

Effect of Splenectomy on Antitumor Immune System in Mice

JUN HIGASHIJIMA, MITSUO SHIMADA, MOTOYA CHIKAKIYO, TOMOHIKO MIYATANI,
KOZO YOSHIKAWA, MASANORI NISHIOKA, TAKASHI IWATA and NOBUHIRO KURITA

*Department of Surgery, Institute of Health Biosciences, The University of Tokushima,
3-18-15 Kuramoto-cho, Tokushima 770-8503, Japan*

Abstract. *Background: The influence on the antitumor immune system after splenectomy in vivo are controversial. CD4⁺CD25⁺Foxp3⁺T-cells (regulatory T-cell: reg T) and natural killer (NK) cells play important roles in immunological tolerance and antitumor immunity. The influence of splenectomy on the antitumor immune system was evaluated in a metastasis induced mouse model. Materials and Methods: Experiment 1, splenectomy in a cancer-free model. The mice were divided into two groups, one control, and the other splenectomy group. At days 4, 7 and 10 after splenectomy, the mesenteric lymph node, the liver and the lung were harvested. The lymph nodes were analyzed by flow cytometric analysis and the number of reg T-cells and NK cells were calculated. Foxp3 mRNA in the liver and the lung was evaluated by reverse transcriptional polymerase chain reaction (RT-PCR). Experiment 2, splenectomy in a liver metastasis model. Colon 26 cells were injected into the spleen of mice and the mice were divided into two groups, a spleen preserved group, and a splenectomy group. Splenectomy was performed at day 4 after injection. At days 7 and 10 after injection, flow cytometric analysis, and at day 10 RT-PCR were performed. Ten days after injection, the number of liver metastases (>1 mm) was counted. Results: Experiment 1, in the splenectomy group the flow cytometric analysis showed a significant decrease in the number of reg T and NK cells in the mesenteric lymph nodes compared with the control group. In the splenectomy group, the Foxp3 mRNA increased significantly in the liver at day 10, and in the lung at days 4 and 7. Experiment 2, liver metastasis was observed in the splenectomy group. Flow cytometric analysis showed that splenectomy did not affect the number of reg T at day 7*

and day 10. The number of NK cells increased in the splenectomy group at day 7, but at day 10, there was no significant difference between the groups. RT-PCR showed that at day 10, the Foxp3 mRNA in liver increased in the splenectomy group. Conclusion: The spleen plays a very important role in the antitumor immune system and splenectomy enhances liver metastasis through the increase of Foxp3 mRNA in the liver.

Colorectal cancer is one of the most common malignancies in the world and hematogenous metastasis to the liver and the lung, as well as lymph node metastases, are the most important factors that influence the prognosis of patients. Hematogenous liver metastases are found in 20% of patients at the time of surgery (1) and in 60-70% after operations (2, 3). Surgical resection of liver metastases is the best treatment for patients with solitary tumors, and the 5-year disease-free survival rate is 25-30% (4-10); on the other hand, for patients with multiple liver metastases, systemic or intraarterial infusion chemotherapy may be performed, but the prognosis is not good. To ascertain the mechanism of metastasis and its relationship with the immune system is the current challenge for improving clinical treatment.

There are many factors that affect cancer metastasis and the antitumor immune system is one such important factors. The spleen is a large lymphoid organ that produces various kinds of cytokines (11), which are known to flow into the liver via the splenic and portal veins and to enhance natural killer (NK) cytotoxicity in the liver (12). NK cells have been known to mediate spontaneous cytotoxicity against tumor cells and their metastases (13). It has also been reported that hepatic NK cells act as the first line of defense in the hepatic metastasis of colon cancer (14, 15). The spleen also plays an important role in the immune system, and a relationship between splenectomy and tumor metastasis has been reported (12, 16, 17). However, the effect of splenectomy on the antitumor immune system *in vivo* remains controversial. It is only known that splenocytes cotraffic with the tumor cells to the liver and facilitate metastatic colony formation (18) but reports of the mechanism of the antitumor immune system in the spleen are limited.

Correspondence to: Mitsuo Shimada, MD, FACS, Professor and Chairman, Department of Surgery, The University of Tokushima 3-18-15 Kuramoto-cho, Tokushima, 770-8503 Japan. Tel: +81 88633 7137, Fax: +81 886319698, e-mail: mshimada@clin.med.tokushima-u.ac.jp

Key Words: Splenectomy, liver metastasis, regulatory T-cell, Foxp3, NK cell.

CD4⁺CD25⁺Foxp3⁺T-cells (regulatory T-cells: reg T-cells) exist in malignant tumors (19-24), and play a role in immunological tolerance to self-antigens and in suppression of antitumor immunity. Foxp3 is a transcriptional factor that regulates the development of reg T-cells (25). We previously reported that the number of reg T-cells in the peripheral blood significantly increased in pancreatic cancer patients compared with healthy donors (26). Moreover, the simultaneous administration of CD4⁺CD25⁺ T-cells with hepatic parenchyma prolonged islet graft survival and induced donor-specific hyporesponsiveness (27). It is known that reg T-cells suppress proliferation of T-cells and autoimmunity in animals (29-31). They are also linked to cancer development because depletion of reg T-cells facilitates tumor rejection (32-35). Reg T-cells preferentially accumulate in tumor-draining lymph nodes and tumor masses and the presence of such cells is correlated with a poor prognosis in patients (36-38). Because the depletion of reg T-cells facilitated tumor rejection in animal studies, identification of the origin of reg T-cells in the tumor microenvironment would provide valuable information for the successful elimination of reg T-cells and thus the suppression of antitumor immunity by these cells (34, 39, 40). Recent evidence (41) has suggested at least four possible sources of these reg T-cells: trafficking (tumor cells produce chemokines that specifically recruit reg T-cells to tumor sites (36)); differentiation (through contact with tumor-induced immature dendritic cells (DCs) (42)); expansion (of reg T-cells through DC stimulation (43)); and conversion (from normal CD4⁺ T-cells in tumor-bearing animals (38)). It appears that there are two distinct types of reg T-cells in the tumor microenvironment: one is naturally occurring reg T-cells that are either recruited to the tumor sites or stimulated to expand and the other is by conversion of normal CD4⁺ T-cells into reg T-cells, although the phenotype of these two types of reg T-cells is virtually indistinguishable (44).

