Phase Ia Study of FoxP3^+ CD4 Treg Depletion by Infusion of a Humanized Anti-CCR4 Antibody, KW-0761, in Cancer Patients

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Abstract

Purpose: FoxP3^+ Tregs inhibit immune responses against tumors. KW-0761 is a humanized anti-human CCR4 monoclonal antibody (mAb) that has antibody-dependent cellular cytotoxicity activity. Depletion of CCR4-expressing FoxP3^+ CD4 Tregs by KW-0761 infusion was investigated in solid cancer patients.

Experimental Design: We conducted a phase I clinical trial of KW-0761 infusion in 7 lung and 3 esophageal cancer patients. Toxicity, clinical efficacy, changes in lymphocyte subpopulations, including Tregs, and induction of immune responses were analyzed.

Results: The results showed that KW-0761 infusion in a dose range between 0.1 mg/kg and 1.0 mg/kg was safe and well tolerated. No dose-limiting toxicity was observed. Four of 10 patients showed stable disease during treatment and were long survivors. The monitoring of FoxP3^+ Tregs in the peripheral blood mononuclear cells during treatment indicated efficient depletion of those cells, even at the lowest dose of 0.1 mg/kg used. The reduction in Th 1 CD4 T cells and CD8 T cells was limited, whereas a significant reduction was observed with Th 2 and Th 17 CD4 T cells. Immune responses to cancer/estis (CT) antigens and an autoantibody response to thyroid peroxidase were observed in some patients.

Conclusions: The findings showed Tregs depletion and the possible occurrence of an immune response following KW-0761 infusion. Combined use of KW-0761 to deplete FoxP3^+ Tregs with other immunotherapies, such as cancer vaccines or checkpoint inhibitors, is a promising approach to augment immune responses. Clin Cancer Res; 21(19); 4327–36. ©2015 AACR.

Introduction

KW-0761 (Mogamulizumab) is a humanized anti-CCR4 immunoglobulin G1 (IgG1) mAb with a defucosylated Fc region, which markedly enhances antibody-dependent cellular cytotoxicity (ADCC; refs. 1, 2). The antibody is capable of efficiently lysing adult T-cell leukemia lymphoma (ATL) cells that express CCR4 on their surfaces and was approved for the treatment of ATL in Japan in 2012 (3–5).

FoxP3 is a key transcription factor for CD4 Tregs (6). Miyara and colleagues (7) reported that human FoxP3^+ CD4 T cells were composed of three functionally and phenotypically distinct subpopulations. CD45RA^− FoxP3^+ CD4 T cells were recently shown in lung cancer (11). FoxP3^+ CD4 T cells in peripheral blood mononuclear cells (PBMC), activated Tregs with strong inhibitory activity express CCR4 at a high level (7, 8).

Infiltration of FoxP3^+ Tregs to local tumor sites has been shown in various murine and human tumors (9). The involvement of CCR4 and CCR4-associated chemokines, TARC/CCL17 and MDC/CCL22, for FoxP3^+ Tregs migration has been documented (10). The accumulation of activated/effecter Tregs in the tumor site and the production of MDC/CCL22 by intratumor myeloid cells were recently shown in lung cancer (11). FoxP3^+ Tregs inhibit immune responses against tumors and also diminish the immunotherapeutic effects which activate host immune responses. The CD8 T cells to FoxP3^+ Tregs ratio correlated with a favorable prognosis in some human cancers (12).

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**Translational Relevance**

KW-0761 is a humanized anti-CCR4 monoclonal antibody that has antibody-dependent cellular cytotoxicity activity. Depletion of CCR4-expressing FoxP3⁺ CD4 Tregs by KW-0761 infusion was investigated in solid cancer patients in a phase 1a clinical trial. The results showed that KW-0761 infusion at a dose range between 0.1 mg/kg and 1.0 mg/kg was safe and well tolerated. Some toxicities, including persistent skin rash, were observed, but no dose-limiting toxicity was observed. The monitoring of FoxP3⁺ Tregs in the peripheral blood mononuclear cells during treatment indicated efficient depletion of those cells even at the lowest dose of 0.1 mg/kg used. Immune responses to cancer/testis antigens and an autoantibody response to thyroid peroxidase were elicited in some patients. The findings showed Tregs depletion and the possible occurrence of an immune response following KW-0761 infusion. Combined use of KW-0761 to deplete FoxP3⁺ Tregs with other immunotherapies, such as cancer vaccines or checkpoint inhibitors, is a promising approach to augment immune responses.

In humans, prolonged elimination of FoxP3⁺ CD25⁺ CD4 T cells together with leukemic cells was observed following treatment with KW-0761 in ATL patients (5). These findings together with recent findings of efficient elimination of FoxP3⁺ Tregs by a chimeric anti-human CCR4 mAb (KM2760) suggested its potential use in solid cancer patients (11).

