

Decreased number and reduced NKG2D expression of V δ 1 $\gamma\delta$ T cells are involved in the impaired function of V δ 1 $\gamma\delta$ T cells in the tissue of gastric cancer

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Abstract

Background In cancer patients, impaired function of immune cells—such as CD8⁺ T cells, NK cells, and dendritic cells—reportedly results in tumor progression. Although $\gamma\delta$ T cells also play a critical role in tumor defense, their function remains unclear in cancer patients.

Methods The frequency and function of $\gamma\delta$ T cells in peripheral blood, normal gastric mucosa, and cancer tissue were evaluated by multicolor flow cytometry. We also determined NKG2D expression on $\gamma\delta$ T cells in gastric cancer patients.

Results The frequency of V δ 1 $\gamma\delta$ T cells in gastric cancer tissue is significantly lower than in normal gastric mucosa; however, differences in the frequencies of V δ 2 and V γ 9 $\gamma\delta$ T cells between normal gastric mucosa and gastric cancer tissue were not statistically significant. The V δ 1 $\gamma\delta$ T cells from gastric cancer tissue produce significantly less IFN- γ than those from normal gastric mucosa do. Expression of NKG2D on V δ 1 $\gamma\delta$ T cells from gastric cancer tissue was significantly lower than in normal gastric mucosa. We also found a significant correlation between NKG2D expression and IFN- γ production of V δ 1 $\gamma\delta$ T cells in gastric cancer tissue.

Conclusion V δ 1 $\gamma\delta$ T cells show decreased frequency and impaired function in gastric cancer tissue, for which decreased NKG2D expression might be one of the mechanisms. Modalities specifically targeting NKG2D in V δ 1 $\gamma\delta$ T cells may provide a breakthrough treatment for gastric cancer patients.

Keywords Gastric cancer · Immune evasion · NKG2D · $\gamma\delta$ T cells

Introduction

T lymphocytes recognize antigens via the heterodimeric T-cell receptor (TCR) molecule, which is noncovalently associated with the CD3 molecular complex. T lymphocytes are classified into two subsets, based on their TCR. Most T cells carry TCRs that consist of an $\alpha\beta$ chain heterodimer. These T cells, referred to as $\alpha\beta$ T cells, can be further subdivided on the basis of the surface markers CD4 and CD8 [1].

$\gamma\delta$ T cells are another type of T cell; these account for 2–5% of CD3⁺ peripheral blood T cells but constitute a dominant fraction of the T cells at other anatomical sites, such as the intestinal epithelia. Most $\gamma\delta$ T cells lack CD4 or CD8 antigens and hence display a “double-negative” phenotype, although a sizeable fraction express CD8. The absence of CD4 or CD8 expression on the majority of circulating $\gamma\delta$ T cells is well in line with the lack of MHC restriction in the antigen recognition of this T-cell subset [2]. In the blood of most healthy individuals, T cells expressing the V δ 2 gene paired with a particular V γ chain (V γ 9) account for 50 to >90% of the $\gamma\delta$ T-cell population. In contrast, intestinal intraepithelial $\gamma\delta$ T cells frequently express the V δ 1 gene, which can associate with different V γ elements [2]. There are no major differences between $\alpha\beta$ and $\gamma\delta$ T cells with regard to effector functions. Thus, activated $\gamma\delta$ T cells have strong cytotoxic effector activity (using both death receptor/death ligand and cytolytic granule pathways), and produce various cytokines (frequently including tumor necrosis factor- α and IFN- γ) [2]. However, $\gamma\delta$ TCRs seem to react to novel ligands that are not recognized by $\alpha\beta$ T cells, thus providing an essential

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additional pathway of local immunosurveillance with immediate relevance for tumor defense [2, 3].

There is accumulating evidence that immune cells such as CD8⁺ T cells, NK cells, and dendritic cells show impaired function in cancer patients, resulting in tumor progression [4]. However, the function of $\gamma\delta$ T cells in cancer patients is unclear. The critical role of $\gamma\delta$ T cells in tumor defense implies that $\gamma\delta$ T-cell function is also impaired by cancer cells. In the current study, we therefore examined the frequency and function of $\gamma\delta$ T cells in peripheral blood, normal gastric mucosa, and cancer tissue. We also determined NKG2D expression on $\gamma\delta$ T cells in gastric cancer patients to show a mechanism that impairs $\gamma\delta$ T-cell function, because NKG2D is required by $\gamma\delta$ T cells to recognize and kill tumor cells.

