Combination Immunotherapy with 4-1BB Activation and PD-1 Blockade Enhances Antitumor Efficacy in a Mouse Model of Subcutaneous Tumor

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Abstract. *Background/Aim: The purpose of the present study was to establish an effective immunotherapy by skewing the cosignal balance to be on the positive side by using the combination of monoclonal antibody (mAb) against 4-1BB also known as Cluster of Differentiation (CD) 137 as a co-stimulatory effector and to programmed death-1 (PD-1) to blockade the immune checkpoint. Materials and Methods: Mice implanted with 1×10^5 CT26 cells were treated with anti 4-1BB mAb alone, anti PD-1 mAb alone, or both anti 4-1BB mAb and anti PD-1 mAb. Immune cell populations were analyzed by flow cytometry. Tumor-infiltrating T-cells were evaluated by immunohistochemistry. Results: Mice treated with the combination therapy had the best antitumor response that resulted in complete tumor rejection. The numbers of $CD4^+$ interferon (IFN)-γ^+ and $CD8^+$ IFN-γ^+ T-cells were significantly higher in the combination group. The number of tumor-infiltrating T-cells was significantly increased in the combination therapy. Conclusion: The therapeutic strategy of targeting co-signal molecules has promising clinical applications in the future.*

T-Cell activation regularly requires two signals. Signal 1 is the interaction between antigenic peptide on major histocompatibility complex (MHC) and the T-cell receptor (TCR). Signal 2 called cosignal, presented by the interaction between antigen-presenting cells (APCs) and T-cells is also necessary to activate T-cells properly (1). Cosignal is composed by the immune system to control the balance between co-stimulatory and co-inhibitory factors. Currently, co-inhibitory factors are known as immune checkpoints (2). The programmed death-1 (PD-1) and cytotoxic T-lymphocyte antigen-4 (CTLA4) pathways are two examples of immune checkpoint pathways with the potential for therapeutic anticancer targeting (3). Knockout of these genes in mouse models leads to two distinct phenotypes: *Ctla4*-deficient mice die within a few weeks because of autoimmune disease, while *PD-1*-deficient mice develop organ-specific immune reactions over the course of several months (4, 5).

PD-1 is expressed on activated T-cells, B-cells, dendritic cells (DCs) and macrophages (3, 6). PD-1 ligand 1 (PD-L1) is expressed on DCs and tumor cells in various types of cancers, and its expression on tumors is thought to contribute to tumor immune evasion (7). Anti-PD-1 monoclonal antibodies (mAbs) have been developed for use in human trials, in which they mediated the regression of several tumor types (8).

To enhance immune responses in immunocompromised hosts with malignancies, use of agonistic mAbs specific to co-stimulatory receptors is a promising approach (9). The protein 4-1BB also known as cluster of differentiation (CD) 137 is transiently up-regulated on both $CD4^+$ and $CD8^+$ T-cells following activation (10). 4-1BB signaling can induce cytokine production, expansion, and functional maturation of T-cells, DCs and monocytes (11, 12). Several studies have reported that agonistic antibodies against 4-1BB can enhance tumor rejection and increase tumor-specific cytotoxicity (13,

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14). Although antibodies against 4-1BB have been developed for use in human trials, concerns about toxicity have led to their termination or withdrawal (15).

Based on the two theories underlying the role of 4-1BB and PD-1 as described above, antibodies activating 4-1BB or blocking PD-1 are expected to comprise the next generation of immunotherapy against human cancer. However, their efficacies as a single agent are insufficient to cause the rejection of established tumors in mice or humans.

The purpose of the present study was to establish an effective antitumor immunotherapy by using the combination of anti-4-1BB mAb and anti-PD-1 mAb.

Materials and Methods

Animal and tumor cell line. BALB/c mice (7-8 weeks old, female) were purchased from Japan SLC, Inc. (Shizuoka, Japan). All mice were maintained under specific pathogen-free conditions in the animal facility at Yamaguchi University. All animal procedures were approved by the Institutional Animal Care and Use Committee of Yamaguchi University. Colon tumor 26 cells (CT26) are murine colon adenocarcinoma cells derived from BALB/c mice (16). 4T1 cells are murine breast carcinoma derived from BALB/c mice (17). Both cell lines were purchased from the American Type Culture Collection (Manassas, VA , USA). Cells were grown in RPMI-1640 (Gibco BRL, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal calf serum as previously described (18).

Antibodies. Agonistic anti-mouse 4-1BB mAb (3H3) and antagonistic anti-mouse PD-1 mAb (RMP1-14) were produced by Dr. Yagita (Juntendo University School of Medicine, Tokyo, Japan) as previously described (13, 19). Rat immunoglobulin (Ig) G used for a control antibody was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Protocol of antibody treatment. BALB/c mice were inoculated subcutaneously in the right flank with 1×10^5 CT26 cells. When the tumors reached 5-8 mm in diameter, approximately 10 days after tumor inoculation, treatment was started. Tumor-bearing mice were treated with rat IgG, agonistic anti-4-1BB and rat IgG, anti-PD-1 and rat IgG, or agonistic anti-4-1BB and anti-PD-1. The dose per injection was 150 μg for rat IgG, 50 μg for anti-4-1BB and 150 μg for anti-PD-1. mAbs were given intraperitoneally on days 10, 14, 17 and 21. Mice were monitored and scored for the formation of palpable tumors twice weekly and sacrificed if tumors became necrotic or exceeded the predetermined size of 2,000 mm^3. Tumor volumes were measured in cubic millimeters with calipers and calculated with the following formula: A×B2 ×0.5, where A is the largest diameter, and B is the smallest diameter.

