SPANDIDOS PUBLICATIONS

Evaluation of immune response according to the metastatic status in the regional lymph nodes in patients with gastric carcinoma

KEISHI NAKAMURA, ITASU NINOMIYA, KATSUNOBU OYAMA, MASAFUMI INOKUCHI, SHINICHI KINAMI, SACHIO FUSHIDA, TAKASHI FUJIMURA, MASATO KAYAHARA and TETSUO OHTA

Division of Cancer Medicine, Department of Oncology, Gastroenterologic Surgery, Graduate School of Medical Science, Kanazawa University, Ishikawa 920-8641, Japan

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Abstract. The immunological response in the regional lymph nodes (LN) of gastric cancer patients who had metastasis was studied by investigating both the degree of metastasis and the maturation status of dendritic cells (DCs). A total of 732 LNs was obtained from 29 gastric cancer patients, including 25 patients whose LNs were pathological metastasis-negative and 4 patients with metastasis-positive LNs. Micrometastasis (MM) in the LNs was analyzed by immunohistochemical staining of cytokeratin (CK) (IHC-MM) and by amplifying CEA and CK19 mRNA by quantitative real-time RT-PCR (PCR-MM). Distribution and density of mature DCs were evaluated in 119 LNs from pathological metastasis-positive cases by examining CD83 expression immunohistochemically. Then, following the examination of PCR-MM, immunological responses were analyzed by amplifying CD83, CD86, CD1a and IFNγ mRNA by quantitative real-time RT-PCR in 613 LNs from 25 pathological metastasis-negative cases. Among 119 LNs from 4 patients with LN metastasis, 20 LNs had histological metastasis and 6 histological metastasis-negative LNs had IHC-MM. The distribution and density of mature DCs were identical in the LNs regardless of metastasis status. Among 613 LNs from 25 patients without histological LN metastasis, 15 LNs (2.45%) from 6 cases (24%) were PCR-MM positive. The expression levels of CD83 and CD86 mRNA were significantly higher in the 15 PCR-MM-positive LNs than in the 598 PCR-MM-negative LNs. The expression levels of CD83 and CD86 mRNA in 136 PCR-MM-negative LNs from 6 PCR-MM-positive cases were also higher than those in 462 LNs from 19 PCR-MM-negative cases. In gastric cancer patients, activation of the immune response by maturation of DCs is wide-spread in regional lymph nodes at a stage before metastasis is detected histologically, including detection by IHC-MM.

Correspondence to: Dr Keishi Nakamura, Division of Cancer Medicine, Department of Oncology, Gastroenterologic Surgery, Graduate School of Medical Science, Kanazawa University, 13-1 Takaramachi, Kanazawa, Ishikawa 920-8641, Japan
E-mail: keic.nakamura@gmail.com

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Introduction

Dendritic cells (DCs) function as professional antigen-presenting cells (APCs) and are important in the initiation of primary immune responses (1). The function of DCs is highly influenced by their level of maturation, and in the T-cell-driven response to tumor presentation the maturations of DCs and T cells are thought to be instrumental.

DCs are heterogeneous in regard to maturation, differentiation and state of activation, (2) which are controlled and regulated by a variety of microenvironmental signals, including cytokines and surface molecules expressed on neighboring cells (3,4). In most tissues, DCs are present in a so-called 'immature' state and are able to capture tumor-specific antigens, when they encounter apoptotic or necrotic tumor cells in the primary tumor. Afterwards, DCs process the antigens into peptides that are presented in the context of MHC class I and class II molecules and migrate from the primary tumor to the regional LNs, where they interact with naïve T-cells (3,5,6). DCs mature during this process by receiving the correct cytokine signals, and peptide-loaded mature DCs activate naïve T-cells and change them to antigen-specific T-cells. CD1a, a cell surface glycoprotein that is structurally related to the MHC molecules and mediates an MHC-independent antigen presentation pathway, is a highly specific and sensitive marker of immature DCs and Langerhans cells, a population of DCs found in the skin (7,8). CD83 is a glycoprotein member of the immunoglobulin superfamily that is strongly up-regulated during DC maturation, thus making it a robust marker for mature DCs (9-12). Activation of naïve T-cell by mature DCs requires two signals. The first signal is transmitted via the MHC molecule/T cell receptor for the recognition of tumor-specific antigen by DCs (13) but is insufficient to induce the immune response. The second signal is required for T-cells to acquire the functions of an effector cell (14) and it is provided by costimulatory molecules such as CD86 and CD80, which are expressed on mature antigen-presenting cells (APC), including the DCs, and bind to the ligands of costimulatory molecules that are expressed on T cells (15,16). In the absence of a costimulatory signals, T cells typically enter a state of anergy.

IFNγ is one of the T helper 1 (Th1) types of cytokines, and its production is inducible by signals from the T-cell receptor and co-stimulatory molecules in the presence of

IL-12. DCs secrete IL-12, which promotes naïve T-cells to differentiate into IFNγ-secreting Th1 cells (17,18).

Current immunological studies have demonstrated that dense infiltration of a variety of malignant tumors by DCs correlates with a favorable prognosis, suggesting that DCs play a critical role in an anti-tumor response (19). A high degree of DC infiltration into the primary tumor was reported to reflect the host immune defense mechanism (3). Similarly, in gastric cancer, DC infiltration into the primary tumor was reported to decrease the progression of nodal involvement and lead to good survival rates (20-25). Lymph node metastasis is a crucial factor that influences prognosis, and patients with nodal involvement in gastric cancer have a poor clinical course compared with those without metastasis (26). However, the clinical significance of micrometastases (MM) still remains controversial. In the 6th edition of the TMN classification, micrometastases were classified as MM or isolated tumor cells (ITC) according to their largest dimensions. Some authors have reported a significant correlation between the presence of MM and prognosis in gastric cancer (27-29), whereas others have not (30,31). Morgagni *et al* (27) defined MM as single cells or small clusters of neoplastic cells identifiable only by immunohistochemical methods and concluded that the presence of MM in early gastric cancer (pN0) does not influence patient prognosis. Fukagawa *et al* (30) also defined MM as the presence of tumor cells detected by cytokeratin-specific immunohistochemistry and not by ordinary HE staining. However, they concluded that the immunohistochemical presence of MM in the regional lymph nodes does not affect the survival of gastric cancer patients. This study had some problems regarding the different antibodies used, the detection technique, the objects and the definition of MM. The main concern was whether a small number of cancer cells dissociated from the primary tumor can survive after being trapped in the regional LNs. Mature DCs activated in the primary tumor might play a role as self-immune defense mediators in the regional LNs. Tsujitani *et al* (32) suggested that DCs in the regional LNs are important for establishing immunological defense mechanisms in cases of gastric cancer. The relation between the extent of LN metastasis, especially MM, and the immune response based on the maturation status of DCs in the regional LNs in gastric cancer patients is still uncertain.

