

The Spleen Plays an Immunosuppressive Role in Patients with Gastric Cancer: Involvement of CD62L⁺ Cells and TGF-beta

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Abstract. CD62L is the human homologue of the murine lymphocyte homing receptor, *mel-14*. We investigated CD62L⁺ cells in the spleen from patients with gastric cancer. Flow cytometric analysis revealed that CD62L⁺ cells were decreased in the peripheral blood, but inversely increased in the spleen in parallel with disease progression in gastric cancer patients. The increased CD62L⁺ cells resided in the CD4⁺ suppressor-inducer phenotype, and the removal of CD62L⁺ cells from spleen cells resulted in a decrease of concanavalin-A-induced suppressor activity in vitro in one-way allogeneic mixed lymphocyte reaction. The CD62L⁺ cells included CD4⁺CD25⁺ regulatory T cells. The culture supernatant of CD62L⁺ cells showed TGF-beta activity that permitted anchorage-independent growth of normal rat kidney (NRK) cells in a soft agar. TGF-beta activity was more significantly detectable in the splenic vein than in the peripheral blood, and TGF-beta mRNA was detectable in the spleen from advanced gastric cancer patients. These results suggest that CD62L⁺ cells migrate into the spleen with disease progression of gastric cancer and serve as suppressor-inducer cells with TGF-beta production to induce regulatory T cells, contributing to disease-associated immunosuppression in advanced gastric cancer patients.

The spleen is the biggest and one of the most important organs in the body's immune system. In children with splenectomy for splenic injury, idiopathic thrombocytopenic purpura is frequently associated with post-splenectomy

sepsis, which can be fatal (1), suggesting the involvement of the spleen in the host defense system. However, this is not the case in adults. Although splenectomy is often performed in patients with gastric cancer to obtain complete removal of the primary tumor in cases of extended invasion penetrating the stomach wall or to achieve sufficient lymph node dissection of the splenic hilus, the incidence of post-splenectomy sepsis is very low (2). Thus, the role of the spleen in the immune system in gastric cancer patients is not fully understood.

We examined the immunological roles of the spleen in gastric cancer patients. Previously, we had shown that the spleen is involved in the immunosuppression mechanism in patients with gastric cancer and that the suppressor precursor cells matured in the spleen in parallel with disease progression (3). To extend this observation, we here examined CD4⁺CD62L⁺ suppressor-inducer cells in the spleen in patients with gastric cancer (4, 5). We also investigated TGF-beta expression in the spleen, which is known to be the most potent immunosuppressive factor (6).

Materials and Methods

Patients. Sixty-three untreated patients with gastric cancer, comprising 40 males and 23 females, whose mean ages were 64 and 61 years old, respectively, were enrolled in this study. The pathological stage distribution was 25 patients in stage I and II, 21 in stage III and 17 in stage IV, according to the Japanese classification for gastric cancer (7). Splenectomy was performed in 6 patients with stage I, II tumor burden and in 9 with stage III, IV tumor burden. Twenty healthy volunteers served as the control group. Written informed consent was obtained from all subjects before enrollment in the study.

Collection of lymphocytes and spleen cells. Heparinized blood was obtained from a peripheral vein, artery and splenic vein, intra-operatively, and buffy coat and plasma were immediately separated by centrifugation (2,000 rpm, 30 minutes). The buffy coat was resuspended in RPMI-1640 medium. The spleen cell suspension was prepared by mincing 1-cm blocks of the resected spleen using scissors and RPMI-1640 medium through a wire mesh (No. 2000).

Abbreviations: CD, cluster differentiation; EDTA, ethylenediaminetetraacetic acid; EGF, epidermal growth factor; RPMI, Roswell Park Memorial Institute; RT-PCR, reverse transcription polymerase chain reaction; TGF, transforming growth factor.

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The buffy coat suspension and spleen cell suspension were layered on Ficoll-Conray. Peripheral blood lymphocytes (PBLs) and spleen cells were isolated by gradient centrifugation (2,000 rpm, 30 min), washed twice and resuspended in the medium containing 2% autologous serum at a density of $1 \times 10^6/\text{ml}$.

