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ORIGINAL ARTICLE

EBV-positive pyothorax-associated lymphoma expresses CXCL9 and CXCL10 chemokines that attract cytotoxic lymphocytes via CXCR3

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Abstract

Epstein–Barr virus (EBV)-positive diffuse large B-cell lymphoma associated with chronic inflammation (DLBCL-CI) develops in the setting of long-standing inflammation. This type of lymphoma may have specific expression profiles of chemokines involved in the pathogenesis of DLBCL-CI. EBV-positive pyothorax-associated lymphoma (PAL) is a prototype of DLBCL-CI and represents a valuable model for the study of this disease category. Using a panel of PAL cell lines, we found that PAL cells expressed and secreted C–X–C motif chemokine ligands 9 and 10 (CXCL9 and CXCL10), the ligands of CXCR3, in contrast to EBV-negative DLBCL cell lines, which did not. Culture supernatants from PAL cell lines attracted CXCR3-expressing $CD4^+$ T cells, $CD8^+$ T cells, and $CD56^+$ natural killer cells from human peripheral blood mononuclear cells. PAL cells injected into mice also attracted CXCR3-positive cytotoxic lymphocytes that expressed interferon-γ. The expression of CXCL9 and CXCL10 was detected in PAL tumor biopsy samples from patients, and CXCR3-positive lymphocytes were abundant in the tissue samples. Collectively, these findings suggest that CXCL9 and CXCL10 are produced by PAL cells and can elicit cytotoxic responses via CXCR3. This chemokine system is also likely to contribute to tissue necrosis, which is a signature histological feature of DLBCL-CI. Further studies are warranted to determine whether the CXCL9–CXCL10/CXCR3 axis exerts antitumor effects in DLBCL-CI.

KEYWORDS
chemokine, cytotoxic lymphocyte, Epstein–Barr virus, inflammation, lymphoma

Abbreviations: CCL, C–C motif chemokine ligand; CCR, C–C motif chemokine receptor; CXCL, C–X–C motif chemokine ligand; CXCR, C–X–C motif chemokine receptor; DLBCL, diffuse large B-cell lymphoma; DLBCL-CI, DLBCL associated with chronic inflammation; EBNA, EBV nuclear antigen; EBV, Epstein–Barr virus; IFN-γ, interferon-gamma; IL, interleukin; LMP, latent membrane protein; LYG, lymphomatoid granulomatosis; NF-κB, nuclear factor-kappa B; NK, natural killer; NOS, not otherwise specified; PAL, pyothorax-associated lymphoma; PD-1, programmed death 1; PD-L1, programmed cell death ligand 1; Th1, T helper type 1; TNF-α, tumor necrosis factor-alpha; Treg, regulatory T.

1 | INTRODUCTION

Diffuse large B-cell lymphoma associated with chronic inflammation (DLBCL-CI) is an Epstein–Barr virus (EBV)-positive type of lymphoid neoplasm that develops in the context of long-standing inflammation usually involving body cavities and narrow spaces.[1] Although patients with this disease have no systemic immunosuppressive condition, DLBCL-CI exhibits type III EBV latency.[2–5] Pyothorax-associated lymphoma (PAL) is recognized as a prototype form of this category and represents a valuable model for the investigation of lymphomagenesis associated with EBV infection and chronic inflammation.

The most important risk factor for PAL is therapeutic pneumothorax for pulmonary tuberculosis or tuberculous pleuritis.[6,7] We and other researchers have shown that PAL is not restricted to pulmonary tuberculosis and can develop from any type of chronic empyema.[8,9] However, the mechanisms that drive the progression from chronic inflammation to the development of PAL remain elusive.

Studies have suggested that an immunosuppressive tumor microenvironment, which includes high levels of interleukin (IL)-6, IL-10, and EBV-encoded microRNA, may aid lymphoma cell survival and lead to the development of overt PAL in immunocompetent individuals.[10–12] We have also shown that PAL cells produce C–C motif chemokine ligands 17 and 22 (CCL17 and CCL22), the ligands of CCR4, and attract CCR4-expressing regulatory T (Treg) cells to evade host antitumor immunity.[13] By contrast, a subset of latent EBV antigens, including EBV nuclear antigen (EBNA) 3A, 3B, 3C, and latent membrane protein 2 (LMP2), is preferentially targeted by cytotoxic lymphocytes.[14] A higher number of $CD8^+$ T cells have been found in PAL than in DLBCL, not otherwise specified (DLBCL, NOS).[15] We have hypothesized that additional mechanisms for eliciting the host response needed to suppress tumor growth by destroying tumor cells and tissues may be operative in the early stage of the disease because variable degrees of necrotic lesions and vascular damage are usually present in affected tissues from patients with PAL.[1,16,17]

C–X–C motif chemokine receptor 3 (CXCR3) is a chemokine receptor that is expressed on effector $CD4^+$ T cells, effector $CD8^+$ T cells, and natural killer (NK) cells, and is strongly associated with T helper type 1 (Th1) and cytotoxic responses against infection and tumor.[18,19] CXCR3 is activated by three CXC chemokines—CXCL9 (formerly known as Mig), CXCL10 (IP-10), and CXCL11 (I-TAC)—and plays an important role in the trafficking of cytotoxic lymphocyte. These ligands are also ELR (Glu-Leu-Arg) motif-lacking angiostatic chemokines.[19–21] Tumor cells expressing CXCL10 have been shown to promote an antitumor immune response in animal models.[22] Other studies using a murine experimental system have reported that inoculation of human CXCL9 and CXCL10 into experimental Burkitt's lymphoma caused tumor regression and tumor necrosis associated with extensive vascular damage.[23,24] These findings suggest that the expression of the CXCR3 ligands exerts antitumor immune effects and promotes tissue necrosis and vascular damage by recruiting cytotoxic lymphocytes. Thus, these chemokines may also be associated with the pathogenesis of tissue necrosis in PAL.

Given this background, this study investigated whether PAL cells produce CXCR3 ligands and whether this leads to the mobilization of CXCR3-expressing effector cells. We found that EBV-positive PAL cells produce abundant CXCL9 and CXCL10, and can thereby attract cytotoxic lymphocytes via CXCR3.

