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Conflict of Interest
The authors declare no potential conflicts of interest.

Enriching CCL3 in the Tumor Microenvironment Facilitates T cell Responses and Improves the Efficacy of Anti-PD-1 Therapy

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ABSTRACT

Chemokines are key factors that influence the migration and maintenance of relevant immune cells into an infected tissue or a tumor microenvironment. Therefore, it is believed that the controlled administration of chemokines in the tumor microenvironment may be an effective immunotherapy against cancer. Previous studies have shown that CCL3, also known as macrophage inflammatory protein 1-alpha, facilitates the recruitment of dendritic cells (DCs) for the presentation of tumor Ags and promotes T cell activation. Here, we investigated the role of CCL3 in regulating the tumor microenvironment using a syngeneic mouse tumor model. We observed that MC38 tumors overexpressing CCL3 (CCL3-OE) showed rapid regression compared with the wild type MC38 tumors. Additionally, these CCL3-OE tumors showed an increase in the proliferative and functional tumor-infiltrating T cells. Furthermore, PD-1 immune checkpoint blockade accelerated tumor regression in the CCL3-OE tumor microenvironment. Next, we generated a modified CCL3 protein for pre-clinical use by fusing recombinant CCL3 (rCCL3) with a non-cytolytic hybrid Fc (HyFc). Administering a controlled dose of rCCL3-HyFc via subcutaneous injections near tumors was effective in tumor regression and improved survival along with activated myeloid cells and augmented T cell responses. Furthermore, combination therapy of rCCL3-HyFc with PD-1 blockade exhibited prominent effect to tumor regression. Collectively, our findings demonstrate that appropriate concentrations of CCL3 in the tumor microenvironment would be an effective adjuvant to promote anti-tumor immune responses, and suggest that administering a long-lasting form of CCL3 in combination with PD-1 blockers can have clinical applications in cancer immunotherapy.

Keywords: Chemokine CCL3; Tumor microenvironment; Immune checkpoint blockade; Immunotherapy

Author Contributions

Conceptualization: Kang TG, Park HJ, Lee JH, Ha SJ; Funding acquisition: Ha SJ; Investigation: Kang TG, Moon J; Methodology: Kang TG, Park HJ, Ha SJ; Supervision: Ha SJ; Validation: Ha SJ; Writing - original draft: Kang TG, Ha SJ; Writing - review & editing: Kang TG, Ha SJ.

INTRODUCTION

Development of immune checkpoint blockades (ICBs) has revolutionized the field of cancer immunotherapy. Tumor cells escape immunosurveillance and evade the host immune system by employing diverse mechanisms, such as up-regulating inhibitory molecules that suppress anti-tumor immune responses (1). ICBs have exhibited significant clinical potential in the treatment of solid tumors, and have extend patient life-span (2). Although these immunotherapies induce anti-tumor immunity in some patients, most cancer patients do not respond to the ICB immunotherapy (3).

A number of studies investigated the factors responsible for the ineffectiveness of ICB immunotherapy, and suggested that the magnitude and composition of the immune cells that infiltrate into the tumor microenvironment is an important factor influencing the efficacy of the ICB immunotherapy (4). Consequently, this has led to the categorization of tumors into hot (inflamed) or cold (non-inflamed) tumors (5). Hot tumors have abundant numbers of CD8+ T cells and Ag-presenting cells (APCs), such as DCs that infiltrate the tumor microenvironment. These tumors display significantly increased responsiveness to ICBs due to the enrichment of immune cells (6). In contrast, cold tumors harbor high numbers of immunologically suppressive cells, such as regulatory T cells and myeloid-derived suppressor cells (MDSCs), instead of CD8+ T cells and APCs, and exhibit poor responsiveness to ICB therapy (7). Therefore, enrichment of tumor-infiltrating CD8+ T cells or DCs could be a promising strategy to elevate responsiveness to immunotherapy.

Chemokines, defined as chemotactic cytokines are small secretory proteins that bind 7 G protein-coupled receptors, which induce intracellular signaling pathways (8,9). Chemokines are required when immune cells migrate for homeostasis, development, and protecting the host from infections or tumors (10,11). A diverse set of chemokines are expressed by tumor cells as well as immune cells, endothelial cells, and stromal cells in the tumor microenvironment. Depending on the level of chemokine expression, the composition of tumor-infiltrating immune cells may change and eventually affect the immune response to tumor regression (12,13). There have been multiple pre-clinical attempts to modulate chemokines and the corresponding receptors to increase responsiveness of anti-tumor effector immune cells to ICB therapy, such as CD8+ T cells. For instance, the CXCL9 and CXCL10 expression in the tumor microenvironment, chemokines that are well known for their ability to recruit T cells and NK cells to target sites via the CXCR3 chemokine receptor, was reported to correlate negatively with cancer metastasis (14-16). Additionally, epigenetic silencing of the CXCL9 and CXCL10 genes has been associated with reduced T cell infiltration. Treating mice with 3-deazaneplanocin A (DZNep), an epigenetic reprogramming drug and enhancer of zeste 2 polycomb repressive complex 2 subunit (EZH2) inhibitor, led to a CXCL9 and CXCL10 dependent increase in tumor infiltration of T cells and a subsequent increase in the efficacy of PD-L1 blockade (17). Another study showed that decitabine (DAC), which is a DNA methyltransferase inhibitor, enhanced CXCL10 expression, and subsequently recruited NK cells and CD8+ T cells into the tumor microenvironment of a murine ovarian cancer model. Furthermore, CTLA-4 blockade therapy was potentiated in combination with DAC (18). In contrast, CXCL12 that binds CXCR4 and is expressed by tumor cells as well as the stromal cells in the tumor microenvironment has been reported to promote tumor angiogenesis together with VEGF (19) and sustain cancer cell proliferation and survival (20). These studies indicated that blockade of CXCR4 using neutralizing Abs repressed tumor growth and metastasis in lymphoma and brain tumor models (21,22). Furthermore,

combination therapy with LY2510924, a CXCR4 peptide antagonist, and durvalumab, a PD-L1 blocking Ab, has been clinically tested for solid tumors (23).

CCL3, also known as macrophage inflammatory protein 1-alpha, is a cytokine belonging to the CC chemokine family. CCL3 is released when CD4^{+} T cells interact with DCs and recruit CCR5-expressing CD8^{+} T cells into specific sites for *in vivo* activation and proliferation (24,25). Moreover, CCL3 was demonstrated to play a dominant role in the activation, maturation, and migration of CD11c^{+}CD11b^{+} cells to cervical draining lymph nodes in a murine hepatitis virus infected mice, an infection model of the central nervous system. The migration of DCs into the LN was inhibited in the absence of CCL3, and resulted in diminished IFN-γ expression by Ag-specific T cells and increased the levels of *in vivo* IL-10 (26). Additionally, CCL3 has been shown to be a potential adjuvant for DNA vaccines. Transfecting the HIV Gag DNA vaccine with CCL3 led to markedly enhanced infiltration of CD11c^{+} DCs, and injecting a CCL3-encoding plasmid protected against viral infection with a nearly 200-fold reduction in virus titers (27). The production of CCL3 by B cells in the tumor microenvironments of patients with human melanoma promoted immune responses, while depletion of CCL3-secreting B cells by anti-CD20 decreased tumor-associated inflammation and CD8^{+} T cell numbers. This indicated that the CCL3 secreted by B cells was important for augmenting immune responses in patients with melanoma (28). Additionally, in the HCmel12 murine tumor model, basophils expressed large amounts of CCL3 and CCL4, and depletion of basophils or blockade of these chemokines inhibited CD8^{+} T cell infiltration in the tumor microenvironment (29).