Materials and methods

Patients and normal donors

Forty-seven patients (37 males and 10 females), treated at Tottori University Hospital and diagnosed with gastric cancer by pathologists, were enrolled in this study. None of the patients received radiotherapy, chemotherapy, or other medical interventions before blood donations. Informed consent for blood donations was obtained from all participants. Patient characteristics are shown in Table 1. Healthy controls ($n = 23$; 16 males and 7 females) were age-matched (67.9 ± 11.0 years for controls; 69.6 ± 10.8 years for cancer patients), and each experiment was performed in parallel. The clinicopathological findings were determined according to the Japanese Classification of Gastric Carcinoma [5].

Preparation of peripheral blood mononuclear cells (PBMCs)

Approximately 30 ml of peripheral blood was drawn from each control, and from each patient before surgery or chemotherapy; blood samples were prepared by centrifugation over a Ficoll-Paque (Pharmacia, Uppsala, Sweden) density gradient.

Isolation of tumor-infiltrating leukocytes

Freshly excised tumor tissues were minced and incubated in 1.5 mg/ml of collagenase D (Wako Pure Chemical Industries, Ltd., Osaka, Japan). Cell suspensions were then filtered through a mesh filter (BD Falcon, Franklin Lakes, NJ, USA). Tumor-infiltrating leukocytes (TILs) were available in 26 patients for the subset analysis of $\gamma\delta$ T cells and in 12 patients for the analysis of NKG2D expression

Table 1 Clinicopathologic characteristics of the gastric cancer patients included in the current study

Clinicopathologic factor	N (%)
Gender	
Male	37 (78.7)
Female	10 (21.3)
Histology ^a	
Differentiated	28 (59.6)
Undifferentiated	19 (40.4)
Depth of invasion	
M/SM (early)	21 (44.7)
MP/SS/SE/SI (advanced)	26 (55.3)
Lymph node metastasis	
Absent	24 (51.1)
Present	23 (48.9)
Lymphatic invasion	
Absent	13 (27.7)
Present	34 (72.3)
Vascular invasion	
Absent	14 (29.8)
Present	33 (70.2)
Liver metastasis	
Absent	45 (95.7)
Present	2 (4.3)
Peritoneal metastasis	
Absent	43 (91.5)
Present	4 (8.5)
Stage of disease	
I/II	30 (63.8)
III/IV	17 (36.2)

M tumor invasion of mucosa, *SM* tumor invasion of submucosa, *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 Differentiated, papillary or tubular adenocarcinoma; undifferentiated, poorly differentiated or mucinous adenocarcinoma; or signet-ring cell carcinoma

and IFN- γ production of V δ 1 $\gamma\delta$ T cells. Since it is necessary to get large piece of tissue sample to have enough TILs for flow cytometry analysis, TILs from most early gastric cancer patients were not available for the analysis of NKG2D expression and IFN- γ production of V δ 1 $\gamma\delta$ T cells.

Flow cytometry analysis

Flow cytometry analysis was performed on a FACSCaliburTM (Becton Dickinson, Franklin Lakes, NJ, USA), using the following antibodies: anti-CD3-PE, anti-CD8-PE-Cy5, anti-CD3-PE-Cy5, anti-NKG2D-PE, anti-TCR $\gamma\delta$ -FITC (BD PharMingen, Franklin Lakes, NJ, USA), anti-V δ 1-FITC,

anti-V δ 2-FITC (Thermo Scientific, Waltham, MA, USA), anti-V γ 9-FITC monoclonal antibody(mAb) (Beckman Coulter, Brea, CA, USA). For intracellular cytokine staining, PBMCs were cultured in the presence of Leukocyte Activation Cocktail (BD PharMingen). The anti-cytokine antibody was anti-IFN- γ -PE (Becton Dickinson). To stain for IFN- γ , cells were fixed and permeabilized with BD Cytotfix/CytopermTM solution (Becton Dickinson).

Statistical analysis

Either paired *t* tests or Mann–Whitney *U* tests were used to determine statistical differences between groups. Correlations were analyzed using the Spearman rank correlation coefficient. $P < 0.05$ was considered significant. GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA, USA) was used for statistical analyses.