To examine selective FoxP3⁺ CD4 Tregs depletion in solid cancer patients, we conducted a phase 1a clinical trial of KW-0761 infusion in 10 patients with CCR4-negative lung and esophageal cancers.

**Patients and Methods**

**Patients**

Patients over 20 years of age with advanced or recurrent CCR4-negative cancer were eligible. CCR4 expression was examined by immunohistochemistry (IHC) using an anti-CCR4 mAb (KM2160; Kyowa Hakko Kirin; ref. 4) and confirmed by the review committee with central evaluation. All patients were required to have an Eastern Cooperative Oncology Group performance status of 0 to 2. Eligibility criteria also included the following laboratory values: an absolute neutrophil count ≥ 1,500/μL, hemoglobin ≥ 8.0 g/dL, platelet count ≥ 75,000/μL, total bilirubin ≤ 2.0 mg/dL, AST ≤ 2.5 × the upper limit of the normal range (UNL), ALT ≤ 2.5 × UNL, serum creatinine ≤ 1.5 mg/dL, and arterial blood oxygen saturation ≥ 93%. All subjects underwent electrocardiography to confirm the absence of abnormalities requiring treatment, and that the left ventricular ejection fraction was at least 50%. Patients were excluded if they had an active infection, a history of organ transplantation, active concurrent cancer, any autoimmune disease, central nervous system involvement, hepatitis B or C virus infection, or HIV infection. The protocol was approved by the institutional review boards at each participating site, and all patients provided written informed consent before enrolment, in accordance with the Declaration of Helsinki.

To examine CCR4 expression on lymphocyte subpopulations, 12 non–small cell lung cancer (NSCLC) patients were enrolled in an independent study from this clinical trial. The patients were recruited into the study of the “Analysis of cancer antigen and host immune response” at the Kawasaki Medical School Hospital (Kurashiki, Japan) with approval of the local ethics committee (number: 603-7). Patients diagnosed with advanced NSCLC were enrolled after obtaining written informed consent in accordance with the Declaration of Helsinki.

**Study design**

This study is a part of an investigator-initiated phase 1a/b clinical trial of KW-0761 (Mogamulizumab) infusion in patients with CCR4-negative advanced or recurrent solid tumors (NCT01929486). The primary end point of the present multicenter dose-escalation study was the safety and pharmacokinetic profile of KW-0761 for patients with advanced or recurrent cancer. The secondary end points included the Treg depletion effect in PBMCs compared with the baseline, and the overall response rate, progression-free survival (PFS), and overall survival (OS). Three to 6 patients were enrolled at each dose level to determine the MTD and estimate the recommended phase Ib dose. Cohorts of patients received KW-0761 at 0.1, 0.5, and 1.0 mg/kg, weekly 8 times followed by monthly intravenous infusion until disease progression. Oral antihistamines and acetaminophen were administered before each KW-0761 infusion, and hydrocortisone was simultaneously injected intravenously for the first KW-0761 dose to prevent infusion reactions. If no dose-limiting toxicity (DLT) was observed in a cohort of 3 patients at a given dose level, the next cohort of 3 new patients could be treated with the next higher dose. If DLT was experienced by 1 or 2 of the 3 patients at any dose, 3 additional patients were treated at the same dose level. If 3 or more patients at a given dose level exhibited DLT, this dose was considered to exceed the MTD and the dose escalation was therefore halted. The recommended phase Ib dose was defined as one dose level below the MTD or the maximum dose level judged to be tolerable.

As an additional research parameter, we investigated the effect on various T-cell subpopulations in PBMCs, immune responses to the CT antigens NY-ESO-1 or XAGE1 (GAGED2a), and autoantigens during and after KW-0761 treatment.

**Toxicity evaluation and definition of DLT**

Patients treated at each dose level were evaluated weekly during therapy and until 24 weeks after the last infusion to assess toxicity. Toxicity was graded according to the National Cancer Institute Common Terminology Criteria for Adverse Events version 4.0. DLT was defined as an adverse event (AE) or a laboratory abnormality that occurred from the first KW-0761 infusion to 7 days later. Patients were enrolled if they had a hematologic toxicity except lymphopenia or CD4-positive lymphopenia, grade 4 symptoms judged to be consistent with an acute infusion reaction/cytokine release syndrome or with tumor lysis syndrome, and grade 3 non-hematologic toxicities. The independent data monitoring committee evaluated the safety data at all dose levels.