In the present study, we aimed to investigate the relation between immune response and metastasis status in the regional LNs by examining the maturation status of DCs and degree of LN metastasis in gastric cancer patients.

Materials and methods

Patients. We studied 731 LNs from 29 gastric cancer patients who underwent gastrectomy at the Department of Gastroenterologic Surgery, Kanazawa University Hospital from April 2003 to 2005, with informed consent. None of the patients had received preoperative chemotherapy or radiotherapy. Our study includes 4 patients with LN metastasis and 25 patients without LN metastasis, which was diagnosed by routine histological examination using haematoxylin and eosin (H&E) staining. Four patients with LN metastasis underwent total or partial gastrectomy with more than D2 LN dissection. A total of 25 LN-negative patients underwent gastrectomy with lymphatic mapping and lymphatic basin dissection (33) followed by segmental gastrectomy in 11 cases, local gastrec-tomy in 2 cases, proximal gastrectomy in 2 cases and distal gastrectomy in 10 cases. The clinicopathological features of the 25 patients without LN metastasis are classified according to the Japanese Classification of Gastric Carcinoma (13th edition) and shown in Table I.

Table I. Clinicopathological features of the 25 gastric cancer patients without LN metastasis.

Median age (years)	62 (44-76)
Gender	
Male	16
Female	9
Median tumor size (mm)	28 (5-55)
Operative procedure	
Segmental gastrectomy	11
Local resection	2
Proximal gastrectomy	2
Distal gastrectomy	10
Lymph node metastasis	
Negative	25
Positive	0
Histological type	
Differentiated	17
Undifferentiated	8
Depth of tumor invasion	
Mucosa	13
Submucosa	10
Muscularis propria	2
Lymphatic invasion	
Negative	7
Positive	18
Venous invasion	
Negative	4
Positive	21

Cancer cell line and normal control LNs. The human gastric cancer cell line MKN45 was used as a positive control for cancer cell detection by a quantitative RT-PCR assay. The cell line was maintained in RPMI-1640 (Nissui Pharma Co., Ltd., Tokyo, Japan) supplemented with 10% heated, inactivated fetal bovine serum (Life Technologies, Inc., Gaithersburg, MD, USA) at 37˚C and 5% CO_2. As a negative control, 22 normal lymph nodes from non-cancerous patients (chronic cholecystitis, ulcerative colitis, diverticulitis and hepatic artery aneurysm) were obtained at the time of surgery (cholecystectomy, partial colectomy, resection of aneurysm) with their

 consents and studied in order to determine the cut-of the quantitative RT-PCR assay.

Distribution and density of mature DCs in the regional LNs in pathological metastasis-positive patients. A total of 119 LNs from 4 LN-positive gastric cancer patients were assessed by immunohistochemistry for the distribution and density of DCs according to the degree of metastasis. All LNs dissected from the patients were cut into halves at the plane of the largest dimension. One half of each LN was fixed in 10% buffered formalin and embedded in paraffin for histological examination. The other half was immediately embedded in O.C.T. (Tissue-Tek Optimal Cutting Temperature) compound (Sakura Finetek, Tokyo, Japan), frozen and stored at -80˚C until immunohistochemical analysis. Each frozen LN was cut into 6-μm slices and incubated with the primary antibody, rabbit polyclonal anti-keratin, (1:600) for 1 min at room temperature and mouse monoclonal anti-CD83 (clone sc-19677, Santa Cruz Biotechnology Inc., Santa Cruz, CA; dilution 1:100) for 4 min at room temperature in order to detect small clusters of metastatic cancer cells and mature DCs, respectively. After washing in PBS, the slides were incubated with diaminobenzidine (Zymed Laboratories, CA) and counterstained with haematoxylin.

The degree of metastasis was grouped into three categories according to keratin immunostaining status: no metastases, immunohistochemical MM (IHC-MM) and histological metastases. IHC-MM was defined as cancer nests <2 mm in size detected immunohistochemically by keratin staining. Mature DC density was defined as the average count of CD83-expressing DCs in ten random fields at a magnification of x400 in a non-cancerous region of the LN medulla. Mature DC density was compared according to the degree of metastasis. LNs that were completely filled with cancer cells were excluded.

Detection of MM by RT-PCR and quantification of mRNA expression of immunologic parameters in LNs from pathological metastasis-negative cases. A total of 613 LNs from 25 gastric cancer patients with pathological metastasis-negative LNs were assessed for the presence of MM by amplification of CEA and CK19 mRNA by quantitative RT-PCR (PCR-MM). The presence of PCR-MM was estimated by the relative amounts of CEA or CK19 mRNA standardized by reference internal control gene expression in the LNs of gastric cancer patient in comparison to that in the LNs from non-cancer patients. The degree of maturation of the DCs was also analyzed by amplifying mRNA of immunological markers by quantitative real-time RT-PCR, including that of CD1a as representing immature DCs, that of CD83 and CD86 as representing mature DCs, and IFNγ mRNA as representing an interaction factor between DCs and naïve T-cells. Special care was taken to prevent contamination of the dissected LNs with cancer cells and normal epithelial cells in the stomach. LNs were separated intraoperatively from the resected stomach on a back table immediately after its resection and were cut in halves as described above. One half of each LN was analyzed by routine histological examination, and the other half was placed in RNA Later (Ambion, Austin, TX) and stored at -80˚C for RNA preservation until RNA extraction. Total RNA was extracted from the tissue using Isogen systems (Nippon Gene Co., Ltd., Tokyo, Japan). After heat denaturation at 68˚C for 15 min with 20 μM oligo(dT) primer, 10 μg RNA was reverse transcribed at 42˚C for 60 min into first-strand cDNA in reverse transcription solution [400 U Moloney murine leukemia virus reverse transcriptase (Invitrogen Japan, Tokyo, Japan), 50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 0.01 M dithiothreitol (DTT), 0.5 mM each deoxynucleoside triphosphate (dNTP) and 16 units of RNasin (Promega, Madison, WI, USA)] with a total volume of 100 μl. A reverse-transcribed cDNA solution corresponding to 100 ng total RNA was quantitatively amplified with primers and a probe specific for the target cDNA using an ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The specific primers were as follows: CEA forward 5'-AATAATTCCATAGTCAAGAGCATCACA-3' and reverse 5'-CCCCAACCAGCACTCCAA-3', CK19 forward 5'-GAAGAACCATGAGGAGGAAATCA-3' and reverse 5'-ACCTCATATTGGCTTCGCATGT-3' (34). The specific PCR primers and probes for CD83, CD86, CD1a and IFNγ were obtained from commercially available kits (Applied Biosystems). Internal standard gene expression was examined using TaqMan human GAPDH control reagents VIC (PE Biosystems Japan, Tokyo, Japan). The PCR solution (20 μl) was composed of 1 μl of cDNA solution corresponding to 100 ng of total RNA, 200 nM of each of the forward and reverse primer, 100 nM of internal probe and TaqMan Universal Master Mix (PE Applied Biosystems, Japan), which contained Ampli-Taq Gold DNA polymerase, a reaction buffer, dNTP, dUTP and AmpErase urasil-N-glycosylase. PCR was carried out using the following thermocycler conditions: 50˚C for 2 min and 95˚C for 5 min, followed by 45 cycles of 95˚C for 15 sec and 60˚C for 1 min. PCR amplification of GAPDH mRNA was conducted according to the manufacturer's instructions for the TaqMan GAPDH Control Reagent (PE Applied Biosystems). Data were analyzed using Sequence Detection Software (PE Biosystems Japan).

