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Original Articles

Epstein–Barr virus-positive pyothorax-associated lymphoma expresses CCL17 and CCL22 chemokines that attract CCR4-expressing regulatory T cells

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ABSTRACT

Epstein–Barr virus (EBV)-positive diffuse large B-cell lymphomas associated with chronic inflammation (DLBCL-CI) develop in patients with chronic inflammation but without any predisposing immunodeficiency. Given the expression of the EBV latent genes, DLBCL-CI should have mechanisms for evasion of host antitumor immunity. EBV-positive pyothorax-associated lymphoma (PAL) is a prototype of DLBCL-CI and may provide a valuable model for the study of immune evasion by DLBCL-CI. This study demonstrates that PAL cell lines express and secrete CCL17 and/or CCL22 chemokines, the ligands of C–C motif chemokine receptor 4 (CCR4), in contrast to EBV-negative DLBCL cell lines. Accordingly, culture supernatants of PAL cell lines efficiently attracted CCR4-positive regulatory T (Treg) cells in human peripheral blood mononuclear cells. PAL cells injected into mice also attracted CCR4-expressing Treg cells. Furthermore, this study confirmed that CCR4-expressing Treg cells were abundantly present in primary PAL tissues. Collectively, these findings provide new insight into the mechanisms of immune evasion by PAL, and further studies are warranted on whether such mechanisms eventually lead to the development of DLBCL-CI.

1. Introduction

Pyothorax-associated lymphoma (PAL) is an Epstein–Barr virus (EBV)-positive type of extranodal diffuse large B-cell lymphoma (DLBCL) that develops in the context of long-standing chronic inflammation usually resulting from a therapeutic artificial pneumothorax for pulmonary tuberculosis or tuberculous pleuritis [1,2]. EBV-positive DLBCLs with features similar to PAL also arise in other settings of chronic inflammation, such as chronic osteomyelitis, metallic implant insertions, surgical mesh implantations, and chronic venous skin ulcers [1]. PAL was included in the 2016 revision of the World Health Organization classification of lymphoid neoplasms as a category of DLBCL associated with chronic inflammation (DLBCL-CI) and is recognized as a prototype form of this new category [1,3]. DLBCL-CI harbors the EBV genome and usually exhibits type III EBV latency with the expression of EBV latent genes such as latent membrane protein 1 (LMP1) and EBV nuclear antigen (EBNA) 2 along with EBNA1 [4–8]. Although this type of EBV latency is seen in immunocompromised hosts such as patients with post-transplant lymphoproliferative disorders and those with AIDS-related lymphomas [9,10], DLBCL-CI usually develops in patients with no predisposing immunodeficiency [5,11]. Previous studies demonstrated that a subset of PALs produces immunosuppressive interleukin (IL)-10 and has reduced levels of MHC class I molecules that are involved in antitumor immune responses by cytotoxic lymphocytes [12–15]. Although these findings may partly explain how PAL cells developing in a confined space with chronic inflammation can escape from the host's immune surveillance, other mechanisms may also be operative in the immune evasion by PAL.

Regulatory T (Treg) cells suppress diverse immune responses and play a key role in maintaining immunological self-tolerance [16,17]. Treg cells may also hinder immune surveillance against tumors in

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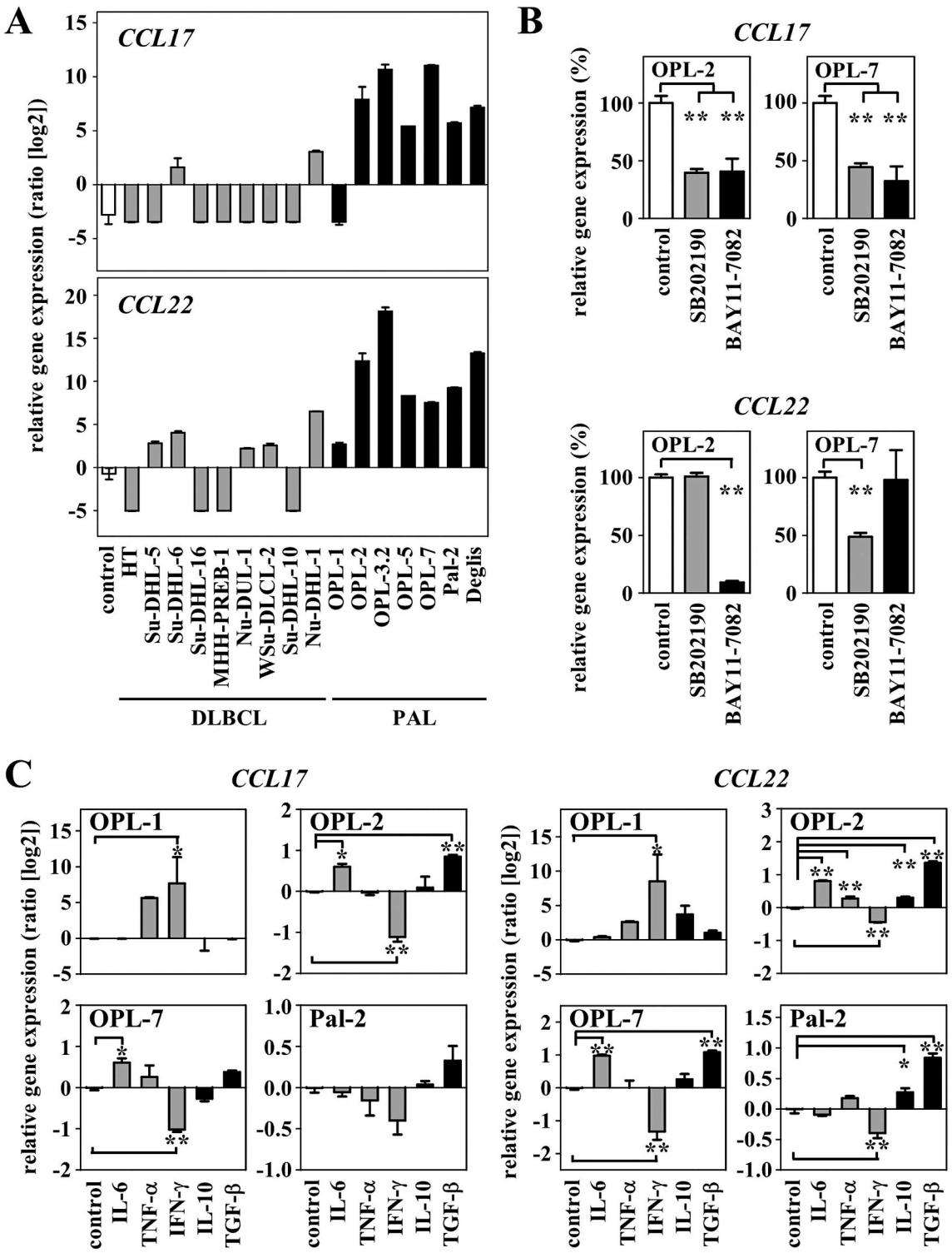