**Pharmacokinetics**

Blood was drawn into a heparin-containing tube, and plasma concentrations of KW-0761 were assessed using an ELISA. The
pharmacokinetic parameters of plasma KW-0761 concentrations were calculated by employing a noncompartment model using WINNonlin (Scientific Consulting) software; plasma maximum (Cmax) and trough (Cthrough) drug concentrations after each administration of KW-0761, and the plasma half-life (t1/2) and area under the blood concentration time curve (AUC0-7 days) after the first and the eighth infusions.

**Clinical responses**

Responses were evaluated at 12 weeks after the first KW-0761 infusion or at the point of study discontinuation using CT scans. The effects were determined according to RECIST (ver. 1.1; ref. 14) and immune-related (ir) RECIST (15). PFS was determined from the day of the first KW-0761 infusion until the date of progressive disease (PD) detection. The tumor response and PFS of each subject were confirmed by the efficacy assessment committee with a central evaluation.

**Treg depletion effect on PBMCs**

Treg depletion was evaluated by flow cytometry. CD45RA−FoxP3 lo resting/naïve Tregs, CD45RA−FoxP3 hi activated/effector Tregs, and CD45RA+FoxP3 lo, non Tregs were analyzed as described previously (7). Intracellular FoxP3 staining was performed using a FoxP3/Transcription Factor Staining Buffer Set (eBioscience) according to the manufacturer’s instructions. Anti-CD4-PerCP (clone SK3; BD Biosciences), anti--CD25-APC (clone 2A3; BD Biosciences), biotinylated anti-CCR4 (clone; 1G1; BD Pharmingen), Streptavidin-PE (BD Biosciences), anti-CD11b-PE/Cy7 (clone M1/70; Beckman Coulter), and anti-FoxP3-PE (clone PCH101; eBioscience) were used for phenotypic analysis of Tregs.

**Overlapping peptides**

Overlapping NY-ESO-1 and XAGE1 (GAGED2a) peptides spanning the entire proteins were synthesized using Fmoc chemistry on a Multiple Peptide Synthesizer (AMS422, ABIMED) at Okayama University and were described in detail previously (16, 17).

**NY-ESO-1 and XAGE1 (GAGED2a) protein**

Production of NY-ESO-1 protein has been described before (18). XAGE1 (GAGED2a) protein was synthesized using a peptide synthetizer by GL Biochemistry.

**IHC**

Expression of CCR4, NY-ESO-1, and XAGE1 (GAGED2a) was examined by IHC as described previously (11, 19–22).

**ELISA for NY-ESO-1 or XAGE1 (GAGED2a) antibodies**

Recombinant NY-ESO-1 and synthetic XAGE1 (GAGED2a) protein (1 μmol/L) in an ELISA Buffer kit (PeproTech) was adsorbed onto a 96-well ELISA plate (Nunc) and incubated overnight at 4°C. Plates were washed with 0.05% Tween 20 in PBS and blocked with 1% BSA/PBS (200 μL/well) for 1 hour at room temperature. After washing, 100 μL of serially diluted serum was added to each well and incubated for 2 hours at room temperature. After washing, horseradish peroxidase (HRP)-conjugated goat anti-human IgG (MBL) was added to the wells, and the plates were incubated for 1 hour at room temperature. After washing and development, absorbance was read at 490 nm (16, 17).

**Flow cytometry**

PBMCs were isolated from heparinized blood by density gradient centrifugation using Ficoll-Faqué Plus (GE Healthcare). The cells were stored in liquid N2 until use. After thawing, PBMCs were incubated with the mAbs for 20 minutes at 4°C. Anti–CD3-V450 (clone UCHT1; BD Horizon), anti–CD4-V500 (clone RPA-T4; BD Horizon), anti–CD6-APC/Cy7 (clone SK1; BD Pharmingen), anti–CD183 (CXR3)-PerCP/Cy5.5 (clone G025H7; BioLegend), anti–CD196 (CCR6)-PE/Cy7 (clone 11A9; BD Pharmingen), anti–CD294 (CRTH2)-PE (clone BM16; BioLegend), and anti–CD185 (CXCR5)–Alexa Fluor 488 (clone RF8B2; BD Pharmingen) were used for phenotypic analysis of CD4.

Analysis of Th1, Th17, Th2, and Tfh cell subsets by the expression of CXC chemokine receptor 3 (CXR3), CC chemokine receptor 6 (CCR6), chemotaxtractant receptor-homologous molecule 2 (CRTH2), and CXC chemokine receptor 5 (CXCR5), respectively, was done as described previously (23).

Anti–CD8-PE (clone T8; Beckman Coulter), anti–CD19-PE (clone B4; Beckman Coulter), anti–CD16-FITC (clone 3G8; Beckman Coulter), and anti–CD56-PE (clone NK1H1-1; Beckman Coulter) were used for phenotypic analysis of CD8, B cells, and natural killer (NK) cells.