The quantification of mRNA levels of the target gene was made using real-time fluorescence detection 'TaqMan™' technology. The amounts of CEA and CK19 mRNA in samples were calculated relative to those in MKN45, and the amounts of CD83, CD86, CD1a and INFγ mRNA in samples were calculated relative to those in non-cancerous lymph nodes. Then the relative quantity of target gene was standardized by that of GAPDH mRNA.

Statistical analysis. The statistical significance of intergroup differences in the number of cells expressing CD83 in immunohistological staining was calculated with Kruskal-Wallis and Mann-Whitney U test. The Student's t-test and Mann-Whitney U test were used to analyze the correlation between the expression level of immunologic parameters and the presence of PCR-MM. All p-values were two-sided. A p-value of <0.05 was considered statistically significant.

Results

Distribution and density of mature DCs in the regional LNs in pathological metastasis-positive patients. A total of 119

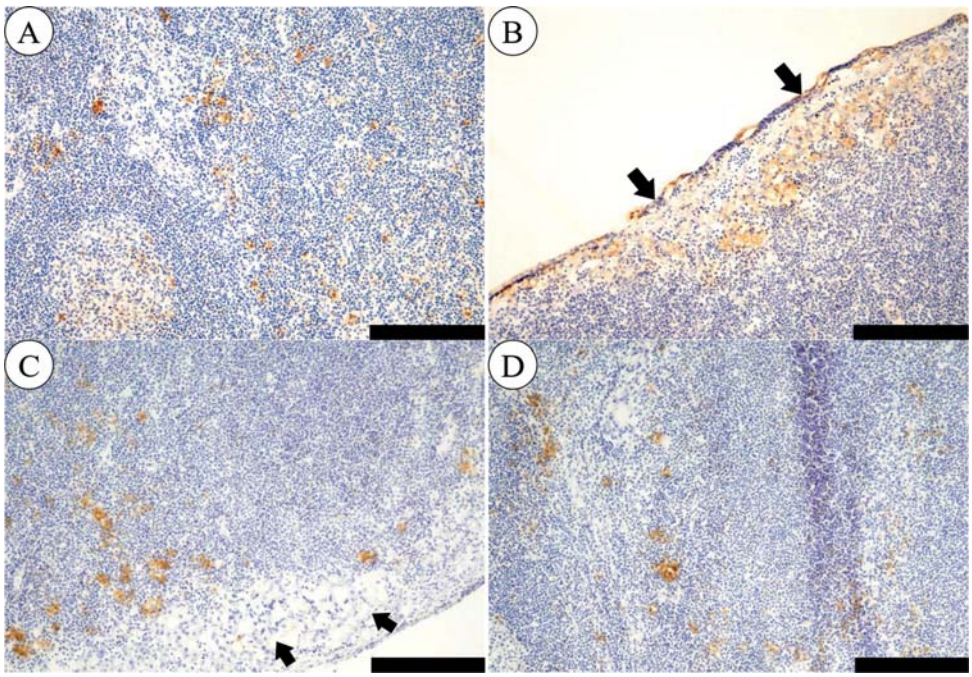

Figure 1. Representative photomicrograph of mature DC and metastatic cancer cell distribution in the regional LNs of gastric cancer. CD83-positive mature DCs were identified as brownish cells in the LNs (A, C and D). Cancer cell metastasis (black arrows) was detected in peripheral sinusoids of the LN [IHC-MM, (B); haematoxylin staining, (C)]. CD83-positive mature DCs were distributed evenly in the LN medulla regardless of the presence of metastatic cancer cells (black arrows) (C and D). Original magnification: (A) and (B) x100, (C) and (D) x400. Scale bar: 200 μm (A) and (B), 50 μm (C) and (D).

LNs from 4 LN metastasis-positive gastric cancer patients were assessed for the presence of cancer cell metastasis and mature DC distribution by haematoxylin and eosin staining and immunohistochemistry. Among 119 LNs from 4 patients, 20 nodes had tumor involvement, as indicated by H&E, as well as tumor cells showing keratin-expression, as indicated by immunohistochemical staining. Moreover, 6 of the histologically negative LNs had occult metastasis, which was indicated only by immunohistochemical staining. The sizes of all 6 keratin-expressing metastatic lesions were <2 mm, and the lesions were considered as IHC-MM. Ninety three LNs had no tumor cells, as indicated by both H&E staining and immunohistochemical staining.

LNs were classified into three groups: 93 nodes without metastasis, 6 nodes with IHC-MM and 20 nodes with pathological metastasis. CD83-positive mature DCs were distributed equally in the LN medulla regardless of the presence of metastatic cancer cells (Fig. 1). We compared the CD83-positive mature DC density among three groups classified according to the degree of metastasis using Kruskal-Wallis analysis of variance. The mature DC density was not different among the three groups (p=0.3208) (Fig. 2).