Fig. 1. Gene expression analysis of *CCL17* and *CCL22* in PAL cell lines. Relative mRNA expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method, and the ΔCt value normalized against the *B2M* gene used as a housekeeping control was expressed as an *n*-fold change relative to that in the control samples. (A) Relative mRNA expression levels in seven PAL cell lines and nine EBV-negative DLBCL cell lines were calculated with the average value in peripheral blood mononuclear cells obtained from 12 healthy donors as a control. (B) Effect of the blockage of the signaling pathways on the expression of CCR4 ligand genes. The cells were treated for 12 h with the p38/ATF2 pathway inhibitor SB202190 at a concentration of 10 μM, the TRAF/NF-κB pathway inhibitor BAY 11-7082 at 2.5 μM, or dimethyl sulfoxide as a control. (C) Effect of cytokines on the expression of CCR4 ligand genes. The cells were treated for 12 h with IL-6 at 10 ng/ml, IL-10 at 10 ng/ml, TNF-α at 50 ng/ml, INF-γ at 100 ng/ml, TGF-β at 10 ng/ml, or phosphate buffered saline as a control. Data are shown as the mean ± standard error of the mean (SEM) of three independent experiments. Statistically significant differences are shown as $^{*}p < 0.05$ and $^{**}p < 0.01$.

tumor-bearing patients. Accordingly, infiltration of Treg cells in tumor tissues is considered to be a predictive factor for poor prognosis of affected patients [18–20]. Therefore, prevention of migration of Treg cells to tumor microenvironments could be a strategy to enhance host antitumor immunity [20,21]. Indeed, the selective depletion of Treg cells evokes strong cytotoxic immune responses against tumors [22]

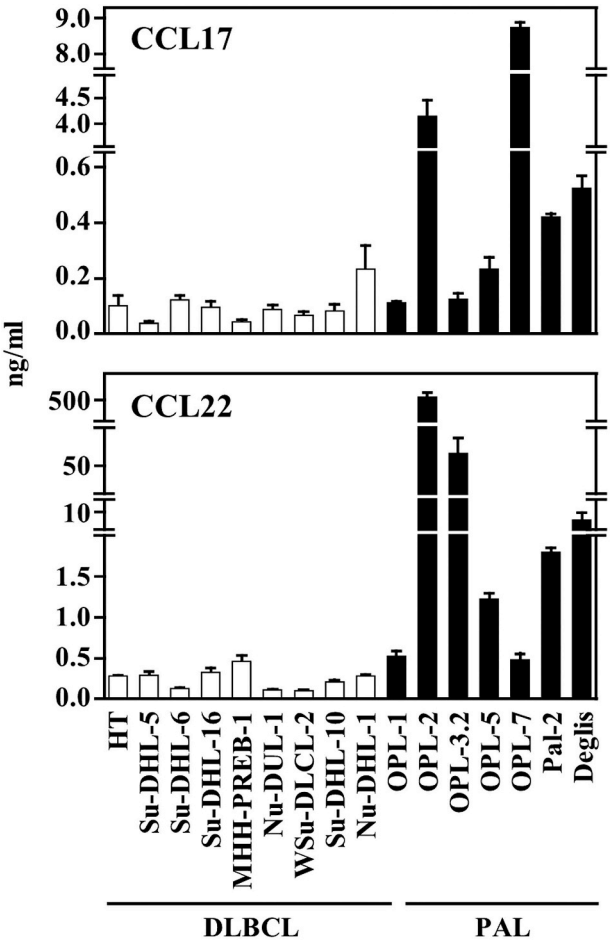

Fig. 2. Secretion of CCL17 and CCL22 from PAL and EBV-negative DLBCL cell lines. The cells were seeded in a 24-well plate at 5×10^5/ml and cultured for 3 days. Concentrations of CCL17 and CCL22 in the culture supernatants were measured by ELISA. Data are shown as the mean ± SEM of three independent experiments.

and augments vaccine-induced antitumor immune responses in animal models [23,24]. Treg cells are known to express C–C motif chemokine receptor 4 (CCR4) and to migrate toward its ligands TARC/CCL17 and MDC/CCL22, which are often produced in tumor microenvironments [18,19]. Most CCR4-expressing Treg cells are effector-type cells with a strong immunosuppressive function [22]. Thus, Treg cells may also be involved in immune evasion by PAL.

Previous studies demonstrated a potent induction of *CCL17* and *CCL22* by one of the EBV latent gene products LMP1 [25]. Given this background, this study investigated whether PAL cells produce CCL17 and CCL22 and whether this leads to an efficient mobilization of CCR4-expressing Treg cells to PAL cells. The findings indeed demonstrate that EBV-positive PAL cells abundantly produce CCL17 and/or CCL22 and thereby efficiently attract Treg cells via CCR4.