Taking into account the protective function of CCL3, we investigated the consequences of administering CCL3 in the tumor microenvironment in combination with PD-1 immune checkpoint blockers. We engineered MC38, a murine colon adenocarcinoma cell line, to overexpress CCL3, and then analyzed tumor growth and changes in the characteristics of immune cells. The CCL3-overexpressed MC38 exhibited delayed tumor growth. Additionally, CD8^{+} T cells proliferated vigorously in the CCL3-enriched tumor microenvironment, tumor-specific CD8^{+} T cells displayed the ability to produce IFN-γ, and there was an increase in the DC population. Furthermore, CCL3-mediated tumor regression was accelerated when mice were treated with Abs that block PD-1. For pre-clinical use, we generated a long-lasting form of CCL3, composed of recombinant CCL3 (rCCL3) and a non-cytolytic hybrid Fc (HyFc). Mice who were subcutaneously administered an appropriate dose of rCCL3-HyFc near tumors exhibited delayed tumor growth and enhanced survival compared to those that had been administered the control HyFc. Also, mice treated with appropriated dose of rCCL3-HyFc had more mature myeloid cells and functionally enhanced T cells than other groups. Surprisingly, mice were combined treated with rCCL3-HyFc and αPD-1 significantly delayed tumor growth compared to each agent alone treatment. Therefore, our results indicate that CCL3 enrichment augments tumor regression and positively reshapes immune cell populations in the tumor microenvironment, suggesting that rCCL3-HyFc might have potential as an adjuvant for enhancing the effect of presently used αPD-1 immunotherapeutic agents.

MATERIALS AND METHODS

Mice
A 6–8 wk old specific-pathogen-free female C57BL/6 (B6) mice were purchased from Orient Bio. Animals. Experiments were performed with the approval of the International Animal

Care and Use Committee at Yonsei University, and in accordance with the Laboratory Animal Act of the Korean Ministry of Food and Drug Safety that improves the ethics and reliability of animal testing through appropriate administration of laboratory animals and animal testing (permit No. IACUC-A-201907-921-03 and IACUC-A-202007-1097-01).

Tumor cell transfection

The MC38 cells were a gift from Seung-woo Lee's laboratory (POSTECH, Pohang, Korea). They were cultured in DMEM (Corning, New York, NY, USA) supplemented with 10% FBS (Thermo Fisher Scientific, Waltham, MA, USA) and 1% penicillin/streptomycin (Thermo Fisher Scientific). To overexpress CCL3, MC38 cells were transfected with the pCDNA3.1 vector (Addgene, Watertown, MA, USA) that encodes the mouse CCL3 gene under the cytomegalovirus promoter and were selected with hygromycin (200 µg/ml). Selected MC38 clones that overexpressed CCL3 were seeded into 6 well plates at a concentration of 10^6 cells/4 ml media, and the culture supernatants were collected after 48 h. CCL3 expression in the supernatants was analyzed using ELISA (Thermo Fisher Scientific).

Mouse tumor model

Wild type (WT), CCL3 overexpressing (CCL3-OE), and control MC38 cells (5×10^5 cells) in PBS were injected subcutaneously into age-matched (6–8 wk) B6 mice. Tumor sizes were measured at the indicated time points with a caliper and the following formula was used for all calculations: $1/2 \times (\text{length} \times \text{width}^2)$. Tumors larger than 2,000 mm^3 in size were considered dead.

Tumor harvest and flow cytometry

Tumors were harvested at the indicated time points and homogenized into small pieces before digestion with 1 mg/ml collagenase type VI (Worthington Biochemical Corporation, Lakewood, NJ, USA) for 20 min at 37°C to obtain single-cell suspensions. For flow cytometric analysis of immune cells, single-cell suspensions were stained with the following Abs that were purchased from BD Biosciences (San Jose, CA, USA): PerCP-Cy5.5-CD4 (clone RM4-5), APC-NK1.1 (clone PK136), PE-cy7-CD11b (clone M1/70), PerCP-Cy5.5-Ly6G (clone 1A8), BV605-Ly6C (clone AL-21), and APC-IFN-γ (clone XMG1.2). Alexa Fluor700-CD8 (clone 53-6.7), BV421-PD-1 (clone 29F.1A12), BV501-CD44 (clone IM7), Alexa Fluor700-CD45.2 (clone 104), BV510-CD11c (clone N418), and BV605-TNF-α (clone MP6-XT22) Abs were purchased from BioLegend. APC-Foxp3 (clone FJK-16s) and PE-TR-F4/80 (clone BM8) Abs were purchased from Thermo Fisher Scientific. The dead cell populations were removed using a Live/Dead fixable Stain Kit (Thermo Fisher Scientific). The intracellular cytokines in the tumor derived T cells were detected by incubating the cells *ex vivo*, with or without the MC38 epitope peptide (p15E, KSPWFTTL, 5 µg/ml) for 6 h in the presence of Golgi plug/Golgi stop (BD Biosciences). Intracellular staining was performed after the surface staining using the BD Cytofix/Cytoperm fixation/permeabilization kit (BD Biosciences) according to the manufacturer's instructions. For Foxp3 staining, the cells were stained with Foxp3/transcription factor staining buffer set (Thermo Fisher Scientific) according to the manufacturer's instructions. Flow cytometric data were collected using CytoFLEX (Beckman Coulter, Brea, CA, USA) and analyzed using FlowJo software (Tree Star Inc., Ashland, OR, USA).

In vivo treatments

Mice were randomly divided into different treatment groups before the tumor size reached 30–50 mm^3. To ensure that the tumor sizes between the different groups were approximately equivalent before the therapy, mice were stratified based on the size of the implanted tumor. Each mouse was treated intraperitoneally with 200 µg of isotype control or anti-PD-1 (RMP1-

14, Bio X cell) Abs, every 3 days. For rCCL3-HyFc treatment, recombinant mouse CCL3-HyFc and its control HyFc was supplied by Genexine, Inc. (Seongnam, Korea). Each mouse was injected subcutaneously with 350 ng HyFc or rCCL3-HyFc at the indicated dose.

Statistical analysis

Statistical analysis was performed using Prism software version 7.0 (GraphPad, San Diego, CA, USA). A 2-tailed unpaired Student's *t*-test was performed to determine differences between 2 groups. Comparisons between multiple groups were performed using 1-way ANOVA with *post hoc* Tukey's test or 2-way ANOVA with *post hoc* Tukey's test. Kaplan-Meier survival curves were analyzed using the Mantel-Cox log-rank test with a 95% confidence interval. Details about the statistical test, exact value of number, precision measure, and statistical significance for each experiment have been reported in the figure legends.