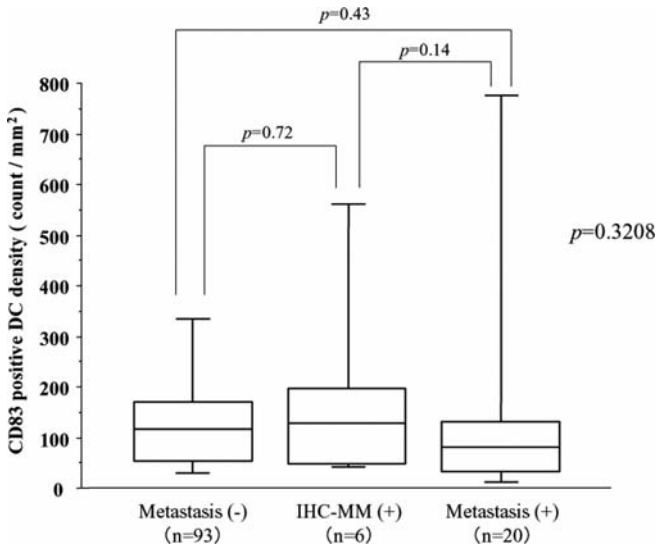

Figure 2. Distribution of CD83-positive mature DCs according to the degree of LN metastasis. The degree of metastasis was separated into three categories according to the keratin immunostaining status: no metastases, IHC-MM and histological metastases. Mature DC density was calculated as the average count of the CD83-expressing DCs in 10 random fields at a magnification of x400 in a non-cancerous region of LN medulla. The density of mature DCs was calculated according to the degree of metastasis. The extent of tumor metastasis had no relation to the mature DC density (p=0.3208).

Detection of PCR-MM and quantification of mRNA expression of immunological parameters in LNs from pathological metastasis-negative cases

Detection of PCR-MM. CEA and CK19 mRNA expression in the 613 lymph nodes from gastric cancer patients ranged from $9.0\text{x}10^{-2}$ to $2.7\text{x}10^{3}$ and $1.0\text{x}10^{-2}$ to $1.7\text{x}10^{5}$, respectively. The corresponding values in normal lymph nodes from non-cancerous patients ranged from 0 to $2.0\text{x}10^{2}$ and 0 to $1.2\text{x}10^{5}$, respectively. In order to exclude false positives, we used the mean values ± 2SD of CEA and CK19 mRNA in normal lymph nodes from non-cancerous patients ($1.3\text{x}10^{2}$ for CEA and $6.1\text{x}10^{4}$ for CK19) as cut-off values. Values above or equal to these cut-off values of CEA and CK19 were defined as CEA and CK19 mRNA positive, respectively. In addition, we defined lymph nodes as PCR-MM positive when either one of CEA mRNA or CK19 mRNA was positive. Using this

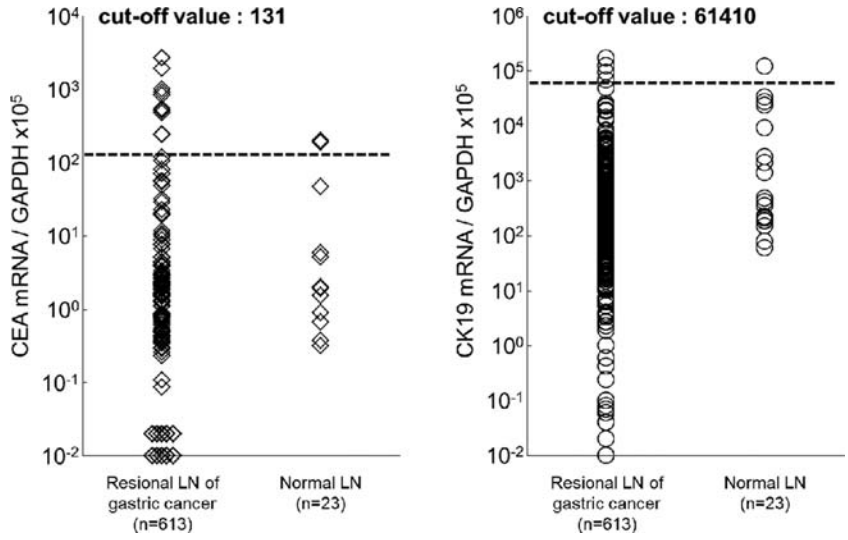

Figure 3. Detection of MM by RT-PCR in the regional pathological metastasis-negative LNs from gastric cancer patients. We defined lymph node PCR-MM when CEA mRNA or CK19 mRNA values were above or equal to the cut-off value: the mean ± 2SD of the target mRNA value in normal lymph nodes from non-cancerous patients ($1.3x10^2$ for CEA and $6.1x10^4$ for CK19).

Table II. Detection of PCR-MM in LNs from pathological metastasis-negative cases.

Parameter	No. of PCR-MM (%)	
	LN (n=613)	Case (n=25)
CK19	4 (0.65)	2 (8)
CEA	11 (1.79)	4 (16)
CK19 or CEA	15 (2.45)	6 (24)

Table III. Expression of immunological parameters in each LN classified by the existence of PCR-MM.

mRNA expression	PCR-MM(+) LN (n=15)	PCR-MM(-) LN (n=598)	P-value
CD1a	0.81 (0-6.7)	0.79 (0-700.0)	0.5864
CD83	2.80 (0-18.7)	1.72 (0-107.1)	0.0242
CD86	2.45 (0-22.8)	1.65 (0-75.5)	0.0421
IFNγ	5.24 (0-819.8)	1.94 (0-376.8)	0.1176

Values are median (range).

cut-off value method, 15 (2.45%) of 613 histologically negative lymph nodes and 6 (24%) of 25 histologically negative cases were determined to be PCR-MM positive (Fig. 3, Table II).

Expression of immunological parameters in PCR-MM-positive LNs. The LNs were separated into two groups based on the presence or absence of PCR-MM as determined by real-time RT-PCR. The expressions of CD83 and CD86 mRNA were significantly higher in PCR-MM-positive than in PCR-MM-negative LNs [2.80 (0-18.7) vs. 1.73 (0-107.1) (p=0.0244), 2.45 (0-22.8) vs. 1.65 (0-75.5) (p=0.0421), respectively]. There was no difference in the expression of CD1a and IFNγ mRNA between the PCR-MM-positive and PCR-MM-negative LNs [0.81 (0-6.7) vs. 0.79 (0-700.0) (p=0.5883), 5.24 (0-819.8) vs. 1.94 (0-376.8) (p=0.1176), respectively] (Table III).

Expression of immunological parameters in LNs from PCR-MM-positive and negative cases. In 151 LNs from 6 PCR-MM-positive cases, 15 LNs were PCR-MM positive and 136 LNs were PCR-MM negative. The expression of immunological parameters in these 151 LNs were compared with 462 LNs from 19 PCR-MM-negative cases. The expression of CD83 and CD86 mRNA was significantly higher in the LNs from PCR-MM-positive cases than in those from PCR-MM-negative cases [2.03 (0-18.7) vs. 1.61 (0-107.1) (p=0.0020), 1.85 (0-29.3) vs. 1.54 (0-75.5) (p=0.0008), respectively]. On the other hand, there was no difference in the expression of CD1a and IFNγ mRNA between the two groups [0.83 (0-617.7) vs. 0.78 (0-700.0) (p=0.3332), 1.84 (0-819.8) vs. 2.03 (0-163.4) (p=0.6941), respectively] (Table IV).

