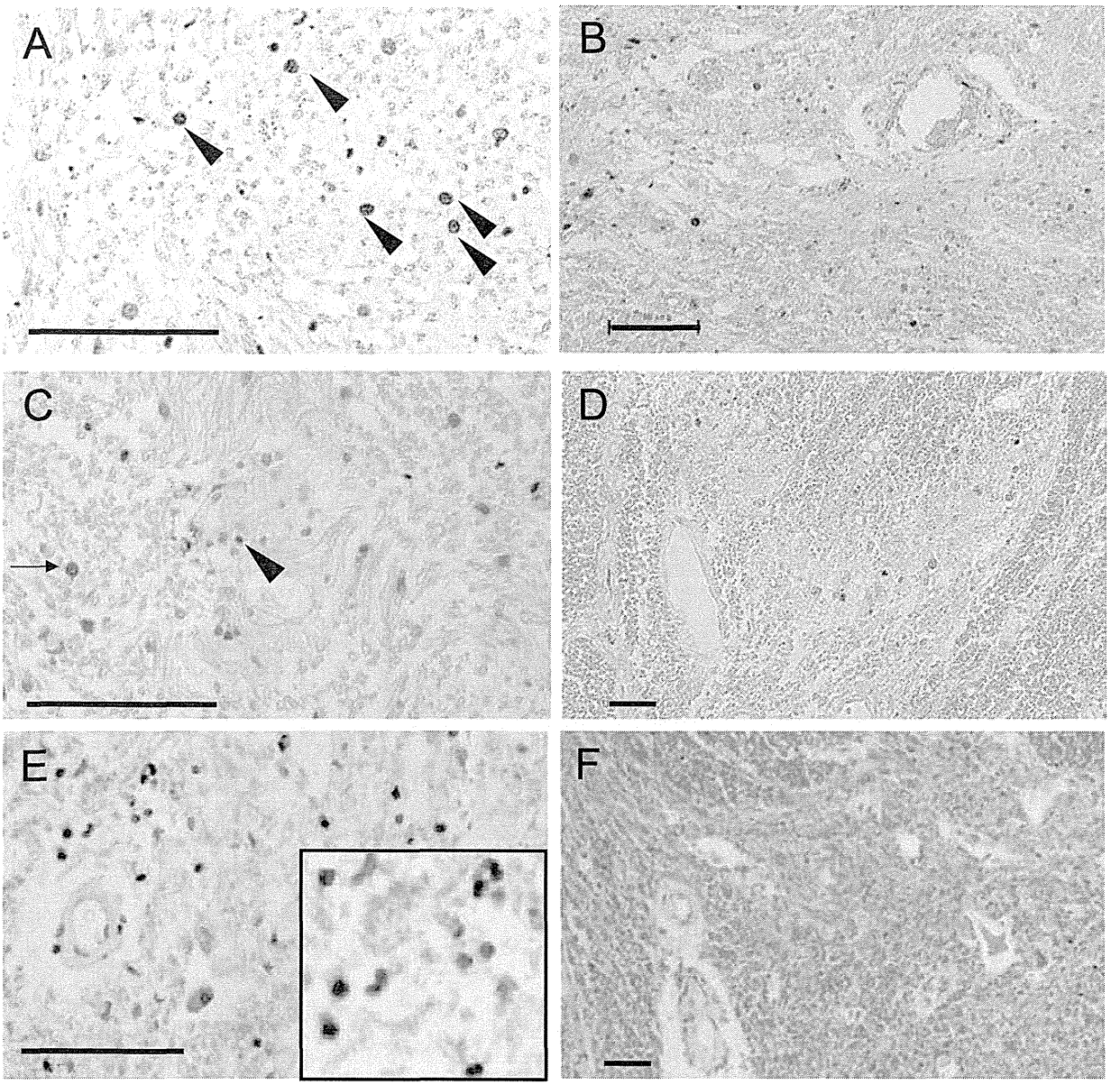

FIGURE 6. Detection of apoptotic cells in the CNS. **(A)** Some small cells are apoptotic (DAB; brown, arrowheads) detected by anti–active caspase-3 antibody (Ab). **(B–D)** TdT-mediated dUTP nick end labeling assay. **(B)** A number of apoptotic cells (DAB; brown) are detected in the spinal cord of a patient with HTLV-1–associated myelopathy/tropical spastic paraparesis (Patient 8624). **(C)** Some infiltrating small cells around a small vessel (arrowhead) and some relatively large cells in the parenchyma (arrow) are apoptotic. **(D)** The apoptotic cells are barely detectable in the control spinal cord from an HTLV-1–seronegative patient with hepatoma. **(E, F)** Anti-single-stranded DNA antibody staining. **(E)** Numerous apoptotic cells (AEC; red) are detected in the spinal cord (Patient 8624). A higher magnification picture in the inset shows apoptotic cells. **(F)** Apoptotic cells are barely detectable in the control patient spinal cord. Scale bar = 100 μm.

HTLV-1–infected cells could express viral antigens anywhere in the body of the infected individuals (14, 32), the expression of HTLV-1 proteins in vivo has remained elusive so far.

In this study, we succeeded in detecting HTLV-1 proteins in the CD4-positive T cells infiltrating the CNS. This is consistent with our previous reports in which HTLV-1–infected cells were determined to be CD4-positive lymphocytes in the CNS by in situ hybridization for HTLV-1 mRNA and in situ polymerase chain reaction for HTLV-1 DNA (33, 34). The infiltrating HTLV-1–infected CD4-positive cells may easily express the viral antigens in the CNS, which in turn facilitates the accumulation of HTLV-1–specific CTLs.

Human T-lymphotropic virus type-1 infection causes several organ-specific inflammatory diseases including HAM/TSP (2, 3). Previous reports demonstrating that HTLV-1 proviral loads are high in affected organs such as the muscles, lungs, and CNS suggest that HTLV-1–infected cells accumulate in the organs (13, 35). The pathogenesis model in which both

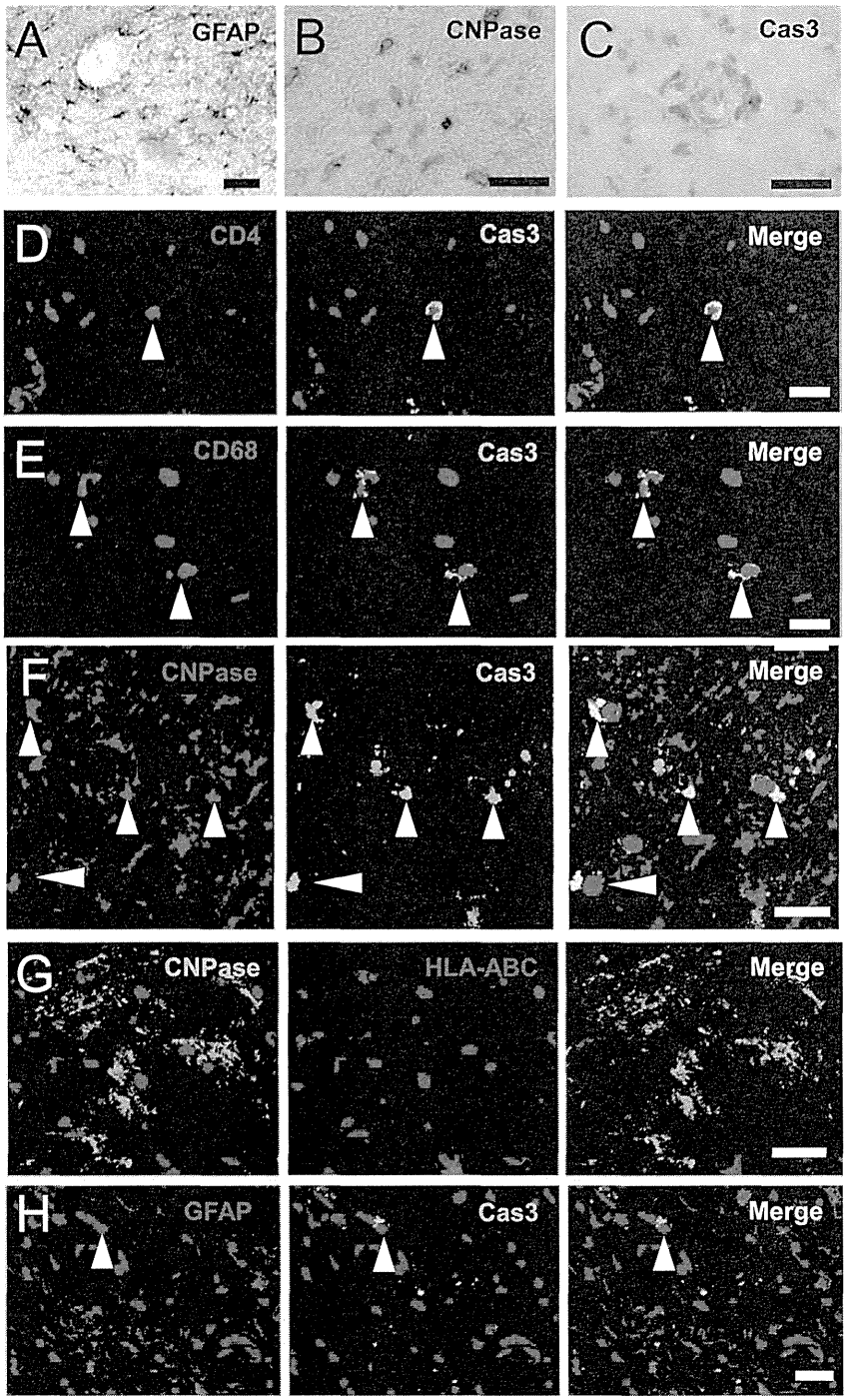

FIGURE 7. Cell identification of apoptotic cells. **(A–C)** Astrocytes **(A)**, oligodendrocytes **(B)**, and apoptotic cells **(C)** were stained with anti–glial fibrillary acidic protein (GFAP) antibody (Ab), anti–2′,3′-cyclic-nucleotide 3′-phosphodiesterase (CNPase) monoclonal antibody (mAb), or anti–active caspase-3 Ab, respectively, in the spinal cord of Patient 8624. Nuclei were counterstained with hematoxylin. **(D–F, H)** Double staining revealed that a CD4-positive cell (red, **D**), a CD68-positive cell (red, **E**), and some oligodendrocytes (red, **F**), but no astrocytes (red, **H**), were apoptotic (green) (arrowheads) in the spinal cord of Patient 8624. **(G)** Double staining with anti-CNPase mAb (green) and anti–HLA-ABC mAb (red) revealed that no oligodendrocyte expresses HLA-ABC. There is no double-positive signal (yellow) in the merged image. White bars indicate 20 μm. Cas3, active caspase-3.

HTLV-1–infected CD4-positive T cells and the virus-specific CD8-positive CTLs infiltrate the organs from the peripheral blood followed by bystander tissue damage may explain why HTLV-1 infection can cause several chronic inflammatory diseases in various organs. Further studies are needed to determine whether the similar immunopathologic model can be applied to HTLV-1–associated inflammatory diseases in other organs.

ACKNOWLEDGMENT

The authors thank Ms. T. Inoue for her excellent technical assistance.

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MAJOR ARTICLE

Mogamulizumab, an Anti-CCR4 Antibody, Targets Human T-Lymphotropic Virus Type 1–infected $CD8^+$ and $CD4^+$ T Cells to Treat Associated Myelopathy

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Background. Human T-lymphotropic virus type 1 (HTLV-1) can cause chronic spinal cord inflammation, known as HTLV-1–associated myelopathy/tropical spastic paraparesis (HAM/TSP). Since $CD4^+CCR4^+$ T cells are the main HTLV-1 reservoir, we evaluated the defucosylated humanized anti-CCR4 antibody mogamulizumab as a treatment for HAM/TSP.

