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

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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 antibody-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. Clin Cancer Res; 21(2): 1–12. © 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 FIUT8 gene, the backbone of this antibody lacks fucose leading to increased antibody-
Regulatory T cells (Tregs) are a subset of immunosuppressive T cells that 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 lymphoma, suggesting that Tregs may weaken antitumor 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 lymphoma, suggesting that Tregs may weaken antitumor immune responses. Depleting Tregs or inhibiting the function of Tregs in patients with cancer has been recently attempted to boost antitumor immunity (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 TGF-β, and they may also express Foxp3 (14, 15). However, these circulating malignant T cells differ from classic Tregs in several aspects. Sézary cells have little to no expression of CD26 or and CD7 (16), usually have normal to low CD45 expression (17), and have normal IL7 receptor (CD127) expression. In contrast, classic Tregs have high CD25 expression and dim or absent CD127 (18). Therefore, depleting Tregs or inhibiting the function of Tregs in patients with cancer has been recently attempted to boost antitumor immunity (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 TGF-β, and they may also express Foxp3 (14, 15). However, these circulating malignant T cells differ from classic Tregs in several aspects. Sézary cells have little to no expression of CD26 or and CD7 (16), usually have normal to low CD45 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/C19+ C19dim−/− 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, 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.

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.

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 (ClinicalTrials.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−/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+/CD127dim−/− 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
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 RNeasy 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.). Preformed 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 IgG1 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 the same DAKO EnVision+ System. The percentages of lymphocytic infiltrates positive for CCR4 and Foxp3 as well as CD16+ cells in skin lesions were recorded.

Chromium release assay

Target K562 cells (ATCC) were radiolabeled with 200 pCi/mL Na[51]Cr (Amersham) at 1 × 10^6/mL for 60 minutes. Labeled K562 cells were suspended at 5 × 10^4 cells/mL, and dispensed in 100 µL aliquots into wells of a V-bottom 96-well plate (Costar). PBMC effector cells were suspended at concentrations of 2.5 × 10^6, 1 × 10^6, and 2.5 × 10^6 cells/mL. Then, 100 µL aliquots of effector cells were added to wells containing target cells to reach effector (E) to target (T) ratios of 5:1, 20:1, and 50:1. Maximum release was determined by incubating target cells with BRJ-35 detergent (Sigma). Spontaneous release was determined by incubating target cells in medium only. After 4 hours of incubation at 37°C, supernatants were harvested and 51Cr release was measured in a gamma counter. Percentages of specific release were calculated as (experimental release – spontaneous release)/maximum release × 100. All samples were analyzed in quadruplicate.

Statistical analysis

For flow cytometry analysis, the percentage of lymphocytes and absolute numbers of each cell population were obtained for all patients at baseline and after one course of treatment. For real-time PCR analysis, fold changes were obtained for all samples. The means and SDs were then calculated for all groups. Statistical significance was determined by χ² test, Student t test, and paired t test as appropriate. The correlation between two parameters was analyzed using the Pearson correlation test. Differences between groups were considered significant if P < 0.05.

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−/CD7+ Tregs at baseline (Fig. 2A). Five Sézary syndrome and one mycosis fungoides patients had no detectable Tregs. The mean percentage and absolute numbers of CCR4+ malignant T cells (of total lymphocytes) were 1.2% and 39.2/μL for all patients with CTCL.
Table 1. The demographics, malignant T cells, Tregs, CD8+ T cells, NK cells, and responses to mogamulizumab in patients with CTCL

<table>
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<th>Primary diagnosis</th>
<th>Patient ID</th>
<th>Stage</th>
<th>Age, y</th>
<th>Gender</th>
<th>Dose/course</th>
<th>Phase</th>
<th>Malignant T cells in peripheral blood (%)</th>
<th>Tregs in peripheral blood (%)</th>
<th>CCR4+ on malignant T cells (%)</th>
<th>CCR4+ on Tregs (%)</th>
<th>CCR4+ on CD8+ T cells (%)</th>
<th>CD8+ T cells in peripheral blood (%)</th>
<th>CCR4+ on NK cells in skin lesions (%)</th>
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<td>11.5</td>
<td>6.2</td>
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Mean ± SD

|                      | 68 (48–81) | 10/1 (M/F) | 1/1/9 | 3/8 | 9.9 ± 11.6 | 63.1 ± 42.3 | 1.7 ± 1.0 | 88.8 ± 12.5 | 18.3 ± 8.3 | 13.3 ± 6.1 | 20.5 ± 11.9 | 44.8 ± 38.2 | 4/6 | 27.3% | —     | 36.4% |