Selection of CD62L⁻ and CD62L⁺ cells. Culture flasks (Sumitomo, Japan) had been pre-coated with 10 $\mu\text{g}/\text{ml}$ anti-CD62L antibody (Becton Dickinson Immune Sys., USA) for 18 hours at 4°C, washed, and further incubated with RPMI-1640 medium containing 1% bovine serum albumin for more than 18 hours at 4°C. Each flask was rinsed 4 times with RPMI-1640 medium before use. Spleen cells ($1 \times 10^6/\text{ml}$) were put in the flask and incubated for 4 hours at room temperature. Uncaptured cells and captured cells were collected as CD62L⁻ and CD62L⁺ cells, respectively, washed, and resuspended in the medium. Flow cytometry revealed the concentration of CD62L⁺ cells to be less than 6% in the CD62L⁻ fraction and more than 85% in the CD62L⁺ fraction. Negative selection was also performed using anti-CD62L antibody plus complement. Spleen cells were incubated with anti-CD62L antibody or control IgG2b (Becton Dickinson Immune Sys.) and rabbit complement (Cedarlane Lab. Lim., USA) for 1 hour at 37°C, and viable cells were collected as CD62L⁻ cells. Flow cytometry revealed that the level of CD62L⁻ cells was more than 89%.

Suppressor-inducer activity. One-way mixed lymphocyte reaction (MLR) was performed, as described in detail elsewhere (3). In brief, spleen cells were stimulated with 0 or 10 $\mu\text{g}/\text{ml}$ concanavalin-A (Con-A, Boehringer, Germany) for 24 hours at 37°C. Cells were treated with 50 $\mu\text{g}/\text{ml}$ mitomycin-C for 1 hour at 37°C, washed 3 times, and resuspended in the medium as effector cells ($5 \times 10^5/\text{ml}$). Responder PBLs ($5 \times 10^5/\text{ml}$) were collected from healthy subjects. Effector cells and responder cells were co-cultured in RPMI-1640 medium containing 10% AB serum for 4 days at 37°C in the presence of 15 $\mu\text{g}/\text{ml}$ phytohemagglutinin (DIFCO, USA). The cells were pulsed with 5 $\mu\text{Ci}/\text{ml}$ ³H-thymidine and incubated for another 8 hours. The cells were harvested and radioactivity was measured. Suppressor-inducer activity (SIA) was calculated using the following formula:

$$\text{SIA (\%)} = \{1 - \text{MLR (cpm)} / \text{responder cells alone (cpm)}\} \times 100$$

Flow cytometry. Fifty microliters each of the lymphocyte suspension and spleen cell suspension (5×10^5) were stained with antibodies, washed, and then analyzed on a Cytoron (Ortho Diagnostic Systems, USA). The antibodies used were peridinin chlorophyll protein-labelled anti-CD4 and -CD8 antibodies, pycoerythrin-labelled anti-CD25 antibody, and fluorescein isothiocyanate-labelled anti-CD62L antibody. All antibodies used were purchased from Becton Dickinson, San Diego, CA, USA.

Bioassay for TGF-beta activity. TGF-beta activity was measured by the bioassay described in detail elsewhere (8). Normal rat kidney (NRK) cells (9) were maintained in DMEM supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM L-glutamine, 100 U/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin (complete medium) in a humidified incubator with 5% CO₂ at 37°C and transferred twice a week using a standard trypsinization. NRK cells (80,000 cells / well) were plated in aliquots of 400 μl of methylcellulose (1.2% w/v)-containing DMEM supplemented with 2% FCS in 11-mm wells of a 24-well culture dish. Each well then

received 10 ng/ml EGF (Wakunaga, Japan) and 40 μl of either patients' sera or culture supernatants or control TGF-beta1 (R & D Systems, MN, USA). Patients' sera and culture supernatant were conditioned under the acidification and neutralization protocol before use (8). After a 5-day incubation, ³H-thymidine was added to a final concentration of 3 $\mu\text{Ci}/\text{ml}$, and the cells were incubated for an additional 24 hours. The methylcellulose was then transferred to Eppendorf tubes, and the cells were pelleted by centrifugation. DNA synthesis was determined from the incorporation of ³H-TdR into TCA-precipitable materials. In some experiments, TGF-beta activity was evaluated by counting the growing colonies of NRK cells.