2 | MATERIALS AND METHODS

2.1 | Cells

We used six EBV-positive PAL cell lines in this study. OPL-1, OPL-2, OPL-5, and OPL-7 were obtained from Dr. Katsuyuki Aozasa (Osaka University Medical School, Japan),[25,26] and Pal-1 and Pal-2 were established by our group.[27,28] Nine of the 10 EBV-negative DLBCL cell lines used here, 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 obtained from Deutsche Sammlung von Migroorganismen und Zellkulturen (Leibniz Institute DSMZ), and DH-My6 has been described elsewhere.[29] Human PBMCs were isolated from heparinized blood samples obtained from healthy adult donors using Ficoll–Paque (GE Healthcare). The mouse pre-B-cell line B300-19 clone that stably expresses human CXCR3 (B300-CXCR3) was generated as previously described.[30,31]

2.2 | Inhibition of cell signaling pathways and cytokine stimulation

Pyothorax-associated lymphoma cells were seeded in a 24-well plate at a density of 2×10^5/mL. To inhibit cell signaling pathways, the cells were treated for 12 h with a signaling inhibitor: the MEK–ERK pathway inhibitor PD98059 (Selleck Chemical) at 50 μM, the p38 MAPK pathway inhibitor SB202190 (Wako) at 10 μM, the JNK pathway inhibitor SP600125 (Selleck Chemical) at 50 μM, the Akt pathway inhibitor MK-2206 2HCl (Selleck Chemical) at 10 μM, the JAK3 pathway inhibitor JAK3 inhibitor 1 (Selleck Chemical) at 50 μM, and the nuclear factor-kappa B (NF-κB) pathway inhibitor BAY11-7082 (Wako) at 2 μM. The doses of the inhibitors were nontoxic to the PAL cell lines in our preliminary experiments (data not shown). For cytokine stimulation, the cells were treated with a recombinant human cytokine: IL-6 (R&D Systems) at 10 ng/ml, tumor necrosis factor α (TNF-α) (R&D Systems) at 50 ng/mL, and interferon (IFN)-γ (R&D Systems) at 100 ng/mL. The optimal concentrations of the cytokines chosen were based on our preliminary experiments (data not shown).

2.3 | Real-time quantitative RT–PCR

Total RNA extraction and real-time quantitative RT–PCR analysis were performed as described previously.[32] The primers and fluorogenic probes for *CXCL9* (Hs00171065_m1), *CXCL10* (Hs01124252_g1), *CXCL11* (Hs00171138_m1), and β2-microglobulin (*B2M*;

Hs00187842_m1) were based on the information provided for TaqMan Gene Expression Assays (Thermo Fisher Scientific).

2.4 | ELISAs

Pyothorax-associated lymphoma cells were seeded in a 24-well plate at a density of 5×10^5/mL and cultured for 3 days. The concentrations of CXCL9 and CXCL10 in the culture supernatants were measured using Quantikine ELISA kits (R&D Systems).

2.5 | Immunohistochemistry

Formalin-fixed paraffin wax-embedded biopsied tissue sections of samples from patients with EBV-positive PAL ($n = 5$) and patients with EBV-negative DLBCL, NOS ($n = 6$) were used for immunohistochemistry. The tissue sections were treated with primary antibodies against human CXCL9, CXCL10, CD4, CD8, CD20, CD56, and CXCR3, or isotype-matched antibodies as negative controls. The antibodies used are listed in Table S1. 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). Immunofluorescent double staining for CD20/LMP1/EBNA2 and CXCL9/CXCL10, and for CXCR3 and CD4/CD8/CD56 was performed using the fluorescently labeled secondary antibodies listed in Table S1.

2.6 | Chemotaxis assay

Chemotaxis assays were performed using a ChemoTx chemotaxis chamber with a 5-μm pore size (Funakoshi). B300-CXCR3 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 CXCL9 (10 nM; R&D Systems), CXCL10 (10 nM; R&D Systems), or culture supernatants of PAL cells with or without a CXCR3 antagonist AMG487 (1 μM; Selleck Chemical) were applied to the lower wells (30 μl/well). For neutralization of CXCR3 ligands, anti-CXCL9 antibody (R&D Systems), anti-CXCL10 antibody (R&D Systems), or normal goat IgG control (R&D Systems) were dissolved at 10 μg/ml in medium containing recombinant CXCL9 (Peprotech), CXCL10, or culture supernatants. After 3 h at 37°C, the cells that migrated into the lower wells were lysed and quantified using PicoGreen dsDNA reagent (Thermo Fisher Scientific).

2.7 | Flow cytometry

Peripheral blood mononuclear cells from healthy donors were treated with TruStain FcX Fc Receptor Blocking Solution (BioLegend) and incubated with a mixture of fluorescently labeled antibodies against human CD3, CD4, CD8a, CD56, and CXCR3, or isotype control. 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 fluorescently labeled antibodies against mouse CD4, CD8a, CD45, CD49b, and CXCR3, or isotype control. For intracellular staining of mouse IFN-γ, the cells were fixed and permeabilized using a Cytofix/Cytoperm kit (BD Biosciences) and stained with the fluorescently labeled antibody. The antibodies used are listed in Table S1. The cells were analyzed using a BD LSRFortessa flow cytometer (BD Biosciences) and FlowJo software (Tree Star Inc.).

2.8 | Cell mobilization experiments in mice

Nine-week-old male BALB/c mice were purchased from Japan SLC. The mice were injected intraperitoneally with 5×10^5 cells suspended in 500 μl of serum-free RPMI1640. For inhibition experiments on cell mobilization, AMG487 at 0.5 or 5.0 mg/kg, 10 μg of anti-CXCL9 antibody, anti-CXCL10 antibody, or normal goat IgG control were added to the cell suspension. 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 using the flow cytometer.

2.9 | Statistical analysis

The Mann–Whitney nonparametric *U* test was used to identify differences between pairs of groups. One-way analysis of variance with the Tukey post hoc test was used for comparisons between multiple groups. A P value <0.05 was considered to be significant.

3 | RESULTS

3.1 | Expression of *CXCL9*, *CXCL10*, and *CXCL11* in PAL cell lines

We first examined the expression of *CXCL9*, *CXCL10*, and *CXCL11* in a panel of six PAL cell lines and 10 EBV-negative DLBCL cell lines. In sharp contrast to the DLBCL cell lines, the PAL cell lines except for Pal-1 expressed *CXCL9* and *CXCL10* at relatively high levels (Figure 1A). Because *CXCL11* expression was barely detectable at a low level near the detection limit in these PAL cell lines, we excluded this chemokine from further studies. The expression levels of *CXCL9* and *CXCL10* in PAL cell lines did not correlate exactly to those of *LMP1* and *EBNA2* (Figure S1).

