Reduction of Regulatory T Cells by Mogamulizumab, a Defucosylated Anti-CC Chemokine Receptor 4 Antibody, in Patients with Aggressive/Refractory Mycosis Fungoides and Sézary Syndrome

Xiao Ni, Jeffrey L. Jorgensen, Meghali Goswami, Pramoda Challagundla, William K. Decker, Youn H. Kim, and Madeleine A. Duvic

Abstract

Purpose: The CC chemokine receptor 4 (CCR4) is expressed on malignant T cells in cutaneous T-cell lymphoma (CTCL) as well as on regulatory T cells (Treg). When mogamulizumab, a defucosylated monoclonal antibody, binds to CCR4, it induces anti-body-dependent cellular cytotoxicity against CCR4+ malignant T cells. The goal of this study was to determine the effect of mogamulizumab on CCR4+ Tregs in patients with CTCL.

Experimental Design: Peripheral blood of 24 patients with CTCL participating in a phase I/II trial was analyzed for CCR4 expression on different T-cell subsets by flow cytometry, before and after one course of mogamulizumab. The number and function of natural killer (NK) cells were also analyzed. Lesional biopsies were examined for CCR4, Foxp3, and CD16 expression by immunohistochemistry.

Results: Malignant T cells in peripheral blood were 20.8%-100% positive for CCR4 at baseline. Fourteen patients who achieved a response in blood had high baseline CCR4 expression on malignant T cells. Tregs in blood were 58.6% to 100% positive for CCR4 at baseline and showed decreased numbers and CCR4 expression after treatment. CD8+ T cells in blood were 3.2% to 23.2% positive for CCR4 at baseline and showed limited reduction of CCR4 expression with increased percentages of CD8+ T cells after treatment. Of 14 patients tested for NK cells in blood, 10 showed increased percentages after treatment. Four of 6 patients tested showed increased NK cell cytotoxicity. Sixteen of 18 patients who had CCR4+ lymphocytes in baseline lesions showed decreased numbers after treatment.

Conclusions: Mogamulizumab reduces levels of CCR4+ malignant T cells and also CCR4+ Tregs in patients with CTCL, which may in turn improve immune profiles. Cancer Res; 21(2); 274–85. ©2014 AACR.

Introduction

Cutaneous T-cell lymphomas (CTCL) are characterized by malignant clonal proliferation of skin-homing T cells. Mycosis fungoides and its leukemic form, Sézary syndrome are the most common of CTCL variants. Mycosis fungoides often begins as indolent skin patches or plaques, but may disseminate to lymph nodes, liver, spleen, lung, and blood at advanced stages. Sézary syndrome is characterized by erythroderma covering at least 80% of the body surface area with at least 1,000/µL malignant T cells in the peripheral blood or with a clonal T-cell receptor gene rearrangement (1). Advanced stage or transformed mycosis fungoides and also Sézary syndrome are considered to be aggressive rather than indolent. Patients with aggressive mycosis fungoides/Sézary syndrome have depressed cellular immunity, poor prognosis, and are often refractory to treatment (2, 3). There is an unmet need for more effective targeted therapies for aggressive/refractory mycosis fungoides/Sézary syndrome that are less immunosuppressive and can induce durable complete remissions.

The CC chemokine receptor type 4 (CCR4) is a seven-transmembrane, G-protein–coupled receptor which is specific for the CC chemokines, macrophage-derived chemokine (MDC or CCL22), and thymus and activation-regulated chemokine (TARC or CCL17). CCR4 is expressed on activated Th2 cells, and is critical for T-cell skin-homing (4, 5). CCR4 is also highly expressed on malignant T cells in mycosis fungoides skin lesions and on circulating malignant T cells in patients with Sézary syndrome, making it an ideal molecule for targeted therapy (5, 6). A defucosylated anti-CCR4 monoclonal antibody, KW-0671 or mogamulizumab, was developed to specifically target CCR4+ malignant T cells in T-cell lymphomas (7, 8). Because of a knockout of the FUT8 gene, the backbone of this antibody lacks fucose leading to increased antibody-
dependent cytotoxicity (ADCC) activity. This antibody binds to the N-terminus of CCR4 and has no neutralizing activity of ligand and no complement-dependent cytotoxicity. Strong ADCC against primary malignant T cells in vitro and antitumor activity was observed in an mycosis fungoides/Sézary syndrome mouse model (7, 8).

Regulatory T cells (Treg) are a subset of immunosuppressive T cells that also have high CCR4 expression (9). Tregs play a key role in maintaining self-tolerance and modulating adaptive immune responses; they inhibit an excessive immune response during inflammation by suppressing effector T cells. Increased numbers of Tregs are correlated with poor prognosis in patients with cancer (10, 11). In Hodgkin lymphoma, CCR4+Foxp3+Tregs created an environment in which Hodgkin lymphoma cells could escape from host immunity (12). Therefore, depleting Tregs or inhibiting the function of Tregs in patients with cancer has been recently attempted to boost antitumor immunity (13). Monoclonal antibodies specific for cell surface molecules predominantly expressed by Tregs are under development (13).