RT-PCR. Total RNA was extracted from 5×10^5 spleen cells and reverse-transcribed with random hexamer, as described previously (10). Aliquots of the cDNA were amplified by polymerase chain reaction (PCR), using TGF-beta-1-specific oligonucleotides on a DNA thermal cycler (Perkin Elmer, Norwalk, CT, USA). Primer sequences used were as follows: TGF-beta1, 5'-AACACATCAG AGCTCCGAGAA-3', 5'-GTCAATGTACAGCTGCCGCAC-3' (11); β -actin (Stratagene, La Jolla, CA, USA). The reaction was carried out on a Perkin-Elmer Cetus thermal cycler, under conditions involving a 3-minute denaturation at 94°C followed by 35 cycles of 1 minute at 94°C, 1 minute at 55°C and 1 minute at 72°C. After amplification, 8 μl of the reaction mixture was removed and analyzed by means of electrophoresis through 2.0% agarose gels in Tris-borate-EDTA buffer, and the gels were then stained with ethidium bromide. The expected lengths of the amplified cDNAs were 500 bp and 514 bp for TGF-beta1 and β -actin, respectively.

Statistical analysis. Statistical analysis was conducted by χ^2 test and paired or un-paired Student's *t*-test using StatView software (Version 5) on a Macintosh computer.

Results

Decrease of CD62L⁺ cells in peripheral blood in gastric cancer patients. We analyzed the CD62L⁺ cell population in peripheral blood on flow cytometry using single-color analysis (Figure 1). It was found that the CD62L⁺ cell population was $59 \pm 8\%$, $53 \pm 6\%$, $39 \pm 9\%$ and $31 \pm 13\%$ in healthy donors, stage I+II, stage III and stage IV gastric cancer patients, respectively. There were significant differences between the CD62L⁺ level of healthy donors and those of stage III ($p < 0.05$) or stage IV ($p < 0.01$) gastric cancer patients.

Increase of CD62L⁺ cells in the spleen from gastric cancer patients. We next analyzed the distribution of CD62L⁺ cells and compared distribution in the artery, spleen, splenic vein and peripheral vein in gastric cancer patients (Figure 2). In stage I, II patients with gastric cancer, the CD62L⁺ cell population decreased in the spleen to one-third compared with those of the artery and veins tested. In contrast, the CD62L⁺ cell population increased in the spleen in stage III, IV gastric cancer patients to a level higher than those of the artery and veins. There was a significant difference in the

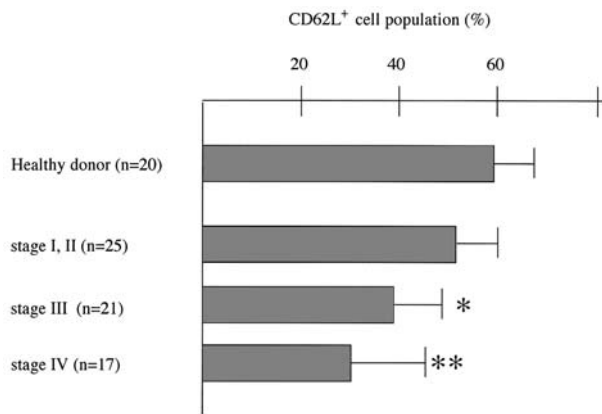


Figure 1. Decrease of CD62L⁺ cells in peripheral blood in gastric cancer patients. PBLs were collected from untreated gastric cancer patients, and flow cytometry was performed to determine CD62L⁺ cells. Significant differences from healthy volunteers, * $p < 0.05$, ** $p < 0.01$.

CD62L⁺ cell population of the spleen between stage I, II and III, IV gastric cancer patients ($p < 0.05$).

CD4⁺ CD62L⁺ cells in the spleen. To analyze, in detail, the increase of the CD62L⁺ cell population in the spleen of stage III, IV gastric cancer patients, we performed two-color flow cytometric analysis (Table I). We could not observe any difference in the CD8⁺CD62L⁺ cell population in the spleen between stage I, II and stage III, IV gastric cancer patients. On the other hand, the CD4⁺CD62⁺ cell population was quite different from the CD8⁺CD62L⁺ cell population. CD4⁺CD62L⁺ cells in the spleen from stage I, II gastric cancer patients were present at an $8.6 \pm 3.2\%$ level, and from stage III, IV patients at $18.2 \pm 6.8\%$, showing an almost double increase in the spleen from stage III, IV gastric cancer patients. There was a significant difference between these values ($p < 0.05$).

