Targeting CCR8 Induces Protective Antitumor Immunity and Enhances Vaccine-Induced Responses in Colon Cancer

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Abstract

CCR8 is a chemokine receptor expressed principally on regulatory T cells (Treg) and is known to be critical for CCR8+ Treg-mediated immunosuppression. Recent studies have demonstrated that CCR8 is uniquely upregulated in human tumor-resident Tregs of patients with breast, colon, and lung cancer when compared with normal tissue-resident Tregs. Therefore, CCR8+ tumor-resident Tregs are rational targets for cancer immunotherapy. Here, we demonstrate that mAb therapy targeting CCR8 significantly suppresses tumor growth and improves long-term survival in colorectal tumor mouse models. This antitumor activity correlated with increased tumor-specific T cells, enhanced infiltration of CD4+ and CD8+ T cells, and a significant decrease in the frequency of tumor-resident CD4+CCR8+ Tregs. Tumor-specific CD8+ T cells displayed lower expression of exhaustion markers as well as increased functionality upon restimulation. Treatment with anti-CCR8 mAb prevented de novo induction and suppressive function of Tregs without affecting CD8+ T cells. Initial studies explored a combinatorial regimen using anti-CCR8 mAb therapy and a Listeria monocytogenes–based immunotherapy. Anti-CCR8 mAb therapy synergized with L. monocytogenes–based immunotherapy to significantly delay growth of established tumors and to prolong survival. Collectively, these findings identify CCR8 as a promising new target for tumor immunotherapy and provide a strong rationale for further development of this approach, either as a monotherapy or in combination with other immunotherapies.

Significance: Inhibition of CCR8 represents a promising new cancer immunotherapy strategy that modulates tumor-resident regulatory T cells to enhance antitumor immunity and prolong patient survival. Cancer Res; 78(18); 5340–8. ©2018 AACR.

Introduction

Tumor-infiltrating CD4+Foxp3+ regulatory T cells (Treg) are a major immune cell population that contribute to the establishment of an immunosuppressive tumor microenvironment (TME; refs. 1–4). Infiltration of large numbers of Tregs into the tumors hamper the development of effective antitumor immunity (1–4) and is often associated with poor prognosis (2–5). Treg modulation strategies have been shown to increase antitumor immunity and reduce tumor burden in both preclinical and clinical settings (4–7). However, although these strategies have demonstrated enhanced antitumor immune responses, there are drawbacks, such as autoimmunity and specificity of targeting (2–4, 8–13). Because Tregs and activated effector lymphocytes both express surface molecules that can be used as therapeutic targets (e.g., anti-CD25), there is the potential for ablation of essential tumor-specific effector cells required to control tumor progression in these types of antibody-mediated immunotherapies (13, 14). Therefore, the development of a more effective approach to specifically and selectively target tumor-infiltrating Tregs is required.

In both humans and mice, the chemokine receptor CCR8 is predominantly expressed on Tregs and on a small portion of Th2 cells, but not on Th1 cells (15, 16). This subset of CD4+Foxp3+ Tregs expressing CCR8 (CCR8+ Tregs) has been demonstrated to be a major driver of immunosuppression and is critical for Treg function and suppression (15–17). More recently, two independent studies characterizing the distinct molecular signature of tumor-resident Tregs demonstrated that CCR8 was a specific marker selectively upregulated by tumor-resident Tregs in several tumor types (18, 19). Interestingly, Plitas and colleagues highlighted that high expression of CCR8+ Tregs was associated with poor prognosis in patients with breast cancer (19). These studies suggest CCR8 may be an effective therapeutic target by which to selectively and specifically modulate a subpopulation of tumor-resident Tregs in the TME to augment antitumor immunity. The extent of antitumor effects resultant from targeting CCR8 and its potential as a promising cancer immunotherapy remains to be determined.

Here, we demonstrate that an anti-CCR8 (αCCR8)–blocking mAb treatment impaired the suppressive character of the TME, markedly reducing tumor-resident CCR8+ Tregs. Ultimately, this contribution enhanced effector T-cell and antitumor immunity in the CT26 colorectal tumor model. Furthermore, our study demonstrates that αCCR8 therapy synergizes with a Listeria monocytogenes (Lm)–based immunotherapy to enhance optimal antitumor efficacy. Collectively, these novel findings highlight
CCR8 as a promising new target for cancer immunotherapy and its potential for broad clinical application.