To examine the signaling pathways involved in the expression of *CXCL9* and *CXCL10* in PAL cells, we next examined the effects of various signaling inhibitors on the expression of these chemokines. For this experiment, we used OPL-2, OPL-7, and Pal-2 cells which expressed *CXCL9* and *CXCL10* at high levels. *CXCL9* and *CXCL10*

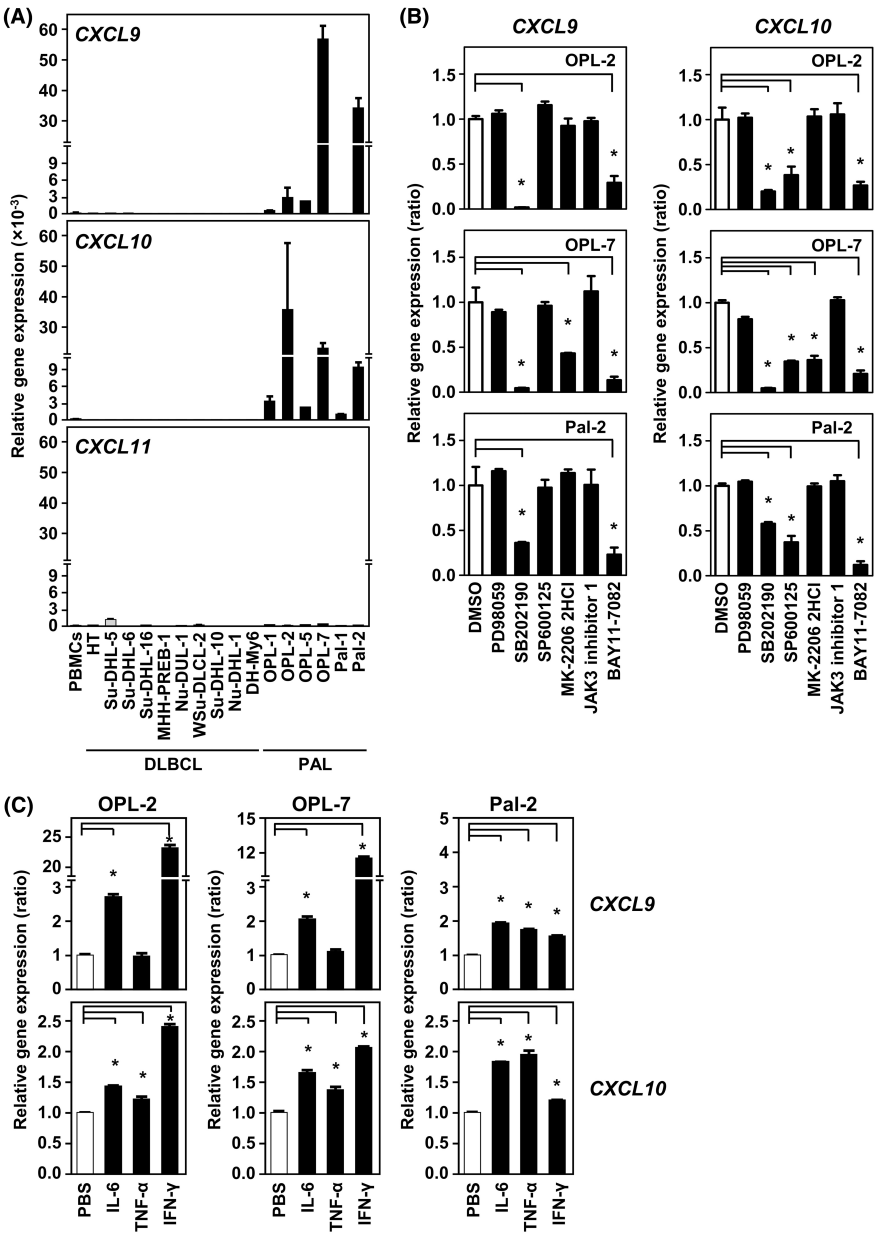

FIGURE 1 Expression analysis of CXCR3 ligand genes in PAL cell lines. Relative mRNA expression levels were calculated using the $2^{-\Delta C_t}$ values or $2^{-\Delta\Delta C_t}$ method with *B2M* used as the housekeeping control. (A) Expression of *CXCL9*, *CXCL10*, and *CXCL11* in six PAL cell lines and 10 EBV-negative DLBCL cell lines. The graph includes the average values in PBMCs obtained from 12 healthy donors as the control. (B) Effects of the blockade of the signaling pathways on the expression of *CXCL9* and *CXCL10*. Cells were treated with various inhibitors of signaling pathways or DMSO as a control. (C) Effects of cytokines on the expression of *CXCL9* and *CXCL10*. Cells were treated with various inflammatory cytokines: IL-6 at 10 ng/mL, TNF-α at 50 ng/mL, and IFN-γ at 100 ng/mL, or PBS as a control. Data are shown as the mean ± SEM of three independent experiments. Significant differences are shown as $^*P < 0.05$.

expression was significantly suppressed by SB202190, an inhibitor of the p38 MAPK pathway, and by BAY11-7082, an inhibitor of the NF-κB pathway (Figure 1B). *CXCL10* but not *CXCL9* expression was suppressed by inhibition of the JNK pathway by SP60012. In the OPL-7 cell line, *CXCL9* and *CXCL10* expression was suppressed by MK-2206 2HCl, an inhibitor of the Akt pathway. However, PD98059, an inhibitor of the MEK/ERK pathway, and JAK3 inhibitor 1, an inhibitor of the JAK3 pathway, had little effect on the expression of these chemokines in any of the PAL cell lines tested. These results suggest that both the p38 MAPK and NF-κB pathways are involved in the expression of *CXCL9* and *CXC10* in PAL cells, although involvement of the Akt pathway may differ between PAL cell lines.

We next examined the effects of various inflammatory cytokines on the expression of *CXCL9* and *CXCL10* given the close association between PAL pathogenesis and chronic inflammation. The expression levels of IL-6, TNF-α, and IFN-γ differed between PAL cell lines, and the expression of *CXCL9* and *CXCL10* was strongly upregulated by certain cytokines (Figure 1C). For example, IFN-γ highly upregulated the expression of both *CXCL9* and *CXCL10*, especially in OPL-2 and OPL-7 cells. IL-6 also upregulated the expression of these chemokines in the PAL cell lines tested. Collectively, these results suggest that the expression of *CXCL9* and *CXCL10* in PAL cells may be highly inducible in chronic inflammatory settings.

3.2 | Secretion of CXCL9 and CXCL10 by PAL cell lines

We next examined the secretion of CXCL9 and CXCL10 by PAL and EBV-negative DLBCL cell lines. CXCL10 was secreted at high levels by PAL cell lines (Figure 2). PAL cells also secreted CXCL9, although the secretion levels differed between cell lines. By contrast,

