CpG oligodeoxynucleotides potentiate the antitumor activity of anti-BST2 antibody

Kosuke Hiramatsu,1,2 Satoshi Serada,3 Kouji Kobiyama,3 Satoshi Nakagawa,1,2 Akiko Morimoto,1 Shinya Matsuzaki,1 Yutaka Ueda,1 Minoru Fujimoto,2 Kiyoshi Yoshino,1 Ken J. Ishii,3 Takayuki Enomoto,4 Tadashi Kimura1 and Tetsuji Naka2

1Department of Obstetrics and Gynecology, Osaka University Graduate School of Medicine, Suita, Japan; 2Laboratory of Immune Signal, National Institute of Biomedical Innovation, Health and Nutrition, Ibaraki, Japan; 3Laboratory of Adjutivant Innovation, National Institute of Biomedical Innovation, Health and Nutrition, Ibaraki, Japan; 4Department of Obstetrics and Gynecology, Niigata University Medical School, Niigata, Japan

Key words
Antitumor antibody, bone marrow stromal antigen 2, CpG oligodeoxynucleotides, macrophage, natural killer cell

Correspondence
Tetsuji Naka, Laboratory of Immune Signal, National Institute of Biomedical Innovation, Health and Nutrition, 7-6-8 Saitoasagi, Ibaraki City, Osaka 567-0085, Japan. Tel: +81-72-641-9843; Fax: +81-72-641-9837; E-mail: tnaka@nibiohn.go.jp

Funding Information
This study was supported by a Grant-in-Aid from the Ministry of Health, Labour and Welfare of Japan.

Received February 23, 2015; Revised June 28, 2015; Accepted July 7, 2015
Cancer Sci 106 (2015) 1474–1478
doi: 10.1111/cas.12738

Numerous monoclonal antibodies (mAb) targeting tumor antigens have recently been developed. Antibody-dependent cellular cytotoxicity (ADCC) and antibody-dependent cellular phagocytosis (ADCP) via effector cells such as tumor-infiltrating natural killer (NK) cells and macrophages are often involved in mediating the antitumor activity of mAb. CpG oligodeoxynucleotides (ODN) have a potent antitumor activity and are considered to increase tumor infiltration of NK cells and macrophages. Our group previously reported significant antitumor activity of anti-bone marrow stromal antigen 2 (BST2) mAb against BST2-positive endometrial cancer cells through ADCC. In this study, we evaluated the synergistic antitumor activity of combination therapy with anti-BST-2 mAb and CpG ODN using SCID mice and elucidated the mechanisms underlying this activity. Anti-BST2 mAb and CpG ODN monotherapy had a significant dose-dependent antitumor activity (P = 0.0135 and P = 0.0196, respectively). Combination therapy with anti-BST2 mAb and CpG ODN had a significant antitumor activity in SCID mice (P < 0.01), but not in NOG mice. FACs analysis revealed significantly increased numbers of NK cells and macrophages in tumors treated with a combination of anti-BST2 mAb and CpG ODN and with CpG ODN alone in SCID mice (P < 0.05 and P < 0.01, respectively). These results suggested that the combination therapy with anti-BST2 mAb and CpG ODN has a significant antitumor activity and induces tumor infiltration of NK cells and macrophages. Combination therapy with CpG ODN and anti-BST2 mAb or other antitumor mAb depending on ADCC may represent a new treatment option for cancer.
Materials and Methods

Cell lines and culture. HEC-88nu cells were obtained from the Japanese Collection of Research Bioresources (JCRB, Osaka, Japan) and maintained in DMEM (Wako Pure Chemical Industries, Osaka, Japan) supplemented with 20% FBS and 1% penicillin-streptomycin (Nacalai Tesque, Kyoto, Japan) at 37°C under a humidified atmosphere with 5% CO2. All experiments are described in Supplementary Data S1.

Results

Anti-bone marrow stromal antigen 2 monoclonal antibody and CpG oligodeoxynucleotides exhibit significant dose-dependent antitumor activity. To determine the optimum concentrations of anti-BST2 mAb and CpG ODN for combination therapy, we evaluated the individual dose-dependent antitumor activity of anti-BST2 mAb and CpG ODN. For the anti-BST2 mAb group, SCID mice xenografted with tumor cells were treated with i.p. injection of 400 μL of PBS or anti-BST2 mAb (12.5, 50 and 200 μg in 400 μL of PBS/mouse). As shown in Figure 1(a), anti-BST2 mAb exhibited a significant dose-dependent reduction in tumor weight ($P = 0.0135$) and a dose-dependent trend toward reduced tumor volume ($P = 0.0552$). In the CpG ODN group, xenografted SCID mice were treated with i.t. injection of PBS or CpG ODN (10, 20 and 40 μg in 10 μL of PBS/mouse). As shown in Figure 1(b), CpG ODN exhibited a significant dose-dependent reduction in tumor volume and tumor weight ($P = 0.0319$ and $P = 0.0196$, respectively). These

![Graph](image-url)
results demonstrate that both anti-BST2 mAb and CpG ODN have a dose-dependent antitumor activities.

