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Adjuvant Postoperative CD40 Agonist and PD-1 Antagonist Combination Therapy in Syngeneic Tongue Cancer Mouse Model

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Abstract. *Background/Aim: Using a syngeneic tongue cancer mouse model, the effect of CD40 agonist and PD-1 antagonist combination therapy for local recurrence after surgery was evaluated in a partially depleted CD4 model. Materials and Methods: C3H/HeN mice were injected with 0.05 mg of the anti-mouse CD4 clone GK1.5, causing partial depletion of CD4 cells. Tongue cancer was induced by injecting the squamous cell carcinoma (SCC) VII cell line, the tumor was resected by partial glossectomy, and CD40 agonist and/or PD-1 antagonist therapy was administered postoperatively. Results: Partial depletion of CD4 cells resulted in faster growth of a recurring tumor in the tongue, faster loss of body weight, and decreased number of CD8a-positive cells in the tumor. Postoperative adjuvant therapy with a combination of CD40 agonist and PD-1 antagonist resulted in a significant increase in survival compared to the CD40 agonist single treatment. Conclusion: CD40 agonist and PD-1 antagonist combination therapy could be an effective postoperative adjuvant treatment, especially in cases with decreased CD4 T cell activity.*

The recurrence of cancer after surgery could be due to microscopic remnants that grow to a clinically significant size. Adjuvant radiation therapy with or without chemotherapy is still the mainstay of treatment of head and neck cancer patients if the microscopic remnant is suspicious after oncological surgery. However, the effect of radiation therapy is limited to the locoregional remnant, and many distant and locoregional recurrences occur even with concurrent chemoradiotherapy.

Another suggestion for treating this remnant can be found in the theory of cancer immunoediting (1). According to this theory, cancer recurrence is not only dependent on the tumorigenic potential of cancer cells but also on the relationship between cancer cells and the immunity of patients. A previous study by our laboratory has shown that decreased expression of CD40L in regional cancer-free lymph nodes is a significant poor prognostic factor (2) and that depletion of CD4 in immunocompetent mice delays the clearance of injected human cancer cells in the tongue (3). These data suggest that the CD40-CD40L interaction can be an important target to improve treatment outcome.

CD40L, which is expressed in activated CD4+ T cells, interacts with CD40, which is a cell surface receptor present on B cells, macrophages, and dendritic cells, and determines the final functional outcome of T-cell signaling (4). A clinical trial performed on a CD40 agonist as a single agent has shown a minimal response. However, there are recent ongoing combinational trials with PD-1, PD-L1, or VEGF and radiotherapy (5). In addition, the effect of immunotherapy or targeted therapy depends on whether the drug is tested in appropriate indications.

In this study, in a syngeneic mouse tongue cancer model, the effect of CD40 agonist and/or PD-1 antagonist was evaluated as postoperative adjuvant treatment in mice with partially depleted CD4 cells, a condition similar to a deficit in CD40L.

Materials and Methods

Partial depletion of CD4. Anti-mouse CD4 clone GK1.5 (BioXCell, West Lebanon, NH, USA) was injected intraperitoneally to deplete CD4 cells in C3H/HeN mice. We used a dosage of 0.05 mg, one-tenth of the normal recommended dose, to cause partial depletion of CD4 cells. The frequency of injection was reduced to once a week, although the manufacturer's recommendation was twice a week for maintenance.

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Postoperatively recurrent tongue cancer in an orthotopic syngeneic mouse model. For this experiment, we used immunocompetent C3H/HeN female mice, 6-8 weeks old. Animal studies were performed in accordance with the protocol approved by our Institutional Animal Care and Use Committee. About 10^6 squamous cell carcinoma (SCC) VII cells, derived from murine oral SCC (6), suspended in 15 μl of phosphate-buffered saline (PBS) were injected in the lateral tongue of C3H/HeN mice using a Hamilton syringe. Two to three weeks later, the tumor mass was resected with minimal margins. The mice were followed up and the size of the recurred tongue mass and their body weight were measured twice a week. The tumor volume was calculated based on the caliper measurement by the modified ellipsoidal formula [tumor volume= 1/2 (length × width^2)]. If the tumor size exceeded 1 cm in its longest diameter, or the body weight was reduced by more than 20% from the time of surgery, the mice were sacrificed. The detailed procedure is described in previous studies (7).

