Anti-CCR4 Monoclonal Antibody Mogamulizumab for the Treatment of EBV-associated T- and NK-Cell Lymphoproliferative Diseases

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TRANSLATIONAL RELEVANCE

Epstein-Barr virus (EBV) occasionally leads to T- and natural killer-cell lymphoproliferative diseases (T/NK-LPDs) that are refractory to conventional chemotherapies. To examine a molecular-targeted therapy for EBV-associated T/NK-LPDs, we investigated whether CC chemokine receptor 4 (CCR4) was expressed on EBV-infected T or NK cells and whether a humanized anti-CCR4 monoclonal antibody, mogamulizumab, was effective. CCR4 was expressed on most EBV-positive T and NK cell lines. Mogamulizumab induced antibody-dependent cellular cytotoxicity (ADCC) activity against CCR4-positive cell lines, and inhibited the growth of EBV-positive NK-cell lymphomas in a murine xenograft model. Furthermore, CCR4 was expressed on EBV-infected cells in 8 of 17 patients with EBV-associated T/NK-LPDs. An ex vivo ADCC assay showed that the γδT cells that were positive for both EBV and CCR4 were killed by mogamulizumab. These finding suggests that mogamulizumab may be a promising therapeutic for patients with EBV-associated T/NK-LPDs.
ABSTRACT

**Purpose:** Epstein-Barr virus (EBV) infects not only B cells but also T cells and natural killer (NK) cells, and T- and NK-cell lymphoproliferative diseases (T/NK-LPDs) that are refractory to conventional chemotherapies may develop. To identify a molecular-targeted therapy for EBV-associated T/NK-LPDs, we investigated whether CC chemokine receptor 4 (CCR4) was expressed on EBV-infected T and/or NK cells and whether a humanized anti-CCR4 monoclonal antibody, mogamulizumab, was effective.

**Experimental design:** CCR4 expression was examined in various cell lines. *In vitro*, the effects of mogamulizumab on cell lines were evaluated in the presence of peripheral blood mononuclear cells from volunteers. *In vivo*, the effects of mogamulizumab were evaluated using a murine xenograft model. CCR4 expression was examined on EBV-infected cells from patients with EBV-associated T/NK-LPDs. *Ex vivo*, the effects of mogamulizumab were evaluated using patient lymphocytes.

**Results:** CCR4 expression was confirmed in most EBV-positive T and NK cell lines. Mogamulizumab induced antibody-dependent cellular cytotoxicity (ADCC) activity against CCR4-positive cell lines, and inhibited the growth of EBV-positive NK-cell lymphomas in a murine xenograft model. Furthermore, CCR4 was expressed on EBV-infected cells in 8/17 patients with EBV-associated T/NK-LPDs. Interestingly, CCR4 was positive in 5/5 patients with hydroa vacciniforme, a photodermatosis caused by clonal expansion of EBV-infected γδT cells. EBV-positive γδT cells were obtained from a patient with hydroa vacciniforme and subjected to an ADCC assay. The γδT cells that were positive for CCR4 were killed by mogamulizumab via ADCC.

**Conclusions:** These results indicate that mogamulizumab may be a therapeutic option against EBV-associated T/NK-LPDs.
INTRODUCTION

Epstein-Barr virus (EBV) infects most individuals by early adulthood and is associated with multiple B-cell lymphoid malignancies, including Burkitt lymphoma, Hodgkin lymphoma, and post-transplant lymphoproliferative disorder (PTLD). EBV also infects T cells and natural killer (NK) cells, and there are several EBV-associated T- and NK-cell malignancies (1-4). Extranodal NK/T-cell lymphomas of the nasal type (ENKL) and aggressive NK-cell leukemia are representative EBV-associated T- or NK-cell malignancies, both of which are well-known and characterized (5, 6). Other than these overt leukemia/lymphomas, a new concept of “EBV-associated T- and NK-cell lymphoproliferative diseases” (T/NK-LPDs) has been proposed (7-9). This encompasses specific clinical diseases of chronic active EBV disease (CAEBV), EBV-associated hemophagocytic lymphohistiocytosis, hypersensitivity to mosquito bites, and hydroa vacciniforme, the distinctions between which are based on clinical manifestations (10, 11). Hydroa vacciniforme is an EBV-positive cutaneous disease associated with photosensitivity (12). Recently, we and others clarified that EBV-infected γδT cells expand with clonality and infiltrate the superficial dermis and subcutaneous tissue in hydroa vacciniforme (13, 14).

