Targeting of CCR8 induces antitumor activity as a monotherapy that is further enhanced in combination with a *Listeria*-based immunotherapy

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ABSTRACT

CCR8 is a chemokine receptor that is expressed principally on regulatory T cells (Tregs) and known to be critical for Treg function, as CCR8$^+$ Tregs can drive immunosuppression. Recent studies have demonstrated that CCR8 is uniquely upregulated in human tumor-resident Tregs of breast, colon, and lung cancer patients compared to normal tissue-resident Tregs. Therefore, CCR8 tumor-resident Tregs are rational targets for cancer immunotherapy. Here, we demonstrate that monoclonal antibody therapy targeting CCR8 significantly suppressed tumor growth and improved long-term survival in two different tumor-bearing mouse models. The antitumor activity could be correlated with an increase in tumor-specific T cells, enhanced infiltration of CD4$^+$ and CD8$^+$ T cells, and a significant decrease in the frequency of intratumoral Tregs. Initial studies explored a combinatorial regimen using anti-CCR8 mAb therapy and a *Listeria*-based cancer immunotherapy. Anti-CCR8 mAb therapy synergized with the *Listeria*-based immunotherapy to significantly delay tumor growth and induce complete regression in 20% of the mice. These results suggest that CCR8 represents a promising target for cancer immunotherapy, either as a single agent or in combination with other forms of immunotherapy.

INTRODUCTION

- Tumor-infiltrating Foxp3$^+$CD4$^+$ regulatory T cells (Tregs) are a major immune cell population that contribute to the establishment of an immunosuppressive TME.
- Chemokine receptor CCR8 is predominantly expressed on Tregs.
- Tregs expressing CCR8, (CCR8$^+$ Tregs) are major drivers of immunosuppression, critical for Treg function and suppression.
- CCR8 has been shown to be a specific marker selectively upregulated by tumor-resident Tregs from several tumor types.
- Advaxis *Lm* platform can induce antigen-specific CD8$^+$ effector T cells in mice.
- *Lm–based* immunotherapy has been shown, in preclinical cancer models and in the clinic, to change the local TME.
- These studies were designed to evaluate whether (1) CCR8 represents a promising new target to selectively reduce tumor-resident Tregs and if (2) aCCR8 mAb would synergize with an *Lm*-based immunotherapy to enhance antitumor activity and prolong survival in tumor-bearing mice.

MATERIALS AND METHODS

Tumor models, tumor vaccine, and treatments: CT26 (300,000) and MC38 (300,000) cells were implanted subcutaneously (s.c.) in the right flank of mice. For therapeutic treatments, mice were treated with intraperitoneal (i.p.) injections of anti-CCR8 (4ug; clone: SA214G2) or control antibody (rat IgG$_{2b,k}$, Clone LTF-2) as indicated. For combination studies, CT26 tumor-bearing mice were immunized i.v. twice on day +1 and +12 post-tumor implantation, with either *Lm*-AH1 (1 x 10^8 CFU), an *Lm* vector expressing the AH1 antigen (gp70$_{423-431}$, SPSYVYHQF) expressed on the CT26 murine tumor cell line that is recognized by CD8$^+$ T cells in the BALB/c mouse or LmddA-274 (1 x 10^8 CFU), an *Lm* vector expressing no tumor-specific antigen.

Flow analysis: Tumors were enzymatically dissociated into single cell suspensions using a Stomacher machine (Steward) with Collagenase IV (Stem Cell Technologies). The resulting single-cell suspensions were immunophenotyped with the following antibodies using standard staining procedures: anti-CD45, anti-CD4, anti-CD8, anti-CCR8 (clone SA214G2; Biolegend), anti-CCR8 (GeneTex), anti-CD44, anti-CD25, anti-CD107a, anti-CTLA4, anti-PD-1, anti-TCRβ, MHC class I peptide AH1 Tetramer, anti-IFNγ, anti-TNFα, annexin V, and Invitrogen LIVE/Dead fixable Violet Fluorescent Reactive Dye. For IFNγ staining, cells were stimulated with either SIINFEKL peptide, AH1 peptide (gp70$_{423-431}$, SPSYVYHQF), and/or cell stimulation cocktail plus protein transport inhibitors (Invitrogen.). Events were acquired using the Attune flow cytometer (Fisher Scientific) and analyzed using FlowJo software (Tree Star). Tumor infiltrating lymphocytes (TILs) were defined as CD45$^+$ cells.