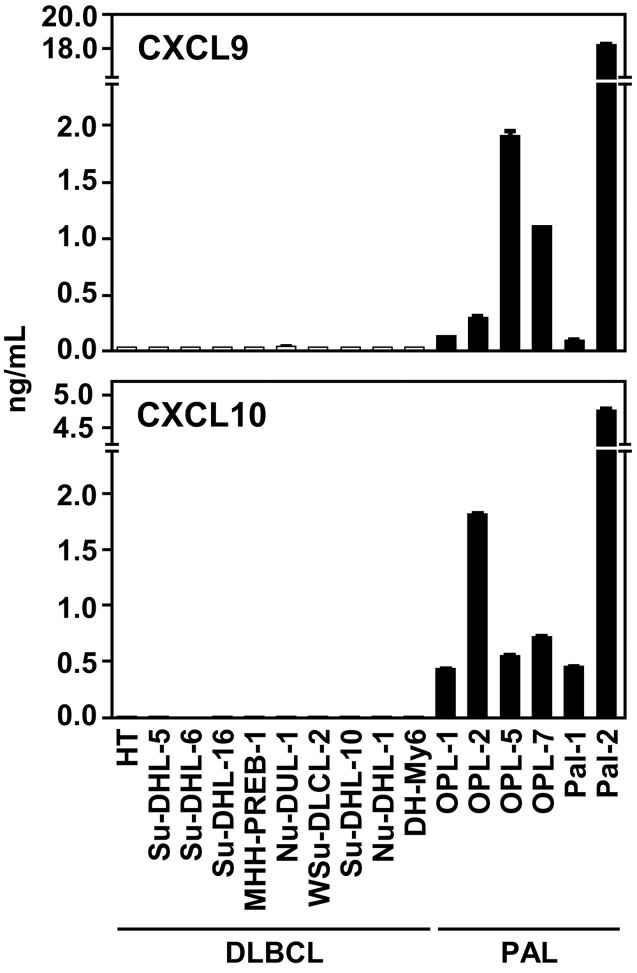

FIGURE 2 Secretion of CXCL9 and CXCL10 by PAL and EBV-negative DLBCL cell lines in vitro. The cells were seeded into 24-well plates at 5×10^5/ml and cultured for 3 days. Concentrations of CXCL9 and CXCL10 in the cell-free culture supernatants were measured by ELISA. Data are shown as the mean ± SEM of three independent experiments.

the DLBC cell lines did not secrete detectable levels of CXCL9 or CXCL10. These results are consistent with those of the qRT–PCR analysis of the expression of *CXCL9* and *CXCL10*.

3.3 | Expression of CXCL9 and CXCL10 in tumor cells in primary PAL tissues

We next performed immunohistochemistry of tumor biopsies obtained from patients with PAL and patients with DLBCL, NOS. All of the biopsies from patients with PAL showed infiltration of lymphoma cells along with necrotic lesions (Figure S2). Staining with CXCL9 and CXCL10 was strong in the PAL tissues but negative or faintly positive in DLBCL, NOS, tissues (Figure 3A). Double staining for CD20 and CXCL9/CXCL10 showed that most $CD20^+$ PAL cells expressed CXCL9 and CXCL10 (Figure 3B). The percentages of $CD20^+$ $CXCL9^+$ and $CD20^+$ $CXCL10^+$ tumor cells were 30%–54% and 13%–65% for five PAL tissues, respectively, and 3%–12% and 2%–15% for six DLBCL, NOS tissues, respectively. We also confirmed the expression of CXCL9 and CXCL10 in $LMP1^+$ $EBNA2^+$ primary PAL cells (Figure 3C,D). The percentages of $LMP1^+$ $CXCL9^+$, $LMP1^+$ $CXCL10^+$, $EBNA2^+$ $CXCL10^+$, and $EBNA2^+$ $CXCL10^+$ tumor cells were 56%–84%, 49%–74%, 54%–73%, and 51%–73% for the PAL tissues, respectively. Neither LMP1 nor EBNA2 was positive in the DLBCL, NOS tissues.

3.4 | Induction of cell migration via CXCR3 by the supernatants from PAL cell lines

We next examined whether the culture supernatants of OPL-2 and OPL-7 cells were chemotactic for CXCR3-expressing B300 cells (B300-CXCR3). The supernatants of both OPL-2 and OPL-7 induced the migration of the cells in a dose-dependent manner (Figure 4A). Furthermore, anti-CXCL9 or anti-CXCL10 antibodies partially suppressed the migration of B300-CXCR3 induced by the culture supernatants, while AMG487, a CXCR3 antagonist, strongly suppressed their migration (Figure 4B) These findings suggest that the CXCR3 ligands secreted into the culture supernatants from PAL cell lines were biologically active.

CXCR3-expressing cells, including cytotoxic T cells and NK cells, are responsible for cell-mediated immunity.[18] We examined whether the culture supernatant from OPL-2 is also chemotactic for primary $CXCR3^+$ $CD4^+$ T cells, $CXCR3^+$ $CD8^+$ T cells, and $CXCR3^+$ $CD56^+$ NK cells derived from human PBMCs. The migration of these CXCR3-expressing lymphocytes was significantly induced by the culture supernatants but was suppressed significantly by AMG487 (Figure 4C). These findings suggest that the CXCR3 ligands secreted by PAL cells can recruit human CXCR3-expressing lymphocytes.

3.5 | In vivo recruitment of cytotoxic lymphocytes via CXCR3 secreted by PAL cells

We next evaluated the in vivo recruitment of CXCR3-expressing cytotoxic lymphocytes by PAL and EBV-negative DLBCL cells. The cells were inoculated into the peritoneal cavity of mice, and the numbers of IFN-γ-expressing $CD8^+$ cells, IFN-γ-expressing $CD4^+$ cells, and $CD49b^+$ NK cells that migrated to the peritoneal cavity were counted. Control mice were injected with medium alone. OPL-2 and OPL-7 cells significantly recruited these cells but Nu-DUL-1 and WSu-DLCL2 cells did not (Figure 5A). Most of the IFN-γ-expressing $CD8^+$ cells, IFN-γ-expressing $CD4^+$ cells, and $CD49b^+$ NK cells were confirmed as CXCR3 positive (Figure 5B). AMG487 significantly suppressed the recruitment of these CXCR3-expressing cells by OPL-2 and OPL-7 (Figure 5C). Anti-CXCL9 or anti-CXCL10 antibodies also significantly suppressed their recruitment by OPL-2 and OPL-7 (Figure 5D). We also confirmed that the PAL cells themselves were negative for CXCR3 (Figure S3). These results suggest that PAL cells efficiently recruited effector cells involved in Th1 immunity and NK cells via CXCR3 in vivo through a paracrine mechanism.