Comparison of immunological parameters among three groups: PCR-MM-positive LNs in PCR-MM-positive cases, PCR-MM-negative LNs in PCR-MM positive cases and PCR-MM-negative LNs in PCR-MM-negative cases. The expression of immunological parameters in LNs were compared among three groups: 15 PCR-MM-positive LNs in 6 PCR-MM-positive cases, 136 PCR-MM-negative LNs in 6 PCR-MM-positive cases and 462 PCR-MM-negative LNs in 19 PCR-MM-negative cases. There were significant differences in the expression of CD83 and CD86 mRNA among the three groups [2.80 (0-18.7) vs. 1.91 (0-12.0) vs. 1.61 (0-107.1) (p=0.0030), 2.45 (0-22.8) vs. 1.80 (0.5-29.3) vs. 1.54 (0-75.5)

Table IV. Expression of immunological parameters in LNs from PCR-MM-negative and positive cases.

mRNA expression	PCR-MM(+) case[a]	PCR-MM(-) case[b]	P-value
CD1a	0.83 (0-617.7)	0.78 (0-700.0)	0.3332
CD83	2.03 (0-18.7)	1.61 (0-107.1)	0.0020
CD86	1.85 (0-29.3)	1.54 (0-75.5)	0.0008
IFNγ	1.84 (0-819.8)	2.03 (0-163.4)	0.6941

Values are median (range). [a]LNs (n=151) from 6 PCR-MM-positive cases were examined. [b]LNs (n=462) from 19 PCR-MM-negative cases were examined.

(p=0.0018), respectively]. There was no difference noted with regard to the expression (values) of CD1a and IFNγ mRNA [0.81 (0-6.7) vs. 0.81 (0-6.7) vs. 0.78 (0-700.0) (p=0.4381), 5.24 (0-819.8) vs. 1.78 (0-376.8) vs. 2.03 (0-163.4) (p=0.2030), respectively] (Table V, Fig. 4). When comparing the three groups in more detail, the expressions of CD83 and CD86 mRNA were significantly higher in both PCR-MM-positive and negative LNs in PCR-MM-positive cases than in PCR-MM-negative LNs in PCR-MM-negative cases (CD83: p=0.0191, p=0.0095, respectively, CD86: p=0.0033, p=0.0311, respectively). However, there was no significant difference in the expressions of CD83 and CD86 mRNA between PCR-MM-positive LNs and PCR-MM-negative LNs in PCR-MM-positive cases (p=0.0656, p=0.1282, respectively).

Table V. Comparison of immunological parameters among three groups according to the PCR-MM status in LNs.

	PCR-MM(+) case[a]		PCR-MM(-) case[b]	
mRNA expression	PCR-MM(+) LN (n=15)	PCR-MM(-) LN (n=136)	PCR-MM(-) LN (n=462)	P-value
CD1a	0.81 (0-6.7)	0.81 (0-6.7)	0.78 (0-700.0)	0.4381
CD83	2.80 (0-18.7)	1.91 (0-12.0)	1.61 (0-107.1)	0.0030
CD86	2.45 (0-22.8)	1.80 (0.5-29.3)	1.54 (0-75.5)	0.0018
IFNγ	5.24 (0-819.8)	1.78 (0-376.8)	2.03 (0-163.4)	0.2030

Values are median (range). [a]LNs (n=151) from 6 PCR-MM-positive cases were classified according to the existence of PCR-MM. [b]LNs (n=462) from 19 PCR-MM-negative cases were examined.

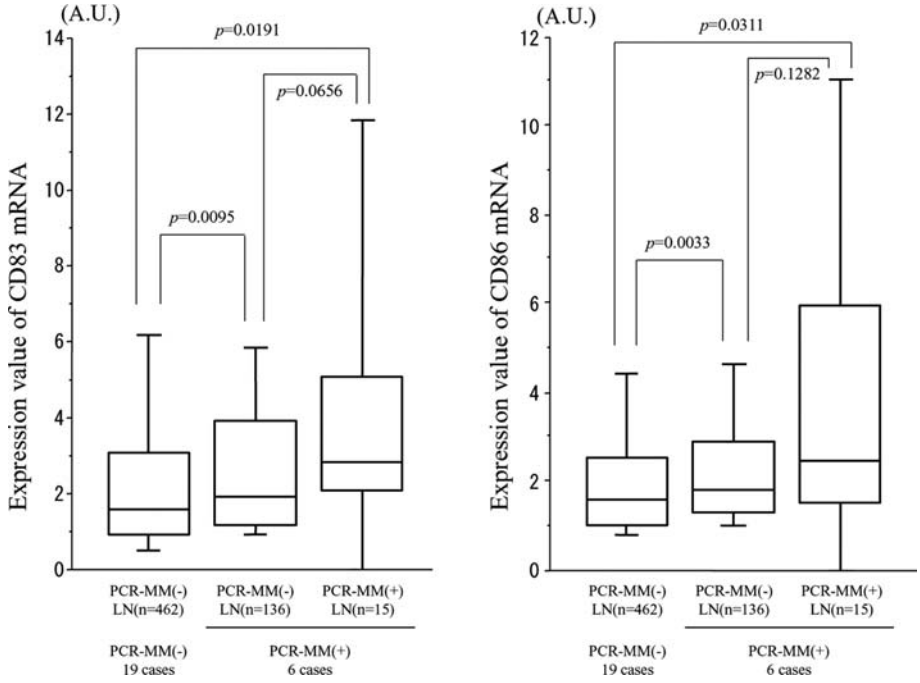

Figure 4. CD83 and CD86 mRNA expression in regional LNs according to the PCR-MM status. CD83 and CD86 mRNAs were quantified by amplifying mRNA by quantitative RT-PCR. The amounts of target mRNA in samples were calculated relative to those in normal lymph nodes from non-cancer patients and standardized according to those of GAPDH mRNA.

In this study, we examined the immunological response including the maturation status of DCs in regional lymph nodes of gastric cancer patients. In metastasis-positive cases, an immunohistochemical study showed that the distribution and density of CD83-positive mature DCs were independent of the extent of metastasis in lymph nodes. In metastasis-negative cases, real-time RT-PCR showed that the expression levels of CD83 and CD86 mRNA were significantly higher in PCR-MM-positive lymph nodes compared with PCR-MM-negative lymph nodes. Comparable results were obtained in comparing PCR-MM-positive cases with negative cases. These results indicate that the immunological response in regional lymph nodes, including the maturation of DCs, is activated when PCR-MM occurs. In addition, further maturation of DCs does not occur after the pathological metastasis is established. This immune response is not restricted to the lymph nodes in which PCR-MM is being developed, but takes place in all of the regional lymph nodes.