Mean ± SD

|                      | 65 (47–86) | 7/6 | 1/1/11 | 4/9 | 6.4 ± 16.2 | 93.2 ± 8.1 | 0.8 ± 1.0 | 87.1 ± 20.5 | 125.6 ± 6.9 | 5.7 ± 2.7 | 57.3 ± 35.8 | 76.9% | 15.4% | 16.7% | 15.4% |

Sum n = 24

|                      | 67.5 (48–86) | 17/7 | 2/2/20 | 7/17 | 39.4 ± 34.4 | 83.7 ± 27.2 | 1.2 ± 1.1 | 88.0 ± 16.3 | 13.4 ± 8.7 | 12.9 ± 6.5 | 131.0 ± 11.4 | 75.8 ± 37.4 | 14/19 | 5/24 | 2/17 | 6/24 |

73.7% 20.8% 11.3% 25.0%

Note: Italics indicate statistical significance.

Abbreviations: M, male; F, female; NS, not significant.

*aCell 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%/C19 to 0.8% or 7.0%/C19 (P < 0.01). In patients with Sézary syndrome from 0.8% or 54.8%/C6 to 0.3% or 6.1%/C6 (P < 0.05).

All 18 patients had decreased CCR4 expression on Tregs as well as decreased absolute CCR4+ Tregs after treatment, regardless of clinical response. The decrease in CCR4+ Tregs was very significant (Table 2). The percentages of CCR4+ cells and absolute counts in patients with mycosis fungoides decreased from 88.8% and 23.5%/C6 to 16.8% and 0.8%/C6, and in patients with Sézary syndrome from 87.1% and 52.9%/C6 to 46.5% and 4.6%/C6.

We also assessed the mRNA expression of Foxp3, a transcription factor important for the development of Tregs. We were able to detect Foxp3 mRNA in PBMCs of all 19 patients tested at baseline. Higher expression of Foxp3 mRNA was present in patients with CTCL (0.57 ± 0.91 fold) compared with healthy donors (0.09 ± 0.04 fold, P < 0.05). Foxp3 mRNA levels were slightly higher in patients with Sézary syndrome (0.79 ± 1.2 fold) than in patients with mycosis fungoides (0.33 ± 0.30 fold, P = 0.27). This may be due to the higher absolute numbers of Tregs in patients with Sézary syndrome (54.8%/C6) compared with mycosis fungoides patients (26.7%/C6 and Foxp3 expression by malignant T cells (15). The levels of Foxp3 mRNA in PBMCs were well correlated with the absolute numbers of CD3+ CD4+CD25+CD127dim/− 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%/C6) among patients, also exhibited the highest Foxp3 mRNA level (4.06-fold). Meanwhile, 5 patients with undetectable Tregs showed low levels of Foxp3 mRNA. After treatment, 14 of 14 patients with follow-up blood samples all showed decreased Foxp3 mRNA levels (Fig. 2D; Table 2).

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 increase after one course of mogamulizumab

The percentages of CD8+ T cells of total lymphocytes 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% ± 6.7%) than in mycosis fungoides patients (18.3% ± 8.3%, P < 0.01). CCR4 was positive on 3.2% to 24.7% of CD8+ T cells, with an average of 12.9% (Table 1).

After one course of mogamulizumab, CCR4 expression was reduced on CD8+ T cells in patients with mycosis fungoides, with an average from 13.3% ± 6.1% to 7.0% ± 5.2% (P < 0.05) but not seen in Sézary syndrome patients (12.5% ± 6.8% to 10.0% ± 6.9%, P > 0.05). Interestingly, the percentages of CD8+ T cells were increased in 23 of 24 patients, an average increase from 13.4% ± 8.7% to 23.1% ± 9.9% in all patients (Table 2, P < 0.01). The increases were seen in both Sézary syndrome patients (20.8 ± 10.9%, P < 0.01) and mycosis fungoides patients (25.9 ± 7.5% P <

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 ± 3,294.5</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>CCR4 expression on lymphoma cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Percentage (1G1, % 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 # (1G1, /μL)</td>
<td>3,934.8 ± 7,411.9</td>
<td>652.6 ± 2,185.9</td>
<td>&lt;0.05</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 # (1G1, /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 (1G1, % of Tregs)</td>
<td>88.0 ± 16.3</td>
<td>30.9 ± 32.0</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Absolute # (1G1, /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 # (1G1, /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 (1G1, % of Tregs)</td>
<td>12.9 ± 6.5</td>
<td>8.6 ± 6.4</td>
<td>NS</td>
</tr>
<tr>
<td>Absolute # (1G1, /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 # (1G1, /μL)</td>
<td>380.9 ± 153.6</td>
<td>362.8 ± 175.4</td>
<td>NS</td>
</tr>
<tr>
<td>Killing (%; E:T, L)</td>
<td>4.9 ± 3.8</td>
<td>8.7 ± 8.5</td>
<td>NS</td>
</tr>
<tr>
<td>Foxp3 mRNA in PBMCs in skin lesions (%)</td>
<td>51.6 ± 37.4</td>
<td>23.9 ± 28.4</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

Abbreviation: NS, not significant.