2. Materials and methods

2.1. Cells and tissues

The seven PAL cell lines used in this study, OPL-1, OPL-2, OPL-3.2, OPL-5, OPL-7, Pal-2, and Deglis, were described previously [26–29]. Nine EBV-negative DLBCL cell lines, HT, MHH-PREB-1, Nu-DHL-1, Nu-DUL-1, Su-DHL-5, Su-DHL-6, Su-DHL-10, Su-DHL-16, and WSu-DLCL-2, were purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany). A mouse pre-B cell line L1.2 was a gift from E. Butcher (Stanford University School of Medicine, Stanford, CA, USA). The L1.2 clone stably expressing human CCR4 (L1.2-CCR4) was described previously [25,30]. Human peripheral blood mononuclear cells (PBMCs) were isolated from heparinized blood samples obtained from healthy adult donors using Ficoll–Paque (GE Healthcare, Tokyo, Japan). Formalin-fixed paraffin wax-embedded (FFPE) biopsied tissues of patients with PAL (n = 4) and EBV-negative DLBCL, not otherwise specified (DLBCL-NOS, n = 6) were obtained from Kochi University Hospital. This study was approved by the Ethics Committee of Kochi Medical School, Kochi University. The human studies were performed in accordance with the relevant guidelines and regulations.

2.2. Inhibition of cell signaling pathways and cytokine stimulation

The cells were seeded in a 24-well plate at a density of 0.5×10^6/ml. For inhibition of the cell signaling pathways, the cells were treated for 12 h with a p38 mitogen-activated protein kinase inhibitor SB202190 (Wako, Osaka, Japan) at 10 μM or a nuclear factor-kappa B (NF-κB) inhibitor BAY11-7082 (Wako) at 2.5 μM. The doses of the inhibitors were nontoxic to the PAL cell lines studied in our preliminary experiments (see Supplementary Fig. S1). For cytokine stimulation, the cells were treated with a recombinant human cytokine: IL-6 (R&D Systems, Minneapolis, MN, USA) at 10 ng/ml; IL-10 (PeproTech, Rocky Hill, NJ, USA) at 10 ng/ml; tumor necrosis factor (TNF)-α (R&D Systems) at 50 ng/ml; interferon (IFN)-γ (R&D Systems) at 100 ng/ml; and transforming growth factor (TGF)-β (PeproTech) at 10 ng/ml. The concentrations of the cytokines were chosen as optimal based on our preliminary experiments (data not shown).

2.3. Real-time quantitative reverse-transcription polymerase chain reaction (RT–qPCR)

The extraction of total RNA and real time RT–qPCR analysis were carried out as described previously [31,32]. The primers and fluorogenic probes for *TARC/CCL17* (Hs00171074_m1), *MDC/CCL22* (Hs01574247_m1), and *β2-microglobulin* (*B2M*; Hs00187842_m1) were based on the information provided for TaqMan Gene Expression Assays (Thermo Fisher Scientific, Tokyo, Japan). All of the experiments were performed in triplicate.

2.4. Enzyme-linked immunosorbent assay (ELISA)

Cells were seeded in a 24-well plate at a density of 0.5×10^6/ml and cultured for 3 days. Concentrations of CCL17 and CCL22 in the culture supernatants were measured using Quantikine ELISA kits (R&D Systems). All of the experiments were performed in triplicate.

2.5. Immunohistochemistry

Immunohistochemistry was performed on FFPE tissue sections as described previously [33]. The sections were treated with goat polyclonal anti-human TARC/CCL17 (R&D Systems), rabbit polyclonal anti-human MDC/CCL22 (Abcam, Cambridge, UK), mouse anti-human CD20 (clone L26, Nichirei Biosciences, Tokyo, Japan), rabbit anti-human CD4 (EPR6855, Abcam), mouse anti-human FOXP3 (236A/E7, Abcam), or anti-human CCR4 in the Poteligeo Test IHC kit (Kyowa Medex, Tokyo, Japan). Isotype-matched goat polyclonal IgG, rabbit polyclonal IgG (Vector Laboratories, Burlingame, CA, USA), mouse IgG_1 (Agilent Technologies, Santa Clara, CA, USA), or rabbit IgG (Cell Signaling Technology, Tokyo, Japan) were used as negative controls. The sections were incubated with Histofine simple stain MAX PO (Nichirei Biosciences), and signals were detected using the Liquid DAB + Substrate Chromogen System (Agilent Technologies). For