Anti–CD3-V450 (clone UCHT1; BD Horizon), anti–CD45-APC (clone H30; BD Pharmingen), anti–CD14-PE/Cy7 (clone M5E2; BD Pharmingen), anti–HLA-DR-APC/Cy7 (clone L243; BioLegend), anti–CD11b-PE (clone ICRF44; BioLegend), anti–CD15-V500 (clone H98; BD Horizon), and anti–CD33-PerCP/Cy5.5 (clone P67.6; BD Biosciences) were used for phenotypic analysis of myeloid-derived suppressor cells (MDSC). MDSCs were defined as a CD45−CD3+CD11b+CD33+HLA-DR+/−population. M-MDSCs and PMN-MDSCs were defined as CD14+MDSCs and CD15+MDSCs, respectively.

Anti–CD3-V450 (BD Horizon), anti–CD4-V500 (BD Horizon), anti–CD8-APC/Cy7 (BD Pharmingen), anti–CD278(UCS)-PE (clone DX29; BD Pharmingen), anti–CD134 (OX40)-PerCP/Cy5.5 (clone Ber-Act35; BioLegend), anti–CD357 (GITR)-Alexa Fluor 488 (clone eBioAITR; eBioscience), anti–CD137 (4-1BB)-APC (clone 4B4-1; BioLegend), anti–CD279 (PD-1)-PE/Cy7 (clone E1H2.2H7; BioLegend), anti–CD272 (BTLA)-PE (clone MH26; BioLegend), anti–CD366 (Tim-3)-APC (clone F38-2E2; eBioscience), and anti–CD244 (2B4)-FITC (clone eBioDM244; eBioscience) were used for analysis of activation and inhibitory molecules on T cells.

After incubation, the cells were washed and analyzed by FACS Canto II (BD Biosciences).

**In vitro stimulation of CD4 and CD8 T cells with NY-ESO-1 and XAGE1 (GAGED2a) antigens and detection of cytokine production**

CD4 (1 × 10⁶/well) and CD8 (1 × 10⁶/well) T cells purified by magnetic cell sorting (Miltenyi Biotec) were cultured with an equal number of irradiated (40 Gy), autologous CD4- and CD8-depleted cells as APC in the presence of a mixture of 18-mer NY-ESO-1 or 16-mer XAGE1 (GAGED2a) overlapping peptides (OLP; 1 μmol/L) for CD4 T cells and in the presence of 18-mer NY-ESO-1 OLPs or synthetic XAGE1 (GAGED2a) protein (1 μmol/L) for CD8 T cells on a 24-well culture plate (BD Falcon).

**References**

(16, 17)
for 12 days at 37°C in a 5% CO2 atmosphere. The medium was ALM-V-Life Technologies) supplemented with 5% pooled human serum, 10 units/ml recombinant IL2 (Takeda Chemical Industries), and 10 ng/ml recombinant IL7 (Peprotech).

After incubation, responder CD4 or CD8 T cells (1 × 10^6) harvested from the stimulation culture were washed and then pulsed with 18-mer NY-ESO-1 or 16-mer XAGE1 (GAGED2a) OLPs for CD4 T cells, or 18-mer NY-ESO-1 or 12-mer XAGE1 (GAGED2a) OLPs for CD8 T cells. After incubation, cytokine production by CD4 and CD8 T cells was detected by an IFNγ capture assay to detect the responses against NY-ESO-1 or by TNFα intracellular cytokine staining (ICS) for XAGE1 (GAGED2a).

IFNγ capture assay

An IFNγ capture assay was carried out according to the manufacturer’s protocol (Miltenyi Biotec) and as described previously (16). The data were analyzed with FlowJo software (version 7.6.5; Tree Star). A net population of IFNγ-captured CD4 and CD8 T cells of more than 0.1% was considered significant.

Intracellular cytokine staining

The cells harvested from culture were washed and stained with anti–CD3-APC/Cy7 (clone HIT3a; BioLegend), anti–CD4-APC/Cy5 (clone; SK3, eBioscience) or anti–CD8-APC/Cy5 (clone RPA-T8; BD Pharmingen), and Fixable Viability Dye-eFluor 506 (eBioscience) for 30 minutes on ice. After incubation, the cells were washed, fixed, and permeabilized with Cytofix/Cytoperm solution (BD Biosciences) for 20 minutes at 4°C. Then, the cells were washed in Perm/Wash solution (BD Biosciences), and pelleted cells were stained (30 minutes at 4°C) for intracellular cytokines using anti–TNFα-Brilliant Violet 421 (clone MAb11; BioLegend). After incubation, the cells were washed and analyzed by FACS Canto II (BD Biosciences). The data were analyzed using FlowJo software (version 7.6.5; Tree Star). A number of TNFα-staining cells stimulated with XAGE1 (GAGED2a) OLPs that was more than 2-fold the number of cytokine-producing cells of the epidermis and in infiltration of CD4 and CD8 T cells without FoxP3-positive cells. The skin rash recovered on application of topical or systemic corticosteroids. With respect to hematologic AEs, grade 2 lymphopenia was observed in 1 patient at 0.1 mg/kg, and grade 3 lymphopenia was observed in 1, 3, and 3 patients at 0.1 mg/kg, 0.5 mg/kg, and 1.0 mg/kg, respectively. Grade 2 leukopenia was observed in 3 patients at 0.5 mg/kg and 2 patients at 1.0 mg/kg. Grade 2 neutropenia was observed in 1 patient at 0.5 mg/kg. All of the AEs were manageable, and all the patients recovered.