***In vitro* assays:** For Treg induction, 2 x 10^6 CD4$^+$ T cells were isolated from spleens of BALB/c mice through negative selection (StemCell). The cells were seeded on plates pretreated with 2 ug/ml anti-CD3. Cells were incubated for 3 days with aCD28 (1ug/mL), IL-2 (100U/mL), and TGF-β1 at 5ng/ml. The percent of converted Tregs was evaluated by flow cytometry on the third day. For inhibition of conversion, aCCR8 was added at 10 ug/ml. To measure suppression, 1 x 10^6 5 day culturally induced Tregs were incubated with freshly isolated CD8$^+$ T cells (2 x 10^6) in a 1:1 ratio, on plates pretreated with 2 ug/ml aCD3. The Fresh isolated CD8$^+$ T cells were stained with 1uM CFSE for 10 minutes, quenched with R10 medium and washed prior to being plated. Co-cultured cells were incubated for 3 days with aCD28 (1ug/mL), IL-2 (100U/mL) and indicated samples were treated with 10ug of aCCR8.

RESULTS

CCR8 is Highly Expressed by Tumor-resident Tregs

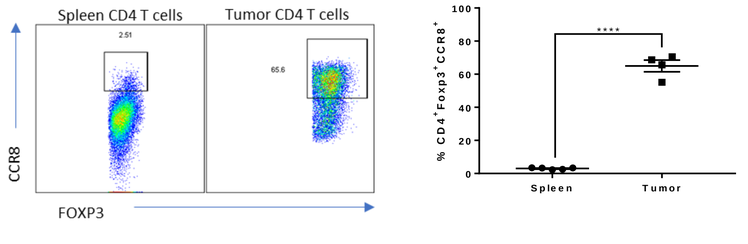

Figure 1. CT26 tumor-infiltrating Tregs exhibit high expression of CCR8. Cells from spleens and tumors of CT26 mice were harvested 17 days after tumor implantation. Representative scatter plots and graphs show frequency of CCR8 expression by Foxp3$^+$CD4$^+$ Tregs.

RESULTS (cont.)

αCCR8 mAb Treatment Impairs Tumor Growth in Solid Tumor Models

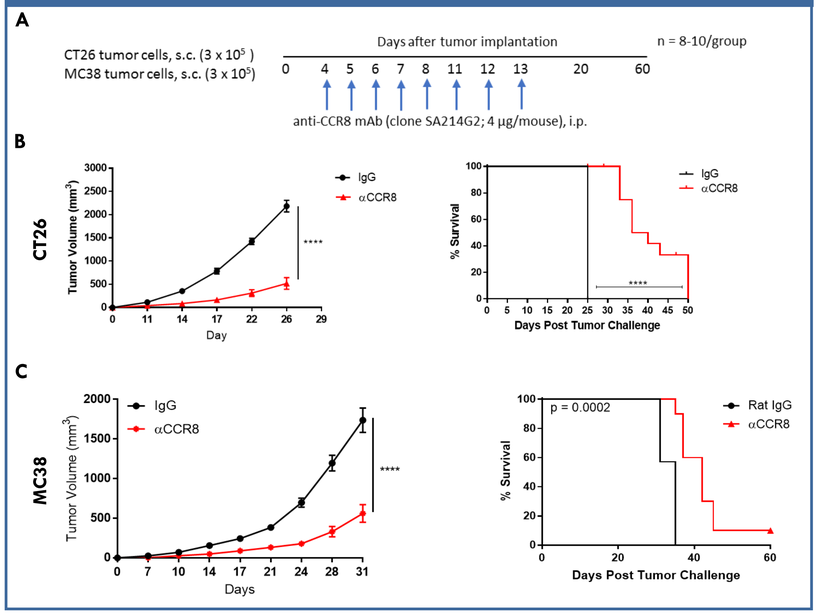

Figure 2. CCR8 mAb therapy enhances antitumor efficacy. Tumor-bearing mice treated with αCCR8 (4ug) decreased rates of tumor growth and improved long-term survival in two different solid tumor models. (A). Schematic representation of the treatment regimen. (B) Tumor growth curve and survival of CT26 implanted mice. (C) Tumor growth curve and survival of MC38 implanted mice.

αCCR8 mAb Treatment Increases the Frequency and Functionality of CD8$^+$ T Cell Responses