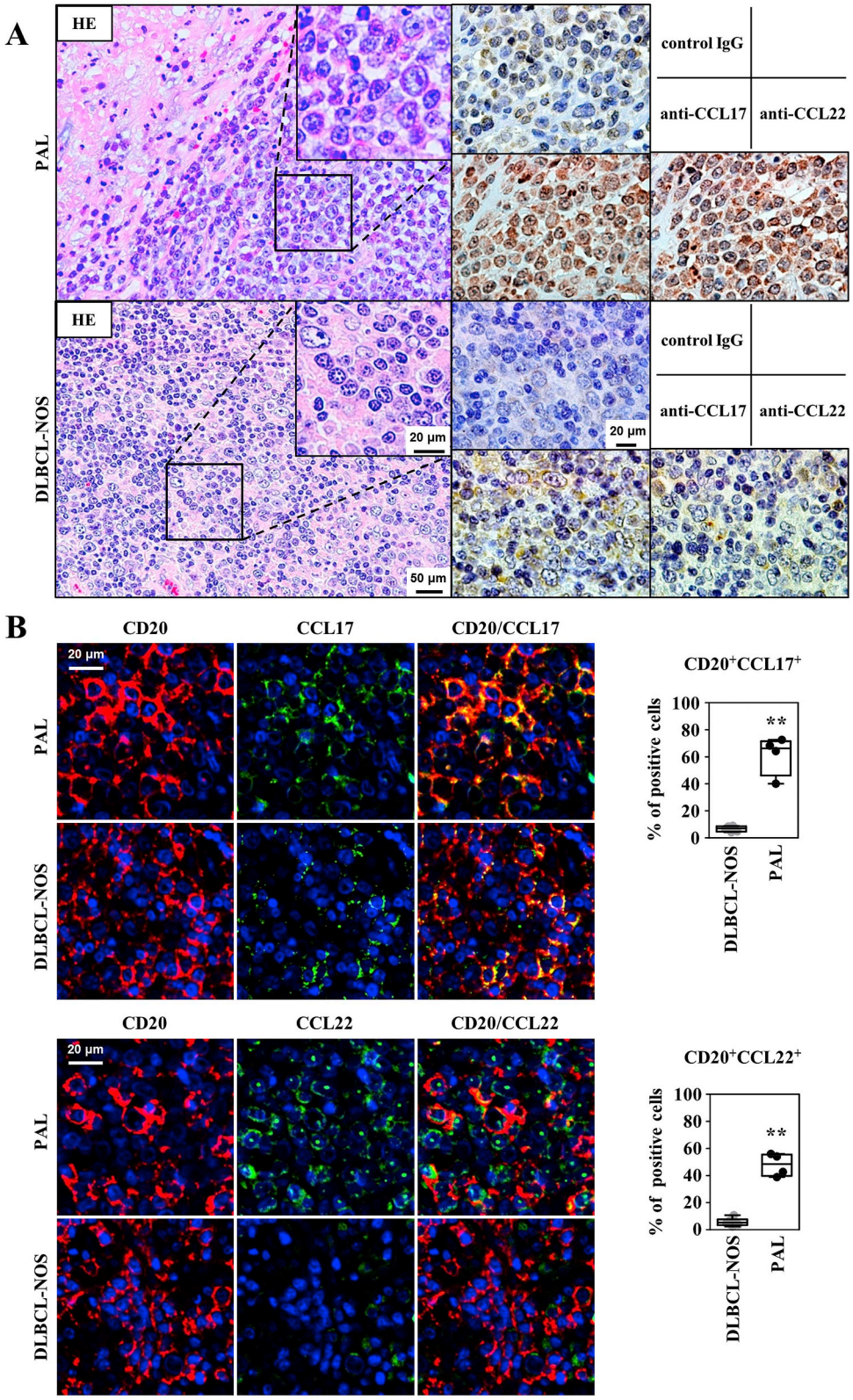

Fig. 3. Expression of CCL17 and CCL22 in primary PAL tissues. (A) Specimens of PAL and DLBCL-NOS were stained with hematoxylin and eosin (HE). Immunohistochemistry was performed on the sections using isotype control IgG antibody, anti-CCL17 antibody, and anti-CCL22 antibody. (B) Immunofluorescent double staining for CD20 (red) and CCL17/CCL22 (green). The nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI, blue). Box plots depicting the percentages of $CD20^+CCL17^+$ cells and $CD20^+CCL22^+$ cells in PAL tissues (n = 4) and DLBCL-NOS tissues (n = 6). For the semi-quantitative assessment of protein expression, the percentage of positive cells was calculated in randomly selected, more than five higher-power fields, including over 100 cells, and the mean was regarded as the labelling index of each lesion. Data are shown as the mean ± standard deviation (SD). Statistically significant differences are shown as $^{**}p < 0.01$. . (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

immunofluorescent double staining for CD20 and CCL17/CCL22, Alexa Fluor 594-labeled goat anti-mouse IgG, Alexa Fluor 488-labeled donkey anti-goat IgG, or Alexa Fluor 488-labeled goat anti-rabbit IgG (Thermo Fisher Scientific) was used as the secondary antibodies. For immunofluorescent double staining for CD4 and FOXP3, Alexa Fluor 488-labeled goat anti-rabbit IgG or Alexa Fluor 594-labeled goat anti-mouse IgG (Thermo Fisher Scientific) was used as the secondary antibodies.

2.6. Chemotaxis assay

Chemotaxis assays were performed using a ChemoTx chemotaxis chamber with a 5-μm pore size (Funakoshi, Tokyo, Japan) [25]. Recombinant MDC/CCL22 was purchased from R&D Systems. A CCR4 antagonist Compound 22 ((R)-{4-{4-[(2,4-dichlorobenzyl)amino] pyrido [2,3-d]pyrimidin-2-yl}piperazin-1-yl}-piperidin-2-yl-

methanone) (99.5% purity) was synthesized based on the published information [34] (see Supplementary Fig. S2 for the structure of Compound 22). L1.2-CCR4 cells were suspended at 8.0×10^6/ml in phenol red-free RPMI 1640 medium and applied to the upper wells of the ChemoTx chemotaxis chambers (25 μl/well). Aliquots of medium containing recombinant MDC/CCL22 or culture supernatants with or without Compound 22 (1 μM) were applied to the lower wells (30 μl/well). After 1 h at 37 °C, the cells that migrated into the lower wells were lysed and quantified using PicoGreen dsDNA reagent (Thermo Fisher Scientific). All of the experiments were performed in triplicate.