Tumor immunity is not only T-cell dependent. Recently, a number of studies in leukemia (45), lymphoma (46) and gastrointestinal stromal tumors (47) has revealed that NK cell activation and cytotoxicity influence patient outcome. NK cells are regulated by cytokines in the environment and when interacting with the tumor directly, there is a delicate balance between inhibitory signals mediated by MHC class I molecules and activating signals triggered by specific ligands (48-50). In particular, the activating NKG2D receptor expressed by NK cells is an important mechanism of tumor recognition and suppression (51-53). Tumors possess many mechanisms by which they might evade NK cell-mediated suppression (49), such as the shedding of soluble ligands for activation receptors (54, 55) and the secretion of inhibitory cytokines such as transforming growth factor (TGF)- β (56-58), however, to date, the role that reg T-cells might play in hampering NK cell activation has been largely ignored.

The present study was carried out to determine whether the host immune system is down-regulated and to investigate the influence on anticancer immunity when splenectomy was performed using splenic injection of tumor cells as a useful method for obtaining liver metastasis (28).

Materials and Methods

Animal preparation. Female Balb/c mice (6 to 8-weeks old) were purchased from Charles River Co. Ltd (Kanagawa, Japan). The animals were provided with water and standard laboratory diet for at least 7 days before use. Throughout the experiment, the animals were maintained behind barriers under controlled conditions and had free access to tap water and diet before and after surgery. The present study was conducted in compliance with the Division for Animal Research Resources, Institute of Health Biosciences, the University of Tokushima. The experiments and procedures were approved by the Animal Care and Use Committee of the University of Tokushima.

Cell line. The standard experimental mouse tumor cell line colon 26 cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin/amphotericin B. All the cells were kept at 37°C, in a humidified 5% CO₂ atmosphere.

Experiment 1: Splenectomy in the cancer-free model. The mice were divided into two groups, a spleen preserved (control) group and a splenectomy group. The splenectomy procedure was as described elsewhere. The mice were sacrificed on days 4, 7 or 10 after splenectomy. The mesenteric lymph nodes, the livers and the lungs were harvested. Reg T-cells and NK cells in the mesenteric lymph nodes were analyzed by flow cytometric analysis. Pieces of the liver and the lung were put in RNA later (RNA Later RNA Stabilization Reagent, QIAGEN) and saved at -80°C until RNA extraction for the reverse transcription-polymerase chain reaction (RT-PCR).

Experiment 2: Splenectomy in liver metastasis model. The outline of the experiment is shown in Figure 1. Under ether anesthesia, a small upper-quadrant incision was made to expose the spleen of each Balb/c mouse and 0.1 ml of the viable cell suspension (1 \times 10⁶ cells/mouse) of colon 26 cells was injected into the lower splenic pole with a 27-gauge needle.

Four days after the injection of tumor cells, the mice were divided into two groups, a spleen-preserved group, and a splenectomy group in which splenectomy was performed with the same procedure as above.

On days 7 and 10 after injection, mice from each group were sacrificed, the mesenteric lymph nodes were harvested and analyzed by flow cytometric analysis. On day 10 after injection, the number of macroscopic metastases on the surface of the liver (>1 mm diameter) was counted and a 3 \times 3 mm piece of liver tissue from the lower edge of the left lobe was removed. The pieces of liver were put in RNA Later and saved at -80°C until RNA extraction for RT-PCR to detect Foxp3.

Flow cytometric analysis. The following antibodies and reagents to detect reg T-cells and NK cells were purchased from BD Biosciences Pharmingen (San Diego, CA, USA): CD4 (FITC anti-mouse CD4), CD25 (APC anti-mouse CD25 (IL-2 Receptor alpha,