RESULTS

Expression of CCL3 in the tumors delays tumor growth and improves survival

Since we aimed to investigate the effect of CCL3 on immune cells and not tumor cells, we first determined the *in vitro* expression of CCL3 receptors CCR1 and CCR5 in 6 murine tumor cell lines. To elaborate, the expression of CCR1 and CCR5 was measured in the C57BL6 derived MC38, LLC1, TC1, B16F10, and EG7 cell lines, and in the CT26 cell line from the BALB/c strain. We confirmed that MC38, TC1, and B16F10 rarely expressed CCR1 and CCR5 via flow cytometric analysis (**Fig. 1A**). In contrast, the LLC1 and CT26 cells did not express CCR5 but showed low levels of CCR1 surface expression. Interestingly, EG7 expressed high levels of CCR1 and low levels of CCR5. Additionally, we concluded that none of the tested tumor cells produced CCL3 because no CCL3 was detected (ELISA analysis) in the culture supernatants (**Fig. 1B**).

Among the 3 tumor cell lines that did not express CCR1 and CCR5, the MC38 tumor cell line was selected and engineered for CCL3 overexpression because MC38 tumors have often been used to determine the efficacy of PD-1 blockers because of abundant expression of PD-L1 in MC38 (30). We transfected MC38 cells with either the plasmid designed to overexpress CCL3 or an empty plasmid, and selected MC38 clones expressing high levels of CCL3 (CCL3-OE) or a mock plasmid (mock) *in vitro* (**Fig. 1C**). The tumor size and survival time of C57BL/6 mice were measured after inoculation with CCL3-OE, mock, or WT MC38 cells (**Fig. 1D**). Mice injected with CCL3-OE MC38 cells exhibited significantly delayed tumor growth compared to mice that had been inoculated with WT- or mock-MC38 cells (**Fig. 1E and F**). Consistent with the reduction in tumor volume, mice inoculated with CCL3-OE displayed enhanced survival rates (**Fig. 1G**). We analyzed the CCL3 levels in blood from CCL3-OE MC38 bearing mice. Although serum CCL3 level seemed to be higher in CCL3-OE MC38-inoculated mice than in WT or Mock MC38-inoculated mice at 8 and 16 days post tumor inoculation, the CCL3 level was below the detectable level in the blood (**Supplementary Fig. 1**). Since CCL3 was produced locally from the tumors, it appears to be difficult to detect enough CCL3 in the blood of CCL3-OE MC38-inoculated mice. Together, these data suggest that CCL3 up-regulation may reshape the tumor microenvironment and enhance anti-tumor immune responses, leading to the efficient control of the *in vivo* tumor growth.
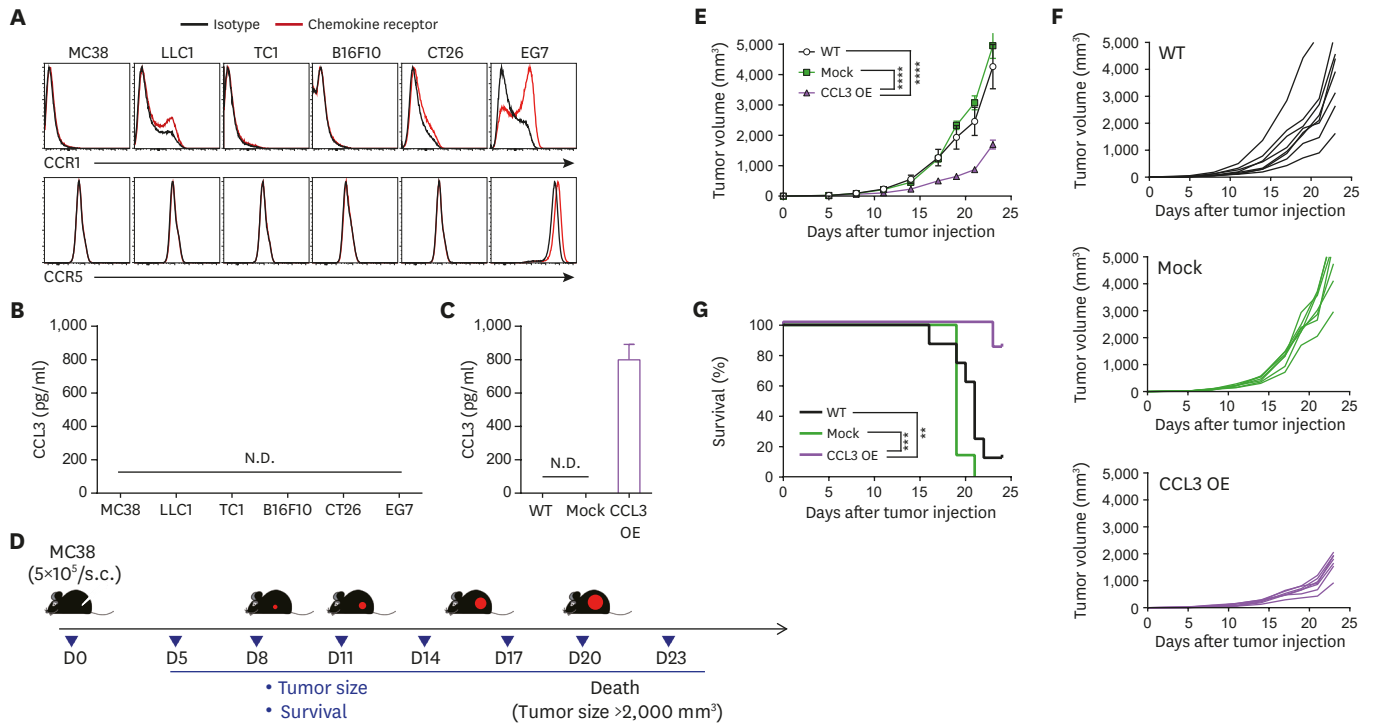
Figure 1. Regression of MC38 tumor growth with high expression of autologous CCL3. (A) The expression levels of CCR1 and CCR5 on indicated murine tumor cell lines were analyzed by flow cytometry. (B) Supernatants of tumor cells were collected and the CCL3 protein content was quantified by ELISA. (C) CCL3-OE MC38 tumor cells were engineered from WT MC38 and expression of CCL3 in supernatants of culture media was analyzed by ELISA. (D) C57BL/6 mice were s.c. inoculated with 5×10^5 WT MC38 or CCL3-OE MC38 cells. (E) The overall tumor growth and (F) individual tumor growth of mouse were represented. (G) Survival of tumor-bearing mice have been represented. Graph shows mean±SEM. Data were analyzed by (E) 2-way ANOVA with Tukey's multiple comparisons test or (G) Mantel-Cox log-rank test. Data are representative of at least 2 experiments (n=6–8 mice/group in each experiment).
ND, not detected; s.c., subcutaneously.
**p<0.01; ***p<0.001; ****p<0.0001.