Some of these EBV-associated lymphoid malignancies are refractory to conventional chemotherapies and have poor prognoses. Rituximab, a humanized monoclonal antibody against CD20, targets B cell-specific surface antigens present on EBV-transformed malignant cells. Currently, rituximab is used for the treatment of B-cell lymphoma and PTLD (15, 16). For effective treatment of T- and NK-cell malignancies, novel approaches using other molecular targets are desirable.

Recently, as a new molecular-targeted therapy, a humanized monoclonal antibody targeting the CC chemokine receptor 4 (CCR4), mogamulizumab, has been developed for the treatment of adult T-cell leukemia/lymphoma (ATLL) (17-22). ATLL is a peripheral T-cell malignancy associated with human T-cell leukemia virus type 1 (HTLV-1) (23). In about 90% of ATLL, malignant cells infected with HTLV-1 express CCR4, so that many ATLL patients
are adaptable to mogamulizumab therapy (24, 25). Mogamulizumab prompts potent antibody-dependent cellular cytotoxicity (ADCC) activity against the malignant cells.

It is unclear whether EBV-associated T/NK-LPDs express CCR4. CCR4 is expressed predominantly on CD4-positive T helper 2 cells and regulatory T cells, but is occasionally also expressed on activated NK cells, CD8-positive cytotoxic T cells, and γδT cells (26-31), all of which are regarded as origins of malignant cells in EBV-associated T/NK-LPDs. This leads to the possibility that tumor cells in some types of EBV-associated T/NK-LPDs may express CCR4 and that mogamulizumab may have an effect against such EBV-associated T/NK-LPDs.

In this study, we screened for CCR4 expression on EBV-positive and -negative T- and NK-cell lines, and evaluated the antitumor effects elicited by mogamulizumab in vitro. We also evaluated in vivo effects of mogamulizumab on EBV-positive lymphoma cells using a murine xenograft model. Furthermore, lymphocytes from patients with EBV-associated T/NK-LPDs were used for estimating CCR4 expression and evaluating the antitumor effects of mogamulizumab.

MATERIALS AND METHODS

Cell lines and reagents

Of the cell lines used, SNT8, SNT13, and SNT16 are EBV-positive T-cell lines (32), Jurkat is an EBV-negative T-cell line (33), SNK1, SNK6 (32), and KAI3 (34) are EBV-positive NK-cell lines, KHYG1 (35) and NKL (36) are EBV-negative NK-cell lines, LCL is an EBV-infected B-lymphoblastoid cell line, Raji, Daudi, Akata, and Namalwa are EBV-positive B-cell lines, and BJAB is an EBV-negative B-cell line (13). KAI3 and KHYG1 were obtained from Japanese Collection of Research Bioresources (Osaka, Japan). SNT8, SNT13, SNT16, SNK1, and SNK6 were kindly provided from Norio Shimizu (Tokyo Medical and Dental University) who had originally established these cell lines. NKL was provided from Yasushi Isobe (St. Marianna University). B-cell lines were obtained from Tatsuya Tsurumi (Aichi
Cancer Institute). These cell lines were regularly authenticated using short tandem repeat profiling. The original diseases for each cell line are shown in Table 1. Mogamulizumab was purchased from Kyowa Hakko Kirin.

