRC diagnosis in these patients was performed according to the Colorectal Cancer Diagnosis Standard (2010) issued by the Ministry of Health, China. The patients were classified as limited CRC or metastatic CRC according to whether lymph node metastases were present. As determined by clinical classification, the patients were stratified into early and advanced CRC. No patients had received chemotherapy and/or radiotherapy prior to sample collection. The clinical characteristics of the enrolled subjects are summarized in Table I. Informed consent was obtained from each participant. The protocol was approved by the Ethics Committee of Shandong Provincial Hospital Affiliated to Shandong University.

Flow cytometry. Tregs were assayed with a regulatory T cell kit (eBioscience, San Diego, CA, USA) according to the manufacturer's instructions. For surface antigen staining, fluorescein isothiocyanate-conjugated anti-human CD4 and allophycocyanin (APC)-conjugated anti-human CD25 antibodies (eBioscience) were added to 100 μl whole blood and incubated for 30 min in the dark at 4˚C. Subsequently, 1X red blood cell (RBC) lysis buffer (eBioscience) was added to whole blood samples at room temperature in order to lyse RBCs. For intracellular antigen staining, fixation/permeabilization working solution (eBioscience) was added to the cells and incubated for 60 min in the dark prior to staining the cells with phycoerythrin (PE)-conjugated anti-human Foxp3 antibodies (eBioscience). Isotype controls were also run in parallel.

For detecting the NKG2D expression levels in NK cells, PE-conjugated anti-human CD3 (BD Biosciences, Missisauga, ON, Canada), APC-conjugated anti-human CD56 (BD Biosciences) and Alexa Fluor 488-conjugated anti-human NKG2D (R&D Systems, Minneapolis, MN, USA) antibodies were used. Isotype controls were concurrently run.

Table I. Clinical characteristics of enrolled subjects.

Group	Patients with CRC	Healthy controls
No. of cases	35	16
Sex (male)	18 (51%)	8 (50%)
Age (years)[a]	58.7±2.3	55.2±4.5
Limited CRC	17	
Metastatic CRC	18	
Early stage (I, II)	20	
Advanced stage (III, IV)	15	
CEA>10 ng/ml	10/29	

[a]Median ± standard deviation; CEA, carcino-embryonic antigen.

At least 10,000 cells were analyzed using a BD FACS Canto II flow cytometer (BD Biosciences).

Serum carcino-embryonic antigen (CEA) assay. Serum CEA protein in CRC patients was routinely assayed using the electrochemiluminescence method with a Roche Cobas e601 system (Roche Diagnostics, Mannheim, Germany) at the Department of Medical Biochemistry, Shandong Provincial Hospital Affiliated to Shandong University. In healthy subjects, CEA<10 ng/ml was considered to indicate a normal level of CEA.

Statistical analysis. The data were analyzed using GraphPad Prism software (GraphPad Software, La Jolla, CA, USA). Student's t-test was used for comparing quantitative variables between two groups. Spearman correlation analysis was performed to determine associations between two variables. $P<0.05$ was considered to indicate a statistically significant difference.

Results

$CD4^+CD25^+Foxp3^+$ Tregs are significantly upregulated in peripheral blood from CRC patients. To evaluate the numbers of Tregs in peripheral blood, flow cytometric analysis was performed on samples from 35 CRC patients and 16 healthy controls. A significant increase was detected in the $CD4^+CD25^+Foxp3^+$ Treg population in the CRC group (7.389±0.5810 in CRC patients vs. 5.169±0.4370 in healthy controls; $P<0.05$; Fig. 1A and B). While no statistically significant difference was observed in Treg numbers between patients with limited and metastatic CRC, the data revealed a tendency for the number of Tregs to be higher in metastatic CRC than in limited CRC (8.233±1.008 vs. 6.435±0.4896, respectively; $P>0.05$; Fig. 1C). Similarly, no significant difference was identified in the Treg numbers between early and advanced CRC patients (data not shown).