Flow cytometry. After three consecutive intraperitoneal injections of anti-mouse CD4 clone GK1.5, the lymph node and peripheral blood were collected for flow cytometry on day 5. The lymph node was mechanically dissociated and filtered through a 40 μm nylon mesh to make a single cell suspension. Peripheral blood was centrifuged after incubation with hemolysis buffer (ammonium chloride solution) and the precipitate was used for experiment. The single cell suspensions were resuspended in flow cytometry buffer (PBS, 0.5% bovine serum albumin, 0.1% sodium azide). The cells were incubated with immunoglobulin Fc receptor blocking agent. Anti-mouse FITC CD4, PreCP-Cy5.5 CD8, APC CD40, PE CD40L, PerCP-Cy5.5 CD11b, and FITC CD19 (BD Bioscience, San Jose, CA, USA) were used for flow cytometry.

Immunohistochemistry. The control and CD4-partially depleted groups were sacrificed after 40 days (approximately 20 days after partial glossectomy) and their tongues were removed for H&E staining and immunohistochemistry. Whole tongue was fixed in 10% neutral formalin solution and embedded in paraffin. Four μm sections were examined with H&E staining and immunohistochemistry was performed according to standard methods (8) with the use of polyclonal antibodies against CD8a (1:20 dilution, Cloud-Clone corp, TX, USA), CD11b (1:1000, Cloud-Clone corp) and PD1 (1:500, Abcam, UK).

Agonistic CD40 and antagonistic PD-1 antibody therapy. Alongside the partial glossectomy, treatments were started and administered intraperitoneally twice weekly until sacrifice. Rat IgG2a isotype control (100 μg) and InVivoPlus Polyclonal Armenian Hamster IgG (100 μg) were injected intraperitoneally in the control group. Agonistic CD40 treatment was administered with the injection of 100 μg of rat anti-mouse CD40 monoclonal Ab, clone FGK 4.5 (BioXCell) and 100 μg of InVivoPlus polyclonal Armenian hamster IgG. The PD-1 antagonist treatment was composed of InVivoPlus polyclonal anti-mouse PD-1 (BioXCell) and rat IgG2a as the isotype control. The combined agonistic CD40 and antagonistic PD-1 treatment included rat anti-mouse CD40 monoclonal antibody clone FGK 4.5 and InVivoPlus polyclonal anti-mouse PD-1. The tumor size and body weight were measured twice weekly and survival was analyzed at the same time.

Statistical analysis. Kaplan–Meier survival analysis was performed using IBM SPSS for windows, version 20.0 (IBM Corp, Armonk, NY, USA) and GraphPad Prism version 5 (GraphPad Software, San Diego, CA, USA) and a *p*-value less than 0.05 was regarded as statistically significant.

Results

Effect of partial CD4 depletion on cancer recurrence. Figure 1A illustrates the scheme of the experimental procedure used to investigate the influence of partial CD4 depletion in tongue cancer recurrence after partial glossectomy. The effect of CD4 depletion with 0.05 mg intraperitoneal injection was confirmed in four mice in whom the blood and lymph node were analyzed by flow cytometry. In the lymph node, the CD4 cell population decreased to 20.56%±2.98% from 65.4%5±3.98% in the control group. Depletion of CD4 cells was more prominent in the peripheral blood, and the CD4 population decreased from 35.16%±6.60% in the control to 2.35%±1.07%. Because of the decrease in CD4 cells, CD8 cells in lymph nodes (Figure 1B) and CD11b-positive cells in the peripheral blood increased significantly (Figure 1C). Moreover, CD40L expression coincided with the expression of CD4 in both the lymph node and peripheral blood. After partial glossectomy, tumor regrowth was faster in the CD4-partially depleted group (Figure 1D). The body weight in the control group showed some recovery after surgery for about 1 week and decreased with tumor regrowth from day 12 after surgery. In comparison, the CD4-depleted group showed a faster decrease in body weight after surgery, with no period of recovery (Figure 1E). All the six control mice survived 19 days after surgery, but CD4-depleted mice were sacrificed between days 12 and 16 after surgery, and only one mouse survived 19 days after surgery.