The role of Tregs in mycosis fungoides/Sézary syndrome is still controversial because Sézary cells share many features with Tregs. Malignant T cells in patients with Sézary syndrome are anergic and immunosuppressive, they secrete IL10 and TGFβ, and they may also express Foxp3 (14, 15). However, these circulating malignant T cells differ from classic Tregs in several aspects. Sézary cells have a low CD26 or/and CD7 (16), usually have normal to low CD25 expression (17), and have normal IL7 receptor (CD127) expression. In contrast, classic Tregs have high CD25 expression and dim or absent CD127 (18). Therefore, it is still possible to distinguish classic Tregs with CD3+CD4+CD25high/CD127dim/s+ phenotypes from malignant T cells in patients with mycosis fungoides/Sézary syndrome.

Mogamulizumab was approved in Japan for adult T-cell lymphoma (ATL; refs. 19, 20). In the United States, a phase I/II multicenter clinical trial was conducted in patients with CTCL with overall response rates of 37% for all patients (21–24). A recent study reports that KM-2760, another defucosylated anti-CCR4 antibody, was able to reduce the number of tumor-infiltrating Foxp3+ Tregs and increase the number of tumor-infiltrating CD56+ NK cells in a Hodgkin lymphoma mouse model (7). Therefore, we hypothesized that mogamulizumab can not only reduce CCR4+ malignant T cells but also deplete CCR4+ Tregs in patients with mycosis fungoides/Sézary syndrome, and subsequently restore NK cell function. Twenty-four patients with aggressive/refractory mycosis fungoides/Sézary syndrome, participating in the phase I/II clinical trial of mogamulizumab, were studied. Flow cytometry was used to analyze the expression of CCR4 on different T-cell subsets before and after one treatment course. Foxp3 and CCR4 mRNA levels were assessed. The number and function of NK cells were also analyzed. Lesional biopsies were examined for CCR4, Foxp3, and CD16 expression.

Materials and Methods

Patients and study design

This translational study was approved by MD Anderson Cancer Center and Stanford Institutional Review Boards and conducted according to the Declaration of Helsinki in parallel with the phase I/II clinical trial of mogamulizumab (Clinical-Trials.gov identifier: NCT00888927; M.A. Duvic; submitted for publication). All patients signed a written consent. Patients were eligible for the phase I/II study if they had a histologically/cytologically confirmed diagnosis of CTCL, and failed at least one prior systemic therapy. Fresh peripheral blood and lesional skin biopsies were collected at baseline and after the first treatment course. In the first treatment course, patients received weekly infusions of 0.1 or 0.3 or 1.0 mg/kg of mogamulizumab for 4 weeks, followed by a 2-week observation period. An early clinical response to mogamulizumab on day 29 was assessed by one of two attending dermatologists (M.A. Duvic and Y.H. Kim). Blood, skin, lymph node, and overall response were graded as complete response (CR), partial response (PR), stable disease (SD), or progressive disease (PD) as previously described (21–23).

Blood and tissue specimens

Peripheral blood mononuclear cells (PBMC) were separated and aliquoted for flow cytometry, total RNA extraction, and NK assays. Peripheral blood samples from normal donors (ND) were obtained from the Department of Transfusion Medicine at our institution. Skin lesion biopsies were fixed with 4% paraformaldehyde and stored in 80% ethanol until paraffin embedding. Five micrometer sections were consecutively cut for hematoxylin and eosin (H&E) and immunohistochemical staining.

Flow cytometry analysis for different T-cell subsets, NK cells, and CCR4 expression

In this study, we defined malignant T cells in peripheral blood as CD3+CD4+CD26− and/or CD7+ by flow cytometry (16). They additionally often showed alteration in levels of CD3 and/or CD4 expression. Fresh peripheral blood and/or PBMCs at baseline and after one course of mogamulizumab were analyzed for malignant T cells, CCR4+ malignant T cells, CD3+CD4+CD25+CD127low/s−/s−Tregs, CCR4+ Tregs, CD3+CD8+ T cells, CCR4+CD3+CD8+ T cells, and CD3+CD16+CD56+ NK cells by multicolor flow cytometry analysis using BD FACSCanto II cytometers (BD Biosciences). Cell surface expression of CCR4 (CD194) on malignant T cells, Tregs, and CD8+ T cells was assessed by a commercial available antibody, 1G1, from BD Biosciences, Pharmingen. The percentage and absolute number

Translational Relevance

The CC chemokine receptor 4 (CCR4) is expressed on malignant T cells in cutaneous T-cell lymphoma (CTCL) as well as on regulatory T cells (Treg). Mogamulizumab (KW-0761), a defucosylated monoclonal antibody, can induce antibody-dependent cellular cytotoxicity against CCR4+ malignant T cells when it binds to CCR4. Our translational study accompanying a phase I/II trial found that mogamulizumab not only eliminates CCR4+ malignant T cells locally and systemically following one course of therapy, but also reduces CCR4+ Tregs that may be instrumental for restoring NK cell antitumor function in patients with CTCL. Thus, besides broad applications for treating patients with CTCL, mogamulizumab therapy may have value in treating many other tumors with immunosuppressive mechanisms involving Tregs.
of each cell population were calculated at baseline and after treatment.