**Flow cytometry**

CCR4 expression was measured by flow cytometry using a phycoerythrin (PE)-labeled anti-human CCR4 antibody (BioLegend). CCR7 and CCR10 expression was examined with a phycoerythrin cyanine (PC) 7-labeled anti-CCR7 antibody (BD Pharmingen) and a PE-labeled anti-CCR10 antibody (R&D Systems), respectively. To stain surface antigens, PE-labeled anti-Vδ2 (BD Pharmingen), PC5-labeled anti-TCRγδ (Immunotech), and PC7-labeled anti-CD3 (BD Biosciences) antibodies were used. Stained cells were analyzed using a FACSCanto II (BD Biosciences) and the FlowJo software (Tree Star). CCR4 expression was defined as positive when the mean fluorescence intensity of anti-CCR4 antibody-treated cells was more than twice that of cells stained with isotype-matched control IgG.

**In vitro ADCC assay**

Cell lines were used as target cells. Human peripheral blood mononuclear cells (PBMCs) derived from healthy volunteers were used as effector cells. Target cells (2.5×10³) and effector cells were cultured in 96-well plates with mogamulizumab or solvent alone (control) for 24 h in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum. After incubation, the supernatant of each well was obtained, and percentage cell death was calculated by measuring the lactate dehydrogenase (LDH) concentration in the supernatant using the CytoTox 96 Non-Radioactive Cytotoxicity Assay (Promega). Each experiment was performed in triplicate, and the mean and standard error were shown.

**Magnetic sorting**
T cell, NK cell, B cell, and γδT cell fractions were separated by magnetic sorting using CD3, CD56, and CD19 Micro Beads or the TCRγ/δ T Cell Isolation kit (Miltenyi Biotec).

**Murine xenograft model**

Female NOD/Shi-scid/IL-2Rγnull (NOG) mice were purchased from the Central Institute for Experimental Animals, Japan. Twelve NOG mice, at an age of 8 weeks, were implanted subcutaneously with SNK6 cells on day 0, as previously described (37). From day 4, saline (control) or mogamulizumab (1 mg/kg in total) was injected intraperitoneally into six mice each twice per week for 4 weeks, and PBMCs (1×10^7 cells/mouse) were injected intraperitoneally into all mice once per week for 2 weeks. The doses of mogamulizumab and the amount of PBMCs used were chosen with reference to earlier reports on the use of mogamulizumab in a similar NOG mouse model (20, 21). Subcutaneous tumor masses were measured with external calipers and the tumor volume was calculated using the formula: \( \pi \times \text{short axis} \times \text{long axis} \times \text{height} / 6 \). After 28 days, all mice were sacrificed and the subcutaneous tumors were removed and the tissues fixed in 10% buffered formalin. Paraffin-wax-embedded tissues were cut into 3-μm serial sections. Sections were then stained with hematoxylin and eosin (H&E). The tumor was also stained for EBV-encoded small RNA (EBER) by in situ hybridization. EBER in situ hybridization was performed using the EBER PNA Probe/FITC (Y5200; Dako) and PNA ISH detection kit (Dako), according to the manufacturer’s protocol. Animal experiments were approved by the University Committee in accordance with the Guidelines for Animal Experimentation at Nagoya University.

**Patients**

In total, samples from 21 patients were used, including five cases of hydroa vacciniforme, eight of CAEBV, four of hemophagocytic lymphohistiocytosis, and four of PTLD. Diagnoses of hydroa vacciniforme and PTLD were made based on biopsy findings according to the World Health Organization (WHO) criteria (12, 38). Diagnoses of hemophagocytic
lymphohistiocytosis were made on the basis of criteria proposed by an international treatment study group (39), while CAEBV was diagnosed using criteria proposed previously (40, 41). Informed consent was obtained from all participants or their guardians according to the Declaration of Helsinki. This study was approved by the Institutional Review Board of Nagoya University Hospital.

