The role of oncolytic virus as an immunomodulator in the microenvironment of colon cancer mouse model

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The role of oncolytic virus as an immunomodulator in the microenvironment of colon cancer mouse model

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ABSTRACT

The role of oncolytic virus as an immunomodulator in the microenvironment of colon cancer mouse model

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Immunotherapy for malignancy, which stimulates the patients' own immune system to treat cancers with low toxicity, has been effective for MSI-high colorectal cancer. MSI-high diseases are less than 10% of overall colorectal cancer.

Recent studies concentrate on synergizing the effect of immune checkpoint inhibitor by combination. Among them is an oncolytic virus. Tumor volumes after local injection of Oncolytic virus (OV) with anti-PD1 antibody decreased compared with OV alone or anti-PD1

antibody alone. Moreover, CD4+ T lymphocyte infiltration markedly increased as well as CD8+T lymphocyte infiltration when combination of OV and anti-PD1 antibody applied.

OV can immunomodulate as well as replicate and kill the cancer cell. The combination of OV and anti-PD1 antibody can benefit for patients with tumors that are resistant to other therapies.

Key words: colorectal cancer, immunotherapy, immune checkpoint inhibitor, oncolytic virus

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I. INTRODUCTION

Colorectal cancer is one of the most common malignancies worldwide, and the radical resection according to the surgical plane is the first treatment option.[1-3] However, the chemotherapy following surgical resection for stage II disease with high risk factors and stage III disease was proven to yield superior oncologic outcomes in terms of recurrence and survival compared with surgery alone.[4-7] In addition, approximately 20% of the patients with colorectal cancer were diagnosed as having stage IV disease, which also needed chemotherapy to increase survival rates.[8]

Chemotherapeutic agents for colorectal cancer have been developed continuously. 5-fluorouracil, the cytotoxic agent, has been widely used with leucovorin for colorectal cancer.[9,10] The addition of the platinum analogue to the 5-fluorouracil showed better oncologic outcomes compared with 5-fluorouracil with leucovorin.[11,12] Moreover, use of targeted agents such as monoclonal antibody to endothelial growth factor receptor (EGFR) or vascular endothelial growth factor (VEGF) for metastatic or recurrent colorectal cancer has improved survival rates.[13-16] However, these chemotherapeutic agents also showed various toxicities that sometimes threatened the patients and subsequently stop the chemotherapy. Additionally, patients with refractory colorectal cancer, which failed to response to any chemotherapy, had no choice but supportive care.

Under these circumstances, the advancement of cancer immunotherapy showed a possibility of an alternative for current chemotherapy. Immunotherapy for malignancy stimulates the patients' own immune system to treat cancers with low toxicity.[17,18] Immune checkpoint inhibitors including anti programmed cell death 1 (PD1) antibody and cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) inhibitor have been approved for various malignancies by the Food and Drug Administration (FDA), based on improved survival for stage IV patients from several trials.[19-21] However, unlike melanoma, lung cancer, and kidney cancer, colorectal cancer showed low response rates to the immune checkpoint inhibitors. Only the microsatellite

instability (MSI)-high tumor (i.e. an inflamed microenvironment that can response to the immune checkpoint inhibitors) responded to the PD1 inhibitor with 80%, and a proportion of MSI-high is less than 10% of overall colorectal cancers.[22]

Therefore, conversion of the tumor microenvironment from the non-inflamed to the inflamed one is required to make or maximize the effect of the immune checkpoint inhibitors for stable MSI (MSS) or MSI-low colorectal cancers. One of the potential immunomodulator to conversion of the tumor microenvironment is oncolytic virus (OV). The anti-cancer effect of oncolytic virus has been proven for malignancies.[23-25] Although other vaccinia virus has a possibility of immunomodulation activating adoptive immunity as well as selective cancer cell killing,[25,26] there is few report of pexastimogene devacirepvec (Pexa-Vec, JX594) as an immunomodulator. The aim of this study is to assess the anti-cancer effect of combination of the anti-PD1 antibody and JX594 in the colon cancer mouse models.

II. MATERIALS AND METHODS

1. Oncolytic viruses

JX594 is a Wyeth strain vaccinia virus genetically engineered by Sillajen (Busan, Korea) to selectively infect and replicate in cancer cells. JX594 was provided by Sillajen and used in this study (Figure 1). Granulocyte-macrophage

colony stimulating factor (GM-CSF) and the lacZ gene were inserted into the viral thymidine kinase (TK) gene. TK gene makes virus replicate in the normal cells, whereas it is disrupted and inactivated in the JX594. Disrupted TK gene can give the virus selectivity for cancer cell and tumor vasculature. GM-CSF activates and stimulates anti-cancer immune response, and lacZ is a kind of markers for monitoring activity of the virus.