Dose escalation and DLT

In all cohorts given 0.1 mg/kg (n = 3), 0.5 mg/kg (n = 3), and 1.0 mg/kg (n = 4) of KW-0761, no DLT was observed during the observation period. The recommended phase Ib dose was determined to be 1.0 mg/kg. The dose of 0.1 mg/kg was the minimal dose, but it efficiently depleted FoxP3+ Tregs (see below) and was also recommended.

Clinical responses

Four of 8 patients obtained stable disease (SD) (Supplementary Table S1). No discrepancy was observed in clinical responses at 12 weeks between RECIST and irRECIST. Median PFS and OS were

Results

Patient characteristics

Seven NSCLC and 3 esophageal cancer patients were enrolled in this phase Ia study (Supplementary Table S1). Four patients were enrolled in cohort 3 because 1 patient (A3-03) died before DLT evaluation.

Pharmacokinetics

KW-0761 exhibited dose-proportional pharmacokinetics. The plasma Cmax and C trough, as well as the AUC0-7 days, increased dose- and frequency-dependently, as presented in Table 1 and Supplementary Fig. S1. The half-life (t1/2) of the KW-0761 infused was also dose-dependent, ranging from 14 to 18 days after the last infusion.

Adverse events

Table 1 lists AEs which were determined as possibly, probably, or definitely KW-0761-related. Grade 3 or higher nonhematologic AEs were grade 3 γ-glutamyl transpeptidase (GGT) elevation in 2 patients given the dose of 1.0 mg/kg and observed after the DLT observation period. All of the other nonhematologic AEs were ≤ grade 2. Skin-related AEs were frequently observed. The skin rash observed in the patients is depicted in Fig. 1. Skin biopsy revealed that the lesions presented vacuolar degeneration in the basal layer cells of the epidermis and infiltration of CD4 and CD8 T cells without FoxP3-positive cells. The skin rash recovered on application of topical or systemic corticosteroids. With respect to hematologic AEs, grade 2 lymphopenia was observed in 1 patient at 0.1 mg/kg, and grade 3 lymphopenia was observed in 1, 3, and 3 patients at 0.1 mg/kg, 0.5 mg/kg, and 1.0 mg/kg, respectively. Grade 2 leukopenia was observed in 3 patients at 0.5 mg/kg and 2 patients at 1.0 mg/kg. Grade 2 neutropenia was observed in 1 patient at 0.5 mg/kg. All of the AEs were manageable, and all the patients recovered.

Table 1. Pharmacokinetic parameters of KW-0761 by cohort

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Cmax (ng/mL)</th>
<th>Ctrough (ng/mL)</th>
<th>AUC0-7 days (µg h/mL)</th>
<th>t1/2 (hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td>by frequency</td>
<td>Mean ± SD</td>
<td>Mean ± SD</td>
<td>Mean ± SD</td>
<td>Mean ± SD</td>
</tr>
<tr>
<td>0.1 1st 3</td>
<td>1,865.0 ± 493.7</td>
<td>585.7 ± 140.6</td>
<td>161.9 ± 74.9</td>
<td>123 ± 11</td>
</tr>
<tr>
<td>8th 2</td>
<td>4,377.8 ± 3,042.9</td>
<td>3,042.9 ± 1,445.4</td>
<td>971.2 ± 308.8</td>
<td>341</td>
</tr>
<tr>
<td>0.5 1st 3</td>
<td>8,835.1 ± 1,445.4</td>
<td>3,551.2 ± 1,086.0</td>
<td>2,614.4 ± 1,388.8</td>
<td>171</td>
</tr>
<tr>
<td>8th 1</td>
<td>19,980.7 ± 12,121.4</td>
<td>12,121.4 ± 6,221.0</td>
<td>622.1 ± 124.0</td>
<td>10</td>
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<tr>
<td>1.0 1st 4</td>
<td>17,918.8 ± 4,171.7</td>
<td>6,039.8 ± 2,990.8</td>
<td>1,656.7 ± 5,720.3</td>
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<tr>
<td>8th 1</td>
<td>42,465.0 ± 32,583.0</td>
<td>18,835.1 ± 15,551.2</td>
<td>8,835.1 ± 7,086.0</td>
<td>11</td>
</tr>
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</table>

NOTE: Values are the mean of those from the indicated numbers of patients.

n = 2.

