CD8-dependent Tumor Growth Inhibition by Tumor Cells Genetically Modified with 4-1BBL

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We previously identified that tumor cells genetically modified with a 4-1BBL co-stimulatory molecule had anticancer effects in a CT26 mouse colorectal tumor model. To identify the distinction between immune cells in a mouse tumor model treated with tumor cells genetically modified with 4-1BBL or β -gal, we examined the immune cells in CT26-WT, CT26- β gal, and CT26-4-1BBL tumor bearing mice 21 days after tumor cell administration. The CD8⁺ T cells population in mice treated with tumor cells genetically modified with 4-1BBL was significantly increased on day 21 compared to that of tumor cells genetically modified with β -gal in the spleen and tumor tissue. The CD4⁺ T cell population was not different between the two mice groups. The Foxp3⁺CD25^{high} CD4 T cell population decreased on day 21 in tumor tissues, but the decrease was not significant. We also found that CD8 T cells had pivotal roles in inhibiting tumor growth by treating mice with anti-CD4 and CD8 antibodies. These results suggest that tumor cells genetically modified with 4-1BBL could inhibit tumor growth by affecting on CD8 T lymphocytes.

Key Words: CD8 T cells, Regulatory T cells, Tumor growth inhibition, 4-1BBL

4-1BBL activates T cells by binding 4-1BB on activated T cells as co-stimulatory molecule and derivess the expansion of T cell and the preservation of long-term memory function (Habib-Agahi et al., 2009; Wang et al., 2009; Vinay and Kwon, 2012; Mock et al., 2020; Trub et al., 2020). 4-1BBL is also related to potent tumor rejection effects in various mouse tumor model (Guinn et al., 2001; Stephan et al., 2007; Ragonnaud et al., 2016). Cytotoxic T cells were reported to be the major immune cell against tumor growth through 4-1BBL in tumor-bearing hosts (Yi et al., 2007; Kim et al., 2009; Lee and Kim, 2012; Sharma et al., 2014; Wang et al., 2015). Some research studies reported that 4-1BBL prevented cancer development via CD4⁺ T and natural killer cells (Madireddi et al., 2012; Barsoumian et

al., 2019).

We previously constructed a lentiviral vector containing 4-1BBL and established tumor cells genetically modified with 4-1BBL and found that 4-1BBL expression on CT26 colorectal cancer cells was associated with tumor growth inhibition (Kim, 2019). In this study, we investigated that tumor growth inhibition by tumor cells genetically modified with 4-1BBL was related to CD8 T cell expansion and reductions in regulatory T cells. To identify this hypothesis, we analyzed the effect of tumor cells genetically modified with 4-1BBL on immune cells related to tumor growth inhibition 21 days after CT26 colorectal cancer cell inoculation and identified the effect of T lymphocytes on tumor growth inhibition using anti-CD4 and anti-CD8 antibody treatments.

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Balb/c female mice (6 to 8-week-old) were obtained from OrientBio (Korea). The mice were nurtured under pathogen-free conditions and maintained by approved institutional animal care protocols. CT26 cells were obtained from ATCC (the American Type Culture Collection, Manassas, VA, USA) and were cultivated in Dulbecco's modified Eagle's medium (DMEM) with 10 mM L-glutamine, 0.1% gentamicin, 100 U/mL penicillin/streptomycin and 10% fetal bovine serum (FBS). CT26 mouse colorectal cancer cells with wild-type (CT26-WT), 4-1BBL (CT26-4-1BBL), and β -gal genes (CT26- β gal) (2×10^5) in 100 μ L phosphate-buffered saline were seeded subcutaneously into the right flank of *Balb/c* mice. For CD4 and CD8 T cell depletion, CT26-4-1BBL tumor bearing mice were intraperitoneal injected with anti-CD4 and anti-CD8 antibodies (100 μ g/mice) (eBioscience, San Diego, CA) six times (two times before tumor cell inoculation and four times after tumor cell inoculation). Tumor size was gauged in two dimensions using calipers and tumor area was calculated as follows: tumor area (mm^2) = length $\times$ width. The mice were sacrificed 21 days after tumor cell injection and the spleen and tumor tissues were aseptically collected. Each sample was homogenized and ran through a mesh to produce single-cell