Methods. We assessed the effects of mogamulizumab on peripheral blood mononuclear cells from 11 patients with HAM/TSP. We also studied how $CD8^+$ T cells, namely $CD8^+$ $CCR4^+$ T cells and cytotoxic T lymphocytes, are involved in HTLV-1 infection and HAM/TSP pathogenesis and how they would be affected by mogamulizumab.

Results. Mogamulizumab effectively reduced the HTLV-1 proviral load (56.4% mean reduction at a minimum effective concentration of 0.01 μg/mL), spontaneous proliferation, and production of proinflammatory cytokines, including interferon γ (IFN-γ). Like $CD4^+CCR4^+$ T cells, $CD8^+CCR4^+$ T cells from patients with HAM/TSP exhibited high proviral loads and spontaneous IFN-γ production, unlike their $CCR4^-$ counterparts. $CD8^+CCR4^+$ T cells from patients with HAM/TSP contained more IFN-γ–expressing cells and fewer interleukin 4–expressing cells than those from healthy donors. Notably, Tax-specific cytotoxic T lymphocytes that may help control the HTLV-1 infection were overwhelmingly $CCR4^-$.

Conclusions. We determined that $CD8^+CCR4^+$ T cells and $CD4^+CCR4^+$ T cells are prime therapeutic targets for treating HAM/TSP and propose mogamulizumab as a new treatment.

Keywords. HTLV-1; HAM/TSP; CCR4; mogamulizumab; CD8.

Human T-lymphotropic virus type 1 (HTLV-1) infects 10–20 million people worldwide, causing HTLV-1–associated myelopathy/tropical spastic paraparesis (HAM/TSP) and adult T-cell leukemia/lymphoma (ATL) in a small fraction of infected individuals [1–3]. HAM/TSP is an inflammatory disease of the central nervous system (CNS) that is thought to develop via so-called bystander damage, meaning that the host immune responses to HTLV-1–infected cells in the CNS damage the spinal cord [4]. Currently established treatments for HAM/TSP, such as corticosteroids [5] and interferon alfa [6], do not effectively reduce the HTLV-1 proviral load, which is well correlated with disease severity [7]. Reverse transcriptase inhibitors, which are used against human immunodeficiency virus type 1, were not effective against HTLV-1 in

Received 6 May 2014; accepted 28 July 2014; electronically published 7 August 2014.

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The Journal of Infectious Diseases® 2015;211:238–48

DOI: 10.1093/infdis/jiu438

clinical trials [8, 9]. These and other existing antiviral drugs usually block the viral replication process [10], but HTLV-1 may escape these drugs by replicating using host cell division [11, 12]. The ideal treatment strategy for HAM/TSP would be selectively targeting and eliminating the HTLV-1–infected cells, but no such treatments currently exist.

Mogamulizumab, a defucosylated humanized anti-CCR4 immunoglobulin G1 (IgG1) monoclonal antibody, was recently approved in Japan as a novel therapy for ATL [13]. Importantly, ATL cells usually express chemokine receptor CCR4 [14]. Mogamulizumab strongly binds to Fcγ receptor IIIa (FcγRIIIa) on natural killer (NK) cells and elicits powerful antibody-dependent cellular cytotoxicity (ADCC) against the $CCR4^+$ ATL cells [15, 16].

Recently, we found that $CD4^+CD25^+CCR4^+$ T cells are the main HTLV-1 reservoir in HAM/TSP [17]. These cells abnormally produce interferon γ (IFN-γ) and are thought to play an important role in producing the chronic inflammation in HAM/TSP [17]. Thus, we began investigating the possibility of treating HAM/TSP and ATL by targeting $CCR4^+$ cells. We have already established that the defucosylated human/mouse chimeric anti-CCR4 antibody KM2760 effectively reduces the HTLV-1 proviral load in cultured peripheral blood mononuclear cells (PBMCs) from patients with HAM/TSP [18]. Here, we evaluate for the first time the effects of the humanized antibody mogamulizumab on cells from patients with HAM/TSP.

There is a population of $CD8^+$ T cells that express CCR4, but these cells have so far received much less attention than $CD4^+$ $CCR4^+$ T cells from HTLV-1 researchers. Although it has been shown that HTLV-1 infects $CD8^+$ T cells [19], it is as of yet unknown which among $CD8^+$ T cells are predominantly infected, as well as whether and how the infection influences the functions of those cells. $CD8^+CCR4^+$ T cells are reported to suppress inflammation and play a beneficial role in controlling chronic inflammatory diseases [20, 21]. It is important to determine whether $CD8^+CCR4^+$ T cells are protective or harmful during HAM/TSP pathogenesis, as well as how these cells would be affected by mogamulizumab.

We hypothesized that $CCR4^+$ cells among $CD8^+$ and $CD4^+$ T cells are highly infected and liable to develop proinflammatory traits. It has been reported that HTLV-1 preferentially transmits to $CCR4^+$ T cells through CCL22 (a CCR4 ligand) production induced by the HTLV-1 protein product Tax [22]. Tax has also been reported to induce IFN-γ production via transcriptional alterations within the infected cells themselves [18].

In the present study, we determined that mogamulizumab is effective at reducing the proviral load and proinflammatory character in PBMCs from patients with HAM/TSP. Next, we revealed that $CD8^+CCR4^+$ T cells are indeed highly infected by HTLV-1 and become proinflammatory. Finally, we determined that the majority of Tax-specific cytotoxic T lymphocytes (CTLs) were $CCR4^-$, indicating that they would not be inadvertently targeted by mogamulizumab. Our results indicate that $CD8^+CCR4^+$ T cells should be considered a key therapeutic target when developing treatments for HAM/TSP and that mogamulizumab represents a viable candidate for such a treatment.

METHODS

Subjects

This study was approved by the Institutional Ethics Committee at St. Marianna University and conducted in compliance with the Declaration of Helsinki. All participants gave written informed consent. Blood samples were obtained from 11 patients with HAM/TSP (8 females and 3 males; median age, 57 years [range, 47–72 years]; proviral load, 4.7 copies/100 cells [range, 1.26–9.71 copies/100 cells]), 8 HTLV-1–positive asymptomatic carriers (6 females and 2 males; median age, 57 years [range, 28–76 years]; proviral load, 4.7 copies/100 cells [range, 2.43–13.19 copies/100 cells]), and 8 HTLV-1–negative healthy volunteers (6 females and 2 males; median age, 59 years [range, 51–72 years]). HTLV-1 seropositivity was determined by a particle agglutination assay (Fujirebio, Tokyo, Japan) and confirmed by Western blot (SRL Inc., Tokyo, Japan). HAM/TSP was diagnosed according to the World Health Organization guidelines [23]. PBMCs were separated by Ficoll-Hypaque density gradient centrifugation (Pancoll; PAN-Biotech, Aidenbach, Germany) and viably cryopreserved in liquid nitrogen with freezing medium (Cell Banker 1; Mitsubishi Chemical Medience Corporation, Tokyo, Japan).

Cell Culture

PBMCs were seeded at 1×10^5 cells/200 μL/well in 96-well round-bottomed plates in the presence or absence of mogamulizumab or KM2760 (gifts from Kyowa Hakko Kirin Co., Ltd., Tokyo, Japan) or human control IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) or prednisolone (LKT Laboratories, Inc., St. Paul, MN) and incubated at 37°C in 5% CO_2. Roswell Park Memorial Institute 1640 medium was supplemented with 10% heat-inactivated fetal bovine serum and 1% penicillin and streptomycin antibiotic solution (Wako Pure Chemical Industries Ltd., Osaka, Japan). The supernatants were collected and stored at −80°C. The cells were harvested for DNA extraction or fluorescence-activated cell-sorter (FACS) analysis. The HTLV-1 proviral load was measured using ABI Prism 7500 SDS (Applied Biosystems, Carlsbad, CA), as described previously [24].

Cell Proliferation Assay

PBMCs from patients with HAM/TSP were cultured for 7 days as described above. PBMCs from healthy donors were stimulated with 4 μg/mL of phytohemagglutinin-P (PHA; Sigma-Aldrich, St. Louis, MO) and cultured in the presence or absence of mogamulizumab or prednisolone for 3 days. During the last 16 hours, 1 μCi of ^{3}H-thymidine was added to each well, and then cells were harvested and counted with a β-plate counter (Wallac-Perkin Elmer, Waltham, MA). The assay was performed in triplicate, and average values were used for analysis.

Measurement of Cytokines

The concentrations of 6 cytokines (IFN-γ, interleukin 2 [IL-2], interleukin 4 [IL-4], interleukin 6 [IL-6], interleukin 10 [IL-10], and tumor necrosis factor α [TNF-α]) in culture supernatants were measured with a cytometric bead array kit (BD Biosciences, San Diego, CA), using a FACSCalibur flow cytometer (BD Biosciences).

Flow Cytometric Analysis

Cells were immunostained with various combinations of the following fluorescence-conjugated antibodies to surface antigens: anti-CD3 (UCHT1), anti-CD4 (RPA-T4), anti-CD8 (RPA-T8), anti-CD14 (61D3), and anti-CD19 (HIB19), from eBiosciences (San Diego, CA); and anti-CD56 (B159) anti-CCR4 (1G1), from BD Biosciences. The epitope of the anti-CCR4 antibody (1G1) is different from that of mogamulizumab and KM2760, and thus these treatments do not affect the binding of 1G1 to CCR4 [16]. In some experiments, allophycocyanin-conjugated HLA-A*2402/HTLV-1 Tax301-309 tetramer (Medical & Biological Laboratories, Nagoya, Japan) was used. To identify HTLV-1–infected cells, cells were fixed and permeabilized using a staining buffer set (eBiosciences) and then intracellularly stained with anti-Tax antibody (Lt-4) [25]. To analyze intracellular effector molecules, cells were fixed and stained with the antibodies to perforin (δG9) and granzyme B (GB11; BD Biosciences). For intracellular cytokine staining, PBMCs were stimulated for 6 hours with phorbol 12-myristate 13-acetate (50 ng/mL) and ionomycin (1 μg/mL, Sigma-Aldrich Japan, Tokyo, Japan) in the presence of monensin (GolgiStop, BD Biosciences). After being harvested, the cells were fixed and stained with the antibodies to IFN-γ (B27) and IL-4 (MP4-25D2; BD Biosciences). The stained cells were analyzed using FACSCalibur, and the data were processed using FlowJo software (TreeStar, San Diego, CA). For cell sorting, $CD8^+$ T cells were negatively selected from PBMCs, using MACS beads (Miltenyi Biotec, Bergisch Gladbach, Germany). The purified $CD8^+$ T cells were stained with anti-CD3, anti-CD8, and anti-CCR4 antibodies, and then $CD3^+CD8^+$, $CD3^+CD8^+CCR4^+$, and $CD3^+CD8^+CCR4^-$ T cells were separated using a cell sorter (JSAN, Bay Bioscience Co., Ltd., Hyogo, Japan). The purity exceeded 95%.

Statistical Analysis

Values are expressed as means ± standard deviations. The paired *t* test or the Wilcoxon signed-rank test was used for within-group comparisons. The Mann–Whitney *U* test was used for comparisons between groups. Repeated-measures analysis of variance (ANOVA) followed by the Dunnett test or the Friedman test followed by the Dunn test were used for paired multiple comparisons. Statistical analyses were performed using GraphPad Prism 5 and Prism statistics (GraphPad Software, Inc., La Jolla, CA), and *P* values of <.05 were considered statistically significant.