One of the 2 patients who was infused with KW-0761 at prolonged intervals due to skin rash was excluded.
Table 2. Adverse events

<table>
<thead>
<tr>
<th>Adverse event</th>
<th>0.1 mg/kg (n = 3)</th>
<th>0.5 mg/kg (n = 3)</th>
<th>1.0 mg/kg (n = 4)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Grade 1</td>
<td>Grade 2</td>
<td>Grade 3</td>
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<td>2</td>
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<td>Malaise</td>
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<tr>
<td>Procedural complication</td>
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<td>Infusion reaction</td>
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<td>Skin and subcutaneous tissue</td>
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<tr>
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<tr>
<td>Erythema multiforme</td>
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<tr>
<td>Rash maculo-papular</td>
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<td>Skin papilloma</td>
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<td>Hypothyroidism</td>
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| Abbreviations: AST, aspartate aminotransferase; CRP, C-reactive protein; LDH, lactate dehydrogenase. |

4.1 and 9.1 months, respectively. Supplementary Figure S2 shows the clinical course with the parameters in patients A1-01, A1-02, and A2-01 with SD.

The effect of KW-0761 infusion on various lymphocyte subpopulations and FoxP3+ CD4 Treg depletion

Figure 2 shows the CCR4 expression on various lymphocyte subpopulations by FACS analysis in 12 NSCLC patients examined in an independent study from this clinical trial. CCR4 expression was observed on CD4 T cells, but not on CD8 T cells, NK cells, or B cells. High expression of CCR4 on FoxP3+ CD4 Tregs was shown previously (8, 11). CCR4 expression on Th17 and Th2 was also high, but that on Th1 cells was low. CCR4 expression on CD45RA+ memory CD4 T cells was likely to be due to FoxP3+ Tregs, Th17, and Th2 cells.

KW-0761 is capable of lysing CCR4 expressing ATL cells in vitro and also in vivo by ADCC. Figure 3 shows the reduction in the absolute cell number in PBMCs during the course of the treatment. Reduction was evident with CD4 T cells and CD16+CD56+ NK cells. The reduction of the latter population was probably caused by consumption due to the main effectors for ADCC. On the other hand, no reduction was evident with CD8 T cells and CD19 B cells. As shown in Fig. 3C, reduction was most evident with CD25+ FoxP3+ CD4 T cells. Within the FoxP3+ CD4 T-cell population, reduction was evident with either activated/effector Treg or non Treg fractions and was less evident with resting/naïve Treg fractions. As shown in Fig. 3D, within the CD4 T-cell population, reduction was evident with CCR6+ Th17 or CRTH2+ Th2 cells. Less evident reduction was observed with CXCR3+ Th1 or CXCR5+ Th17 cells.
Supplementary Figure S3 shows the changes in the specific populations. Within PBMCs, lymphocytes were decreased while MDSCs were increased (Supplementary Fig. S3A). Within the lymphocyte population, CD4 T cells and CD16+CD56+ NK cells were decreased, whereas CD8 T cells and CD19 B cells were increased. CD25+FoxP3+ CD4 T cells, including fractions of...
activated/effector Tregs or non Tregs, were markedly decreased. Within the CD4 T-cell population, the reduction in Th2 was evident while that of Th1, Th17, or Tfh cells was less evident. The results of the analyses of the absolute cell numbers show in Fig. 3a and those of the population in PBMCs, lymphocytes, and CD4 T cells shown in Supplementary Fig. S3 of various lymphocyte subpopulations indicated marked reduction of FoxP3⁺ CD4 Tregs with only marginal reduction of Th1 cells and CD8 T cells in PBMCs after KW-0761 infusion.

We then investigated the changes in CD4 and CD8 T cells expressing inhibitory or activation molecules on their surfaces. As shown in Fig. 4, with CD4 T cells, an increase in PD-1, TIM-3, LAG-3, ICOS, or GITR-expressing cells was evident. The BTLA, OX-40, or 4-1BB-expressing cells were decreased in most cases. With CD8 T cells, the PD-1 or ICOS-expressing cells were increased in most cases. The LAG-3 or GITR-expressing cells were retained. The TIM-3, BTLA, OX-40, or 4-1BB-expressing cells were decreased in most cases.

The number of granulocytes
The number of granulocytes was also estimated during the treatment. No reduction was observed (Fig. 3E).

