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Original Articles

CCR4 is critically involved in effective antitumor immunity in mice bearing intradermal B16 melanoma

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ABSTRACT

CCR4 is a major chemokine receptor expressed by Treg cells and Th17 cells. While Treg cells are known to suppress antitumor immunity, Th17 cells have recently been shown to enhance the induction of antitumor cytotoxic T lymphocytes. Here, CCR4-deficient mice displayed enhanced tumor growth upon intradermal inoculation of B16-F10 melanoma cells. In CCR4-deficient mice, while IFN-γ+CD8+ effector T cells were decreased in tumor sites, IFN-γ+CD8+ T cells and Th17 cells were decreased in regional lymph nodes. In wild-type mice, CD4+IL-17A+ cells, which were identified as CCR4+CD44+ memory Th17, were found to be clustered around dendritic cells expressing MDC/CCL22, a ligand for CCR4, in regional lymph nodes. Compound 22, a CCR4 antagonist, also enhanced tumor growth and decreased Th17 cells in regional lymph nodes in tumor-bearing mice treated with Dacarbazine. In contrast, CCR6 deficiency did not affect the tumor growth and the numbers of Th17 cells in regional lymph nodes. These findings indicate that CCR4 is critically involved in regional lymph node DC-Th17 cell interactions that are necessary for Th17 cell-mediated induction of antitumor CD8+ effector T cells in mice bearing B16 melanoma.

Introduction

Chemokines are small secreted polypeptides that play important roles in a wide range of inflammatory and immunological processes by recruiting selected subsets of leukocytes. CC chemokine receptor 4 (CCR4) is the receptor for MDC/CCL22 and TARC/CCL17 [1,2]. Initial reports suggested that CCR4 was selectively expressed by Th2 cells [3]; however, regulatory T (Treg) cells, Th17 cells, and Th22 cells have also been shown to dominantly express CCR4 [4–6].

Treg cells are the dedicated suppressors of diverse immune responses and are critically involved in immunological self-tolerance and maintenance of homeostasis [7,8]. Furthermore, tumor-associated Treg cells are associated with reduced antitumor immunity and the accumulation of Treg cells in tumor tissues is predictive of a reduction in patient survival [4,9]. Treg cells suppress tumor-specific T cell responses and contribute to tumor growth. Previous studies have suggested that blocking Treg cell trafficking to tumors represents a potent strategy for enhancing host antitumor immunity [10]. Tumor Treg cells are known to express CCR4 and to migrate toward CCL22 and CCL17 produced in the tumor microenvironment [4,9]. Recently, CCR4 antagonists have been shown to transiently inhibit the recruitment of Treg cells to the site of immunization and have a significant adjuvant activity in cancer vaccines [11].

Th17 cells are a CD4+ T-helper cell subset, characterized by production of IL-17 which is a potent inflammatory cytokine [12]. Th17 cells share CCR4 as a major trafficking receptor with Treg cells [5]. Th17 cells mediate inflammation associated with various autoimmune diseases, such as rheumatoid arthritis, multiple sclerosis, and inflammatory bowel disease [13,14]. In addition, it was only recently shown that Th17 cells directly expanded tumor-specific CD8+ T-cell responses via peptide:MHC I and IL-2 signaling, indicating that

Abbreviations: ADCC, antibody-dependent cellular cytotoxicity; ATL, adult T-cell leukemia/lymphoma; BSA, bovine serum albumin; CCR, CC chemokine receptor; CXCR, CXC chemokine receptor; DC, dendritic cell; DMEM, Dulbecco's modified Eagle's medium; DTIC, dacarbazine; FBS, fetal bovine serum; TBS, Tris-buffered saline; TBS-T, TBS containing 0.1% (v/v) Triton X-100; Treg, regulatory T; WT, wild-type.

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Th17 cells play an important role in preventive and therapeutic antitumor immunity by the induction of CD8+ effector T cells [15,16].

Both Treg cells and Th17 cells are known to express CCR4 as a major trafficking receptor. Thus, for the development of cancer therapy by targeting CCR4, the understanding of the roles of CCR4 in host antitumor immunity is important. In this study, we investigated the role of CCR4 in antitumor immunity of mice bearing B16 melanoma.

Materials and methods

Reagents

Compound 22 ((R)-{4-{4-[(2,4-dichlorobenzyl)amino]pyrido[2,3-d]pyrimidin-2-yl}piperazin-1-yl}-piperidin-2-yl-methanone) (99.5% purity) was synthesized based on the published information [17] with the help of the Division of Computational Drug Design and Discovery, Kindai University Faculty of Pharmacy (Osaka, Japan).