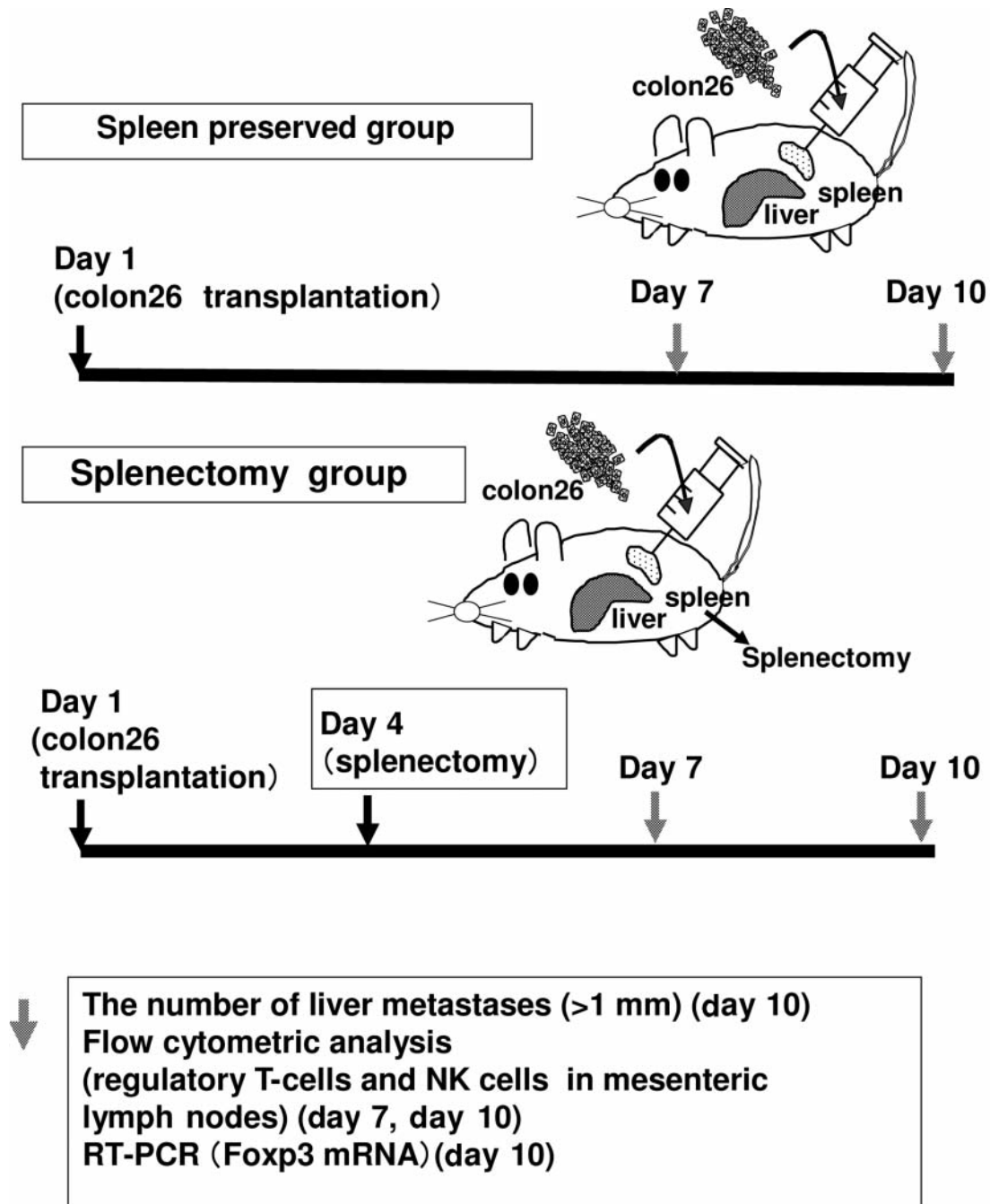


Figure 1. Experimental protocol of liver metastasis model. 1×10^6 colon 26 cells were injected into spleen of Balb/c mice. Splenectomy was performed 4 days after cancer cell implantation in the splenectomy group. Reg T-cells and NK cells were detected by flow cytometric analysis and Foxp3 mRNA by RT-PCR on Day 7 and Day 10. Macroscopic metastases on the surface of the liver (>1 mm) were counted on Day 10.

p55)), Foxp3 (PE anti-mouse/rat Foxp3; e-Bioscience, San Diego, CA, USA). The reagent for NK cells (PE CD49b/Pan-NK Cells; e-Bioscience, San Diego, CA, USA) was obtained from BD Biosciences Pharmingen. A PE anti-Foxp3 staining set (e-Bioscience) was used to stain intracellular Foxp3, and Via-Probe

(BD Biosciences Pharmingen) was used to discriminate viable from non-viable cells according to the manufacturer's instructions. Immunofluorescence was analyzed with CellQuest software (BD Biosciences Pharmingen) with a FACS Calibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA).

RT-PCR. The mRNA in liver was evaluated using Foxp3-specific primers. Total RNA was extracted from the mouse liver and lung tissue using RNeasy Mini Kit (Qiagen, Hilden, Germany). The amount of purified RNA was measured by UV spectra at 260 nm, and its purity was determined by calculating the ratio of Foxp3 to β -actin by UV activity at 260/280nm. Using the total RNA isolated from each sample, the synthesis of cDNA was carried out with M-MLV Reverse Transcriptase (Promega Co., Madison, WI, USA), and then PCR was conducted using Foxp3-specific primers (TaqMan Gene Expression Assays; Applied Biosystems).

Statistical analysis. All the results are presented as mean \pm SD. Comparisons between two or multiple groups were performed with Student's *t*-test using StatView-J 5.0 software (SAS, CA, USA). A *p*-value less than 0.05 was considered statistically significant.

Results

Experiment 1. In the splenectomy group, the percentage of reg T-cells in the mesenteric lymph nodes decreased compared with the control (spleen preserved) group on day 4 and day 10 as shown by flow cytometric analysis (Figure 2A, control group: day 4: day 10=6.144 \pm 0.375: 4.328 \pm 0.439: 4.984 \pm 0.389, *p*<0.01). In the splenectomy group, the percentage of NK cells in the mesenteric lymph nodes also decreased on days 4, 7 and 10 compared with the control group (Figure 2B, control group: day 4: day 7: day 10=1.542 \pm 0.089: 0.898 \pm 0.117: 0.795 \pm 0.099: 1.296 \pm 0.083, *p*<0.01).

RT-PCR showed that splenectomy significantly up-regulated the Foxp3 mRNA in the liver on day 10 (Figure 3A, *p*<0.01) and in the lung on day 4 (Figure 3B, *p*<0.05) and day 7 (Figure 3B, *p*<0.01).

Experiment 2. Macroscopic metastasis on the surface of the liver was seen in all cases, in both groups, and the number of hepatic metastases significantly increased in the splenectomy group (spleen-preserved group: splenectomy group=1.5 \pm 1.3: 19.2 \pm 9.1) (Figure 4).

The flow cytometric analysis showed that splenectomy did not affect the percentage of reg T-cells on both days 7 and 10 compared with the untreated control group (day 7, control group: spleen-preserved group: splenectomy group=6.144 \pm 0.375: 5.198 \pm 0.646: 3.890 \pm 1.075; day 10, control group: spleen-preserved group: splenectomy group=6.144 \pm 0.375: 5.988 \pm 0.857: 5.415 \pm 1.250) (Figure 5). The percentage of NK cells decreased in both the spleen-preserved group and the splenectomy group on day 7 and the spleen-preserved group showed a significant decrease in the number of NK cells compared with the splenectomy group (control group: spleen-preserved group: splenectomy group=1.542 \pm 0.089: 0.560 \pm 0.177: 0.8875 \pm 0.127, *p*<0.05). On the other hand, on day 10, both the spleen-preserved group and the splenectomy group showed a low percentage of NK cells, but there was no significant difference between the groups (Figure 6). RT-PCR on day 10 showed that

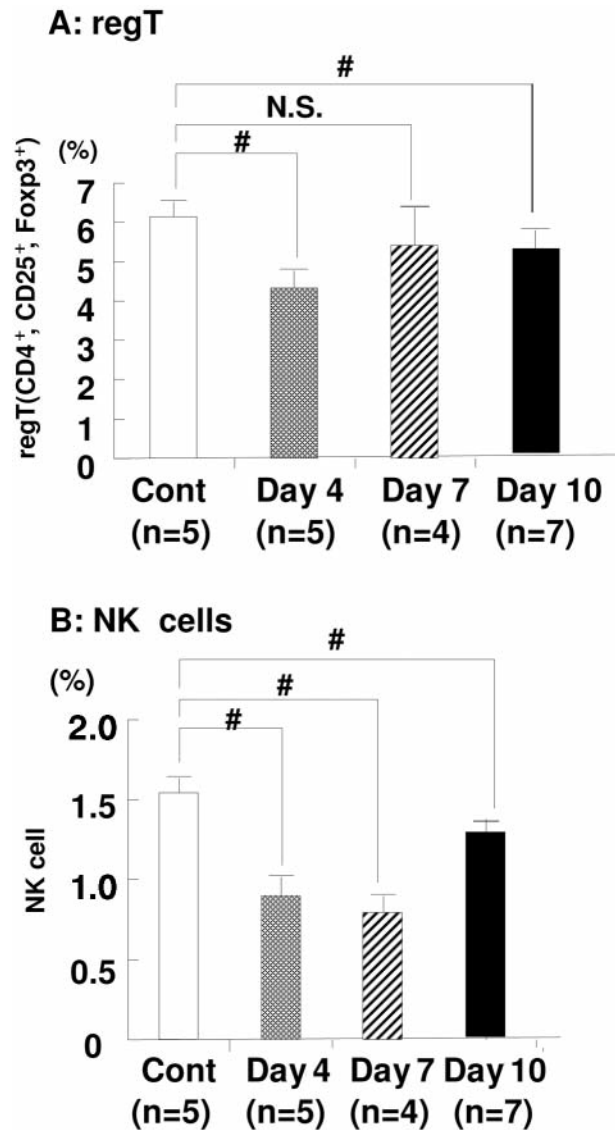


Figure 2. Flow cytometric analysis of reg T and NK cells in mesenteric lymph nodes. The percentage of (A) reg T-cells and (B) of NK cells in the spleen-preserved animals (control) and on days 4, 7 and 10 after splenectomy. #*p*<0.01.