Discussion
In this phase Ia study, we showed the safety of KW-0761 infusions in 7 lung and 3 esophageal cancer patients. Based on the previous results with ATL patients similarly infused with KW-0761 (2, 5), we used 3 dose regimens of 0.1 mg/kg, 0.5 mg/kg, and 1.0 mg/kg in this study. Some toxicities, including persistent skin rash related to the KW-0761 infusion, were observed, but no DLT was observed. The patients recovered from either toxicity eventually. Infusion reactions were prevented by using corticosteroids at the first infusion. The skin rash observed in this study was much less severe than that observed in ATL patients reported previously (2, 5). The induction of a strong autoimmune response to the skin was suggested to be involved following the destruction of CCR4⁺ CD4 Tregs in the skin together with FoxP3⁺ CD4 Tregs. The lesions were heavily infiltrated with CD8 T cells. An ATL case that showed Stevens-Johnson's syndrome was reported (24). In the lung or esophageal cancer patients in this study, the lesions were superficial and restricted in terms of eruptions. Infiltration of lymphocytes in the lesions was comparably low. Although we did not find any DLT and did not establish Treg depletion in the local tumor site, no dose escalation exceeding 1 mg/kg was applied. The reasons were that we
observed serious skin toxicities in ATL patients during prolonged treatment for more than a year with 1 mg/kg, and because complete elimination of Tregs in PBMCs was easily obtained even with 0.1 mg/kg (see below).

This study showed that FoxP3\(^+\) CD4 Tregs were efficiently depleted in PBMCs after KW-0761 infusion. This was observed even at the lowest dose of 0.1 mg/kg KW-0761 infusion. The effect was generally durable for more than 6 months after finishing 8 infusions of KW-0761. However, a slight elevation of the baseline FoxP3\(^+\) CD4 Tregs was noticed during the course of additional infusions at a frequency of once a month. The half-life of the KW-0761 infused at 0.1 to 1.0 mg/kg ranged between 14 and 18 days. A one month interval for the infusion may be too long to achieve sustained complete depletion of FoxP3\(^+\) CD4 Tregs. As shown in the clinical course of 3 SD patients (Supplementary Fig. S2), the disease was stable for the initial 8 weeks during the course of KW-0761 infusions on a weekly basis. However, the disease gradually progressed again during a period of additional infusions given once a month. It is difficult to conclude, but possible, that SD resulted from weekly injections. The expression of CCR4 on CD8 T cells was augmented when the cells were activated (25). Although we intended selective depletion of FoxP3\(^+\) CD4 Tregs by the infusion of KW-0761, the findings indicated the possible occurrence of off-target effects.

Figure 4.

A, percentage of baseline for inhibitory and activation molecule-expressing cells in CD4 and CD8 T cells. In the box at the bottom, patient ID with a clinical response evaluated by iRECIST is shown. B, representative cytometry dot plots of ICOS and GITR-expressing, CD4 and CD8 T cells from patient A3-04 during treatment. Analysis was done by FACSCanto II.

CCR4 is differentially expressed on the surface of various types of lymphocyte subpopulations (10). No expression of CCR4 was observed in myeloid cells. Within lymphocytes, no expression of CCR4 was observed on CD56\(^+\) NK cells, whereas high expression of CCR4 was observed on FoxP3\(^+\) CD4 Tregs (8, 11), Th2, and Th17 CD4 T cells. Low expression of CCR4 was observed on Th1 CD4 T cells and CD8 T cells. The expression of CCR4 on CD8 T cells was augmented when the cells were activated (25). Although we intended selective depletion of FoxP3\(^+\) CD4 Tregs by the infusion of KW-0761, the findings indicated the possible occurrence of off-target effects. However, this study showed that the reduction in the number of Th1 CD4 T cells or CD8 T cells was limited. Differential depletion between FoxP3\(^+\) CD4 Tregs, and Th1 CD4 T cells and CD8 T cells, shows that KW-0761 infusion is a promising therapeutic option for FoxP3\(^+\) CD4 Tregs depletion.

It is unlikely that NK-cell reduction resulted from KW-0761 cytotoxicity. Rather, it is likely to be due to their involvement as the main effector cells for ADCC. Because NK cells mediate killing of HLA-negative tumor cells and secrete IFN\(\gamma\), their reduction in PBMCs may be unfavorable for antitumor immune responses (26). Further study is necessary to clarify these matters.

We observed an increase in PD-1, TIM-3, LAG-3, ICOS, or GITR-expressing CD4 T cells, and PD-1 or ICOS-expressing CD8 T cells, and a decrease in BTLA, OX-40, and 4-1BB–expressing CD4 and CD8 T cells during the course of KW-0761 infusion. Although the findings suggest that increases or decreases in those cells resulted
from depletion of Tregs, the mechanism was not fully investigated in this study.