Results

Prevalence of circulating $\gamma\delta$ T cells in gastric cancer patients

We first determined the frequency of each subtype of $\gamma\delta$ T cells in the peripheral blood of healthy controls and gastric cancer patients. Frequencies of V δ 1, V δ 2, and V γ 9 $\gamma\delta$ T cells in healthy controls were 1.9 ± 1.3 , 3.8 ± 3.4 , and $4.1 \pm 3.5\%$, respectively. Those in gastric cancer patients were 1.8 ± 1.5 , 2.6 ± 3.5 , and $2.7 \pm 3.6\%$, respectively. The frequency of V γ 9 $\gamma\delta$ T cells in gastric cancer patients was significantly lower than in healthy controls ($P = 0.048$). Furthermore, the frequency of V δ 2 in gastric cancer patients tended to be lower than that in healthy controls (Fig. 1).

Frequency of $\gamma\delta$ T cells in the tissue of normal gastric mucosa and gastric cancer

We then investigated the frequencies of V δ 1, V δ 2, and V γ 9 $\gamma\delta$ T cells in both normal gastric mucosa and gastric cancer tissue. The frequencies of V δ 1, V δ 2, and V γ 9 $\gamma\delta$ T cells in normal gastric mucosa were 51.4 ± 15.3 , 4.9 ± 4.6 , and $2.7 \pm 3.0\%$, respectively (Fig. 2a). The frequencies of V δ 1, V δ 2, and V γ 9 $\gamma\delta$ T cells in gastric cancer tissue were 41.4 ± 16.0 , 3.2 ± 2.9 , and $2.4 \pm 2.5\%$, respectively (Fig. 2b). The frequency of V δ 1 $\gamma\delta$ T cells in gastric cancer tissue was significant lower than that in normal gastric mucosa ($P = 0.015$; Fig. 2c). However, there were no statistically significant differences in the frequencies of V δ 2 and V γ 9 $\gamma\delta$ T cells between normal gastric mucosa and gastric cancer tissue. On the other hand, the frequency of V δ 1 $\gamma\delta$ T cells in gastric cancer tissue was 41.0 ± 8.8