Increasing CCL3 expression reshapes the T cell and DC populations in the tumor microenvironment

To investigate the influence of CCL3 on immune cell populations, we sacrificed tumor-bearing mice 20 days after inoculation with MC38 cells. We defined CD11b$^+$ cells, T cells, and NK cells after serial sub-gating different immune cell populations (**Supplementary Fig. 2**). While there was no difference in the frequency of CD11b$^+$Ly6C$^+$, CD11b$^+$Ly6G$^+$, and CD11b$^+$F4/80$^+$ cells between mock and CCL3-OE groups, the frequency of CD4$^+$ and CD8$^+$ T cells had decreased in CCL3-OE mice (**Fig. 2A**). The regulatory T cells and NK cells displayed similar frequencies between mock and CCL3-OE groups (**Fig. 2A**). Interestingly, there was tendency to up-regulation of frequency of Ki67$^+$ cells among tumor-infiltrating T cells in the CCL3-OE group compared to the mock group. (**Fig. 2B**). Additionally, when the tumor-infiltrating CD8$^+$ T cells were re-stimulated *ex vivo* with the P15E peptide, an H-2K^b-restricted CD8$^+$ T cell Ag epitope expressed in H-2^b haplotype tumors, we observed that the MC38 and CD8$^+$ T cells from the CCL3-OE group exhibited a significantly enhanced capacity to produce IFN-γ compared to those from the mock group (**Fig. 2C**). Furthermore, the frequency of DCs co-expressing CD11c and MHC class II was also significantly elevated in the CCL3-OE group (**Fig. 2D**). Given the previous report showing the role of CCL3 in activating CD8$^+$ T cells, our data suggest that CCL3 enrichment facilitates DC recruitment to the tumor microenvironment, and enables tumor-specific CD8$^+$ T cells to proliferate and function. Collectively, we hypothesized that CCL3-mediated rewiring of immune cells, such as T cells and DCs in the tumor microenvironment, might contribute to delayed tumor growth and enhanced survival *in vivo*.
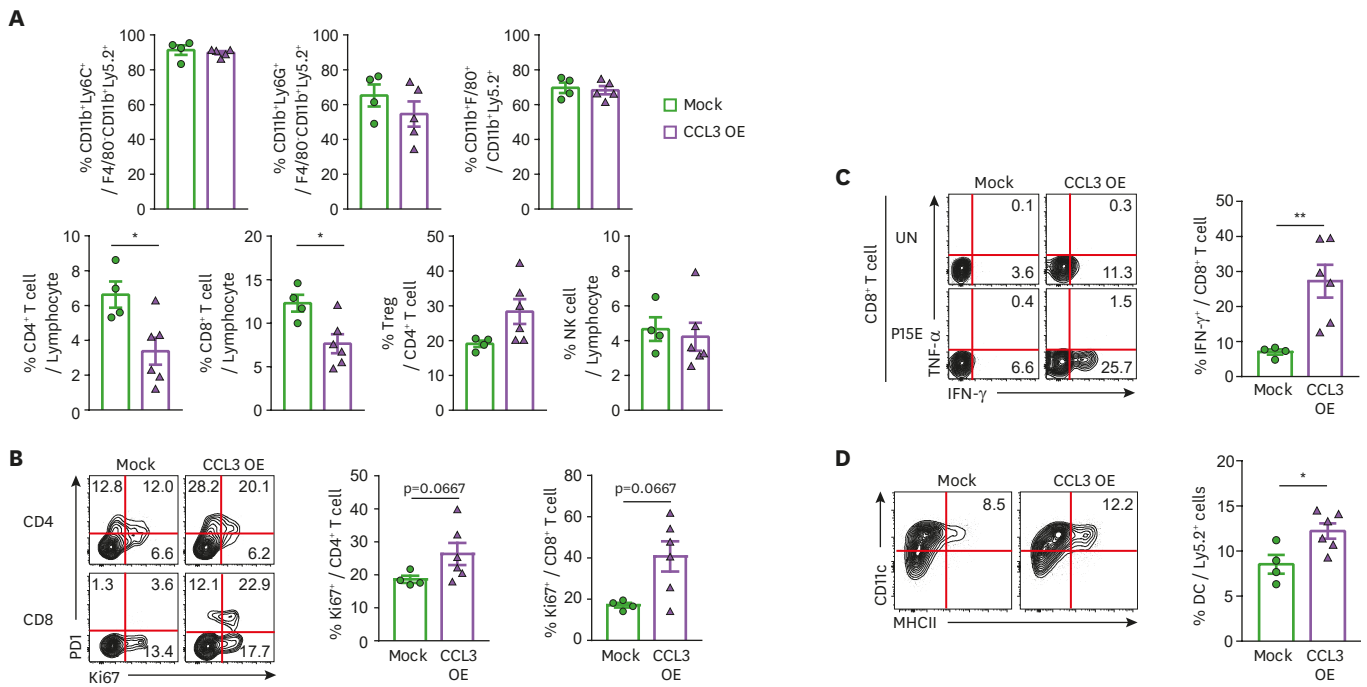
Figure 2. Enriching CCL3 in the tumor microenvironment changes the characteristics of immune cells. Tumor-infiltrating immune cells in each tumor-bearing mice were analyzed by flow cytometry, and after 20 days of tumor cell inoculation. (A) The frequencies of immune cells have been summarized in the bar graph. (B) The graph summarizes the frequencies of Ki67+CD8+ or Ki67+CD4+ T cells as observed after analysis. Numbers in plots indicate the percentage of Ki67+PD1+, Ki67+PD1+, and Ki67+PD1− cells in CD8+ or CD4+ T cell populations. (C) Isolated tumor-infiltrating lymphocytes were re-stimulated *ex vivo* with p15E peptide and stained for CD8+ T cells. The graph summarizes frequencies of IFN-γ positive CD8+ T cells as observed after analysis. Numbers in the plots indicate the percentage of TNF-α+ and TNF-α− CD8+ T cells producing IFN-γ. (D) The graph summarizes the frequency of DCs as observed after analysis. Numbers in the plots indicate the percentages of DCs. Data were analyzed by Mann-Whitney *U* test. Graphs show mean±SEM. Data are representative of a single experiment (n=4–5 mice/group).
*p<0.05; **p<0.01.

PD-1 blockade immunotherapy enhanced the effect of CCL3-induced tumor regression

Since CCL3 rewired the immune cells in the tumor microenvironment, we expected that PD-1 blockade in CCL3-enriched microenvironments would further accelerate anti-tumor T cell responses and tumor regression. To test this hypothesis, mice were intraperitoneally treated with monoclonal PD-1 blocking (αPD-1) or isotype control Abs, starting 5 days post tumor inoculation, and after every 3 days (**Fig. 3A**). In the CCL3-enriched tumor microenvironment, tumor growth was significantly delayed by PD-1 blockade compared to the isotype Ab treatment (**Fig. 3B and C**). Similarly, mice injected with PD-1 blockade Ab lived longer than the mice that belonged to the isotype-injected control group (**Fig. 3D**). Therefore, our data demonstrate that a tumor microenvironment where CCL3 is stably maintained can support the PD-1 blockade mediated control of the tumor growth. This suggests that CCL3 could be used clinically in combination with PD-1 blockers.