Tumor re-challenge. One hundred days after tumor inoculation, the surviving mice (n=8) treated with anti-4-1BB mAb and anti-PD-1 mAb (referred to as the immunized mice) were injected again with 1×10^5 CT26 cells (n=4) or 1×10^5 4T1 cells (n=4) respectively as an independent experiment. Each 4 (total of 8) nonimmunized (naïve) mice were injected in the same manner as a control, respectively. A total of 1×10^5 4T1 cells was also injected into both naïve (n=4) and immunized (n=4) mice. All mice were observed until the tumor volume reached 2,000 mm^3 and were then euthanized.

Flow cytometric analysis. Lymphocytes from the draining lymph node (LN) and spleens were isolated and processed on day 25 as previously described (20). For intracellular staining, cells were stained according to the manufacturer's instructions (eBiosciece, San Diego, CA, USA).

Cells were stained with the following antibodies: Fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD3e (clone 145-2C11) (Biolegend, San Diego, CA, USA), Gr1 (clone RB6-8C5), and IL4 (clone BVD6-24G2) (both from eBioscience); Phycoerythrin (PE)-conjugated anti-mouse CD11b (clone M1/70) and CD8a (clone 53-6.7) (both from Biolegend); PE-conjugated Gr1 (clone RB6-8C5), interferon (IFN)-γ (clone XMG1.2), FOXP3 (clone NRRF-30), and CD4 (clone GK1.5) (all from eBioscience). AH1 is tumor-associated immunodominant MHC class I–restricted antigen for CT26 (21). AH1 tumor-specific $CD8^+$ T-cells were analyzed by using Ld-AH1 tetramer loaded with AH1 (SPSYVYHQF) purchased from MBL (Nagoya, Japan). All flow cytometric data were acquired on a MACSQuant flow cytometer (Miltenyi Biotec, Bergisch Gladbach, Germany) and analyzed using the MACSQuantify software.

Immunohistochemistry. On days 17 and 24 after inoculation, tumors were dissected. Scar-like tissues were dissected in the mice treated with the combination of anti-4-1BBand anti-PD-1 because the mice rejected tumors. Zinc-fixed, paraffin-embedded tissues of primary tumors were processed and stained as previously described (22). Purified rabbit anti-CD3 (ab5690; Abcam, Tokyo, Japan) was used for staining.

Evaluation of $CD3^+$ cells. Evaluation of $CD3^+$ cells in the tumor-infiltrating lymphocytes was performed as previously described (23). The number of $CD3^+$ cells was counted by using a computerized image analysis system composed of a DP70 charge-coupled device camera (Olympus, Tokyo, Japan) mounted on an Olympus AX70 light microscope. Under ×400 magnification, three independent and intact computerized microscopic fields in duplicates of each tissue sample were used for counting of the number of $CD3^+$ cells. The evaluation of $CD3^+$ cells was performed by two independent observers in a blinded manner.

Statistical analysis. Results are expressed as the means±standard error. All data were analyzed with GraphPad Prism V5.0 (GraphPad Software, Inc., San Diego, CA). One- and two-way analyses of variances (ANOVA) were used for data analysis of more than two groups and a Bonferroni *post-hoc* test was utilized to determine significant differences between treatment groups. *p*-Values of less than 0.05 were considered statistically significant.

Results

Antitumor effects of a combination of 4-1BB activation and PD-1 blockade. Anti-4-1BB significantly inhibited tumor growth ($p<0.05$). Anti-PD-1 slightly inhibited tumor growth, but the inhibition was not significant. Furthermore, mice treated with the combination of anti-4-1BB and anti-PD-1 had the best antitumor response, which resulted in complete tumor rejection in all mice injected with CT26 cells ($p<0.01$) (Figure 1A). There were no overt toxicities in treated mice. Tumor re-challenge. In all re-challenged immunized mice,