$CD4^+CD25^{high}Foxp3^+$ cells are upregulated in peripheral blood samples from CRC patients. Since $CD4^+CD25^{high}Foxp3^+$ cells have been commonly identified as Tregs, the numbers of this subpopulation of cells were also analyzed. The

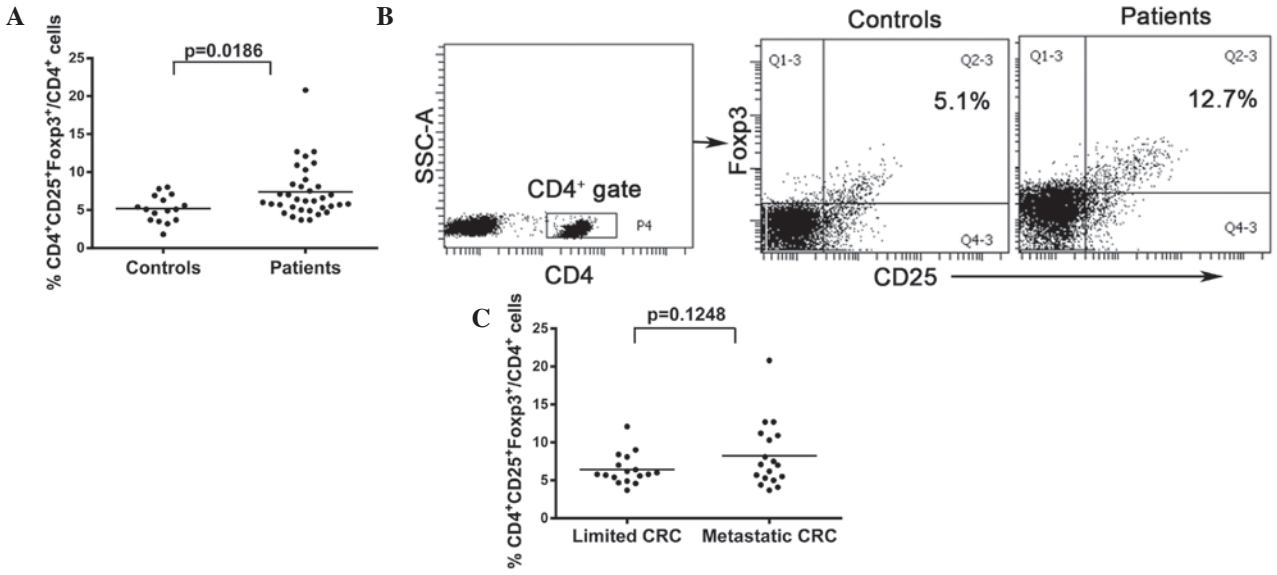

Figure 1. Numbers of $CD4^+CD25^+Foxp3^+$ Tregs in peripheral blood from patients with colorectal cancer (CRC) and healthy controls (A) Percentages of $CD4^+CD25^+Foxp3^+$ cells/$CD4^+$ cells from CRC patients (n=35) and healthy controls (n=16) as determined by flow cytometry. Each dot represents a subject. (B) Representative plots of Tregs from CRC patients and healthy controls. (C) Percentages of $CD4^+CD25^+Foxp3^+$ cells/$CD4^+$ cells from limited (n=17) and metastatic (n=18) CRC patients. Values are presented as the mean ± standard deviation.

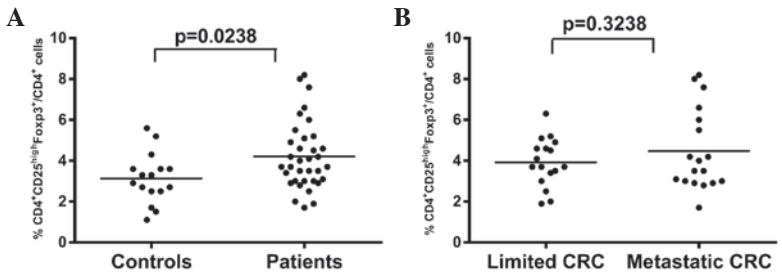

Figure 2. Numbers of peripheral $CD4^+CD25^{high}Foxp3^+$ cells from patients with colorectal cancer (CRC) and healthy controls (A) Percentages of $CD4^+CD25^{high}Foxp3^+$ cells/$CD4^+$ cells from CRC patients (n=35) and healthy controls (n=16) were compared. (B) Percentages of $CD4^+CD25^{high}Foxp3^+$ cells/$CD4^+$ cells from limited (n=17) and metastatic (n=18) CRC patients were also compared. Each dot represents a subject. Values are presented as the mean ± standard deviation.