Quantitative real-time PCR for Foxp3 and CCR4 mRNA expression

Total RNA was extracted from PBMCs with RNaseasy Mini Kit (Qiagen). First strand cDNA was synthesized from 400 ng of total RNA with an oligo (dT) 12–18 primer using SuperScript III reverse transcriptase (Life Technologies Inc.). Preformulated TaqMan primers and probes for Foxp3 and CCR4 (Hs99999919-m1) and GAPDH were used. Quantitative PCR was performed and the relative fold changes were calculated as described previously (15, 25).

Immunohistochemical staining for CCR4, Foxp3, and CD16 in CTCL skin lesions

Mycosis fungoides/Sézary syndrome tissue sections were stained with H&E for pathologic diagnosis and confirmation (26). The expression of CCR4 was assessed by immunohistochemical staining with DAKO EnVision+ System (DAB; Dako North America, Inc.) using the murine parent monoclonal antibody, KM2160 (2.0 µg/mL, Kyowa Hakko Kirin Pharma, Inc.). KM2160 binds to the same epitope as KW-761. HH cell mouse xenograft sections served as a positive control and the SR cell mouse xenograft sections and f(3G1 served as negative controls. The expressions of Foxp3 and CD16 were assessed using anti-Foxp3 monoclonal antibody, 236A/E7 (1.0 mg/mL, 1:10, Abcam Inc.) and prediluted anti-CD16 antibody (2H7; Abcam Inc.) with IgG1 served as negative controls. Xenograft sections served as a positive control and the SR cell

Results

The early blood response is seen after one course of treatment with mogamulizumab

Demographics and baseline laboratory data for 24 patients with CTCL in this translational study are summarized in Table 1. Eleven patients with mycosis fungoides and 13 Sézary syndrome participating in the Phase I/II clinical trial were enrolled. Seven patients participated in phase I, and 17 were participants of phase II. Twenty patients (including 17 in phase II) received four doses of mogamulizumab at 1.0 mg/kg. Two patients received 0.1 mg/kg, and two received 0.3 mg/kg in phase I. After completing one course of treatment, 14 of 19 patients (73.7%) with initial blood involvement had responses in blood, including 10 of 13 Sézary syndrome patients (76.9%) and 4 of 6 in mycosis fungoides patients (66.7%). A response in skin was seen in only 5 of 24 (20.8%), including 2 of 13 Sézary syndrome patients (15.4%), and 3 of 11 mycosis fungoides patients (27.3%). Responses in blood were more common than responses in skin (P < 0.01), suggesting that an early blood response after one course of mogamulizumab occurs in most patients with CTCL, especially patients with Sézary syndrome who have high blood burden.

Decreased circulating CCR4+ malignant T cells are seen after one course of mogamulizumab

The percentages of circulating malignant T cells (out of total lymphocytes) at baseline for all patients are shown in Table 1. All 13 Sézary syndrome patients and 6 of 11 mycosis fungoides patients had detectable circulating malignant T cells (Fig. 1A). The mean percentages and absolute numbers of malignant T cells in patients with CTCL were 39.4% and 4154.4/µL. As expected, patients with Sézary syndrome had higher numbers of circulating malignant T cells than patients with mycosis fungoides who had limited blood involvement (Table 1).

Further flow cytometry analysis for CCR4 expression on malignant T cells was performed with 1G1 anti-CCR4 antibody. Before treatment, the average percentage of malignant T cells positive for CCR4 was 83.7% (Fig. 1B; Table 1). Interestingly, although similar blood malignant T cells at baseline, 14 patients who achieved responses in blood had higher baseline CCR4 expression on malignant T cells (93.5% ± 9.0%) than 5 nonresponders (56.2% ± 42.2%; P < 0.01, Fig. 1C).

After one course of mogamulizumab treatment, malignant T-cell numbers were decreased in 16 of 19 patients, and 14 patients reached >50% reduction for a response in blood (Table 1; Fig. 1A). The average decrease was from 39.4% to 19.7% (Table 2, P < 0.01). Twelve of 13 Sézary syndrome patients had an average reduction from 64.3% to 32.8% (P < 0.01), and 4 of 6 patients with mycosis fungoides with 9.9% to 4.3% (P = 0.12).

As shown in Fig. 1B, the decrease in malignant T cells appears to correspond to elimination of CCR4+ malignant T cells. The average fraction of CCR4+ cells in 19 patients decreased from 83.7% to 25.2% of malignant T cells (P < 0.01, Table 2). All 13 patients with Sézary syndrome showed decreases in CCR4 expression on residual lymphoma cells, with an average reduction from 93.2% to 34.4% (P < 0.01). Five of 6 patients with mycosis fungoides also showed a decrease in CCR4 expression, with an average reduction from 63.5% to 5.2% (P < 0.05).