CD4⁺CD25⁺ cells in CD62L⁺ spleen cells. Next, three-color flow cytometry was performed to analyze the existence of CD4⁺CD25⁺ cells in CD62L⁺ spleen cells (Figure 3). When gated on CD4⁺ cells, CD25⁺CD62L⁺ and CD25⁺CD62L⁻ cells were 7.7% and 2.5%, respectively, indicating that approximately 70% of the CD4⁺CD25⁺ cells expressed CD62L in spleen cells from stage III, IV gastric cancer patients.

Suppressor-inducer activity of CD62L⁺ cells in the spleen. Next, we investigated the suppressor-inducer activity using spleen cells from stage III, IV gastric cancer patients in a one-way allogeneic mixed lymphocyte reaction (Table II). Con-A stimulation of effector lymphocytes generated

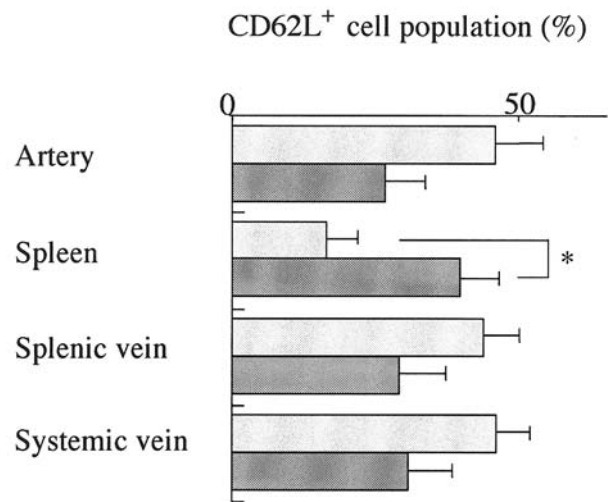


Figure 2. Distribution of CD62L⁺ cells in gastric cancer patients. Lymphocytes were collected from the artery, spleen, splenic vein and peripheral vein in 6 and 9 stage I, II and stage III, IV gastric cancer patients, respectively, and flow cytometry was performed to determine CD62L⁺ cells. Significant difference, * $p < 0.05$.

suppressor-inducer activity (59.2 ± 15.1) in unfractionated spleen cells. When spleen cells were fractionated using anti-CD62L antibody-coated flask, CD62L⁺ cells (79.8 ± 11.4) showed significantly higher suppressor-inducer activity with Con-A stimulation than did CD62L⁻ cells (39.5 ± 14.0) ($p < 0.001$). The CD62L⁻ cells and CD62L⁺ cells showed significantly lower and higher Con-A-stimulated suppressor-inducer activity, respectively, as compared with that of unfractionated spleen cells ($p < 0.01$). When CD62L⁻ cells were selected using anti-CD62L antibody plus complement, they also showed significantly lower suppressor-inducer activity (32.5 ± 14.4) ($p < 0.01$), which was very similar to the activity of CD62L⁻ cells fractionated with the antibody-coated flask. Treatment of spleen cells with control antibody plus complement, however, did not affect Con-A-induced suppressor-inducer activity at all.

TGF-beta activity in culture supernatant of spleen cells. We cultured fractionated spleen cells and determined TGF-beta activity in the supernatant using NRK cells (Figure 4). Culture supernatants on day 1 from unfractionated and CD62L⁻ spleen cells failed to stimulate growth of NRK cells on a soft agar. Culture supernatants on day 1 from CD62L⁺ cells, on the other hand, showed significant growth of NRK cells on a soft agar compared with those of unfractionated and CD62L⁻ spleen cells ($p < 0.05$). This TGF-beta activity increased day by day, and culture supernatant on day 3 of CD62L⁺ cells contained approximately 10 ng/ml TGF-beta as calculated by the control TGF-beta.

Table I. CD62L⁺ cell population in the spleen from gastric cancer patients.

Cell population	Stage I, II (n=6)	Stage III, IV (n=9)
CD4 ⁺ CD62L ⁻	24.7±6.4	20.4±6.4
CD4 ⁺ CD62L ⁺	8.6±3.2	18.2±6.8*
CD8 ⁺ CD62L ⁻	16.4±3.8	18.3±8.9
CD8 ⁺ CD62L ⁺	8.5±2.4	8.8±3.5

Spleen cells were collected from gastric cancer patients, and two-color flow cytometry was performed. Significant difference in the values between the spleen from stage I, II and stage III, IV gastric cancer patients, $p < 0.05$.