Immunohistochemistry showed a dense infiltration of CD8a-positive cells in the tumor tissue of the control mice, which was decreased in the tumor of the CD4-depleted mice. PD-1 expression could be identified in tumors of the control group, and the expression of PD-1 appears to coincide with the infiltration of CD8a-positive cells. In CD4-depleted mice, the expression of PD-1 decreased along with a decrease in CD8a-positive cells in the tumor (Figure 2).

CD40 agonist and PD-1 antagonist therapy. Treatment was initiated just after partial glossectomy, with twice weekly intraperitoneal injections (Figure 3A). The tumor regrowth was slightly delayed in the CD40 agonist or PD-1 antagonist single treatment groups compared to the control group. However. the CD40 agonist and PD-1 antagonist combination treatment resulted in a significant delay in recurrence (Figure 3B). Changes in body weight showed the same trend as tumor recurrence, with the combination therapy group gaining weight until 14 days after surgery, which then decreased with tumor recurrence. Compared to the combination therapy and control groups, the single

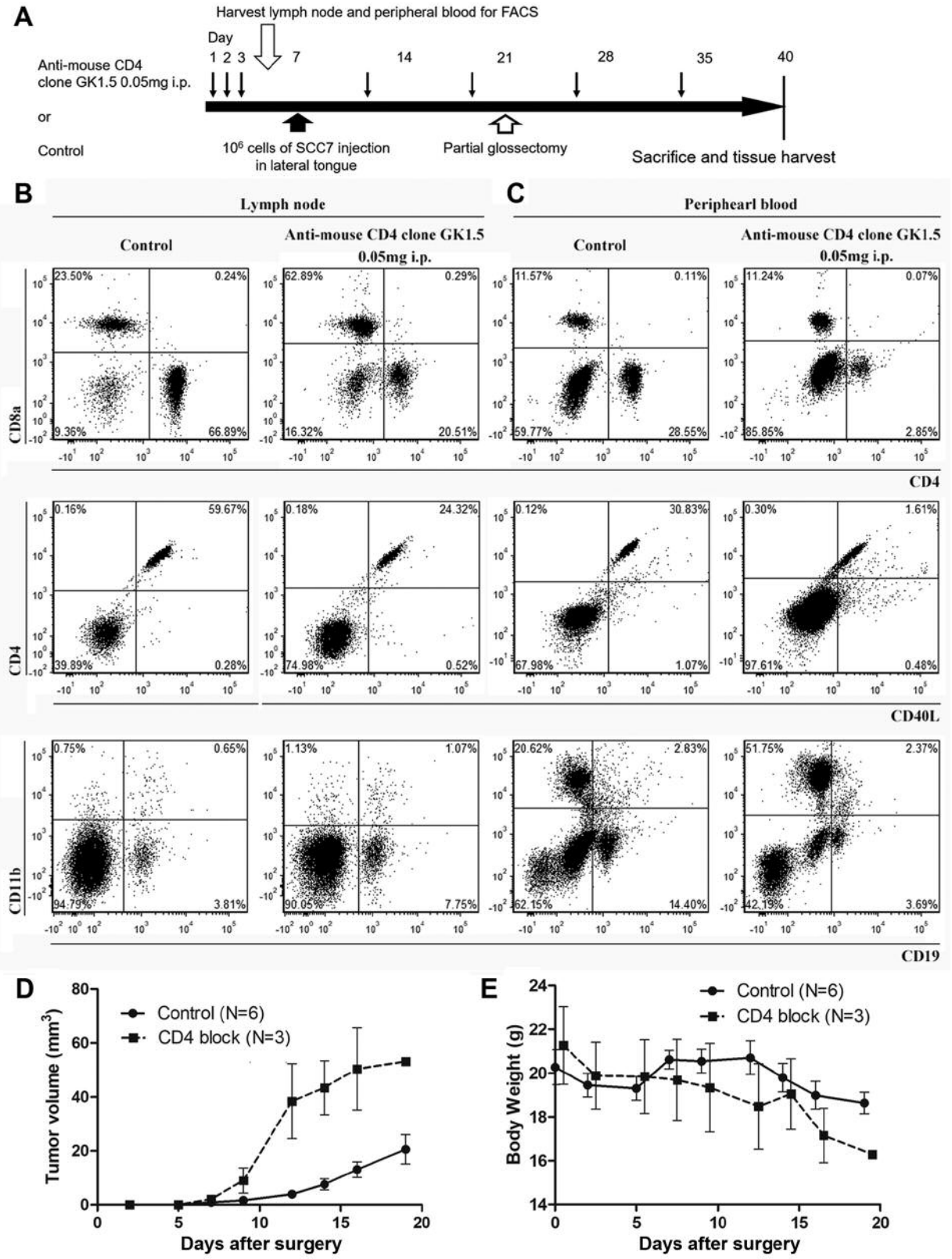


Figure 1. *Effect of partial depletion of CD4 on postoperative local recurrence in the tongue cancer model. A. Experimental design scheme. B. Flow cytometry analysis after 0.05 mg injection of the anti-mouse CD4 clone GK1.5 in lymph node. C. Flow cytometry analysis in the peripheral blood. D. Tumor volume changes after partial glossectomy. E. Changes in body weight postoperatively.*