**Flow cytometric in situ hybridization (flow-FISH) assay**

Since all patients with EBV-associated T/NK-LPDs had very high numbers of EBV-infected cells in peripheral blood, PBMCs were subjected to this assay. The flow-FISH assay was performed as described previously (13, 42, 43). First, for surface marker staining, 5×10^5 PBMCs were stained with a PE-labeled anti-CCR4 antibody (R&D Systems), and PC5-labeled anti-CD4 (Immunotech), anti-TCRγδ (Immunotech), anti-CD8 (Immunotech), anti-CD20 (DAKO), and PC7-labeled anti-CD3 (BD Biosciences) antibodies for 1 h at 4°C. For CD56 staining, a biotin-labeled anti-CD56 (eBioscience) antibody was used, followed by application of PE-conjugated streptavidin (eBioscience). Isotype-matched monoclonal mouse IgG antibodies were used as controls. Cells were fixed, permeabilized, and hybridized with EBER PNA Probe/FITC (Y5200; Dako) or Negative Control PNA Probe/FITC (Dako) (13). An Alexa Fluor 488 Signal Amplification Kit (Molecular Probes) was used to enhance fluorescence and photostability. Stained cells were analyzed using a FACSCanto II and the FlowJo software.

**Ex vivo ADCC assay**

PBMCs were obtained from a patient with hydroa vacciniforme, and γδT cells and NK cells were separated by magnetic sorting. In a similar way, NK cells were isolated from two healthy volunteers. γδT cells were used as the target, while NK cells were used as the effector. Target (1.0×10^4) and effector cell were cultured in 96-well plates with or without 10 μg/mL mogamulizumab for 24 h. The ratio of target/effector was fixed at 1:10. Percentage of cell
death was calculated by the same procedure as the in vitro ADCC assay. Each experiment was performed in triplicate, and the mean and standard error were shown.

Statistical analysis
Statistical analyses were performed using the SPSS software (ver. 18.0 for Windows; IBM Corporation). In vivo experimental data are shown as means and standard errors. To compare therapeutic results between groups, the Mann-Whitney U-test was used.

RESULTS
CCR4 expression in EBV-positive and -negative T-, NK-, and B-cell lines
To determine CCR4 expression in EBV-positive and -negative cell lines, flow cytometry was performed. All results for CCR4 expression are shown in Figure 1A. EBV-positive T-, and NK-cell lines, except SNT13, expressed CCR4 on their surface. However, of the EBV-positive B-cell lines, none expressed CCR4. In four EBV-negative cell lines, only NKL expressed CCR4. Similarly, another CC chemokine receptor, CCR7, was expressed in most T/NK-cell lines, while CCR10 was not. Representative results of CCR7 and CCR10 are shown in Figure 1B and the results are summarized in Table 1.

Effects of mogamulizumab against CCR4-positive and -negative T- and NK-cell lines in vitro
To evaluate the effects of mogamulizumab, CCR4-positive T-and NK-cell lines (SNT8, SNT16, SNK6, and KAI3) and CCR4-negative cell lines (SNT13, Jurkat, and LCL) were assessed using an in vitro ADCC assay. As shown in Figure 2A, PBMCs from a volunteer showed cytotoxicity against all CCR4-positive cell lines in the presence of mogamulizumab, and percentage of cell death increased depending on the ratio of target/effector. In contrast, CCR4-positive cells were not killed in the absence of mogamulizumab. However, the % cell lysis of CCR4-negative cell lines was not affected by the presence of mogamulizumab.
Next, a cytotoxicity assay against SNK6 was performed with PBMCs derived from three healthy volunteers with various doses of mogamulizumab. This experiment revealed that cell death increased in a mogamulizumab dose-dependent manner and there was a difference in killing activity among individual PBMCs (Fig. 2B). To determine which cell fraction in PBMCs played a major role of cytotoxicity, CD56-, CD3-, and CD19-positive cells were then used as effector cells. The highest percentage of cell death was observed when target cells were incubated with CD56-positive cells, showing that NK-cells played a central role (Fig. 2C). These results indicated that mogamulizumab killed CCR4-positive T- and NK-cells via ADCC.