The TNM classification stipulates that MM are metastases that measure ≤2 mm but >0.2 mm. First, we examined the immunological response by investigating the maturation status of the DCs histologically in metastasis-positive cases. We found that the distribution and density of mature DCs in LNs were independent of metastasis status in pathological metastasis-positive patients. It was assumed that DCs had been activated in an earlier stage of metastasis than the stage of histologically detectable MM. Therefore, we examined the immunological response by RT-PCR at the stage before pathological MM was established. RT-PCR is a highly sensitive molecular technique to detect the minute epithelial mRNA of cancer cells that could not be detected by immunohistochemical staining. In the present study, we defined the MM detected only by RT-PCR as PCR-MM and that detected by IHC as IHC-MM. We distinguished PCR-MM as the initial state of MM, in contrast to IHC-MM in which metastatic foci was <0.2 mm but could be detected histologically. The relationship between the histological metastasis in lymph nodes and the immune response was reported in previous studies. Focusing on gastric cancer, Tsujitani *et al* (32) investigated the infiltration of DCs into regional lymph nodes by using immunohistochemical staining against S-100 protein. They found that there was a significant correlation between the extent of infiltration by DCs in the primary tumor and that of the peritumoral nodes. They also established that when the primary nodes were involved, the extent of infiltration by DCs was elevated in both the primary and secondary nodes. These authors concluded that DCs in the regional lymph nodes can set up immunological defense mechanisms against lymph node metastasis when metastasis is absent or limited to the primary nodes. Our data demonstrated that the distribution and density of mature DCs in IHC-MM-positive LNs were not different from those in histological metastasis-negative and positive LNs. This observation suggests that the immune response may show a tendency toward immunotolerance or immunosuppression and that the defensive mechanism against cancer may decrease when the histological metastasis, including IHC-MM, is established. The different results in the two studies may result from the different surface markers used to detect the mature DCs. Tsujitani *et al* used S-100 protein as a marker of DCs. However, S-100 protein is expressed in several types of DCs, including immature and mature DCs. In contrast, most of the recent immunological studies, including the present study, used CD83 as a marker of mature DCs. Moreover, the possibility that the primary tumor or its metastasis may cause immune suppression is shown from the examination of several cytokine profiles and the function of DCs (35). Therefore, we propose the hypothesis that the immunological activation, based on DC maturation, had already occurred at a stage before a histological metastasis was detected, and that immune suppression was induced as a result. As far as we know, no information is available on DC maturation accompanied by PCR-MM in the regional lymph nodes of cancer cases. To investigate the relationship between metastasis and the immune response at the earlier stage, we examined the relation between PCR-MM and the maturation status of DCs. The term, 'PCR-MM-positive' means the existence of minute epithelial mRNA. It is uncertain whether or not the cancer cells metastasize to the lymph nodes at the stage of PCR-MM. Because DCs capture tumor-specific antigens in the primary tumor, process the antigens into peptides and migrate to the regional lymph nodes, the possibility that the fragments of mRNA processed and presented by DCs were amplified and recognized as 'PCR-MM positive' cannot be excluded. In any case, we established that the immune response caused by the maturation of the DCs was initiated when small amounts of tumor-specific antigens or cancer cells were admitted into the regional lymph nodes, and that the activation of the immune response was almost ending at the stage when the metastasis in the lymph nodes was observed as IHC-MM. It is uncertain whether the PCR-MM can grow to be recognized as histological metastasis or is excluded by the subsequent immune response. In addition, it is still undetermined whether the DC maturation is triggered after establishment of PCR-MM or whether the immune response is already activated by the signal from the primary tumor before the entry of PCR-MM into the lymph nodes. Di Girolamo *et al* (36) investigated the immune response by immunohistochemistry on $CD4^+$ T cells, $CD8^+$ T cells, B lymphocytes, DCs and NK cells in metastasis-free regional lymph nodes draining different human epithelial tumors and compared the results with those of control lymph nodes from patients with no malignancy. The results indicated that regional lymph nodes contained reduced numbers of almost all immune cells, except $CD57^+$ cells, and the authors concluded that the decrease of several immunocytes might be related to a deficient anti-tumor immune response and subsequent tumor progression in patients with cancer. Laguens *et al* (37) also demonstrated that lymph nodes draining cancer sites had significantly fewer $CD1a^+$ DCs and $CD86^+$ DCs than did lymph nodes from patients without malignancy. In addition, Kiertscher *et al* (35) showed that tumors can produce a number of immunosuppressive factors that block DC maturation and induce early apoptosis of monocyte-derived DCs. These previous reports indicated that immunosuppression took place in the regional lymph nodes draining cancer sites. In the present study, we found that the maturation of DCs in regional lymph nodes is activated when PCR-MM is present. The tumor-specific immune response might be

triggered by the generation of metastasis to regional lymph nodes, but only at the PCR-MM stage. Further activation of DCs and subsequent immunological response in the lymph nodes might decrease because immunosuppressive signals are produced by the primary cancer at the stage after IHC-MM formation.

In order for naïve T-cells to acquire the functions of effector cells, not only tumor-specific antigens associated with MHC molecules but also a second signal provided by co-stimulatory molecules, which are expressed on T-cells, are needed (14). Two co-stimulatory molecules designated CD80 and CD86 have been identified on professional APC, including DCs (38). CD80 and CD86 share two receptors on T cells, which have been designated CD28 and CTLA-4, respectively (39,40). When either CD80 or CD86 binds to the CD28 receptor, a co-stimulatory signal is produced that results in a Th1 immune response. In contrast, binding of either CD80 or CD86 to CTLA-4 appears to activate a Th2 immune response, which results in T-cell anergy (41,42). We demonstrated that CD83 and CD86 mRNA expression is elevated in PCR-MM-positive LNs and patients. These results suggest that when PCR-MM occurs the DCs become activated and mature, and they are then able to present antigens to T-cells. However, there was no difference between the expression levels of IFNγ in PCR-MM-positive and negative LNs. IFNγ is a Th1 cytokine secreted by naïve T-cells, but it is possible that the signal to secrete does not transmit to naïve T-cells although the DCs have already been activated. Another possibility might be that the Th1 response is controlled by binding of CD86 to the CTLA-4 receptor instead of the CD28 receptor. CTLA-4 behaves as a negative regulator of the proliferation and the effector function of T-cells.