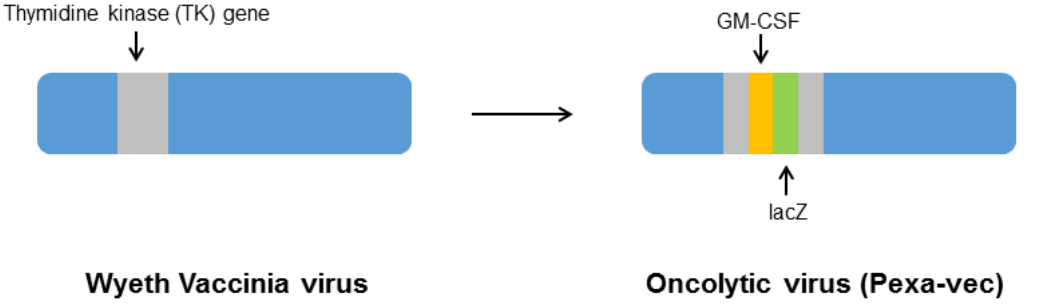

Figure 1. The structure of oncolytic vaccinia virus

2. Cell lines and animals

CT26 cancer cell lines were purchased from American Type Culture Collection (Manassas, VA, USA). They were maintained in RPMI-1640 or DMEM medium supplemented with 10% fetal bovine serum with 1% penicillin and streptomycin in 37℃, 5% CO_2 incubators.

Male BALB/c mice between 6 to 8 weeks of age were obtained from Orient

Bio Inc. (Gyeonggi-do, Korea) and housed in pathogen-free animal facility of CHA University, Bundang, Korea. All animal experiments were performed according to the protocols approved by the Institutional Animal Care and Use Committee (IACUC) of CHA University.

3. Tumor models and treatment regimens

2 x 10^5 tumor cells were injected to the subcutaneous layers at the flank of mice: CT26 cells for wild type BALB/c mice. Intratumoral injection of either phosphate buffered saline (PBS) or 1 x 10^7 plaque-forming units (PFU) of OV was performed every 3 days for 4 times (day 0, 3, 6, and 9) after the size of tumor grew up to 50 mm^3. For immune checkpoint inhibition, 10 mg/kg of anti-PD1 antibody (J43) were injected intraperitoneally with or without OV according to the dosing schedule. Therefore there were four groups of experimental animals according to the intratumoral injection regimens: PBS alone (n=8), OV alone (n=6), J43 alone (n=6), and OV with J43 (n=6), respectively. Tumor length and width were measured every 3 days by digital caliper, and tumor volumes were calculated by the modified ellipsoid formula (1/2 x length x $width^2$). Mice were euthanized when the tumor size reached 15mm in diameter or ulceration or moribund occurred.

4. NanoString gene expression analysis

On day 12 after treatment, the mice were killed, and the tumors were excised,

placed in TRIzol reagent (Invitrogen, CA, USA) and homogenized. The samples were purified with ethanol and checked quality with Fragment Analyzer (Advanced Analytical Technologies, IA, USA). The digital multiplexed NanoString nCounter PanCancer Immune Profiling mouse panel (NanoString Technologies, WA, USA) was performed with 100 ng total RNA isolated from tumor tissues. Hybridizations were carried out by combining 5 ul of each RNA sample with 8 ul of nCounter Reporter probes in hybridization buffer and 2 ul of nCounter Capture probes (for a total reaction volume of 15 ul) overnight at 65 ℃ for 16-30 hrs. Excess probes were removed using two-step magnetic bead based purification on the nCounter Prep Station (NanoString Technologies, WA, USA). Abundances of specific target molecules were quantified on the nCounter Digital Analyzer by counting the individual fluorescent barcodes and assessing the target molecules. For each assay, a high-density scan encompassing 280 fields of view was performed. The data was collected using the nCounter Digital Analyzer after taking images of the immobilized fluorescent reporters in the sample cartridge with a CCD camera. Data analysis was performed using the nSolver software analysis, freely available from NanoString Technologies. The mRNA profiling data was normalized using housekeeping genes. R software was used for the analysis.

5. Flow cytometry analysis of tumor-infiltrating immune cells

Tumors from the four groups were incubated with Collagenase D (20mg/ml)

and DNase I (2mg/ml) for 1 hour at 37℃ in shaking incubator at the day 12. Cell suspensions were generated by repeated pipetting, filtered through a 70 um cell strainer, and then lysed to remove red blood cells. Those suspended cells were filtered through a nylon mesh after washed with PBS. Single cells from tumor tissues were blocked with anti-CD16/32 antibody (clone 2.4G2, BD Pharmingen, NJ, USA) and stained with fixable viability dye eFlouor450 (eBioscience, Seoul, Korea) that used to distinguished the live cells. For analysis of surface markers, cells were stained in PBS containing 1% FBS, with CD4 (RM4-5, BD Pharmingen, NJ, USA) and CD8 (54-6.7, BD Pharmingen, NJ, USA) on ice for 30 minutes. Flow cytometry data were acquired on Beckman Coulter CytoFLEX and analyzed using FlowJo software (Tree Star Inc., Ashland, OR).

6. RNA isolation and quantitative real-time PCR

Total RNA was extracted from whole-cell lysates with a High Pure RNA Isolation Kit (Roche, Basel, Switzerland) and was reverse-transcribed with a Transcriptor First Strand cDNA Synthesis Kit (Roche, Basel, Switzerland). Quantitative real-time polymerase chain reaction (PCR) was performed in triplicate with FastStart Essential DNA Green Master (Roche, Basel, Switzerland) and LightCycler® 96 Instrument (Roche, Basel, Switzerland) using gene-specific primers. Relative expression was normalized to the levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

7. Histologic analysis

Frozen sections (50 um thick) were fixed with 1% paraformaldehyde and blocked with 5% goat serum (FBS) for 1 hour at room temperature. OV and CD8 antibody were incubated for overnight at 4℃. FITC and Cy3 conjugate antibodies were used as secondary antibody. DAPI was used as the nuclear counterstain. Selected fields at x20 magnification were quantitated. Images were taken on a Zeiss LSM880 microscope and Zen software.