Materials and Methods

Mice and tumor cell lines
Female, 6- to 8-week-old BALB/c and C57BL/6 (B6) mice were purchased from The Jackson Laboratory. All mouse procedures were performed in accordance with protocols approved by Advasix Immunotherapies IACUC. CT26 WT cells (CRM-2638) were purchased from the ATCC and authenticated by ATCC using COI analysis. The MC38 colon carcinoma cells were purchased from Kerafast and were authenticated by simple sequence length polymorphism. CT26 cells were maintained in RPMI medium from Kerafast and were authenticated by simple sequence length polymorphism. CT26 cells were maintained in RPMI medium supplemented with 10% FBS in a humidified atmosphere with 5% CO2 at 37°C. The MC38 cell lines were maintained in DMEM medium supplemented with 10% FBS. All cell lines were passaged twice prior to storage and thawed and passaged twice prior to implantation for all described tumor experiments. All cell lines were determined to be free of Mycoplasma (Sigma-Aldrich).

Tumor models, tumor vaccine, and αCCR8 treatment

CT26 (3 × 10⁶) and MC38 (3 × 10⁶) cells were implanted subcutaneously in the right flank of mice. Tumor vaccine consisted of LmddA-274 (1 × 10⁶) or AH1-21mer (1 × 10⁶) diluted in PBS. Details of plasmid construction are described in the Supplementary Materials and Methods. For therapeutic treatments, mice were treated with intraperitoneal injections of purified αCCR8 (4 μg; clone: SA214G2; BioLegend) and control antibody (rat IgG2b, Clone RTK4530; BioLegend). Tumor growth was monitored using electronic calipers and calculated according to the formula: V = (length x width²)/2. Mice were immunized with 200 μL of vaccine intravenously on day +12 and +19 after tumor implantation. To analyze tumor-infiltrating T cells upon vaccine/anti-CCR8 combination treatment, tumors from all groups were harvested for analysis +22 days after tumor implantation. For survival experiments, mice were euthanized when tumor size reached 2,000 mm³ or when tumors become necrotic.

In vitro assays

Treg induction. Naïve 2 × 10⁶ CD4⁺ T cells were isolated from spleens of BALB/c mice through negative selection using the StemCell EasySep Mouse naïve CD4⁺ T Cell Isolation Kit (catalog no. 19765) according to the manufacturer’s instructions. The cells were seeded on plates pretreated with 2 μg/mL αCD3. Cells were incubated for 3 to 5 days with αCD28 (1 μg/mL), IL2 (100 U/mL), and TGFB1 at 5 ng/mL. The percent of converted Tregs was evaluated by flow cytometry on the third day. For inhibition of conversion, αCCR8 was added at 10 μg/mL.

Proliferation of CD8⁺ and CD4⁺ T cells from αCCR8-treated mice.

A total of 2 × 10⁶ CD8⁺ and CD4⁺ T cells were isolated from spleens of BALB/c mice through negative selection using the StemCell EasySep Mouse naïve CD8⁺ or naïve CD4⁺ T Cell Isolation Kits (catalog no. 19853; catalog no. 19765, respectively). To measure proliferation, both CD8⁺ and CD4⁺ T cells were stained with carboxyfluorescein diacetate succinimidyl ester (CFSE) at a concentration of 0.5 μmol/L for 10 minutes. Cells were then washed with RPMI medium supplemented with 10% FBS and washed twice. The cells were seeded on pretreated αCD3 plates and incubated at 37°C for 3 days with αCD28 (1 μg/mL) and IL2 (100 U/mL) and in the presence or absence of αCCR8 (10 μg/mL) and TGFB (5 ng/mL).