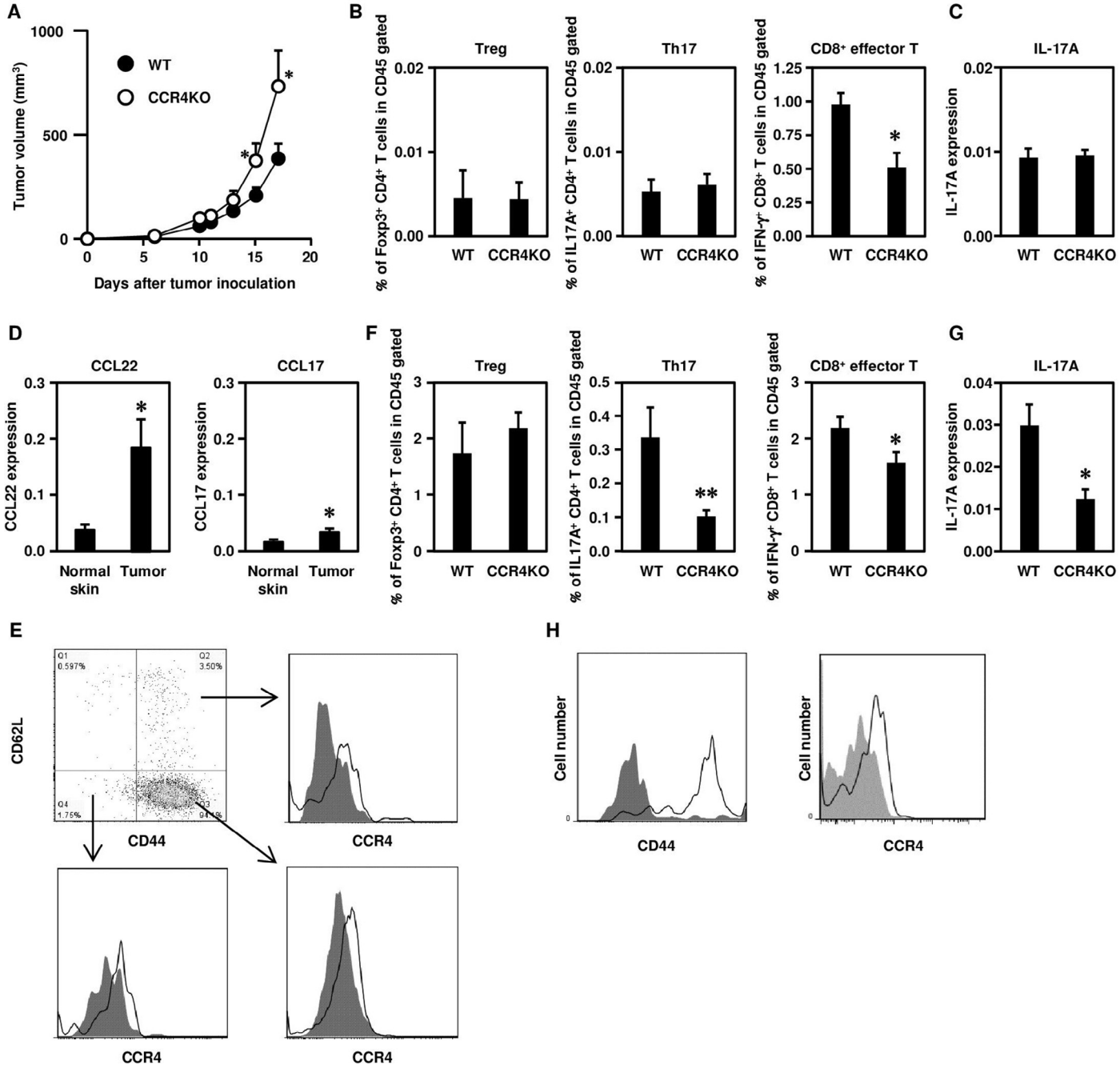

Fig. 1. The impact of CCR4-deficiency on antitumor immunity. CCR4-deficient (CCR4KO) or wild-type (WT) mice were inoculated with B16-F10 tumor cells. (A) Tumor volume was monitored. (B, F) Fifteen to eighteen days after tumor inoculation, the tumor tissues and the regional lymph nodes were harvested from B16-F10-bearing mice. (B) Foxp3+CD4+ Treg cells, IL-17A+CD4+ T cells, and IFN-γ+CD8+ T cells in the CD45 gate of the tumor tissues were analyzed by flow cytometry. (C) IL-17A expression in the tumor tissues was investigated by real-time PCR. (D) CCL22 and CCL17 expression in the tumor tissues was examined by real-time PCR. (E) CCR4 expression on CD44+CD62L+CD8+ T cells, CD44+CD62L-CD8+ T cells, and CD44-CD62L-CD8+ T cells in the tumor tissues was analyzed by flow cytometry. (F) Foxp3+CD4+ Treg cells, IL-17A+CD4+ T cells, and IFN-γ+CD8+ T cells in the regional lymph nodes were analyzed by flow cytometry. (G) IL-17A expression in the regional lymph nodes was examined by real-time PCR. (H) CD44 expression on IL-17A+CD4+ cells and CCR4 expression on IL-17A+CD44+CD4+ T cells were analyzed by flow cytometry. The data are expressed as mean ± SE of results from 15 mice (A) or 5 mice (B, C, D, F, G). The representative data are shown from at least three independent experiments (E, H). $^{*}p < 0.05$. $^{**}p < 0.01$.

Animals and cell line

B16-F10 cells (a mouse melanoma cell line) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). A mouse pre-B cell line L1.2 was kindly provided by Dr. E. Butcher (Stanford University School of Medicine, Stanford, CA). The panels of L1.2 cell lines that stably express mouse chemokine receptors were generated using a retroviral vector pMX-IRES-EGFP as described previously [18]. Wild-type (WT) C57BL/6J mice were purchased from Japan Clea (Tokyo, Japan). CCR4- and CCR6-deficient mice were purchased from The Jackson laboratory (Bar Harbor, ME) and backcrossed with C57BL/6J for at least 12 generations. Mice were maintained in specific pathogen-free conditions. Female mice of 6 weeks old were used for the experiments. All animal experiments in the present study were approved by the Center of Animal Experiments, Kindai University, and performed in accordance with the institutional guidelines.