Next, we used real-time PCR to assess the expression of CCR4 mRNA in PBMCs of 19 patients. Patients with CTCL had much higher CCR4 mRNA levels (23.39 ± 33.50 fold) compared with
healthy donors (2.08 ± 0.64-fold, P < 0.05). As expected, higher CCR4 mRNA levels were seen in patients with Sézary syndrome than in patients with mycosis fungoides. The levels of CCR4 mRNA were correlated with the absolute numbers of circulating CCR4+ malignant T cells in these patients (Fig. 1D, r = 0.731; P < 0.001). The highest CCR4 mRNA level (134.0-fold) was found in Sézary syndrome patient #22 who also had the highest numbers of malignant T cells with 99.5% positive for CCR4 expression. Of 5 mycosis fungoides patients who had no detectable malignant T cells, 4 showed low levels of CCR4 mRNA and one patient (#9) had a moderate level of CCR4 mRNA which could be attributed to increased CCR4+ Tregs. After treatment, all 14 patients showed decreased CCR4 mRNA levels (Fig. 1D) with an average fold change from 23.39- to 1.73-fold (P < 0.05, Table 2), supporting our finding of reduced CCR4+ malignant T cells following mogamulizumab.

The results confirm that the majority of circulating malignant T cells in patients with CTCL, especially those with Sézary syndrome, were CCR4 positive and sensitive to mogamulizumab treatment. Higher CCR4 expression on malignant T cells was associated with an early blood response.

Circulating CCR4+ Tregs also decrease after one course of mogamulizumab

To determine whether mogamulizumab also reduces the numbers of Tregs, we simultaneously monitored the numbers of circulating Tregs during therapy. As shown in Table 1, 18 of 24 patients (75%) had detectable CD3+CD4+CD26– and/or CD7+ malignant T cells in the blood before and after one course of mogamulizumab. A, representative flow plots of lymphocytes from patient #24 before and after mogamulizumab treatment are shown. The percentages of malignant T cells before and after treatment are also shown beneath. B, CCR4 expression on malignant T cells was further analyzed with 1G1 anti-CCR4 antibody. Representative flow plots of CD3+CD4+CD26– cells from patient #24 before and after mogamulizumab are shown. The CCR4-positive cell fractions are circled in each plot. The percentages of CCR4+ malignant T cells before and after treatment are also shown beneath. C, the percentages of malignant T cells (% of lymphocytes) as well as malignant T cells positive for CCR4 (% of malignant T cells) at baseline in blood responders (R, n = 14) versus nonresponders (NR, n = 5) are shown. NS, not significant; **, P < 0.01. D, the correlation between CCR4 mRNA levels (fold changes) and absolute numbers of CCR4+ malignant T cells (μL) by 1G1 anti-CCR4 antibody is shown; *, dots for patient #22; Pearson correlation, r = 0.741, P < 0.001. The levels of CCR4 mRNA in PBMCs of 19 patients at baseline and 14 patients after one course of mogamulizumab are shown.
Table 1. The demographics, malignant T cells, Tregs, CD8+ T cells, NK cells, and responses to mogamulizumab in patients with CTCL

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<td>II</td>
<td>0</td>
<td>23.9</td>
<td>90.5</td>
<td>14.3</td>
<td>20.5</td>
<td></td>
<td>90</td>
<td>16.6</td>
<td>0</td>
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<tr>
<td></td>
<td>10</td>
<td>IVA</td>
<td>67</td>
<td>M</td>
<td>1.0 mg/kg/l</td>
<td>II</td>
<td>13.7</td>
<td>20.8</td>
<td>15</td>
<td>58.6</td>
<td>22</td>
<td></td>
<td>45</td>
<td>15.7</td>
<td>36.4</td>
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<tr>
<td></td>
<td>11</td>
<td>IVA</td>
<td>67</td>
<td>M</td>
<td>1.0 mg/kg/l</td>
<td>II</td>
<td>13.7</td>
<td>20.8</td>
<td>15</td>
<td>58.6</td>
<td>22</td>
<td></td>
<td>45</td>
<td>15.7</td>
<td>36.4</td>
</tr>
</tbody>
</table>

NOTE: Italic indicates statistical significance.
Abbreviations: M, male; F, female; NS, not significant.

*Cell numbers as a percentage of total lymphocytes.
Patients with mycosis fungoides had higher percentages of Tregs than patients with Sézary syndrome. Further flow cytometry analysis using 1G1 anti-CCR4 antibody found that CCR4 was positive on 58.6%–100% of Tregs, with a mean of 88.0% (Fig. 2B; Table 1). Fourteen of 18 patients had more than 90% of Tregs positive for CCR4.

After treatment, there was a clear trend towards decreased Tregs in all 18 patients who had detectable Tregs at baseline, regardless of their clinical response. The decrease was very significant (Table 2). Tregs in patients with mycosis fungoides was decreased from 1.7% or 26.7/μL to 0.4% or 6.1/μL (P < 0.001) in patients with Sézary syndrome from 0.8% or 54.8/μL to 0.3% or 6.1/μL (P < 0.001). These results indicate that the majority of Tregs in the blood of mycosis fungoides/Sézary syndrome were CCR4 positive and were very sensitive to mogamulizumab treatment.