Subcutaneous administration of long-lasting CCL3 near the tumors was therapeutically beneficial at a specific dose

Since our previous data demonstrate that continuous expression and enrichment of CCL3 in the tumor microenvironment supported the inhibition of tumor growth, we attempted to generate an *in vivo* long-lasting form of rCCL3 and investigated the *in vivo* efficacy of the protein when administered exogenously near tumors. We first designed a plasmid to express mouse rCCL3 linked to HyFc (rCCL3-HyFc) and purified the recombinant fusion protein from
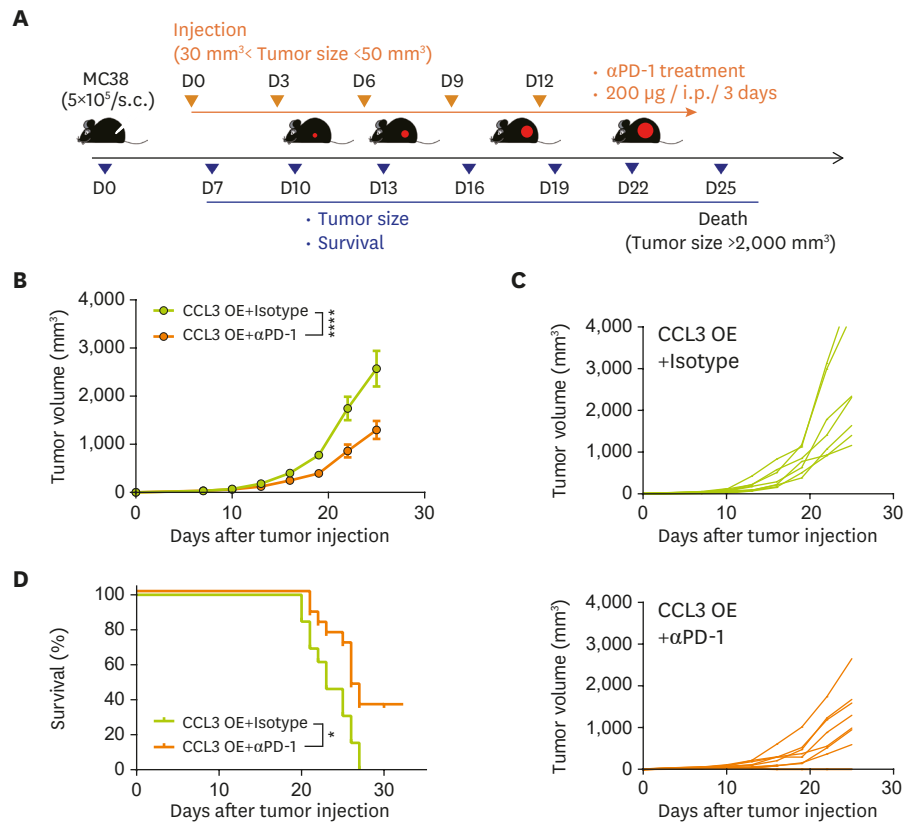
Figure 3. PD-1 blockade immunotherapy inhibits tumor growth in a tumor microenvironment with CCL3 overexpression. (A) C57BL/6 mice were s.c. inoculated 5×10^5 CCL3-OE MC38 cells. After 5 days of tumor inoculation, some mice were i.p. injected with 200 μg of anti-PD-1 monoclonal Ab. (B) The overall tumor growth and (C) individual tumor growth in mice is represented. (D) Survival of each tumor-bearing mice have been represented. Graph shows mean±SEM. Data were analyzed by (B) 2-way ANOVA with Bonferroni's multiple comparison test or (D) Mantel-Cox log-rank test. Data are pooled 2 independent experiments (n=7–8 mice/group in each experiment).
s.c., subcutaneously; i.p., intraperitoneally.
*p<0.05; ****p<0.0001.

cell supernatants after the transient transfection of MC38 cells with the plasmid. Purified rCCL3-HyFc was injected subcutaneously near the tumors every 3 days to maintain CCL3 concentration in the tumor microenvironment. Additionally, to test the dose-dependency of rCCL3-HyFc, tumor-bearing mice were injected with 2 different doses of rCCL3-HyFc or HyFc as a control (**Fig. 4A**). In previous reports, mice were subcutaneously injected with rCCL3 with 100 ng dose and they exhibited the slowed tumor growth in established tumors (31). So, we injected higher doses of rCCL3-HyFc in order to better effect than previous reports. Interestingly, treatment with 500 ng of rCCL3-HyFc led to a significant decrease in tumor growth, whereas treatment with 5,000 ng of rCCL3-HyFc did not have any beneficial effect on tumor growth inhibition compared to HyFc control treatment (**Fig. 4B and C**). In addition to tumor growth, mice treated with 500 ng of rCCL3-HyFc also showed improved survival compared to those treated with HyFc or 5,000 ng of rCCL3-HyFc (**Fig. 4D**). Furthermore, to optimize the appropriate concentration of rCCL3-HyFc, we injected 150 ng and 1,500 ng of rCCL3-HyFc to mice with the same experimental scheme and conditions. When mice were injected with 150 ng and 1,500 ng of rCCL3-HyFc, they exhibited similar tumor growth and survival rated with control HyFc injected group. But, in this repeated experiment, mice treated with 500 ng of rCCL3-HyFc displayed delayed tumor growth *in vivo* (**Supplementary Fig. 3**). In
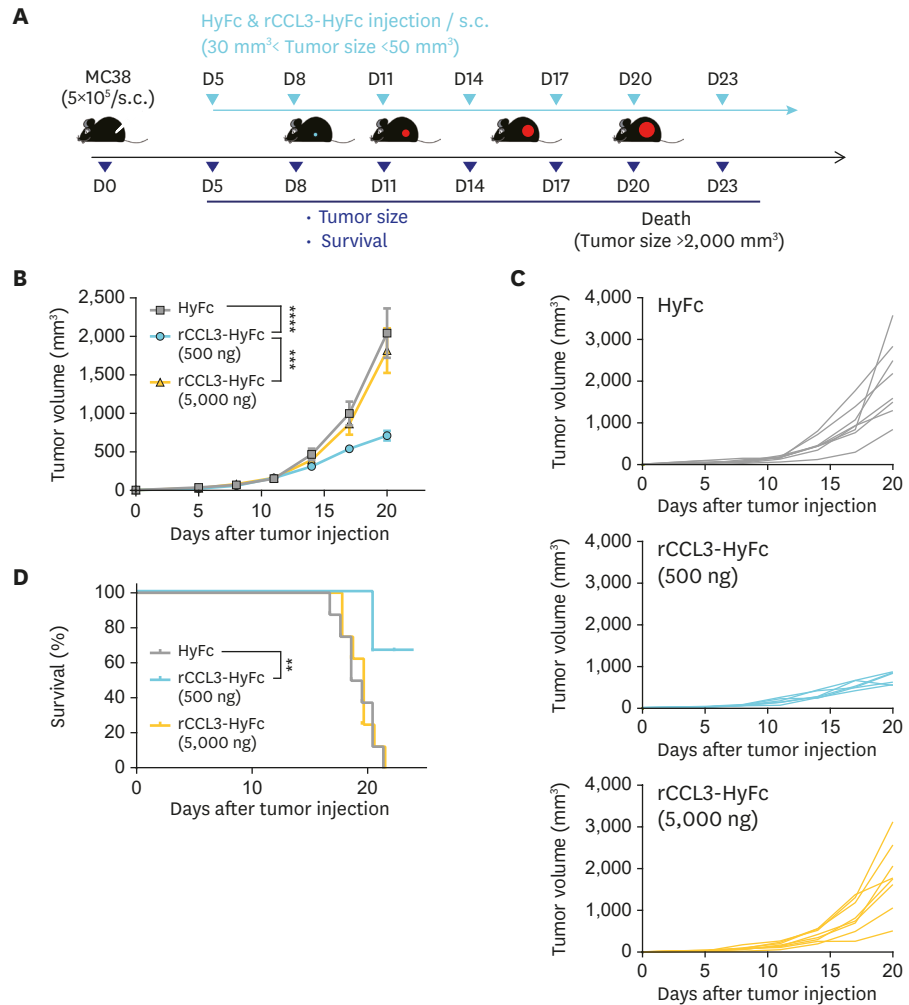
Figure 4. Effect of rCCL3-HyFc administration on tumor growth. (A) C57BL/6 mice were s.c. inoculated with 5×10^5 WT MC38 cells. Five days after tumor inoculation, some mice were s.c. injected with an indicated dose of HyFc or rCCL3-HyFc. (B) The overall tumor growth and (C) individual tumor growth of mouse are represented. (D) Survival of each of the tumor-bearing mice have been represented. Graph shows mean±SEM. Data were analyzed by (B) 2-way ANOVA with Tukey's multiple comparison test or (D) Mantel-Cox log-rank test. Data are representative of at least 2 experiments (n=6–8 mice/group in each experiment).
s.c., subcutaneously.
**p<0.01; ***p<0.001; ****p<0.0001.

