Expression of immune checkpoint molecules of T cell immunoglobulin and mucin protein 3/galectin-9 for NK cell suppression in human gastrointestinal stromal tumors

HIDEO KOMITA[1], SHIGEO KOIDO[1,2], KAZUMI HAYASHI[2], SHIN KAN[2], MASAKI ITO[2], YUKO KAMATA[2], MASAFUMI SUZUKI[3] and SADAMU HOMMA[2]

[1]Division of Gastroenterology and Hepatology, Department of Internal Medicine, [2]Division of Oncology, Research Center for Medical Science and [3]Department of Pathology, Jikei University School of Medicine, Tokyo, Japan

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Abstract. Monoclonal antibody therapy for immune checkpoint blockade has achieved promising results for several types of malignant tumors. For the future treatment of gastrointestinal stromal tumors (GISTs) by immune checkpoint blockade, expression of immune checkpoint-related molecules that suppress antitumor immunity in GISTs was examined. Infiltration of immune cell types into 19 GIST tissues was analyzed by immunohistochemistry, and expression of T cell immunoglobulin and mucin protein 3 (Tim-3) and programmed cell death-1 (PD-1) in the infiltrated immune cells was examined by immunofluorescence microscopy. The expression status of galectin-9 in the GIST tumor cells was also determined by immunohistochemistry. All the GIST tissues showed $CD8^+$ T cell infiltration and 8 showed $CD56^+$ natural killer (NK) cell infiltration, and the numbers of infiltrated $CD8^+$ T and NK cells were strongly correlated. However, these $CD8^+$ T and NK cells were CD69-negative inactivated cells. Tim-3 was expressed in the infiltrated NK cells in 6/8 (75%) of the GIST tissues. Expression of galectin-9, a ligand of Tim-3, was observed in 13/19 (68.4%) GIST tissues and all of the GIST tissues with $Tim\text{-}3^+$ NK cell infiltration showed positive galectin-9 expression. No PD-1 expression in the infiltrated NK cells and neither Tim-3 nor PD-1 expression was observed in the infiltrated $CD8^+$ T cells. Interaction between Tim-3 in infiltrated NK cells and galectin-9 in tumor cells may be involved in an immune checkpoint mechanism for suppression of antitumor immunity in GISTs. Blockade of the Tim-3/galectin-9 pathway may become a new strategy for GIST treatment.

Correspondence to: Professor Sadamu Homma, Division of Oncology, Research Center for Medical Science, Jikei University School of Medicine, 3-25-8 Nishi-shimbashi, Minato-ku, Tokyo 105-8461, Japan
E-mail: sahya@jikei.ac.jp

Abbreviations: GIST, gastrointestinal stromal tumor; NK cell, natural killer cell; CTL, cytotoxic T lymphocyte, PD-1, programmed death-1; Tim-3, T cell immunoglobulin and mucin protein 3; mAb, monoclonal antibody; pAb, polyclonal antibody

Key words: gastrointestinal stromal tumor, immune checkpoint, galectin-9, T cell immunoglobulin and mucin protein 3, natural killer cell

Introduction

Gastrointestinal stromal tumor (GIST) is the most common mesenchymal tumor of the gastrointestinal tract derived from the interstitial cells of Cajal (1). GIST develops in the stomach most frequently, followed by the small intestine, colon and esophagus. Expression of receptor tyrosine kinase KIT seems to be closely associated with GIST development, and 75-90% of GISTs elicit functional mutations of the KIT gene at exon 9, 11, 13 and 17 (2-4). Although tyrosine kinase inhibitors (TKIs) such as imatinib mesylate or sunitinib malate have been applied for the treatment of inoperable GISTs, tolerance to such agents has often been induced and the antitumor effects by such agents on GISTs is restricted (5,6). Accordingly, new therapeutic modalities for GISTs which are based on new biological mechanisms are urgently required.

Studies indicate that GIST has immunogenic properties which attract the antitumor immune response. Infiltration of immune cells into GIST tissues has been shown as possible immune responses between the tumor and immune cells (7,8). It has also been reported that NK cells and the interferon-γ status of GIST patients predict prognosis (9), and imatinib mesylate induced NK cell activation and promoted antitumor immunity to GIST (10). Of note, GISTs express various types of tumor-associated antigens recognized by specific cytotoxic T lymphocytes (11-14). However, there is no evidence indicating that host antitumor immunity inhibits the development of GISTs and no therapeutic modalities which could suppress GISTs by activation of antitumor immunity have been developed.