RESULTS

Mogamulizumab and KM2760 Reduce the HTLV-1 Proviral Load and Inhibit Spontaneous Proliferation of PBMCs From Patients With HAM/TSP

The effects of mogamulizumab and KM2760 were assessed by measuring proviral loads in treated PBMCs from patients with HAM/TSP. ^{3}H-thymidine incorporation was used to assess the inhibitory effects of the antibodies on spontaneous cell proliferation, a distinctive phenomenon associated with PBMCs from HTLV-1–infected individuals by which the cells proliferate without mitogens or stimuli in vitro [26]. Mogamulizumab and KM2760 both reduced proviral load in a dose-dependent manner at concentrations of ≥0.01 μg/mL (mean reduction [±SD], 56.4% ± 21.1% and 61.1% ± 17.0%, respectively; $P < .01$ and $P < .001$, respectively; Figure 1*A*). Notably, there was a mean reduction (±SD) of 66.4% ± 20.2% in the proviral load with 10 μg/mL mogamulizumab ($P < .001$), which is the blood concentration of the antibody in patients with ATL treated with 1 mg/kg mogamulizumab [13]. Mogamulizumab and KM2760 similarly inhibited spontaneous proliferation in a dose-dependent manner at concentrations of ≥0.01 μg/mL (mean inhibition [±SD], 25.6% ± 31.9% and 22.1% ± 35.9%, respectively; $P < .01$ and $P < .05$, respectively; Figure 1*B*). Because mogamulizumab and KM2760 showed such similar results, only mogamulizumab was used in the next experiments as representative of the 2. Mogamulizumab was also tested against human IgG to control for nonspecific antibody effects and more effectively reduced the proviral load and spontaneous proliferation (Supplementary Figure 1*A* and 1*B*). Mogamulizumab reduced the HTLV-1 proviral load in cells from asymptomatic carriers, as well, in a dose-dependent manner (Figure 1*C*). Finally, mogamulizumab inhibited PHA-stimulated proliferation of PBMCs from healthy donors at concentrations of ≥0.1 μg/mL ($P < .01$), but the effects of prednisolone were much more pronounced than those of mogamulizumab (prednisolone vs mogamulizumab, $P < .001$; Figure 1*D*).

Prednisolone suppressed spontaneous proliferation (mean inhibition [±SD], 37.4% ± 35.2%; $P < .001$; Figure 1*B*) but did not decrease proviral load (Figure 1*A*). The combination of mogamulizumab and 0.1 μg/mL of prednisolone, which corresponds to the serum concentration achieved when 5 mg of prednisolone is administered orally [27], reduced proviral load as much as but no more than did mogamulizumab alone (Supplementary Figure 2*A*). On the other hand, ^{3}H-thymidine incorporation was substantially more inhibited by the combination treatment than with mogamulizumab alone (mean inhibition [±SD], 81.3% ± 18.3% vs 71.3% ± 19.3%; $P = .01$; Supplementary Figure 2*B*).

Mogamulizumab and KM2760 Inhibit Proinflammatory Cytokine Production in PBMCs From HAM/TSP Patients

Here we examined the effects of mogamulizumab and KM2760 on cytokine production in PBMCs from patients with HAM/

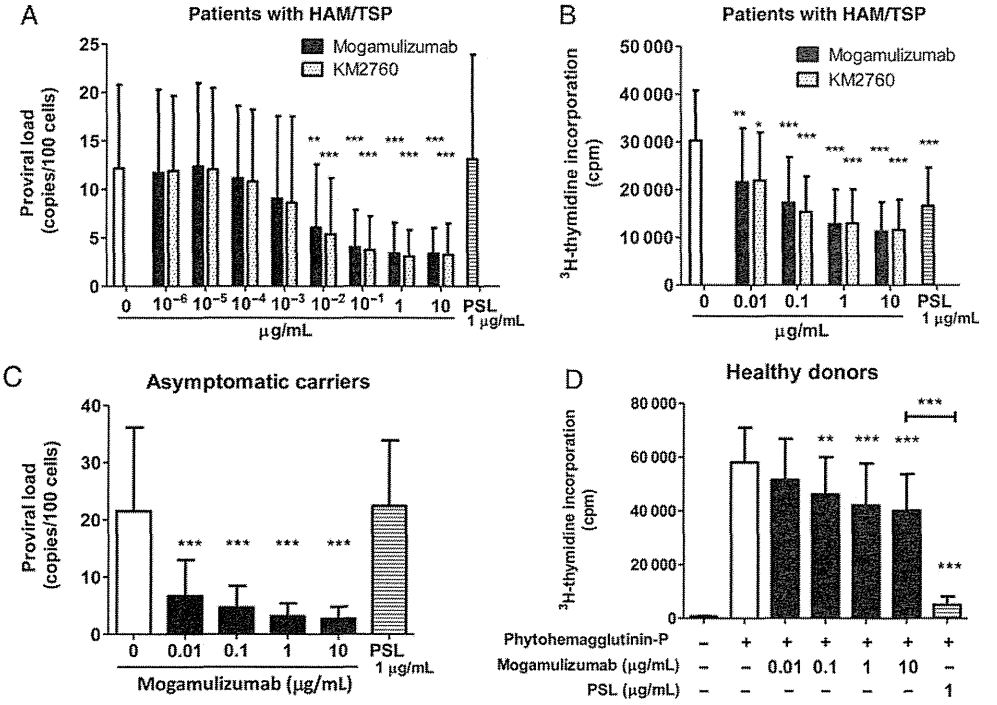

Figure 1. Mogamulizumab and KM2760 reduce the human T-lymphotropic virus type 1 (HTLV-1) proviral load and inhibit spontaneous proliferation of peripheral blood mononuclear cells (PBMCs) from patients with HTLV-1–associated myelopathy/tropical spastic paraparesis (HAM/TSP). *A* and *B*, PBMCs from 11 patients with HAM/TSP were cultured for 7 days without stimuli and without treatment or in the presence of mogamulizumab, KM2760, or prednisolone (PSL). Cells were harvested, and the proviral load was measured using real-time polymerase chain reaction (*A*). ^{3}H-thymidine was added during the last 16 hours of culturing. Cells were then harvested and analyzed for ^{3}H-thymidine incorporation (*B*). Because mogamulizumab and KM2760 were equally effective, only mogamulizumab was used thereafter as representative of the 2. *C*, PBMCs from 8 asymptomatic carriers were cultured for 7 days without treatment or in the presence of mogamulizumab or PSL, and the proviral load was measured as described above. *D*, PBMCs from 8 healthy donors were stimulated with 4 μg/mL of phytohemagglutinin-P and cultured for 3 days without treatment or in the presence of mogamulizumab or PSL. ^{3}H-thymidine incorporation was analyzed as described above. Data are presented as the mean ± SD. Statistical analyses were performed using repeated-measures analysis of variance, followed by the Dunnett test, for comparison with PBMCs alone (*A–C*) or with PBMCs stimulated with PHA (*D*). The paired *t* test was used to compare 10 μg/mL of mogamulizumab and PSL (*D*). **P* < .05, ***P* < .01, and ****P* < .001. Abbreviation: SD, standard deviation.

TSP. In line with previous reports [28], PBMCs produced various cytokines, most notably IFN-γ, in 7-day cultures without stimuli (Figure 2*A*). Mogamulizumab and KM2760 both reduced the production of the proinflammatory cytokines IFN-γ, IL-6, IL-2, and TNF-α, as well as the immunosuppressive cytokine IL-10 (Figure 2*B–F*). Mogamulizumab reduced IFN-γ production more than did human IgG (Supplementary Figure 1*C*). Prednisolone at a concentration of 1 μg/mL effectively reduced IFN-γ and TNF-α but not IL-2, IL-6, or IL-10 production.

Mogamulizumab Eliminates CCR4^{+} Cells Among Both CD4^{+} and CD8^{+} T cells

Mogamulizumab effectively eliminated the CD4^{+}CCR4^{+} T cells in cultured PBMCs from patients with HAM/TSP (Figure 3*A* and 3*B*). FACS analysis also revealed a population of CD4^{-} CCR4^{+} cells similarly affected by the antibody, and these cells were found to be CD8^{+} T cells (Figure 3*C*). Detailed investigation confirmed that CCR4^{+} T cells among the CD8^{+} subset were eliminated by mogamulizumab just as they were from the CD4^{+} subset (Figure 3*D–E*).

The ADCC Activity of Mogamulizumab Is Fast Acting and Specific

FACS analysis showed that mogamulizumab reduced the frequency of CCR4^{+} T cells among both CD4^{+} and CD8^{+} subsets within 6 hours ($P = .0003$ and $P = .004$, respectively), with a similar reduction in Tax^{+} T cells observed within 24 hours ($P = .01$ and $P = .03$, respectively; Figure 4*A–C* and Supplementary Figure 3). By contrast, mogamulizumab did not reduce the frequency of B cells, NK cells, or monocytes after 24 hours (Figure 4*D*).

CD8^{+}CCR4^{+} T Cells From Patients With HAM/TSP Are Numerous and Highly Infected by HTLV-1

CD8^{+}CCR4^{+} T cells were then further analyzed to assess their role in HAM/TSP and predict the potential benefits and risks of eradicating them with mogamulizumab. Samples from patients with HAM/TSP, compared with those from age-matched healthy donors, contained a higher proportion of CCR4^{+} cells among both the CD4^{+} T-cell subset ($P = .003$) and the CD8^{+} T-cell subset ($P = .02$; Figure 5*A*). In addition, the proviral

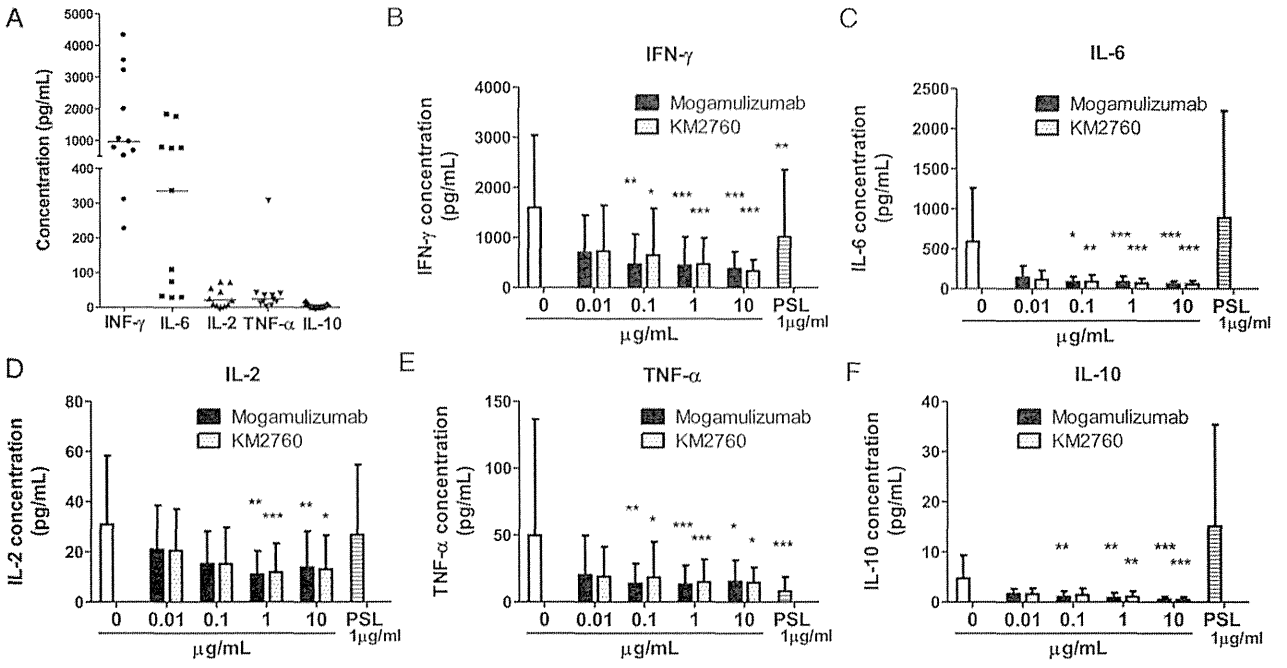

Figure 2. Mogamulizumab and KM2760 inhibit cytokine production in peripheral blood mononuclear cells (PBMCs) from patients with human T-lymphotropic virus type 1–associated myelopathy/tropical spastic paraparesis (HAM/TSP). PBMCs from 11 patients with HAM/TSP were cultured for 7 days without stimuli and without treatment or in the presence of mogamulizumab, KM2760, or prednisolone (PSL). The concentrations of cytokines (interferon γ [IFN-γ], interleukin 6 [IL-6], interleukin 2 [IL-2], tumor necrosis factor α [TNF-α], and interleukin 10 [IL-10]) in the supernatants were then measured. *A*, Direct comparison of the concentrations of these cytokines in the supernatants of untreated PBMC cultures. Horizontal bars represent the median values. *B–F*, The effects of the treatments on the concentrations of these cytokines. Data are presented as the mean ± SD. Statistical analyses were performed using the Friedman test followed by the Dunn test for comparison with PBMCs alone. $^{*}P < .05$, $^{**}P < .01$, and $^{***}P < .001$. Abbreviation: SD, standard deviation.

load was significantly higher in $CD8^+CCR4^+$ T cells than in $CD8^+CCR4^-$ T cells (mean load [±SD], 13.6 ± 7.9 copies/100 cells and 0.72 ± 0.65 copies/100 cells, respectively; $P = .0002$; Figure 5*B*).