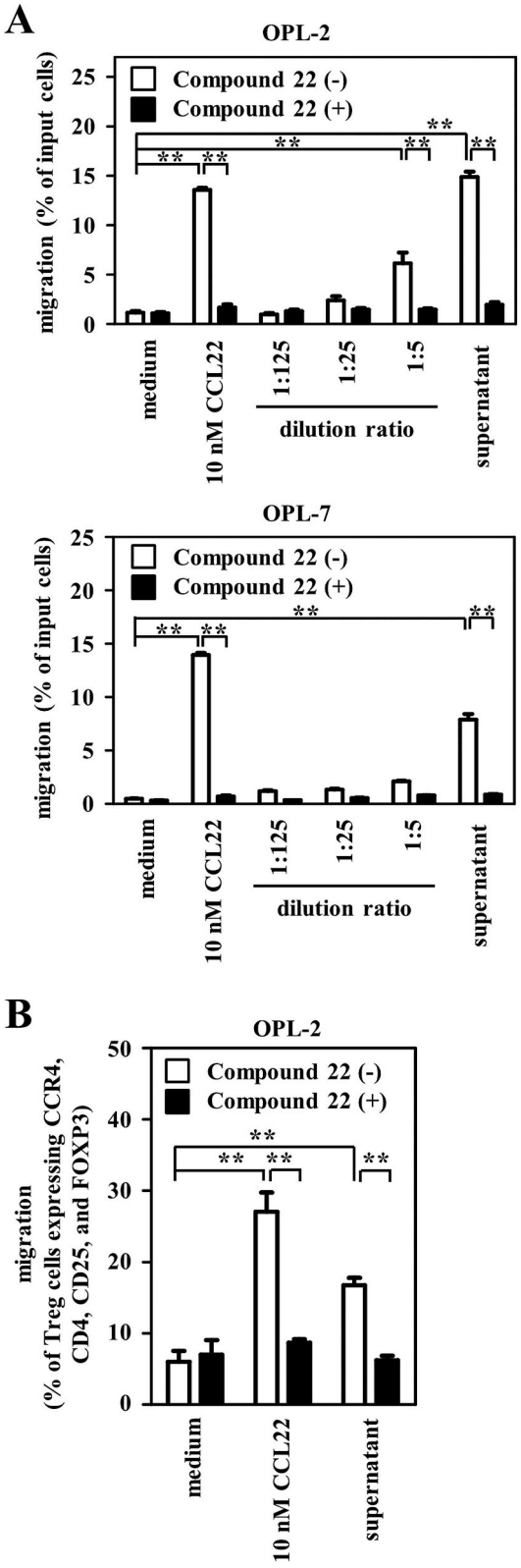

Fig. 4. Induction of CCR4-mediated cell migration by supernatants of PAL cell lines. (A) Culture supernatants of OPL-2 and OPL-7 cells were tested for chemotactic activity using CCR4-expressing L1.2 cells in the absence or presence of the CCR4 antagonist Compound 22 (1 μM). Serially diluted culture supernatants were also tested for chemotactic activity. Cell migration was expressed as a percentage of the input cells. (B) Human peripheral blood mononuclear cells were examined for cell migration induced by the culture supernatant of OPL-2 cells. The cells that migrated into lower wells were stained for CD4, CD25, FOXP3, and CCR4 and quantified by flow cytometry. Recombinant CCL22 (10 nM) and medium alone were used as positive and negative controls, respectively. Results from three separate experiments are shown as the mean ± SEM. Statistically significant differences are shown as $^{**}p < 0.01$.

2.7. Flow cytometry

PBMCs from healthy donors were stained using a Zombie Aqua Fixable Viability kit (Biolegend, San Diego, CA, USA) to distinguish between the live and dead cell populations. The cells were first treated with TruStain FcX Fc Receptor Blocking Solution (BioLegend). Staining of CD4, CD25, and FOXP3 was performed using eBioscience Human Regulatory T Cell Staining kit (Thermo Fisher Scientific). The expression of CCR4 was analyzed with Brilliant Violet 510-labeled anti-human CCR4 antibody (clone L291H4, BioLegend). In the mouse experiments, the cells were treated with anti-mouse CD16/32 (2.4G2, BioLegend) to block the Fc receptors and incubated with a mixture of antibodies consisting of PerCP/Cy5.5-labeled anti-mouse CD45 (30-F11, BioLegend), fluorescein isothiocyanate (FITC)-labeled anti-mouse CD4 (RM4-5, BioLegend), and phycoerythrin (PE)-labeled anti-mouse CCR4 (2G12, BioLegend) or PE-labeled isotype control (HTK888, BioLegend). For intracellular staining, the cells were fixed, and permeabilized using Cytofix/Cytoperm kits (BD Biosciences, San Jose, CA, USA) and stained with Alexa Fluor 647-labeled anti-mouse FOXP3 (150D, BioLegend). The cells were analyzed using a BD LSRFortessa flow cytometer (BD Biosciences) and FlowJo software (Tree Star Inc., Ashland, OR, USA).

2.8. Cell mobilization in mice

Nine-week-old male BALB/c mice were purchased from Japan SLC (Shizuoka, Japan). The mice were injected intraperitoneally with 5×10^5 cells suspended in 500 μl serum-free RPMI1640 with or without Compound 22 at 0.5 or 5.0 mg/kg. At 24 h, the mice were euthanized and their peritoneal exudate cells were isolated. The cell suspensions were filtered through a 70-μm cell strainer and the cell numbers were counted. The cells were subjected to the flow cytometry analysis as previously described. All of the animal experiments were performed in accordance with the guidelines of the Center for Animal Experiments, Kindai University Faculty of Pharmacy.

2.9. Statistical analysis

Mann–Whitney nonparametric *U* tests were used to analyze differences between the pairs of groups. One-way analysis of variance with the Tukey post hoc test was used for multiple groups. We considered $p < 0.05$ statistically significant.