Suppression assay. A total of 1 × 10⁶ five-day culturally induced Tregs were incubated with freshly isolated CD8⁺ T cells (1 × 10⁶) in a 1:1 ratio on plates pretreated with 2 μg/mL of αCD3. The freshly isolated CD8⁺ T cells were stained with 1 μmol/L CFSE for 10 minutes, quenched with RPMI medium supplemented with 10% FBS, and washed prior to being plated. Cocultured cells were incubated for 3 days with αCD28 (1 μg/mL), IL2 (100 U/mL), and indicated samples were treated with 10 μg of αCCR8. Cells were then harvested and stained with viability dye for 3 minutes, washed, and stained for CD3, CD4, and CD8 for 30 minutes at 4°C. Cells were then washed and analyzed by flow cytometry. Proliferation was assessed on live CD3⁺ CD8⁺ CD4⁻ T cells only.

All in vitro assays are representative experiments of independent triplicate determinations.

Statistical analysis

Statistical significance was determined either by unpaired Student t test (two-tailed) or two-way ANOVA. For tumor survival analysis, Kaplan–Meier test was used. Error bars indicate SEM. All graphs and statistical analyses were generated using Prism 6 software (GraphPad Software, Inc). All data are representative of 2 to 3 independent experiments.

Results

CCR8 mAb treatment impairs tumor growth in solid tumor models

Considering that CCR8 is highly expressed on human colorectal tumor-resident Tregs (18, 19) and is principally expressed by Tregs as part of their immunosuppressive arsenal (15–17), we first looked at CCR8 expression on Tregs in CT26 tumor-bearing mice. We found that CCR8 was highly expressed mainly on tumor-infiltrating Tregs, with very little expression on splenic Tregs (Fig 1A), but is not expressed on CT26 tumor cells (data not shown). This suggests that CCR8 Foxp³ Tregs within the TME play a role in suppressing tumor-specific immunity. Therefore, given that CCR8 Tregs have been suggested to be potent drivers of immunosuppression (17), we hypothesized that targeting CCR8 Tregs with an αCCR8-blocking mAb would enhance antitumor immunity. We examined the impact of CCR8 mAb treatment in the CT26 colorectal tumor model. CT26 tumor cells were implanted into groups of naïve recipient BALB/c mice (n = 10/group). Four days after tumor implantation, αCCR8 or IgG control therapy was initiated and given daily for a total of 8 days as outlined in Fig 1B. Interestingly, we found that treatment with αCCR8 generated significant antitumor activity and improved long-term survival (P < 0.0001; Fig 1B). Tumor growth curves show that αCCR8 treatment markedly delayed tumor growth over control groups. To verify these results in a different tumor model, we performed a similar experiment using the αCCR8 mAb therapy in the MC38 colon tumor model (Supplementary Fig S1). We implanted C57Bl/6 mice with MC38 tumor cells, and αCCR8 or IgG therapy was administered from day 4 to day 13 (8 days total) after tumor implantation (Supplementary Fig S1A). Similarly, we found that treatment with αCCR8 showed significant MC38 tumor growth inhibition and prolonged survival (Supplementary Fig S1B). Collectively, these results show
that blocking CCR8 can result in reduced tumor burden and improve long-term survival in tumor-bearing animals.

CCR8 mAb therapy induces robust antigen-specific tumor-infiltrating CD8⁺ T cells

Our observation that αCCR8 treatment enhances antitumor immunity prompted us to next examine the antigen (Ag)-specific CD8⁺ T responses systemically and in the tumors of αCCR8-treated tumor-bearing mice. For the immune analysis in tumor-bearing mice, total leukocytes were isolated from the spleens and tumors 17 days after tumor implantation. Splenocyte cultures from tumor-bearing mice treated with αCCR8 generated a robust splenic Ag-specific IFNɤ ELISpot response when stimulated with the classical MHC class I-restricted AH1 peptide used to monitor CD8⁺ T-cell responses (Fig. 2A). By direct staining with an AH1 H2-L1-restricted tetramer, αCCR8-treated mice significantly amplified the frequency of AH1 tetramer-specific CD8⁺ T-cell responses in the spleen (Fig. 2B). Similarly, αCCR8 therapy increased the frequency of AH1 tetramer-specific CD8⁺ T cells infiltrating into the tumors, indicating trafficking of target effector T cells to the site of malignancy and initiating effector function (Fig. 2C; ref. 20). CCR8 mAb therapy significantly increased the frequency of IFNɤ-positive effector CD8⁺ T cells within the tumor compared with the control group (Fig. 2D). Furthermore, a similar trend was seen with the frequency of T cells secreting IFNɤ when stimulated with PMA/ION, indicating that treatment with CCR8 mAb induces more functional CD4⁺ and CD8⁺ T-cell responses overall (Fig. 2E). Finally, we observed that treatment with CCR8 mAb significantly increased the level of polyfunctional effector CD8⁺ T cells coexpressing CD107a/IFNɤ within the tumor (Fig. 2E), indicating that these tumor-infiltrating T cells comprised an effector CD8⁺ T-cell population with a cytolytic phenotype. These results demonstrate the ability of CCR8 mAb therapy to alter the suppressive signature of the TME and promote tumor-specific effector T-cell function, which likely contributed to significantly delayed tumor growth.

