CCR5 is a novel target for the treatment of experimental alopecia areata

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Abstract

Background: Alopecia areata (AA) is an organ-specific and cell-mediated autoimmune disease. Hair follicle autoantigens are disclosed to these autoreactive NKG2D+CD8+ T cells. A Th1 chemokine, CXCL10, is highly expressed on hair follicle keratinocytes and leads to the infiltration of CXCR3+ and CCR5+ Th1 or Tc1 cells around anagen hair follicles in AA lesions.

Aim: To evaluate CCR5 as a new candidate target for the treatment of experimental AA.

Methods: We initiated 7-month-old female C3H/HeJ mice with intracutaneous injections of lymph node (LN) cells, which were activated by IL-2, IL-7, IL-15, and CD3/CD25 Dynabeads, from C3H/HeJ mice with spontaneous AA. Then, 330 μg/d of maraviroc, a negative allosteric modulator of the CCR5 receptor that is already used for treating patients with human immunodeficiency virus, was orally administrated for 28 days.

Results: We successfully treated the experimental AA lesions of C3H/HeJ mice with maraviroc. In addition, CCR5 blockade prevented the development of AA in activated T cell-transferred C3H/HeJ mice. Interestingly, maraviroc-treated C3H/HeJ mice with experimental AA showed improvement of hair loss lesions after 2 weeks. Immunohistological assessments and FACS analysis revealed a decreased number of CD4+CCR5+ and CD8+CCR5+ T cells in the lesions after maraviroc treatment. A real-time horizontal chemotaxis assay showed that maraviroc significantly inhibited the chemotactic activity of CD8+ LN cells toward RANTES. Furthermore, CCR5 blockade prevented experimental AA development when compared to phosphate-buffered saline.

Conclusion: CCR5 appears to be a promising new candidate target for the treatment of AA. CCR5 blockade may prevent the development of AA as well as improve AA lesions.

Keywords
alopecia areata, CCR5, chemotaxis, maraviroc, RANTES
1 | INTRODUCTION

Recent clinical and experimental studies have revealed that alopecia areata (AA) is an organ-specific and cell-mediated autoimmune disease.1,2 Tyrosinase-related protein, tyrosinase, and trichohyalin are strongly regarded as the autoantigens in anagen hair follicles (HFs), and NKG2D⁺CD8⁺ T cells may recognize these antigens, resulting in the apoptosis in the proximal portion of anagen HFs.3,4 Normally, anagen hair bulbs maintain an immunoprivileged milieu that protects anagen HFs from autoimmune reactions.5-7 HF-IP is established and maintained by the downregulation or absence of classical major histocompatibility complex (MHC) class I expression, which results in the sequestering of (auto)antigens in tissue sites, hindering their presentation to CD8⁺ T cells with a matching T-cell receptor, and by the local production of potent immunosuppressants such as transforming growth factor-β1, interleukin (IL)-10, and α-melanocyte-stimulating hormone.8-14 The collapse of HF-IP is considered to be a critical step of AA induction, and type 1 inflammation is regarded as a key mediator of the autoimmune reactions in AA lesions. Th1 cytokines, interferon (IFN)-α and IFN-γ, are key cytokines that initiate the collapse of HF-IP with the upregulation of MHC class I expression at the proximal outer root sheath (ORS) and matrix cells, and these IFNs also stimulate NKG2D⁺CD8⁺ Tc1 cells.15,16 In addition, Th1/Tc1 chemokines, such as CXCL9 and CXCL10, are upregulated at the proximal ORS and matrix cells in AA lesions. “Swarm of bees” is a characteristic feature of the histopathological change observed in the lesions of acute-phase AA.17 This unique feature can be explained by the accumulation of CXCR3⁺ and CCR5⁺ Th1/Tc1 cells, which are chemokine receptors for CXCL9 and CXCL10. The proportion of CXCR3⁺ T cells is significantly higher in the peripheral blood mononuclear cells and skin-infiltrating cells of patients with acute-phase AA than in healthy controls. CCR5⁺ cells also densely infiltrate around human hair bulbs in AA lesions.18 In addition, the chemotactic velocity of CXCR3⁺ Th1/Tc1 cells toward CXCL10 is significantly higher in AA patients than in control subjects.17 Dai et al.19 have reported the effect of a blocking antibody to CXCR3 that prevented the development of AA in a C3H/HeJ mouse graft model and inhibited the accumulation of NKG2D⁺CD8⁺ T cells in the skin and cutaneous lymph nodes (LNs); this indicated that AA onset may be prevented by hindering the Tc1 response in AA via a blockade of IFN-inducible chemokines.