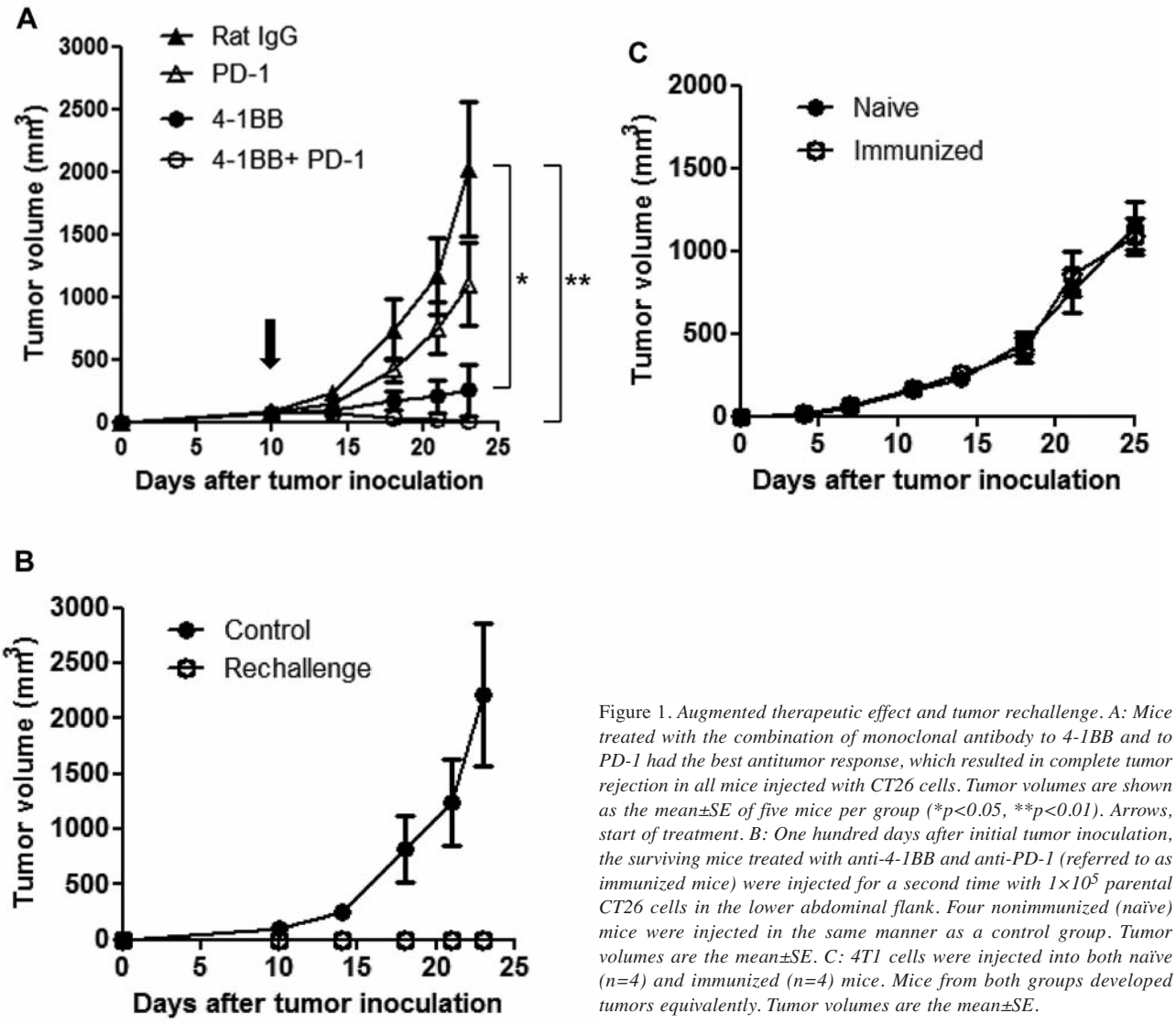

Figure 1. *Augmented therapeutic effect and tumor rechallenge. A: Mice treated with the combination of monoclonal antibody to 4-1BB and to PD-1 had the best antitumor response, which resulted in complete tumor rejection in all mice injected with CT26 cells. Tumor volumes are shown as the mean±SE of five mice per group (*p<0.05, **p<0.01). Arrows, start of treatment. B: One hundred days after initial tumor inoculation, the surviving mice treated with anti-4-1BB and anti-PD-1 (referred to as immunized mice) were injected for a second time with 1×10^5 parental CT26 cells in the lower abdominal flank. Four nonimmunized (naïve) mice were injected in the same manner as a control group. Tumor volumes are the mean±SE. C: 4T1 cells were injected into both naïve (n=4) and immunized (n=4) mice. Mice from both groups developed tumors equivalently. Tumor volumes are the mean±SE.*

CT26 tumors were rejected, whereas tumors in naïve mice grew progressively (Figure 1B). There was no significant difference in the growth of implanted 4T1 cells between naïve mice and immunized mice (Figure 1C).

Analysis of $CD4^+$ T-cells in the spleen. A significant increase in the number of $CD4^+$ T-cells was observed in mice treated with any mAb compared to naïve mice (Figure 2A). The number of $CD4^+$ IFN-γ^+ T-cells was significantly higher in the mice treated with combination immunotherapy ($p<0.0001$) (Figure 2B). The number of $CD4^+$ $IL4^+$ T cells was significantly increased in the mice treated with control mAb and anti-PD-1 ($p<0.01$) (Figure 2C).

Analysis of $CD8^+$ T-cells in the spleen. The number of $CD8^+$ T-cells was also significantly increased in mice treated with anti-4-1BB and those with combination therapy (Figure 3A). The mice treated with combination therapy had the most $CD8^+$ IFN-γ^+ T-cells, and the activation of these cells was antigen-specific ($p<0.0001$) (Figure 3B). The mice treated with combination therapy also had an increased number of AH1-specific $CD8^+$ T-cells (Figure 3C).

Regulatory T-cells (Tregs) and myeloid-derived suppressor cells (MDSCs) in the LN and spleen. We next assessed negative factors of immune effector cells focusing on $CD4^+$ $FOXP3^+$ T-cells as Tregs and $CD11b^+$ $Gr\text{-}1^+$ cells as MDSCs.