CCR8 mAb therapy reprograms the tumor immune milieu

To better define the mechanism of action, we next examined the changes in the CT26 TME elicited by αCCR8 therapy. CCR8 mAb treatment altered the tumor-infiltrating lymphocyte (TIL) composition in the CT26 model. We observed that αCCR8 administration increased total CD45⁺ TILs compared with the control treatment group (Fig. 3A). As expected, within the CD45⁺ TIL population, αCCR8 treatment profoundly increased the percentage of tumor-infiltrating CD8⁺ and CD4⁺ T cells (Fig. 3A). Interestingly, both tumor-infiltrating CD8⁺ and CD4⁺ T cells displayed significantly lower expression of PD-1 and CTLA-4 exhaustion markers (Figs. 3B and C). This suggests that αCCR8 therapy may help retain functional tumor-reactive effector T cells.

Figure 1. CCR8 mAb treatment mediates antitumor activity, suppressing CT26 tumor growth and improving long-term survival. A, Cells obtained at day 17 postinjection from tumors and spleens. Representative density plots and graphs show frequency of CCR8⁺ Foxp3⁺ cells gated on CD45⁺ CD3⁺ CD4⁺ Foxp3⁺ T cells. B, Data illustrate treatment regimen, group tumor measurements, and survival of CT26-implanted mice following treatment as indicated. ****, P < 0.0001.
by limiting T-cell exhaustion. Furthermore, although not significant, αCCR8 mAb treatment slightly decreased the percentage of G-MDSCs (CD11b+Ly6G−/Ly6C+) in the tumor (Fig. 3D).

Given that CCR8 is principally expressed on Tregs (17–19), we next examined whether αCCR8 mAb treatment altered the tumor-resident Treg population and depleted the CCR8+ Treg target population. As expected, αCCR8 blockade could significantly reduce the frequency of tumor-infiltrating CD4+ Tregs (Foxp3+CD4+; Fig. 3E), and, more strikingly, deplete the frequency of infiltrating tumor-resident CCR8+Foxp3+CD4+ Tregs (Fig. 3F). Moreover, the CD8:Treg ratio within the tumor increased significantly in αCCR8-treated tumors (Fig. 3E), further suggesting a tipping of immune balance in favor of antitumor immunity (21). In addition, αCCR8 did not significantly reduce Foxp3−CD4+, but did significantly reduce the CCR8+ Treg target population in the periphery, demonstrating the target-specific effects of this agent (Supplementary Fig. 2A and 2B). These observations indicate that αCCR8 can reprogram the suppressive TME by specifically targeting and reducing suppressive tumor-resident CCR8+CD4+ Tregs, thus inducing a more favorable antitumor TME with enhanced effector T-cell function.