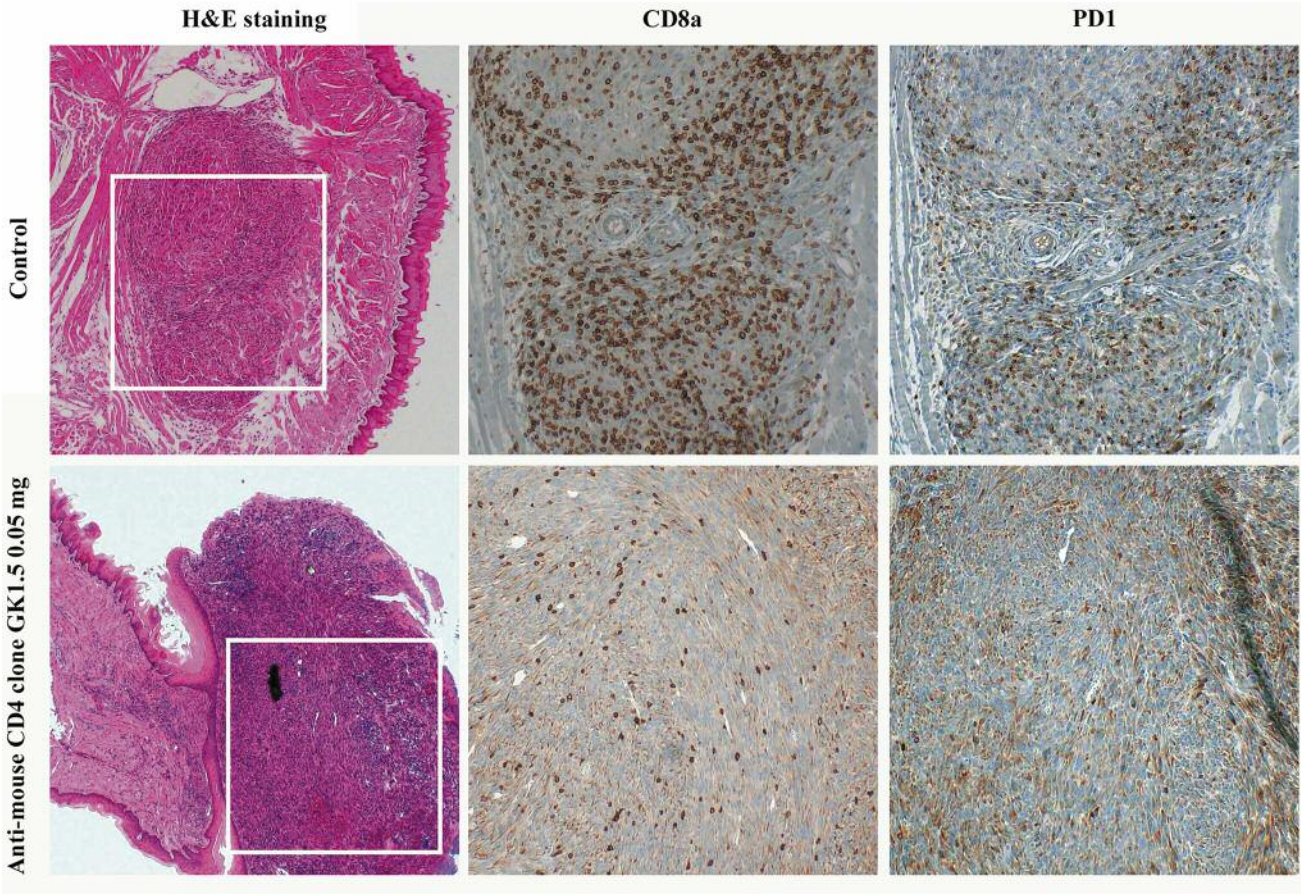