Recently, treatment with immune checkpoint inhibitors has provided monumental progress in cancer treatment (15,16). Some melanoma patients showed marked responses to treatment with a monoclonal antibody to cytotoxic T lymphocyte antigen-4 (CTLA-4) (17). Marked tumor regression, durable

antitumor effect and no autoimmune-associated adverse events of the therapy indicated that inhibition of tumor-associated immune-suppression could become a potential therapeutic modality to suppress tumor development (18). Recent studies have shown that the neo-antigens generated by somatic mutations may become targets recognized by specific CTLs (19). Blockade between programmed cell death-1 (PD-1) on T cells and programmed cell death ligand-1 (PD-L1) on tumor cells by monoclonal antibody treatment also showed a significant antitumor effect against melanomas as well as lung and renal cancers (20,21). Combined treatment with anti-CTLA-4 and anti-PD-1 treatment of melanomas may show high therapeutic efficacy which has never been observed before and may decrease the toxicity of anti-CTAL-4 by decreasing its dose (18). Besides these molecules, T cell immunoglobulin and mucin protein 3 (Tim-3) on immune cells and galectin-9 on tumor cells play an important role in immune checkpoint mechanism in malignancies and the blockade of the Tim-3/galectin-9 pathway is expected for future immune checkpoint therapy against malignant tumors (22,23). Exciting results by immune-checkpoint blockade has opened a new era for cancer therapy, and patients with various malignant tumors other than melanomas may be able to receive the benefits in the near future.

Considering that GISTs have immunogenic properties, it is possible that antitumor immune activity of GISTs may be inhibited by immune checkpoint mechanisms. Although immune checkpoint blockade would be expected as a noted method for GIST treatment, no data concerning the immune checkpoint mechanism of GISTs have been reported. In the present study, we demonstrated that the Tim-3/galectin-9 pathway may be involved in the immune checkpoint mechanism of GISTs.

Materials and methods

Patient characteristics. Human GIST tumors investigated in the present study were obtained from 2006 to 2014 from 12 male and 7 female patients ranging from 40 to 82 years of age (median 60 years). All samples were primary GIST tissues of the stomach, whose diameters were >30 mm in 16 cases and from 30-100 mm in 3 cases. All of the GIST tissue samples were obtained by surgical procedure, and no distant metastatic lesions were noted. All the GIST tissues were structured with spindle or epithelioid cells with positive c-kit expression. None of the GIST cases were treated with tyrosine kinase inhibitors. Analyses of the GIST tissues were approved by the institutional review board according to the guidelines of the Jikei University School of Medicine and the Jikei University Hospital (25-299, 7434).

Antibodies. The primary antibodies for immunohistochemistry were as follows: anti-human CD69 rabbit polyclonal antibody (pAb), anti-galectin-9 rabbit pAb and anti-programmed death-1 (PD-1) rabbit pAb (all from Abcam, Cambridge, UK), anti-Tim-3 rabbit pAb (BioVision, Milpitas, CA, USA), anti-human CD4 mouse monoclonal antibody (mAb), 1F6, anti-human CD8 mouse mAb, C8/144B and anti-human CD56 mouse mAb, 1B6 (all from Nichirei Biosciences, Tokyo, Japan), anti-FOXP3 mouse mAb, 236A/E7 (Abcam) and anti-human CD68 mouse mAb, PG-M1 (Dako, Glostrup, Denmark).

Secondary antibodies for immunohistochemistry were anti-mouse immunoglobulin goat pAb conjugated with peroxidase in Max-PO(M) and anti-rabbit immunoglobulin goat pAb conjugated with alkaline-phospatase in Max-PO(R) (both from Nichirei Biosciences).

Secondary antibodies for immune-fluorescence microscopy were goat anti-mouse immunoglobulin conjugated with Alexa Fluor 488 and donkey anti-rabbit immunoglobulin conjugated with Alexa Fluor 594 (both from Thermo Fischer Scientific, Waltham, MA, USA).

Immunohistochemistry by immune-peroxidase or alkaline phosphatase method. All stainings of tissue sections were performed on 4-μm formalin-fixed and paraffin-embedded tumor sections. Tissue sections were de-waxed in xylene and re-hydrated through decreasing concentrations of ethanol. Before immunostaining with the primary antibody, the sections were washed with phosphatase-buffered saline (PBS) and incubated with 10% bovine serum in PBS for 60 min. They were then washed extensively with PBS and incubated with each primary antibody at a dilution of 1:100 at room temperature for 1 h. The slides were washed three times with PBS and incubated with secondary antibodies at room temperature according to the manufacturer's instructions. Each slide was immersed in hematoxylin bath for 1 min and washed with water. Tissue sections were re-hydrated in ethanol and cleared in xylene and mounted with permanent mounting media.

Enumeration of the immune cells in the GIST tissues. Numbers of $CD8^+$ T, $CD4^+$ T and $CD56^+$ NK cells, $CD68^+$ macrophages and $FOXP3^+$ regulatory T cells were counted in 10 randomly chosen x40 microscopic fields.

Immunofluorescence microscopy. Formalin-fixed and de-waxed GIST tissue sections were incubated with the primary antibodies at a dilution of 1:100-1:200 at 4˚C overnight. After being washed, they were incubated with goat anti-mouse IgG (H+L) conjugated with Alexa Fluor 488 or donkey anti-rabbit IgG (H+L) conjugated with Alexa Fluor 594 (Thermo Fischer Scientific) at room temperature for 1 h. Immunofluorescence images were detected with a immunofluorescence microscope (BZ-9000 All-in-One; Keyence, Tokyo, Japan).

Correlation analysis. Correlation analyses were performed using Microsoft Office Excel 2007 (Microsoft Corporation, Redmond, WA, USA).