CCR8 mAb reduces Treg induction and Treg function

Studies have demonstrated that the CCR8−CCL1 axis plays a role in the process of Treg induction to a suppressive phenotype as well as Treg survival (22, 23). We speculated that αCCR8-mediated blockade was preventing de novo conversion of Tregs to a suppressive phenotype. To understand the mechanism of CCR8-mediated suppression, we first assessed whether αCCR8 played a role in Treg conversion. We demonstrated that αCCR8 mAb therapy inhibited Treg induction, whereas the isotype Ab control had no effect (Fig. 4A). Nevertheless, we also observed that TILs harvested from CT26 tumors and cultured ex vivo in the presence of αCCR8 demonstrated a substantial reduction of Tregs compared with control (45% vs. 10%, respectively) after 72 hours (Fig. 4B). We did observe an increase in the frequency of CD8+ T cells within the ex vivo αCCR8-treated TILs (Supplementary Fig. 2C), likely due to the reduction of suppressive Tregs (Fig. 4B).
The presence of Tregs reduced CD8+ T-cell proliferation and the addition of αCCR8 to Tregs/CD8+ T-cell cocultures significantly attenuated the suppressive capacity of Tregs (Fig. 4C). To confirm that targeting CCR8 did not directly affect T effector cells, we also measured CD8+ T-cell proliferation in the presence of αCCR8 and found no changes from basal proliferation rates (Fig. 4C), nor did they affect CD4+ T-cell proliferation (Fig. 4D). Taken together, these data show that blocking CCR8 with an αCCR8 mAb results in both reduced Treg induction and diminished Treg-suppressive function, without affecting the proliferation of effector CD4+ and CD8+ T cells.

**CCR8 mAb therapy with a Listeria-based tumor vaccine induces antitumor immunity**

We have demonstrated that Listeria-based vaccines can be potent cancer immunotherapies by inhibiting suppressive cell function in the TME while simultaneously enhancing antitumor effector immune cell activity (24–25). Given that αCCR8 therapy alone increases the frequency of AH1-specific effector CD8+ T cells in the TME (Fig. 2), we sought to increase the magnitude of the tumor-specific immune response by combining CCR8 mAb therapy with a Listeria-based cancer immunotherapy targeting the classical AH1 tumor-associated antigen (Supplementary Fig. S3). Combination Lm-AH1/αCCR8 therapy was administered in a sequential dosing strategy: αCCR8 mAb treatment was first administered to modulate the tumor immune milieu (reduce CCR8+ Tregs) as described in Fig. 1A with dosing started on day 4 after tumor implantation followed by Lm-AH1 immunization beginning on day 11 after tumor implantation. Combinatorial treatment showed significantly improved suppression of tumor growth and led to approximately 20% long-term survival compared with control group (Fig. 5A). Analysis of tumor-infiltrating leukocytes showed synergistically enhanced responses against the immunizing AH1 antigenic target (Fig. 5B). Combination therapy significantly increased IFNγ and TNFα production by effector CD8+ and CD4+ TILs and reduced tumor-resident Tregs, indicating that the enhanced development of T-cell–mediated immune responses might be critical for the efficacy of the combination therapy (Fig. 5C and D). In addition, we observed that the CD8/Treg ratio increased significantly within the tumor (Fig. 5C). This successful augmentation of effector CD4+ and CD8+ T cells in the TME indicates that the combination of *L. monocytogenes* and αCCR8 could be an ideal strategy for immunotherapy for a variety of cancers. These results set the stage for additional studies designed to evaluate combination therapies with αCCR8 mAb.

**Discussion**

Accumulating evidence indicates that effective cancer immunotherapy will need to control Treg infiltration into tumor tissues (2–7, 12–24). Tumor-infiltrating Tregs represent major obstacles for driving effective tumor-specific immune responses. Several Treg depletion strategies, such as anti-CD25 and...
addition of CD4 B, cytometry.

cyclophosphamide, have been explored to enhance immunotherapy (2–7, 12–14). However, there are serious drawbacks to these treatments such as (1) autoimmunity resulting from a reduction in Tregs systemically and/or (2) attenuation of antitumor immunity by depletion of effector T cells due to off-target effects. Therefore, effective new cancer immunotherapies are needed to specifically target Tregs that are abundantly and specifically located in tumor tissues. Recent evidence indicates that CCR8 Tregs are critical for Treg proliferation and suppression (17). Furthermore, Plitas and colleagues and De Somine and colleagues showed that intratumoral Tregs of human patients with cancer predominantly expressed CCR8, highlighting their therapeutic potential as a target to manipulate tumor-resident Tregs (18, 19). In this study, we report for the first time that CCR8 can serve as a novel candidate target to inhibit tumor-resident Tregs, thereby enhancing the efficacy of immunotherapy.