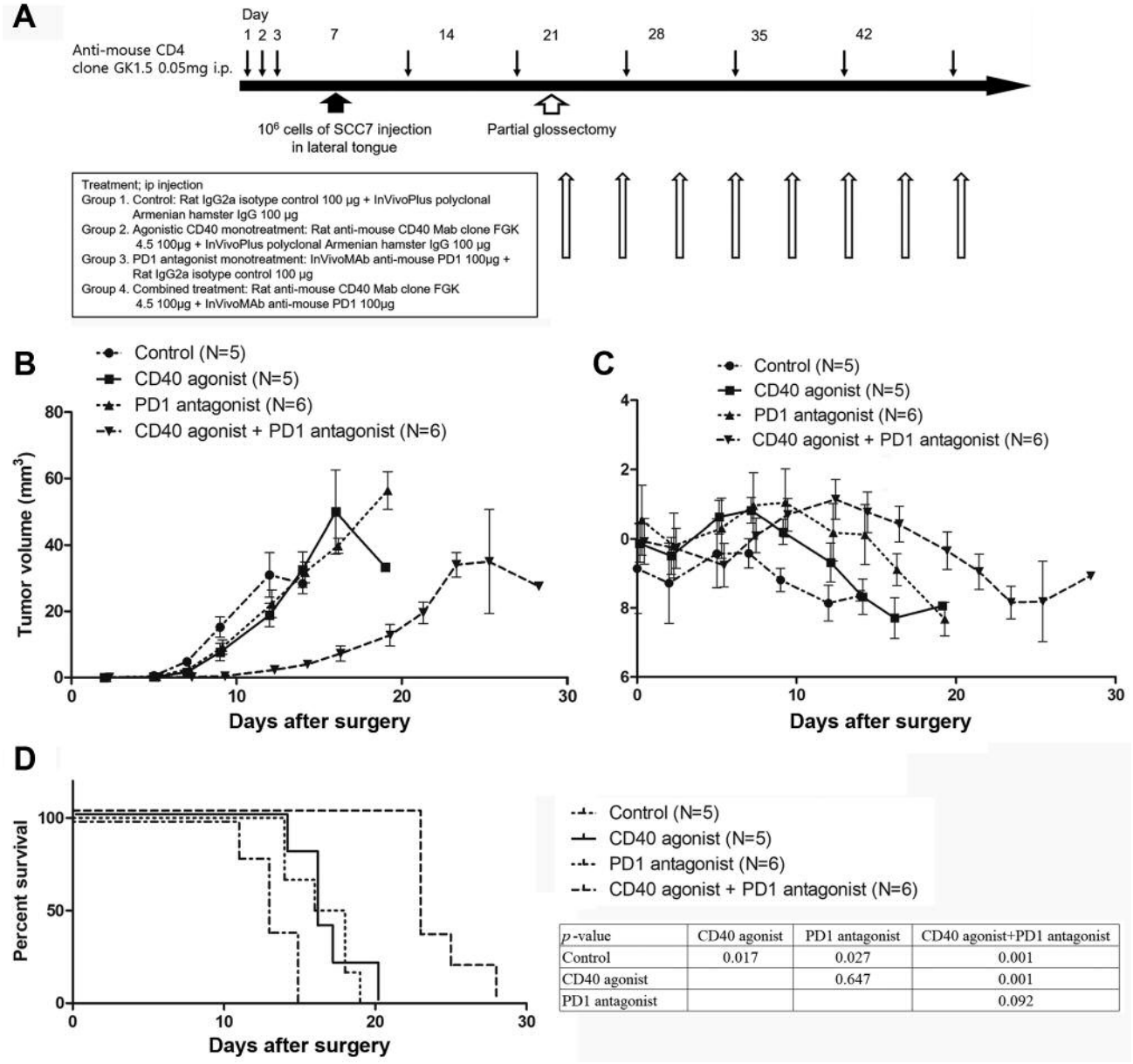