Effect of mogamulizumab in a murine xenograft model

Recently, we reported a murine xenograft model using the immunodeficient NOG mouse and the EBV-positive NK-cell lymphoma cell line, SNK6 (37). We used this model to determine the in vivo effects of mogamulizumab. After subcutaneous inoculation with SNK6, mogamulizumab and PBMCs from healthy volunteers were administered. Tumor growth was suppressed significantly in the mogamulizumab-treated group, versus the control group (P < 0.05; Fig. 3A). A representative image of a tumor-bearing mouse is shown in Fig. 3B. H&E staining and EBER in situ hybridization showed the extent of the tumor in each mouse (Fig. 3C, 3D). In the mogamulizumab-treated mouse, the tumor was regressed with vacuolar degeneration. On the other hand, tumor expansion and massive infiltration in the dermis were seen in the control mouse.

CCR4 expression in patients with EBV-associated LPDs

We next examined the expression of CCR4 on EBV-infected cells from 17 patients with various EBV-associated T/NK-LPDs using a flow-FISH assay. For comparison, four patients with PTLD were also examined. Characteristics of each patient are summarized in the Supplementary Table. CCR4 was expressed on EBV-infected T- and NK-cells in 5/5 hydroa
vacciniforme, 2/8 CAEBV, and 1/4 hemophagocytic lymphohistiocytosis cases. CCR4 was expressed on EBV-infected B-cells in 1/4 PTLD patients. Representative results of the flow-FISH assay are shown in Figure 4A. In patients with hydroa vacciniforme (Patients 1, 2), TCRγδ Vδ2+ γδT cells were positive for EBER, and CCR4 was expressed on the EBER-positive cells. However, in Patient 8 with NK cell-type CAEBV (CD3-CD56+) and Patient 14 with hemophagocytic lymphohistiocytosis (CD3+CD8+), the EBER-positive cells did not express CCR4.

The fluorescence peak of CCR4-treated cells was not clearly separated from that of control IgG-treated cells (Fig. 4A). This is likely because the fluorescence signals were weakened under the harsh conditions of the flow-FISH assay (13). To further confirm CCR4 expression, we stained the presumed EBV-infected population with an anti-CCR4 antibody, without in situ hybridization. In Patient 1, Vδ2-positive γδT cells harbored EBV (Fig. 4A). Expression of CCR4 was clearly recognized in the Vδ2-positive fraction (Fig. 4B), confirming CCR4 expression on EBV-infected γδT cells in the patient.

**Effects of mogamulizumab against tumor cells from a patient with hydroa vacciniforme**

To examine the effects of mogamulizumab against tumor cells from patients, an ex vivo ADCC assay was performed. First, γδT cells were isolated from Patient 2 by magnetic sorting. In this patient, the γδT cells harbored EBV (Fig. 4A). Next, we confirmed that over 90% of the isolated cells were positive for TCRγδ, and that these cells expressed CCR4 (Fig. 5A). Then, the γδT cells were incubated with mogamulizumab and NK cells from either Patient 2 or healthy volunteer controls. The γδT cells were killed by NK cells from the patient in the presence of mogamulizumab (Fig. 5B). Cytotoxicity was also seen when NK cells from healthy controls were used, and the ADCC activity was similar between NK cells from the patient and those from controls.

**DISCUSSION**
EBV-associated T/NK-LPDs, initially proposed by Kawa et al. and subsequently noted by other researchers (8, 10, 11, 44), are prevalent in eastern Asian countries and are characterized by clonal expansion of EBV-infected T or NK cells. Because EBV-associated T/NK-LPDs are refractory to conventional chemotherapies and have poor prognoses, there is a continuing need for novel, effective treatments. In this study, we clarified that CCR4 was expressed on EBV-positive T and NK cells in EBV-associated T/NK-LPDs. In particular, in hydroa vacciniforme, CCR4 was expressed on EBV-positive γδT cells in all (5/5) patients tested. Furthermore, the ex vivo ADCC assay showed that γδT cells isolated from a patient with hydroa vacciniforme were killed by mogamulizumab. Importantly, ADCC activity was similar between NK cells from the patient and those from healthy controls, indicating that the patient’s NK cells were capable of killing the tumor cells. Taken together with results from in vitro experiments and the in vivo mouse xenograft model, our results indicate that mogamulizumab has potential as a therapeutic agent for EBV-associated T/NK-LPDs, at least in CCR4-positive cases.