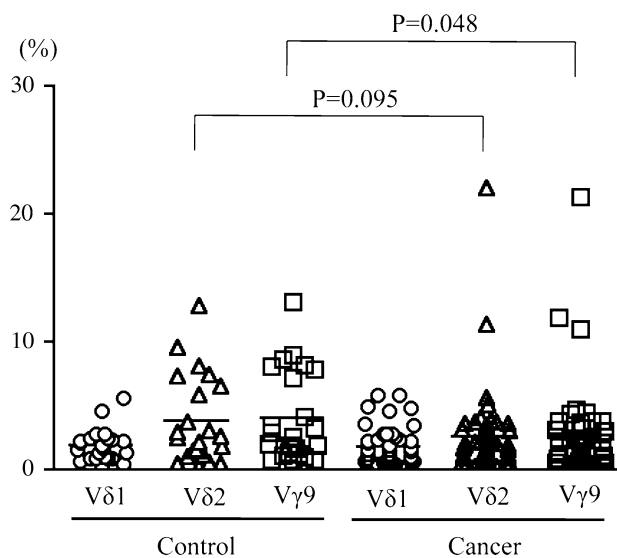


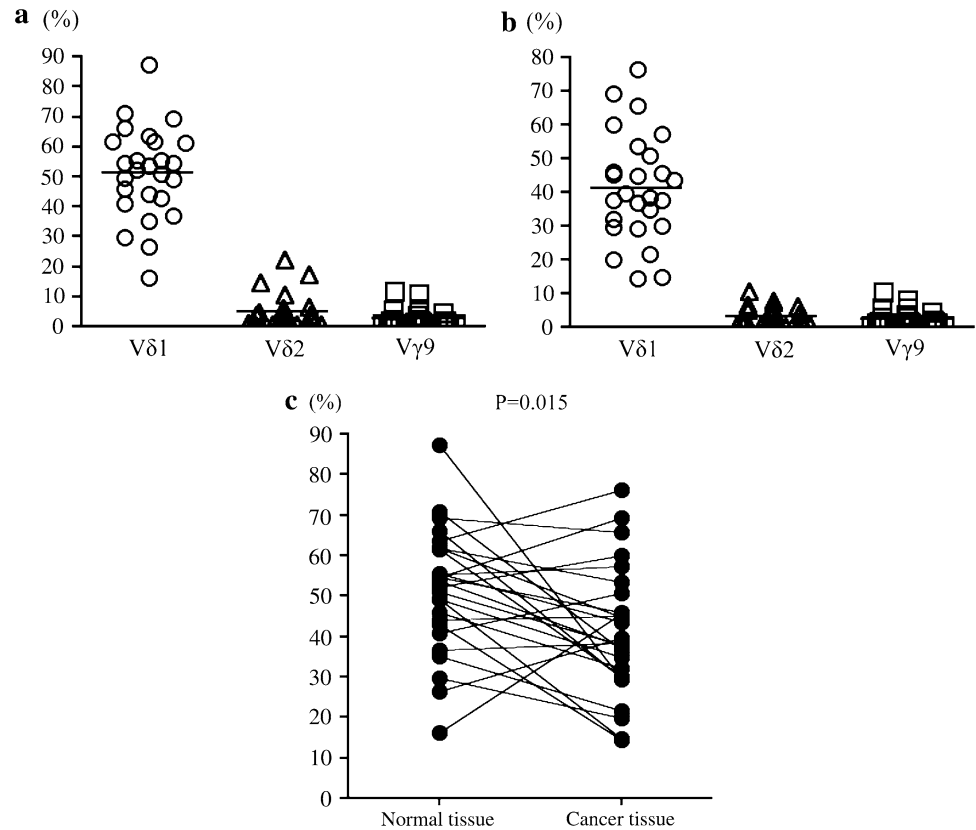
Fig. 1 Frequencies of V δ 1, V δ 2, and V γ 9 $\gamma\delta$ T cells in peripheral blood from healthy control and gastric cancer patients. The frequency of V γ 9 $\gamma\delta$ T cells in gastric cancer patients was significantly lower than that in healthy controls ($P = 0.048$); the frequency of V δ 2 cells in gastric cancer patients also tended to be lower than that in healthy controls ($P = 0.095$)

and $41.6 \pm 18.5\%$ in early and advanced gastric cancer, respectively, and this difference was not significant ($P = 0.98$). Furthermore, the frequency of V δ 1 $\gamma\delta$ T cells in gastric cancer tissue was 37.2 ± 11.1 and $45.0 \pm 18.9\%$ in stages I/II and III/IV, respectively, and this difference was not significant ($P = 0.23$).

NKG2D expression on V δ 1 $\gamma\delta$ T cells decreased in gastric cancer tissue

Our data showed that $\gamma\delta$ T cells are abundant in gastric cancer tissue compared with peripheral blood. Moreover, because V δ 1 $\gamma\delta$ T cells predominate among $\gamma\delta$ T cells in gastric cancer tissue, we investigated the function of such cells in gastric cancer patients. There was no statistical difference in IFN- γ production from circulating V δ 1 $\gamma\delta$ T cells between normal controls and cancer patients. On the other hand, V δ 1 $\gamma\delta$ T cells obtained from gastric cancer tissue produced significantly less IFN- γ than those obtained from normal gastric mucosa ($P = 0.0097$; Fig. 3). Since $\gamma\delta$ T cells require NKG2D to recognize and kill target cells, we examined NKG2D expression on V δ 1 $\gamma\delta$ T cells in both normal gastric mucosa and gastric cancer tissue, and found it to be 60.0 ± 13.8 and $45.6 \pm 10.1\%$, respectively; this difference was statistically significant ($P = 0.0009$; Fig. 4). With regard to the correlation between NKG2D expression and IFN- γ production of V δ 1 $\gamma\delta$ T cells, IFN- γ was mainly produced by NKG2D-expressing V δ 1 $\gamma\delta$ T cells (Fig. 5a). Furthermore, we also found that the NKG2D expression and IFN- γ production of V δ 1 $\gamma\delta$ T cells in

Fig. 2 Frequencies of V δ 1, V δ 2, and V γ 9 $\gamma\delta$ T cells in both normal gastric mucosa (a) and gastric cancer tissue (b). c The frequency of V δ 1 $\gamma\delta$ T cells in gastric cancer tissue was significantly less than that in normal gastric mucosa ($P = 0.015$). On the other hand, there were no statistically significant differences in the frequencies of V δ 2 and V γ 9 $\gamma\delta$ T cells between normal gastric mucosa and gastric cancer tissue



gastric cancer tissue were significantly correlated ($r = 0.75$, $P = 0.007$; Fig. 5b).