Evaluation of tumor growth

Mice were intradermally inoculated with 1×10^6 B16-F10 cells in the right flank. Tumor growth was monitored two or three times a week by measuring the major and minor axes of the tumors using microcalipers, and tumor volume was calculated by the following formula: (tumor volume; mm^3) = (major axis; mm) × (minor axis; mm)^2 × 0.5236. The mice were euthanized when one of the two measurements was >20 mm. For evaluating the effect of Compound 22 [17], a CCR4 antagonist, on anticancer drug treatment, mice bearing tumors with the diameter of 5–6 mm were intraperitoneally injected with 100 µl 0.1% DMSO/PBS containing Dacarbazine (DTIC; Sigma-Aldrich) without or with Compound 22.

Cell isolation

To isolate tumor-infiltrating lymphocytes, tumor tissues were removed from mice and were incubated at 37 °C for 60 min in RPMI1640 supplemented with 10% FBS, 0.24 mg/ml collagenase A (Roche; Basel, Switzerland), and 40 U/ml DNase I (Thermo Fisher Scientific Inc.). After shaking vigorously for 10 s, the resulting suspension was filtered through a 70-µm cell strainer, centrifuged, and the cell pellet was suspended in 40% Percoll in 0.9% NaCl. This cell suspension was overlaid onto 80% Percoll in 0.9% NaCl and centrifuged for 20 min. Lymphocytes were collected at the interface between the discontinuous Percoll gradient. Cells from spleen and lymph nodes were mechanically separated by passing through a 70-µm cell strainer.

Flow cytometry

PerCP/Cy5.5-labeled anti-CD45 (clone 30-F11), AlexaFluor488-labeled anti-CD4 (clone GK1.5), AlexaFluor488-labeled anti-CD8 (clone 53-6.7), AlexaFluor647-labeled anti-Foxp3 (clone 150D), PE-labeled anti-IL-17A (TC11-18H10.1), APC/Cy7-labeled anti-CD44 (clone IM7), APC-labeled anti-CD62L (clone MEL-14), APC-labeled anti-IFN-γ (clone), APC-labeled anti-CCR4 (clone 2G12), and purified anti-mouse CD16/32 (clone 2.4G2) were purchased from Biolegend (San Diego, CA). Cells were suspended in ice-cold 0.1% bovine serum albumin (BSA) and 0.05% sodium azide (staining buffer) and treated with anti-mouse CD16/32 for 20 min to block the Fc receptors. After washing, cells were incubated for 30 min with the mixture of anti-CD45, anti-CD4, anti-CD8, anti-CD44, or anti-CD62L. For intracellular staining, cells were then fixed and permeabilized (Cytofix/Cytoperm kit; BD Biosciences, San Diego, CA) and subsequently stained intracellularly with anti-IFN-γ, anti-IL17A, or anti-Foxp3. After washing, cells were immediately analyzed on a FACSFortessa (BD Biosciences) and analyzed with FlowJo software (Tree Star Inc., Ashland, OR). For staining of IL-17A and IFN-γ, cells were stimulated with leukocyte activation cocktail (BD Biosciences) for 4 hours.

Double immunofluorescence staining

Regional lymph nodes were removed fifteen to eighteen days after tumor inoculation and stored in 4% (w/v) paraformaldehyde. Frozen thin sections were washed and blocked with 50 mM glycine in Tris-buffered saline (TBS; 0.1 M Tris–HCl, pH 7.5, 0.15 M NaCl) at 37 °C for 2 h to reduce non-specific fluorescence staining. The sections were washed with TBS containing 0.1% (v/v) Triton X-100 (TBS-T), blocked with 3% (v/v) BSA in TBS-T, and incubated with the mouse monoclonal anti-CD209 antibody (1:100) or mouse monoclonal IL-17A antibody (1:100) at room temperature overnight. Following extensive washing, sections were incubated with Alexa Fluor 488 or 555 anti-mouse IgG (1:300; BD Biosciences Pharmingen, San Diego, CA, USA) at room temperature for 80 min. Next, sections were washed and incubated with a rabbit polyclonal anti-CCL22 antibody (1:100) at room temperature overnight. Following extensive washing, sections were further incubated with Alexa Fluor 488 or 555 anti-rabbit IgG (1:300; BD Biosciences Pharmingen) at room temperature for 80 min.

Real-time PCR

Quantitative real-time PCR was performed on an ABI7000 (Applied Biosystems, Foster City, CA) using a Kapa SYBR Fast qPCR Kit (Kapa Biosystems, Woburn, MA). The conditions of PCR were 60 °C for 20 min, 94 °C for 5 min, and then 40 cycles of