Among the cell lines used here, SNK1, SNK6, and SNT8 originated from ENKL. All three cell lines expressed CCR4. However, Ishida et al. reported that CCR4 was expressed in only 3.7% of tissue samples from ENKL patients (18). In the present study, CCR4 was expressed in only one of six patients with NK-cell infection. Thus, there was a discrepancy between established cell lines and patient samples. A previous report showed that expression of CCR4 on naïve NK-cells was limited but that ex vivo culture, in medium supplemented with interleukin-2, enhanced CCR4 expression (30). Thus, CCR4 expression may have been induced in ENKL cells in establishing or maintaining these cell lines.

Hydroa vacciniforme is characterized by recurrent vesiculopapules, usually occurring on sun-exposed areas, and is seen in children and adolescents (45). In some of these patients, systemic symptoms, including fever, wasting, lymphadenopathy, and hepatosplenomegaly, develop and such systemic disease has been defined as “hydroa vacciniforme-like lymphoma” in the 4th WHO classification of tumours of hematopoietic and
lymphoid tissues (12). EBV-infected γδT cells infiltrate the superficial dermis and subcutaneous tissue in hydroa vacciniforme (13, 14). If CCR4 expression is seen in γδT cells, what is the role of CCR4 in the pathogenesis of hydroa vacciniforme? CCR4 plays a major role in chemotaxis, and is attracted to its ligands, CCL17 and CCL22, that are largely expressed in the epidermis (46). ATLL frequently involves the skin, and CCR4 expression in ATLL is considered to be closely associated with skin involvement (24). Like ATLL, CCR4 expression on tumor cells may be related to the clinical features of hydroa vacciniforme.

In addition to hydroa vacciniforme, CCR4 was expressed on EBV-infected T-cells in some patients with CAEBV and hemophagocytic lymphohistiocytosis, and even on B-cells in a PTLD patient. From these findings, we suggest an association between CCR4 expression and EBV infection. Nakayama et al. reported that HTLV-1 promoted CCR4 expression via activation of the AP-1 family, especially Fra-2 (47). It is also known that LMP-1, an EBV oncoprotein, activates the AP-1 pathway in nasopharyngeal carcinoma (48). These reports support our hypothesis raise the possibility that EBV infection indirectly promotes CCR4 expression. If the CCR4 expression promoted by EBV plays an important role in the pathogenesis of EBV-associated T/NK-LPDs, targeting CCR4 may be an ideal- and definitive-therapy.

In conclusion, mogamulizumab has potential as a new therapeutic against EBV-associated T/NK-LPDs, particularly against hydroa vacciniforme. Furthermore, this drug may be effective in other types of EBV-associated T/NK-LPDs, if CCR4 expression can be confirmed. In fact, the use of mogamulizumab is now expanding from ATLL to peripheral T-cell lymphoma and cutaneous T-cell lymphoma, which are positive for CCR4 (22, 49). Further studies with larger samples may expand the range of EBV-associated T/NK-LPDs suitable for mogamulizumab therapy.
DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

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REFERENCES


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LCL, lymphoblastoid cell line; ENKL, extranodal NK/T-cell lymphoma; CAEBV, chronic active EBV disease; ALL, acute lymphoblastic leukemia; ANKL, aggressive NK-cell leukemia; LGLL, large granular lymphocyte leukemia; BL, Burkitt lymphoma.
Figure legends

**Figure 1.** Expression of CC chemokine receptors (CCRs) on various cell lines. **A:** CCR4. All data are presented. **B:** CCR7 and CCR10. Representative data are shown. Black shading: anti-CCRs antibody, white: isotype-matched IgG antibody.