$CD8^+CCR4^+$ T Cells From Patients With HAM/TSP Possess Proinflammatory Properties

Here we investigated the functional differences between $CD8^+CCR4^+$ T cells from patients with HAM/TSP and those from healthy donors. $CD8^+CCR4^+$ cells from both groups expressed minimal perforin and granzyme B (Figure 5*C* and 5*D*). In the $CD8^+CCR4^-$ T-cell subset, the frequency of perforin-expressing cells was unremarkable, but the frequency of granzyme B–expressing cells was higher in patients with HAM/TSP than in healthy donors ($P = .04$; Figure 5*C* and 5*D*). Next, cytokine expression was evaluated in PBMCs stimulated with phorbol 12-myristate 13-acetate and ionomycin in the presence of monensin. Interestingly, samples from patients with HAM/TSP included more IFN-γ–producing cells ($P = .02$; Figure 5*E*) but fewer IL-4–producing cells ($P = .01$; Figure 5*F*) in the $CD8^+CCR4^+$ T-cell subset than did samples from healthy donors. On the other hand, there were no such significant differences among $CD8^+CCR4^-$ T cells (Figure 5*E* and 5*F*). Finally, the concentrations of cytokines in the supernatants of unstimulated cultures of isolated total $CD8^+$, $CD8^+CCR4^-$, and $CD8^+CCR4^+$ T cells were measured to assess spontaneous cytokine production in these cell populations. Spontaneous IFN-γ production, like spontaneous proliferation, is a hallmark of PBMCs from patients with HAM/TSP [29, 30]. Unsurprisingly, IFN-γ was detected in no cell population from healthy donors. Among samples from patients with HAM/TSP, $CD8^+CCR4^+$ T cells produced remarkably more IFN-γ than did $CD8^+CCR4^-$ cells (mean level [±SD], 364.0 ± 445.3 pg/mL vs 1.9 ± 4.5 pg/mL; $P = .001$; Figure 5*G*). IL-4 was not detected in any of the samples (data not shown).

The Majority of HTLV-1 Tax-Specific Cytotoxic T Lymphocytes Are $CCR4^-$

We analyzed CCR4 expression in Tax-specific CTLs to determine whether CTLs against HTLV-1 also become targets of mogamulizumab. Among the 11 patients studied, 7 had HLA-A*2402 and were analyzed using the HLA-A*2402/HTLV-1 Tax301-309 tetramer. The majority of Tax-specific CTLs did not express CCR4, and the percentage of $CCR4^+$ cells was lower in CTLs than in total $CD8^+$ T cells (mean frequency [±SD], 2.3% ± 1.0% and 8.5 ± 4.7%, respectively; $P = .02$; Figure 6).

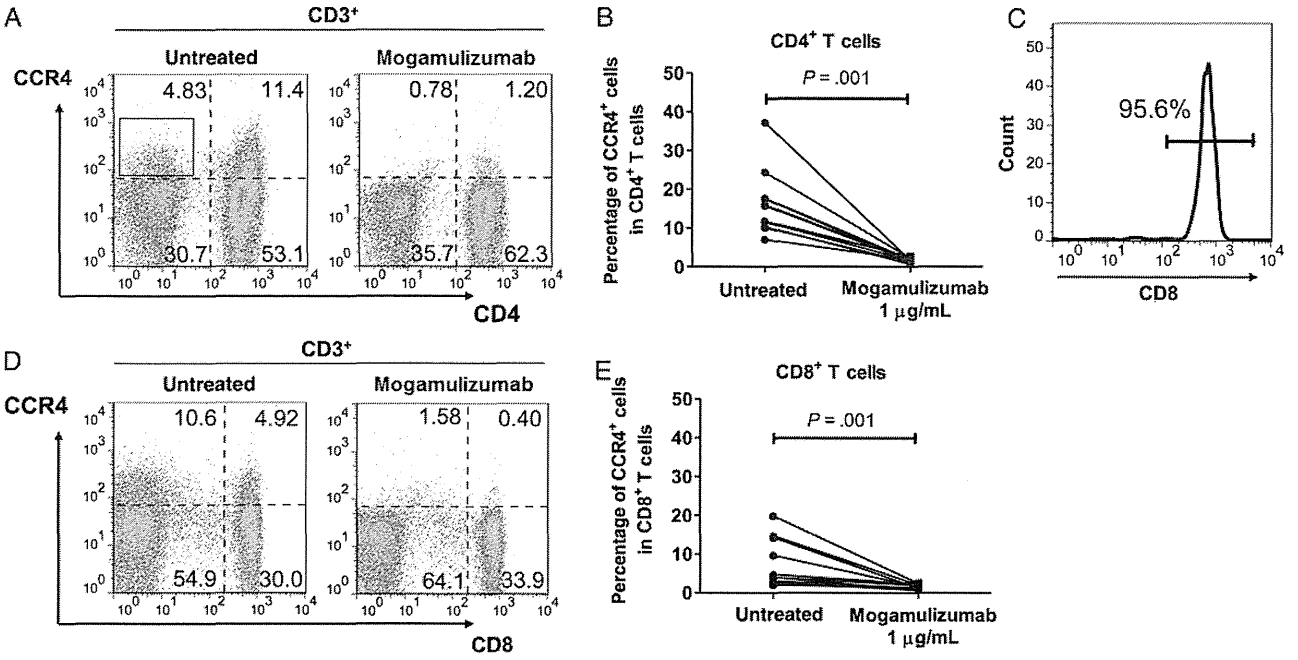

Figure 3. Mogamulizumab eliminates CCR4$^+$ cells in both CD4$^+$ and CD8$^+$ T cells. *A*, Representative dot plots of fluorescence-activated cell-sorter analysis of CCR4 and CD4 expression in CD3$^+$ T cells among peripheral blood mononuclear cells from patients with human T-lymphotropic virus type 1–associated myelopathy/tropical spastic paraparesis after 5-day culture in the presence or absence of 1 μg/mL of mogamulizumab. *B*, Percentages of CCR4$^+$ cells in CD3$^+$CD4$^+$ T cells were compared between the untreated and mogamulizumab groups (n = 11). Statistical analysis was performed using the Wilcoxon signed-rank test. *C*, The population enclosed in the box in panel *A* (the CD3$^+$CD4$^-$CCR4$^+$ subset) was gated and analyzed for the expression of CD8. The percentage of CD8$^+$ cells is shown. *D* and *E*, CCR4 and CD8 expression in CD3$^+$ T cells was analyzed as described above.

DISCUSSION

In this study, we established mogamulizumab as a novel candidate treatment for HAM/TSP that targets infected cells by marking CCR4$^+$ T cells for elimination. Mogamulizumab reduced the number of infected cells, as measured via the proviral load, and thus inhibited the excessive immune responses such as spontaneous proliferation and proinflammatory cytokine production that are attributed to those infected cells (Figures 1 and 2). Effects of mogamulizumab-induced ADCC activity were detectable by FACS after as little as 6 hours of culturing (Figure 4*A*–*C*).

The remaining proviral load after mogamulizumab therapy was higher than expected (mean load [±SD], 3.25 ± 2.58 copies/100 cells; Figure 1*A*). CD4$^+$CCR4$^-$ T cells [18] and CD8$^+$CCR4$^-$ T cells (Figure 5*B*) from patients with HAM/TSP were predominantly uninfected, and the antibody therapy should have destroyed the vast majority of the infected CCR4$^+$ T cells (Figure 3), yielding an expected proviral load of <1.0 copy/100 cells. It is possible that some CCR4$^-$ T cells became infected while the samples were being cultured, which is a potential limitation of such in vitro experiments.

The inhibitory effects of mogamulizumab on PHA-stimulated proliferation in PBMCs from healthy donors were statistically significant but still minimal, compared with those of prednisolone (Figure 1*D*), indicating that mogamulizumab, in contrast to immunosuppressive agents, acts via specific reduction of infected cells rather than via nonspecific immune suppression. Interestingly, prednisolone was considerably less effective at suppressing the proliferation of T cells from patients with HAM/TSP (Figure 1*B*) than from healthy donors. Although we cannot be sure of the reasons behind this discrimination, it appears that spontaneous proliferation is less vulnerable to suppression by steroids since it is not a simple T-cell response to antigens [31, 32]. Nevertheless, compared with mogamulizumab alone, the combination with prednisolone enhanced the suppressive effect of mogamulizumab on spontaneous proliferation without hampering proviral load reduction (Supplementary Figure 2).

Mogamulizumab also reduced the proviral load in PBMCs from asymptomatic carriers (Figure 1*C*), which suggests that it can be administered as a preventive treatment to asymptomatic carriers with high proviral loads who are at risk for developing HAM/TSP or ATL. It is well established that high proviral load is associated with the onset and progression of HAM/TSP [7, 33], as well as with the development of ATL [34].