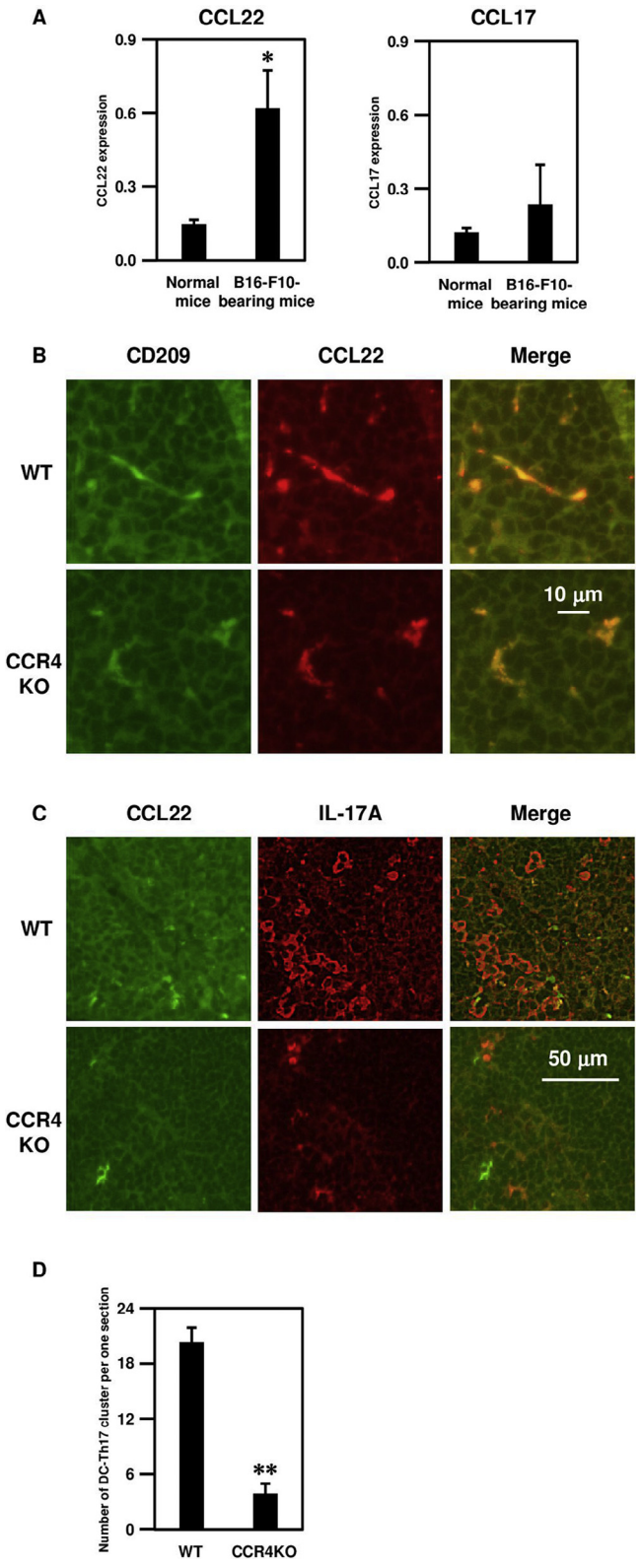

Fig. 2. Fluorescent immunohistochemistry of the regional lymph nodes. (A) The expression levels of CCL22 and CCL17 mRNAs in the lymph nodes of WT mice and B16-F10-bearing mice were examined by real-time PCR. (B, C) CCR4-deficient (CCR4KO) or wild-type (WT) mice were intradermally inoculated with B16-F10 cells. Frozen sections of the draining lymph nodes from these mice were prepared 15–18 days after tumor inoculation. The frozen lymph node sections were double stained for CD209 (green) and CCL22 (red) (B), and CCL22 (green) and IL-17A (red) (C). Representative data are shown. (D) The number of CCL22+DC-Il-17A+ Th17 cell clusters per one lymph node section was counted. The data are expressed as mean ± SE of results from at least three frozen lymph node sections. $^*p < 0.05$. $^{**}p < 0.01$.

94 °C for 30 s (denaturation) and 55 °C for 30 s (annealing extension). The primers used were as follows:

+5′-TCTGATGCAGGTCCCTATGGT-3′ and –5′-TTATGGAGTAGCTTCTTCAC-3′ for CCL22;
+5′-TGAGGTCACTTCAGATGCTGC-3′ and –5′-ACCAATCTGATGGCCTTCTTC-3′ for CCL17;
+5′-CAGCAGCGATCATCCCTCAAAG-3′ and –5′-CAGGACCAGGATCTCTTGCTG for IL-17A;
+5′-ACCACAGTCCATGCCATCAC-3′ and –5′-TCCACCACCCTGTTGCTGTA-3′ for GAPDH.

CCL22 expression was normalized to the amount of GAPDH mRNA and quantified according to the $2^{-\Delta Ct}$ method.

Chemotaxis assay

Chemotaxis assays were performed using 96-well chemoTx chamber (Neuroprobe, Gaithersburg, MD). Cells that migrated into the lower wells were lysed with 0.1% Triton X-100 and quantified using PicoGreen dsDNA reagent (Thermo Fisher Scientific, Waltham, MA). All the recombinant human and mouse chemokines were all purchased from R&D Systems (Minneapolis, MN).

Statistical analysis

Student's t test was used for statistical analysis, considering $p < 0.05$ as being statistically significant.

Results

To investigate the role of CCR4 in antitumor immunity, we monitored the tumor growth after intradermal inoculation of B16-F10 melanoma cells in WT mice and CCR4-deficient mice. As shown in Fig. 1A, tumor growth was much enhanced in CCR4-deficient mice compared to WT mice. This suggested that CCR4 contributed to induction of antitumor immunity.