In the current study, we demonstrated that CCR8 was predominantly upregulated in tumor-resident Tregs in comparison with peripheral Tregs (Fig. 1A). These data recapitulate those from clinical studies, demonstrating in several different tumor types (colorectal, breast, lung, melanoma) that CCR8 is highly expressed predominantly on tumor-resident Tregs relative to peripheral tissue-resident Tregs (18, 19). As such, CCR8 represents a valuable target, as specifically limiting the CCR8 Treg population to the tumor in vivo can promote better-primed immune responses and effective antitumor immunity. The high expression of CCR8 on Tregs within the TME suggests that tumor-resident CCR8 Tregs suppressed tumor-infiltrating effector T cells. This hypothesis is supported by the enhanced expansion, activation, and effector function of tumor-infiltrating T cells in αCCR8 mAb-treated mice (Fig. 2). The observation of higher tumor-infiltrating CCR8+ Tregs in the tumors of nontreated mice (Fig. 3F) correlated with poor tumor control. Plitas and colleagues support these results in a clinical setting, demonstrating that high CCR8 to Foxp3 mRNA ratios correlated with poor prognosis in breast cancer (19). We observed that CCR8 blockade altered the suppressive cellular signature of the TME by reducing tumor-infiltrating CCR8 Foxp3+ Tregs, while increasing the frequency of infiltrating tumor-specific effector T cells (Figs. 2 and 3). Recently, a study by Barsheshet and colleagues demonstrated that CCR8+ Treg proliferation and suppression is dependent on interaction of CCR8–CCL1 axis (17). Therefore, the number of CCR8+ Tregs could be reduced by targeting CCR8 with an αCCR8 antibody.

Our results demonstrated that CCR8 blocking by αCCR8 mAb could deplete the infiltrating CCR8+ Tregs and enhance antitumor immunity. Thus, this mechanism of reducing suppressive CCR8+ Tregs in the tumor may account for the robust increase in the frequency of Ag-specific tumor-infiltrating effector CD8+ T-cell responses with cytolytic potential. The increase of effector CD8+ T-cell-to-Treg ratios within the tumor is considered to be essential for controlling and eliminating established tumors (1–4, 21, 26). The fact that treatment with αCCR8 induced more functional Ag-specific CD8+ T cells (Fig. 2) suggests that αCCR8 treatment reduced tumor-resident CCR8+ Tregs cells while sparing CD8+ effector T cells. The selective effect on Tregs and not CD8+ T cells most likely results from the higher expression levels of CCR8 on Tregs (17–19). Taken together, these results indicate that αCCR8 therapy augments antitumor immunity by targeting CCR8+ Tregs in tumor tissues. Thus, these effects of αCCR8 treatment, along with the increase in the number of Th1 cytokine-producing T cells, facilitates the establishment of a proinflammatory TME, which may lead to improved antitumor immunity.

Figure 4. Blocking CCR8 reduces Treg induction and Treg function. A, αCCR8 mAb reduces Treg induction, whereas the isotype control (IgG) does not. The 1 × 10^6 naïve CD4+ T cells were cultured under specific conditions (including αCD28 and IL2) and Treg induction (frequency of Foxp3+ CD4+ cells) was measured by flow cytometry. B, TILs were harvested from day 17 implanted CT26 nontreated tumors and cultured for 72 hours in the presence or absence of αCCR8 (10 μg) on plates coated with or without αCD3 in the presence of αCD28a and IL2. C, αCCR8 reduces the capacity of Tregs to suppress CDB T-cell proliferation. Cultured induced Tregs were cocultured with purified CD8+ T cells (1:1 ratio) with or without αCCR8 (10 μg); control included CDB T cells alone. The purified CDB+ T cells were cultured for 3 days on plates coated with αCD3 in the presence or absence of Tregs or αCCR8. Addition of αCCR8 significantly reversed the suppressive function of Tregs. C and D, αCCR8 did not affect CDB+ T-cell proliferation (C), nor did it affect CD4+ T-cell proliferation (D). The effect of the addition of αCCR8 antibody on proliferation was evaluated by measuring CFSE by flow cytometry. *** P < 0.001; **** P < 0.0001; ns, not significant.
Summary data showing positive CD8 and CD4 T cells releasing IFN-\(\gamma\), 8 consecutive days) and on day 22, followed by analysis of frequency of CD45\(^+\) leukocyte infiltrate and CD8\(^+\) and CD4\(^+\) TILs as percentage of total CD45\(^+\) cells. C, Summary data show the frequency of CD4\(^+\) Tregs (Foxp3\(^+\)/CD25\(^+\)) and the ratio of CD8 effector T cells to Tregs in the tumors. D, Summary data showing positive CD8 and CD4 T cells releasing IFN\(\gamma\), TNF\(\alpha\), and/or IL2 following AHI peptide incubation with PMA/ION stimulation. Error bars, SEM of \(n = 4\)-5/group. *, \(P < 0.05\); **, \(P < 0.01\); ***, \(P < 0.001\); ****, \(P < 0.0001\).