Discussion

In the current study, we demonstrated that V δ 1 $\gamma\delta$ T cells predominate among $\gamma\delta$ T cells in both normal gastric mucosa and gastric cancer, whereas V δ 2 and V γ 9 $\gamma\delta$ T cells predominate in peripheral blood. The V δ 1 $\gamma\delta$ T cells isolated from TILs of patients with colorectal cancer have been shown to lyse not only autologous but also various allogeneic epithelial tumor cells, indicating the importance of V δ 1 $\gamma\delta$ T cells in tumor surveillance [6]. In the current study, we found that the frequency of V δ 1 $\gamma\delta$ T cells in gastric cancer tissue was significantly less than in normal gastric mucosa. Furthermore, IFN- γ production by V δ 1 $\gamma\delta$ T cells in gastric cancer tissue was significantly less than that in normal gastric mucosa, indicating that V δ 1 $\gamma\delta$ T cell function in gastric cancer tissue is impaired. On the other hand, the frequency of V δ 1 $\gamma\delta$ T cells in the tissue of gastric cancer was not related to disease progression. Since we determined the frequency of V δ 1 $\gamma\delta$ T cells in the tissue of gastric cancer, those cells are considered to have been already affected by cancer cells and their stromal cells, even in early gastric cancer.

The MHC class I chain-related molecules A and B (MICA and MICB) and the UL-16 binding proteins 1–3 (ULBP1–3) can be induced on epithelial cells by heat shock or oxidative stress, and are constitutively expressed to variable levels on many epithelial tumor cells and also on some leukemias and lymphomas. Such MICA/MICB- or ULBP-expressing tumor and lymphoma cells are killed by V δ 1 $\gamma\delta$ T cells through recognition by NKG2D, which is the receptor for MICA, MICB, and ULBP [7, 8]. 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 [9]. 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 [10–13]. In cancer patients, tumor-infiltrating and systemic NK cells and CD8 $^{+}$ T cells often express little NKG2D and are functionally compromised, indicating that decreased NKG2D expression on NK cells and CD8 $^{+}$ T cells is closely related to impaired function of those cells [14]. However, NKG2D expression on $\gamma\delta$ T cells and its relationship to impaired $\gamma\delta$ T-cell function in cancer patients remains unclear. In the current study, therefore, we studied NKG2D expression in V δ 1 $\gamma\delta$ T cells to identify a mechanism responsible for impaired $\gamma\delta$ T-cell function in gastric cancer patients. NKG2D expression on V δ 1 $\gamma\delta$ T cells is

Fig. 3 Comparison of IFN- γ production by V δ 1 $\gamma\delta$ T cells from normal gastric mucosa and from gastric cancer tissue. **a** The representative results for IFN- γ production by V δ 1 $\gamma\delta$ T cells from normal gastric mucosa and from gastric cancer tissue by FACS. **b** V δ 1 $\gamma\delta$ T cells from gastric cancer tissue produced significantly less IFN- γ than those from normal gastric mucosa ($P = 0.0097$)