Next, we analyzed T cell subsets in the tumor tissues and the regional lymph nodes. As shown in Fig. 1B, there were no significant differences in the percentages of Foxp3+CD4+ Treg cells and IL-17A+CD4+ Th17 cells in tumor-infiltrating lymphocytes between CCR4-deficient mice and WT mice. We also examined IL-17A mRNA expression in tumor tissues by real-time PCR analysis. As shown in Fig. 1C, the expression levels of IL-17A mRNA in B16-F10 tumor tissues were quite similar between CCR4-deficient mice and WT mice. On the other hand, the percentages of IFN-γ+CD8+ T cells, which have a direct cytotoxic activity toward tumor cells, were significantly decreased in the tumor tissues of CCR4-deficient mice (Fig. 1B). Chemokine receptors implicated in skin homing include CCR4 and CCR10, and CCR4 is known to be highly expressed on the surface of most of skin-homing cells [19,20]. However, there was little evidence about a role of CCR4 in CD8+ T cells infiltrating skin-resident tumor tissues. It was reported that CCL22 and CCL17, CCR4 ligands, were expressed in melanoma environment [21], and CD11c+ conventional dendritic cells (DCs) were the major source of CCL22 in melanoma tumor tissues [22]. We confirmed the increased expression of CCL22 and CCL17 mRNAs in B16-F10 tumor tissues compared with normal skin tissues by real-time PCR analysis (Fig. 1D). Furthermore, we investigated the expression of CCR4 on the tumor-infiltrating CD8+ T cells. As shown in Fig. 1E, most of the tumor-infiltrating CD8+ T cells were CD44+CD62L– effector memory T cells and all subsets of the tumor-infiltrating CD8+ T cells weakly expressed CCR4.

Next, we examined lymphocytes in the regional lymph nodes where CD8+ effector T cells were induced. In a murine melanoma model, CD8+ effector T cells have recently been reported to be induced by Th17 cells in the regional lymph nodes [15,16]. Therefore, we investigated Treg cells, Th17 cells, and CD8+ effector T cells in the regional lymph nodes of tumor bearing mice. The percentages of IL-17A+CD4+ Th17 cells and IFN-γ+CD8+ T cells, but not Treg cells, were significantly reduced in B16-F10-bearing CCR4-deficient mice (Fig. 1F). We also examined IL-17A mRNA expression in the regional lymph nodes by real-time PCR analysis. As shown in Fig. 1G, IL-17A mRNA expression in the regional lymph nodes decreased in CCR4-deficient mice compared to WT mice, consistent with the results of flow cytometric analyses. We also confirmed that most of the CD4+IL-17A+ cells were positive for CCR4 and expressed a

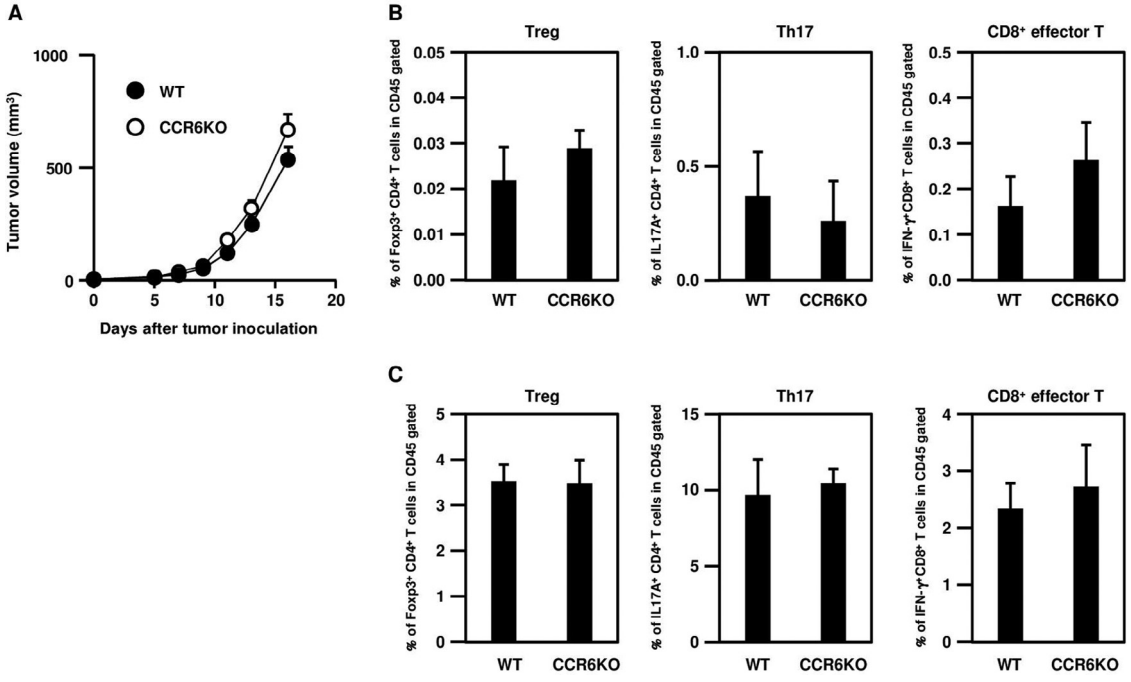

Fig. 3. The impact of CCR6-deficiency on antitumor immunity. CCR6-deficient (CCR6KO) or wild-type (WT) mice were inoculated with B16-F10 tumor cells. (A) Tumor volume was monitored. (B, C) Fifteen to eighteen days after tumor inoculation, the tumor tissues and the regional lymph nodes were harvested from B16-F10-bearing mice. Foxp3+CD4+ Treg cells, IL-17A+CD4+ T cells, and IFN-γ+CD8+ T cells in the CD45 gate of the tumor tissues (B) and the regional lymph nodes (C) were analyzed by flow cytometry. The data are expressed as mean ± SE of results from 20 mice (A) or 5 mice (B, C).

memory marker, CD44, in the regional lymph nodes (Fig. 1H). These results suggested that CCR4 played an important role in induction of Th17 cells but not Treg cells in regional lymph nodes, and the reduction of Th17 cells in the regional lymph nodes resulted in less efficient induction of CD8+ effector T cells.