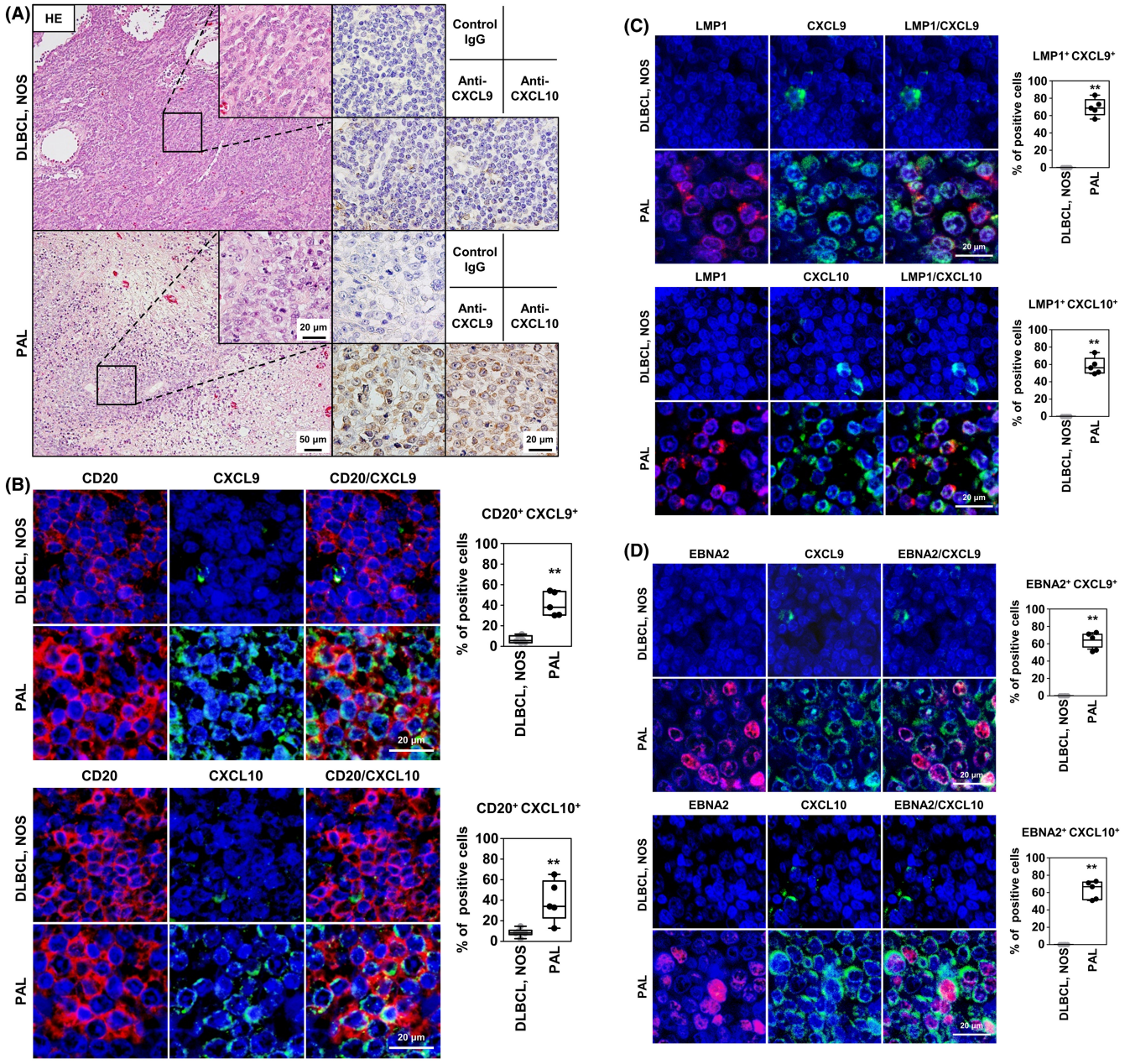

FIGURE 3 Expression of CXCL9 and CXCL10 in primary PAL tissues. (A) Specimens of PAL and DLBCL, NOS, were stained with hematoxylin and eosin. Immunohistochemistry was performed on the sections using isotype control IgG antibody, anti-CXCL9 antibody, and anti-CXCL10 antibody. Samples from representative patients are shown. (B) Immunofluorescent double staining for CD20 (red) and CXCL9/CXCL10 (green). (C) Immunofluorescent double staining for LMP1 (red) and CXCL9/CXCL10 (green). (D) Immunofluorescent double staining for EBNA2 (red) and CXCL9/CXCL10 (green). The nuclei were counterstained with DAPI (blue). Box plots depict the percentages of the double staining-positive cells in PAL tissues ($n = 5$) and DLBCL, NOS, tissues ($n = 6$). For the semiquantitative assessment of protein expression, the percentage of positive cells was calculated in more than five randomly selected higher-power fields that included over 100 cells each, and the mean was used as the labeling index for each lesion. Data are shown as the mean ± SD. Significant differences are shown as $^{**}P < 0.01$.

3.6 | Infiltration of CXCR3-expressing cells in PAL tissues

Finally, using immunohistochemistry, we evaluated the accumulation of CXCR3-expressing lymphocytes in primary PAL tissues. Double staining with antibodies against CXCR3/CD8, CXCR3/CD4, and CXCR3/CD56 revealed the presence of a large number of $CXCR3^+$ $CD8^+$, $CXCR3^+$ $CD4^+$, and $CXCR3^+$ $CD56^+$ cells in PAL tissues (Figure 6).

4 | DISCUSSION

To our knowledge, our study is the first to examine the expression of CXCR3 ligands in PAL. We found that CXCL9 and CXCL10 were expressed and produced in most of the PAL cell lines tested but were never expressed in EBV-negative DLBCL cell lines. We also found that the expression of these chemokines was mediated by both the NF-κB and p38 MAPK signaling pathways. The EBV