Results

Infiltration of the immune cells into GIST tissues. $CD8^+$ T cells were infiltrated into all of the GIST tissues at various degrees of infiltration (Fig. 1). $CD4^+$ T cells were also infiltrated into all of the GIST tissues, but fewer than that in the $CD8^+$ T cells. Significant infiltration of $CD56^+$ NK cells was observed in 8 GIST tissues (Fig. 1). Several cases showed macrophage infiltration. The number of $Foxp3^+$ cells was extremely low in all of the GIST tissues examined (Fig. 1).

Correlation of the infiltration between each immune cell type was examined. Infiltration of $CD8^+$ T and NK cells was found to be strongly correlated (Fig. 2, correlation coefficient

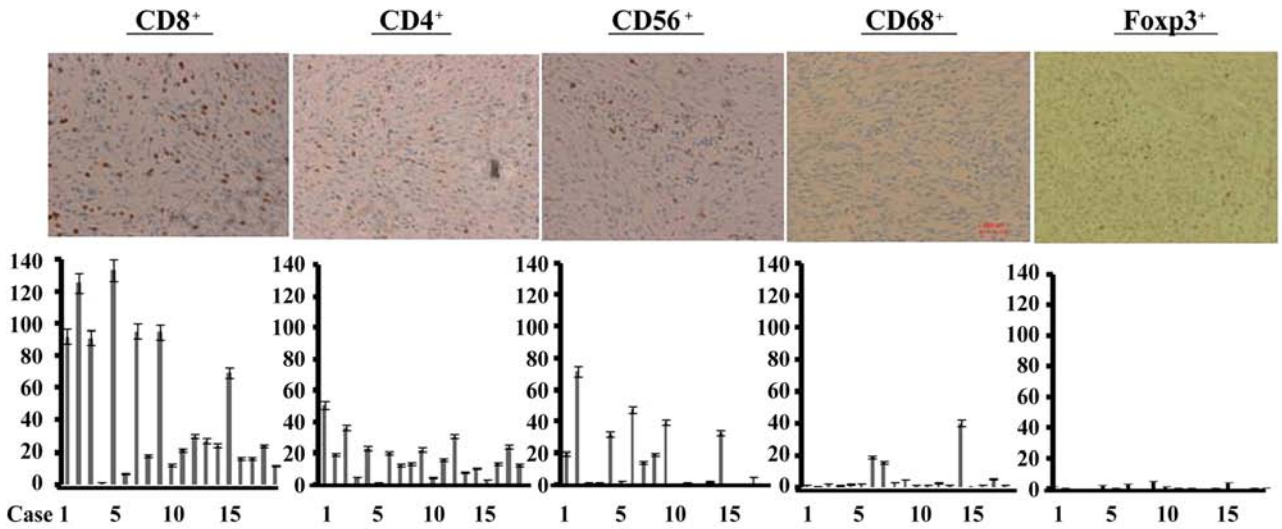

Figure 1. Upper images, immunohistochemical staining of $CD8^+$, $CD4^+$ T cells and $CD56^+$ NK cells, $CD68^+$ macrophages and $Foxp3^+$ regulatory T cells. Lower histograms, numbers of infiltrated immune cells into GIST tissues. Vertical axis shows the number of infiltrated immune cells in a x40 microscopic field. Horizontal axis shows the case number.

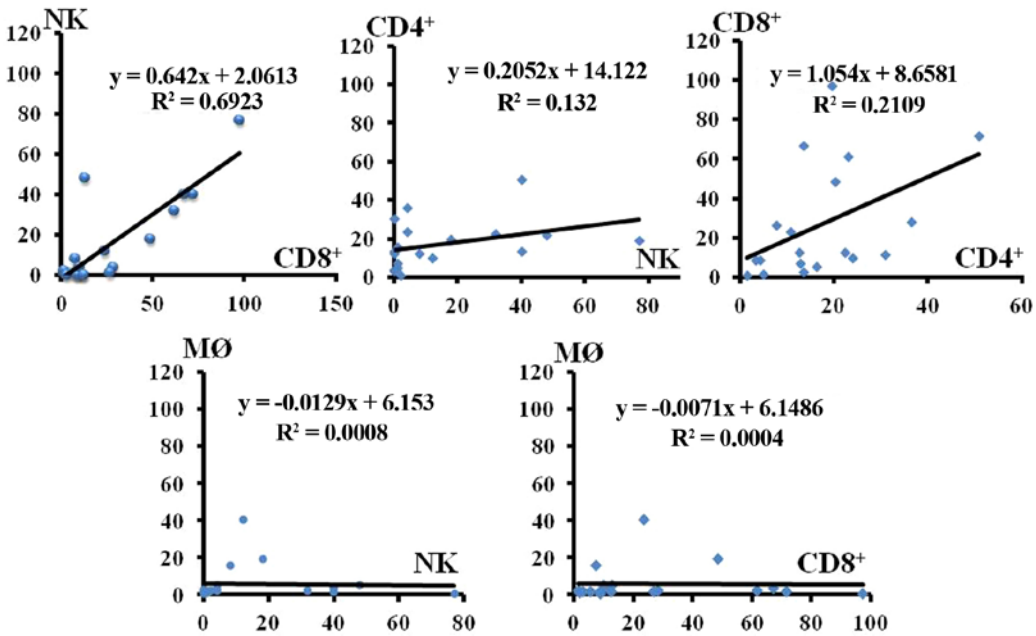

Figure 2. Correlation of the number of infiltrated immune cells in the GIST tissues.