We next investigated the mechanism for the decrease of Th17 cells in the regional lymph nodes of tumor-bearing CCR4-deficient mice. It has been reported that CCL22 and CCL17 were expressed by antigen-loaded DCs and CCR4 was expressed on effector/memory T cells including Th17 cells [23–25], suggesting that the CCL22 and CCL17/CCR4 axes were involved in the formation of DC-T cell clusters and expansion of Th17 cells in regional lymph nodes. We confirmed the increased expression of CCL22 and CCL17 in the regional lymph nodes of tumor bearing mice by real-time PCR analysis (Fig. 2A). Furthermore, the expression levels of CCL22 mRNA were much higher than those of CCL17 mRNA in the regional lymph nodes (Fig. 2A) and B16-F10 tumor tissues (Fig. 1D). Since CCL22 binds CCR4 with a higher affinity and has a stronger chemotactic activity than CCL17 [1,2], CCL22 rather than CCL17 may play the major role in the present experimental conditions. Therefore, we focused here on CCL22. We performed immunofluorescence staining of the regional lymph nodes to compare the formation of CCL22+DC-Th17 cell clusters between B16-F10-bearing WT mice and CCR4-deficient mice. We first confirmed CCL22 expression by CD209+ DCs in both WT and CCR4-deficient mice by double staining of CD209 and CCL22 in the regional lymph nodes (Fig. 2B). Next, we performed immunofluorescence staining of IL-17A and found that IL-17A was expressed in small round cells (probably lymphocytes) (Fig. 2C). Finally, by double staining of CCL22 and IL-17A, we demonstrated that IL-17A+ cells interacted with CCL22+ DCs in WT mice, supporting that the CCL22+DC-Th17 cell clusters were formed in regional lymph nodes. Conversely, in CCR4-deficient mice, the numbers of IL-17A+ cells and the clusters of CCL22+DC-Th17 cells significantly decreased (Fig. 2D). These results suggested that memory Th17 cells contacted with DCs via the CCR4/CCL22 axis, which led to their expansion.

Since Th17 cells are known to express CCR6 in addition to CCR4 [5], we next examined the development of antitumor immunity in CCR6-deficient mice. There were no significant differences in B16 melanoma tumor growth between CCR6-deficient mice and WT mice (Fig. 3A). Additionally, the percentages of Th17 cells, Treg cells, and CD8+ effector T cells in the tumor tissues and the regional lymph nodes of CCR6-deficient mice were not significantly different from those of WT mice (Fig. 3B and C). These findings demonstrated that CCR6 is not involved in the expansion of memory Th17 cells in regional lymph nodes through the formation of DC-Th17 cell clusters, which was necessary for Th17 cell-mediated induction of CD8+ effector T cells.

Finally, we investigated the effect of Compound 22, a CCR4 antagonist, on mice bearing B16-F10 melanoma and being treated with DTIC. Recently, Compound 22 has been reported to efficiently inhibit the CCR4-mediated Ca2+ mobilization and chemotaxis in vitro, and Compound 22 is a >500-fold less effective inhibitor of other chemokine receptors (CCR2, CCR3, and CXCR3) [17,26]. Our previous report demonstrated that Compound 22 selectively inhibited CCR4-mediated cell migration by using a panel of murine L1.2 cells stably expressing the whole set of human chemokine receptors (CCR1 to 10, CXCR1 to 4, CX3CR1, and XCR1), and Compound 22 suppressed Th2-mediated allergic inflammation by blocking infiltration of Th2 cells which expressed CCR4 [26]. However, the specificity of Compound 22 to mouse chemokine receptors has not been clarified. Therefore, we examined the inhibitory specificity of Compound 22 using a panel of L1.2 cells stably expressing the following mouse chemokine receptors: CCR1 to 10, CXCR2 to 4, CX3CR1, and XCR1. As shown in Fig. 4A, Compound 22 exhibited selective inhibitory activity on cell migration via CCR4, and the inhibitory concentration