numbers of $CD4^+CD25^{high}Foxp3^+$ cells in CRC patients were significantly increased compared with those in healthy controls (4.211±0.2793 vs. 3.131±0.3063, respectively; P<0.05; Fig. 2A). However, no significant differences in the numbers of $CD4^+CD25^{high}Foxp3^+$ cells between limited and metastatic CRC patients were identified (3.924±0.2855 vs. 4.483±0.4712, respectively; P>0.05; Fig. 2B). Thus, the data demonstrate that peripheral $CD4^+CD25^{high}Foxp3^+$ cells were increased in CRC patients, while no significant differences were observed between CRC patients with and without lymph node metastases.

$CD4^+CD25^{high}Foxp3^+$ cells are more frequent than $CD4^+CD25^{low}Foxp3^+$ cells in CRC patients and healthy controls. As $CD4^+CD25^+Foxp3^+$ cells may be divided into $CD4^+CD25^{high}Foxp3^+$ and $CD4^+CD25^{low}Foxp3^+$ subsets, the numbers of cells in each of these subsets were compared in peripheral blood samples from CRC patients and healthy controls. The numbers of $CD4^+CD25^{high}Foxp3^+$ cells were significantly higher than those of $CD4^+CD25^{low}Foxp3^+$ cells in CRC patients (P<0.001) and healthy controls (P<0.001; Fig. 3). However, the levels of $CD4^+CD25^{low}Foxp3^+$ cells did not differ significantly between CRC patients and healthy controls (1.483±0.1357 vs. 1.238±0.1455, respectively; P>0.05; Fig. 3). These data reveal that $CD4^+CD25^{high}Foxp3^+$ cells were the dominant cell type in the $CD4^+CD25^+Foxp3^+$ population in CRC patients and healthy controls.

Peripheral NKG2D expression is significantly downregulated in NK cells from CRC patients. NKG2D is an important activating receptor in NK cells and may be critical in the process of NK cytotoxicity. To investigate the expression levels of NKG2D in NK cells, flow cytometric analysis of samples from 20 CRC patients and 10 healthy controls was performed. NKG2D expression levels in NK cells were significantly lower in CRC patients than in healthy controls (73.14±1.758 vs. 82.56±1.569, respectively; P<0.01; Fig. 4A and B). However, no differences in NKG2D expression levels were observed between NK cells collected from limited and metastatic CRC patients (Fig. 4C).

$CD4^+CD25^+Foxp3^+$ and $CD4^+CD25^{high}Foxp3^+$ Tregs are not correlated with NKG2D expression levels in NK cells collected