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The absolute numbers of CD8^+ T cells remained pretty even in Sézary syndrome patients (baseline: 325.7 ± 219.5/μL; post: 383.5 ± 374.2/μL, P = 0.55) and mycosis fungoides patients (baseline: 344.9 ± 275.1/μL; post: 339.9 ± 223.1/μL, 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.

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. Average
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 (\( n = 5, 42.7\% \pm 35.9\% \)) and patients without a response in skin (\( n = 17, 54.3\% \pm 36.4\%, P = 0.57 \)). 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 (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 healthy donors (\( n = 5, 14.6 \pm 12.7\% \)). However, much lower percentages and absolute numbers of NK cells were present in Sézary syndrome patients (\( n = 7, 5.7 \pm 2.7\% \) or 357.6 ± 124.3/µL) than in mycosis fungoides patients (\( n = 7, 20.5 \pm 11.9\%, P < 0.01 \) 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

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**Figure 3.**

CCR4 and Foxp3 expression by immunohistochemical staining (IHC) in mycosis fungoides/Sézary syndrome skin lesions before and after mogamulizumab. A, mycosis fungoides/Sézary syndrome skin specimens before and after treatments were stained with hematoxylin and eosin (H&E) for pathologic diagnosis and confirmation, patient #15; magnification, \( \times 200 \). B, by IHC, CCR4 protein was examined using the murine parent anti-CCR4 antibody (KM2160, 2.0 µL/mL) in skin lesions before and after treatment, from patient #15; magnification, \( \times 200 \). C, CCR4⁺-infiltrating lymphocytes (% of total infiltrating lymphocytes) in skin lesions from 18 patients before and after treatment are presented, paired t test; **, \( P < 0.01 \). D, by IHC, Foxp3 protein was examined using antibody (236A/E7, 1.0 mg/mL, 1:10) in skin lesion before and after treatment, from patient #19; magnification, \( \times 200 \).
NK cells were increased in 3 of 7 mycosis fungoides patients, but not in the remaining 4 mycosis fungoides patients (average from 20.5% to 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 51Cr 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...
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 and PLCL (19, 20) and for mycosis fungoides/Sézary syndrome (19–21, 29).

This translational study ran in parallel with the phase I/II trial, but clinical responses were assessed after only one course or four weekly doses of treatment. A high response rate was seen in blood in 14 of 19 or 73.7% of patients studied, compared with one-fifth of patients with responses in skin (5/24, 20.8%). The difference between the responses in blood and in skin may be attributed to the intravenous administration of mogamulizumab and to the high numbers of CCR4+ cells in blood of Sézary syndrome patients (average of 61.8% in Sézary syndrome and 10.5% in mycosis fungoides). CCR4+ cells were decreased in 16 of 18 skin lesions. A longer treatment time may be required to eliminate CCR4+ cells from skin lesions. In the completed phase I/II clinical trial, skin responses were seen in more than 40% of patients 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 on B lymphocytes, NK cells, and dendritic cells (30). Targeting these markers may deplete all T cells, normal helper T cells, or other innate immune cells, raising the risk of infection and other adverse events (31, 32). In contrast, CCR4 is highly expressed on skin-homing T cells which give rise to malignant T-cell clones in CTCL (6). Moreover, CCR4 is rarely expressed on CD8+ T cells, B cells, NK cells, monocytes, and/or macrophages. Although CCR4 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, but 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: effectors Tregs (eTregs), naive Tregs, and nonclassic Tregs (35, 40, 41). Effector Tregs are differentiated and highly suppressive, and they are characterized by a Foxp3hi CD45RA − CD25hi phenotype. Naive Tregs are Foxp3lo CD45RA+CD25lo and can differentiate into eTregs after antigenic stimulation. Nonclassic Tregs are Foxp3lo+ CD45RA− CD25lo− 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 naive Tregs in peripheral blood of healthy donors and patients with melanoma (35). They also found that anti-CCR4 antibody treatment resulted in selective reduction of eTregs, while preserving naive 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 denileukin difitox, an IL2–diptheria 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 maybe 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 (mAb) 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 IL-12, IFN-α2, and IFN-γ (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/Sezary 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
I.L. Jorgensen reports receiving a commercial research grant from Kyowa Hakko Kirin. M.A. Duvic reports receiving commercial research grants from Eisai, Calderma, Kyowa Hakko Kirin, Millennium, and Seattle Genetics and is a consultant/advisory board member for Celgene, Eisai, Kyowa Hakko Kirin, and Millenium. No potential conflicts of interest were disclosed by the other authors.

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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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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

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