8. Statistical analysis

All statistical analysis was performed with Prism 7.0 (GraphPad software, CA, USA). Less than 0.05 of P-value was considered to be statistically significant. Test, group sizes, and P-values were given in the corresponding figure legends.

III. RESULTS

1. Establishment of syngeneic tumor models for immunotherapy

High expression of the markers related to immune system including CD3, 4, 8, 11, and 45, was noted more in the CT26 cells (colon cancer) compared with other kinds of malignancies (Figure 2). Stimulatory markers were highly expressed in the CT26 cells, whereas suppressive markers were not expressed. Markers for angiogenesis were expressed in the CT26, RENCA, 4T1, LLC, and

B16. Therefore, colon cancer model from CT26 cells were appropriate for the study of immunotherapy.

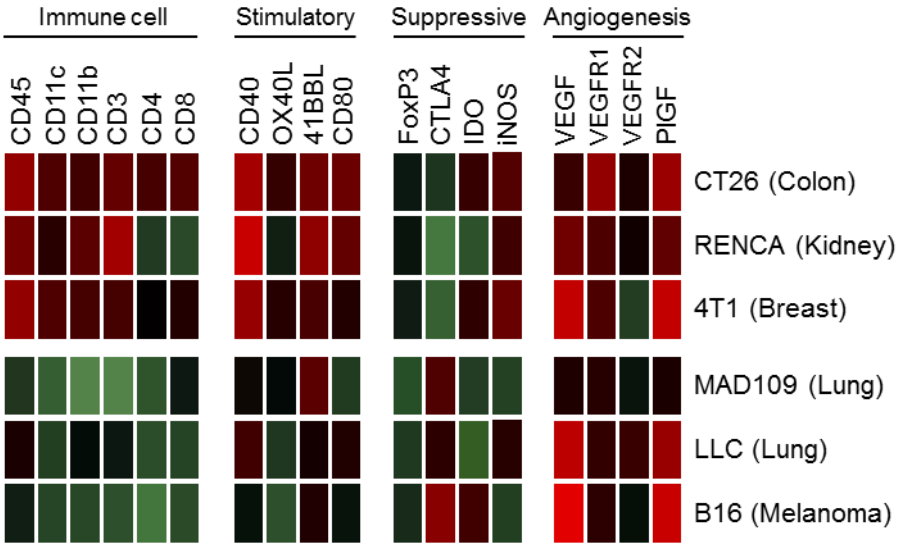

Figure 2. Syngeneic tumor models with distinct immune-related gene expression

2. Anti-PD1 therapy in animal model of colon cancer

Mean tumor volumes after intraperitoneal injection of the anti-PD1 treatment group were less than the control group (Figure 3). The mean volume of tumor in the control group was 69.9 mm^3, 161 mm^3, 374.8 mm^3, 736 mm^3, and 1298.9 mm^3 at the day 7, 10, 13, 16, and 20 after injection, whereas the volume of tumor in the anti-PD1 treatment group was 40.1 mm^3, 90.9 mm^3, 197.2 mm^3,

376.6 mm^3, and 670.8mm^3, respectively. However, each volume of tumor showed variations. There were various responses to the anti-PD1 treatment, while the control group increased consistently. The study to assess what characteristics of tumor response to anti-PD1 treatment needed.

Fluorescence-activated cell sorting (FACS) revealed that the efficacy of PD1 blockade was inversely proportional to intratumoral CD8+ T lymphocyte infiltration, which is responsible for anti-cancer immunity (Figure 4). It suggested that the tumors which have high infiltration of CD8+ T cell response to anti-PD1 treatment better than the tumors have not.