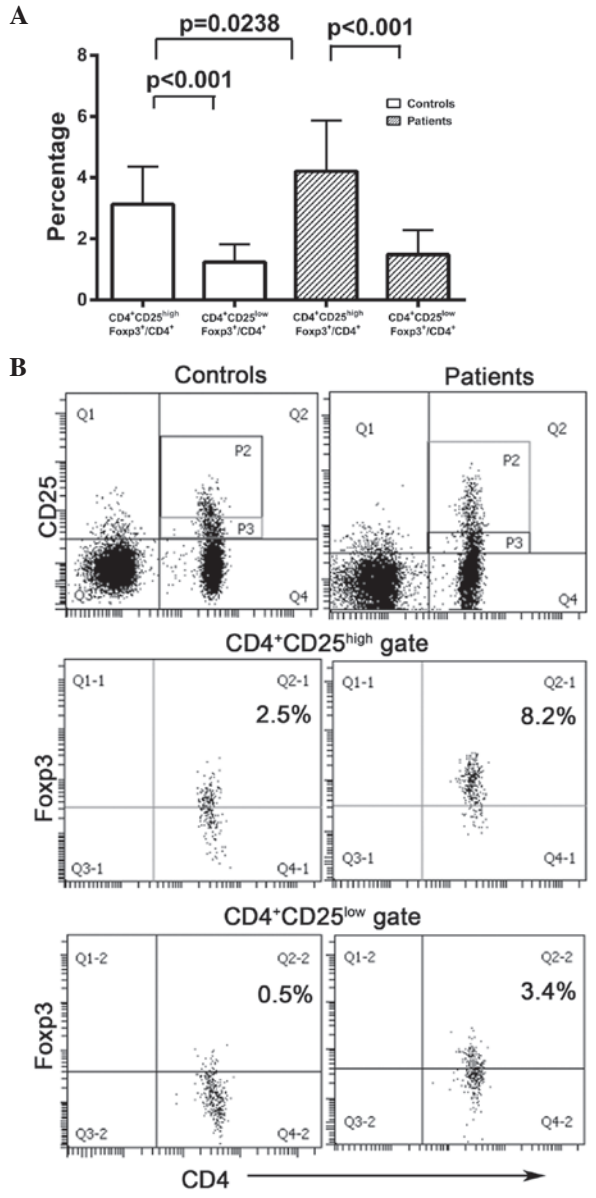

Figure 3. Comparison of numbers of peripheral $CD4^+CD25^{high}Foxp3^+$ cells and $CD4^+CD25^{low}Foxp3^+$ cells from patients with colorectal cancer (CRC) and healthy controls (A) Percentages of $CD4^+CD25^{high}Foxp3^+$ cells/$CD4^+$ cells and $CD4^+CD25^{low}Foxp3^+$ cells/$CD4^+$ cells were compared from CRC patients (n=35) and healthy controls (n=16). (B) Representative plots of $CD4^+CD25^{high}Foxp3^+$ cells and $CD4^+CD25^{low}Foxp3^+$ cells from CRC patients and healthy controls.

from the peripheral blood of CRC patients. The above results indicate that increased Treg numbers and reduced NKG2D expression levels in NK cells are found in CRC patients in comparison with healthy controls. Therefore, the association between these two variables in CRC patients was evaluated. Spearman correlation analysis identified no significant correlations between $CD4^+CD25^+Foxp3^+$ Treg numbers and NKG2D expression levels in NK cells (P>0.05; Fig. 5A) or between $CD4^+CD25^{high}Foxp3^+$ Treg numbers and NKG2D expression levels in NK cells (P>0.05; Fig. 5B) in CRC patients. In conclusion, these results indicate that upregulation of Tregs was not correlated with downregulation of NKG2D expression in NK cells from the peripheral blood of CRC patients.

Peripheral $CD4^+CD25^+Foxp3^+$ and $CD4^+CD25^{high}Foxp3^+$ Tregs are not correlated with serum CEA protein in CRC patients. Serum CEA protein is detected at a high frequency in CRC patients at Shandong Provincial Hospital Affiliated to Shandong University. Among 29 CRC patients analyzed prior to surgery, 10 exhibited increased serum CEA levels (>10 ng/ml) and the highest CEA level was 1,000 ng/ml. Spearman correlation analysis was performed to analyze the association between Tregs and CEA. No statistical significance was observed in the full dataset (P>0.05; Fig. 6). However, in advanced CRC patients, elevated Treg numbers and CEA levels were observed (data not shown). Due to the small sample size of the present study, the possibility that these two variables are correlated in advanced CRC patients cannot be ruled out.