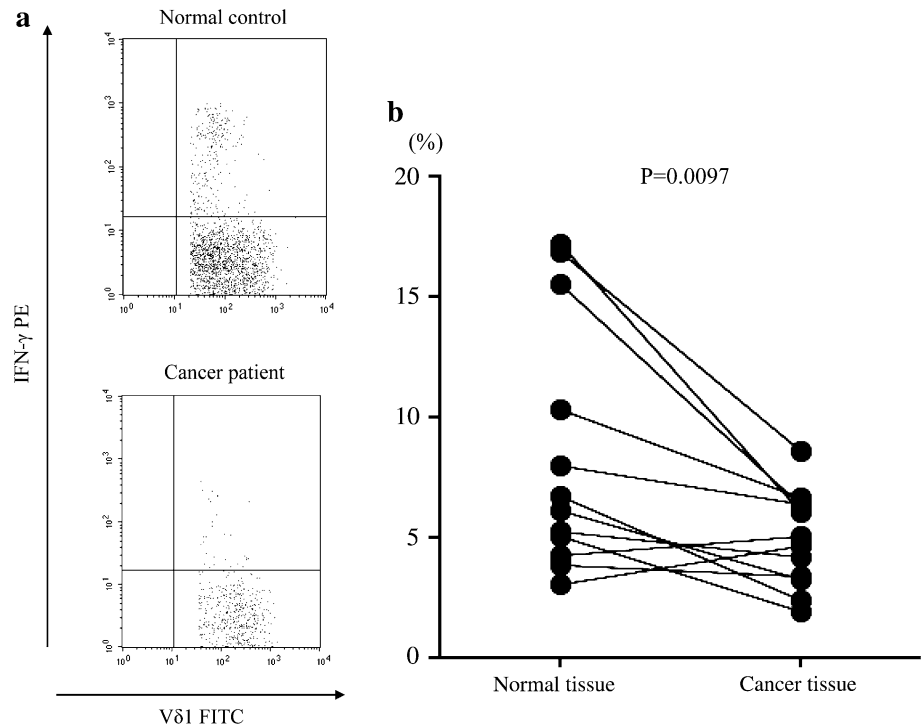
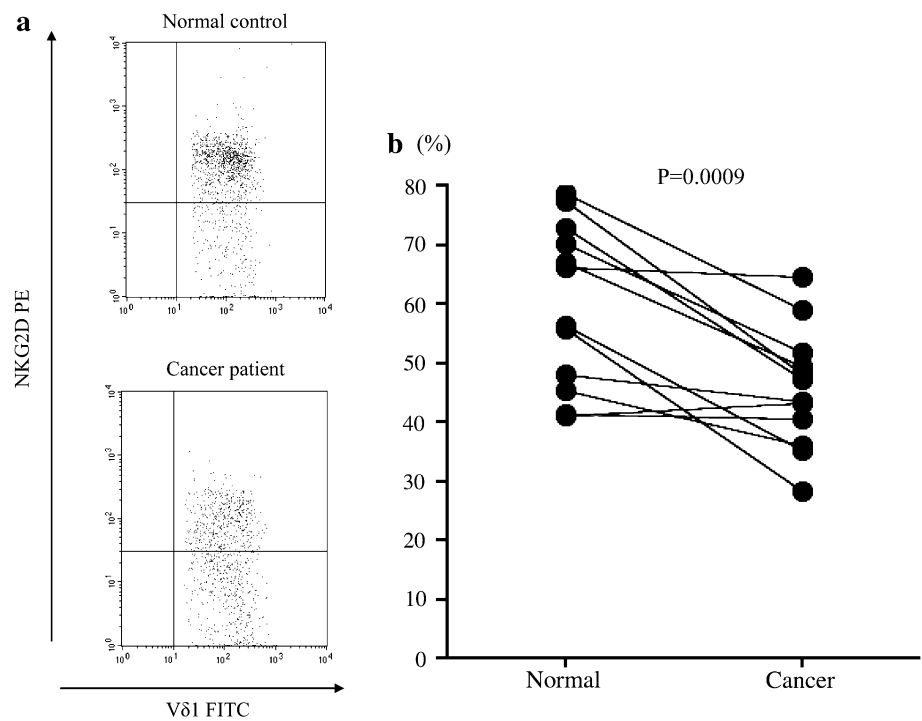


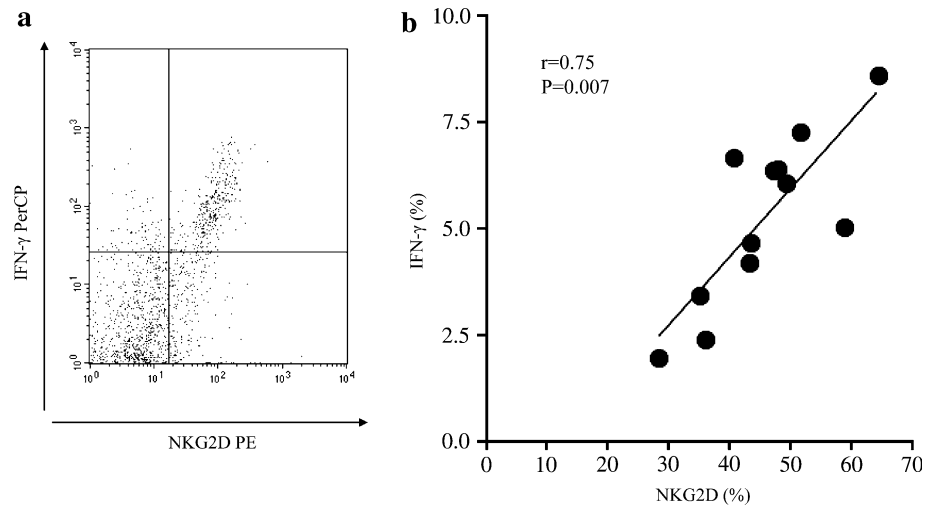
Fig. 4 Comparison of NKG2D expression on V δ 1 $\gamma\delta$ T cells in normal gastric mucosa and gastric cancer tissue. **a** The representative results for NKG2D expression on V δ 1 $\gamma\delta$ T cells from normal gastric mucosa and from gastric cancer tissue by FACS. **b** NKG2D expression on V δ 1 $\gamma\delta$ T cells in gastric cancer tissue was significantly lower than in normal gastric mucosa ($P = 0.0009$)