**Combination therapy with anti-bone marrow stromal antigen 2 monoclonal antibody and CpG oligodeoxynucleotide exhibits synergistic activity in xenografted SCID mice but not in NOG mice.**

To evaluate the synergistic effect of anti-BST2 mAb and CpG ODN, xenografted SCID mice were treated by i.p./i.t. injection of (A) PBS/PBS, (B) anti-BST2 mAb (12.5 µg/mouse)/PBS, (C) anti-BST2 mAb (200 µg/mouse)/PBS, (D) PBS/CpG ODN (10 µg/mouse) and (E) anti-BST2 mAb (12.5 µg/mouse)/CpG ODN (10 µg/mouse), respectively. In SCID mice, treatment with regimen (e) resulted in a significant antitumor activity compared with other regimens in terms of tumor volume and tumor weight (*P* < 0.01 and *P* < 0.05, respectively; Fig. 2a,b). To reveal whether the synergistic effect of anti-BST2 mAb and CpG ODN is dependent on NK cells, NOG mice that have the complete defect of NK cells and the dysfunction of macrophages were used. In NOG mice xenografted with tumors, all regimes showed no antitumor effects in tumor volume and tumor weight (*P* = 1.00 and *P* = 1.00, respectively; Fig. 2c,d). These results demonstrate a synergistic antitumor effect of combination therapy with anti-BST2 mAb and CpG ODN via NK cells and macrophages.

**FACS analysis demonstrates natural killer cell and macrophage infiltration in tumors treated with anti-bone marrow stromal antigen 2 monoclonal antibody and CpG oligodeoxynucleotide.** For FACS analysis, xenografted SCID mice were treated by i.p./i.t. injection of (A) PBS/PBS, (B) anti-BST2 mAb (12.5 µg/mouse)/PBS, (C) PBS/CpG ODN (10 µg/mouse) and (D) anti-BST2 mAb (12.5 µg/mouse)/CpG ODN (10 µg/mouse), respectively. Treatment with regimen (E) caused a significant decrease in tumor volume compared with that of other regimens in SCID mice (*P* < 0.01).
the representative results of FACS analysis of F4/80+ macrophages and CD49b+ NK cells in tumors treated with regimen (A) to (D).

In tumors from SCID mice treated with regimens (A) and (B), NK cells accounted for 8% of all lymphocytes, whereas the proportion of NK cells was significantly increased in tumors from SCID mice treated with regimens (C) and (D) than in those treated with regimens (A) and (B) \((P < 0.05); \text{ Fig. 3b}\). Similarly, the number of macrophages was significantly higher in tumors from SCID mice treated with regimens (C) and (D) than in those treated with regimens (A) and (B) \((P < 0.01); \text{ Fig. 3c}\).

Discussion

In this study, we demonstrated a significant therapeutic activity of combination therapy with anti-BST2 mAb and CpG ODN in a BST2-positive endometrial cancer xenograft model. We further demonstrated increased tumor infiltration of NK cells and macrophages in SCID mice treated with CpG ODN.

The preclinical efficacy of combination therapy with CpG ODN and antitumor agents has been reported\(^6\); however, its antitumor activity has not been fully investigated in vivo. In our study, combination therapy with anti-BST2 mAb and CpG ODN also exhibited a potent antitumor activity in a SCID mouse xenograft model. Anti-BST2 mAb exhibit a dose-dependent antitumor effect via ADCC; therefore, greater concentrations of mAb are required to achieve greater efficacy. However, there is concern regarding increased adverse effects with greater doses of anti-BST2 mAb. BST2 is known to be expressed not only on the cell surface of dendritic cells but also in other normal tissues, including spleen, gallbladder and stomach.\(^1\) The anti-BST2 mAb used in the present study recognizes human BST2, but not mouse BST2 (data not shown), suggesting that the humanized form of anti-BST2 mAb may induce cytotoxicity against these cells and tissues. However, CpG ODN increased the activity of anti-BST2 mAb at low doses, suggesting that combination therapy may allow decreased therapeutic doses of anti-BST2 mAb, thereby reducing adverse effects.