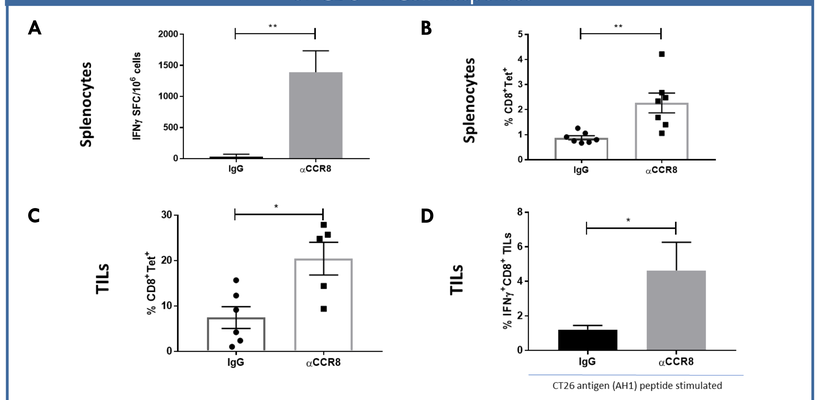

Figure 3. αCCR8 mAb treatment increases the frequency of CT26 antigen-specific CD8$^+$ T cells. Spleens and TILs from tumor-bearing CT26 mice were harvested 17 days after tumor implantation. (A) The frequency of AH1-specific IFNγ (spot forming cells/10^6 splenocytes) responses determined by IFNγ ELISpot assay in response to stimulation with CT26 AH1 peptide (SPSYVYHQF). (B-C) Bar scatter plot graphs show the percentages of AH1 peptide tetramer-positive CD8$^+$ T cells in the spleens and in the tumors of tumor-bearing mice. (D) Column graphs showing intracellular cytokine staining for IFNγ in CD8$^+$ TILs following AH1 peptide stimulation or (E) PMA/ION stimulation.

αCCR8 mAb Treatment Alters the TME in the CT26 Tumor Model

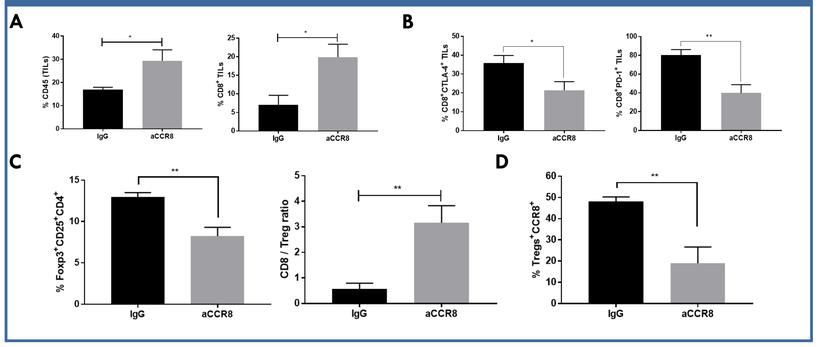

Figure 4. CCR8 mAb treatment alters the TME. TILs (n = 5-6 per group) from tumors mice were harvested 17 days after CT26 tumor implantation. (A) CD45$^+$ leukocyte infiltrate and CD8$^+$ T cells as a percentage of total CD45$^+$ cells are shown in treated versus untreated groups. (B) Frequency of CTLA-4 and PD-1 expression on tumor infiltrating CD8$^+$ T cells (C) Tumor-resident CD4$^+$ Tregs (Foxp3$^+$CD25$^+$) and the ratio of CD8/Tregs in the tumor. (D) CCR8 expression by tumor-resident Tregs (Foxp3$^+$CD4$^+$). *P<0.05; **P<0.01.

RESULTS (cont.)