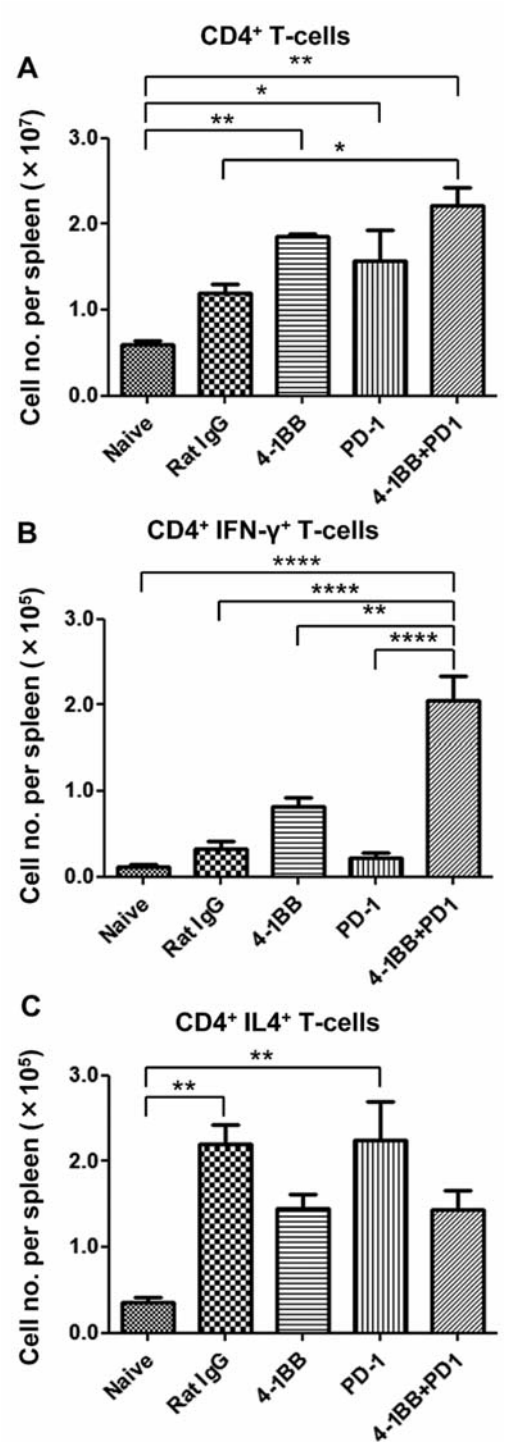

Figure 2. *CD4+ T-cells in the spleen after treatment. A: A significant increase in the number of CD4+ T-cells was observed in mice treated with any monoclonal antibody compared to naïve mice. B: A significant increase in the number of CD4+ IFN-γ+ T-cells was observed in the combination-therapy group. C: A significant increase in the number of CD4+ IL4+ T-cells was observed in the control and anti-PD-1-treated groups. Statistical significance was calculated by using one-way ANOVA with the Bonferroni post-hoc test (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).*

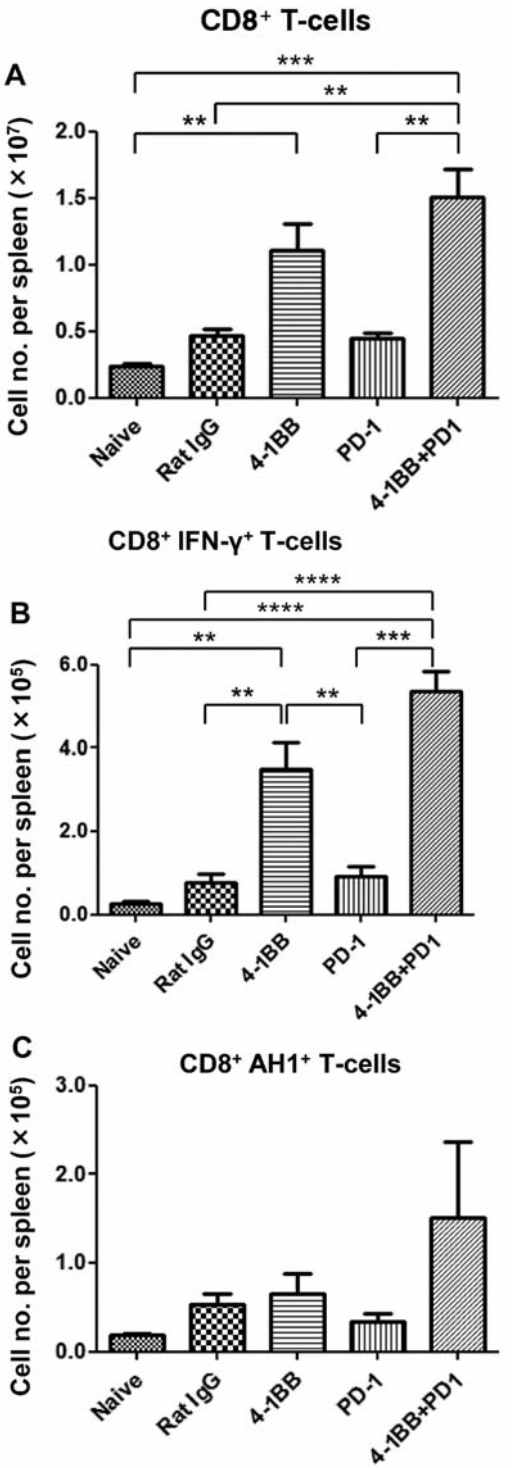

Figure 3. *CD8+ T-cells in the spleen after treatment. A: A significant increase in the number of CD8+ T-cells was observed in mice treated with monoclonal antibody to 4-1BB alone and those treated with combination therapy. B: The mice treated with the combination therapy had most CD8+ IFN-γ+ T-cells. C: The mice treated with the combination therapy had an increased number of AH1-specific CD8+ T-cells. Statistical significance was calculated by using one-way ANOVA with the Bonferroni post-hoc test (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).*