Recently, the immune status in the lymph nodes, especially in sentinel lymph nodes (SLNs), was investigated in several malignancies. The immune status of SLNs seems to be suppressed in the absence of cancer cells, although this idea is still controversial (43). Matsuura *et al* (44) investigated the antigen-specific immunity of SLNs based on the maturation of DCs, Th1 and Th2 responses, and T-reg cell responses in lymph node-negative breast carcinoma patients. By using flow cytometry and RT-PCR analysis they observed that the immunological status of SLNs, including DC maturation and Th1 responses, is depressed in SLNs before metastasis. They also observed that the expression levels of CD83, IL-12p40 and IFNγ were higher in metastasis-positive SLNs than in metastasis-negative SLNs, and they concluded that once metastasis was established in SLNs, DC maturation was triggered and followed by the up-regulation of Th1 responses, which may reflect antigen-specific immune responses in SLNs. However, they observed that the immunological status of metastasis-positive SLNs was up-regulated in comparison with metastasis-negative SLNs and was augmented when the values were corrected for MM by using RT-PCR. Their results are in conflict with our suggestions that the immunological response is activated at the PCR-MM stage and that the immune status is not up-regulated when a histological metastasis is present. In melanoma studies, Leong *et al* (45) investigated the cytokine profiles of SLNs draining the primary melanoma and compared them with those of adjacent non-SLNs. They showed that patients with no metastasis had an increased secretion of cytokines in the SLNs, whereas patients harboring lymph-node MM showed no increase of cytokine secretion in the SLNs. This finding suggested that the lymphocytes in tumor-free SLNs could have been stimulated by a very small number of tumor cells that were not detectable by haematoxylin and eosin or immunohistochemical staining. Thus, it seems that a large immune response can be induced in SLNs that are melanoma-free, and that this response might be stimulated by submicroscopic tumor cells or soluble tumor antigens, resulting in the production of cytokines (43). Their suggestion that the immune response might be stimulated in the initial stage of histological or immunohistochemical metastasis development appears to be consistent with our data. Sentinel node biopsy in early gastric cancer is now widely used to predict nodal status. In our institution, lymphatic basin dissection with limited gastrectomy is performed in early-stage gastric carcinoma patients, especially if there is no metastasis detected in SLNs on frozen sections. Lymphatic basins are defined as the lymphatic zones divided by the stream of the stained lymphatic canals. Because it is difficult to identify the SLNs with certainty, we regard the lymphatic basin as the most important lymphatic area in which lymph node metastasis first develops (33,46). We have confirmed the progression pattern of lymph node metastasis: nodal metastasis occurred first in SLNs, then spread to non-SLNs within the lymphatic basin, and rarely extended outside the lymphatic basin. In this study, we also compared the immune response pattern in SLNs with that of non-SLNs. The DC maturation status in SLNs was no different from that in non-SLNs (data not shown). On the other hand, an activated immune response is not restricted to the lymph nodes in which PCR-MM is developing, but takes place in all regional lymph nodes within the lymphatic basin. This suggests that when PCR-MM occurs the immune response develops in all lymph nodes in the same lymphatic basin, regardless of whether the lymph node is an SLN or not.

In summary, we conclude that in gastric cancer the activation of the immune response by maturation of DCs takes place at the stage before metastasis has occurred, especially the stage of MM, and that it develops in the regional lymph nodes. Our results might provide a step toward clarifying the self-defense mechanism against lymph node metastasis in carcinoma. Further studies are needed to clarify whether immunological defense mechanisms work effectively to prevent lymph node metastasis.

References

1. Hart DN: Dendritic cells: unique leukocyte populations which control the primary immune response. Blood 90: 3245-3287, 1997.
2. Becker Y: Anticancer role of dendritic cells (DC) in human and experimental cancers - a review. Anticancer Res 12: 511-520, 1992.
3. Banchereau J and Steinman RM: Dendritic cells and the control of immunity. Nature 392: 245-252, 1998.
4. Tazi A, Bouchonnet F, Grandsaigne M, Boumsell L, Hance AJ and Soler P: Evidence that granulocyte macrophage-colony-stimulating factor regulates the distribution and differentiated state of dendritic cells/langerhans cells in human lung and lung cancers. J Clin Invest 91: 566-576, 1993.
5. Cella M, Sallusto F and Lanzavecchia A: Origin, maturation and antigen presenting function of dendritic cells. Curr Opin Immunol 9: 10-16, 1997.