It was well known that although the main reservoir for HTLV-1 is CD4$^+$ T cells, the virus also infects CD8$^+$ T cells in patients

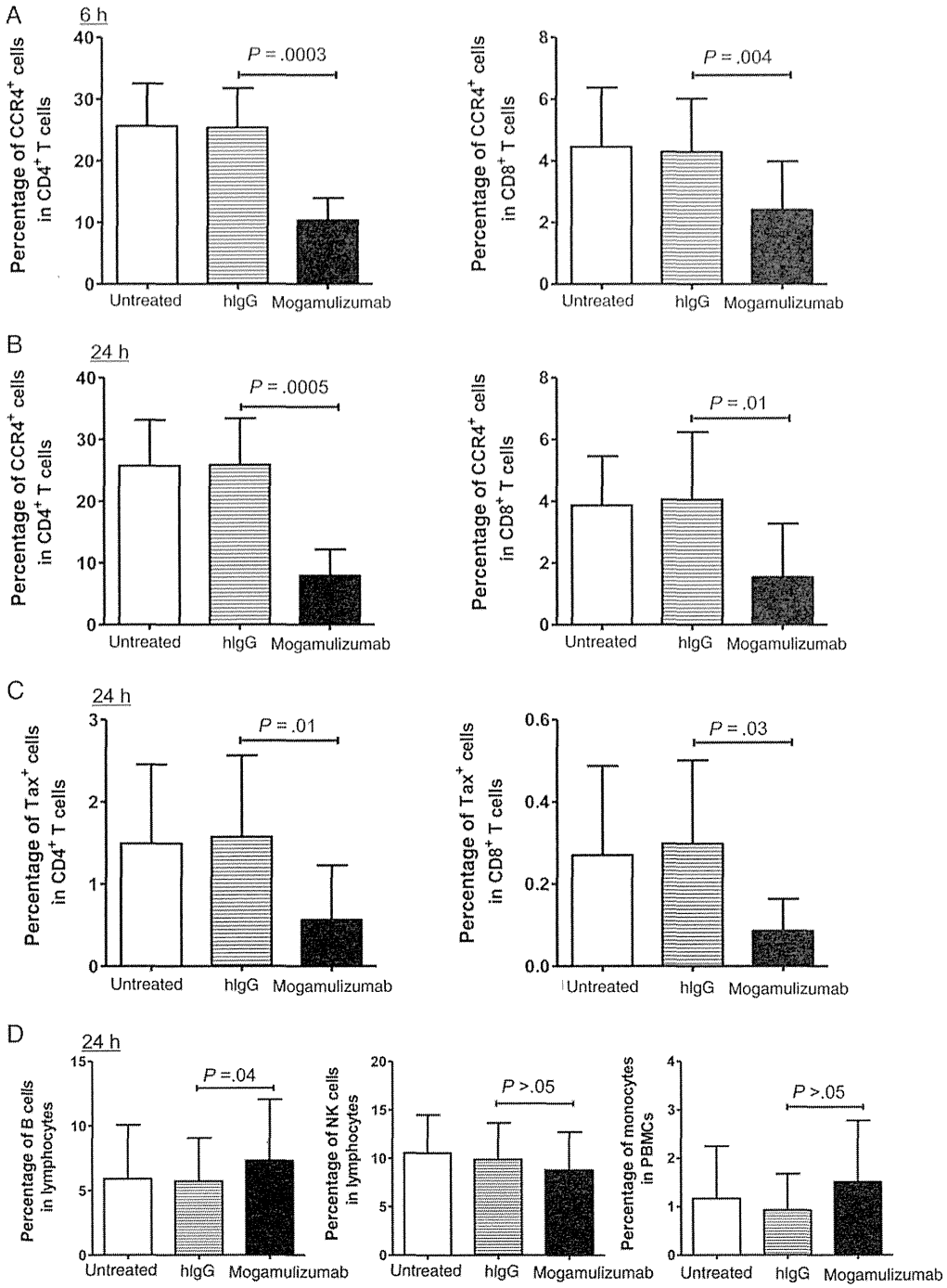

Figure 4. The antibody-dependent cellular cytotoxicity activity of mogamulizumab is fast acting and specific. Peripheral blood mononuclear cells (PBMCs) from 6 patients with human T-lymphotropic virus type–associated myelopathy/tropical spastic paraparesis were cultured in the presence of 1 μg/mL of mogamulizumab or human immunoglobulin G (hIgG) or without treatment. $CD4^+$ and $CD8^+$ T cells were analyzed using fluorescence-activated cell-sorter analysis, and the frequencies of $CCR4^+$ cells after 6 hours (*A*) and 24 hours (*B*), as well as that of Tax^+ cells (*C*) after 24 hours, are shown here. The frequencies of $CD19^+$ B cells and $CD3^-CD56^+$ natural killer (NK) cells among lymphocytes, as well as $CD14^+$ monocytes among PBMCs after 24 hours are also shown (*D*). Data are presented as the mean ± SD. The paired *t* test was used to compare the effects of mogamulizumab and hIgG. Abbreviation: SD, standard deviation.

with HAM/TSP [19]. Here we revealed for the first time that the overwhelming majority of infected $CD8^+$ T cells also expressed CCR4 (Figure 5*B*). Our findings in this study suggest that it is important to eliminate both $CD4^+CCR4^+$ and $CD8^+CCR4^+$ T-cell subsets because both have elevated proviral loads and a tendency to develop proinflammatory traits (Figure 5).

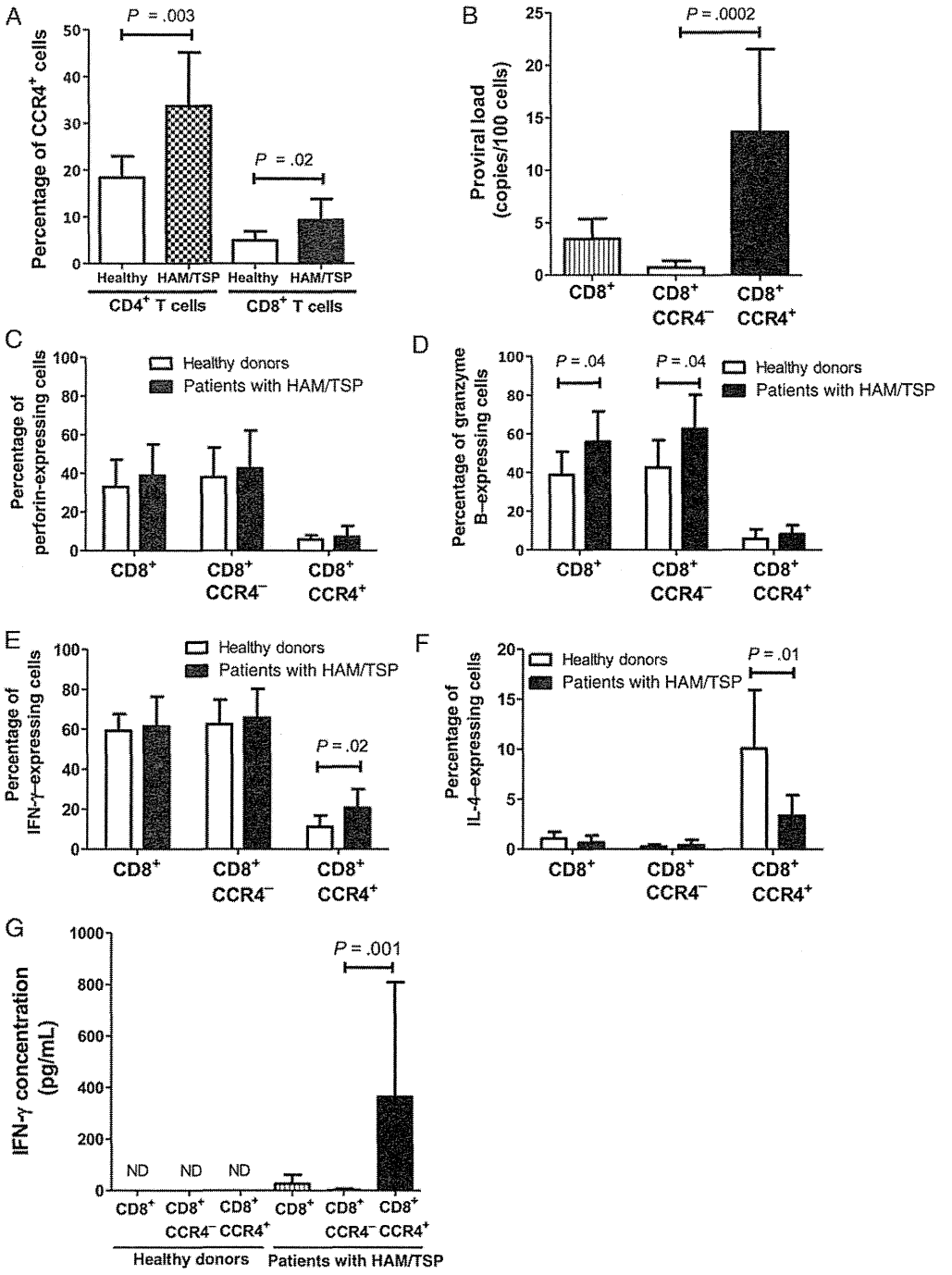

Figure 5. $CD8^+CCR4^+$ T cells from patients with human T-lymphotropic virus type 1 (HTLV-1)–associated myelopathy/tropical spastic paraparesis (HAM/TSP) are numerous, highly HTLV-1 infected, and proinflammatory. *A*, Proportions of $CCR4^+$ cells among $CD4^+$ and $CD8^+$ T cells in 8 healthy donors and 11 patients with HAM/TSP were analyzed by fluorescence-activated cell-sorter (FACS) analysis. *B*, The HTLV-1 proviral load in total $CD3^+CD8^+$, $CD3^+CD8^+CCR4^-$, and $CD3^+CD8^+CCR4^+$ T-cell subsets. $CD8^+$ T cells from 11 patients with HAM/TSP were isolated using negative separation with magnetic beads, and then $CD3^+CD8^+$, $CD3^+CD8^+CCR4^-$ and $CD3^+CD8^+CCR4^+$ T cells were separated with FACS analysis. Proviral loads in each subset were measured using real-time polymerase chain reaction. *C–F*, Peripheral blood mononuclear cells (PBMCs) from 8 healthy donors and 11 patients with HAM/TSP were stained for CD8 and CCR4, as well as intracellular perforin or granzyme B, and analyzed using FACS analysis. PBMCs from the same individuals were stimulated with phorbol 12-myristate 13-acetate (50 ng/mL) and ionomycin (1 μg/mL) in the presence of monensin for 6 hours. The cells were then analyzed for CD8, CCR4, and intracellular interferon γ (IFN-γ) or interleukin 4 (IL-4) expressions. The percentages of perforin-expressing (*C*), granzyme B–expressing (*D*), IFN-γ–expressing (*E*), and IL-4–expressing (*F*) cells in total $CD8^+$, $CD8^+CCR4^-$, and $CD8^+CCR4^+$ T-cell subsets from healthy donors versus patients with HAM/TSP are shown. *P* values are indicated only when <.05. *G*, $CD3^+CD8^+$, $CD3^+CD8^+CCR4^-$, and $CD3^+CD8^+CCR4^+$ T cells were isolated from 6 healthy donors and 11 patients with HAM/TSP as described above. These cells (3×10^4 cells/well) were cultured for 3 days without stimuli, and the concentration of IFN-γ in the supernatants was measured. Statistical analysis was performed using the Mann–Whitney *U*-test (*A* and *C–F*) or the Wilcoxon signed-rank test (*B* and *G*). Data are presented as the mean ± SD. Abbreviation: SD, standard deviation.

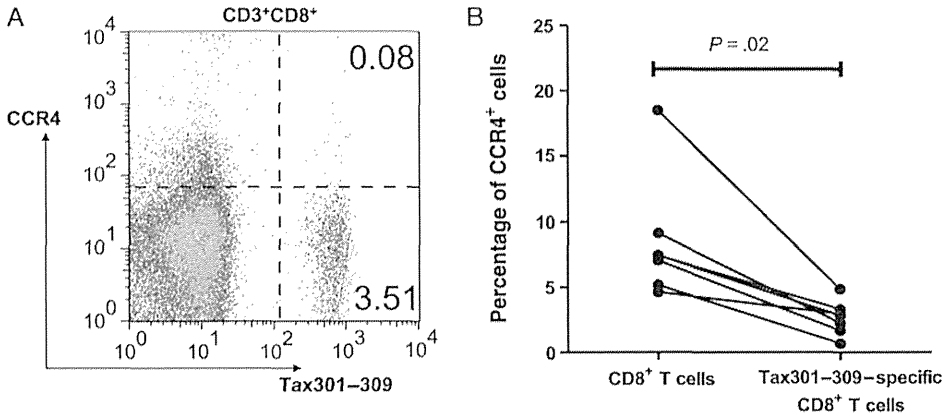

Figure 6. $CD8^+CCR4^+$ T cells from patients with human T-lymphotropic virus type 1 (HTLV-1)–associated myelopathy/tropical spastic paraparesis (HAM/TSP) do not include many Tax-specific cytotoxic T lymphocytes (CTLs). *A*, A representative dot plot of fluorescence-activated cell-sorter (FACS) analysis of the expression of CCR4 in HTLV-1 Tax-specific CTLs. Peripheral blood mononuclear cells from a patient with HAM/TSP and HLA-A*2402 were stained with antibodies for CD3, CD8, CCR4, and HLA-A*2402-restricted Tax301-309–specific tetramer. The $CD3^+CD8^+$ subset was gated. The values in the upper and lower right quadrants indicate the percentages of $CCR4^+$ and $CCR4^-$ Tax-specific CTLs among $CD3^+CD8^+$ T cells, respectively. *B*, Proportions of $CCR4^+$ cells among total $CD8^+$ T cells and Tax301-309–specific $CD8^+$ T cells are compared (n = 7). Statistical analysis was performed using the Wilcoxon signed-rank test.