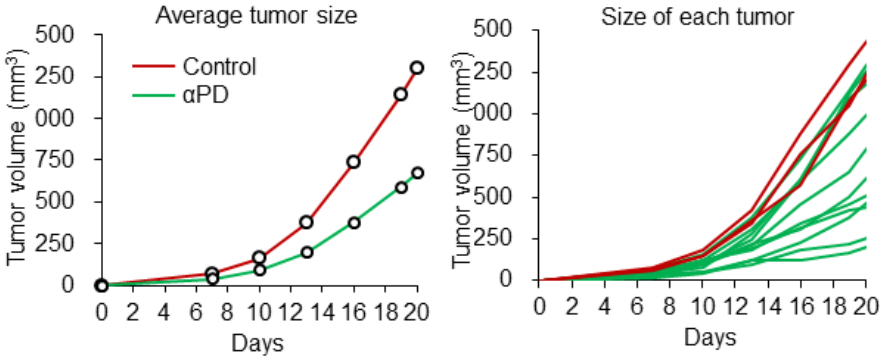

Figure 3. The efficacy of anti-PD1 treatment in CT26 colon cancer

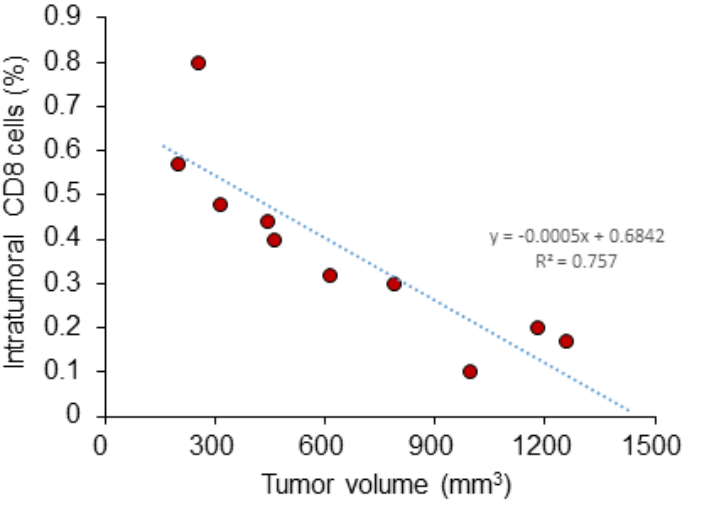

Figure 4. The relationship between the efficacy of PD1 blockade and intratumoral CD8+ T cell infiltration

3. Tumor-specific replication and oncolysis of OV in colon cancer

5 days after intratumoral injection of 1 x 10^7 PFU of OV, the virus has actively replicated, while tumor vasculature was destroyed (Figure 5), although vasculature recovered after 10 days (data not shown). In addition, Caspase3, which is a marker for cell apoptosis, was highly expressed after OV treatment (Figure 6).

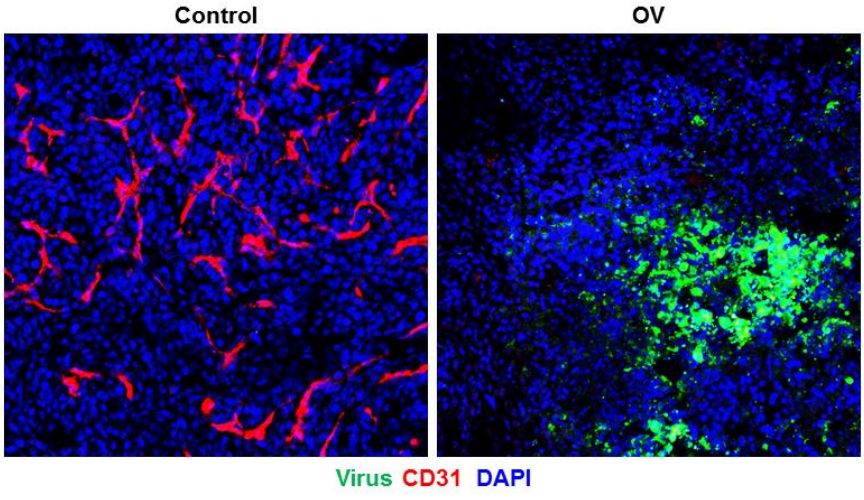

Figure 5. OV selectively replicated in tumor cells and disrupted tumor vasculature

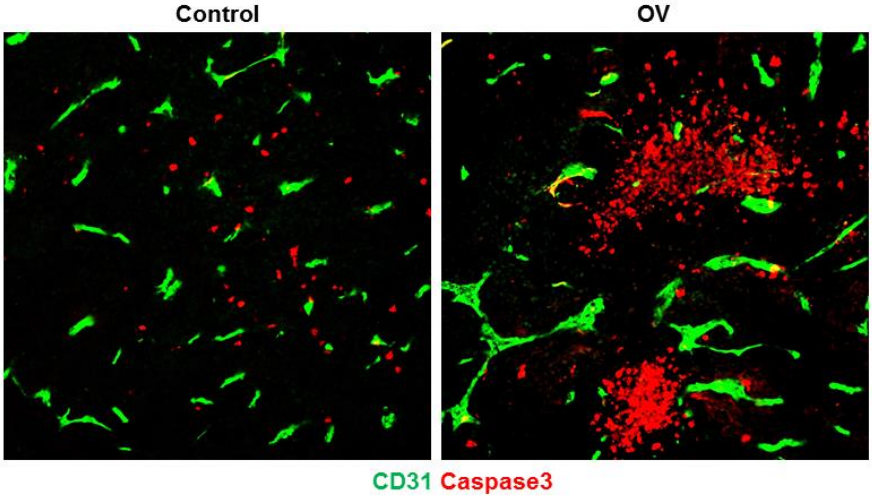

Figure 6. OV led to extensive intratumoral apoptosis in colon cancer

4. OV regulates immune-related genes and remodels tumor immune microenvironment in colon cancer

Induction of immune-related genes after OV treatment was noted on the volcano plot from NanoString and immune profiling (Figure 7). CD3d, CD3e, and CD3g, T cell; CD8, cytotoxic T cell; Gzma, Gzmb, and Gzmk, for granzyme, were significantly increased. It means that T cell infiltration increased as a total number but activity.