Figure 5.
CCR8 mAb therapy with a Listeria-based tumor vaccine enhances antitumor immunity. A, Naive mice (\(n = 8\)/group) were inoculated with CT26 tumor cells (3 \(\times\) 10\(^5\)) on the right flank. Combination therapy was given in a sequential setting. On day +4 postinoculation, mice were treated with \(\alpha\)CCR8 (administered for 8 consecutive days) and on day +11 postinoculation mice were treated with Listeria-based vaccine or the combination as indicated. Mean tumor growth and survival are depicted. B, TILs (\(n = 4\)-5/group) were harvested at day +22, followed by analysis of frequency of CD45\(^+\) leukocyte infiltrate and CD8\(^+\) and CD4\(^+\) TILs as percentage of total CD45\(^+\) cells. C, Summary data show the frequency of CD4\(^+\) Tregs (Foxp3\(^+\)/CD25\(^+\)) and the ratio of CD8 effector T cells to Tregs in the tumors. D, Summary data showing positive CD8 and CD4 T cells releasing IFN\(\gamma\), TNF\(\alpha\), and/or IL2 following AHI peptide incubation with PMA/ION stimulation. Error bars, SEM of \(n = 4\)-5/group. *, \(P < 0.05\); **, \(P < 0.01\); ***, \(P < 0.001\); ****, \(P < 0.0001\).

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Tregs have been shown to play a key role in controlling autoimmunity and in the regulation of pathologic and physiologic immune responses (17, 23, 27). Therefore, the reduction of a particular Treg cell population (in this case, CCR8\(^+\) Tregs) rather than the entirety of the Foxp3\(^+\) T-cell population can be exploited to augment antitumor immunity without the risk of inducing autoimmunity. In this study, we showed that \(\alpha\)CCR8 therapy selectively reduced intratumoral Tregs (Fig. 3E), but spared Tregs in the periphery, demonstrating the tumor-specific effects of these agents. In our studies, no autoimmunity or toxicity were observed in the treated mice. In fact, several studies that support this notion have shown that CCR8-deficient mice do not exhibit any of the characteristics of severe autoimmune and lymphoproliferative disorders resulting from Foxp3 deficiency (17, 23, 28–30). In addition, it is likely that, although \(\alpha\)CCR8 therapy reduced CCR8\(^+\) Tregs during treatment, peripheral CCR8\(^+\) Foxp3\(^+\) Tregs can be sufficient to prevent deleterious autoimmunity. CCR8 blocking can therefore be a unique cancer immunotherapy aimed at depleting tumor-resident Tregs without the severe clinical adverse events that would arise from systemic Treg depletion. Nevertheless, determining whether or not in vivo \(\alpha\)CCR8 therapy will elicit autoimmune disease in the clinic warrants investigation.