Table I. Expression of CD69, Tim-3 and PD-1 in the $CD56^+$ cells in the GIST tissues.

	CD69		Tim-3		PD-1	
	Positive	Negative	Positive	Negative	Positive	Negative
$CD56^+$ cells	0	8	6	2	0	8

0.6923). The correlation of infiltration between $CD4^+$ T and NK cells was low. Although the infiltration of $CD8^+$ T cells was correlated with that of $CD4^+$ T cells, the correlation was lower than that between $CD8^+$ T and NK cells (Fig. 2, correlation coefficient 0.2109).

Positive Tim-3 but negative PD-1 expression is noted in the infiltrated NK cells in the GIST tissues. The characteristics of the NK cells infiltrated into the GIST tissues were examined. No CD69-positive NK cells were found in all of the GIST tissues (Table I). Tim-3 was significantly expressed in the

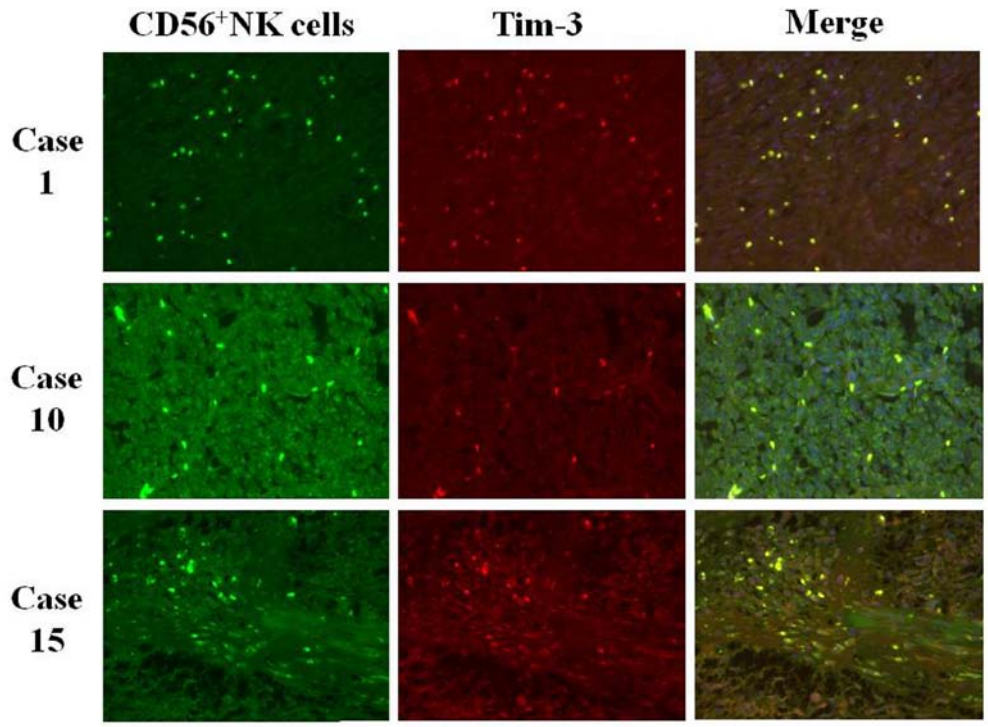

Figure 3. Expression of Tim-3 in CD56+ NK cells in the GIST tissues.

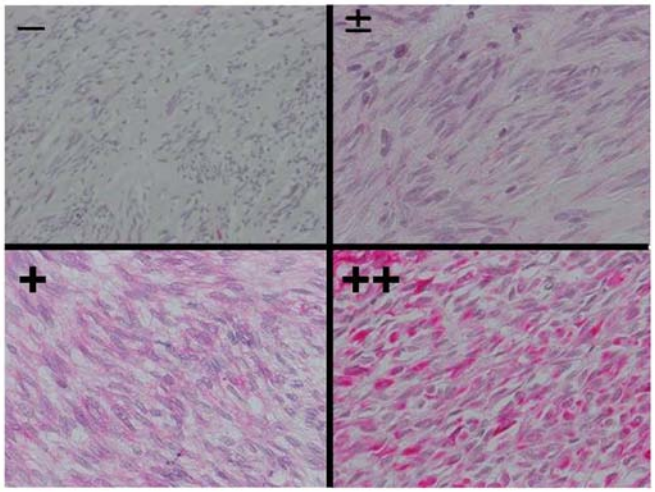

Figure 4. Expression of galectin-9 in the GIST tissues.

infiltrated NK cells in 6 out of 8 GIST tissues (Table I and Fig. 3), but no GIST tissue showed positive PD-1 staining in the infiltrated NK cells (Table I).