Discussion

Since Foxp3 is considered to be the best marker of naturally occurring Tregs (2-4), the two $CD4^+CD25^+Foxp3^+$ and $CD4^+CD25^{high}Foxp3^+$ populations were defined as Tregs. In the present study, a significant increase in Treg numbers was observed in peripheral blood from CRC patients compared with the numbers of Treg cells in healthy controls (P<0.05), which is in accordance with a previous study (13). However, no statistically significant difference in Treg numbers between CRC patients with and without lymph node metastases was identified, although increased Treg numbers in CRC patients with lymph node metastases were observed. Similarly, no significant difference was found in Treg numbers from patients with different clinical stages of CRC (data not shown). However, certain studies have reported that increased frequency of Tregs in peripheral blood and enhanced numbers of tumor-filtrating lymphocytes have marked prognostic significance in CRC (13-15). Another study has demonstrated that accumulation of Tregs in draining lymph nodes is correlated with disease progression in CRC (28).

$CD4^+CD25^+Foxp3^+$ Tregs are divided into two subpopulations: $CD4^+CD25^{high}Foxp3^+$ cells and $CD4^+CD25^{low}Foxp3^+$ cells (29). Notably, in the present study, Foxp3 expression levels were found to be markedly higher in $CD4^+CD25^{high}$ cells than in $CD4^+CD25^{low}$ cells in CRC patients and healthy controls. As Foxp3 mediates the function of regulatory T cells (2,3), $CD4^+CD25^{high}Foxp3^+$ cells have commonly been investigated as Tregs exhibiting suppressive functions (17,29). Furthermore, $CD4^+CD25^{low}Foxp3^+$ cells have been found to function differently from $CD4^+CD25^{high}Foxp3^+$ cells (29,30).

NKG2D is a predominant activating receptor in NK cells. As previously reported, the NKG2D receptor is critical for immunosurveillance of primary tumors in mouse models (24,26). A previous study suggested that decreased expression levels of NKG2D may be involved in the suppression of NK activity in CRC (27). Another study observed that depletion of Tregs enhanced NK cell-mediated suppression of tumors expressing NKG2D ligands in a $Rae1^+$ experimental metastatic mouse model (31). The Treg cells were able to suppress NK cell-mediated cytolysis of $Rae1^+$ target cells *in vitro*. Therefore, Tregs may inhibit the cytotoxicity of NK cells via an NKG2D-NKG2D ligand pathway. However, in the present study, no significant correlation was identified between peripheral Treg numbers and NKG2D expression levels in NK cells collected from CRC patients.