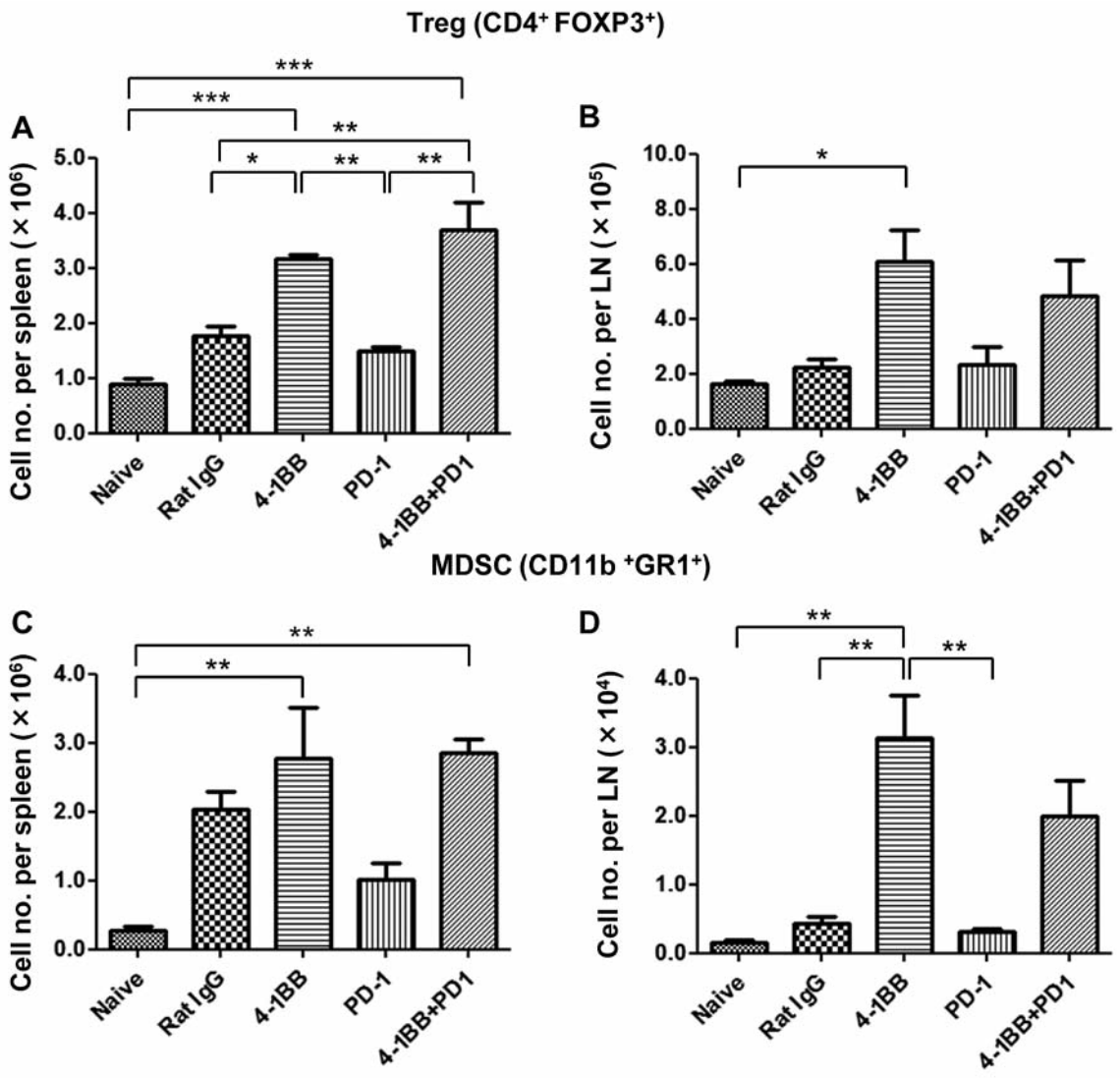

Figure 4. *Tregs and MDSCs in the LNs and spleen. The number of Tregs (A) and MDSCs (C) in the spleen was increased in mice treated with monoclonal antibody to 4-1BB mAb and combination therapy as compared with the other groups. The number of Tregs (B) and MDSCs (D) in the LN was the most increased in mice treated with anti-4-1BB. The number of Tregs and MDSCs are the average (±SE) number of cells derived from three mice. Statistical significance was calculated by using one-way ANOVA with the Bonferroni post-hoc test (*p<0.05, **p<0.01, ***p<0.001).*

The number of Tregs in the spleen was increased in mice treated with anti-4-1BB and combination therapy compared to the other groups (Figure 4A). Anti-4-1BB resulted in the most pronounced increase in the number of Tregs in LNs (Figure 4B). The number of MDSCs in the spleen was increased in mice treated with anti-4-1BB and those treated with the combination therapy as compared to the other groups (Figure 4C). Anti-4-1BB resulted in the most pronounced increase in the number of MDSCs in LNs (Figure 4D).

Tumor-infiltrating T-cells. Figure 5A and B show tissue stained for tumor-infiltrating $CD3^+$ cells in each group on days 17 and day 24, respectively. The presence of tumor-infiltrating $CD3^+$ cells was significantly increased on day 17 (Figure 5C) and day 24 (Figure 5D) in the group treated with combination therapy.