In this study, we treated AA model mice, C3H/HeJ mice, with maraviroc (MVC), which is a blocking antibody to CCR5. CCR5 is a G-protein-coupled receptor that has been identified as the receptor for CCL3/macrophage inflammatory protein (MIP)-1α, CCL4/ MIP-1β and CCL5/regulated on activation, normal T cell expressed and secreted (RANTES).20-22 CCR5 regulates the migration of monocytes, natural killer cells, and Th1 cells into inflammation sites.23 Although the alopecic lesion of C3H/HeJ mice is not completely identical to that of human AA, they share several common features, including the clinical types (AA simplex, totalis, and universalis), wax and wane of disease activity, spontaneous hair loss and regrowth, dermoscopic observations, peribulbar infiltration of lymphocytes, and good response to topical immunotherapy with squaric acid dibutylester.24-25 MVC, a small molecule C-C chemokine receptor antagonist, has already been established as part of a combination antiretroviral therapy for treating patients infected by human immunodeficiency virus type 1.26 Therefore, a CCR5 blockade could easily be adapted clinically for treating human AA if MVC shows effectiveness for experimental AA in C3H/HeJ mice.

2 | METHODS

2.1 | Mice and induction of experimental AA by adoptive transfer of stimulated LN cells

Seven-month-old C3H/HeJ mice (SLC) were maintained under specific pathogen-free conditions at the animal facility of the Hamamatsu University School of Medicine. Experimental AA was induced in C3H/HeJ mice by the adoptive transfer of stimulated LN cells.27 Briefly, cervical and inguinal LNs were removed from C3H/HeJ mice with AA. The LNs were then passed through a 70-μm mesh cell strainer (Thermo Fisher Scientific) until the LN clump was dispersed and the medium had turned cloudy. After washing, the LN cells (2 × 10⁶ cells/mL) were cultured in complete RPMI supplemented with recombinant human IL-2 (30 U/mL; Roche Applied Science), recombinant mouse IL-7 (25 ng/mL; R&D Systems), and recombinant mouse IL-15 (50 ng/mL; R&D Systems). Dynabeads T-Activator CD3/CD28 (500 μL; Invitrogen) was then added into 1 mL of the culture medium (final volume of 1.5 mL/well). The stimulated LN cells were washed to remove all of the beads from the cell suspension, and the washed cells were then sucked up into an insulin syringe. These stimulated LN cells were then intradermally injected into the back skin of 6-week-old C3H/HeJ mice.

2.2 | Antibodies and reagents

For the immunofluorescence staining of murine tissue sections, we used antibodies against CCR5 (polyclonal; NB1-41434; Novus Biologicals), CD4 (GK1.5; BioLegend) and CDBa (53.6.7; BioLegend). Multiparameter flow cytometric analysis of murine immune cell phenotype was performed by staining with the following antibodies: CCR5 (C34-3448; BD Pharmingen), CD4 (GK1.5; BD Pharmingen), and CDBa (53.6.7; BD Pharmingen).

2.3 | Treatment and prevention studies using MVC

Maraviroc was purchased from AdooQ.com Bioscience. For the treatment of AA lesions in C3H/HeJ mice, affected mice were orally treated with 330 μg/d of MVC for 5 d/wk (the treatment group) or with phosphate-buffered saline (PBS; the control group). For prevention studies, treatment was started on the day of the adoptive transfer of stimulated LN cells (3 mice/group). MVC
(330 μg/d) or PBS was orally administrated five times weekly for 12 weeks.

2.4 | Treatment of infiltrated cells in experimental AA lesions of C3H/HeJ mice with MVC

Dermal cell suspensions were prepared from the whole trunk skin. To separate the dermal sheets from the epidermal sheets, shaved trunk skin from mice was floated at 37°C for 45 minutes with the dermal side down in complete RPMI 1640 (Sigma-Aldrich) containing 10% heat-inactivated fetal calf serum, 5 × 10⁻³ mol/L 2-mercaptoethanol, 2 mmol/L L-glutamine, 25 mmol/L HEPES, 1 mmol/L nonessential amino acids, 1 mmol/L sodium pyruvate, 100 units/mL penicillin, and 100 μg/mL streptomycin with 0.25% trypsin and 0.27 mmol/L ethylendiaminetetraacetic acid. The dermis was scraped off in complete RPMI, then incubated in PBS with 1% bovine serum albumin containing collagenase I and collagenase II (Liberase TL Research Grade; Roche) for 60 minutes at 37°C, and finally washed and filtered through a 70-μm cell strainer (Becton Dickinson). Next, isolated cells were treated with PBS, 1.0 or 10 μmol/L MVC for 12 hours in complete RPMI supplemented with recombinant human IL-2 (30 U/mL; Roche Applied Science).