Figure 2. *Pathological findings of recurred tongue cancer in control and partially depleted CD4 mouse model.*

therapy groups showed moderate changes in body weight (Figure 3C). Mice in the control group showed signs of morbidity earlier and were sacrificed between days 11 and 15 after surgery. The time of sacrifice was between days 14 and 19 in the single therapy group, and between days 23 to 28 in the combination therapy group. The survival analysis showed a statistically significant difference between groups, except between the single therapy PD1 antagonist and CD40 agonist groups as well as between the PD-1 antagonist single therapy and the combined therapy groups (Figure 3D).

Discussion

Even in similar clinical situations, certain patients experience an unexpectedly rapid recurrence after successful treatment, and the decreased immune function in these patients may have a significant impact. In our previous clinical experiment, tumor-free regional lymph nodes in oral cavity cancer patients were evaluated, decreased expression of many genes related to T cell activity were observed by cDNA microarray analysis, and decreased CD40L expression was found to be an independent poor prognostic factor (2). Accordingly, experiments with CD40 agonist that could replace the reduced CD40L were conducted. In our previous experiment on the elimination of human cancer cells in immunocompetent mice, depletion of CD4 delayed the elimination of injected cancer cells in the tongue, which could be reversed by CD40 agonist antibody treatment (3). However, injection of 0.5 mg of anti-mouse CD4 clone GK1.5 as per manufacturer's instructions, led to a complete depletion of CD4 cells in the lymph nodes, spleen, and peripheral blood. To generate a more realistic model, a partially depleted CD4 animal model was used in this experiment. Using a tenth of the recommended dose, intraperitoneal injection of 0.05 mg, a partially depleted CD4 model was generated, in which the CD4 cells in the lymph nodes and peripheral blood decreased from 65.5% to 20.6% and from 31.4% to 2.4%, respectively. Although it is still an exaggerated situation, an animal model that is closer to reality than a complete depletion was made. In this situation, the recurred tongue cancer after partial glossectomy showed faster growth, and the loss of body weight progressed faster than in the normal control indicating the negative effect of partial depletion of CD4 in postoperative local recurrence.



p-value	CD40 agonist	PD1 antagonist	CD40 agonist+PD1 antagonist
Control	0.017	0.027	0.001
CD40 agonist		0.647	0.001
PD1 antagonist			0.092