Discussion

In the present study, we focused on 4-1BB, which is a promising target for an immunostimulatory molecule. Furthermore, we combined anti-4-1BB treatment with that against PD-1, a well-investigated immune checkpoint molecule in cancer immunotherapy. Anti-4-1BB inhibited

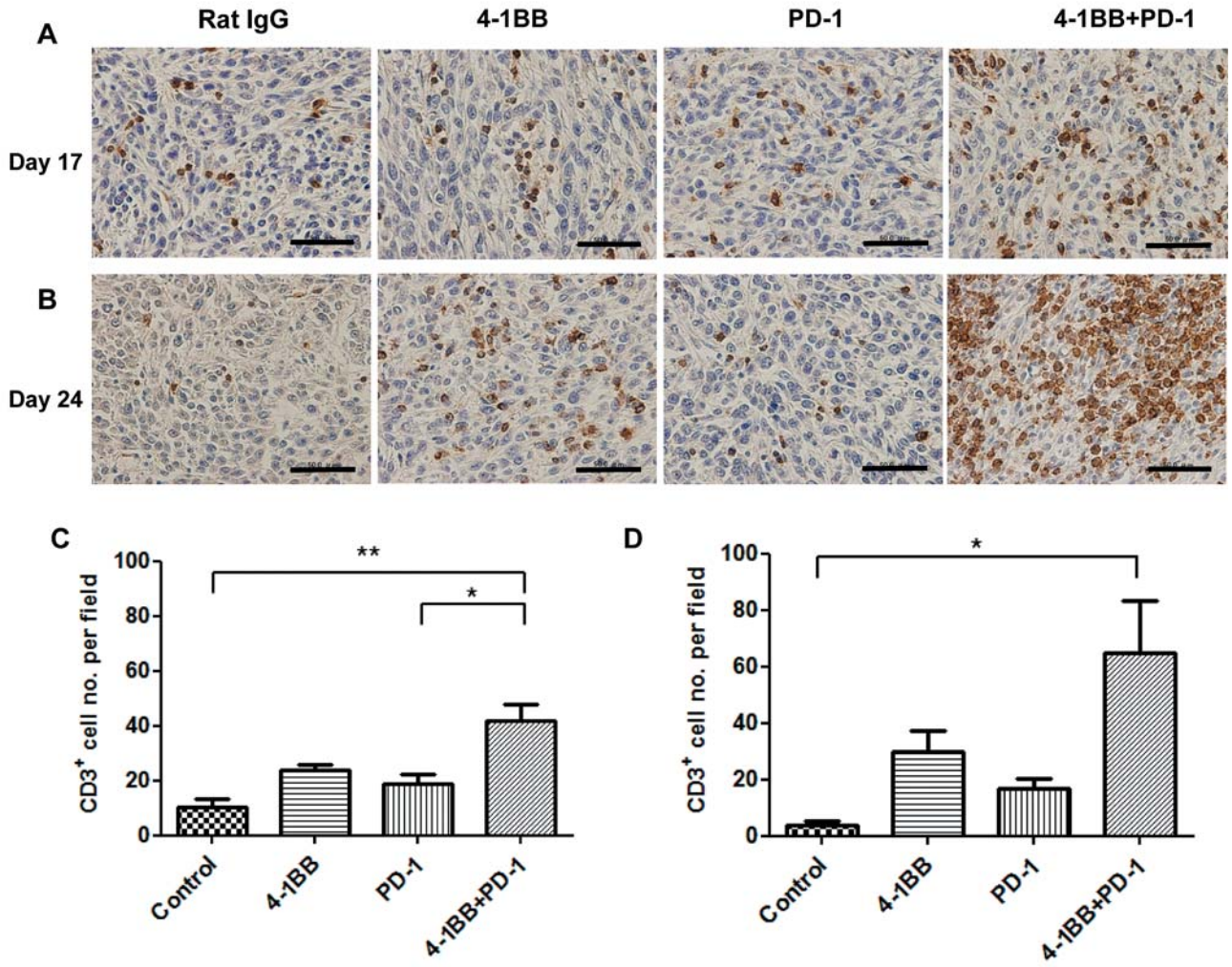

Figure 5. *Pathological analysis of immune infiltration after therapy. Tumor-infiltrating T-cells were identified by immunohistochemical analysis on day 17 (A) and day 24 (B) after tumor inoculation. The presence of tumor-infiltrating T-cells was significantly increased on day 17 (C) and day 24 (D) after inoculation in mice treated with combination therapy. Tumor-infiltrating T-cells were counted at ×400 magnification. Photographs representative of three mice in each group are shown. Data are the mean±SE (*p<0.05; **p<0.01). Scale bar, 50 μm.*

tumor growth more significantly than did anti-PD-1. The anti-tumor effect of anti-4-1BB might depend on increased tumor-specific cytotoxic T-lymphocyte activity and IFN-γ production by $CD4^+$ and $CD8^+$ T-cells. Moreover, the combination therapy led to increased numbers of $CD4^+$ IFN-γ^+ T-cells (type 1 helper T-cells: Th1 cells) and $CD8^+$ IFN-γ^+ cells, resulting in complete tumor rejection in all mice. The presence of high numbers of tumor-infiltrating T-cells are consistent with a role for adaptive antitumor immunity in the prevention of tumor progression (24). We found that high numbers of T-cells were infiltrated at the sites of tumor rejection in the mice treated with combination therapy.