2.5 | Treatment of LN cells obtained from C3H/HeJ mice with MVC

Cervical and inguinal LNs were removed from C3H/HeJ mice with experimental AA (n = 5). The LNs were then passed through a 70-μm mesh cell strainer (Thermo Fisher Scientific) until the LN clump was dispersed and the medium had turned cloudy. After washing, the LN cells (2 × 10⁶ cells/mL) were treated with 0, 1, or 10 μmol/L MVC for 12 hours in complete RPMI supplemented with recombinant human IL-2 (30 U/mL; Roche Applied Science).

2.6 | Flow cytometric analysis

Flow cytometric analysis was employed to study the distribution of CCR5+ T cells obtained from healthy and lesioned skin, and LNs from healthy and AA like lesion-affected C3H/HeJ mice. Briefly, isolated LN cells were immediately stained with FITC-conjugated rat anti-mouse CD8a antibody (53-6.7; BD Pharmingen) and PE-conjugated rat anti-mouse CCR5 monoclonal antibody (C34-3448; BD Pharmingen). Skin-infiltrated cells were immediately stained with FITC-conjugated rat anti-mouse CD3 antibody (17A2; BD Pharmingen) and PE-conjugated rat anti-mouse CCR5 monoclonal antibody (C34-3448; BD Pharmingen). All monoclonal antibodies were applied at 1-5 μg/10⁶ cells and incubated for 30 minutes at 4°C, followed by two washes in PBS (pH 7.4) supplemented with 5% fetal calf serum and 0.02% sodium azide. Nonspecific staining was performed with the appropriate same-class immunoglobulin for each specific monoclonal antibody. Fluorescent profiles were generated using FACSCanto II (Becton Dickinson). Cells were first incubated with anti-mouse Fcγ II/III receptor monoclonal antibody for 10 minutes to prevent nonspecific binding of the subsequent reagents to Fc receptors. Dead cells were excluded by adding 7-amino-actinomycin D.

2.7 | Double immunofluorescent staining

Obtained acetone-fixed cryosections of murine skin were stained with rabbit polyclonal anti-mouse CCR5 antibody (Novus Biologicals) followed by donkey anti-rabbit Alexa Fluor 488 antibody (BioLegend). In addition, PE-conjugated anti-mouse CD4 or CD8a antibody (Becton Dickinson) was also applied on the same tissue. The infiltrating cell number is calculated within the square around hair follicles.

2.8 | T-cell isolation and real-time horizontal chemotaxis assay

CD8+ T cells were isolated from LNs by negative selection with a CD8+ T Cell Isolation Kit II (Miltenyi Biotec). The CD8+ T cells were then cultured with MVC in complete RPMI for 12 hours. Time-lapse images of cell migration were observed directly with an optically accessible horizontal chemotaxis apparatus, EZ-TAXIScan (Effector Cell Institute), via a charge-coupled device camera (GE Healthcare) as described previously. The extent of T-cell chemotraction was plotted and analyzed with ImageJ software (National Institutes of Health, https://imagej.nih.gov/ij/). The apparatus consisted of front and back chambers connected by a microchannel. A 1-μL suspension of CD8+ T cells (5 × 10⁶ cells/mL) from murine LN cells was placed in the front chamber, and 1 μL of recombinant mouse RANTES (0-100 μg/mL) was injected into the back six chambers to initiate chemotaxis under the concentration gradient in the channel. The length of the terrace between the two microchannels was 260 μm. Data were analyzed with ImageJ software (National Institutes of Health) and the Manual Tracking plug-in produced by FP Cordelieres (Institut Curie, Orsay, France).

2.9 | Statistical analysis

All data are presented as the mean ± SD. Groups of data were compared using a two-tailed Student’s t-test. P values < .05 were considered to be significant.

2.10 | Ethical approval

All experiments were performed in compliance with institutional guidelines as approved by the Institutional Animal Care and Use Committee of Hamamatsu University School of Medicine.
**3 | RESULTS**

3.1 | MVC significantly decreased the frequency of CCR5 on CD8+ T cells obtained from LN of C3H/HeJ mice with experimental AA

T cells obtained from the LN cells of C3H/HeJ mice with experimental AA were cultured with 0, 1.0, or 10 μmol/L MVC for 12 hours. Then, the expression of CCR5 was analyzed in the LN cells by flow cytometric analysis. As shown in Figure 1, the frequency of CCR5+ cells was 44% lower in the 10 μmol/L MVC-treated cells (0.56%) than in the PBS-treated cells (1.0%), and the frequency of CD8+CCR5+ cells was 2.2-fold lower in the 10 μmol/L MVC-treated cells (0.23%) than in the PBS-treated cells (0.51%).