$CD8^+CCR4^+$ T cells normally produce IL-4 more often than IFN-γ and hardly produce any cytotoxic granules [35, 36]; these cells are thought to be protective against type 1-skewed inflammation [21, 37]. In patients with HAM/TSP, these $CD8^+CCR4^+$ but not $CD8^+CCR4^-$ T cells are altered to produce IFN-γ rather than IL-4 (Figure 5*E* and 5*F*). $CD8^+CCR4^+$ T cells cultured alone exhibited spontaneous IFN-γ production (Figure 5*G*), a hallmark of PBMCs from patients with HAM/TSP [29, 30]. These results suggest that abnormal cells contributing to the pathogenesis of HAM/TSP exist not only among $CD4^+CCR4^+$ T cells but also among $CD8^+CCR4^+$ T cells. It is thought that the functional abnormalities of these cells may arise through transformations occurring within the infected cells themselves, whereby HTLV-1 Tax induces transcriptional alterations via T box transcription factor [18].

In the present study, HTLV-1 infection did not influence cytotoxic granule production in $CD8^+CCR4^+$ T cells (Figure 5*C* and 5*D*). The slightly increased fraction of granzyme B^+ cells in $CD8^+CCR4^-$ T cells from patients with HAM/TSP is presumably attributable to the immune activation resulting from the chronic viral infection [38–40].

Although eliminating the abnormal immune responses of the infected cells should alleviate inflammation and related symptoms of the infection, it is also true that immune responses against HTLV-1 are important for controlling said infection [41]. We evaluated CCR4 expression in HTLV-1 Tax-specific CTLs for fear that use of mogamulizumab might inadvertently destroy CTLs that would have helped to control the infection [42, 43]. Since Tax-specific CTLs have been reported to be preferentially infected by HTLV-1 [44], there was some concern that our finding that infected $CD8^+$ T cells are predominantly $CCR4^+$ meant that these CTLs would also be targeted by mogamulizumab. However, we found that the majority of Tax-specific CTLs do not express CCR4 (Figure 6), meaning that they should essentially be spared during mogamulizumab treatment.

Also concerning is that mogamulizumab is expected to target $CD4^+CCR4^+$ regulatory T (Treg) cells [45], which could elicit autoimmune problems and even exacerbate the chronic inflammation plaguing patients with HAM/TSP. However, there are also $CCR4^-$ Treg cells [45], which would be spared, and there have been no reports of increased incidence of autoimmune disease in patients with ATL treated with mogamulizumab. Furthermore, reducing the number of Treg cells may benefit patients with HAM/TSP by preventing abundant Treg cells from dampening immune control of the HTLV-1 infection [28, 46].

We expect that eliminating HTLV-1–infected cells in the peripheral blood with mogamulizumab would reduce the number of proinflammatory cells and mitigate the inflammation in the CNS. Although HAM/TSP is a disease of the CNS, recent reports suggest that it is indeed effective to target HTLV-1–infected cells in the peripheral blood because continued migration of infected cells from the peripheral blood maintains and even exacerbates the inflammation in the CNS [30].

Based on the results of this study, we have begun conducting a clinical trial to test the efficacy of mogamulizumab on patients with HAM/TSP (UMIN000012655). Our data suggest that as little as one thousandth of the dose administered to patients with ATL (1 mg/kg body weight [13]) may be effective for patients with HAM/TSP. In contrast to patients with an aggressive cancer such as ATL, those with a chronic inflammatory disorder like HAM/TSP would benefit from a more conservative approach that is safer but still effective.

In conclusion, we have demonstrated that mogamulizumab shows promise as a novel treatment for HAM/TSP. Our results indicate that $CD8^+CCR4^+$ T cells and $CD4^+CCR4^+$ T cells are key therapeutic targets and, thus, that the CCR4-targeting therapy mogamulizumab can be expected to effectively ameliorate chronic inflammation in patients with HAM/TSP. The lack of success with classic antiviral therapies [8, 9] suggests that blocking viral replication is ineffective against HTLV-1, which mainly spreads by cell division [11, 12]. Targeting the infected cells themselves on the basis of their characteristic markers may be the key to combating this tricky virus. If successful, mogamulizumab would become the first treatment for a chronic viral infection that effectively targets infected cells.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (http://jid.oxfordjournals.org). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

Acknowledgments. We thank M. Koike, Y. Hasegawa, Y. Suzuki-Ishikura, and Y. Saito, for technical assistance; and Kyowa Hakko Kirin, Japan, for kindly providing monoclonal antibodies (mogamulizumab and KM2760).

J. Y. performed most of the experiments, performed data analysis, created the figures, and wrote the manuscript. A. C. R. performed data interpretation and wrote the manuscript. T. S., N. A., N. Y., H. A., Y. K., and K. T. performed data analysis and interpretation. Y. T., Y. S., K. N., T. N., Y. H., A. U., and K. K. reviewed and edited the manuscript. Y. Y. developed the project, performed data analysis, and wrote the manuscript. All authors approved the final manuscript.

Financial support. This work was supported by the Ministry of Health Labor and Welfare (matching-fund subsidy for the Research on Measures for Intractable Disease project), JSPS KAKENHI (grant 25461294), and the MEXT-Supported Program for the Strategic Research Foundation at Private Universities, 2008–2012.

Potential conflicts of interest. Y. Y. has 1 established patent and another pending for the use of anti-CCR4 antibodies as a treatment for HAM/TSP. All other authors report no potential conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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bjh research paper

Clinical outcomes of a novel therapeutic vaccine with Tax peptide-pulsed dendritic cells for adult T cell leukaemia/lymphoma in a pilot study

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Received 13 November 2014; accepted for publication 17 December 2014

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Summary

Adult T cell leukaemia/lymphoma (ATL) is a human T cell leukaemia virus type-I (HTLV-I)-infected T cell malignancy with poor prognosis. We herein developed a novel therapeutic vaccine designed to augment an HTLV-I Tax-specific cytotoxic T lymphocyte (CTL) response that has been implicated in anti-ATL effects, and conducted a pilot study to investigate its safety and efficacy. Three previously treated ATL patients, classified as intermediate- to high-risk, were subcutaneously administered with the vaccine, consisting of autologous dendritic cells (DCs) pulsed with Tax peptides corresponding to the CTL epitopes. In all patients, the performance status improved after vaccination without severe adverse events, and Tax-specific CTL responses were observed with peaks at 16–20 weeks. Two patients achieved partial remission in the first 8 weeks, one of whom later achieved complete remission, maintaining their remission status without any additional chemotherapy 24 and 19 months after vaccination, respectively. The third patient, whose tumour cells lacked the ability to express Tax at biopsy, obtained stable disease in the first 8 weeks and later developed slowly progressive disease although additional therapy was not required for 14 months. The clinical outcomes of this pilot study indicate that the Tax peptide-pulsed DC vaccine is a safe and promising immunotherapy for ATL.

Keywords: adult T cell leukaemia/lymphoma, tumour vaccine, dendritic cell, human T cell leukaemia virus type-I, cytotoxic T lymphocyte.

Adult T cell leukaemia/lymphoma (ATL) is an aggressive lymphoproliferative disease caused by human T cell leukaemia virus type-I (HTLV-I) infection (Uchiyama *et al*, 1977; Poiesz *et al*, 1980; Hinuma *et al*, 1981). In particular, the acute and lymphoma types of ATL are characterized by a poor prognosis. Although the chronic and smouldering types

doi: 10.1111/bjh.13302

of ATL exhibit milder disease progression, these diseases also result in poor clinical outcome once they have converted to the acute or lymphoma types.

One reason for the poor clinical outcome associated with ATL is rapid progression of the disease at onset, which requires a prompt diagnosis and effective first-line therapy. Currently available first-line therapies for ATL include intensive multi-agent chemotherapy (Tsukasaki *et al*, 2012), interferon-α combined with zidovudine (Gill *et al*, 1995; Hermine *et al*, 1995) and an anti-CCR4 antibody (mogamulizumab) (Ishida *et al*, 2012).

Frequent relapse is another reason for the poor prognosis of ATL, requiring subsequent administration of second-line therapy that can produce a long-lasting anti-ATL effect. Haematopoietic stem cell transplantation (HSCT) has been reported to achieve a long-lasting remission in 30–40% of ATL patients, although it occasionally induces treatment-related mortality in a similar percentage of recipients (Utsunomiya *et al*, 2001; Okamura *et al*, 2005; Hishizawa *et al*, 2010; Ishida *et al*, 2013). In addition to the graft-*versus*-host response (Tanosaki *et al*, 2008), the actions of Tax-specific cytotoxic T lymphocytes (CTLs) have been implicated in the graft-*versus*-ATL effects of HSCT. This is based on our previous finding that ATL patients who obtained complete remission following HSCT often exhibit activation of $CD8^+$ CTLs specific for HTLV-I Tax (Harashima *et al*, 2004).

In untreated ATL patients, Tax-specific CTLs are either undetectable or dysfunctional, if present (Takamori *et al*, 2011). Although ATL patients are in a severe immune suppressive state, the impaired CTL response is not merely a result of general immune suppression in the advanced disease, but also observed in the patients with earlier stages of the disease in a selective manner for HTLV-I-specific responses (Takamori *et al*, 2011). The anti-tumour effects of Tax-specific T cells have been well characterized in animal models, where Tax-coding DNA and Tax-peptide vaccines have been shown to induce T cell immunity, thus eradicating HTLV-I-infected lymphomas in rats (Ohashi *et al*, 2000; Hanabuchi *et al*, 2001).

The efficacy of the vaccine targeting Tax in human ATL patients remains unclear, and no such treatment has ever been attempted as an actual therapy. This is partly because the HTLV-I gene expression levels are believed to be very low *in vivo* (Kurihara *et al*, 2005; Rende *et al*, 2011), and ATL cells occasionally lack the ability to express Tax (Takeda *et al*, 2004). However, our previous finding of the Tax-specific CTL activation in ATL patients following HSCT from uninfected donors indicated the presence of a sufficient level of Tax expression for the CTL response *in vivo* (Harashima *et al*, 2004).

These findings prompted us to attempt to develop a therapeutic anti-ATL vaccine designed to augment a Tax-specific CTL response that may partly reproduce the long-lasting anti-tumour effects of HSCT as second-line therapy for ATL. For the vaccine antigen, we used synthetic oligopeptides corresponding to the major epitopes recognized by Tax-specific CTL identified in our previous studies of post-HSCT ATL patients (Harashima *et al*, 2004, 2005). These epitopes are restricted to HLA-A2, A24 or A11, all of which are common in the Japanese population. For the vaccine adjuvant, we used autologous dendritic cells (DCs) induced from the peripheral monocytes. Although previous reports suggested dysfunctions of DCs in ATL patients (Makino *et al*, 2000; Hishizawa *et al*, 2004), the monocyte-derived DCs obtained from ATL patients retained the ability of antigen presentation in our preliminary experiments. The use of autologous DCs loaded with tumour antigens have been reported in various tumour vaccine trials of different tumours (Nagayama *et al*, 2003; Ueda *et al*, 2004; Linette *et al*, 2005; Fuessel *et al*, 2006; Thomas-Kaskel *et al*, 2006; Wierecky *et al*, 2006).