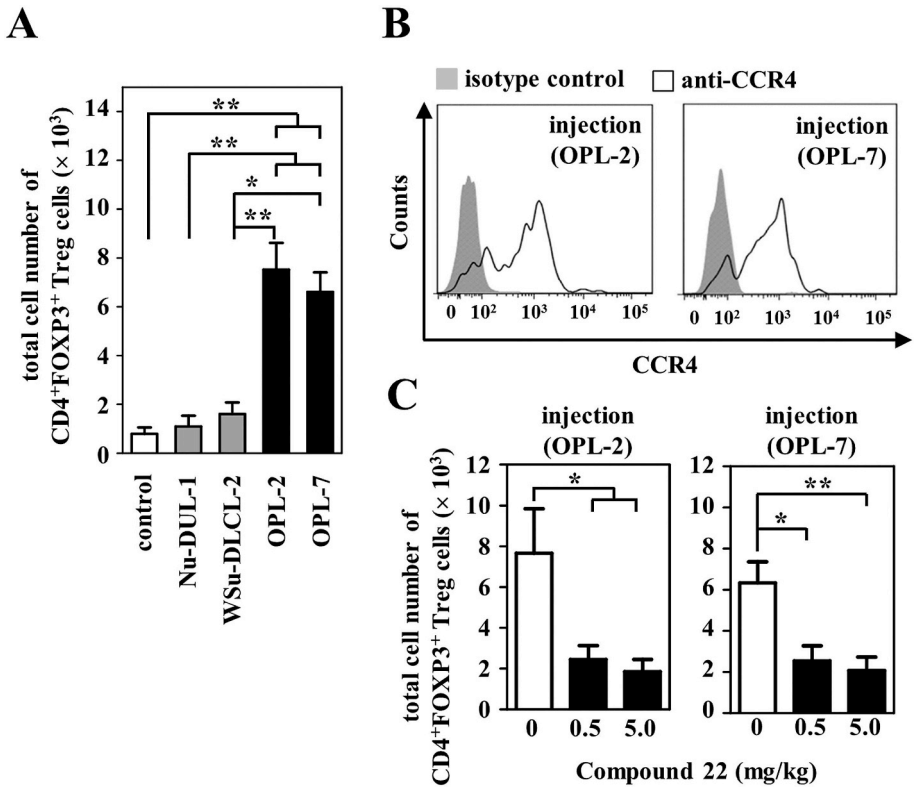

Fig. 5. *In vivo* recruitment of Treg cells via CCR4 by PAL cells. (A) OPL-2, OPL-7, Nu-DUL-1, or WSu-DLCL-2 cells (5×10^5 cells) were injected intraperitoneally into BALB/c mice. After 24 h, cells were isolated from the mice peritoneal cavity and the cell numbers were counted. The cells were stained for CD4, FOXP3, CD45, and CCR4. The $CD4^+FOXP3^+$ cells were counted by flow cytometry using the CD45 gate. Total $CD4^+FOXP3^+$ Treg cell numbers were determined using the following formula: total Treg cell numbers = numbers of total peritoneal exudate cells enumerated by cell counting × the ratio of $CD4^+$ $FOXP3^+$ cells analyzed by flow cytometry. The data are expressed as the SEM of the results obtained from seven to 10 mice. (B) The $CD4^+FOXP3^+$ Treg cells were further analyzed for CCR4 expression by flow cytometry. Representative data are shown from at least three independent experiments. (C) OPL-2 or OPL-7 cells (5×10^5) were injected intraperitoneally into BALB/c mice with or without Compound 22 at concentrations of 0.5 or 5.0 mg/kg. Total $CD4^+FOXP3^+$ Treg cell numbers were determined as previously described. The data are expressed as the SEM of the results obtained from five to 10 mice. Statistically significant differences are shown as $^*p < 0.05$ and $^{**}p < 0.01$.

3. Results

3.1. Expression of CCL17 and CCL22 by PAL cell lines

We first examined the expression of *CCL17* and *CCL22* in a panel of seven PAL cell lines and nine EBV-negative DLBCL cell lines. PBMCs obtained from 12 healthy donors were used as the baseline controls. Compared with the DLBCL-NOS cell lines, the PAL cell line except for OPL-1 were found to express *CCL17* and *CCL22* at relatively high levels (Fig. 1A). In the case of EBV-immortalized B cell lines, *CCL17* and *CCL22* have been shown to be induced by LMP-1 via p38/ATF2 and TRAF/NF-κB pathways [25]. We therefore addressed whether these signaling pathways are also involved in the expression of *CCL17* and *CCL22* in PAL cell lines. For this experiment, we used OPL-2 and OPL-7 that expressed *CCL17* and *CCL22* at high levels. The *CCL17* expression in both cell lines was significantly suppressed by SB202190, an inhibitor of the p38/ATF2 pathway, and by BAY11-7082, an inhibitor of the TRAF/NF-κB pathway (Fig. 1B). However, the *CCL22* expression in the OPL-2 and OPL-7 cells was significantly suppressed by BAY11-7082 and SB202190, respectively (Fig. 1B). These results support that both the p38/ATF2 and TRAF/NF-κB pathways are indeed involved in the expression of *CCL17* and *CCL22* in PAL cell lines, although in the case of *CCL22* expression, the dominant signaling pathway may be different according to individual PAL cell lines.

We next examined the effects of various cytokines on the expression of *CCL17* and *CCL22* in PAL cell lines, taking into account the close association of PAL pathogenesis with chronic inflammation. Although the effects of cytokines were quite variable according to the individual PAL cell lines, the expression of *CCL17* and *CCL22* was strongly upregulated by some cytokines (Fig. 1C). For example, IFN-γ upregulated the expression of *CCL17* and *CCL22* in OPL-1 cells, but downregulated their expression in OPL-2, OPL-7, and Pal-2 cells. Conversely, TGF-β upregulated the expression of *CCL17* and *CCL22* in OPL-2, OPL-7, and Pal-2 cells, but had little effect on their expression in OPL-1 cells. Similarly, IL-6 upregulated the expression of *CCL17* and *CCL22* in OPL-2 and OPL-7, but did not affect their expression in OPL-1 and PAL-2. Collectively, these results suggest that the expression of *CCL17* and *CCL22* in PAL cells could be highly inducible in chronic inflammatory settings.

3.2. Secretion of CCL17 and CCL22 by PAL cell lines

We next examined the secretion of CCL17 and CCL22 from PAL and EBV-negative DLBCL cell lines. As shown in Fig. 2, CCL22 was secreted by the PAL cell lines mostly at higher levels than by the DLBCL cell lines. CCL17 was also secreted by some PAL cell lines. These results were mostly consistent with those of the RT–qPCR analysis for *CCL22* expression (Fig. 1A). However, there was a discrepancy between the levels of mRNA expression and protein secretion for *CCL17* in OPL-3.2 and OPL-5. Such discrepancies might be due to posttranscriptional regulations [35].

3.3. Expression of CCL17 and CCL22 in tumor cells of in primary PAL tissues

We next performed immunohistochemistry of tumor biopsies from PAL and DLBCL-NOS patients using antibodies against CCL17 and CCL22 and isotype-matched IgGs as controls (Fig. 3A). Both CCL17 and CCL22 were strongly stained in the PAL tissues, whereas they were negative or faintly positive in the DLBCL-NOS tissues. Double staining for CD20 and CCL17/CCL22 demonstrated that the majority of $CD20^+$ PAL cells expressed CCL17 and CCL22 (Fig. 3B). Fig. 3B also shows the semi-quantitative assessment of $CD20^+CCL17^+$ and $CD20^+CCL22^+$ tumor cells in four PAL samples (CCL17, 40–73%; CCL22, 36–56%) and six DLBCL-NOS samples (CCL17, 4–9%; CCL22, 2–11%). These results support the strong expression of *CCL17* and *CCL22* in primary PAL cells.