TGF-beta activity in the splenic vein. The TGF-beta activity in sera from splenic veins was analyzed and compared with that in peripheral veins (Figure 5). We could obtain no significant increase of growing colonies of NRK cells on a soft agar with sera from splenic veins compared with that from peripheral veins in stage I, II gastric cancer patients. In stage III, IV gastric cancer patients, on the other hand, a significant increase of growing colonies of NRK cells on a soft agar was observed in the presence of sera from splenic veins (74.5 ± 7.6) compared with peripheral veins (38.8 ± 11.8) ($p < 0.01$).

TGF-beta expression in the spleen. Finally, we used RT-PCR to analyze TGF-beta expression in the spleen (Figure 6). Spleen cells from stage I, II gastric cancer patients showed a positive band for TGF-beta in one (patient 1) of 4 patients tested. On the other hand, TGF-beta mRNA was detected in 4 (patients 5, 6, 8, 11) of 7 spleens from stage III, IV gastric cancer patients.

Discussion

CD62L has been used to identify suppressor-inducer CD4⁺ T-lymphocyte subsets (4, 5). In this study, we showed that CD62L⁺ cells decreased in the peripheral blood, but inversely increased in the spleen, in relation to disease progression in patients with gastric cancer. Two-color analysis showed that this increase of CD62L⁺ cells in the spleen resulted from an increase of the CD4⁺CD62L⁺ suppressor-inducer phenotype. Moreover, we observed that CD62L⁺ cells were involved in the Con-A-stimulated suppressor-inducer activity that was determined with allogeneic one-way mixed lymphocyte reaction. Independently of this investigation, we previously reported similar results of the disease-associated increase in the spleen from gastric cancer patients of suppressor-inducer cells that were identified with

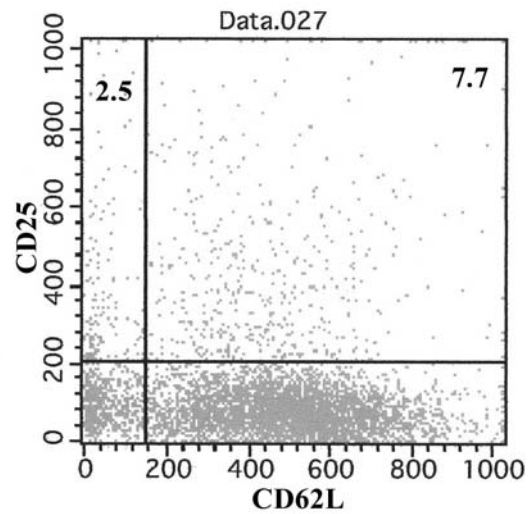


Figure 3. Three-color analysis of spleen cells using anti-CD4, CD25 and CD62L antibodies. Spleen cells were stained with antibodies. CD25⁺CD62L⁺ cells and CD25⁺CD62L⁻ cells, which were gated on CD4⁺ cells, were displayed.

CD4⁺CD45RA⁺ naïve T cells (12), indicating the involvement of the spleen in the immunosuppression mechanism of gastric cancer patients. Importantly, CD62L is the human homolog of the mouse mel-14 homing receptor, a molecule that promotes the initial adhesion of blood-borne lymphocytes to the specialized post-capillary endothelium of lymphoid organs (13). It is present on the surface membrane of all leukocytes, where it plays a critical role in the early weak adhesive "rolling" interactions between white cells and vascular wall (14). CD62L mediates homing of naïve T-lymphocytes (CD45RA⁺) to the high endothelial venules of peripheral lymph nodes and serves as the primary site of binding for circulating lymphocytes into organized peripheral lymphoid tissues (15). Collectively, it is suggested that naïve T cells migrate into the spleen by using CD62L, a human homolog of homing receptor, and function as suppressor-inducer cells to negatively regulate immune responses in advanced gastric cancer patients.

Our CD62L⁺ cells in the spleen expressed a CD4⁺CD25⁺ phenotype, which has recently been highlighted as regulatory T (Treg) cells (16-18). The CD4⁺CD25⁺ Treg cells are highly involved in tumor immunity and autoimmunity. Onizuka *et al.* (16) reported, in murine tumor models, that *in vivo* elimination of CD25⁺ cells using the administration of anti-CD25 monoclonal antibody caused the regression of tumors that grew progressively in syngeneic mice. Fu *et al.* (17) divided CD4⁺CD25⁺ Tregs into two subsets according to CD62L expression and demonstrated that the CD62L⁺ subset was a more potent suppressor than the CD62L⁻ population or

Table II. Suppressor-inducer activity of spleen cells from gastric cancer patients.