We observed that blockade with CCR8 prevented the differentiation of naive CD4\(^+\) T cells into Tregs and inhibited their suppressive function (Fig. 4), which likely attenuated immunosuppression, thereby enhancing efficacy of immunotherapy (Fig 1). Furthermore, treatment with \(\alpha\)CCR8 did not affect CD8\(^+\) or CD4\(^+\) T-cell proliferation (Fig. 4C and D), nor did it affect the induction of CD8\(^+\) effector T cells (Figs. 2 and 3). Taken together, these results indicate that treatment with \(\alpha\)CCR8 exclusively targeted CCR8\(^+\) Tregs, disrupting the function of these cells, without affecting the function of effector CD8\(^+\) T cells. In addition, how \(\alpha\)CCR8 therapy may affect the immune function of other cell populations that express CCR8 cannot be ruled out and requires further study (17). Collectively, the results reveal that accordant with the reduction of CCR8\(^+\) Tregs and enhanced effector CTL responses, administration of \(\alpha\)CCR8 led to strong inhibition of tumor growth and prolonged survival. Similarly, Hoelzinger and colleagues reports that neutralizing the CCR8 ligand, CCL1, is also effective in blocking Treg conversion and inhibiting Treg-suppressive function (22). Overall, our results further add to the pool of information that the CCR8–CCL1 axis plays a role in Treg function and that targeting this axis can improve antitumor immunity (17, 22).

CCR8 may have multiple effects on Treg biology (17, 23, 28, 31, 32). Therefore, targeting CCR8 on Tregs may also have multiple endpoints: (i) It has been reported that CCR8 has anti-apoptotic effects and is essential for Treg survival (23, 31). Thus, targeting CCR8 may increase Treg susceptibility to cell death via apoptosis; (ii) given that CCL1 is unique in potentiating CCR8\(^+\) Tregs (17–19, 22), targeting its receptor, CCR8, may block its activity for expansion and suppression; (iii) because CCL1 can...
drive CCR8 Treg motility (32), it is plausible to speculate that the decrease in number of tumor-infiltrating regulatory cells by targeting the CCR8–CCL1 axis could also be the result of an alteration in their migration properties to the tumor (28, 32). Studies to elucidate the specific role of CCR8 on Treg biology warrants further investigation and are currently under way in our laboratory.

Combining multiple immunotherapies for cancer will be critical for improving the therapeutic outcome for future clinical trials. In this study, our data show promising synergistic effects of combining αCCR8 mAb with a Listeria-based immunotherapy. We report that targeting CCR8 can increase the infiltration of vaccine-induced effector T cells into tumors and increase their functionality, thus leading to improved antitumor immunity, tumor regression, and prolonged survival in established tumor-bearing mice (Fig. 5). Our studies appear to be supported by previous work showing that eliciting higher vaccine-induced effector T cells in the tumor correlated with better protective immunity for controlling tumor growth (33). In addition, the increased production of TGFβ cytokine-producing CD4+ and CD8+ tumor-infiltrating T cells likely helps shift the TME from a suppressive to a more inflammatory antitumor state (34). In future studies, it will be interesting to test whether combining αCCR8 with additional immunotherapies such as checkpoint inhibitors or costimulatory molecules can achieve better antitumor immunity. In addition, understanding how CCR8 functions, in what step of the cancer immunity cycle, will allow us to best design αCCR8 immunotherapies and target them to the patients who are most likely to respond. Overall, our findings provide initial in vivo evidence that a vaccine can be coupled with αCCR8 mAb and support the use of this combination strategy for use in future clinical trials. We expect that data from this study can serve to leverage the design of clinical trials that would deliver αCCR8 as monotherapy or in combination with other modalities such as vaccines or checkpoint inhibitors for the treatment of cancer.

Our study provides the first compelling evidence for targeting CCR8 as a therapy to reduce tumor-resident Tregs to control immunity against cancer. The high specificity of CCR8 expression on tumor-resident Tregs in various types of human cancers, such as colorectal, breast, lung, melanoma, and angiosarcoma (18–19), highlights the broad clinical applicability for a CCR8 blockade or depletion antibody in the treatment of a variety of cancers. Overall, we present CCR8 as a promising new target for cancer immunotherapy, either as a single agent or in combination with other forms of cancer immunotherapies and, therefore, it has direct relevance for the design of future therapeutic trials in patients.

Disclosure of Potential Conflicts of Interest

D.O. Villarreal has ownership interest (including stock, patents, etc.) in Advaxis, Inc (Stock). C. Mottershead has ownership interest (including stock, patents, etc.) in Advaxis Stock. B.D. Coder has ownership interest (including stock, patents, etc.) in Advaxis Inc. M.F. Princiotta has ownership Interest (including stock, patents, etc.) in Advaxis Inc. No potential conflicts of interest were disclosed by the other authors.

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