3.4. Induction of cell migration via CCR4 by the supernatants of PAL cell lines

We next examined whether the culture supernatants of OPL-2 and OPL-7 were chemotactic for CCR4-expressing L1.2 (L1.2-CCR4). As

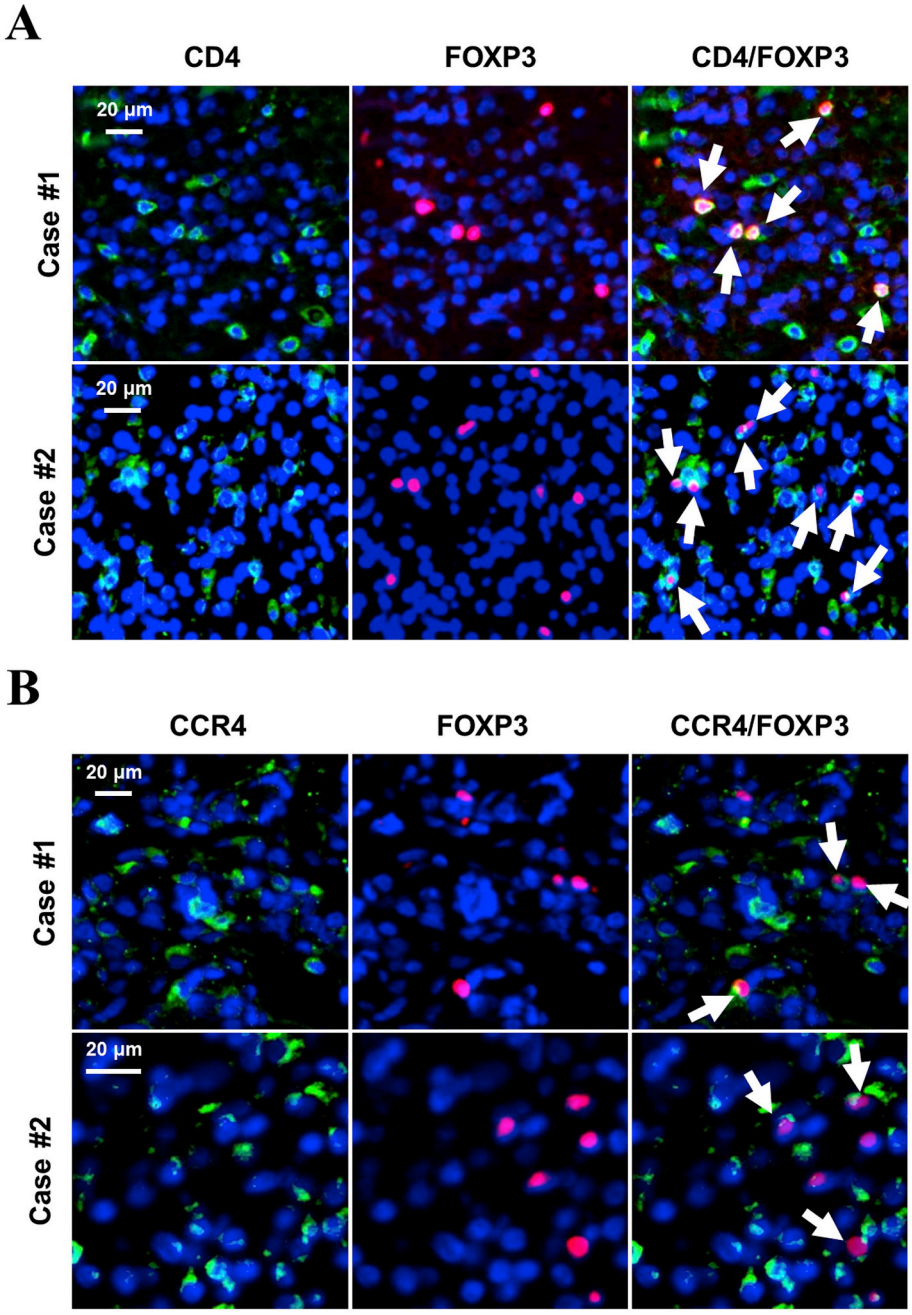

Fig. 6. Infiltration of FOXP3$^+$ cells and CCR4$^+$ cells in tumor tissues of patients with PAL. (A) Immunofluorescent double staining for CD4 and FOXP3 on PAL tissue sections. (B) Immunofluorescent double staining for CCR4 and FOXP3 on PAL tissue sections. The expressions of CD4 (green) and CCR4 (green) were observed on the cell surfaces, while the expression of FOXP3 (red) was in the nuclei. The nuclei were counterstained with DAPI (blue). The arrows indicate cells with colocalization of the two indicated proteins. The results of two representative PAL cases are shown. . (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

shown in Fig. 4A, the supernatants of both OPL-2 and OPL-7 efficiently induced the migration of L1.2-CCR4 cells in a dose-dependent manner. To evaluate the effect of Compound 22, a CCR4 antagonist, on the chemotaxis, we used this compound at a 1 μM dose because previous studies demonstrated that it highly inhibited the migration of L1.2 cells expressing CCR4, but not other chemokine receptors including CCR1 to 10, CXCR1 to 4, CX3CR1, and XCR1 [30,36]. We confirmed that Compound 22 suppressed the migration of L1.2-CCR4 induced by the culture supernatants (Fig. 4A). These findings showed that the CCR4-ligands secreted in the culture supernatants of PAL cell lines were biologically active.

CD4$^+$CD25$^+$FOXP3$^+$ Treg cells including CCR4$^+$ Treg cells, which account for 5–10% of the peripheral CD4$^+$ T cells of normal humans and mice, have an immunosuppressive function [37]. We examined whether the culture supernatant of OPL-2 was chemotactic for primary CCR4$^+$ Treg cells derived from human peripheral blood. As shown in Fig. 4B, the culture supernatant significantly induced the migration of CCR4$^+$ Treg cells, while Compound 22 suppressed the migration. These findings support that the CCR4-ligands secreted by PAL cells can recruit CCR4$^+$ Treg cells.

3.5. *In vivo recruitment of Treg cells via CCR4 by PAL cells*

We next evaluated the *in vivo* recruitment of CCR4-expressing Treg cells by PAL and EBV-negative DLBCL cells. The cells were inoculated into the peritoneal cavity of mice, and the numbers of CD4$^+$FOXP3$^+$ Treg cells that migrated to the peritoneal cavity were determined. The control mice were injected with medium alone. OPL-2 and OPL-7 cells significantly recruited CD4$^+$FOXP3$^+$ Treg cells in contract to Nu-DUL-1 and WSu-DLCL2 cells (Fig. 5A). Most of the CD4$^+$FOXP3$^+$ Treg cells were confirmed to be CCR4-positive (Fig. 5B). Previous studies showed that intraperitoneal injection of Compound 22 at doses 0.5–5 mg/kg in mice inhibited the migration of CCR4-expressing Th2 cells and Th17 cells [30,38]. In our mouse model, Compound 22 also

significantly suppressed the recruitment of Treg cells by OPL-2 and OPL-7 (Fig. 5C). Thus, PAL cells efficiently recruited Treg cells via CCR4 *in vivo*.