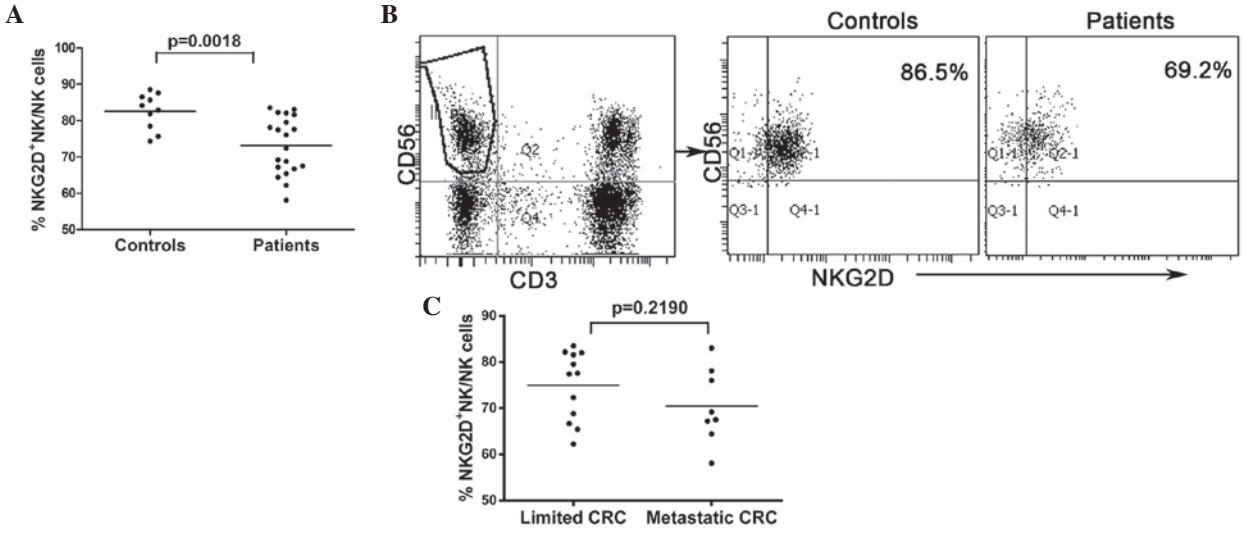

Figure 4. Natural killer group 2, member D (NKG2D) expression levels in peripheral natural killer (NK) cells from colorectal cancer (CRC) patients and healthy controls. (A) Percentages of NKG2D$^+$ NK cells/NK cells from patients (n=20) and healthy controls (n=10) as determined by flow cytometry. Each dot represents a subject. (B) Representative plots of NKG2D expression levels in NK cells from the CRC patient and the healthy control. (C) Percentages of NKG2D$^+$ NK cells/NK cells from patients with limited (n=12) and metastatic CRC (n=8). Values are presented as the mean ± standard deviation.

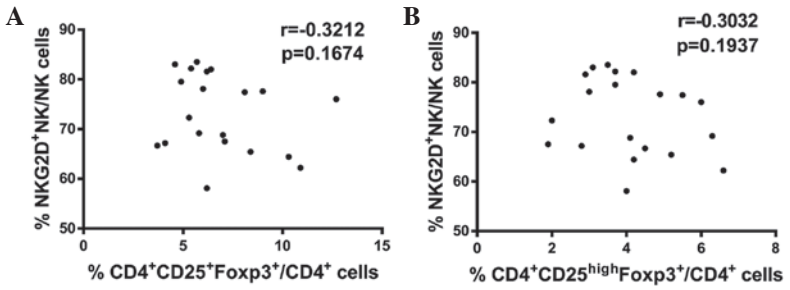

Figure 5. Correlations of peripheral regulatory T cell (Treg) numbers and natural killer group 2, member D (NKG2D) expression levels in natural killer (NK) cells from colorectal cancer (CRC) patients. Tregs and NKG2D in NK cells in peripheral blood from CRC patients were examined in parallel. (A) The correlation between the number of CD4$^+$CD25$^+$Foxp3$^+$ Tregs and the NKG2D expression levels in NK cells was analyzed in the patient group (n=20). (B) The correlation between the number of CD4$^+$CD25^{high}Foxp3$^+$ cells and the NKG2D expression levels in NK cells was also analyzed in the patient group (n=20). Each dot represents a patient with CRC.

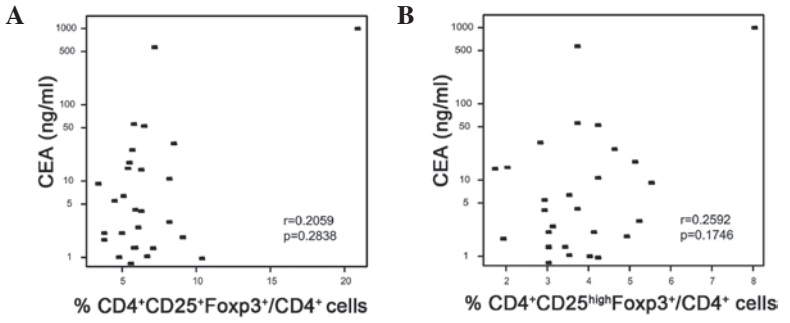

Figure 6. Correlations of peripheral Tregs and serum carcino-embryonic antigen (CEA) protein. Peripheral Tregs and serum CEA were detected in parallel. (A) Correlation between the number of CD4$^+$CD25$^+$Foxp3$^+$ Treg and serum CEA levels in colorectal cancer (CRC) patients (n=29). (B) Correlation between the number of CD4$^+$CD25^{high}Foxp3$^+$ cells and serum CEA levels in CRC patients (n=29). Each dot represents a patient with CRC.