The percentages of CD8⁺ T cells in peripheral blood and the expression of CCR4 on CD8⁺ T cells for all patients at baseline are shown in Table 1. As expected, there were lower percentages of CD8⁺ T cells in Sézary syndrome patients (9.3% ± 6.8%) than in mycosis fungoides patients (18.3% ± 8.5%, P < 0.01). CCR4 was positive on 3.2% to 5.2% circulating Tregs in our patients (Fig. 2C; r = 0.804, P < 0.001). For example, Sézary syndrome patient #18, who had the highest number of Tregs (198.9/μL) among patients, also exhibited the highest Fox3 mRNA level (4.06-fold). Meanwhile, 5 patients with undetectable Tregs showed only low levels of Fox3 mRNA. After treatment, 14 of 14 patients with follow-up blood samples all showed decreased Fox3 mRNA levels (Fig. 2D; Table 2).

Abbreviation: NS, not significant.

### Table 2. Effects of mogamulizumab on lymphoma cells, Tregs, CD8⁺ T cells, CCR4 expression, Foxp3 expression, in peripheral blood and skin lesions of patients with CTCL

<table>
<thead>
<tr>
<th>Patients with CTCL (n = 24)</th>
<th>Before treatment (Mean ± SD)</th>
<th>After treatment (Mean ± SD)</th>
<th>P (t test)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymphoma cells in peripheral blood</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Percentage of total lymphocytes (%)</td>
<td>39.4 ± 34.4</td>
<td>19.7 ± 26.2</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Absolute # (μL)</td>
<td>4,154.4 ± 7,646.3</td>
<td>1,089.6 ± 5,294.5</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>CCR4 expression on lymphoma cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Percentage (IGI, % of total lymphoma cells)</td>
<td>83.7 ± 27.2</td>
<td>25.2 ± 29.2</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Absolute # (IGI, μL)</td>
<td>3,934.8 ± 7,411.9</td>
<td>652.6 ± 2,185.9</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Tregs in peripheral blood</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Percentage of total lymphocytes (%)</td>
<td>1.2 ± 1.1</td>
<td>0.3 ± 0.5</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Absolute # (μL)</td>
<td>39.2 ± 46.8</td>
<td>5.6 ± 8.7</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>CCR4 expression on Tregs</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Percentage (IGI, % of Tregs)</td>
<td>88.0 ± 16.3</td>
<td>30.9 ± 32.0</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Absolute # (IGI, μL)</td>
<td>36.5 ± 46.3</td>
<td>2.7 ± 6.8</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>CD8⁺ T cells in peripheral blood</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Percentage of total lymphocytes (%)</td>
<td>13.4 ± 8.7</td>
<td>23.1 ± 9.9</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Absolute # (μL)</td>
<td>334.5 ± 246.7</td>
<td>363.5 ± 314.8</td>
<td>NS</td>
</tr>
<tr>
<td>CCR4 expression on CD8⁺ T cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Percentage (IGI, % of Tregs)</td>
<td>12.9 ± 6.5</td>
<td>8.6 ± 6.4</td>
<td>NS</td>
</tr>
<tr>
<td>Absolute # (IGI, μL)</td>
<td>45.3 ± 46.6</td>
<td>36.9 ± 60.3</td>
<td>NS</td>
</tr>
<tr>
<td>Foxp3 mRNA in PBMCs (fold)</td>
<td>0.57 ± 0.91</td>
<td>0.07 ± 0.08</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>CCR4 mRNA in PBMCs (fold)</td>
<td>23.39 ± 33.50</td>
<td>1.73 ± 2.35</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>NK cells in peripheral blood (n = 14)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Percentage of total lymphocytes (%)</td>
<td>13.1 ± 11.4</td>
<td>21.4 ± 10.5</td>
<td>0.05</td>
</tr>
<tr>
<td>Absolute # (μL)</td>
<td>380.9 ± 153.6</td>
<td>362.8 ± 175.4</td>
<td>NS</td>
</tr>
<tr>
<td>Killing (%; E:T ratio = 50:1; n = 6)</td>
<td>4.9 ± 3.8</td>
<td>8.7 ± 8.5</td>
<td>NS</td>
</tr>
<tr>
<td>CCR4⁺ lymphocytes in skin lesions (%; n = 18)</td>
<td>51.6 ± 37.4</td>
<td>23.9 ± 28.4</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

Patients with mycosis fungoides had higher percentages of CCR4 expression on lymphoma cells and Tregs in peripheral blood and skin lesions of patients with CTCL. The percentages of CD8⁺ T cells, CCR4 expression, Foxp3 expression, in peripheral blood and skin lesions of patients with CTCL.
The absolute numbers of CD8\(^+\) T cells remained pretty even in Sézary syndrome patients (baseline: 325.7/\(\mu\)L; post: 383.5/\(\mu\)L, \(P = 0.55\)) and mycosis fungoides patients (baseline: 344.9/\(\mu\)L; post: 339.9/\(\mu\)L, \(P = 0.95\)). The results suggest that the expression of CCR4 on CD8\(^+\) T cells was relatively low compared with CCR4 on malignant T cells and Tregs, and that CD8\(^+\) T cells are less sensitive to mogamulizumab treatment.

**Figure 2.** Circulating Tregs, CCR4\(^+\) Tregs, and Foxp3 mRNA in aggressive/refractory mycosis fungoides/Sézary syndrome patients before and after mogamulizumab.