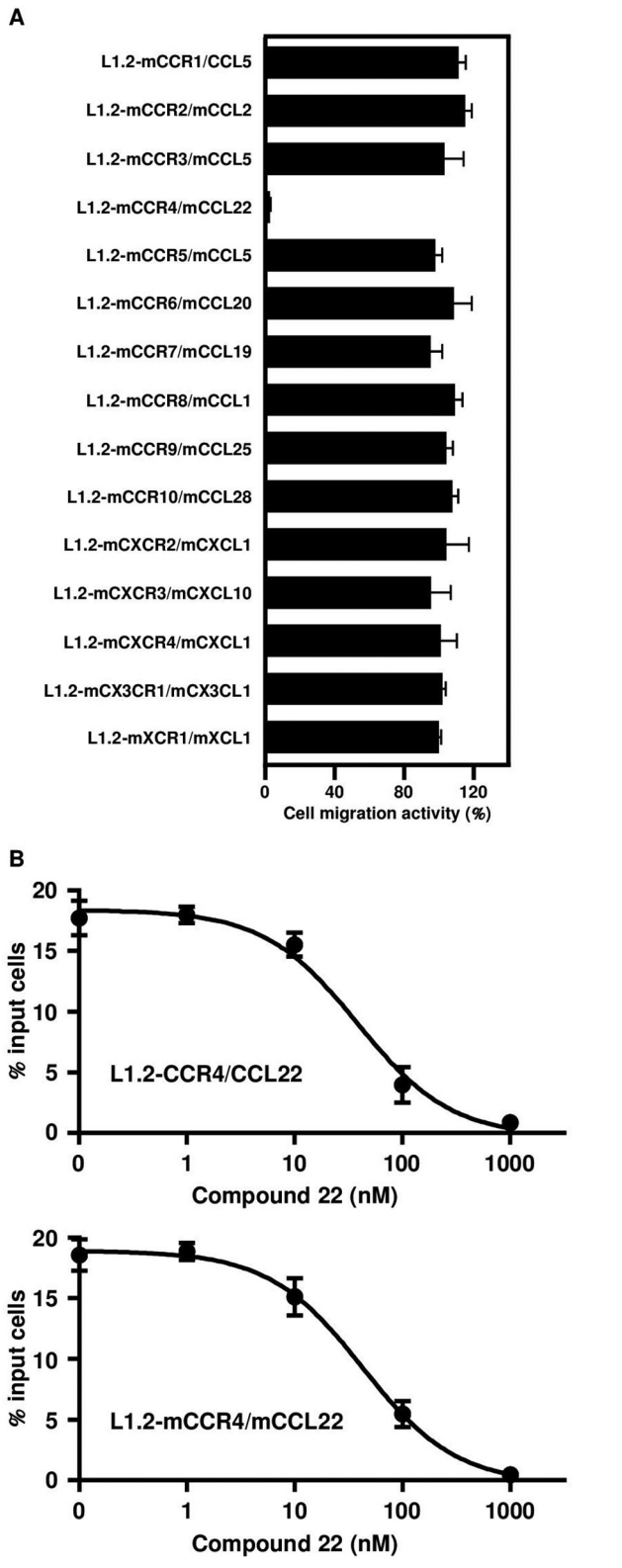

Fig. 4. Compound 22 selectively inhibits chemotaxis via mouse CCR4. (A) Chemotaxis assay was performed using the following cells and corresponding chemokines in the presence of Compound 22 at 1 μM: L1.2-mCCR1/mCCL5, L1.2-mCCR2/mCCL2, L1.2-mCCR3/mCCL5, L1.2-mCCR4/mCCL22, L1.2-mCCR5/mCCL5, L1.2-mCCR6/mCCL20, L1.2-mCCR7/mCCL19, L1.2-mCCR8/mCCL1, L1.2-mCCR9/mCCL25, L1.2-mCCR10/mCCL28, L1.2-mCXCR2/mCXCL1, L1.2-mCXCR3/mCXCL10, L1.2-mCXCR4/mCXCL12, L1.2-mCX3CR1/mCX3CL1, and L1.2-mXCR1/mXCL1. (B) Chemotaxis assay was performed using L1.2-CCR4 and L1.2-mCCR4 in the presence of Compound 22 at the indicated concentrations. IC_{50} was calculated. Each chemokine was used at 10 nM. Cell migration was expressed as % input cells. Results from three separate experiments are shown as mean ± SE.

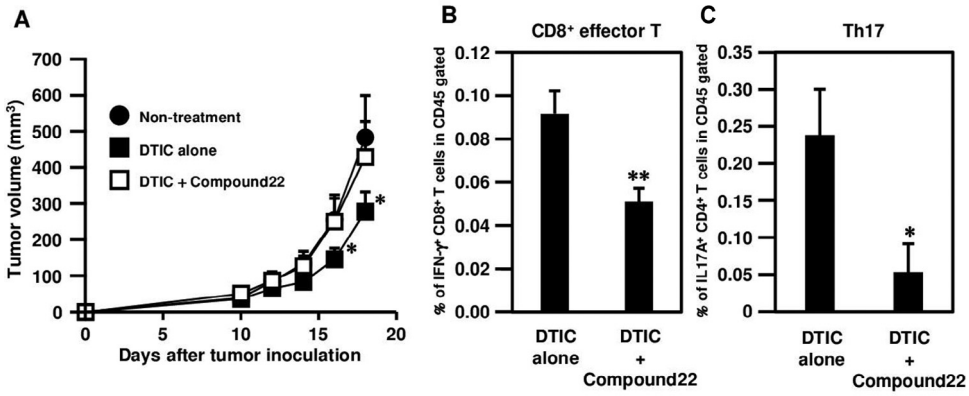

Fig. 5. The effect of a CCR4 antagonist on anticancer drug treatment in a murine melanoma model. C57BL/6J mice were intradermally inoculated with B16-F10 cells. DTIC alone or DTIC plus Compound 22 as a CCR4 antagonist were intraperitoneally injected into mice 10 times every other day. Tumor volume was monitored (A). Fifteen to eighteen days after tumor inoculation, the tumor tissues and the regional lymph nodes were harvested. IFN-γ+CD8+ T cells in the tumor tissues (B) and IL-17A+CD4+ T cells in the regional lymph nodes (C) were analyzed by flow cytometry. The data are expressed as mean ± SE of results from 15 mice (A) or 5 mice (B, C). $^{**}p < 0.01$ vs. DTIC alone.

was almost equivalent between human and mouse CCR4 (human CCR4: $IC_{50} = 38.2$ nM; mouse CCR4: $IC_{50} = 42.3$ nM) (Fig. 4B).