Chemokines and their receptors that are associated with Th1 response and T cell migration were upregulated after OV treatment (Figure 8). CCL5, which induces macrophage migration and interaction of T cell and dendritic cell, showed 4.474 fold changes. CXCL9, known as inductor of CD8+ T cell and natural killer cell migration, showed 2.899 fold changes. Moreover, both of CD8+ and CD4+ T cell infiltrations were augmented by OV treatment after 14 days (Figure 9). According to the amount of T cell infiltration, PD1 molecules showed 7.019 fold changes after OV treatment compared with the control group (Figure 10). CTLA-4-mRNA, LAG3-mRNA, and TIGIT-mRNA, which are thought to be related with the mechanism of resistance, also showed 2.656-3.741 fold changes.

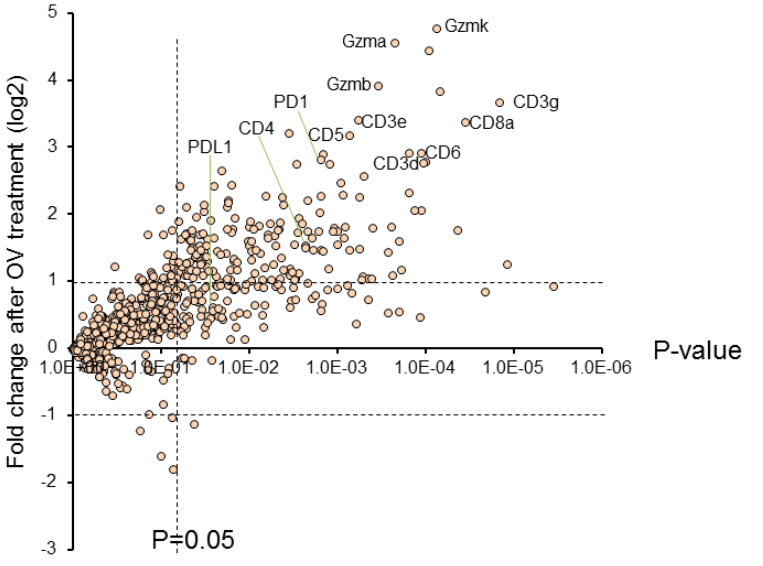

Figure 7. OV induced distinct change of immune-related genes in colon cancer

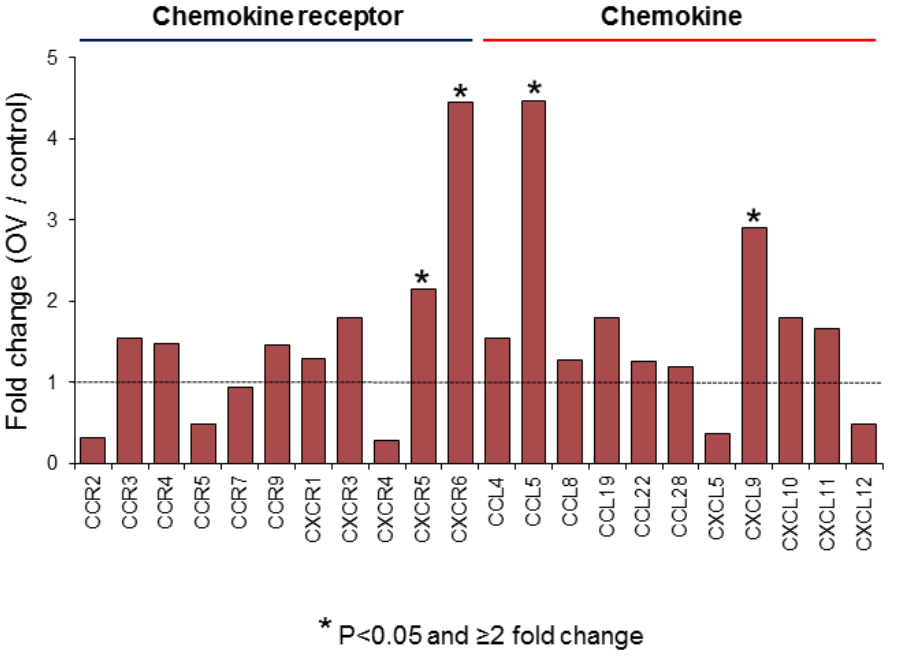

Figure 8. OV upregulated chemokine and their receptors which are associated with Th1 response and T cell migration