We observed a reduction in the number of lymphocytes, but an increase in the number of MDSCs, after KW-0761 infusions. These findings may simply suggest compensation in the balance of the number of lymphocytes and MDSCs in PBMCs. Alternatively, it could be functional compensation by MDSCs for depletion of Tregs. Involvement of MDSCs in disease progression in various cancer types and after immunotherapy has been reported in many studies (27–29).

As for the number of granulocytes responsible for protection from bacterial infection, no change was observed after KW-0761 infusion.

We observed an increase in antibody response in 2 of 3 patients with NY-ESO-1–expressing lung cancers and also 2 of 3 patients with XAGE1 (GAGED2a)-expressing lung cancers, although the increase was modest (Supplementary Fig. S4 and Supplementary Table S2). Appearance of CD4 and CD8 T-cell responses against those antigens was also observed in the patients. Vaccination of patients with NY-ESO-1–expressing cancers using various preparations of NY-ESO-1 antigens has been extensively studied by many investigators, including us (30–32), and has been shown to elicit vigorous immune responses of antibody, and CD4 and CD8 T cells in patients. Vigorous augmentation or induction of immune responses to NY-ESO-1 or XAGE1 (GAGED2a) of which localization is primarily in the nucleus of the tumor cells may not be easily achievable by simply depleting FoxP3+ CD4 Tregs without immunization. In this regard, combination therapy of FoxP3+ CD4 Tregs depletion by KW-0761 infusion and vaccination with tumor antigens should be a worthwhile challenge for a future study (33).

It should be noted that an increase in autoantibodies against thyroid peroxidase was observed in 2 of 5 patients examined (Supplementary Table S3). Those 2 patients showed stable disease during infusions, and this favorable disease course may be related to the autoimmune response. Further study of a larger number of patients will be necessary to elucidate the relation between autoimmune responses and tumor immune responses.

Treg depletion and resulting tumor regression were first found by us with anti-CD25 (IL-2Rα) mAb administration in the mouse (13). However, the treatment was effective only in a prophylactic, but not a therapeutic, tumor model probably because of high expression of CD25 on Tregs and also effector T cells, both of which were subjected to depletion (34). Recently, it has been shown that Treg depletion is the mechanism of the antitumor effect in anti–CTLA-4 therapy used as a checkpoint inhibitor (35), and also in anti–OX-40 therapy used as a costimulatory receptor agonist (36) in the mouse, both involving FcR-expressing immune cells. Both of these molecules highly express on both effector T cells and Tregs, so the mechanisms of selective depletion of Tregs by ADCC require further study. The present study showed selective depletion of activated Tregs in PBMCs and the possible occurrence of immune responses by KW-0761 (anti-CCR4) with enhanced ADCC activity in humans. Combined use of KW-0761 for depleting FoxP3+ Tregs and other immunotherapies, such as cancer vaccines or checkpoint inhibitors, is promising to augment immune responses.

**Disclosure of Potential Conflicts of Interest**

S. Ishida reports receiving commercial research grants from Kyowa Hakko Kirin Co. Ltd. T. Ishida reports receiving speakers bureau honoraria from Kyowa Hakko Kirin Co. Ltd. and reports receiving commercial research support from Bayer Pharma AG, Celgene K.K. and Kyowa Hakko Kirin Co. Ltd. S. Suzuki is a consultant for Medical & Biological Laboratories Co. Ltd. H. Nishikawa reports receiving commercial research grants from Bristol-Myers Squibb and Kyowa Hakko Kirin. R. Ueda reports receiving speakers bureau honoraria from Chugai Pharmaceutical Co., Ltd and Kyowa Hakko Kirin Co. Ltd., is a consultant/ advisory board member for Mundipharma KK, and reports receiving commercial research grants from Chugai Pharmaceutical Co., Ltd., Kyowa Hakko Kirin Co., Ltd., Medical & Biological Laboratories Co., Ltd., and Rikakom Co., Ltd. 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):** K. Kurose, Y. Ohue, T. Ishida, K. Kakimi, H. Nishikawa, H. Udono, M. Oka, R. Ueda

**Writing, review, and/or revision of the manuscript (e.g., statistical analysis, data processing, construction of tables, figures, artwork, in vitro, animal studies, clinical studies):** Y. Ohue, T. Doi, S. Suzuki, M. Isebe, M. Oka, R. Ueda

**Study supervision:** R. Ueda, E. Nakayama

**Other (representative of this phase of trial (NCT01929486)):** R. Ueda

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Phase Ia Study of FoxP3+ CD4 Treg Depletion by Infusion of a Humanized Anti-CCR4 Antibody, KW-0761, in Cancer Patients

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