Foxp3 mRNA significantly increased (*p*<0.01) in both the spleen-preserved group and the splenectomy group compared to the control group, with the splenectomy group showing a significantly higher ratio compared with the spleen-preserved group (Figure 7, *p*<0.05).

Discussion

Our first hypothesis was that the proportion of CD4⁺CD25⁺Foxp3⁺ reg T-cells would be reduced after splenectomy, which would induce the up-regulation of NK cells. However, the splenectomy group showed a significant decrease

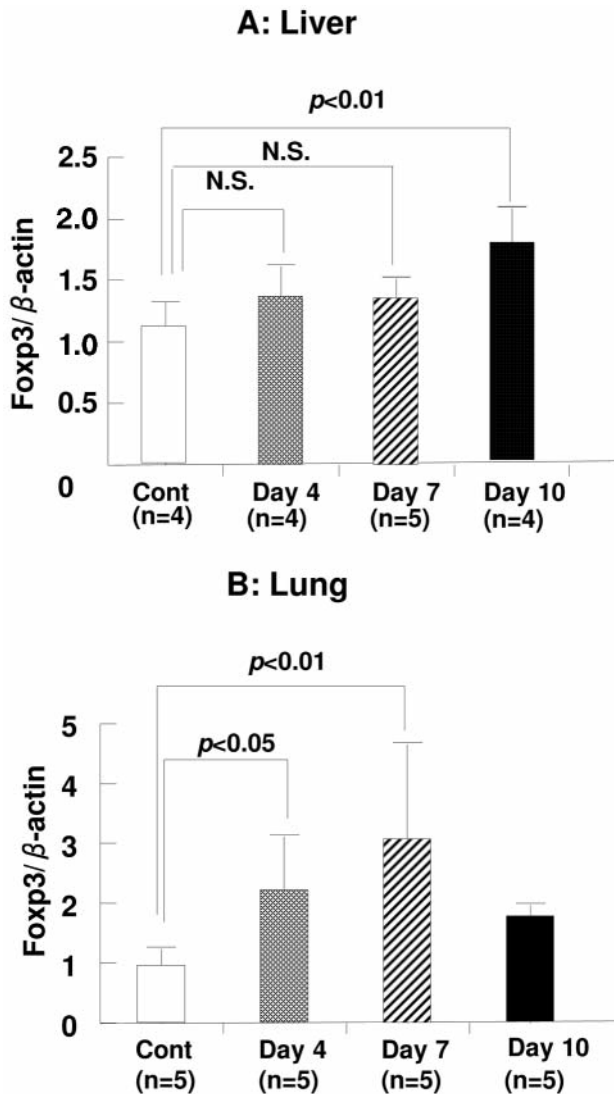


Figure 3. *Fcpx3* to β -actin mRNA ratio by RT-PCR in the liver (A) and lung (B) of the spleen-preserved animals (control) and days 4, 7 and 10 after splenectomy.

in the percentage of both reg T- and NK cells in the mesenteric lymph nodes, and also revealed a significant increase in the *Fcpx3* to β -actin mRNA ratio both in the liver and the lung compared with the control group. The present results were in contrast to those of Smyth *et al.* (59). But several reports have indicated a parallel decrease of reg T-cells and NK cells after splenectomy, so further investigation is needed to explain the meaning of these phenomena. A possible explanation as to why both reg T-cells and NK cells were reduced after splenectomy is the specificity of origin of the $CD4^+CD25^+Fcpx3^+$ reg T-cells. Some of the $CD4^+CD25^+$ reg T-cells may be generated from the $CD4^+CD25^-$ population in the spleen. Recent studies have shown that $CD4^+CD25^+Fcpx3^+$ reg T-cells are generated from

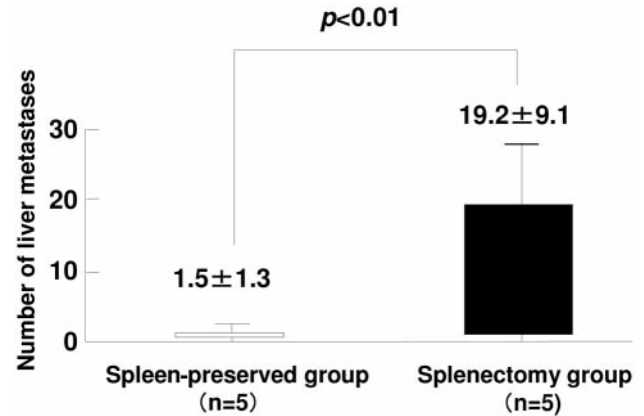


Figure 4. Macroscopic metastases on the surface of the liver. Number of macroscopic (>1 mm diameters) metastases on the surface of the liver in the spleen preserved and splenectomy mice 10 days after tumor cell injection.

the $CD4^+CD25^-$ population in the adoptive transfer system. Karim *et al.* demonstrated that alloantigen-specific $CD4^+CD25^+$ reg T-cells could be generated in the spleen of thymectomized mice. Chosa *et al.* reported that splenectomy in conjunction with anti- $CD25$ antibody therapy on post-heart-transplanted C57BL/6 mice resulted in rejection, which indicated that the $CD4^+CD25^-$ population in the periphery (other than the spleen) could not maintain alloantigen-specific reg T-cells. The $CD4^+CD25^-$ population present in the spleen might decrease after splenectomy, so the reg T-cells might be reduced.

The spleen has several unique functions in the immune system concerning not only $CD4^+CD25^+Fcpx3^+$ reg T-cells, but also NK cells and expression of TGF- β (transforming growth factor- β). It is well known that TGF- β suppresses the activation of $CD4^+CD25^+Fcpx3^+$ reg T-cells and $CD4^+CD25^+Fcpx3^+$ reg T-cells suppress the activation of NK cells. Smyth MJ *et al.* reported that activated reg T-cells directly suppressed NK cell function *via* the NKG2D pathway *in vivo* (59). They have extended the implications further by illustrating that the relief of that reg T-cell suppression could greatly enhance the functional activity of NK cells responding to the activating interleukin (IL)-12 cytokine, which is known to enhance the NKG2D pathway of NK cell activation (60). The suppressive effect of reg T-cell-expressed TGF- β on NK cell cytotoxicity was brought about by the soluble TGF- β reducing NK cell cytotoxicity and perforin gene transcription (57) and the role of perforin downstream of the NKG2D receptor function (60).