A, multicolor flow cytometry analysis was done to detect CD3\(^+\)CD4\(^+\)CD25\(^+\)CD127\(^+\) Tregs in the blood before and after one course of mogamulizumab. Representative flow plots from patient #6 before and after mogamulizumab are shown. The percentages (% of total lymphocytes) and absolute numbers (/\(\mu\)L) of Tregs from 18 patients before and after treatment are shown. B, CCR4 expression on Tregs was further measured with 1G1 anti-CCR4 antibody before and after mogamulizumab. Representative flow plots from patient #6 before and after mogamulizumab are shown. The CCR4-positive cell fractions are circled in each plot. The percentages of CCR4\(^+\) cells (% of total Tregs) and absolute numbers (/\(\mu\)L) of CCR4\(^+\) Tregs from 18 patients before and after treatment are shown. **P < 0.01. C, the correlation between Foxp3 mRNA levels (fold changes) and absolute numbers of CD3\(^+\)CD4\(^+\)CD25\(^+\)CD127\(^-\) Tregs (/\(\mu\)L) is shown;  \(*\) dots for patient #18; Pearson correlation, \(r = 0.804, P < 0.001\). D, the levels of Foxp3 mRNA in PBMCs of 19 patients at baseline and in 14 patients after one course of mogamulizumab are shown.

CCR4\(^+\) lymphocytes in CTCL skin lesions decrease after one course of mogamulizumab

Using the murine parent anti-CCR4 antibody, KM2160, we assessed CCR4\(^+\) infiltrating lymphocytes in skin lesions from 22 patients with CTCL by immunohistochemistry. Eighteen of 22 baseline lesions had CCR4\(^+\) infiltrating lymphocytes, ranging from 1% to 100% (Fig. 3A–C). Four lesions (2 mycosis fungoides and 2 Sézary syndrome) had no detectable CCR4\(^+\) cells.
percentages of CCR4+ cells in Sézary syndrome lesions (57.3%) and mycosis fungoides lesions (44.8%, P = 0.46, Table 1) were similar. There was no difference in CCR4+ cell numbers in the baseline lesions between patients who had responses in skin (n = 5, 42.7% ± 35.9%) and patients without a response in skin (n = 17, 54.3% ± 36.4%, P = 0.57). Interestingly, after just one course of treatment, all 5 patients who showed clinical partial responses in skin had a decrease in CCR4+ cells. In addition, another 11 patients with stable disease in skin also showed decreased CCR4+ cells to various degrees, with a decrease in the mean from 51.6% to 23.9% (16/18, P < 0.01, Fig. 3C; Table 2). Only one treated patient (#24) retained a high percentage of CCR4+ cells in the lesion, and showed no clinical improvement in skin lesions. A posttreatment lesional biopsy was not available in another patient. Four patients with skin lesions negative for CCR4 at baseline remained negative and none of them showed skin improvement.

Foxp3+-infiltrating lymphocytes were found in only 10 of 22 baseline skin lesions. Eight lesions showed single or scattered Foxp3+ cells, and only 2 lesions (#19 and #24) had countable numbers of Foxp3+ cells. Patient #19 had 10% Foxp3+ cells in the baseline lesion that dropped to 5% positivity following treatment (Fig. 3D), with decreased CCR4+ cells as well. Patient #24 had 2% Foxp3+ cells in both baseline and posttreatment lesions while CCR4+ cells were also unchanged. There were various CD16+ cells in baseline lesions which showed little change after treatment (data not included).

**NK cell numbers and function are restored after treatment with mogamulizumab**

To assess the subsequent effect of mogamulizumab on NK cells, we examined the numbers of CD3−CD56−CD16+ NK cells in the blood and their cytotoxicity before and after treatment. Flow cytometry analysis showed that the percentages of NK cells in 14 patients with CTCL tested (13.1 ± 11.4%, Fig. 4A; Tables 1 and 2) was comparable with those in healthy donors (n = 5, 14.6 ± 12.7%). However, much lower percentages and absolute numbers of NK cells were present in Sézary syndrome patients (n = 7, 5.7 ± 2.7% or 357.6 ± 124.3/µL) than in mycosis fungoides patients (n = 7, 20.5 ± 11.9%, P < 0.01 or 404.2 ± 178.0/µL).

After treatment, 10 of 14 patients demonstrated increased NK cell percentages with an average of 13.1% at baseline versus 21.4% after treatment (P = 0.05), but with similar absolute numbers.
NK cells were increased in 3 of 7 mycosis fungoides patients, but not in the remaining 4 mycosis fungoides patients (average from 20.5% or 404.2/μL to 21.5% or 402.5/μL, \( P = 0.99 \)). In contrast, increases in NK cell percentages were seen in all 7 tested Sézary syndrome patients, with an average increase from 5.7% to 21.3% (\( P < 0.01 \)). All 7 Sézary syndrome patients also had decreased levels of both malignant T cells and Tregs, with 5 of 7 patients achieving responses in blood.