As shown in Fig. 5A, Compound 22 effectively suppressed the antitumor effect of DTIC. Furthermore, Compound 22 significantly decreased IFN-γ+CD8+ T cells in the tumor tissues and IL-17A+CD4+ Th17 cells in the regional lymph nodes in tumor-bearing mice treated with DTIC (Fig. 5B and C). These results suggested that the CCR4 antagonists suppressed DC-Th17 cell interactions in regional lymph nodes, which resulted in decreases of Th17 cell-mediated CD8+ effector T cell induction in the regional lymph nodes and infiltration of CD8+ effector T cells into tumor tissues, as we observed in CCR4-deficient mice, leading to enhanced tumor growth in mice inoculated with B16 melanoma cells.

Discussion

CCR4 is a major trafficking receptor for Treg cells and thus is considered to be involved in suppression of diverse immunological and inflammatory responses. Therefore, CCR4 is regarded as a promising therapeutic target for tumor immunology. However, CCR4, together with CCR6, is also expressed by Th17 cells, which have recently been shown to have an opposite role to Treg cells in antitumor immunity. In this study, we therefore explored the role of CCR4 using the B16 melanoma model. We have demonstrated that CCR4, but not CCR6, contributes to the promotion of antitumor immunity via Th17 cells rather than to the suppression of antitumor immunity via Treg cells. Mechanistically, we have demonstrated that the numbers of CCL22+DC-Th17 cell clusters were significantly decreased in the absence of CCR4 and thus Th17 cells were unable to fully expand in the regional lymph nodes of B16-F10-bearing CCR4-deficient mice.

It has been known that IL-23 is required for Th17 cell expansion [27], while TGF-β and IL-2 are required for Treg cell expansion [28,29]. Thus, their expansions need different cytokines. Although we still do not know exactly how Th17 cells, but not Treg cells, were decreased in CCR4-deficient mice, the deficiency of CCR4 may selectively affect the cytokine environment for the Th17 cell expansion. In addition, the deficiency of CCR6 did not affect the Th17 expansion. This may be due to the fact that antigen-loaded DCs express CCL22, the ligand for CCR4, but not LARC/CCL20, the ligand for CCR6. Taken together, these results suggest that CCR4 and CCR6 on Th17 cells have a different role in antitumor immunity, and that CCR4 plays an important role in Th17 cell expansion and induction of Th17 cell-mediated CD8+ effector T cells, leading to enhanced antitumor immunity.

The numbers of tumor-infiltrating CD8+ effector T cells were reduced in CCR4-deficient mice compared to WT mice. On the other hand, although the numbers of Th17 cells were reduced in regional lymph nodes of CCR4-deficient mice, we could not confirm the decrease of Th17 cells in the tumor sites. These results suggest that Th17 cells in regional lymph nodes play important roles in the induction of CD8+ effector T cells. In addition, CCR4 may also be involved in the recruitment of CD8+ effector T cells into tumor tissues. Indeed, CCR4 was previously reported to be expressed on activated CD8+ T cells and a subset of CD8+ T cells that produce multiple cytokines [30]. A recent study reported that not only CXC chemokine receptor 3 (CXCR3) expression but also CCR4 expression by CD8+ effector T cells were correlative to poor prognosis of melanoma patients [31]. Our present findings may also support that CCR4 is implicated in the infiltration of CD8+ effector T cells into tumor tissues and the pathophysiology of human melanoma. However, the *in vivo* role of CCR4-expressing CD8+ effector T cells in tumor immunity remains to be seen.

Checkpoint blocking antibodies targeting regulatory molecules on T cells such as CTLA-4 and PD-1 have demonstrated clinical efficacy across a variety of tumor types [32]. Previously, we have shown that CCR4 is highly expressed in adult T-cell leukemia/lymphoma (ATL) where its expression is highly upregulated by the activity of FRA-2 proto-oncogene [33,34]. Accordingly, humanized defucosylated anti-CCR4 monoclonal antibody, mogamulizumab, has been developed with a high antibody-dependent cellular cytotoxicity (ADCC) activity and has been approved as a potent treatment option for refractory ATL [35,36]. It has also been shown that the *in vivo* administration of anti-CCR4 monoclonal antibody markedly reduces the Treg cell fraction and augments NY-ESO-1-specific CD8+ T-cell responses in an ATL patient whose leukemic cells expressed NY-ESO-1 [37]. Furthermore, since increased numbers of Treg cells and a decreased ratio of tumor-infiltrating CD8+ T cells to Treg cells are well correlated with poor prognosis in various types of cancer in humans, a phase Ia study of cancer immunotherapy that aims to enhance antitumor immunity by depletion of Treg cells using anti-CCR4 monoclonal antibody in cancer patients has recently been commenced [38]. Thus, CCR4 is considered as a promising molecule target for cancer immunotherapies. However, our present results show that CCR4-deficiency enhanced tumor growth by suppressing antitumor immunity via Th17 cells rather than abrogating Treg cell activity. This suggests that the infusion of CCR4 antagonists aiming at inhibition of Treg cell recruitment via CCR4 may not always enhance antitumor immunity but may rather promote tumor growth depending on individual tumor types and conditions. In the thera-

pies using anti-CTLA4 and anti-PD1 antibodies, there are also different response rates among tumor types. Therapies targeting CCR4 may also have different outcomes depending on tumor types and patients. Further investigations on the role of CCR4 in various tumor types are required; however, such studies will provide important information about the safety and the efficacy of CCR4-targeting cancer immunotherapies.

In conclusion, we have revealed that CCR4 contributes to the activation of antitumor immunity in a murine B16 melanoma model via Th17.

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Conflict of interest

None.

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