The infiltration of NK cells and macrophages plays an important role in inducing ADCC and ADCP. Previously, tumor infiltration of NK cells and macrophages has been analyzed by immunohistochemistry.\(^1\) This is the first report of FACS analysis revealing increased infiltration of NK cells and macrophages using whole-tumor tissues.

Intra-tumoral injection is required for the effective treatment of CpG ODN.\(^6\) In gynecological cancer, i.t. injection is possible for localized cervical cancer, endometrial cancer and recurrent tumor of the vaginal stump, allowing the use of CpG ODN in gynecological cancer. Our study specifically demonstrated the efficacy of combination therapy
with anti-BST2 mAb and CpG ODN; however, CpG ODN could be combined with gynecological antitumor antibodies routinely used in clinical practice. Moreover, in recent studies, a systemically administered CpG ODN-conjugated antitumor antibody demonstrated antitumor activity, suggesting that CpG ODN could also be used in gynecological cancers where deep location prevents direct administration.

In summary, combination therapy with anti-BST2 mAb and CpG ODN exerts significant antitumor activity and induces infiltration of NK cells and macrophages in CpG ODN-treated tumors. Combination therapy with CpG ODN and anti-BST2 mAb or other antitumor agents depending on ADCC may represent a novel treatment option for cancer.

Acknowledgments
We thank Y. Kanazawa and J. Yamagishi for their secretarial assistance and M. Ako, E. Harada and K. Sakiyama for technical assistance. This study was supported by a Grant-in-Aid from the Ministry of Health, Labour and Welfare of Japan.

Disclosure Statement
The authors have no conflict of interest to declare.

References
1 Cobleigh MA, Vogel CL, Tripathy D et al. Multinational study of the efficacy and safety of humanized anti-HER2 monoclonal antibody in women who have HER2-overexpressing metastatic breast cancer that has progressed after chemotherapy for metastatic disease. J Clin Oncol 1999; 17: 2639–48.
2 Villegas FR, Coca S, Villanubia VG et al. Prognostic significance of tumor infiltrating natural killer cells subset CD57 in patients with squamous cell lung cancer. Lung Cancer 2002; 35: 23–8.
3 Ishigami S, Natsugoe S, Tokuda K et al. Prognostic value of intratumoral natural killer cells in gastric carcinoma. Cancer 2000; 88: 577–83.
4 Terme M, Ullrich E, Aymere L et al. Cancer-induced immunosuppression: IL-18-elicited immunoablative NK cells. Cancer Res 2012; 72: 2757–67.
5 Akira S, Takeda K, Kaisho T. Toll-like receptors: critical proteins linking innate and acquired immunity. Nat Immunol 2001; 2: 675–80.
6 Ishii KJ, Kawakami K, Gursel I et al. Antitumor therapy with bacterial DNA and toxin: complete regression of established tumor induced by liposomal CpG oligodeoxynucleotides plus interleukin-13 cytotoxin. Clin Cancer Res 2003; 9: 6516–22.
7 Yokoyama T, Enomoto T, Serada S et al. Plasma membrane proteomics identifies bone marrow stromal antigen 2 as a potential therapeutic target in endometrial cancer. Int J Cancer 2013; 132: 472–84.
8 Harada T, Ozaki S, Oda A et al. Combination with a defucosylated anti-HM1.24 monoclonal antibody plus lenalidomide induces marked ADCC against myeloma cells and their progenitors. PLoS One 2013; 8: e83905.
9 Sommariva M, de Cesare M, Meini A et al. High efficacy of CpG-ODN, cetuximab and cisplatin combination for very advanced ovarian xenograft tumors. J Transl Med 2013; 11: 25.
10 Erikson E, Adam T, Schmidt S et al. In vivo expression profile of the antiviral restriction factor and tumor-targeting antigen CD317/BST-2/HM1.24/tetherin in humans. Proc Natl Acad Sci U S A 2011; 108: 13688–93.
11 Halama N, Braun M, Kahlert C et al. Natural killer cells are scarce in colorectal carcinoma tissue despite high levels of chemokines and cytokines. Clin Cancer Res 2011; 17: 678–89.
12 Li Z, Jang JK, Lechner MG et al. Generation of tumor-targeted antibody-CpG conjugates. J Immunol Methods 2013; 389: 45–51.

Supporting Information
Additional supporting information may be found in the online version of this article:
Data S1. Supplementary Materials and Methods.