Liver metastasis by the intrasplenic injection of colon cancer cells as used in this study would appear to involve locomotion to distant tissue, invasion from blood vessels (extravasation) into the hepatic tissue and metastatic colonization and subsequent growth. It would seem that host

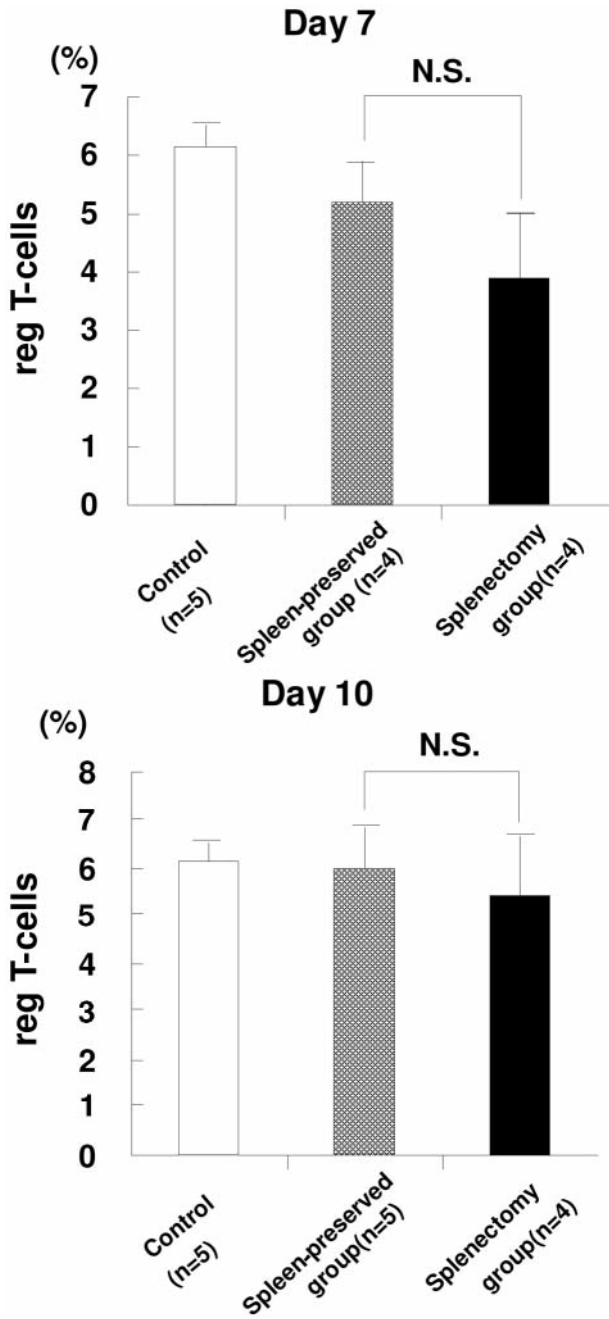


Figure 5. Percentage of reg T-cells in mesenteric lymph nodes by flow cytometry.

cells could have a strong promoting effect on the formation of liver metastasis. The inhibition of liver metastasis by hydrodynamics-based gene expression of NK4 cells indicated that NK4 might inhibit these distinct processes leading to metastatic colony formation and growth in the liver. As NK4 inhibited *in vitro* invasion of MC-38 colon cancer cells, NK4 gene expression inhibited invasive

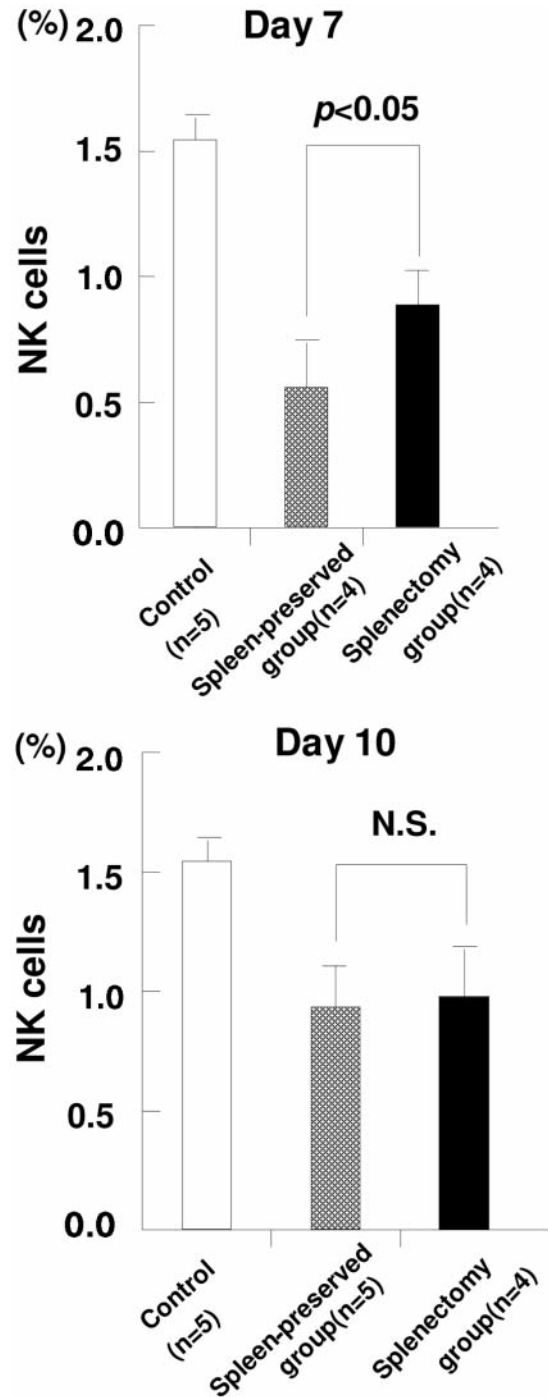


Figure 6. Percentage of NK cells in mesenteric lymph nodes by flow cytometry.

behavior of cells in the liver, and NK4 gene expression suppressed tumor growth primarily due to angiogenesis inhibition; NK4 gene expression may inhibit both invasion and colonization of colon cancer cells and the subsequent growth of colonies in the liver (61).