Fractionation	Suppressor -inducer activity (%)					Mean \pm S.D.
	Con-A	Pt. 1	Pt. 2	Pt. 3	Pt. 4	
None	-	18.4	62.8	54.9	60.9	49.3 \pm 20.8*
	+	37.3	64.3	63.0	72.1	59.2 \pm 15.1**
CD62L ⁻	+	18.6	42.3	42.8	54.3	39.5 \pm 14.0#
CD62L ⁺	+	62.7	85.6	84.3	86.5	79.8 \pm 11.4##
Anti-CD62L ⁺ C'	+	12.2	41.8	32.4	43.6	32.5 \pm 14.4¥
IgG ⁺ C'	+	53.5	62.1	60.1	68.9	61.2 \pm 6.3¥¥

Spleen cells were collected, and CD62L⁻ cells were fractionated. Cells were stimulated with Con-A, and suppressor-inducer activity was evaluated using one-way mixed lymphocyte reaction analysis. Statistical difference between * and **, $p=0.078$; # and ##, $p=0.0007$; ¥ and ¥¥, $p=0.0078$; ** and #, $p=0.0002$; ** and ##, $p=0.0029$; ** and ¥, $p=0.0007$; ** and ¥¥, $p=0.7053$.

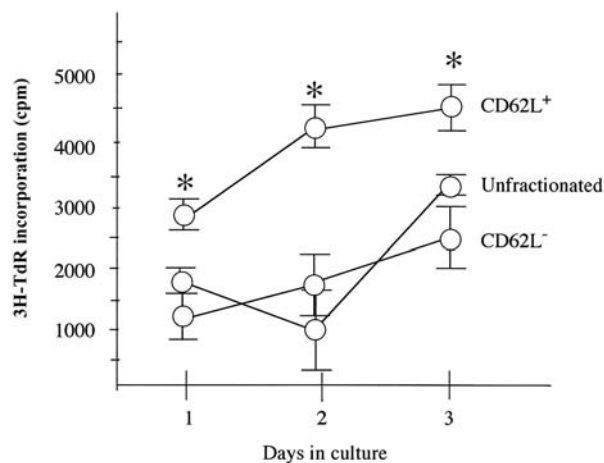


Figure 4. TGF-beta activity of CD62L⁺ and CD62L⁻ spleen cells. Spleen cells were collected from stage III, IV gastric cancer patients and fractionated using anti-CD62L antibody. Cells were cultured for 3 days, and TGF-beta activity in the supernatant was determined using NRK cell growth in a soft agar.

unfractionated CD4⁺CD25⁺ Treg cells. Moreover, Szanya *et al.* (18) showed that CD4⁺CD25⁺ splenocytes inhibited diabetes in cotransfer with islet-infiltrating cells, and that CD62L expression was necessary for this disease-delaying effect of CD4⁺CD25⁺ cells in a nonobese diabetic mouse model. Taken together, these findings strongly suggest that CD62L⁺ cells in the spleen serve, in part, as regulatory T cells and contribute to disease-associated immunosuppression in advanced gastric cancer patients.

Finally, we investigated the involvement of TGF-beta, which is the most potent soluble immunosuppressive factor (6), in the spleen from advanced gastric cancer patients. We observed that CD62L⁺ cells in the spleen were responsible for TGF-beta production, and that TGF-beta activity and TGF-beta