Immunomodulatory mAbs can provoke immune rejection of the tumor without identification of tumor antigens that may be unique to an individual tumor. This approach is advantageous because it has the potential for developing immunological memory to the tumor, preventing its recurrence (25). Based on this theory, we also attempted to establish the circumstances developing immunological memory to the tumor after immunotherapy. In fact, the mice treated with the combination therapy that survived more than 100 days after tumor inoculation rapidly rejected tumors when rechallenged by the same tumor. To demonstrate the specificity of the protective immunity, 4T1 cells were injected into both naïve and immunized mice. There was no significant difference in the growth of implanted 4T1 cells between naïve mice and immunized mice. These results suggest that long-lasting tumor antigen-specific memory had been established.

PD-1 is an inhibitory receptor expressed by T-cells with long-term exposure to antigens (26). PD-1 is preferentially expressed on Tregs and directly promotes Treg-mediated suppression of immune responses (27). PD-1 negatively regulates the effector phase of T-cell response after ligation of PD-L1 expressed within the tumor. Thus, this immune checkpoint receptor plays multiple roles in distinct cell types to quantitatively regulate immunity (28). In this study, although anti-PD-1 therapy alone did not improve antitumor efficacy, anti-PD-1 reduced the number of Tregs and MDSCs in the LNs and the spleen (not significantly). Our results also demonstrate the interesting fact that anti-4-1BB induced Tregs and MDSCs in the LNs and the spleen, suggesting that overstimulation of the immune system resulted in the induction of immune inhibitors. These results support our strategy that the blockade of immune-inhibitory factor, such as PD-1, under stimulation by 4-1BB induces strong immunity in the cancer microenvironment. In fact, the combination therapy reduced the number of Tregs and MDSCs in the LNs as compared with use of anti-4-1BB alone. These data support the theory that combination therapy with anti-4-1BB and anti-PD-1 shifted an immunosuppressive tumor environment to an immunostimulatory state, which favorably contributes to a durable antitumor effect.

In conclusion, this combination therapy systemically and effectively enriched the number of activated $CD4^+$ and $CD8^+$ T-cells. In addition, this therapy strongly recruited $CD3^+$ T-cells around the tumor. As a result, in mice treated with the combination therapy, inoculated tumors were eradicated, indicating that the therapeutic strategy of targeting cosignal has promising clinical applications.

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References

1 Harris NL and Ronchese F: The role of B7 co-stimulation in T-cell immunity. Immunol Cell Biol *77*: 304-311, 1999.

2 Topalian SL, Drake CG and Pardoll DM: Targeting the PD-1/B7-H1 (PD-L1) pathway to activate anti-tumor immunity. Curr Opin Immunol *24*: 207-212, 2012.

3 Pentcheva-Hoang T, Corse E and Allison JP: Negative regulators of T-cell activation: potential targets for therapeutic intervention in cancer, autoimmune disease, and persistent infections. Immunol Rev *229*: 67-87, 2009.

4 Fife BT, Guleria I, Gubbels Bupp M, Eagar TN, Tang Q, Bour-Jordan H, Yagita H, Azuma M, Sayegh MH, and Bluestone JA: Insulin-induced remission in new-onset NOD mice is maintained by the PD-1-PD-L1 pathway. J Exp Med *203*: 2737-2747, 2006.

5 Tivol EA, Borriello F, Schweitzer AN, Lynch WP, Bluestone JA, and Sharpe AH: Loss of CTLA-4 leads to massive lymphoproliferation and fatal multiorgan tissue destruction, revealing a critical negative regulatory role of CTLA-4. Immunity *3*: 541-547, 1995.

6 Carreno BM and Collins M: The B7 family of ligands and its receptors: new pathways for costimulation and inhibition of immune responses. Annu Rev Immunol *20*: 29-53, 2002.

7 Blank C and Mackensen A: Contribution of the PD-L1/PD-1 pathway to T-cell exhaustion: an update on implications for chronic infections and tumor evasion. Cancer Immunol Immunother *56*: 739-745, 2007.

8 Topalian SL, Hodi FS, Brahmer JR, Gettinger SN, Smith DC, McDermott DF, Powderly JD, Carvajal RD, Sosman JA, Atkins MB, Leming PD, Spigel DR, Antonia SJ, Horn L, Drake CG, Pardoll DM, Chen L, Sharfman WH, Anders RA, Taube JM, McMiller TL, Xu H, Korman AJ, Jure-Kunkel M, Agrawal S, McDonald D, Kollia GD, Gupta A, Wigginton JM and Sznol M: Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. N Engl J Med *366*: 2443-2454, 2012.

9 Wu YL, Liang J, Zhang W, Tanaka Y and Sugiyama H: Immunotherapies: the blockade of inhibitory signals. Int J Biol Sci *8*: 1420-1430, 2012.

10 Shuford WW, Klussman K, Tritchler DD, Loo DT, Chalupny J, Siadak AW, Brown TJ, Emswiler J, Raecho H, Larsen CP, Pearson TC, Ledbetter JA, Aruffo A and Mittler RS: 4-1BB costimulatory signals preferentially induce $CD8^+$ T-cell proliferation and lead to the amplification *in vivo* of cytotoxic T-cell responses. J Exp Med *186*: 47-55, 1997.

11 Wilcox RA, Tamada K, Strome SE and Chen L: Signaling through NK cell-associated CD137 promotes both helper function for $CD8^+$ cytolytic T-cells and responsiveness to IL-2 but not cytolytic activity. J Immunol *169*: 4230-4236, 2002.

12 Cannons JL, Lau P, Ghumman B, DeBenedette MA, Yagita H, Okumura K and Watts TH: 4-1BB ligand induces cell division, sustains survival, and enhances effector function of CD4 and CD8 T-cells with similar efficacy. J Immunol *167*: 1313-1324, 2001.