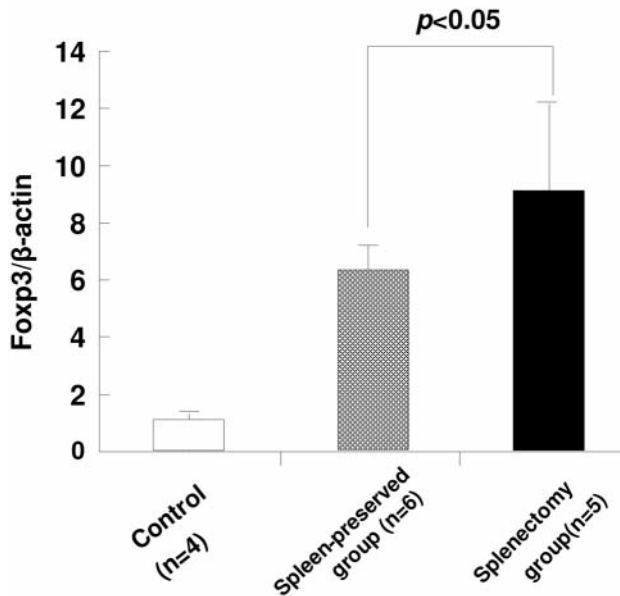


Figure 7. *Fxp3* mRNA ratio in the liver by RT-PCR.

Regarding the enhanced liver metastasis by splenectomy, Shiratori *et al.* (12) and Imai *et al.* (16) reported that the number of hepatic and lung metastases increased in splenectomized mice. They focused on the relationship between the spleen and the NK cells, and explained the mechanism of up-regulation of liver and pulmonary metastasis as being due to decreased activity of NK cells after splenectomy. These results were compatible with our present results. NK activity per hepatic asialo GM-1 (ganglioside)-positive cell in the splenectomized mice was less than that of the sham-operated mice. This result suggested that the spleen was one of the sources of NK cells that can migrate to the liver or that the spleen might be a source of factors that allow stimulation of the endothelium in the liver.

On the other hand, using a metastasis mice model Sonoda *et al.* reported that the lung metastatic nodules were significantly smaller in size and number in the splenectomy group than in the control group (17) and concluded that this might be associated with a decrease in serum levels of vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) induced by splenectomy. The results were in contrast to the present study, but in the present study VEGF and bFGF were not evaluated. These results suggest organ specificity in the influence of splenectomy on pulmonary and liver metastasis.

The present study suggested that one of the mechanisms by which splenectomy enhanced liver metastasis was by an increase of reg T-cells in the liver. Several reports were

incompatible with these results including that of Shiratori *et al.* who reported that the NK activity of hepatic mononuclear cells in the splenectomized mice was less than that of the sham-operated mice (12).

In the present study, the reg T-cells and NK cells in the mesenteric lymph nodes decreased in parallel after splenectomy. Tumor cells were not transplanted in experiment 1, and the number and activity of NK cells and reg T-cells in mesenteric lymph nodes decreased. In experiment 2, splenectomy did not significantly affect the number of NK cells and reg T-cells in the mesenteric lymph nodes. There might be some difference in the role of reg T-cells in each organ.

In conclusion, the spleen has a very important role in the antitumor immune system, and splenectomy enhances liver metastasis through the increase of *Fxp3* mRNA in the liver.

Acknowledgements

This study was supported in part by grants-in-aid for Scientific Research from the Japan Society for the Promotion of Science (grant no. 17591407 to M.S.).

References

- 1 Wanebo H, Semaglou C, Attiyeh F and Stearns M: Surgical management of patients with primary operable colorectal cancer and synchronous liver metastases. *Am J Surg* 135: 81-85, 1978.
- 2 Welch JP and Donaldson GA: The clinical correlation of an autopsy study of recurrent colorectal cancer. *Ann Surg* 189(4): 496-502, 1979.
- 3 Gilbert H and Kagan R: Metastases: incidence, detection, and evaluation without histologic confirmation. In: *Fundamental Aspects of Metastasis*. Weiss L (ed.) Amsterdam: North-Holland, pp. 385-389, 1976.
- 4 Scheele J, Stang R, Altendorf-Hofmann A and Paul M: Resection of colorectal liver metastases. *World J Surg* 19(1): 59-71, 1995.
- 5 Rosen CB, Nagorney DM, Taswell HF, Helgeson SL, Ilstrup DM, van Heerden JA and Adson MA: Perioperative blood transfusion and determinants of survival after liver resection for metastatic colorectal carcinoma. *Ann Surg* 216(4): 493-504, 1992.
- 6 Pedersen I K, Burcharth F, Roikjaer O and Baden H: Resection of liver metastases from colorectal cancer. Indications and results. *Dis Colon Rectum* 37(11): 1078-1082, 1994.
- 7 Sesto ME, Vogt DP and Hermann RE: Hepatic resection in 128 patients: a 24-year experience. *Surgery* 102(5): 846-851, 1987.
- 8 Doci R, Gennari L, Bignami P, Montalto F, Morabito A and Bozzetti F: One hundred patients with hepatic metastases from colorectal cancer treated by resection: analysis of prognostic determinants. *Br J Surg* 78(7): 797-801, 1991.
- 9 Hughes KS, Simon R, Songhorabodi S, Adson MA, Ilstrup DM, Fortner JG, Maclean BJ, Foster JH, Daly JM, Fitzherbert D *et al*: Resection of the liver for colorectal carcinoma metastases: a multi-institutional study of patterns of recurrence. *Surgery* 100(2): 278-284, 1986.