We also assessed NK cell cytotoxicity by a \(^{51}\)Cr release assay. Among 6 patients assessed, 4 patients demonstrated an increase in NK cell killing at different E:T ratios (Fig. 4C). Three of these 4 patients exhibited responses in blood and one mycosis fungoides patient, without blood involvement, achieved a partial response in skin and overall assessment. The average specific killing at the 50:1 ratio was up from 4.9% at baseline to 8.7% after treatment but significant (Table 2; \( P = 0.27 \)). NK cell killing in one patient was similar before and after treatment (#14). Another patient whose NK cell numbers remained unchanged exhibited a decrease in NK cell killing (#6). Blood and skin improvement were not present after one course of treatment in these 2 patients.

The results suggest that NK cell percentages and function may be restored after anti-CCR4 treatment, especially in patients with Sézary syndrome, and could be attributable to the removal of malignant T cells and/or Tregs.

Discussion

We studied 24 aggressive/refractory mycosis fungoides/Sézary syndrome patients participating in a phase I/II clinical trial of mogamulizumab and found that majority of circulating malignant T cells in these patients were positive for CCR4, and decreased after only one 4-week course of mogamulizumab infusion. Indeed, a high proportion of CCR4⁺ malignant T cells in the blood are associated with the early blood response. Circulating Tregs were also positive for CCR4, and were decreased following therapy, confirming the effect of mogamulizumab on Tregs in vivo. The expression levels of Foxp3 and CCR4 mRNA in PBMCs of these patients were consistent with the observed numbers of Tregs and malignant T cells. Meanwhile, CCR4⁻ infiltrating lymphocytes in skin lesions were also reduced after 4 weeks of treatment. A posttreatment increase in NK cell percentages in the remaining 4 mycosis fungoides patients (average from 20.5% or 404.2/μL to 21.5% or 402.5/μL, \( P = 0.99 \)).
Sézary syndrome patients and NK cell cytotoxicity in some patients suggests that potential restoration of NK cell function may also be achieved with 4 weeks of therapy. Thus, mogamulizumab treatment not only eliminates CCR4+ malignant T cells locally and systemically, but also reduces CCR4+ Tregs that may be instrumental for restoring NK cell antitumor function in patients with mycosis fungoides/Sézary syndrome. To our knowledge, studies monitoring the effects of mogamulizumab on different T-cell subsets and NK cells in patients with mycosis fungoides/Sézary syndrome in vivo have not been reported.

Multiple strategies have been attempted to target CCR4 in CTCL. Han and colleagues described an anti-CCR4 gene transfer system in a xenograft mouse model. This approach showed in vivo antitumor activity in mice model and a reduction of CCR4+ malignant T cells and human cell lines in vitro (27, 28), but human trials have not been reported. Mogamulizumab or KW-0761, a humanized antibody given by intravenous infusion, has shown promising results in clinical trials for refractory ATLL committed for publication). Continuing on biweekly treatment schedules (M.A. Duvic; submitted for publication).

Among multiple cell surface markers on malignant T cells of CTCL, CCR4 is a superior target in comparison with other markers like CD3, CD4, or CD52 for therapeutic targeting. CD3 is expressed on all T cells, CD4 is present on all helper T cells, and CD52 is expressed not only on all mature T lymphocytes, but also expressed on all T cells, CD4 is present on all helper T cells, and may be expressed on platelets and important for platelet activation (33, 34), we found a small effect of mogamulizumab on platelets in our study (Supplementary Fig. S1). Thus, CCR4 is a highly specific target on the surface of CTCL malignant T cells, and targeting CCR4 may protect innate immune cells from destruction (35). In addition, CCR4+ malignant T cells are not only found mycosis fungoides and Sézary syndrome, and also in other types of CTCL such as primary cutaneous anaplastic large cell lymphoma, and some peripheral T-cell lymphomas not otherwise specified (6, 36, 37). Hence, broad clinical applications for anti-CCR4 treatment are expected.

High expression of CCR4 on Tregs is reported to be related to tumor escape from immune surveillance. The expression of CCR4 on the surface of Tregs allows them migrate into the tumor site where CCR4 ligands, including CCL17 (TARC) and CCL22 (MDC) are increased (11, 38). High levels of CCL17 and CCL22 were found in CTCL lesions, and could attract CCR4+ malignant T cells as well as CCR4+ Tregs into the skin (6). A recent mouse study suggests that high levels of CCL17 and CCL22 were found in inflammatory lesions where there was a heavily skewed Th2-type cytokine response (39). Advanced CTCL, especially Sézary syndrome, are malignancies with skewed Th2-type cytokine profiles. Therefore, anti-CCR4 treatment in CTCL could not only remove CCR4+ malignant T cells but also deplete immunosuppressive CCR4+ Tregs. In this study, circulating CCR4+ Tregs as well as Foxp3 mRNA levels in PBMCs were reduced in all patients studied after treatment. In CTCL skin lesions, CCR4+ infiltrating lymphocytes were decreased in most skin lesions after treatment. A few Foxp3+ cells were seen in half of lesions. We postulate that Tregs comprise a portion of the CCR4+ cells in CTCL lesions. A more sensitive assay for detecting Tregs in skin lesions could be used in future studies.