3.2 | MVC significantly decreased the frequency of CCR5 on CD8+ T cells obtained from experimental AA lesions of C3H/HeJ mice

T cells obtained from experimental AA lesions of C3H/HeJ mice were cultured with PBS or 10 μmol/L MVC for 12 hours. Then, the expression of CCR5 was analyzed in the T cells obtained from the AA lesions and in nonlesional skin by flow cytometric analysis. As shown in Figure 2, the frequency of CD8+CCR5+ cells was lower in the 10 μmol/L MVC-treated cells (2.63%) than in the PBS-treated cells (3.79%), although the frequency of CCR5+ cells was the lowest in nonlesional skin (0.936%). In addition, the frequency of CD8+CCR5+ cells was lower in the 10 μmol/L MVC-treated cells (1.75%) than in the PBS-treated cells (2.52%; Figure 2).

3.3 | MVC strongly decreased the chemotactic velocity of CD8+ T cells toward RANTES

The real-time chemotaxis assay revealed that the chemotactic activity of CD8+ T cells toward RANTES was significantly lower in the MVC-treated CD8+ T cells obtained from LNs (10 mmol/L: 0.108 ± 0.146 μm/s; 1 mmol/L: 0.179 ± 0.198 μm/s) than in the PBS-treated CD8+ T cells obtained from LNs (0.321 ± 0.24 μm/s) in a dose-dependent manner (Figure 3A) (*P < .01). Representative pictures of the real-time chemotaxis assay are shown in Figure 3B.
3.4 Improvement of alopecic lesions by treatment with MVC

After the induction of experimental AA in C3H/HeJ mice by the adoptive transfer of stimulated LN cells, the mice were orally treated with PBS (n = 5) or 330 μg/d (n = 5) of MVC for 5 d/wk (the treatment group) or with PBS (the control group). As shown in Figure 4A-F, 3 of 5 mice treated with 330 μg/d of MVC showed improvement of hair loss on day 15 (Figure 4D,E) when compared to day 0 (Figure 4A,B). In contrast, none of the PBS-treated mice

![Graph showing frequency of CCR5+ T cells in nonlesion and AA lesion skin with treatment groups](graph1)

**Figure 2** Frequency of CCR5+ T cells obtained from experimental AA lesions of C3H/HeJ mice. T cells obtained from experimental AA lesions of C3H/HeJ mice were cultured with PBS or 10 μmol/L MVC for 12 h. Then, the expression of CCR5 was analyzed in the T cells obtained from the experimental AA lesions and in nonlesional skin by flow cytometric analysis. The frequency of CD8+CCR5+ cells is 0.936%, 2.53%, and 1.75% in the skin obtained from nonlesion, PBS, and 10 μmol/L MVC-treated C3H/HeJ mice, respectively.

![Graph showing real-time chemotaxis assay](graph2)

**Figure 3** The real-time chemotaxis assay. The chemotactic activity of CD8+ T cells toward RANTES is 0.108 ± 0.146, 0.179 ± 0.198, and 0.321 ± 0.24 μm/s in the 10 μmol/L MVC-treated CD8+ T cells, 1 μmol/L MVC-treated CD8+ T cells, and PBS-treated CD8+ T cells of LN cells obtained from C3H/HeJ mice, respectively. Representative pictures of the real-time chemotaxis assay are shown in B.
Treatment study of experimental AA by MVC administration in C3H/HeJ mice. Representative pictures of alopecic lesions before treatment (A-C) and after treatment (D-F). Skin samples obtained from C3H/HeJ mice after treatment are stained with H&E (G-I), Alexa Fluor 488 (CCR5⁺ cell)/PE (CD8⁺ cell) (J-L), and Alexa Fluor 488 (NKG2D⁺ cell)/PE (CD8⁺ cell) (M-O). Regrowth area (%)
ITOE et al. showed improvement of hair loss on day 15 (Figure 4F) when compared to day 0 (Figure 4C). Skin biopsy revealed a decreased number of inflammatory mononuclear cells around the hair bulbs of mice treated with 330 μg/d of MVC (Figure 4G,H) when compared to mice treated with PBS (Figure 4I). Immunohistochemical staining also showed decreased infiltration of CCR5^+CD8^+ cells in the MVC-treated mice (Figure 4J,K) when compared to the PBS-treated mice (Figure 4L). The number of infiltrating NKG2D^+CD8^+ T cells was also decreased in the MVC-treated mice (Figure 4M,N) when compared to the PBS-treated mice (Figure 4O). Statistical analysis shows significant difference between MVC-treated mice and PBS-treated mice (The number of CCR5^+CD8^+ cells [square] in the MVC-treated mice vs in the PBS-treated mice: 3.6 ± 0.84 vs 7.8 ± 1.48, P < .01; The number of NKG2D^+CD8^+ cells [square] in the MVC-treated mice vs in the PBS-treated mice: 2.1 ± 1.37 vs 6.2 ± 1.32, P < .01) (Figure 4O,P). The regrowth area (%) was significantly larger in the MVC-treated group than in the PBS-treated group on day 15 (*P = .008) (Figure 4Q).