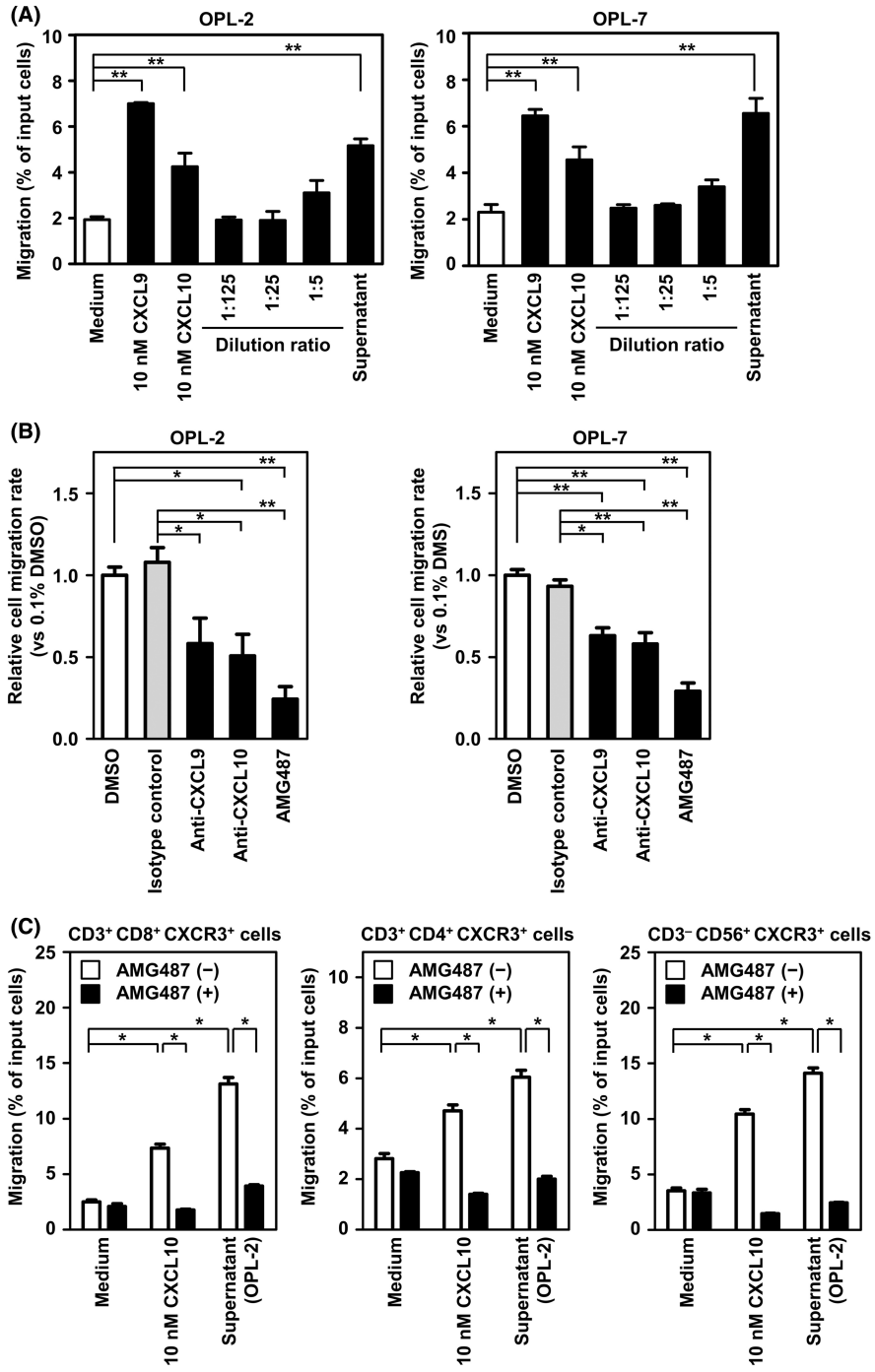

FIGURE 4 Induction of CXCR3-mediated cell migration by secretions from PAL cells. (A) Culture supernatants of OPL-2 and OPL-7 cells were tested for chemotactic activity using CXCR3-expressing B300-19 cells. Serially diluted culture supernatants were also tested for chemotactic activity. Cell migration is expressed as a percentage of the input cells. Recombinant CXCL9/CXCL10 and medium alone were used as positive and negative controls, respectively. (B) Effect of anti-CXCL9 antibody (1 μg/ml), anti-CXCL10 antibody (1 μg/ml), and AMG487 (1 μM) on CXCR3-mediated cell migration. (C) Cell migration of human PBMCs induced by the culture supernatants from OPL-2 cells was measured. The cells that migrated into the lower wells were stained for CD3, CD4, CD8, CD56, and CXCR3, and quantified by flow cytometry. Results from three to four separate experiments are shown as the mean ± SEM. Significant differences are shown as $^{*}P < 0.05$ and $^{**}P < 0.01$.

oncogenic LMP1 is known to activate the NF-κB and p38 MAPK pathways.[33–35] Cherney et al.[36] showed that transfection with *LMP1* into Burkitt's lymphoma cells led to the expression of *CXCL9* and *CXCL10*. Vockerodt et al.[37] used EBV-negative lymphoma lines transfected with *LMP1* and found that LMP1 induced CXCL10 expression through transcriptional (NF-κB) and posttranscriptional (p38 MAPK) mechanisms. Because all PAL cell lines used here express *LMP1* (Figure S1), it is possible that LMP1 signaling is also involved in inducing CXCL9 and CXCL10 in PAL cells. Further understanding of the regulation of the LMP1–NF-κB and LMP1–p38 MAPK axes may help to elucidate the precise mechanisms of CXCL9 and CXCL10 induction in PAL cells.

We found here that the expression of *CXCL9* and *CXCL10* was upregulated in PAL cells by stimulation with inflammatory cytokines, particularly IFN-γ. Previous studies have shown that IFN-γ stimulation induces the expression of *CXCL9* and *CXCL10*.[38] It is therefore conceivable that PAL cells can express these chemokines in response to exogenous proinflammatory cytokine stimulation. Collectively, our findings suggest that *CXCL9* and *CXCL10* are expressed constitutively and inducibly by PAL cells. Importantly, we confirmed the expression of CXCL9 and CXCL10 in primary tumor cells in the PAL tissues obtained from patients.

In this study, the CXCL9 and CXCL10 produced by PAL cells were functional because the culture supernatants from PAL cell