Galectin-9 expression in the GIST tumor tissues and corresponding expression of Tim-3 in the infiltrated NK cells. Expression of galectin-9 in the GIST tissues was classified according to 4 grades: -, +/-, + and ++ (Fig. 4). All of the GIST tissues with positive galectin-9 expression showed a cytoplasmic staining pattern. Thirteen out of 19 GIST tissues (68.4%) were significantly positive (+ to ++) for galectin-9 immunohistochemical staining (Table II). Six GIST tissues with Tim-3+ NK cell infiltration exhibited positive galectin-9 expression (Table III).

Table II. Expression of galectin-9 in human GIST tissues of the stomach.

	Galectin-9				
	-	+/-	+	++	Total
No. of tissues	2	4	8	5	19

Table III. Expression of galectin-9 in GIST tumor cells and Tim-3 in infiltrated NK cells in 8 GIST tissues with significant NK cell infiltration.

	Case							
	1	2	5	7	8	9	10	15
Galectin-9	+	+	++	++	+	++	++	+
Tim-3	+	+	-	+	+	-	+	+

Negative Tim-3 or PD-1 expression in the infiltrated CD8+ T cells in the GIST tissues. All of the CD8+ T cells in the 19 GIST tissues were negative for CD69 staining (Table IV and Fig. 5). Furthermore, none of the infiltrated CD8+ T cells in the GIST tissues showed positive staining for Tim-3 or PD-1 (Table IV).

Discussion

In the present study, we found that galectin-9 was expressed in 68.4% of the human GISTs. Galectins elicit β-galactoside

Table IV. Expression of CD69, Tim-3 and PD-1 in $CD8^+$ cells in the GIST tissues.

	CD69		Tim-3		PD-1	
	Positive	Negative	Positive	Negative	Positive	Negative
$CD8^+$ cells	0	19	0	19	0	19

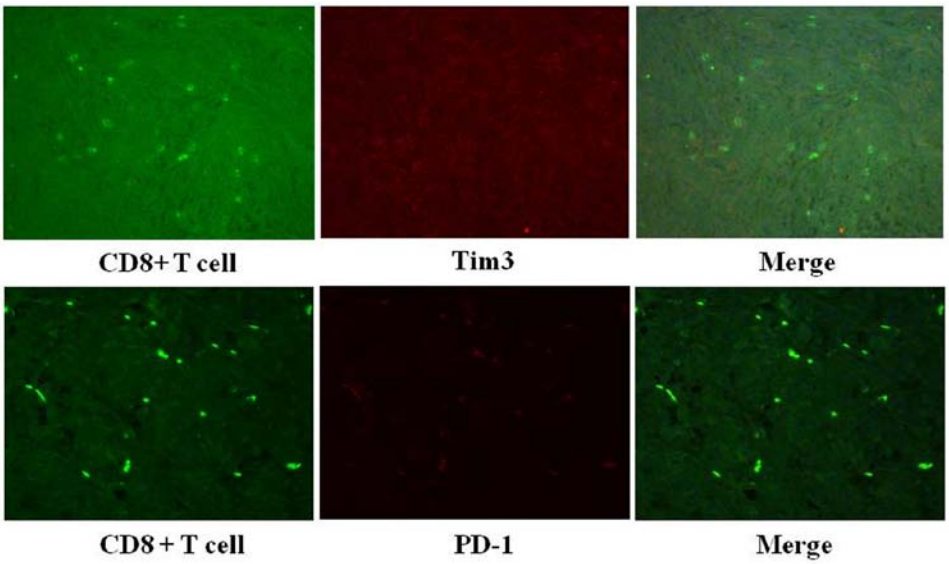

Figure 5. Expression of PD-1 and Tim-3 in the $CD8^+$ T cells in the GIST tissues.

binding capacity with the conserved carbohydrate recognition domains (CRDs). Fifteen mammalian galectins have been identified to date with three subtypes among which galectin-9 shows tandem-repeat with two CRDs joined by a linker peptide (24). The multi-faceted and occasionally conflicting roles of galectin-9 in tumor biology have been demonstrated, and tumor progression, tumor cell adhesion, metastasis and immune escape are the most likely involved (25). Galectin-9 expression has been shown as a prognostic marker of malignancies indicating that galectin-9-negative malignancies show frequent metastasis and those with high galectin-9 expression are associated with prolonged survival (26,27). In fact, GISTs are associated with a rare incidence of recurrence after surgical resection and infrequent distant metastases (1,4). It remains unclear whether this low aggressiveness of GISTs is associated with galectin-9 expression and whether GISTs showing distant metastasis lack galectin-9 expression. Another important biological implication of galectin-9 is its association with cell cycle control and induction of apoptosis (28,29). Galectin-9 induces the apoptosis of various types of blood cancer cells (30,31). Galectin-9 may contribute to the prevention of tumor metastasis by blocking adhesion to the endothelium and extracellular matrix (32).

Previous studies have found that GISTs express known tumor-associated antigens such as Wilms' tumor-1 (11-14), suggesting that GISTs are immunogenic tumors (7). However, in the present study, all of the $CD8^+$ T and NK cells infiltrated into the GIST tissues were CD69-negative inactivated cells. In spite of the immune cell infiltration, no immune-related tumor cell destructions were observed in the GIST tissues. These results suggest that antitumor immunity is strongly suppressed in GIST tissues. Immune suppression in GIST tissues by a macrophage-mediated mechanism or others may be involved (33,34).