The present pilot study investigated the safety and efficacy of the Tax peptide-pulsed dendritic cell (Tax-DC) vaccine when administered to augment Tax-specific CTL responses in ATL patients.

Materials and methods

Study design

This clinical study was approved by the institutional ethics committee and registered as UMIN000011423. Three ATL patients possessing HLA-A*02:01, A*24:02 and/or A*11:01, in stable condition at least 4 weeks after the administration of previous therapy, provided their written informed consent and were enrolled in this study, which investigated the safety and efficacy of the Tax peptide-pulsed DC (Tax-DC) vaccine between September 2012 and February 2013.

HTLV-I proviruses in the peripheral blood mononuclear cells (PBMCs) were examined for the potential Tax expression and conservation of targeted CTL epitopes by analysing their nucleotide sequences beforehand. All patients were subcutaneously administered with Tax peptide-pulsed autologous DCs (5×10^6) three times at 2-week intervals (Fig 1A) at Kyushu University Hospital.

Patients

Patient 1 was a 69-year-old male who was diagnosed with acute ATL in August 2011. After receiving four courses of multi-agent chemotherapy, he achieved stable disease (SD). Although additional treatment with lenalidomide was administered for a few weeks, it was discontinued due to the development of thrombocytopenia. The patient was registered to the study in September 2012.

Patient 2 was a 67-year-old female who was diagnosed with acute ATL in December 2011. She presented with remarkable systemic lymphadenopathy and splenomegaly, in addition to an extremely high level of soluble interleukin-2 receptor (sIL2R; 57 815 u/ml). She received four courses of

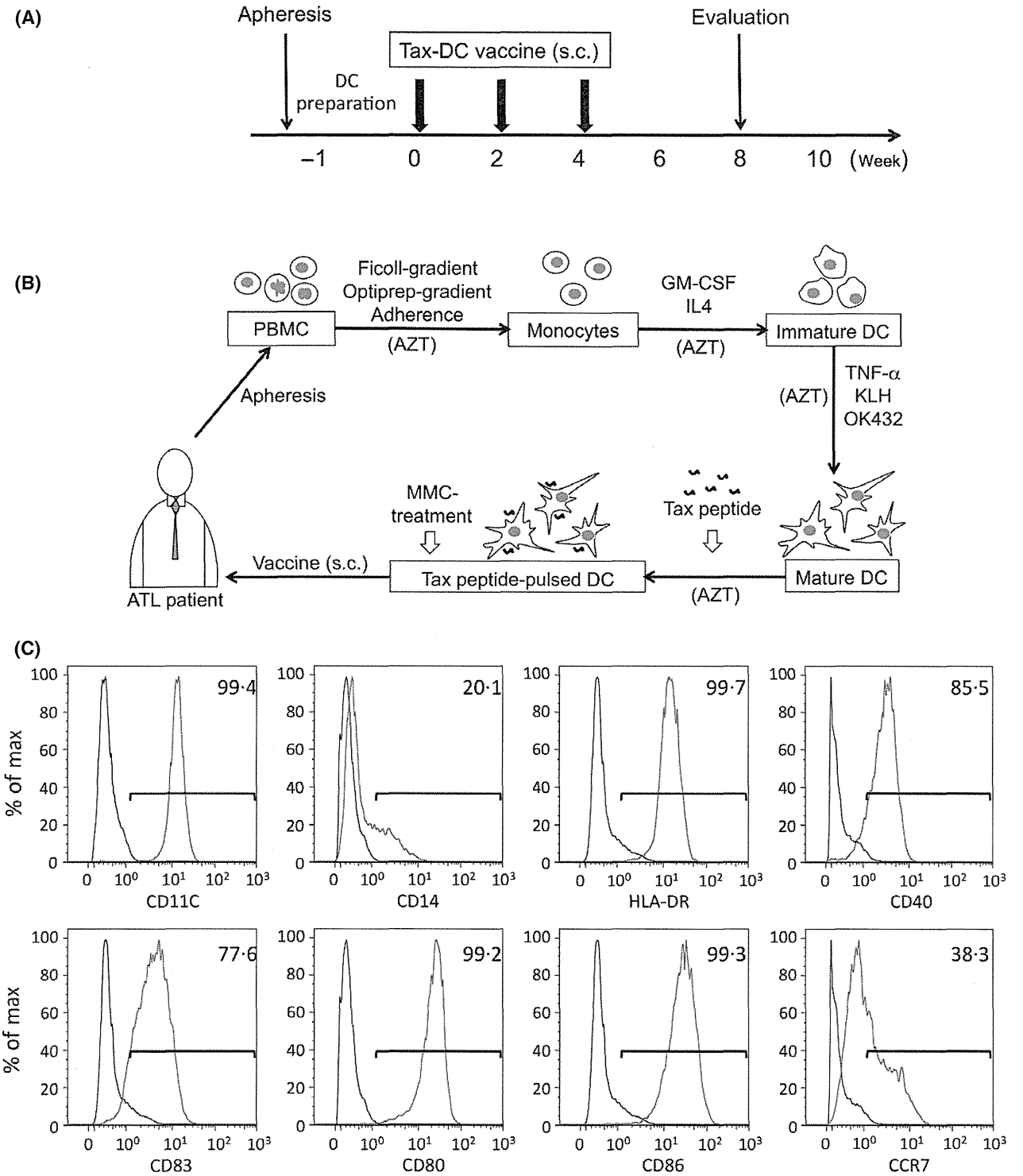

Fig 1. Outline of the Tax-DC vaccine therapy. (A) Schedule for the Tax-DC vaccine therapy. (B) Preparation of the monocyte-derived dendritic cells (DCs). Monocytes were enriched via serial density gradient centrifugation, and the adherent cells were cultured in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF) and interleukin 4 (IL4) for 5 d, followed by 48 h of culture with TNF-α, keyhole limpet haemocyanin (KLH), and OK432. A total of 10 μmol/l of zidovudine (AZT) was added whole throughout the culture. The matured DCs were pulsed with synthetic Tax peptides, treated with Mitomycin C (MMC), and then cryopreserved prior to subcutaneous injection. (C) Representative phenotype of mature dendritic cells prepared from Patient 1 prior to administration, as evaluated using flow cytometry. The red histograms indicate the results of staining with monoclonal antibodies for the indicated molecules, while the black histograms indicate the results of staining with control antibodies. ATL, Adult T cell leukaemia/lymphoma; PBMC, peripheral blood mononuclear cells.

multi-agent chemotherapy and achieved a partial remission (PR). Due to the development of disease recurrence with rapid progression after 2 months, treatment with mogamulizumab and low-dose chemotherapy (sobuzoxane + etoposide) was added. After obtaining a second PR, the patient was registered to the study in November 2012.

Patient 3 was a 56-year-old female diagnosed with acute ATL who presented with severe pneumocystis pneumonia in August 2012. After receiving two courses of multi-agent chemotherapy followed by two courses of mogamulizumab combined with chemotherapy, she achieved a PR. Further intensive treatment was not planned due to the development of severe respiratory dysfunction. The patient was registered to the study in February 2013.

The clinical information of the patients at enrollment is summarized in Table I.

Preparation of Tax peptide-pulsed DCs

Monocyte-derived DCs were generated from apheresis samples collected from the peripheral blood (6 l) of ATL patients at institutional cell processing facilities according to the good manufacturing practice (GMP) standard using a previously reported method, with some modifications (Nagayama *et al*, 2003) (Fig 1B). Briefly, monocytes enriched via serial density gradient centrifugation on Ficoll-Paque Plus (GE Healthcare, Uppsala, Sweden) and density-adjusted Optiprep (1·073 g/ml; Axis-Shield PoC, Oslo, Norway) were cultured at 37°C for 2 h, after which the adherent cells were cultured in CellGro DC medium (CellGenix GmbH, Freiburg, Germany) with 1000 iu/ml of granulocyte-macrophage colony-stimulating factor (Leukine; Bayer HealthCare Pharmaceuticals, Seattle, WA, USA) and 100 iu/ml of IL4 (Miltenyi Biotec, Bergisch Gladbach, Germany) for 5 d. The resulting monocyte-derived DCs were matured in the presence of 10 ng/ml of TNF-α (Miltenyi Biotec) and 12·5 μg/ml of keyhole limpet haemocyanin (KLH; Calbiochem, La Jolla, CA, USA) for 48 h, with 0·1 Clinical unit (Klinische Einheit; KE)/ml of OK432 (Picibanil; Chugai Pharmaceutical Co. Ltd., Tokyo, Japan) for the last 24 h. The matured DCs were pulsed with 2 μg/ml of synthetic peptides (NeoMPS; PolyPeptide Laboratories Group, San Diego, CA, USA), including Tax11-19 (LLFGYPVYV) (Kannagi *et al*, 1992) or Tax301-309 (SFHSLHLLY) (Harashima *et al*, 2004) restricted to HLA-A*02:01 or -A*24:02 respectively, and treated with Mitomycin C (MMC; Kyowa Hakko Kirin Co. Ltd., Tokyo, Japan) (50 μg/ml) in order to inactivate the ATL cells potentially contained in the preparation. As DCs are reported to be susceptible for HTLV-I infection (Jones *et al*, 2008), 10 μmol/l of zidovudine (Retrovir, AZT; GlaxoSmithKline, Research Triangle Park, NC, USA) was added whole throughout the culture to avoid *de novo* infection. The peptide-pulsed DCs were then washed and examined for safety by checking for contamination with bacteria, fungi, mycoplasma and/or endotoxins, then cryopreserved until use. The cells (5×10^6) were subsequently thawed and washed prior to administration.

Evaluation of adverse events and the clinical response

Toxic effects were graded according to the Common Terminology Criteria for Adverse Events version 3.0 (http://ctep.cancer.gov/protocolDevelopment/electronic_applications/docs/ctcaev3.pdf). The clinical response was evaluated according to the criteria proposed by the international consensus meetings that led to the modification of the Japan Clinical Oncology Group criteria (Tsukasaki *et al*, 2009). Briefly, complete remission (CR) was defined as the disappearance of all clinical, microscopic and radiographic evidence of disease. PR was defined as a ≥50% reduction in the level of measurable disease without the appearance of new lesions. In addition, the diagnosis of a PR was required to satisfy a 50% or greater reduction in the absolute abnormal lymphocyte count in the peripheral blood. Progressive disease (PD) or relapsed disease was defined as a ≥50% increase from the nadir in the sum of the products of measurable disease or the appearance of new lesions, excluding the skin. Stable disease (SD) was defined as the failure to attain CR/PR nor PD.

The soluble IL2 receptor (sIL2R) level, HTLV-I proviral load and Tax-specific CTL response were monitored in addition to the results of general laboratory tests. Adverse effects

Table I. Patient characteristics at enrollment.