summary, these data demonstrate that a continuous exogenous supply of CCL3 near tumors may have therapeutically beneficial anti-tumor response at a controlled and appropriate dosage, suggesting that rCCL3-HyFc may be used clinically as a therapeutic agent.

Therapeutically injection of rCCL3-HyFc augmented maturation of myeloid cells and function of T cells *in vivo*

In previous results, administration of specific dose of rCCL3-HyFc only exhibited the delayed tumor growth. To address the immunological differences, which triggered disparate tumor growth, between 500 ng and 5,000 ng of rCCL3-HyFc injected mice, we analyzed the tumor-infiltrating immune cell populations and their characteristics. In myeloid cell populations, they exhibited similar number of CD11b⁺Ly6C⁺, CD11b⁺Ly6G⁺, and CD11b⁺F4/80⁺ cells among 3 groups (**Fig. 5A**). In HyFc and 500 ng of rCCL3-HyFc injected group, myeloid cells dominantly consisted of CD11b⁺Ly6C⁺ and CD11b⁺F4/80⁺ cells (**Fig. 5B**). In addition,

these dominant CD11b⁺Ly6C⁺ and CD11b⁺F4/80⁺ cells in 500 ng of rCCL3-HyFc injected group displayed more activated and matured phenotype, such as up-regulation of MHCII and CD86, than these cells in other control group (**Fig. 5C**). Next, we further analyzed the tumor-infiltrating T cell responses. Although the total numbers of CD4⁺ and CD8⁺ T cells
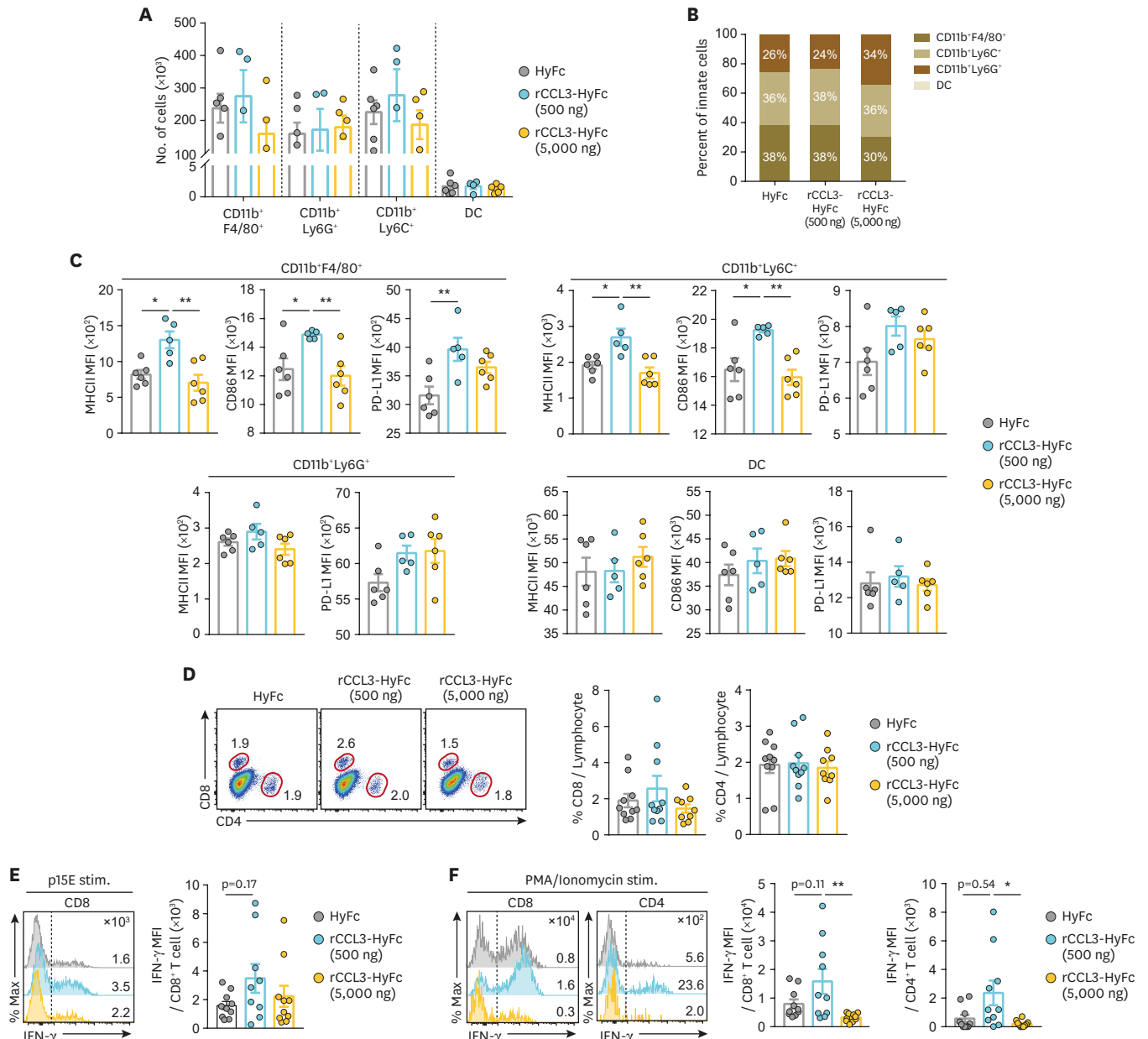