Human Tregs are divided into three subsets: effector Tregs (eTregs), naïve Tregs, and nonclassic Tregs (35, 40, 41). Effector Tregs are differentiated and highly suppressive, and they are characterized by a Foxp3hiCD45RAflCD25lo phenotype. Naïve Tregs are Foxp3loCD45RAloCD25hi+ and can differentiate into eTregs after antigenic stimulation. Nonclassic Tregs are Foxp3hiCD45RAloCD25lo and lack suppressive functional activity but do secrete proinflammatory cytokines (35, 40, 41). Sugiyama and colleagues found that CCR4 was predominantly expressed on eTregs but not on naïve Tregs in peripheral blood of healthy controls and patients with melanoma (35). They also found that anti-CCR4 antibody treatment resulted in selective reduction of eTregs, while preserving naïve Tregs. Our study confirms that CCR4+ Tregs in mycosis fungoides/Sézary syndrome patients account for about 90% of Tregs in peripheral blood and they are very sensitive to mogamulizumab. Therefore, mogamulizumab treatment may lead to selective elimination of CCR4+ eTregs in patients with CTCL.

Numerous strategies have or are being investigated to eliminate or inhibit Tregs in patients with cancer. These include anti-CD25 antibody and demileukin diltux, an IL2–diphtheria fusion protein (42). However, elimination of Tregs by these approaches can elicit harmful autoimmunity. If anti-CCR4 treatment reduces only eTregs during treatment, such severe autoimmunity may be avoided because naïve Tregs are still available to differentiate into eTregs. Of note, none of mycosis fungoides/Sézary syndrome patients treated with mogamulizumab in this study experienced a severe immune-related adverse event, except mild skin rash (M.A. Duvic; submitted for publication). Therefore, anti-CCR4 therapy may be a unique cancer immunotherapy by depleting eTregs without eliciting serious autoimmunity.

We found that there are low numbers of CD8+ T cells and NK cells in Sézary syndrome patients compared with mycosis fungoides patients, which may contribute to more severe immune suppression in patients with Sézary syndrome. Interestingly, CD8+ T cells and NK cell numbers rebounded in patients with Sézary syndrome after treatment and NK cell function was increased in some patients. Removal of immunosuppressive Tregs and/or malignant T cells by mogamulizumab may be instrumental for restoration of NK cell numbers and function in patients with mycosis fungoides/Sézary syndrome. The improvement in the immune profiles of CD8+ T cells and NK cells was correlated with clinical responses in blood in patients with Sézary syndrome.
Future studies with longer follow-up are warranted. We see a rationale for use of mogamulizumab to deplete Tregs in vaccination trials in patients with CTCL (25, 43, 44).

Similar to other monoclonal antibodies (mAbs) used in cancer immunotherapy, mogamulizumab works by ADCC. Therefore, any strategy which stimulates NK cell function and enhance ADCC can be synergistic with mogamulizumab therapy. A study reported that an agonistic anti-CD137 (4-1BB) mAb boosted NK cell function when combined with tumor-specific mAbs in xenotransplant models of lymphoma and breast cancer (45–47). Another study reported that ADCC induced by KW2760, another defucosylated anti-CCR4 antibody, is greatly enhanced by the immunomodulatory cytokines IL12, IFNα, and CCR4/CCR5/C0 and IFNγ (7). Future studies combining mogamulizumab with other reagents and ADCC enhancers can test this potential for synergy.

In summary, our study demonstrates that mogamulizumab can concurrently reduce circulating CCR4+ malignant T cells and CCR4+ Tregs in patients with aggressive/refractory mycosis fungoides/Sézary syndrome in vivo. These findings will be further confirmed during the ongoing phase III randomized multicenter clinical trial. We speculate that mogamulizumab therapy may not only have broad applications for treating patients with CTCL, but also may have value in treating many other tumors with immunosuppressive mechanisms involving Tregs.

Disclosure of Potential Conflicts of Interest

J.L. Jorgensen reports receiving a commercial research grant from Kyowa Hakko Kirin. M.A. Duvic reports receiving commercial research grants from Eisai, Galderma, Kyowa Hakko Kirin, Millennium, and Seattle Genetics and is a consultant/advisory board member for Celgene, Eisai, Kyowa Hakko Kirin, and Millennium. No potential conflicts of interest were disclosed by the other authors.

Authors’ Contributions

Conception and design: X. Ni, M. Goswami, M.A. Duvic
Development of methodology: X. Ni, J.L. Jorgensen, M. Goswami, W.K. Decker
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): X. Ni, M. Goswami, P. Challagundla, W.K. Decker, Y.H. Kim, M.A. Duvic
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): X. Ni, J.L. Jorgensen, M. Goswami, P. Challagundla, Y. H. Kim, M.A. Duvic
Writing, review, and/or revision of the manuscript: X. Ni, J.L. Jorgensen, W.K. Decker, Y.H. Kim, M.A. Duvic
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): X. Ni, M.A. Duvic
Study supervision: X. Ni, M.A. Duvic

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References


Reduction of Regulatory T Cells by Mogamulizumab, a Defucosylated Anti-CC Chemokine Receptor 4 Antibody, in Patients with Aggressive/Refractory Mycosis Fungoides and Sézary Syndrome

Xiao Ni, Jeffrey L. Jorgensen, Meghali Goswami, et al.


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