13 Melero I, Shuford WW, Newby SA, Aruffo A, Ledbetter JA, Hellstrom KE, Mittler RS and Chen L: Monoclonal antibodies against the 4-1BB T-cell activation molecule eradicate established tumors. Nat Med *3*: 682-685, 1997.

14 Taraban VY, Rowley TF, O'Brien L, Chan HT, Haswell LE, Green MH, Tutt AL, Glennie MJ and Al-Shamkhani A: Expression and co-stimulatory effects of the TNF receptor superfamily members CD134 (OX40) and CD137 (4-1BB), and their role in the generation of antitumor immune responses. Eur J Immunol *32*: 3617-3627, 2002.

15 Ascierto PA, Simeone E, Sznol M, Fu YX and Melero I: Clinical experiences with anti-CD137 and anti-PD-1 therapeutic antibodies. Semin Oncol *37*: 508-516, 2010.

16 Corbett TH, Griswold DP Jr., Roberts BJ, Peckham JC and Schabel FM Jr.: Tumor induction relationships in development of transplantable cancers of the colon in mice for chemotherapy assays, with a note on carcinogen structure. Cancer Res *35*: 2434-2439, 1975.

17 Aslakson CJ and Miller FR: Selective events in the metastatic process defined by analysis of the sequential dissemination of subpopulations of a mouse mammary tumor. Cancer Res *52*: 1399-1405, 1992.

18 Yoshimura K, Hazama S, Iizuka N, Yoshino S, Yamamoto K, Muraguchi M, Ohmoto Y, Noma T and Oka M: Successful immunogene therapy using colon cancer cells (colon 26) transfected with plasmid vector containing mature interleukin-18 cDNA and the Igkappa leader sequence. Cancer Gene Ther *8*: 9-16, 2001.

19 Yamazaki T, Akiba H, Koyanagi A, Azuma M, Yagita H and Okumura K: Blockade of B7-H1 on macrophages suppresses CD4+ T-cell proliferation by augmenting IFN-gamma-induced nitric oxide production. J Immunol *175*: 1586-1592, 2005.

20 Yoshimura K, Laird LS, Chia CY, Meckel KF, Slansky JE, Thompson JM, Jain A, Pardoll DM and Schulick RD: Live attenuated *Listeria monocytogenes* effectively treats hepatic colorectal cancer metastases and is strongly enhanced by depletion of regulatory T-cells. Cancer Res *67*: 10058-10066, 2007.

21 Huang AY, Gulden PH, Woods AS, Thomas MC, Tong CD, Wang W, Engelhard VH, Pasternack G, Cotter R, Hunt D, Pardoll DM and Jaffee EM: The immunodominant major histocompatibility complex class I-restricted antigen of a murine colon tumor derives from an endogenous retroviral gene product. Proc Natl Acad Sci USA *93*: 9730-9735, 1996.

22 Maeda N, Yoshimura K, Yamamoto S, Kuramasu A, Inoue M, Suzuki N, Watanabe Y, Maeda Y, Kamei R, Tsunedomi R, Shindo Y, Inui M, Tamada K, Yoshino S, Hazama S and Oka M: Expression of B7-H3, a potential factor of tumor immune evasion in combination with the number of regulatory T-cells, affects against recurrence-free survival in breast cancer patients. Ann Surg Oncol 2014.

23 Cho Y, Miyamoto M, Kato K, Fukunaga A, Shichinohe T, Kawarada Y, Hida Y, Oshikiri T, Kurokawa T, Suzuoki M, Nakakubo Y, Hiraoka K, Murakami S, Shinohara T, Itoh T, Okushiba S, Kondo S and Katoh H: $CD4^+$ and $CD8^+$ T-cells cooperate to improve prognosis of patients with esophageal squamous cell carcinoma. Cancer Res *63*: 1555-1559, 2003.

24 Berghuis D, Santos SJ, Baelde HJ, Taminiau AH, Egeler RM, Schilham MW, Hogendoorn PC and Lankester AC: Pro-inflammatory chemokine-chemokine receptor interactions within the Ewing sarcoma microenvironment determine CD8(+) T-lymphocyte infiltration and affect tumour progression. J Pathol *223*: 347-357, 2011.

25 Lee CS, Cragg M, Glennie M and Johnson P: Novel antibodies targeting immune regulatory checkpoints for cancer therapy. Br J Clin Pharmacol *76*: 233-247, 2013.

26 Hamid O, Robert C, Daud A, Hodi FS, Hwu WJ, Kefford R, Wolchok JD, Hersey P, Joseph RW, Weber JS, Dronca R, Gangadhar TC, Patnaik A, Zarour H, Joshua AM, Gergich K, Elassaiss-Schaap J, Algazi A, Mateus C, Boasberg P, Tumeh PC, Chmielowski B, Ebbinghaus SW, Li XN, Kang SP and Ribas A: Safety and tumor responses with lambrolizumab (anti-PD-1) in melanoma. N Engl J Med *369*: 134-144, 2013.

27 Francisco LM, Salinas VH, Brown KE, Vanguri VK, Freeman GJ, Kuchroo VK and Sharpe AH: PD-L1 regulates the development, maintenance, and function of induced regulatory T-cells. J Exp Med *206*: 3015-3029, 2009.

28 Pardoll D and Drake C: Immunotherapy earns its spot in the ranks of cancer therapy. J Exp Med *209*: 201-209, 2012.

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