Figure 5. Matured myeloid cells and functionally augmented T cells are exhibited in rCCL3-HyFc injected mice. (A-C) At 12 days post tumor inoculation, tumor-infiltrating immune cells in each tumor-bearing mice were analyzed by flow cytometry. (A) The number of each immune cell has been summarized in the bar graph. (B) The graph summarizes the percent of indicted immune cells among 4 different immune cells. (C) The MFI of indicated marker had been summarized in the bar graph. (D-F) At 15 days post tumor inoculation, tumor-infiltrating T cells in each tumor-bearing mice were analyzed by flow cytometry. (D) The T cells were analyzed by flow cytometry. The frequencies of T cells have been summarized in the bar graph. Numbers in the plots indicated the percentage of CD4⁺ and CD8⁺ T cells. (E) Isolated tumor-infiltrating CD8⁺ T cells were re-stimulated *ex vivo* with p15E peptide and stained for CD8⁺ T cells. The graph summarizes MFI of IFN-γ in CD8⁺ T cells. Numbers in the plots indicate the MFI of IFN-γ in CD8⁺ T cells. (F) Isolated tumor-infiltrating T cells were re-stimulated *ex vivo* with PMA/Ionomycin and stained for CD8⁺ and CD4⁺ T cells. The graph summarizes MFI of IFN-γ in CD8⁺ T cells. Numbers in the plots indicate the MFI of IFN-γ in CD8⁺ T cells. Data were analyzed by 1-way ANOVA with Tukey's test. Graphs show mean±SEM. Data are representative of a single experiment (n=4–10 mice/group). MFI, mean fluorescence intensity; PMA, phorbol 12-myristate 13-acetate.
*p<0.05; **p<0.01.

were similar among 3 groups (**Fig. 5D**), the function of IFN-γ production in CD8+ T cells was elevated in 500 ng of rCCL3-HyFc injected group when they were re-stimulated with p15E peptide *ex vivo* (**Fig. 5E**). Also, when CD4+ and CD8+ T cells were re-stimulated with PMA/Ionomycin *ex vivo*, the T cells in 500 ng of rCCL3-HyFc injected group exhibited augmented function and this tendency was down-regulated in 5,000 ng of rCCL3-HyFc injected group (**Fig. 5F**). In summary, when mice were administrated with specific dose of 500 ng of rCCL3-HyFc, they had myeloid cells with increased MHCII and CD86, and functionally enhanced T cells. The immunological improvement might help to delay tumor progression. In contrast, *in vivo* treatment with high amount of rCCL3-HyFc did not result in the maturation of myeloid cells and the functional enhancement of tumor-infiltrating T cells. It would be still required to investigate how different doses of rCCL3-HyFc can control anti-tumor immune response and tumor progression differently *in vivo*.

Combination therapy of rCCL3-HyFc with PD-1 blockade exhibited substantial delayed tumor growth *in vivo*

In CCL3-enriched tumor microenvironment, we confirmed that tumor growth was delayed when mice were additionally injected with PD-1 monoclonal Ab (**Fig. 3**). Since rCCL3-HyFc also therapeutically reduced tumor growth, we expected that combination therapy of rCCL3-HyFc with PD-1 blockade would further delayed tumor growth. To validate this hypothesis, mice were intraperitoneally injected with αPD-1 or subcutaneously treated with rCCL3-HyFc or combination of both agents *in vivo* (**Fig. 6A**). When mice were treated with rCCL3-HyFc or αPD-1 alone, they exhibited a delayed tumor growth to a similar extent (**Fig. 6B and C**). Interestingly, combination of rCCL3-HyFc and αPD-1 led to more dramatic control of tumor growth and better survival than rCCL3-HyFc or αPD-1 alone, which seemed to be synergistic (**Fig. 6B-D**). This data indicates that the anti-tumor effect caused by rCCL3-HyFc or αPD-1 is mechanistically distinct, suggesting the clinical use of rCCL3-HyFc combined with αPD-1 to improve the efficacy of current αPD-1 therapy.

DISCUSSION

Chemokines are important for the *in vivo* migration and homeostasis of immune cells. Because of their ability to alter the profile of immune cells, they are involved in the protection of the host from infections or tumors. Here, we focused on the role of CCL3 in reshaping immune cell populations and in improving the anti-tumor immune response. We observed that a CCL3-enriched tumor microenvironment not only reduced tumor growth but also improved the survival rate compared to the parental tumor microenvironment, suggesting CCL3-mediated modulation of immune cell populations. Indeed, CD8+ T cells from tumor microenvironments where CCL3 was overexpressed showed enhanced proliferation and function. Additionally, these tumor microenvironments exhibited an increase in the DC numbers. Furthermore, we observed that PD-1 blockade had a synergistic effect on tumor growth inhibition in CCL3-enriched niches. To reproduce *the in vivo* CCL3-enriched tumor microenvironment, we generated long-lasting rCCL3-HyFc proteins and injected them near the tumors. Therefore, an appropriate dose of rCCL3-HyFc could augment tumor regression and survival rate along with matured myeloid cells and functionally enhanced T cells, and suggests that an optimized dosage of rCCL3-HyFc may have clinical applications as a potential adjuvant for boosting present immunotherapies.
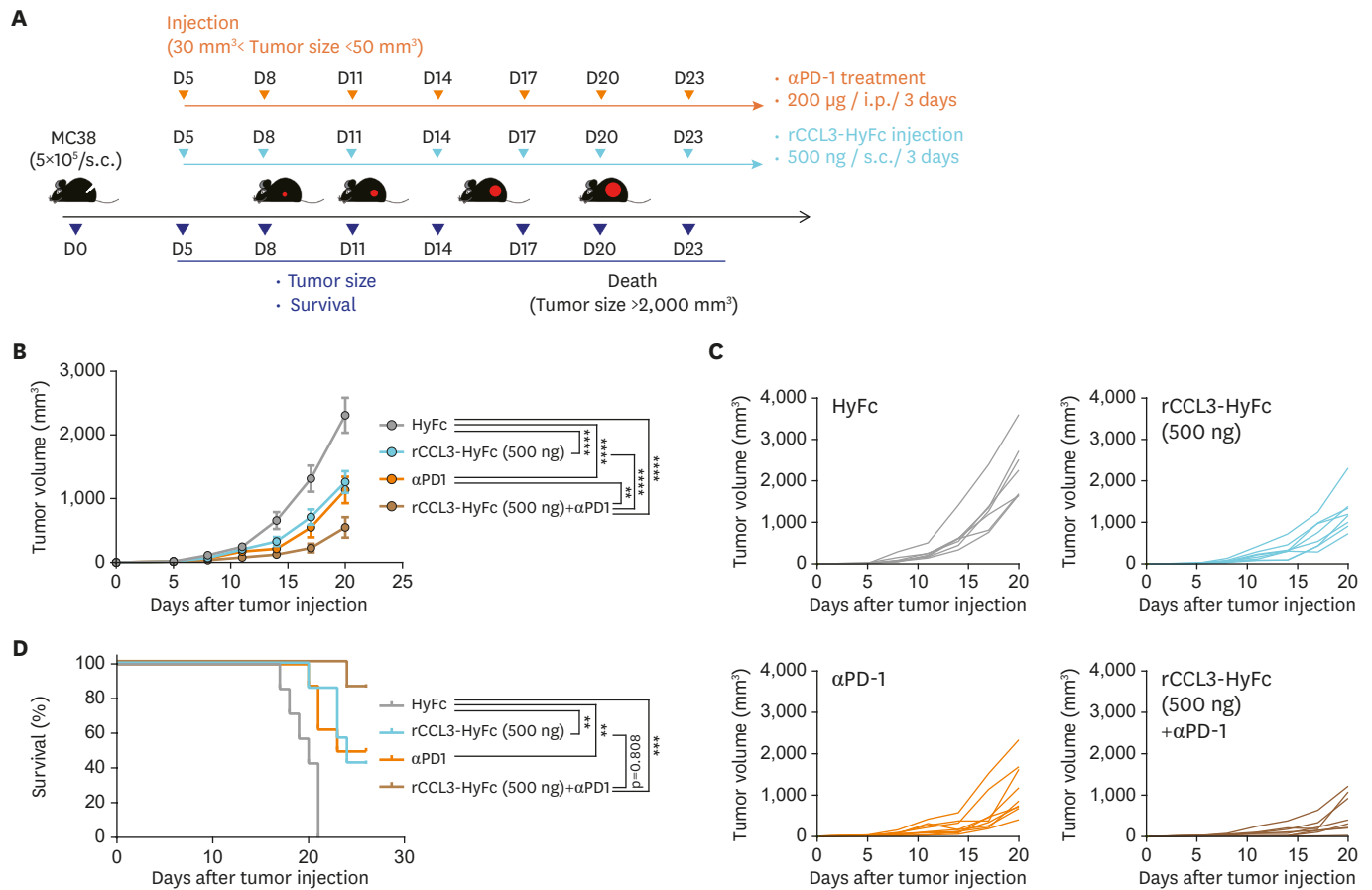
Figure 6. A rCCL3-HyFc immunotherapy is significantly enhanced by combination with PD-1 ICB therapy *in vivo*. (A) C57BL/6 mice were s.c. inoculated 5×10⁵ WT MC38 cells. After 5 days of tumor inoculation, some mice were i.p. injected with 200 μg of anti-PD-1 monoclonal Ab or s.c. with an indicated dose of rCCL3-HyFc or combination therapy. (B) The overall tumor growth and (C) individual tumor growth of mouse are represented. (D) Survival of each of the tumor-bearing mice have been represented. Graph shows mean±SEM. Data were analyzed by (B) 2-way ANOVA with Tukey's multiple comparison test or (D) Mantel-Cox log-rank test. Data are representative of a single experiment (n=7–9 mice/group in each experiment).
s.c., subcutaneously; i.p., intraperitoneally.
**p<0.01; ***p<0.001; ****p<0.0001.