- 10 Nordlinger B, Guiguet M, Vaillant J C, Balladur P, Boudjema K, Bachellier P and Jaeck D: Surgical resection of colorectal carcinoma metastases to the liver. A prognostic scoring system to improve case selection, based on 1,568 patients. Association Francaise de Chirurgie. *Cancer* 77(7): 1254-1262, 1996.
- 11 Hood LE, Weissman IL and Wood WB: Immunology. Menlo Park, The Benjamin/Cummings Publishing Co., 1978.
- 12 Shiratori Y, Kawase T, Nakata R, Tanaka M, Hikiba Y, Okano K, Matsumura M, Niwa Y, Komatsu Y and Shiina S: Effect of splenectomy on hepatic metastasis of colon carcinoma and natural killer activity in the liver. *Dig Dis Sci* 40(11): 2398-2406, 1995.
- 13 Gorelik E, Wiltout RH, Okumura K, Habu S and Herberman RB: Role of NK cells in the control of metastatic spread and growth of tumor cells in mice. *Int J Cancer* 20: 107-112, 1982.
- 14 Shiratori Y, Nakata R, Okano K, Komatsu Y, Shiina S, Kawase T, Sugimoto T, Omata M, and Tanaka M: Inhibition of hepatic metastasis of colon carcinoma by asialo GM1-positive cells in the liver. *Hepatology* 16: 469-478, 1992.
- 15 Bouwens L, Jacobs R, Remels L and Wisse E: Natural cytotoxicity of rat hepatic natural killer cells and macrophages against a syngeneic colon adenocarcinoma. *Cancer Immunol Immunother* 27: 137-141, 1998.
- 16 Imai S, Nio Y, Shiraishi T, Tsubono M, Morimoto H, Tseng CC, Kawabata K, Masai Y and Tobe T: Effects of splenectomy on pulmonary metastasis and growth of SC42 carcinoma transplanted into mouse liver. *J Surg Oncol* 47(3): 178-187, 1991.
- 17 Sonoda K, Izumi K, Matsui Y, Inomata M, Shiraishi N and Kitano S: Decreased growth rate of lung metastatic lesions after splenectomy in mice. *Eur Surg Res* 38(5): 469-475, 2006.
- 18 Bouvet M, Tsuji K, Yang M, Jiang P, Moossa AR and Hoffman RM: *In vivo* color-coded imaging of the interaction of colon cancer cells and splenocytes in the formation of liver metastases. *Cancer Res* 66(23): 11293-11297, 2006.
- 19 Ichihara F, Kono K, Takahashi A, Kawaida H, Sugai H and Fujii H: Increased populations of regulatory T-cells in peripheral blood and tumor-infiltrating lymphocytes in patients with gastric and esophageal cancers. *Clin Cancer Res* 9: 4404-4408, 2003.
- 20 Wolf AM, Wolf D, Steurer M, Gastl G, Gunsilius E and Grubeck-Loebenstien B: Increase of regulatory T-cells in the peripheral blood of cancer patients. *Clin Cancer Res* 9: 606-612, 2003.
- 21 Sasada T, Kimura M, Yoshida Y, Kanai M and Takabayashi A: CD4⁺CD25⁺ regulatory T-cells in patients with gastrointestinal malignancies: possible involvement of regulatory T-cells in disease progression. *Cancer* 98: 1089-1099, 2003.
- 22 Liyanage UK, Moore TT, Joo HG, Tanaka Y, Herrmann V, Doherty G, Drebin JA, Strasberg SM, Eberlein TJ, Goedegebuure PS and Linehan DC: Prevalence of regulatory T-cells is increased in peripheral blood and tumor microenvironment of patients with pancreas or breast adenocarcinoma. *J Immunol* 169: 2756-2761, 2002.
- 23 Woo EY, Chu CS, Goletz TJ, Schlienger K, Yeh H, Coukos G, Rubin SC, Kaiser LR and June CH: Regulatory CD4⁺CD25⁺ T-cells in tumors from patients with early stage non-small cell lung cancer and late-stage ovarian cancer. *Cancer Res* 61(12): 4766-4772, 2001.
- 24 Yang X H, Yamagiwa S, Ichida T, Matsuda Y, Sugahara S, Watanabe H, Sato Y, Abo T, Horwitz DA and Aoyagi Y: Increase of CD4⁺CD25⁺ regulatory T-cells in the liver of patients with hepatocellular carcinoma. *J Hepatol* 45: 254-262, 2006.
- 25 Hori S, Nomura T, Sakaguchi S: Control of regulatory T-cell development by the transcriptional factor Foxp3. *Science* 299: 14, 2003.
- 26 Ikemoto T, Yamaguchi T, Morine Y, Imura S, Soejima Y, Fujii M, Maekawa Y, Yasutomo K and Shimada M: Clinical roles of increased populations of Foxp3⁺CD4⁺ T-cells in peripheral blood from advanced pancreatic cancer patients. *Pancreas* 33(4): 386-390, 2006.
- 27 Ikemoto T, Tashiro S, Yasutomo K, Kishihara K, Kurita N and Miyake H: Donor-specific tolerance induced by simultaneous allogeneic islet transplantation with CD4⁺CD25⁺ T-cells into hepatic parenchyma in mice. *J Med Invest* 51(3-4): 178-185, 2004.
- 28 Fidler IJ: Critical factors in the biology of human cancer metastasis: twenty-eighth G.H.A. Clowes Memorial Award Lecture. *Cancer Res* 50(19): 6130-6138, 1990.
- 29 Suri-Payer E and Cantor H: Differential cytokine requirements for regulation of autoimmune gastritis and colitis by CD4⁺CD25⁺ T-cells. *J Autoimmun* 16: 115-123, 2001.
- 30 Seddon B and Mason D: Regulatory T-cells in the control of autoimmunity: the essential role of transforming growth factor β and interleukin 4 in the prevention of autoimmune thyroiditis in rats by peripheral CD4⁺CD45RC⁻ cells and CD4⁺CD8⁻ thymocytes. *J Exp Med* 189: 279-288, 1999.
- 31 Asseman C, Mauze S, Leach MW, Coffman RL and Powrie F: An essential role for interleukin 10 in the function of regulatory T-cells that inhibit intestinal inflammation. *J Exp Med* 190: 995-1004, 1999.
- 32 Shimizu J, Yamazaki S and Sakaguchi S: Induction of tumor immunity by removing CD25⁺CD4⁺ T-cells: a common basis between tumor immunity and autoimmunity. *J Immunol* 163: 5211-5218, 1999.
- 33 Golgher D, Jones E, Powrie F, Elliott T and Gallimore A: Depletion of CD25⁺ regulatory cells uncovers immune responses to shared murine tumor rejection antigens. *Eur J Immunol* 32: 3267-3275, 2002.
- 34 Tanaka H, Tanaka J, Kjaergaard J and Shu S: Depletion of CD4⁺CD25⁺ regulatory cells augments the generation of specific immune T-cells in tumor-draining lymph nodes. *J Immunother* 25: 207-217, 2002.
- 35 Akbar AN, Taams LS, Salmon M and Vukmanovic-Stejić M: The peripheral generation of CD4⁺CD25⁺ regulatory T-cells. *Immunology* 109: 319-325, 2003.
- 36 Curiel TJ, Coukos G, Zou L, Alvarez X, Cheng P, Mottram P, Evdemon-Hogan M, Conejo-Garcia JR, Zhang L, Burow M *et al*: Specific recruitment of regulatory T-cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. *Nat Med* 10: 942-949, 2004.
- 37 Sasada T, Kimura M, Yoshida Y, Kanai M and Takabayashi A: CD4⁺CD25⁺ regulatory T-cells in patients with gastrointestinal malignancies: possible involvement of regulatory T-cells in disease progression. *Cancer* 98: 1089-1099, 2003.
- 38 Valzasina B, Piconese S, Guiducci C and Colombo M P: Tumor-induced expansion of regulatory T-cells by conversion of CD4⁺CD25⁻ lymphocytes is thymus and proliferation independent. *Cancer Res* 66: 4488-4495, 2006.
- 39 Steitz J, Bruck J, Lenz J, Knop J and Tuting T: Depletion of CD25⁺ CD4⁺ T-cells and treatment with tyrosinase-related protein 2-transduced dendritic cells enhance the interferon α -induced, CD8⁺ T-cell-dependent immune defense of B16 melanoma. *Cancer Res* 61: 8643-8646, 2001.