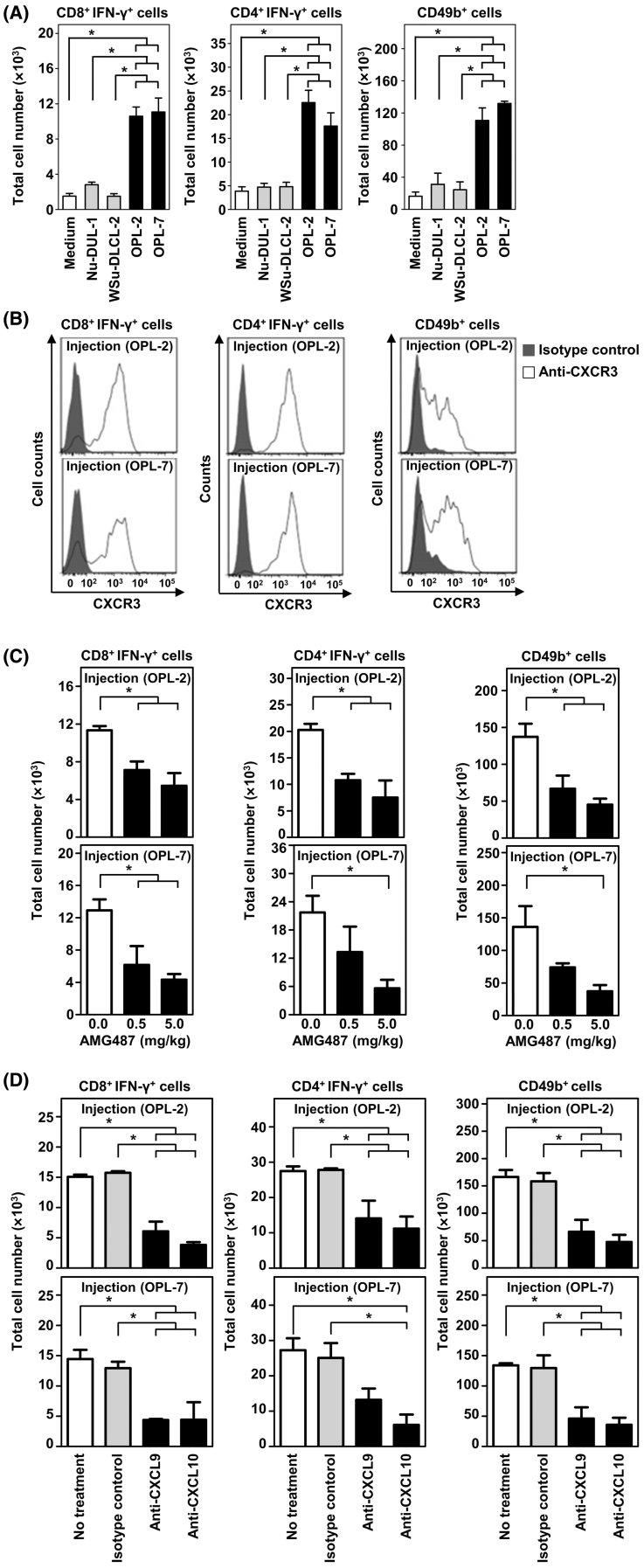

FIGURE 5 In vivo recruitment of cytotoxic effector lymphocytes via CXCR3 secreted by PAL cells. (A) OPL-2, OPL-7, Nu-DUL-1, or WSu-DLCL-2 cells (5×10^5) were injected intraperitoneally into BALB/c mice. After 24 h, cells were isolated from the peritoneal cavity and the cell numbers were counted. The cells were stained for CD4, CD8, CD49b, and IFN-γ. The $CD4^+$ IFN-γ^+ cells, $CD8^+$ IFN-γ^+ cells, and $CD49b^+$ NK cells were counted by flow cytometry using the CD45 gate. Total cell number was calculated using the following formula: total cell number = number of total peritoneal exudate cells enumerated by cell counting × the ratio of $CD4^+$ IFN-γ^+ cells, $CD8^+$ IFN-γ^+ cells, or $CD49b^+$ cells analyzed by flow cytometry. Control mice were injected with medium alone. The data are expressed as the SEM of the results obtained from three to 10 mice. (B) The $CD4^+$ IFN-γ^+ cells, $CD8^+$ IFN-γ^+ cells, and $CD49b^+$ cells were analyzed further for CXCR3 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 AMG487 at a concentration of 0.5 or 5.0 mg/kg. (D) OPL-2 or OPL-7 cells (5×10^5) were injected intraperitoneally into BALB/c mice with 10 μg of isotype control, anti-CXCL9 antibody, or anti-CXCL10 antibody. The data are expressed as the SEM of the results obtained from three to four mice. Significant differences are shown as $^*P<0.05$.

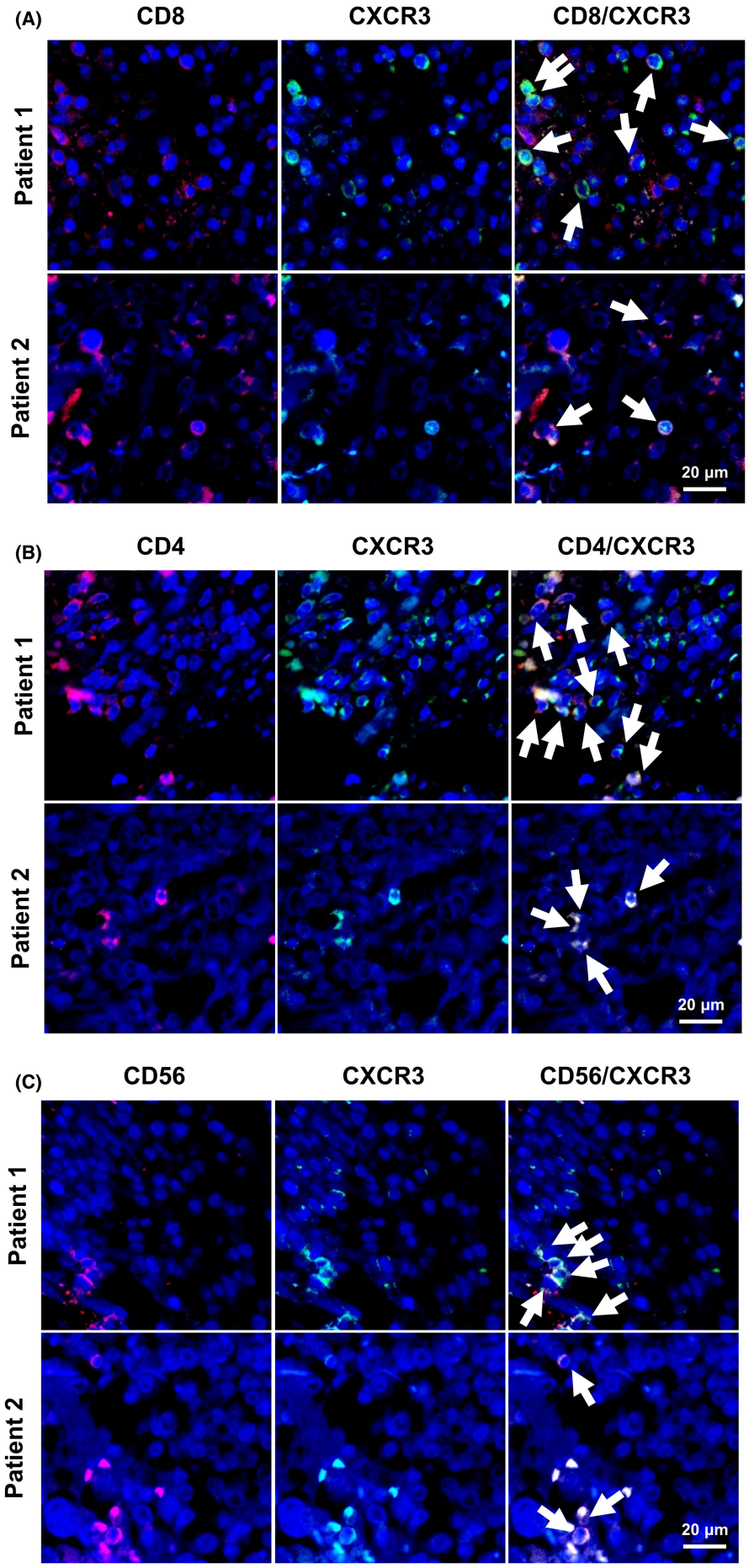

FIGURE 6 Infiltration of CXCR3-expressing cells in tumor tissues from patients with PAL. Immunofluorescent double staining for CD8 and CXCR3 (A), CD4 and CXCR3 (B), and CD56 and CXCR3 (C) was performed. Expression of CD4 (red), CD8 (red), CD56 (red), and CXCR3 (green) was observed on the cell surfaces. The nuclei were counterstained with DAPI (blue). The arrows indicate cells with colocalization of the two indicated proteins. Samples from two representative PAL patients are shown.