3.5 | Inhibition of experimental AA induction by MVC administration

C3H/HeJ mice were orally treated with 330 μg/d of MVC (n = 3) or PBS (n = 3) after the injection of LN cells activated by IL-2, IL-7, IL-15, and Dynabeads T-Activator CD3/CD28. MVC completely inhibited the induction of AA after 2 months of injections (Figure 5A-C). In contrast, PBS-treated C3H/HeJ mice showed hair loss lesions on the trunk (Figure 5D-F).

4 | DISCUSSION

The pathogenesis of AA stems mainly from the recognition of HF autoantigens by NKG2D^+CD8^+ T cells accumulated in and around hair bulbs. Therefore, inhibition of the chemotactic activity of T cells toward hair bulbs may be a possible treatment for AA. Basically, AA is a type 1 inflammatory disease. IFN-α-producing plasmacytoid dendritic cells are a strong initiator of AA that upregulate MHC class I expression on proximal ORS and matrix cells, leading to the infiltration of CXCR3/CCR5^+Th1/Tc1 cells that migrate toward the CXCL10 expressed on proximal ORS and matrix cells. After the initiation of AA, IFN-γ-producing CXCR3/CCR5^+Th1/Tc1 cells may establish AA lesions. Thus far, the inhibition of CXCR3 or its ligand, CXCL10, has shown favorable results as a treatment in some models of autoimmune diseases, such as AA, rheumatoid arthritis, and vitiligo. The pathogenesis of vitiligo is similar to that of AA. For example, vitiligo patients have increased numbers of autoreactive melanocyte-specific CXCR3^+CD8^+ T cells in the lesional skin that promote the expression of CXCL10. Treatment with CXCL10-neutralizing antibody led to the repigmentation of murine tails after 8 weeks. In rheumatoid arthritis, extensive lymphocyte infiltration of the joint space, synovium, and periarticular tissues results in joint destruction. These infiltrating lymphocytes express chemokine receptors such as CXCR3, CCR2, CCR4, CCR5, and CXCR6. Injection of anti-CXCR3 monoclonal antibody X3.2 (2 mg) intraperitoneally markedly inhibited mycobacterial antigen-activated T-cell migration to synovial tissues. In AA model C3H/HeJ mice, treatment with neutralizing anti-CXCR3 monoclonal antibody prevented experimental AA induction by AA grafting. MHC class I/II expression, the accumulation of IFN-γ-producing CD8^+NKG2D^+ T cells in the skin, and the expression of CXCL10 mRNA were also decreased by treatment with neutralizing anti-CXCR3 monoclonal antibody.

In this study, we targeted CCR5 for the treatment of experimental AA. Anti-CCR5 antibody, MVC, has been widely used for the treatment of human immunodeficiency virus infection, and it can be easily applied for the treatment of experimental AA. In this study, CCR5 blockade not only improved AA lesions, but also prevented the initiation of experimental AA. As such, MVC can be used for patients who often suffer from relapses of AA lesions. This study also demonstrated the inhibitory effect of MVC against the chemotactic activity of CD8^+ T cells toward RANTES, which is highly expressed in murine experimental AA lesions. This finding indicated that AA initiation can be inhibited by preventing the
accumulation of CCR5+ Th1/Tc1 cells around hair bulbs even when RANTES is overexpressed AA lesions. Furthermore, the inhibition of T-cell accumulation may impede the interaction between autoreactive T cells and the HF-keratinocytes/autoantigens that stabilize these T cells, leading to the downregulation of IFN-γ production from Th1/Tc1 cells.

Our data suggest that a C-C chemokine receptor blockade could inhibit disease progression by preventing the recruitment to HF and the sensitization and reactivation of autoreactive T cells. Actually, this study lacks the data bridging of the current findings to human bona fide AA, small sample size, and no functional data, and further experiments are required to conclude the efficacy of maraviroc on AA. In the future, CCR5 and its ligands should be investigated further as promising candidate novel targets for the treatment of human AA.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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