Galectin-9 induces inactivation and apoptosis of T cells via Tim-3 receptor signaling (35,36) and also suppresses NK cell function via Tim-3/galectin-9 interaction (37-40). On the contrary, a promoting effect of galectin-9 on NK cell-mediated antitumor activity was also reported (41), Tim-3/galectin-9 interaction seems to be complicated. The Tim-3 receptor has been implicated as a negative regulator of adaptive immune responses and has been linked to T-cell dysfunction in chronic viral infections or cancer (42) and the regulatory roles of Tim-3 on innate immune responses have also been shown (38). In the present study, we found that NK cells that infiltrated into the GIST tissues were inactive and frequently expressed Tim-3. Considering that galectin-9, a ligand of Tim-3, was frequently expressed in the GIST tumor cells and Tim-3 was expressed in the infiltrated NK cells corresponding to galectin-9 expression in the GIST tumor cells, it is quite likely that the functions of NK cells in the GIST tissues were suppressed by an immune checkpoint mechanism mediated by the Tim-3/galectin-9 pathway. This suppressive effect may be achieved through a complex mosaic of inhibitory or activating receptors probably depending on the signal intensity, and Tim-3 signaling acts as a suppressor for NK-cell response in GISTs (38,43).

$CD8^+$ T cells that infiltrated into the GIST tissues were all CD69-negative inactivated cells and no $CD8^+$ T cells expressed Tim-3 or PD-1. Unfortunately, expression of PD-L1,

a ligand of PD-1, in the GIST tissues was undefined, since the results of PD-L1 staining of the GIST tissues was quite different depending on the type of anti-PD-L1 antibody used for immunohistochemistry. Considering the absence of PD-1 expression in the infiltrated $CD8^+$ T and $CD56^+$ NK cells, the PD-1/PD-L1 pathway is not involved in the immune checkpoint mechanism in GISTs.

Although tumor-associated antigens recognized by specific CTLs were expressed in the GISTs, adaptive antitumor immunity was deceased. We performed primary cultures of resected GIST tissues in IL-2-containing medium in some cases and observed vigorously proliferating mononuclear cells migrating from the GIST tissues. They mainly consisted of $CD56^+$ NK and $CD8^+$ T cells, and HLA-A24 restricted WT1 tetramer-positive $CD8^+$ T cells were present. These findings indicate that adaptive immunity targeting known tumor antigens may have been primed but suppressed in the GIST tissues. It has been stressed that innate immune responses including NK cell response are an important process for the induction of antitumor immune activity (44) and that activation of NK cell-mediated immune response is closely associated with the following activation of adaptive immunity (45,46). Suppression of NK cell activity may be correlated with the impaired $CD8^+$ T cell-mediated adaptive antitumor immunity in GIST tissues. Finally, blockade of the immune checkpoint mediated by Tim-3/Galectin-9 interaction may potentially re-activate NK cell activity and may be beneficial for the treatment of advanced GISTs.