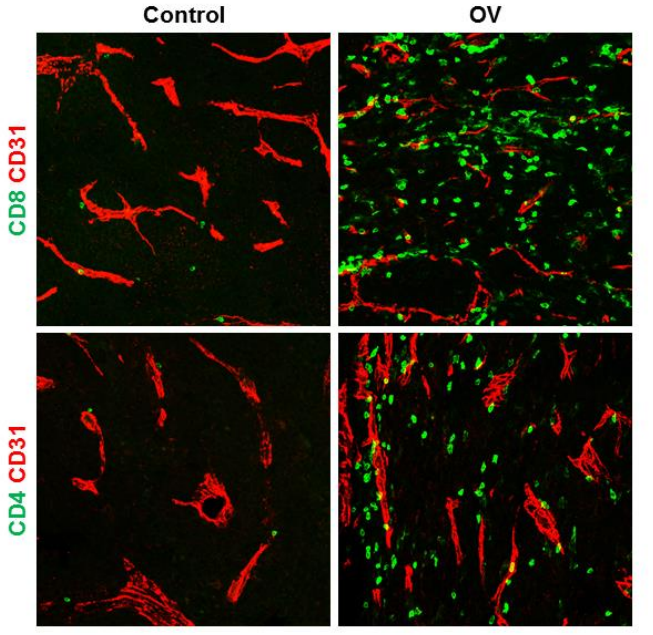

Figure 9. OV augmented the infiltration of CD8+ or CD4+T cell into tumor

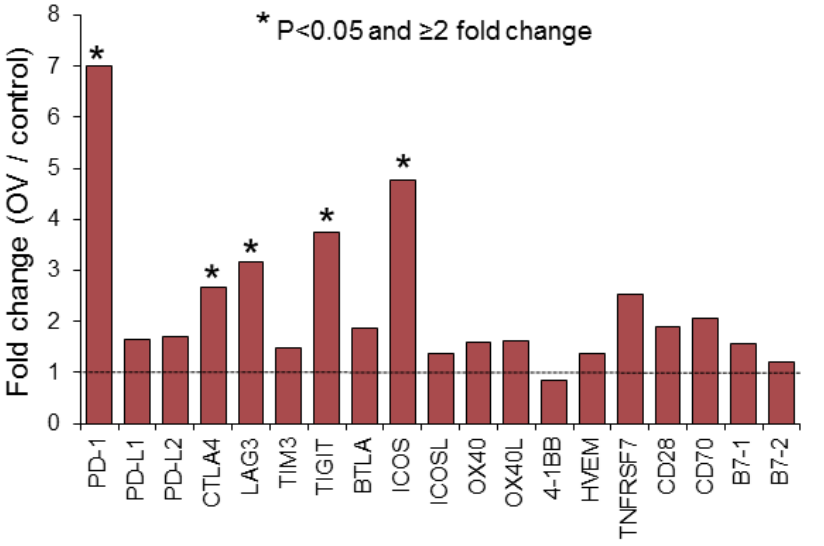

Figure 10. OV regulated immune checkpoints molecules in colon cancer

5. OV synergizes with PD1 blockade to suppress tumor growth in colon cancer

Treatment regimens of combination therapy of OV and anti-PD1 antibody are depicted in the Figure 11. Tumor volumes after intraperitoneal injection of OV, anti-PD1, and OV with anti-PD1 decreased compared with control group (Figure 12 and 13). The mean volumes of tumor at the day 3, 6, 9, and 12 after injection were as follows: 109.8 mm^3, 171.9 mm^3, 462.3 mm^3, and 647.9 mm^3 in the OV group; 140.9 mm^3, 313.5 mm^3, 515.8 mm^3, and 868.9 mm^3 in the anti-PD1 group; 125 mm^3, 171.9 mm^3, 283.3 mm^3, and 464.1 mm^3 in the combination treatment group; 137.7 mm^3, 357.9 mm^3, 736.5 mm^3, and 1022.8 mm^3 in the control group. Combination of OV and J43 showed mostly decreased tumor volume, followed by anti-PD1 alone and OV alone.

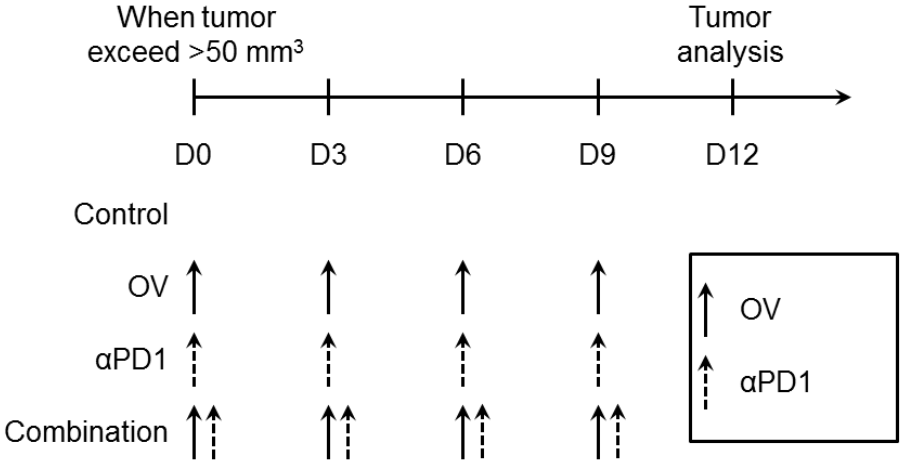

Figure 11. Scheme of combination therapy

Figure 12. Gross picture of representative tumors after combination treatment

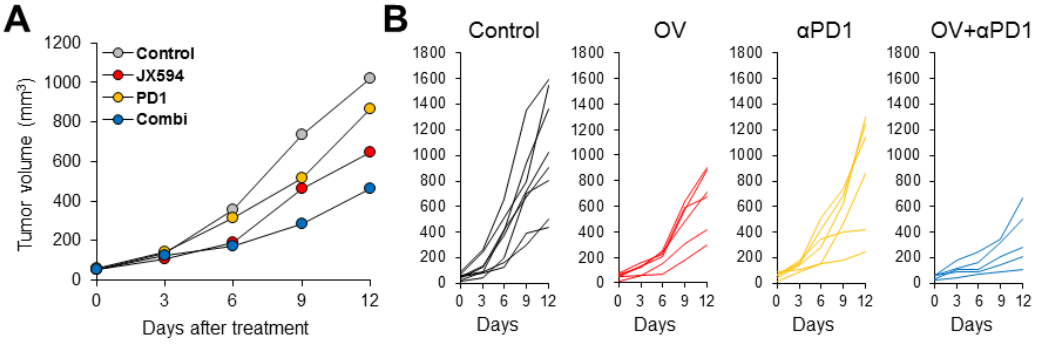

Figure 13. Colon growth was markedly suppressed with combination treatment of OV anti-PD1.