In our present study, we observed that treating mice with 5,000 ng of rCCL3-HyFc did not attenuate tumor growth or improve survival. However, simply increasing the dosage may not be an effective alternative for treating patients with tumor, because these chemokines may exert adverse effects on immune cells during infection or tumor metastasis. The CCL2 and CCR2 axis promoted migration of CCR2 expressing inflammatory monocytes and macrophages into the tumor niche. Moreover, CCL2 expression correlated with the infiltration of tumor-associated macrophages (TAMs), which was associated with poor prognosis in breast cancer patients (32,33). Additionally, CCL2 may also activate TAMs to secrete CCL3, which in turn may lead to the recruitment of additional TAMs and promote tumor metastasis (34). During simian immunodeficiency virus and HIV infections, CCL3 was reported to play a dominant role in the chemotactic recruitment of MDSCs (31). Furthermore, CCL2 and CCL3 can induce the production of matrix metalloproteinase 9 in monocytes. Matrix metalloproteinase 9 induces degradation of the matrix and facilitates tumor cell extravasation (35,36). Therefore, treating mice with a high dose of rCCL3-HyFc (5,000 ng) in us *in vivo* experiments might have induced pro-tumorigenic effects, such as recruitment of MDSCs to suppress immune cells involved in tumor regression and promote tumor extravasation. Therefore, for clinical applications of chemokine therapy, administering

an appropriate dose that has clear anti-tumorigenic effects on immune cells may be an important factor for successful therapy.

As mentioned above, the injection dosage may have important implications on treatment when chemokine therapy is used alone. Therefore, administering chemokines at low doses as adjuvants to induce immune activation, in combination with other therapeutic agents, might be a suitable alternative. Intravenous injection of CCL3 in mice increased the *in vivo* frequency of DCs (37). Another study revealed that among the different DC populations, CD103^{+} DCs were most effective in transporting tumor Ags to the draining lymph nodes and in activating tumor-specific CD8^{+} T cells in the murine melanoma environment. Furthermore, administering FLT3 ligand (FLT3L) and Poly I:C in combination with the BRAF and PD-L1 immunotherapy expanded CD103^{+} DC populations elevated the *in vivo* efficacy of BRAF and PD-L1 immunotherapy (38). Additionally, tumor cell-derived VEGF potently inhibits FLT3L activity and negatively affects the differentiation of conventional DCs (39). Therefore, blocking VEGF signaling might augment DC differentiation and FLT3L activity in the tumor microenvironment. The improved activity of PD-1 blockers in a CCL3-enriched microenvironment may be further elevated by administering FLT3L, Poly I:C, and VEGF blockers, which are strong activators of CD103^{+} DCs that are recruited into tumors by CCL3. However, a detailed analysis of the immunological factors that are altered would be required for such an investigation.

In summary, our study focused on the positive role of CCL3 in controlling tumor growth, and examined the possibility of using rCCL3-HyFc as a clinical therapeutic for cancer. We conclude that rCCL3-HyFc can be a promising therapeutic agent if the appropriate dosage and effectiveness of the combination therapy are verified in clinical phase. In addition to validating effectiveness, a detailed immunological analysis of the patient being treated with rCCL3-HyFc proteins would be helpful in developing a more effective immunotherapy for cancer patients.

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SUPPLEMENTARY MATERIALS

Supplementary Figure 1
The CCL3 levels in blood from CCL3-OE bearing mice. At indicated time points after tumor inoculation, the serum was collected from CCL3-OE bearing mice. Then, the protein level of CCL3 was analyzed by ELISA. The dashed line indicated the detectable level of CCL3 by ELISA (15.6 pg/ml).

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Supplementary Figure 2

Flow cytometry-gating strategy for elucidation of DC and myeloid cell populations in the tumor microenvironment. The tumor was harvested and cells were stained for expression of the indicated cell surface markers. To define immune cells in tumors, total immune cells were pre-gated on a single-cell, FSC-A^{high} SSC-A^{high} (leukocytoke), a live cell, and then on Ly5.2$^+$ cells.

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Supplementary Figure 3

Effect of diverse doses of rCCL3-HyFc injection to tumor growth. C57BL/6 mice were subcutaneously inoculated with 5×10^5 WT MC38 cells. Five days after tumor inoculation, some mice were subcutaneously injected with an indicated dose of HyFc or rCCL3-HyFc. (A) The overall tumor growth and (B) individual tumor growth of mouse are represented. (C) Survival of each of the tumor-bearing mice have been represented. Graph shows mean±SEM. Data were analyzed by (A) 2-way ANOVA with Tukey's multiple comparison test or (C) Mantel-Cox log-rank test. Data are representative of single experiment (n=9–11 mice/group in each experiment).

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