significantly lower in gastric cancer tissue than in normal gastric mucosa. Furthermore, in gastric cancer tissue, NKG2D expression in V δ 1 $\gamma\delta$ T cells significantly correlates with IFN- γ production by V δ 1 $\gamma\delta$ T cells, indicating that the impaired $\gamma\delta$ T-cell function observed in these tissues is due to decreased NKG2D expression on V δ 1 $\gamma\delta$ T

cells. The reason for this phenomenon remains unclear. We previously showed that NKG2D expression on CD8⁺ T cells is also downregulated; this decreased NKG2D expression correlates significantly with reduced IFN- γ production by CD8⁺ T cells [15]. We have also shown that MICA expressed in gastric cancer cells is responsible for

Fig. 5 Correlation between NKG2D expression and IFN- γ production of V δ 1 $\gamma\delta$ T cells in gastric cancer tissue. **a** The representative results for the correlation between NKG2D expression and IFN- γ production of V δ 1 $\gamma\delta$ T cells from gastric cancer tissue by FACS. Cells are gated on CD3⁺ V δ 1⁺ cells. **b** IFN- γ production of V δ 1 $\gamma\delta$ T cells was significantly related to NKG2D expression on V δ 1 $\gamma\delta$ T cells ($r = 0.75$, $P = 0.007$)



decreased NKG2D expression on CD8⁺ T cells. Further studies are urgently required to solve this problem.

Immune cells with negative immune regulatory function at tumor sites have recently attracted considerable attention, since these cells are thought to play extremely important roles in the progression of tumors through the suppression of immune responses and induction of immune tolerance. The best-studied immunosuppressive cell population is that of CD4⁺/CD25⁺ regulatory T cells (Treg cells) [16–18]. Human Treg cells [19, 20] disable TAA-specific T-cell immunity [21, 22]. Increased numbers of Treg cells are correlated with poor survival in hepatocellular carcinoma patients [23] and ovarian cancer patients [19]. Treg cells also increase in peripheral blood and tumor-infiltrating lymphocytes in patients with gastric cancer [24]. Furthermore, myeloid-derived suppressor cells [25, 26] and CD4⁺/NKG2D⁺ T cells [27] can diminish immunity against cancer cells in cancer patients. These accumulating lines of evidence indicate that immunosuppressive cells play important roles in tumor progression. Notably, $\gamma\delta$ T cells also have an immune regulatory function. In this regard, Peng et al. [28] showed that a dominant V δ 1 $\gamma\delta$ T cell population among lymphocytes infiltrating breast tumors was strongly able to suppress naive and effector T-cell responses, and to block the maturation and function of dendritic cells. Furthermore, they showed that these cells have suppressive activity *in vivo* through adoptive cotransfer experiments. Although the dominant subpopulation of $\gamma\delta$ T cells in gastric cancer tissue was also V δ 1 $\gamma\delta$ T cells in the current study, the frequency of V δ 1 $\gamma\delta$ T cells in the tissue of gastric cancer was significantly lower than that in normal gastric mucosa. Therefore, it is unlikely that the abundant V δ 1 $\gamma\delta$ T cells seen in the current study have an immune regulatory function, although their immune regulatory activity was not determined in the current study.

In conclusion, our results show that the frequency of V δ 1 $\gamma\delta$ T cells was decreased and that their function was impaired in gastric cancer tissue. Decreased NKG2D expression may be one of the mechanisms responsible for impaired V δ 1 $\gamma\delta$ T-cell function. Development of specific treatments targeting NKG2D on V δ 1 $\gamma\delta$ T cells may provide a clinical breakthrough for gastric cancer patients.

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