**Figure 2.** Mogamulizumab-induced antibody-dependent cellular cytotoxicity (ADCC). **A:** Four CCR4-positive and three CCR4-negative cell lines were examined. Cytotoxicity was measured using the lactate dehydrogenase assay in the presence of effector cells (PBMCs; peripheral blood mononuclear cells) obtained from healthy volunteers and mogamulizumab (10 µg/mL) or the same volume of solvent (control). **B:** Dose-dependent effects of mogamulizumab against SNK6. Various doses of mogamulizumab and PBMCs from three healthy individuals were prepared. The ratio of target/effector was fixed at 1:50. **C:** Identification of the fraction responsible for ADCC against SNK6. Separated CD56-, CD3-, and CD19-positive cells were used as effector cells, respectively. The ratio of target/effector was fixed at 1:10. The mogamulizumab dose was fixed at 10 µg/mL. Error bars indicate the standard error of the mean.

**Figure 3.** Mogamulizumab induced potent antitumor activity against EBV-positive NK-cell lymphoma in the murine xenograft model. **A:** NOG mice were implanted subcutaneously with SNK6 cells on day 0. From day 4, human PBMCs with mogamulizumab or saline (control) were injected intraperitoneally (6 mice/group). *P < 0.05 (Mann-Whitney U-test). Error bars indicate the standard error of the mean. **B:** Tumor-bearing mice at day 28. Tumors are outlined by the red dotted line. **C:** H&E-stained section showing tumor infiltration into the subcutaneous lesion wall. **D:** EBV-encoded small RNA (EBER) in situ hybridization for subcutaneous tumor. Scale bars; 300 µm.
**Figure 4.** CCR4 expression on EBV-infected cells in representative patients with EBV-associated T/NK lymphoproliferative diseases. **A:** Multiple surface antigens and nuclear EBER were stained simultaneously using a flow cytometric *in situ* hybridization assay and shown in two-dimensional dot plots. EBER-positive fractions were gated and their CCR4 expression is shown in the histograms. EBER-negative and CD4-negative fractions are also shown as controls. **B:** CCR4 expression by “classic” flow cytometry. Patient 1 in whom Vδ2-positive γδT cells harbored EBV was tested. Each histogram shows CCR4 fluorescence intensity of gated cells (black shading: anti-CCR4 antibody, white: isotype-matched IgG antibody).

**Figure 5.** *Ex vivo* antibody-dependent cellular cytotoxicity (ADCC) assay. **A:** γδT cells were isolated by magnetic sorting from the peripheral blood of Patient 2. Expression of surface antigens (TCRγδ, CD3, V2δ, and CCR4) was examined by flow cytometry and is shown in the two-dimensional dot plots. TCRγδ and CD3 double-positive cells were gated (indicated with a rectangle box in the left figure) and plotted on a dot-plot with V2δ and CCR4 (right). **B:** ADCC activity against sorted γδT cells. TCRγδ-positive cells isolated from the patient were used as target cells, and NK-cells from the patient or two healthy individuals were used as effector cells. The ratio of target/effector was at 1:10. Percentage of cell lysis was calculated using the lactate dehydrogenase assay. Error bars indicate the standard error of the mean.
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Fig. 1

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Fig. 2

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[**CCR4-positive cell lines**]

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B

![Graph](image)

C

![Bar chart](image)
Fig. 3

A) Tumor volume (mm$^3$) over time post cell injection for Mogamulizumab and Control groups.

B) Visual comparison of tumors in the Mogamulizumab and Control groups.

C) Histological images at low-power (top) and high-power (bottom) magnification for Mogamulizumab and Control groups.

D) Detailed histological images at high-power magnification for Mogamulizumab and Control groups.
Fig. 5

A

B

% cell lysis

Patient
Anti-CCR4 Monoclonal Antibody Mogamulizumab for the Treatment of EBV-associated T- and NK-Cell Lymphoproliferative Diseases

Tetsuhiro Kanazawa, Yutaka Hiramatsu, Seiko Iwata, et al.

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