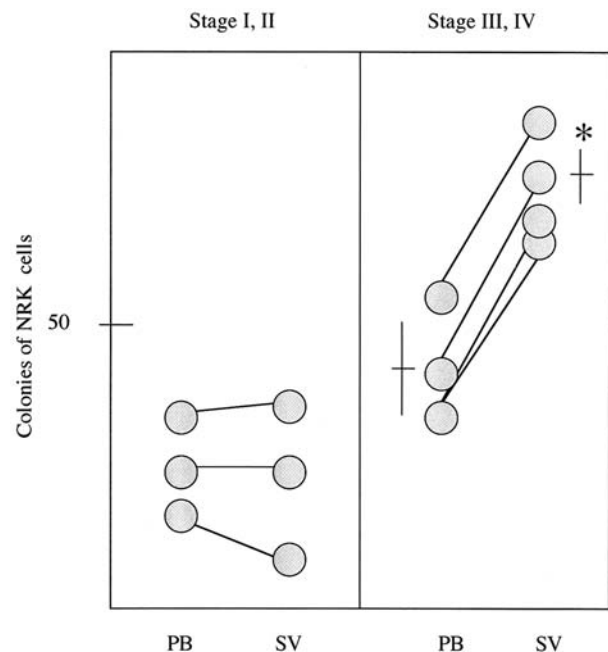


Figure 5. TGF-beta activity in sera from a peripheral vein and a splenic vein. Sera were collected from peripheral blood (PB) and a splenic vein (SV) from 3 and 4 stage I, II and stage III, IV gastric cancer patients, respectively, and TGF-beta activity in the sera was determined using NRK cell growth in a soft agar. Significant difference, * $p<0.01$.

mRNA expression were detectable in the splenic vein and the spleen, respectively, in relation to disease progression. Ueda *et al.* (19) also reported the production of TGF-beta in the spleen, which contributed to growth inhibition of hepatocyte regeneration. Saito *et al.* (20) reported that the serum level of TGF-beta was elevated and correlated with lymph node metastasis and poor prognosis in patients with gastric cancer, suggesting that TGF-beta produced in the spleen is also

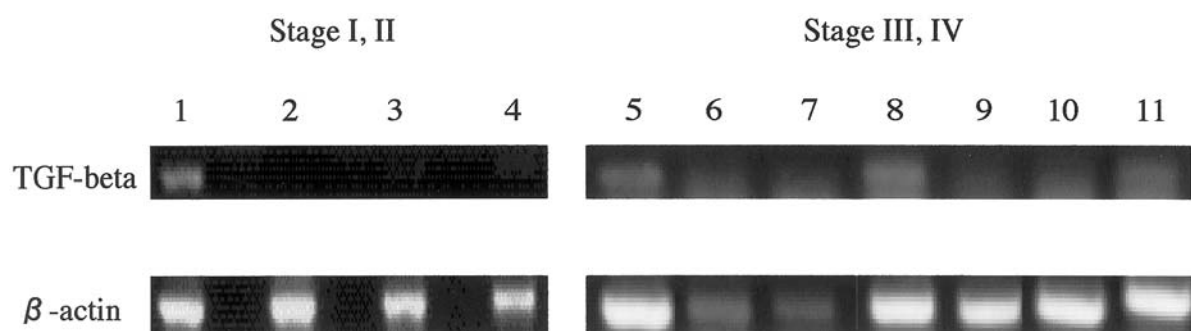


Figure 6. *TGF-beta* expression in the spleen. Spleen cells were collected from 4 and 7 stage I, II and stage III, IV gastric cancer patients, respectively, and *TGF-beta* expression was determined using RT-PCR analysis.

involved in the immunosuppression mechanism in gastric cancer patients. Recently, it was reported that TGF-beta induced a regulatory phenotype in CD4⁺CD25⁻ T cells through the induction of Foxp3 and a positive autoregulatory loop of TGF-beta signaling due to the absence of Smad7 (21). Moreover, Zheng *et al.* (22) reported that CD4⁺CD25⁺ cells were the targets of the costimulatory effects of IL-2 and TGF-beta, and increased the numbers of CD4⁺CD25⁻ cells that became CD25⁺ cytokine-independent suppressor cells. They also described that CD4⁺CD25⁺ Treg cells induced other allo-activated CD4⁺CD25⁻ cells to become potent suppressor cells by mechanisms that require TGF-beta, and that the suppressive effects of these secondary CD4⁺CD25⁺ cells depended upon TGF-beta (22). Thus, TGF-beta may not only be a potent soluble immunosuppressor itself, but it may also induce CD4⁺CD25⁺ Treg cells in the spleen, contributing to disease-associated immunosuppression in advanced gastric cancer patients.

In conclusion, we propose that the spleen is involved in immunosuppression in advanced gastric cancer patients. Naïve T cells accumulate in the spleen by using CD62L, the novel homing receptor, and function as suppressor-inducer cells. CD62L⁺ cells produce TGF-beta and TGF-beta induces CD4⁺CD25⁺ Treg cells in the spleen, both of which may contribute to gastric cancer-associated immunosuppression. This may imply the necessity for splenectomy in patients with advanced gastric cancer.

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