lines attracted $CD4^+$ T cells, $CD8^+$ T cells, and NK cells via CXCR3 in human PBMCs. Moreover, PAL cells injected into mice attracted CXCR3-expressing lymphocytes. We also found that the CXCR3-expressing lymphocytes mobilized by PAL cells in mice expressed IFN-γ, a marker of cytotoxic immune cells.[39,40] The major function of CXCL9 and CXCL10 is the recruitment of CXCR3-expressing cytotoxic lymphocytes to the inflammatory and tumor microenvironments.[38,41] Such infiltrating cytotoxic lymphocytes can cause tissue destruction via cell death mechanisms involving the perforin–granzyme pathway and the Fas–Fas ligand pathway.[42,43] CXCL9 and CXCL10 can act directly as antiangiogenic factors,[41,44] which also cause tissue necrosis and vascular damage.[23,24]

A study of lymphomatoid granulomatosis (LYG) and extranodal NK/T-cell lymphoma, nasal type, which are other EBV-associated disorders characterized by progressive tissue necrosis, found that CXCL9 and CXCL10 are associated with tissue destruction.[45] In that study, CXCL9 and CXCL10 expression was observed in endothelial cells, macrophages, and fibroblasts in the viable tissues surrounding areas of necrosis, which indicated that the reactive cells were the principal source of these chemokines.[45] By contrast, Moriai et al.[46] found coexpression of CXCL10 and CXCR3 in nasal NK/T-cell lymphoma cells and suggested that CXCL10 may act as an autocrine cell invasion factor. Unlike LYG and nasal NK/T-cell lymphoma, the PAL tumor cells examined in our study expressed both CXCL9 and CXCL10, but not CXCR3, and abundant CXCR3-expressing lymphocytes, including $CD4^+$ T cells, $CD8^+$ T cells, and $CD56^+$ NK cells, were present in the tumor microenvironment in which extensive necrosis lesions were noted. Although the major therapeutic source of cytotoxic T cells is $CD8^+$ T cells, EBV-induced $CD4^+$ T cells can also exhibit cytotoxicity.[47,48] Thus, the immune response to EBV in PAL appears to be a principal cause of tissue necrosis and, presumably, vascular damage, which are typical histological features of PAL.

It is possible that the recruitment of cytotoxic T lymphocytes to the tumor microenvironment confers a proliferative disadvantage to PAL cells. However, PAL usually has a progressive clinical course. The mechanisms favoring tumor progression may be operative in PAL. This type of lymphoma usually develops in an immunosuppressive confined space and is considered to escape from the host immune surveillance. Studies have shown that PAL produces immunosuppressive IL-10 and has reduced levels of MHC class I molecules, which are involved in antitumor immune responses by cytotoxic lymphocytes.[11,49] We have also reported that CCL17 and CCL22 chemokines produced by PAL cells recruit CCR4-expressing Treg cells to the tumor microenvironment.[13] EBV can induce programmed cell death ligand 1 (PD-L1) expression by the LMP1 pathway and IFN-γ stimulation, which may also contribute to tumor immunosuppression.[50] We confirmed the expression of high levels of *PD-L1* in most of the PAL cell lines studied here (Figure S4). It is plausible that PAL cells can recruit effector T cells and NK cells via the CXCL9 and CXCL10/CXCR3 axis, which inhibit tumor development, as well as Treg cells via the CCL17 and CCL22/CCR4 axis, which suppress immune function. This dual role of the immune system in suppressing or promoting tumor growth is termed "cancer immunoediting" and comprises three phases: elimination, equilibrium, and escape.[51,52] The role of chemokines produced in PAL may vary between the phases. During long-standing inflammation in PAL, the function of chronically stimulated effector T cells may decrease progressively (e.g., loss of cytokine production) as they enter a state of hyporesponsiveness called "$CD8^+$ T-cell exhaustion" in the escape phase or the tumor cells may become unsusceptible to cytotoxic T-lymphocyte-mediated lysis.[53,54] This may cause a failure to eliminate tumor cells and eventually lead to overt PAL.

The optimal management of PAL remains unresolved. Chheda et al.[55] reported a critical correlation between CXCR3-mediated cytotoxic T-cell homing to the tumor site and the effect of anti-PD-1 treatment in an in vivo model. The anti-PD-1 antibody failed to shrink the tumor in CXCR3-knockout mice, which implies that anti-PD-1 therapy is not effective without the CXCL9 and CXCL10/CXCR3 axis.[55] Moreover, high levels of CXCL9 and CXCL10 expression in the tumor microenvironment are positive indicators of the responsiveness to anti-PD-1 therapy.[38,56] In this context, our findings suggest that new approaches to stimulate or reinvigorate the antitumor immune responses, such as anti-PD-1 therapy, may be beneficial for patients with PAL, although the clinical efficacy needs to be evaluated.

Although our clinical studies on patients with PAL included a small sample size, our data obtained from the PAL cell lines were consistent across the biopsied PAL tissues. However, the potential association between CXCL9 and CXCL10 expression and the extent of effector T-cell infiltration during disease progression awaits further investigation. Similarly, because of the retrospective design of our study, we could not evaluate whether serum levels of these chemokines correlate with disease progression.

In summary, our study suggests a novel feature of PAL, that is, the constitutive and inducible production of CXCL9 and CXCL10, and the attraction of cytotoxic lymphocytes via CXCR3, which may be associated with the pathogenesis of tissue necrosis, a conspicuous histological feature of DLBCL-CI. This axis is likely to play a role in the activation of antitumor immunity through paracrine signaling. Our findings should stimulate further studies to identify more effective therapeutic strategies to stimulate or reactivate the antitumor immune responses in refractory DLBCL-CI.

AUTHOR CONTRIBUTIONS

Tomonori Higuchi: conceptualization, formal analysis, investigation, methodology, visualization, writing—original draft (supporting). Yumiko Hashida: investigation. Kazuhiko Matsuo: investigation. Kosuke Kitahata: investigation. Takako Ujihara: investigation. Ichiro Murakami: resources. Takashi Nakayama: conceptualization, formal analysis, methodology, resources, visualization, writing—review and editing. Masanori Daibata: conceptualization, formal analysis, funding acquisition, methodology, project administration, supervision, resources, writing—original draft (lead), writing—review and editing.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

ETHICS STATEMENTS

Approval of the research protocol by an Institutional Reviewer Board: This study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Ethics Committee of Kochi Medical School, Kochi University (approval no. 28-80).

Informed Consent: Because this study was retrospective, the requirement for written informed consent from patients was waived for the immunohistochemistry experiments. Written informed consent was obtained from all volunteers for the donation of peripheral blood.

Registry and the Registration No. of the study/trial: N/A

Animal Studies: All of the animal experiments were performed in accordance with the guideline of the Center for Animal Experiments, Kindai University Faculty of Pharmacy.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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