Blocking CCR8 Reduces Treg Conversion and Treg Function

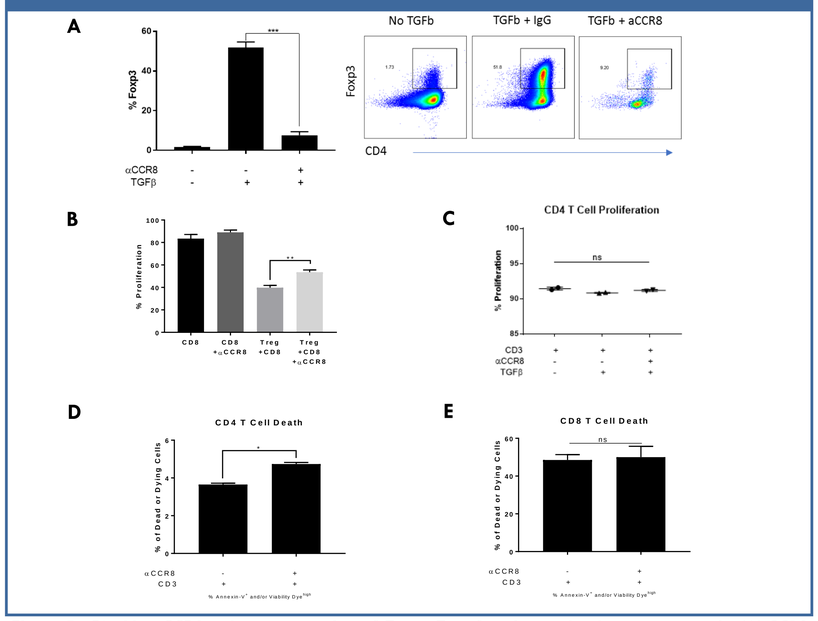

Figure 5. Blocking CCR8 reduces generation of Tregs, Treg function and enhances apoptosis. (A) CCR8 blocking mAb (αCCR8) reduces generation of Tregs from Foxp3$^-$CD4$^+$ T cells; whereas, the isotype control (IgG) does not. Naïve CD4$^+$ T cells (1x10^6) were cultured under specific conditions (including αCD28 and IL-2) and generation of Tregs (frequency of Foxp3$^+$CD4$^+$ T cells) was measured by flow cytometry. (B) αCCR8 reduces the capacity of Tregs to suppress CD8$^+$ T cell proliferation. Culture induced Tregs were cocultured with purified CD8$^+$ T cells (1:1 ratio) with or without αCCR8 (10 ug). CD8$^+$ T cells were cultured for 3 d on plates coated with αCD3 in the presence or absence of Tregs or αCCR8. Addition of αCCR8 strongly reversed Treg suppressive function. (C) Blocking CCR8 using an αCCR8 mAb does not affect CD4$^+$ T cell proliferation as determined by CFSE staining and flow cytometry. (D) The effects of 72 h treatment with αCCR8 on Treg apoptosis and (E) on CD8$^+$ T cell apoptosis were determined by staining with Annexin V and viability dye using flow cytometry. *P<0.01; ***P<0.001; ****P<0.0001.

Lm-Ah1 and αCCR8 Combination Therapy Enhanced Antitumor Efficacy

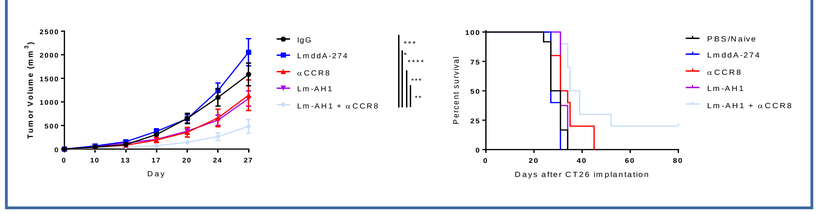

Figure 6. αCCR8 mAb synergizes with a *Listeria*-based therapy to enhance therapeutic outcome. Naïve mice (n=10 per group) were implanted with CT26 tumor cells (300,000) on the right flank. Combination therapy was given sequentially. On day 4 post-inoculation, mice were treated with αCCR8 (administered for eight consecutive days) and on day 11 post-inoculation mice were treated with Lm-AH1, alone or in combination with αCCR8, as indicated.

Combination *Lm*-AH1/αCCR8 Therapy Increases CD8$^+$ T cell Responses in the TME

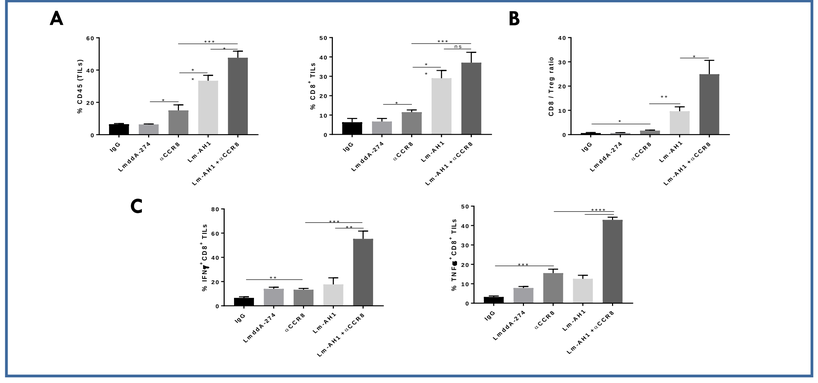

Figure 7. *Lm*-AH1 and αCCR8 combination therapy increases effector CD8$^+$ TIL numbers. (A) TILs (n = 4-5 per group) were harvested at day +22, followed by analysis of frequency of CD45$^+$ leukocyte infiltrate and CD8$^+$ as percentage of total CD45$^+$ cells. (B) The ratio of CD8$^+$ effector T cells to Tregs (CD4$^+$Foxp3$^+$CD25$^+$) in the tumors. (C) IFNγ and TNFα expression by CD8+ TILs following peptide incubation with PMA/ION stimulation. Error bars indicate SEM. *P<0.01; ***P<0.001; ****P<0.0001.

SUMMARY

- αCCR8 mAb therapy enhanced antitumor efficacy and improved long-term survival.
- αCCR8 mAb increased tumor-specific T cells and significantly decreased tumor-resident CCR8$^+$ Tregs.
- CCR8 blocking mAb prevented generation and suppressive function of Tregs.
- *Lm*-AH1 and αCCR8 combination therapy enhanced antitumor efficacy and prolonged survival relative to *Lm*-AH1 or αCCR8 alone.
- *Lm*-AH1 and αCCR8 combination therapy increased CD8$^+$ T cell responses and induced changes in the immune effectors in the TME.