References

1. Miettinen M and Lasota J: Gastrointestinal stromal tumors - definition, clinical, histological, immunohistochemical, and molecular genetic features and differential diagnosis. Virchows Arch 438: 1-12, 2001.
2. Hirota S, Isozaki K, Moriyama Y, Hashimoto K, Nishida T, Ishiguro S, Kawano K, Hanada M, Kurata A, Takeda M, *et al*: Gain-of-function mutations of c-kit in human gastrointestinal stromal tumors. Science 279: 577-580, 1998.
3. Rubin BP, Singer S, Tsao C, Duensing A, Lux ML, Ruiz R, Hibbard MK, Chen CJ, Xiao S, Tuveson DA, *et al*: KIT activation is a ubiquitous feature of gastrointestinal stromal tumors. Cancer Res 61: 8118-8121, 2001.
4. Corless CL, Fletcher JA and Heinrich MC: Biology of gastrointestinal stromal tumors. J Clin Oncol 22: 3813-3825, 2004.
5. Demetri GD, von Mehren M, Blanke CD, Van den Abbeele AD, Eisenberg B, Roberts PJ, Heinrich MC, Tuveson DA, Singer S, Janicek M, *et al*: Efficacy and safety of imatinib mesylate in advanced gastrointestinal stromal tumors. N Engl J Med 347: 472-480, 2002.
6. Le Cesne A, Ray-Coquard I, Bui BN, Adenis A, Rios M, Bertucci F, Duffaud F, Chevreau C, Cupissol D, Cioffi A, *et al*; French Sarcoma Group: Discontinuation of imatinib in patients with advanced gastrointestinal stromal tumours after 3 years of treatment: An open-label multicentre randomised phase 3 trial. Lancet Oncol 11: 942-949, 2010.
7. Rusakiewicz S, Semeraro M, Sarabi M, Desbois M, Locher C, Mendez R, Vimond N, Concha A, Garrido F, Isambert N, *et al*: Immune infiltrates are prognostic factors in localized gastrointestinal stromal tumors. Cancer Res 73: 3499-3510, 2013.
8. Rubin BP, Heinrich MC and Corless CL: Gastrointestinal stromal tumour. Lancet 369: 1731-1741, 2007.
9. Ménard C, Blay JY, Borg C, Michiels S, Ghiringhelli F, Robert C, Nonn C, Chaput N, Taïeb J, Delahaye NF, *et al*: Natural killer cell IFN-gamma levels predict long-term survival with imatinib mesylate therapy in gastrointestinal stromal tumor-bearing patients. Cancer Res 69: 3563-3569, 2009.
10. Borg C, Terme M, Taïeb J, Ménard 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.
11. Perez D, Herrmann T, Jungbluth AA, Samartzis P, Spagnoli G, Demartines N, Clavien PA, Marino S, Seifert B and Jaeger D: Cancer testis antigen expression in gastrointestinal stromal tumors: New markers for early recurrence. Int J Cancer 123: 1551-1555, 2008.
12. Perez D, Hauswirth F, Jäger D, Metzger U, Samartzis EP, Went P and Jungbluth A: Protein expression of cancer testis antigens predicts tumor recurrence and treatment response to imatinib in gastrointestinal stromal tumors. Int J Cancer 128: 2947-2952, 2011.
13. Ghadban T, Perez DR, Vashist YK, Bockhorn M, Koenig AM, El Gammal AT, Izbicki JR, Metzger U, Hauswirth F, Frosina D, *et al*: Expression of cancer testis antigens CT10 (MAGE-C2) and GAGE in gastrointestinal stromal tumors. Eur J Surg Oncol 40: 1307-1312, 2014.
14. Kang GH, Kim KM, Noh JH, Sohn TS, Kim S, Park CK, Lee CS and Kang DY: WT-1 expression in gastrointestinal stromal tumours. Pathology 42: 54-57, 2010.
15. Pardoll DM: The blockade of immune checkpoints in cancer immunotherapy. Nat Rev Cancer 12: 252-264, 2012.
16. Ramsay AG: Immune checkpoint blockade immunotherapy to activate anti-tumour T-cell immunity. Br J Haematol 162: 313-325, 2013.
17. Hodi FS, O'Day SJ, McDermott DF, Weber RW, Sosman JA, Haanen JB, Gonzalez R, Robert C, Schadendorf D, Hassel JC, *et al*: Improved survival with ipilimumab in patients with metastatic melanoma. N Engl J Med 363: 711-723, 2010.
18. Wolchok JD, Kluger H, Callahan MK, Postow MA, Rizvi NA, Lesokhin AM, Segal NH, Ariyan CE, Gordon RA, Reed K, *et al*: Nivolumab plus ipilimumab in advanced melanoma. N Engl J Med 369: 122-133, 2013.
19. Snyder A, Makarov V, Merghoub T, Yuan J, Zaretsky JM, Desrichard A, Walsh LA, Postow MA, Wong P, Ho TS, *et al*: Genetic basis for clinical response to CTLA-4 blockade in melanoma. N Engl J Med 371: 2189-2199, 2014.
20. Topalian SL, Hodi FS, Brahmer JR, Gettinger SN, Smith DC, McDermott DF, Powderly JD, Carvajal RD, Sosman JA, Atkins MB, *et al*: Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. N Engl J Med 366: 2443-2454, 2012.
21. Brahmer JR, Tykodi SS, Chow LQ, Hwu WJ, Topalian SL, Hwu P, Drake CG, Camacho LH, Kauh J, Odunsi K, *et al*: Safety and activity of anti-PD-L1 antibody in patients with advanced cancer. N Engl J Med 366: 2455-2465, 2012.
22. da Silva IP, Gallois A, Jimenez-Baranda S, Khan S, Anderson AC, Kuchroo VK, Osman I and Bhardwaj N: Reversal of NK-cell exhaustion in advanced melanoma by Tim-3 blockade. Cancer Immunol Res 2: 410-422, 2014.
23. Fujihara S, Mori H, Kobara H, Rafiq K, Niki T, Hirashima M and Masaki T: Galectin-9 in cancer therapy. Recent Pat Endocr Metab Immune Drug Discov 7: 130-137, 2013.
24. Leffler H, Carlsson S, Hedlund M, Qian Y and Poirier F: Introduction to galectins. Glycoconj J 19: 433-440, 2004.
25. Heusschen R, Griffioen AW and Thijssen VL: Galectin-9 in tumor biology: A jack of multiple trades. Biochim Biophys Acta 1836: 177-185, 2013.
26. Irie A, Yamauchi A, Kontani K, Kihara M, Liu D, Shirato Y, Seki M, Nishi N, Nakamura T, Yokomise H, *et al*: Galectin-9 as a prognostic factor with antimetastatic potential in breast cancer. Clin Cancer Res 11: 2962-2968, 2005.
27. van den Brûle F, Califice S and Castronovo V: Expression of galectins in cancer: A critical review. Glycoconj J 19: 537-542, 2004.
28. Yang RY and Liu FT: Galectins in cell growth and apoptosis. Cell Mol Life Sci 60: 267-276, 2003.
29. Liu FT, Yang RY, Saegusa J, Chen HY and Hsu DK: Galectins in regulation of apoptosis. Adv Exp Med Biol 705: 431-442, 2011.
30. Kageshita T, Kashio Y, Yamauchi A, Seki M, Abedin MJ, Nishi N, Shoji H, Nakamura T, Ono T and Hirashima M: Possible role of galectin-9 in cell aggregation and apoptosis of human melanoma cell lines and its clinical significance. Int J Cancer 99: 809-816, 2002.
31. Kobayashi T, Kuroda J, Ashihara E, Oomizu S, Terui Y, Taniyama A, Adachi S, Takagi T, Yamamoto M, Sasaki N, *et al*: Galectin-9 exhibits anti-myeloma activity through JNK and p38 MAP kinase pathways. Leukemia 24: 843-850, 2010.
32. Nobumoto A, Nagahara K, Oomizu S, Katoh S, Nishi N, Takeshita K, Niki T, Tominaga A, Yamauchi A and Hirashima M: Galectin-9 suppresses tumor metastasis by blocking adhesion to endothelium and extracellular matrices. Glycobiology 18: 735-744, 2008.