	Patient 1	Patient 2	Patient 3
Age (years)/gender	70/ male	68/ female	57/ female
HLA-A allele	24:02, 31:01	24:02, 26:03	02:01, 11:01
Subtype of ATL	Acute	Acute	Acute
Previous therapy	mEPOCH, lenalidomide	mEPOCH, mogamulizumab + PVP	mEPOCH, mogamulizumab + PVP
Disease status	SD	PR	PR
Interval from previous therapy	2·5 months	1·5 months	2 months
Duration since diagnosis	14 months	11 months	6 months
Complication	Allergic dermatitis	Breast cancer, DM, NASH	Interstitial pneumonia

mEPOCH, modified combination chemotherapy with etoposide + prednisone + vincristine + doxorubicin + carboplatin; PVP, combination chemotherapy with sobuzoxane + etoposide; SD, stable disease; PR, partial remission; DM, diabetes mellitus; NASH, nonalcoholic steatohepatitis.

and the clinical response were monitored and evaluated at 8 weeks after the initiation of the Tax-DC vaccine therapy.

Tax-specific CTL analysis

Phycoerythrin (PE)-conjugated HLA-A*0201/Tax11-19, HLA-A*1101/Tax88-96 and HLA-A*2402/Tax301-309 tetramers were purchased from Medical & Biological Laboratories, Co., Ltd. (Nagoya, Japan). Whole blood samples or PBMCs were stained with PE-conjugated Tax/HLA tetramers, together with fluorescein isothiocyanate (FITC)-conjugated anti-human CD3 and PE/cyanin 5 (Cy5)-conjugated anti-human CD8 monoclonal antibodies (mAbs) (BioLegend, San Diego, CA, USA), then fixed in Becton Dickinson (BD) FACS lysing solution (BD Biosciences, San Jose, CA, USA), followed by analysis on the FACS Calibur system using the CELLQUEST software program (BD Biosciences). For staining intracellular IFN-γ production, PBMCs pre-stained with PE-conjugated Tax/HLA tetramers and anti-human CD8-PE/Cy5 mAb were incubated at 37°C for 6 h in the presence of cognate Tax peptides (10 μmol/l), with brefeldin A (10 μg/ml; Sigma Aldrich, St. Louis, MO, USA) for the last 5 h. The cells were then permeablized using BD Cytofix/Cytoperm Fixation/Permeabilization Kit (BD Biosciences) and stained with FITC-conjugated anti-human IFN-γ mAb (4S.B3; BioLegend).

Detection of HTLV-I gene expression

To detect intracellular HTLV-I antigens, cells were serially treated with 4% paraformaldehyde for 10 min and 100% methanol for 10 min on ice, and then stained with Alexa Fluor 488-labelled anti-Tax Lt-4 (Lee *et al*, 1989) or isotype control mAbs followed by flow cytometry.

To quantify HTLV-I *pX* mRNA, total RNA extracted by using Isogen (Nippon Gene, Tokyo, Japan) were treated with DNase (Ambion, Austin, TX, USA), and subjected to quantitative reverse transcription polymerase chain reaction (RT-PCR) with the primer sets specific for HTLV-I *pX* (forward, 5′-CGG ATA CCC AGT CTA CGT GTT TGG AGA CT-3′; reverse, 5′-GAG CCG ATA ACG CGT CCA TCG ATG GGG TCC-3′) and *GAPDH* (forward, 5′-TGA TTT TGG AGG GAT CTC GCT CCT GGA AGA-3′; reverse, 5′-GTG AAG GTC GGA GTC AAC GGA TTT GGT CGT-3′) by using LightCycler Fast Start DNA Master SYBR Green I (Roche Diagnostics, Mannheim, Germany) after reverse transcription with oligo(dT)20 primers. The *pX* mRNA levels were standardized against *GAPDH* mRNA copy numbers.

Results

Feasibility of the DC preparation in ATL patients

We obtained 4·3–10·6 × 10^7 DCs with 72·2–91·3% purity. The cells exhibited the phenotype of mature DCs ($CD11c^+$, $CD80^+$, $CD86^+$, $CD83^+$, $CD40^+$, $HLA\text{-}DR^+$). The representative results obtained in Patient 1 are shown in Fig 1C. The HTLV-I proviral load of the PBMCs in the input apheresis samples were 114·8, 36·7 and 25·5 copies/1000 cells in the three patients respectively, with final loads in the DCs of 5·9, 5·0 and 10·3 copies/1000 cells, respectively.

Clinical courses after the Tax-DC vaccine therapy in the ATL patients

The clinical outcomes of the Tax-DC vaccine therapy in the three patients are summarized in Table II.

Table II. Clinical responses after the Tax-DC vaccine therapy in the three ATL patients.

Clinical response in 8 weeks after initiation of the vaccine therapy	Patient 1*		Patient 2†		Patient 3‡	
Time at evaluation	Pre-therapy	8 weeks	Pre-therapy	8 weeks	Pre-therapy	8 weeks
KPS (%)	70	90	70	80	70	90
LDH (iu/l)	473	245	250	326	329	268
sIL2R (u/ml)	19 056	1866	806	1462	1739	871
HTLV-I PVL (copies/1000 PBMCs)	114·8	12·4	36·7	14·9	17·7	29·6
Clinical response	–	PR	–	SD	–	PR
Long-term outcomes						
TTNT (months from registration)	25+		15		20+	
Survival (months from diagnosis)	39+		34		26+	

KPS, Karnofsky performance status; LDH, lactate dehydrogenase; HTLV-I PVL, human T cell leukaemia virus type-I proviral load, PBMCs, peripheral blood mononuclear cells; SD, stable disease, PR, partial remission; TTNT, time to next anti-tumour therapy.

*The size of the lymph nodes in Patient 1 repeatedly increased and decreased, especially at time points later than 6 months after initiation of vaccine therapy.

†Patient 2 was considered to have developed a progressive disease at 6 months after the initiation of the vaccine therapy.

‡Patient 3 achieved complete remission at 6 months after the initiation of the vaccine therapy.

Patient 1 was positive for HLA-A*24:02 and vaccinated with Tax 301-309 peptide-pulsed DCs. Following the first administration of the Tax-DC vaccine, he developed a fever (grade 2), dermatitis (grade 2) and diarrhoea (grade 1). The white blood cell count, level of ATL cells in the peripheral blood and LDH level in the serum showed remarkable fluctuation during the vaccination, and then stabilized after the third administration of the vaccine (Fig 2A). In Patient 1, the level of sIL2R, which is a sensitive tumour marker for ATL, decreased from 19 056 to 1866 u/ml (normal range: <570 u/ml) by 8 weeks of therapy (Fig 2B). In addition, his surface lymph nodes decreased in size (Fig 2C), and he achieved a partial remission (PR) that persisted for at least 24 weeks. He returned to his normal life, and his Karnofsky performance status (KPS) improved from 70% to 100%. Although the size of the patient's lymph nodes and the level of sIL2R fluctuated at later time points, he has remained in remission for more than 24 months after the completion of the Tax-DC vaccine therapy, without any additional anti-tumour treatment.

Patient 2 had HLA-A*24:02 and was vaccinated with Tax 301–309 peptide-pulsed DCs. She developed a low-grade fever and dermatitis (grade 2) after each vaccine administration. However, no severe adverse events were observed during her clinical course. At 8 weeks of therapy, she was considered to have achieved SD. Although there was no objective response, an improvement in the KPS was noted. She was subsequently considered to have developed PD 6 months after the initiation of the Tax-DC vaccine therapy. Nevertheless, due to slow progression of the disease and her stable general condition, she was followed without any additional anti-tumour therapy until 14 months after the completion of vaccination. The patient died of infection 23 months after the initiation of the vaccine therapy.

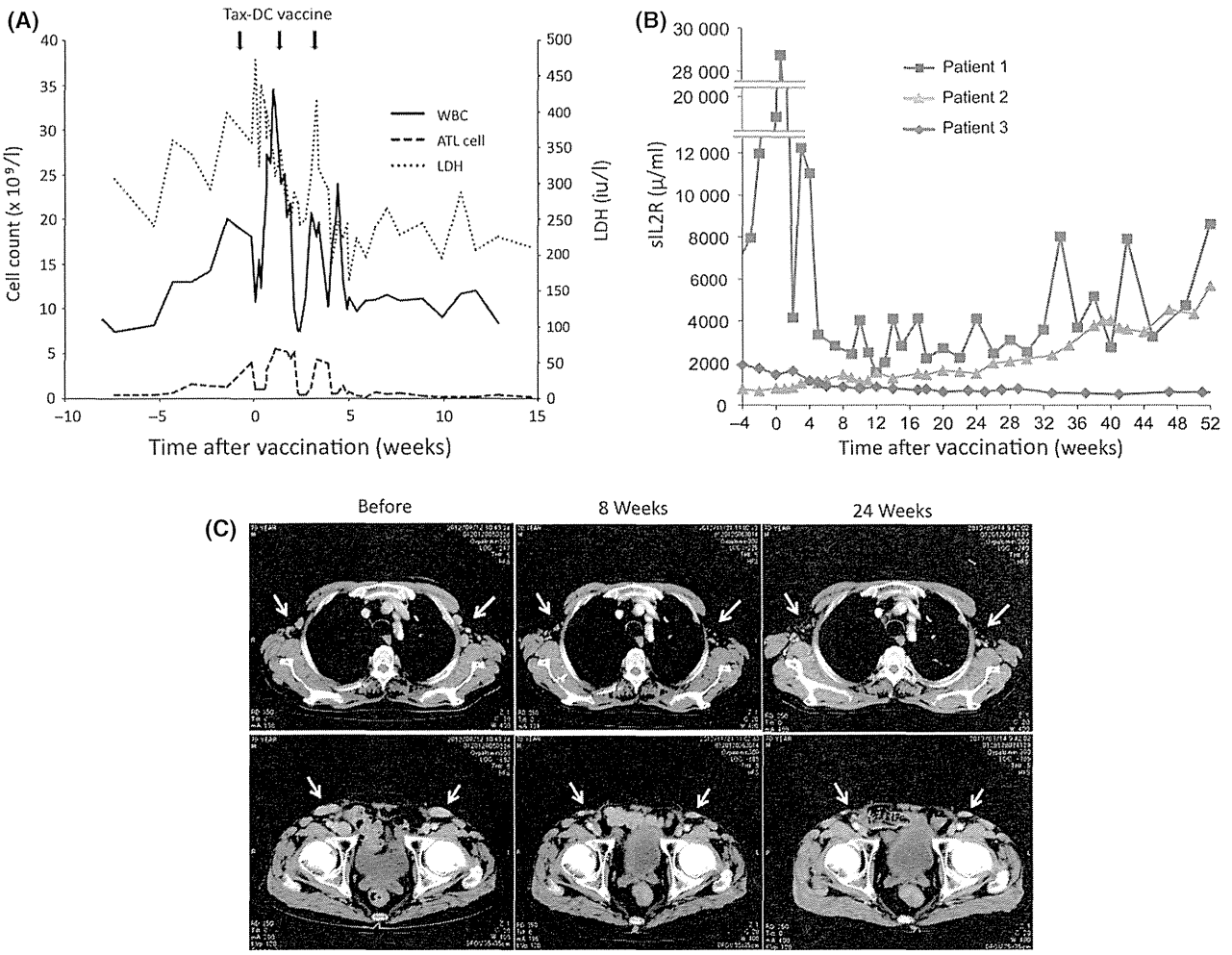

Fig 2. Clinical courses of the patients after the Tax-DC vaccine therapy. (A) Changes in the peripheral white blood cell count (WBC, solid line), ATL cell count (dashed line) and lactate dehydrogenase (LDH) level (fine dotted line) during the initial 15 weeks in Patient 1. The arrows indicate the days of Tax-DC vaccine administration. (B) Kinetics of the sIL2R levels in the sera obtained from Patients 1 (red), 2 (green) and 3 (blue) during the long-term observation period after the initiation of the Tax-DC vaccine therapy. (C) Computerized tomography images of the axillary (top) and inguinal (bottom) lymph nodes (arrows) of Patient 1 before and 8 and 24 weeks after the initiation of the Tax-DC vaccine therapy.