te R, Toso JF, Butts C, Young HA and Hwu P: Human itic cells require multiple activation signals for the efficient generation of tumor antigen-specific T lymphocytes. Eur J Immunol 30: 3291-3298, 2000.
7. Bell D, Chomarat P, Broyles D, *et al*: In breast carcinoma tissue, immature dendritic cells reside within the tumor, whereas mature dendritic cells are located in peritumoral areas. J Exp Med 190: 1417-1426, 1999.
8. Krenacs L, Tiszalvicz L, Krenacs T and Boumsell L: Immunohistochemical detection of CD1A antigen in formalin-fixed and paraffin-embedded tissue sections with monoclonal antibody 010. J Pathol 171: 99-104, 1993.
9. Lechmann M, Berchtold S, Hauber J and Steinkasserer A: CD83 on dendritic cells: more than just a marker for maturation. Trends Immunol 23: 273-275, 2002.
10. Zhou LJ, Schwarting R, Smith HM and Tedder TF: A novel cell-surface molecule expressed by human interdigitating reticulum cells, langerhans cells, and activated lymphocytes is a new member of the ig superfamily. J Immunol 149: 735-742, 1992.
11. Zhou LJ and Tedder TF: $CD14^+$ blood monocytes can differentiate into functionally mature $CD83^+$ dendritic cells. Proc Natl Acad Sci USA 93: 2588-2592, 1996.
12. Lohmann S, Galle K, Knop J and Enk AH: $CD83^+$ human dendritic cells transfected with tumor peptide cDNA by electroporation induce specific T-cell responses: a potential tool for gene immunotherapy. Cancer Gene Ther 7: 605-614, 2000.
13. Marrack P and Kappler J: The T cell receptor. Science 238: 1073-1079, 1987.
14. Geppert TD, Davis LS, Gur H, Wacholtz MC and Lipsky PE: Accessory cell signals involved in T-cell activation. Immunol Rev 117: 5-66, 1990.
15. Linsley PS and Ledbetter JA: The role of the CD28 receptor during T cell responses to antigen. Annu Rev Immunol 11: 191-212, 1993.
16. June CH, Bluestone JA, Nadler LM and Thompson CB: The B7 and CD28 receptor families. Immunol Today 15: 321-331, 1994.
17. Freidlin IS: Cells of immune system: development, activation, effector functions. Russ J Immunol 4: 220-223, 1999.
18. McRae BL, Semnani RT, Hayes MP and van Seventer GA: Type I IFNs inhibit human dendritic cell IL-12 production and Th1 cell development. J Immunol 160: 4298-4304, 1998.
19. Lespagnard L, Gancberg D, Rouas G, *et al*: Tumor-infiltrating dendritic cells in adenocarcinomas of the breast: a study of 143 neoplasms with a correlation to usual prognostic factors and to clinical outcome. Int J Cancer 84: 309-314, 1999.
20. Ishigami S, Aikou T, Natsugoe S, *et al*: Prognostic value of HLA-DR expression and dendritic cell infiltration in gastric cancer. Oncology 55: 65-69, 1998.
21. Maehara Y, Kabashima A, Tokunaga E, *et al*: Recurrences and relation to tumor growth potential and local immune response in node-negative advanced gastric cancer. Oncology 56: 322-327, 1999.
22. Ishigami S, Natsugoe S, Tokuda K, *et al*: Clinical impact of intratumoral natural killer cell and dendritic cell infiltration in gastric cancer. Cancer Lett 159: 103-108, 2000.
23. Maehara Y, Tomisaki S, Oda S, *et al*: Lymph node metastasis and relation to tumor growth potential and local immune response in advanced gastric cancer. Int J Cancer 74: 224-228, 1997.
24. Tsujitani S, Kakeji Y, Watanabe A, Kohnoe S, Maehara Y and Sugimachi K: Infiltration of dendritic cells in relation to tumor invasion and lymph node metastasis in human gastric cancer. Cancer 66: 2012-2016, 1990.
25. Ishigami S, Natsugoe S, Hokita S, *et al*: Intranodal antitumor immunocyte infiltration in node-negative gastric cancers. Clin Cancer Res 6: 2611-2617, 2000.
26. Adashek K, Sanger J and Longmire WP Jr: Cancer of the stomach. Review of consecutive ten year intervals. Ann Surg 189: 6-10, 1979.
27. Morgagni P, Saragoni L, Scarpi E, *et al*: Lymph node micrometastases in early gastric cancer and their impact on prognosis. World J Surg 27: 558-561, 2003.
28. Yasuda K, Adachi Y, Shiraishi N, Inomata M, Takeuchi H and Kitano S: Prognostic effect of lymph node micrometastasis in patients with histologically node-negative gastric cancer. Ann Surg Oncol 9: 771-774, 2002.
29. Cai J, Ikeguchi M, Tsujitani S, Maeta M, Liu J and Kaibara N: Significant correlation between micrometastasis in the lymph nodes and reduced expression of E-cadherin in early gastric cancer. Gastric Cancer 4: 66-74, 2001.
30. Fukagawa T, Sasako M, Mann GB, *et al*: Immunohistochemically detected micrometastases of the lymph nodes in patients with gastric carcinoma. Cancer 92: 753-760, 2001.
31. Morgagni P, Saragoni L, Folli S, *et al*: Lymph node micrometastases in patients with early gastric cancer: experience with 139 patients. Ann Surg Oncol 8: 170-174, 2001.
32. Tsujitani S, Oka A, Kondo A, Ikeguchi M, Maeta M and Kaibara N: Infiltration of dendritic cells into regional lymph nodes in gastric cancer. Cancer 75: 1478-1483, 1995.
33. Miwa K, Kinami S, Taniguchi K, Fushida S, Fujimura T and Nonomura A: Mapping sentinel nodes in patients with early-stage gastric carcinoma. Br J Surg 90: 178-182, 2003.
34. Inokuchi M, Ninomiya I, Tsugawa K, Terada I and Miwa K: Quantitative evaluation of metastases in axillary lymph nodes of breast cancer. Br J Cancer 89: 1750-1756, 2003.
35. Kiertscher SM, Luo J, Dubinett SM and Roth MD: Tumors promote altered maturation and early apoptosis of monocyte-derived dendritic cells. J Immunol 164: 1269-1276, 2000.
36. Di Girolamo W, Coronato S, Portiansky E and Laguens G: Profile of immune cells in lymph nodes draining human malignant tumors. Medicina (B Aires) 68: 423-427, 2008.
37. Laguens G, Coronato S, Laguens R, Portiansky E and Di Girolamo V: Human regional lymph nodes draining cancer exhibit a profound dendritic cell depletion as comparing to those from patients without malignancies. Immunol Lett 84: 159-162, 2002.
38. Caux C, Vanbervliet B, Massacrier C, *et al*: B70/B7-2 is identical to CD86 and is the major functional ligand for CD28 expressed on human dendritic cells. J Exp Med 180: 1841-1847, 1994.
39. Lenschow DJ, Walunas TL and Bluestone JA: CD28/B7 system of T cell costimulation. Annu Rev Immunol 14: 233-258, 1996.
40. Boussiotis VA, Freeman GJ, Gribben JG and Nadler LM: The role of B7-1/B7-2:CD28/CLTA-4 pathways in the prevention of anergy, induction of productive immunity and down-regulation of the immune response. Immunol Rev 153: 5-26, 1996.
41. Krummel MF and Allison JP: CD28 and CTLA-4 have opposing effects on the response of T cells to stimulation. J Exp Med 182: 459-465, 1995.
42. Marengere LE, Waterhouse P, Duncan GS, Mittrucker HW, Feng GS and Mak TW: Regulation of T cell receptor signaling by tyrosine phosphatase SYP association with CTLA-4. Science 272: 1170-1173, 1996.
43. Kim R, Emi M, Tanabe K and Arihiro K: Immunobiology of the sentinel lymph node and its potential role for antitumour immunity. Lancet Oncol 7: 1006-1016, 2006.
44. Matsuura K, Yamaguchi Y, Ueno H, Osaki A, Arihiro K and Toge T: Maturation of dendritic cells and T-cell responses in sentinel lymph nodes from patients with breast carcinoma. Cancer 106: 1227-1236, 2006.
45. Leong SP, Peng M, Zhou YM, Vaquerano JE and Chang JW: Cytokine profiles of sentinel lymph nodes draining the primary melanoma. Ann Surg Oncol 9: 82-87, 2002.
46. Kinami S, Fujimura T, Ojima E, *et al*: PTD classification: proposal for a new classification of gastric cancer location based on physiological lymphatic flow. Int J Clin Oncol 13: 320-329, 2008.