6. Combination of OV and PD1 blockade elicited potent anti-tumor immunity

Intratumoral infiltration of CD8+ cytotoxic T cells, mostly increased after combination treatment on the confocal microscope (Figure 14). They rose much more in the peripheral area of the tumor compared with in the central area. Although OV injection alone showed more infiltration of CD8+ T cell than the control group, they did not reach the result of combination treatment.

OV led changes of intratumoral CD8+ and CD4+ T cells as 5.54 fold and 3.3 fold (Figure 15). However, the changes were 12.1 fold and 7.07 fold after combination treatment. However, there was no significant change in intratumoral myeloid cells including monocytes, granulocytes, and tumor-associated macrophage (Figure 16).

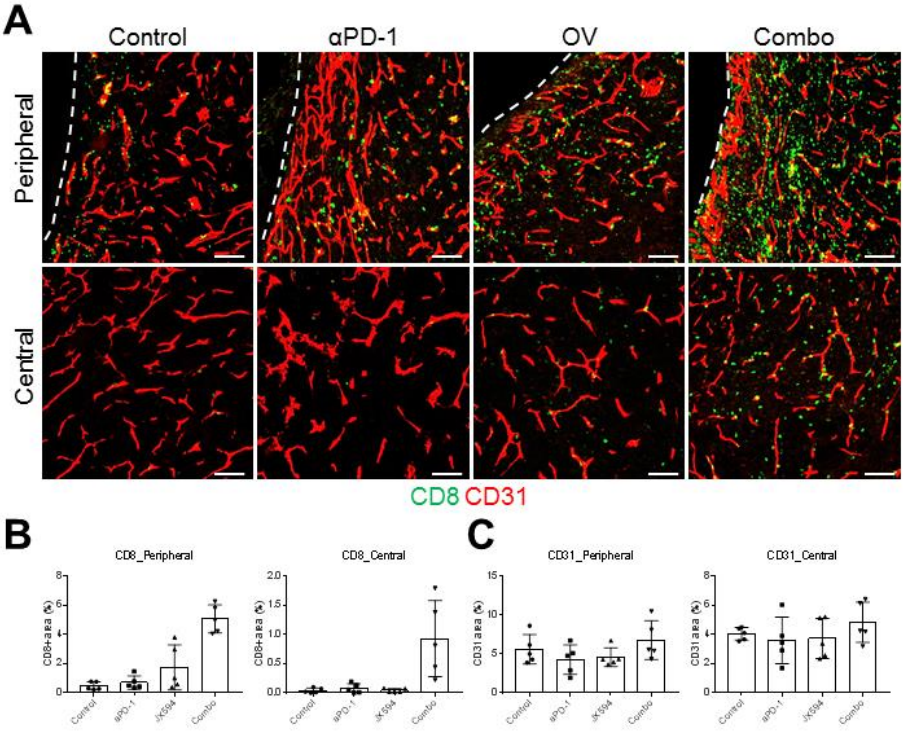

Figure 14. Changes of tumor microenvironment after combination treatment

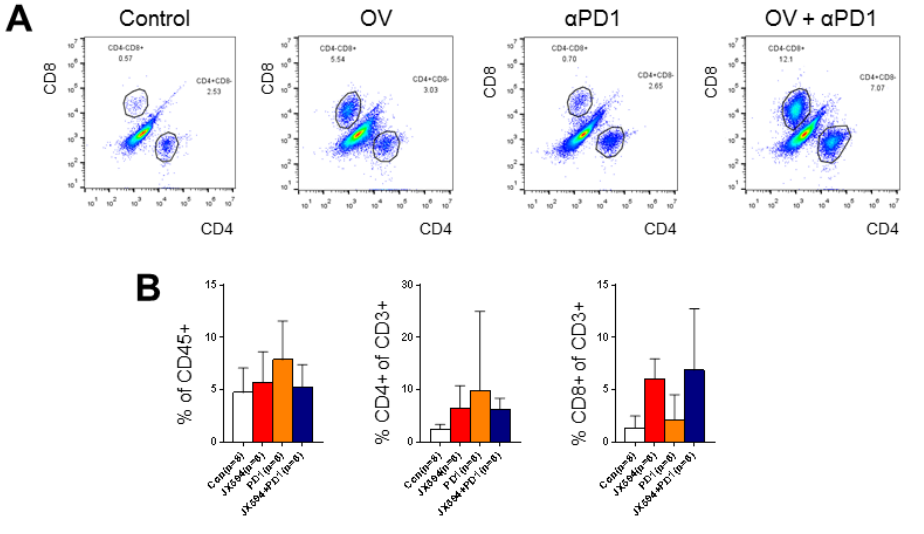

Figure 15. Changes of intratumoral lymphoid cells after combination treatment

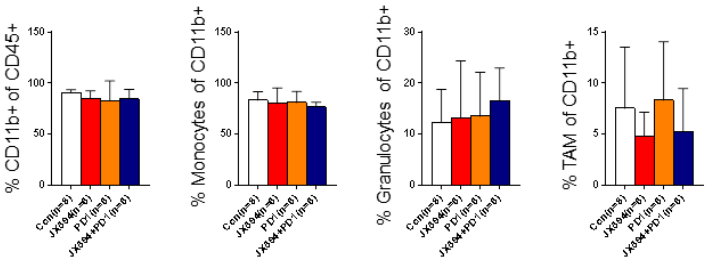

Figure 16. Changes of intratumoral myeloid cells after combination treatment

IV. DISCUSSION

Immune checkpoint inhibitors that block PD1 or CTLA-4 have shown benefits in survival for various malignancies such as lung, kidney, and skin cancer. However, despite promising effect with less toxicity of them, some cancers never respond to immune checkpoint inhibitors. Especially for colorectal cancer, only a portion of MSI-high diseases, which are less than 10% of overall colorectal cancers, responses to the treatment using immune checkpoint inhibitors. Therefore, the innovative treatment option to improve response rates to immune checkpoint inhibitors, and new combination strategies has been tried using anti-PD1 or CTLA-4 antibodies to overcome the immune checkpoint inhibitors alone.

Since 2014, FDA has approved combination immunotherapies of agents that have different mechanisms to treat advanced melanoma. In addition,

combination immunotherapy has gathered much interest owing to the patients who did not respond to immune checkpoint inhibitors alone. Most trials aim to convert the non-responders into the responders to immune checkpoint inhibitors using proper partners. Oncolytic virus is one of the potential partners which can combine with immune checkpoint inhibitors. It selectively infects, amplifies within and destroys cancer cells, thereby representing a novel class of anti-cancer therapy.[27] However, recent studies revealed that oncolytic virus might act as an immunomodulator.[28] The combination of T-vec and anti-PD1 antibody may allow greater response rates in immune sensitive tumors and may render immune checkpoint inhibitor-resistant tumors more sensitive to treatment. We also hypothesized that OV may play a role like T-vec, converting the non-inflamed tumor microenvironment into the inflamed one.