3.6. Infiltration of FOXP3$^+$ cells and CCR4$^+$ cells in PAL tissues

Finally, we evaluated the accumulation of Treg cells in primary PAL tissues. Double staining with anti-CD4 and anti-FOXP3 antibodies revealed that a large number of CD4$^+$FOXP3$^+$ Treg cells were present in PAL tissues (Fig. 6A). By double staining with CCR4, we further confirmed the presence of CCR4$^+$FOXP3$^+$ Treg cells in the primary PAL tissues (Fig. 6B).

4. Discussion

This study investigated the expression of CCR4 ligands in PAL, a prototype lymphoma of DLBCL-CI with the type III EBV latency. Despite this, patients with DLBCL-CI have no apparent predisposing immunodeficiency conditions [4–8], suggesting that DLBCL-CI developing under an environment of chronic inflammation might have mechanisms to escape the host's immune surveillance. However, the immune evasion of DLBCL-CI has not been well elucidated, possibly because of the insufficiency of suitable *in vitro* and *in vivo* models. To address this issue, we used a panel of PAL cell lines as a model and have revealed for the first time that PAL cells express CCL17 and/or CCL22, the CCR4-ligand chemokines that are known to attract regulatory T cells [39].

The *CCL17* expression in PAL cell lines is mediated by both p38/ATF2 and TRAF/NF-κB signaling pathways, whereas the *CCL22* expression is dominantly mediated by either of the pathways according to individual PAL cell lines. Previous studies have shown that the EBV LMP1 protein induces the expression of its target genes, including *CCL17* and *CCL22*, via the activation of the p38/ATF2 and TRAF/NF-κB signaling pathways [25,40]. Because all of the PAL cell lines used in this study express the *LMP1* gene (see Supplementary Fig. S3), other factors may affect the signaling pathways involved in the *CCL22* expression. In fact, the expression of either or both *CCL17* and *CCL22* is observed in cells persistently infected with tumor viruses, such as human T-cell leukemia virus type 1, hepatitis C virus, human papillomavirus, and Merkel cell polyomavirus [41–44]. Thus, the signaling mechanisms involved in their expression could be divergent. Moreover, we have demonstrated that the expression of *CCL17* and *CCL22* in PAL cells can be upregulated by stimulations with inflammatory and/or anti-inflammatory cytokines. Previous studies have shown that cytokines such as IFN-γ, IL-4, and IL-13 are capable of inducing the expression of *CCL17* or *CCL22* in cell-type dependent manners [45–47]. It is therefore conceivable that PAL cells express these chemokines in response to exogenous cytokine stimulations while adapting to a chronic inflammatory microenvironment. Collectively, our findings demonstrate the constitutive and inducible expression of *CCL17* and *CCL22* by PAL cells. Importantly, we also confirmed a strong expression of these chemokines by primary tumor cells in PAL tissues.

CCL17 and CCL22 expressed by PAL cells were functional because the culture supernatants of PAL cell lines were able to attract immunosuppressive FOXP3$^+$ Treg cells via CCR4, as shown by both *in vitro* transwell migration assays and an *in vivo* mouse model (Figs. 4B and 5). We also confirmed the abundant infiltration of CCR4$^+$ Treg cells in primary PAL tissues. Previous studies have shown the strong induction of *CCL17* and *CCL22* by EBV LMP1 in EBV-immortalized human B cell lines [25] and the infiltration of CCR4$^+$ cells in a subset of EBV-positive B-cell lymphoproliferative disorders of elderly individuals [48]. This study presents the first data on the attraction of CCR4-expressing Treg cells to the site of PAL tumor cells that express CCL17 and CCL22. A suppressive effect of Treg cells on antitumor immune responses has been well reported in various types of tumors, such as ovarian cancers and oral squamous cell carcinomas [18,19]. Thus, the production of CCL17 and CCL22 by PAL cells may also contribute to the immune evasion of tumor cells by attracting Treg cells via CCR4 despite the expression of the EBV latency III genes.

One advantage of this study was the use of all the PAL cell lines available to date. Most cases of PAL have been reported in Asia, with a few reports from Western countries [4,49]. Moreover, these tumors are often confined to the thoracic cavity, and it can be difficult to obtain sufficient materials for cell culture. Thus, the insufficiency of PAL cell lines might have hampered the detailed study of pathophysiology of this rare disease. Although PAL develops in the setting of chronic inflammation associated with pulmonary tuberculosis, it is not restricted to this disease and can occur in any types of empyema [50]; thus, PAL represents a model of DLBCL-CI [1]. Although our clinical studies on patients with PAL were limited by a small sample size, our data obtained from the PAL cell lines are highly reproduced by the biopsied PAL tissues.

PAL is often resistant to chemotherapy with poor overall prognosis [2,51]. Although the combination therapy with anti-CD20 antibody rituximab plus cyclophosphamide, doxorubicin, vincristine, and prednisone remains the widely used treatment regimen for patients with DLBCL, the optimal management of PAL remains unresolved. Conversely, therapy with an anti-CCR4 antibody has been used for adult T-cell leukemia/lymphoma and cutaneous T-cell lymphoma [52–54]. The findings presented here suggest that the strategy of targeting the CCL17/CCL22–CCR4 axis using either small-molecule antagonists or monoclonal antibodies such as anti-CCR4 could be beneficial for patients with PAL in order to enhance their antitumor immune responses, although the efficacy needs to be evaluated in a preclinical model.

In summary, this report demonstrated a novel feature of PAL: the constitutive and inducible production of CCL17 and CCL22 and the attraction of Treg cells via CCR4. This study provides insights into the mechanism of tumor immune evasion by PAL. The findings are expected to stimulate studies on the mechanism of immune evasion by other forms of DLBCL-CI.

Conflicts of interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.canlet.2019.03.053.

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