suspensions. After the cells were washed two times with PBS, RBC lysis buffer (140 mM ammonium chloride and 20 mM Tris-HCl) was treated by removing erythrocytes at 37°C for 5 min and the cells were stained with the following mAbs: phycoerythrin (PE)-conjugated CD8 and CD25; allophycocyanin (APC)-conjugated anti-CD4; and fluorescein isothiocyanate (FITC)-conjugated Foxp3. All antibodies were acquired from eBiosciences (San Diego, CA, USA). Foxp3 Staining Buffer kit (eBioscience) was used for labeling splenocytes. Samples were examined using CELLquest™ software and a FACScalibur™ flow cytometer, and FACS data were analyzed by the Flowjo program and presented using Graphpad Prism6. The data are presented as the means $\pm$ SEM. Significance among the percentage of each cell were expressed using the two-tailed student's *t*-test. $P < 0.05$ were determined significant. To identify changes in systemic and tumor tissue immune cells in mice with tumor cells, we examined the lymphocytic populations (CD8, CD4, and Treg) in the spleen and tumor tissue. We collected splenocytes and tumor-infiltrating immune cells from tumor-bearing mice 21 days after tumor cell injection and implemented FACS analysis. In the spleen, the percentage of CD4+ and regulatory T cell populations in the CT26-WT,

Fig. 1. Immune cell phenotype after gene modified CT26 cell inoculation on spleen. CT26-WT (wildtype), CT26- β gal, CT26-4-1BBL were subcutaneously administrated on the flank of mice. The splenocytes were collected on 21 days after tumor cell seeding, and the percentage of each immune cell (CD8+, CD4+, and Treg population) was analyzed by flow cytometry. The percentage of (A) CD4+, (B) CD8+, and (C) T reg (CD4+Foxp3+CD25^{high}) cell population. The results are depicted by GraphPad Prism. CD26- β gal versus CT26-4-1BBL; *: $P < 0.05$.

Fig. 2. Immune cell phenotype after gene modified CT26 cell inoculation on tumor tissue. Mice were treated by described Fig. 1. The tumor tissues were collected on 21 days after tumor cell seeding and the percentage of each immune cells was analyzed through flow cytometry. The percentage of (A) CD4+, (B) CD8+, and (C) T reg cell population. The results are depicted by GraphPad Prism. CD26-βgal versus CT26-4-1BBL; **: $P < 0.01$.

Fig. 3. Tumor growth pattern of CT26-4-1BBL bearing mice after antibody treatment. Before subcutaneous implantation of CT26-4-1BBL tumor cells (2×10^5 cell/100 μ L PBS) on the flank of *Balb/c* mice, antibody was intraperitoneally treated two times every other day. Two days after tumor cell seeding, antibody was treated four times every week. The splenocytes were collected on 27 days after tumor cell seeding and the percentage of each immune cells was analyzed through flow cytometry. (A) The percentage of CD4+ and CD8+ T cell population. Tumor size was measured every third day. (B) The average tumor volume of each group. The results are defined as mean $\pm$ SEM. ***: $P < 0.0001$.

CT26-βgal, and CT26-4-1BBL tumor-bearing mice were not significantly different (Figs. 1 A and 1C), but the percentage of CD8+ T cells in CT26-4-1BBL-bearing mice were significantly increased on day 21 compared to the CT26-βgal-bearing mice (Fig. 1B). In tumor tissues, the percentage of CD4+ and Treg cells were not significantly

different among the CT26-WT, CT26-βgal, and CT26-4-1BBL tumor-bearing mice (Figs. 2 A and 2C), but CT26-4-1BBL mice showed a tendency toward decreased regulatory T cells compared to CT26-βgal mice (Fig. 2C). The percentage of CD8+ T cells in CT26-4-1BBL-bearing mice showed significant increases compare to the CT26-WT and

CD26-βgal-bearing mice (Fig. 2B). These data indicated that CT26-4-BBL induced CD8⁺ T cell population expansion in the spleen and tumor tissue. To identify the effect of CD8⁺ and CD4⁺ T cell population on tumor growth inhibition in CT26-4-1BBL-bearing mice, we treated them with anti-CD4 and anti-CD8 antibodies and identified the tumor growth patterns. On day 28, we identified immune cell depletion by antibody treatment (Fig. 3A). In the case of CD8 T cell-depleted mice, there was no tumor growth inhibition. The results indicated that CD8 T cells had a pivotal role in tumor growth inhibition in CT26-4-1BBL-bearing mice. In the case of CD4 T cell-depleted mice, there was significant tumor growth inhibition. Also, CD4 T cell-depleted mice showed better results compared to mice not treated with antibodies (Fig. 3B). These data represented that tumor growth inhibition in CT26-4-1BBL-tumor bearing mice was mainly dependent upon the existence of CD8⁺ T cells, especially in tumor tissues, and showed that CD4⁺ T cells could induce tumor growth in the CT26-4-1BBL system. In this study, we analyzed CD8, CD4, and Treg cells in mice with CT26-4-1BBL-bearing tumors. But there are many kinds of immune cells related to tumor growth inhibition including MDSC, NK cell, etc. In the future, we will investigate CD8 T cell subpopulations and other immune cells related to tumor growth inhibition.

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CONFLICT OF INTEREST

No potential conflict of interest relevant to this article was reported.

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