As a nonspecific tumor marker, serum CEA protein is commonly increased in CRC patients and regularly serves as a treatment response and prognostic indicator for CRC (32,33). Nevertheless, in the present study, no correlation between Treg numbers and serum CEA protein levels in CRC patients was observed.

In conclusion, the results of the present study demonstrated that the number of peripheral Tregs was upregulated in CRC, but was not correlated with lymph node metastasis or clinical stage. Additionally, NKG2D expression was found to be downregulated in NK cells collected from patients with CRC, but was not correlated with lymph node metastasis. No

significant correlations were identified between increased Treg numbers and reduced NKG2D expression levels in NK cells in CRC patients. Similarly, no significant correlations between peripheral Treg numbers and serum CEA levels were observed. Therefore, further studies are required to clarify the mechanisms through which Tregs and NKG2D may regulate immune escape in CRC.

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References

1. Sakaguchi S, Miyara M, Costantino CM and Hafler DA: FOXP3+ regulatory T cells in the human immune system. Nat Rev Immunol 10: 490-500, 2010.
2. Hori S, Nomura T and Sakaguchi S: Control of regulatory T cell development by the transcription factor Foxp3. Science 299: 1057-1061, 2003.
3. Fontenot JD, Gavin MA and Rudensky AY: Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. Nat Immunol 4: 330-336, 2003.
4. Ghiringhelli F, Ménard C, Martin F and Zitvogel L: The role of regulatory T cells in the control of natural killer cells: relevance during tumor progression. Immunol Rev 214: 229-238, 2006.
5. Kohrt HE, Pillai AB, Lowsky R and Strober S: NKT cells, Treg, and their interactions in bone marrow transplantation. Eur J Immunol 40: 1862-1869, 2010.
6. Clarke SL, Betts GJ, Plant A, *et al*: CD4+CD25+FOXP3+ regulatory T cells suppress anti-tumor immune responses in patients with colorectal cancer. PLoS One 1: e129, 2006.
7. Trzonkowski P, Szmit E, Myśliwska J, Dobyszuk A and Myśliwski A: CD4+CD25+ T regulatory cells inhibit cytotoxic activity of T CD8+ and NK lymphocytes in the direct cell-to-cell interaction. Clin Immunol 112: 258-267, 2004.
8. Zhou H, Chen L, You Y, Zou L and Zou P: Foxp3-transduced polyclonal regulatory T cells suppress NK cell functions in a TGF-beta dependent manner. Autoimmunity 43: 299-307, 2010.
9. Lundqvist A, Yokoyama H, Smith A, Berg M and Childs R: Bortezomib treatment and regulatory T-cell depletion enhance the antitumor effects of adoptively infused NK cells. Blood 113: 6120-6127, 2009.
10. Salagianni M, Lekka E, Moustaki A, *et al*: NK cell adoptive transfer combined with Ontak-mediated regulatory T cell elimination induces effective adaptive antitumor immune responses. J Immunol 186: 3327-3335, 2011.
11. Ramos RN, Oliveira CE, Gasparoto TH, *et al*: CD25+ T cell depletion impairs murine squamous cell carcinoma development via modulation of antitumor immune responses. Carcinogenesis 33: 902-909, 2012.
12. Chen YL, Chang MC, Chen CA, Lin HW, Cheng WF and Chien CL: Depletion of regulatory T lymphocytes reverses the imbalance between pro- and anti-tumor immunities via enhancing antigen-specific T cell immune responses. PLoS One 7: e47190, 2012.
13. Ling KL, Pratap SE, Bates GJ, *et al*: Increased frequency of regulatory T cells in peripheral blood and tumour infiltrating lymphocytes in colorectal cancer patients. Cancer Immun 7: 7, 2007.
14. Salama P, Phillips M, Grieu F, *et al*: Tumor-infiltrating FOXP3+ T regulatory cells show strong prognostic significance in colorectal cancer. J Clin Oncol 27: 186-192, 2009.