We found that OV synergized with anti-PD1 antibody to delay colon cancer growth. Combination of OV and anti-PD1 antibody increased intratumoral CD8+ T lymphocytes. These imply that the immunity-related mechanism of OV accounts for more among anti-cancer efficacy, because the infiltration of CD8+ cytotoxic T lymphocytes is responsible for anti-cancer immunity. Additionally, we found that consistent anti-cancer immunity of OV regardless of the schedule or location of the injection, whereas some previous reports showed different therapeutic efficacy according to the schedule or location of the injection. Local injection of OV with systemic injection of anti-PD1 antibody might be the reasonable treatment option to maximize the therapeutic efficacy. However,

further work is required to evaluate the exact mechanism and to determine the best combinations.

There are several limitations in this study. First, relatively small sample size of animals is inevitable. Second, we found the possibility of colon cancer to response the combination therapy of OV and anti-PD1 antibody, not for MSI-high colon cancer specifically. Because there is no syngeneic animal model that expressed characteristics of MSI-high colon cancer wholly, another colon cancer cell lines or transgenic animal models are required to give more clear answers. Last, clinical trials including colon cancer patients are warranted to prove correlations between preclinical results and clinical outcomes.

V. CONCLUSION

OV can immunomodulate as well as replicate and kill the colon cancer cell. The combination of OV and anti-PD1 antibody can benefit particularly for patients with colon cancer that are resistant to other therapies.

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ABSTRACT (IN KOREAN)

논문제목

대장암에서 항암 바이러스의 면역 미세환경 변화 기전에 대한 연구

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김창우

면역치료는 인체의 면역체계를 활성화시켜 종양세포를 공격함으로서 다양한 암종에서 반응을 보이고 지속 가능한 항암 효과를 나타낼 것으로 기대되었으나 대장암에서는 현미부수체 불안정성(MSI) high 중에서만 치료 반응이 나타났다. 하지만 MSI-high 는 전체 대장암의 일부에 불과하므로 면역치료에 반응하지 않는 대장암의 치료법 개발이 필요하다.

면역치료의 반응률을 높일 수 있는 방법으로 면역체크포인트 억제제와 다른 후보 물질들을 병합하는 연구들이 진행되고 있는데, 그 중 하나가 항암 바이러스이다. 항암 바이러스는 단독으로 사용했을 때보다 면역체크포인트 억제제와 병합하였을 때 가장 많은 종양 감소를 보였고, CD8+ T세포 뿐 아니라 CD4+ T세포의 증가 또한 관찰되었다. 항암바이러스는 단순히 암세포 내에서 증식하고 암세포를 사멸하는 역할 뿐 아니라 면역조절 기능을 하고 그로 인해 면역체크포인트 억제제의 반응률을 상승시키는 것으로 여겨지므로, 향후 추가 연구가 필요하다.

핵심되는 말: 대장암, 면역치료, 면역 체크포인트 억제제, 항암 바이러스