Figure 3. *Postoperative adjuvant treatment with a CD40 agonist and a PD-1 antagonist. A. Experimental design scheme. B. Tumor volume of recurred tongue cancer after surgery. C. Changes in body weight postoperatively. D. Kaplan–Meier survival analysis of the different treatment groups.*

The immunohistochemistry of the recurred tongue mass showed a significant decrease in CD8a-positive cells when CD4 was partially depleted. The expression of PD-1 was found in CD8a cells infiltrating the tumors, and PD-1 expression was decreased together with CD8a cells in the CD4-depleted group. The decreased number of CD8a cells in the tumor due to the depletion of the CD4 cells, reconfirms the well-known role of CD4 cells in *in vivo* priming of CD8 cytotoxic T lymphocytes, and the CD40-CD40L interaction is known to mediate this process (9, 10). Moreover, as PD-1 is mainly expressed on the surface of T cells, a decrease in the expression of PD-1 in CD4-depleted mice was expected.

Postoperative CD40 agonist and PD-1 antagonist combination therapy resulted in a significant delay in the growth of the recurred tumor, maintenance of the body weight for a longer period of time, and a significantly longer survival compared to the control or CD40 agonist single therapy groups. Combination therapy showed better results than PD-1 antagonist single therapy, although the survival analysis failed to show statistical significance. Since the first report on

the role of the CD40-CD40L interaction in 1998 (9, 10), there has been an increasing interest in CD40 agonists, which can replace the role of CD40L expressed in T helper cells. In mice, expansion of antigen specific CD8 T cell could be observed by CD40 activation (11) and in tumor models with prominent tumor antigen, CD40 agonist alone could achieve tumor regression (12). There have been some clinical trials on CD40 agonist therapy. In the first phase I study using recombinant human CD40L (Avrend, Seattle, WA, USA), a laryngeal cancer patient was completely cured (13). Thereafter, various CD40 agonists have been tested and among them, the largest trial was performed with selicrelumab (14). Its antitumor effect was expected, but only 27% of the advanced melanoma cases showed partial response and no solid tumor showed a response (15). However, with the innovative development of immunotherapy targeting checkpoint inhibitors, CD40 agonists have been receiving increasing attention, and research on the possibility of combination therapy has been conducted (5). Clinical trial have been conducted to examine the efficacy of combination therapy with a CD40 agonist and PD-1 antagonist since there were findings suggesting supporting this approach (5). In mice colon and breast cancer models, CD40 agonist treatment induced PD-L1 up-regulation in tumor-infiltrating monocytes and macrophages and resulted in resistance to CD40 agonist treatment, and combination treatment with blockers of the PD-1/PD-L1 axis showed a synergistic effect (16). In addition, a decrease in tumor-infiltrating cytotoxic T lymphocytes has been identified as a cause of resistance to PD-1 treatment, and CD40 agonist treatment can reverse this resistance (17). Therefore, combination therapy with CD40 agonists and PD-1 antagonists can provide a mechanism that could overcome resistance to each drug.

It should be emphasized that in this study, the combination therapy was used as a postoperative adjuvant treatment. Contrast to the most existing studies which tried to evaluate the effect of immunotherapy in advanced cancer, this study evaluated its role in the treatment of microscopic residual cancers in patients who can be operated. Another important point is that we tested this treatment in mice with partially depleted CD4, a weak immune system according to the cancer immunoediting theory. As found in previous studies, some patients with a decreased expression of CD40L showed poor prognosis even after successful surgery and postoperative radiotherapy (2), and in these patients, immunotherapy, which can up-regulate the T-cell system, will result in better outcomes. This experiment therefore showed that the combination of a CD40 agonist and a PD-1 antagonist exerts a synergistic effect in postoperative settings.

Conflicts of Interest

The Authors have no conflicts of interest to declare regarding this study.

Authors' Contributions

SH Ahn designed the work, analysis of data, wrote and edited the manuscript. S Song carried immunohistochemistry and analysis, SR Kim carried out *in vivo* and *in vitro* experiments.

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