33. van Dongen M, Savage ND, Jordanova ES, Briaire-de Bruijn IH, Walburg KV, Ottenhoff TH, Hogendoorn PC, van der Burg SH, Gelderblom H and van Hall T: Anti-inflammatory M2 type macrophages characterize metastasized and tyrosine kinase inhibitor-treated gastrointestinal stromal tumors. Int J Cancer 127: 899-909, 2010.
34. Cameron S, Haller F, Dudas J, Moriconi F, Gunawan B, Armbrust T, Langer C, Füzesi L and Ramadori G: Immune cells in primary gastrointestinal stromal tumors. Eur J Gastroenterol Hepatol 20: 327-334, 2008.
35. Zhu C, Anderson AC, Schubart A, Xiong H, Imitola J, Khoury SJ, Zheng XX, Strom TB and Kuchroo VK: The Tim-3 ligand galectin-9 negatively regulates T helper type 1 immunity. Nat Immunol 6: 1245-1252, 2005.
36. Li H, Wu K, Tao K, Chen L, Zheng Q, Lu X, Liu J, Shi L, Liu C, Wang G, *et al*: Tim-3/galectin-9 signaling pathway mediates T-cell dysfunction and predicts poor prognosis in patients with hepatitis B virus-associated hepatocellular carcinoma. Hepatology 56: 1342-1351, 2012.
37. Ju Y, Hou N, Meng J, Wang X, Zhang X, Zhao D, Liu Y, Zhu F, Zhang L, Sun W, *et al*: T cell immunoglobulin - and mucin-domain-containing molecule-3 (Tim-3) mediates natural killer cell suppression in chronic hepatitis B. J Hepatol 52: 322-329, 2010.
38. Han G, Chen G, Shen B and Li Y: Tim-3: An activation marker and activation limiter of innate immune cells. Front Immunol 4: 449, 2013.
39. Hou H, Liu W, Wu S, Lu Y, Peng J, Zhu Y, Lu Y, Wang F and Sun Z: Tim-3 negatively mediates natural killer cell function in LPS-induced endotoxic shock. PLoS One 9: e110585, 2014.
40. Golden-Mason L, McMahan RH, Strong M, Reisdorph R, Mahaffey S, Palmer BE, Cheng L, Kulesza C, Hirashima M, Niki T, *et al*: Galectin-9 functionally impairs natural killer cells in humans and mice. J Virol 87: 4835-4845, 2013.
41. Gleason MK, Lenvik TR, McCullar V, Felices M, O'Brien MS, Cooley SA, Verneris MR, Cichocki F, Holman CJ, Panoskaltsis-Mortari A, *et al*: Tim-3 is an inducible human natural killer cell receptor that enhances interferon gamma production in response to galectin-9. Blood 119: 3064-3072, 2012.
42. Ferris RL, Lu B and Kane LP: Too much of a good thing? Tim-3 and TCR signaling in T cell exhaustion. J Immunol 193: 1525-1530, 2014.
43. Ndhlovu LC, Lopez-Vergès S, Barbour JD, Jones RB, Jha AR, Long BR, Schoeffler EC, Fujita T, Nixon DF and Lanier LL: Tim-3 marks human natural killer cell maturation and suppresses cell-mediated cytotoxicity. Blood 119: 3734-3743, 2012.
44. Campbell KS and Hasegawa J: Natural killer cell biology: An update and future directions. J Allergy Clin Immunol 132: 536-544, 2013.
45. Lee KM, Bhawan S, Majima T, Wei H, Nishimura MI, Yagita H and Kumar V: Cutting edge: The NK cell receptor 2B4 augments antigen-specific T cell cytotoxicity through CD48 ligation on neighboring T cells. J Immunol 170: 4881-4885, 2003.
46. Mocikat R, Braumüller H, Gumy A, Egeter O, Ziegler H, Reusch U, Bubeck A, Louis J, Mailhammer R, Riethmüller G, *et al*: Natural killer cells activated by MHC class I(low) targets prime dendritic cells to induce protective CD8 T cell responses. Immunity 19: 561-569, 2003.