- 40 Jones E, Dahm-Vicker M, Simon A K, Green A, Powrie F, Cerundolo V and Gallimore A: Depletion of CD25⁺ regulatory cells results in suppression of melanoma growth and induction of autoreactivity in mice. *Cancer Immun* 2: 1, 2002.
- 41 Zou W: Regulatory T-cells, tumor immunity and immunotherapy. *Nat Rev Immunol* 6: 295-307, 2006.
- 42 Ghiringhelli F, Puig P E, Roux S, Parcellier A, Schmitt E, Solary E, Kroemer G, Martin F, Chauffert B and Zitvogel L: Tumor cells convert immature myeloid dendritic cells into TGF- β -secreting cells inducing CD4⁺CD25⁺ regulatory T-cell proliferation. *J Exp Med* 202: 919-929, 2005.
- 43 Yamazaki S, Iyoda T, Tarbell K, Olson K, Velinzon K, Inaba K and Steinman RM: Direct expansion of functional CD25⁺CD4⁺ regulatory T-cells by antigen-processing dendritic cells. *J Exp Med* 198: 235-247, 2003.
- 44 Wing K, Fehervari Z and Sakaguchi S: Emerging possibilities in the development and function of regulatory T-cells. *Int Immunol* 18: 991-1000, 2006.
- 45 Ruggeri L, Capanni M, Urbani E, Perruccio K, Shlomchik W D, Tosti A, Posati S, Rogaia D, Frassoni F, Aversa F *et al*: Effectiveness of donor natural killer cell alloreactivity in mismatched hematopoietic transplants. *Science* 295: 2097-2100, 2002.
- 46 O'Hanlon LH: Natural born killers: NK cells drafted into the cancer fight. *J Natl Cancer Inst* 96: 651-653, 2004.
- 47 Borg C, Terme M, Taieb J, Menard C, Flament C, Robert C, Maruyama K, Wakasugi H, Angevin E, Thielemans K *et al*: Novel mode of action of c-kit tyrosine kinase inhibitors leading to NK cell-dependent antitumor effects. *J Clin Invest* 114: 379-388, 2004.
- 48 Moretta A, Bottino C, Mingari MC, Biassoni R and Moretta L: What is a natural killer cell? *Nat Immunol* 3: 6-8, 2002.
- 49 Smyth MJ, Hayakawa Y, Takeda K and Yagita H: New aspects of natural killer cell surveillance and therapy of cancer. *Nat Rev Cancer* 2: 850-861, 2002.
- 50 Lanier LL: NK cell recognition. *Annu Rev Immunol* 23: 225-274, 2005.
- 51 Diefenbach A, Jensen ER, Jamieson AM and Raulet DH: Rae1 and H60 ligands of the NKG2D receptor stimulate tumour immunity. *Nature* 413: 165-171, 2001.
- 52 Hayakawa Y, Kelly JM, Westwood JA, Darcy PK, Diefenbach A, Raulet D and Smyth MJ: Cutting edge: tumor rejection mediated by NKG2D receptor-ligand interaction is dependent upon perforin. *J Immunol* 169: 5377-5381, 2002.
- 53 Cerwenka A, Baron JL and Lanier LL: Ectopic expression of retinoic acid early inducible-1 gene (*RAE-1*) permits natural killer cell-mediated rejection of a MHC class I-bearing tumor *in vivo*. *Proc Natl Acad Sci USA* 98: 11521-11526, 2001.
- 54 Wu JD, Higgins LM, Steinle A, Cosman D, Haugk K and Plymate SR: Prevalent expression of the immunostimulatory MHC class I chain-related molecule is counteracted by shedding in prostate cancer. *J Clin Invest* 114: 560-568, 2004.
- 55 Groh V, Wu J, Yee C and Spies T: Tumour-derived soluble MIC ligands impair expression of NKG2D and T-cell activation. *Nature* 419: 734-738, 2002.
- 56 Loeffler CM, Smyth MJ, Longo DL, Kopp WC, Harvey LK, Tribble HR, Tase JE, Urba WJ, Leonard AS, Young HA *et al*: Immunoregulation in cancer-bearing hosts: down-regulation of gene expression and cytotoxic function in CD8⁺ T-cells. *J Immunol* 149: 949-956, 1992.
- 57 Smyth MJ, Strobl SL, Young HA, Ortaldo JR and Ochoa AC: Regulation of lymphokine-activated killer activity and pore-forming protein gene expression in human peripheral blood CD8⁺ T lymphocytes. Inhibition by transforming growth factor- β . *J Immunol* 146: 3289-3297, 1991.
- 58 Lee JC, Lee KM, Kim DW and Heo DS: Elevated TGF- β 1 secretion and down-modulation of NKG2D underlies impaired NK cytotoxicity in cancer patients. *J Immunol* 172: 7335-7340, 2004.
- 59 Smyth MJ, Teng MW, Swann J, Kyriakoudis K, Godfrey DI and Hayakawa Y: CD4⁺CD25⁺ T regulatory cells suppress NK cell-mediated immunotherapy of cancer. *J Immunol* 176(3): 1582-1587, 2006.
- 60 Smyth MJ, Swann J, Kelly JM, Cretney E, Yokoyama WM, Diefenbach A, Sayers TJ and Hayakawa Y: NKG2D recognition and perforin effector function mediate effective cytokine immunotherapy of cancer. *J Exp Med* 200: 1325-1335, 2004.
- 61 Wen J, Matsumoto K, Taniura N, Tomioka D and Nakamura T: Hepatic gene expression of NK4, an HGF-antagonist/angiogenesis inhibitor, suppresses liver metastasis and invasive growth of colon cancer in mice. *Cancer Gene Ther* 11(6): 419-430, 2004.

Received May 17, 2008

Revised November 18, 2008

Accepted December 1, 2008