15. Brudvik KW, Henjum K, Aandahl EM, Bjørnbeth BA and Taskén K: Regulatory T-cell-mediated inhibition of antitumor immune responses is associated with clinical outcome in patients with liver metastasis from colorectal cancer. Cancer Immunol Immunother 61: 1045-1053, 2012.
16. Curiel TJ, Coukos G, Zou L, *et al*: Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. Nat Med 10: 942-949, 2004.
17. Miller AM, Lundberg K, Ozenci V, *et al*: CD4+CD25high T cells are enriched in the tumor and peripheral blood of prostate cancer patients. J Immunol 177: 7398-7405, 2006.
18. Ormandy LA, Hillemann T, Wedemeyer H, Manns MP, Greten TF and Korangy F: Increased populations of regulatory T cells in peripheral blood of patients with hepatocellular carcinoma. Cancer Res 65: 2457-2464, 2005.
19. de Vries IJ, Castelli C, Huygens C, *et al*: Frequency of circulating Tregs with demethylated FOXP3 intron 1 in melanoma patients receiving tumor vaccines and potentially Treg-depleting agents. Clin Cancer Res 17: 841-848, 2011.
20. Sampson JH, Schmittling RJ, Archer GE, *et al*: A pilot study of IL-2Ralpha blockade during lymphopenia depletes regulatory T-cells and correlates with enhanced immunity in patients with glioblastoma. PLoS One 7: e31046, 2012.
21. Rech AJ, Mick R, Martin S, *et al*: CD25 blockade depletes and selectively reprograms regulatory T cells in concert with immunotherapy in cancer patients. Sci Transl Med 4: 134ra162, 2012.
22. Lanier LL: NK cell receptors. Annu Rev Immunol 16: 359-393, 1998.
23. Bauer S, Groh V, Wu J, *et al*: Activation of NK cells and T cells by NKG2D, a receptor for stress-inducible MICA. Science 285: 727-729, 1999.
24. Pende D, Cantoni C, Rivera P, *et al*: Role of NKG2D in tumor cell lysis mediated by human NK cells: cooperation with natural cytotoxicity receptors and capability of recognizing tumors of nonepithelial origin. Eur J Immunol 31: 1076-1086, 2001.
25. Ljunggren HG: Cancer immunosurveillance: NKG2D breaks cover. Immunity 28: 492-494, 2008.
26. López-Soto A, Huergo-Zapico L, Galván JA, *et al*: Epithelial-mesenchymal transition induces an antitumor immune response mediated by NKG2D receptor. J Immunol 190: 4408-4419, 2013.
27. Shen Y, Lu C, Tian W, *et al*: Possible association of decreased NKG2D expression levels and suppression of the activity of natural killer cells in patients with colorectal cancer. Int J Oncol 40: 1285-1290, 2012.
28. Deng L, Zhang H, Luan Y, *et al*: Accumulation of foxp3+ T regulatory cells in draining lymph nodes correlates with disease progression and immune suppression in colorectal cancer patients. Clin Cancer Res 16: 4105-4112, 2010.
29. Okita R, Yamaguchi Y, Ohara M, *et al*: Targeting of CD4+CD25high cells while preserving CD4+CD25low cells with low-dose chimeric anti-CD25 antibody in adoptive immunotherapy of cancer. Int J Oncol 34: 563-572, 2009.
30. Xu Q, Lee J, Jankowska-Gan E, *et al*: Human CD4+CD25low adaptive T regulatory cells suppress delayed-type hypersensitivity during transplant tolerance. J Immunol 178: 3983-3995, 2007.
31. Guerra N, Tan YX, Joncker NT, *et al*: NKG2D-deficient mice are defective in tumor surveillance in models of spontaneous malignancy. Immunity 28: 571-580, 2008.
32. Kanellos I, Zacharakis E, Kanellos D, *et al*: Prognostic significance of CEA levels and detection of CEA mRNA in draining venous blood in patients with colorectal cancer. J Surg Oncol 94: 3-8, 2006.
33. Aggarwal C, Meropol NJ, Punt CJ, *et al*: Relationship among circulating tumor cells, CEA and overall survival in patients with metastatic colorectal cancer. Ann Oncol 24: 420-428, 2013.