Expression of CCL17 and CCL22 by latent membrane protein 1-positive tumor cells in age-related Epstein–Barr virus-associated B-cell lymphoproliferative disorder

Sumio Takegawa,[1,6] Zhe Jin,[1,6] Takashi Nakayama,[1] Takashi Oyama,[2] Kunio Hieshima,[1] Daisuke Nagakubo,[1] Aiko-Konno Shirakawa,[1] Toyonori Tsuzuki,[3] Shigeo Nakamura[4] and Osamu Yoshie[1,5]

[1]Department of Microbiology, Kinki University School of Medicine, Osaka-Sayama, 589-8511; [2]Department of Hematology and [3]Department of Pathology and Laboratory Medicine, Nagoya Daini Red Cross Hospital, Nagoya, 466-8650; [4]Department of Pathology and Clinical Laboratories, Nagoya University Graduate School of Medicine, Nagoya, 466-8550, Japan

(Received August 23, 2007/Revised October 17, 2007/Accepted October 22, 2007/Online publication February 4, 2008)

Age-related Epstein–Barr virus-positive (EBV$^+$) B-cell lymphoproliferative disorder (ALPD) is a disease entity identified from a large-scale re-survey of cases diagnosed as diffuse large B-cell lymphoma. ALPD is a group of EBV$^+$ polymorphic B-cell lymphoma typically seen in elderly patients. An age-associated decline in host immunity against EBV might be partly responsible for the pathogenesis of ALPD. Histologically, ALPD is often characterized by a minor proportion of EBV-encoded RNA-positive tumor cells in a background of extensive cellular infiltration, similar to that of classical Hodgkin's lymphoma. In contrast to Hodgkin and Reed–Sternberg cells, ALPD tumor cells are clearly positive for B cell markers CD20 and/or CD79a. Hodgkin and Reed–Sternberg cells produce various chemokines, including CCL17 and CCL22, that attract chemokine receptor CCR4-expressing Th2 cells and regulatory T cells. Previously, we have shown that EBV-immortalized B cells also produce CCL17 and CCL22 through latent membrane protein 1 (LMP1)-mediated activation of nuclear factor κB. Here we examined expression of CCL17 and CCL22 in ALPD. ALPD tumor cells were often heterogeneous in size in accordance with the differential expression of EBV latent genes at the single cell level. LMP1-expressing tumor cells were typically large in size and selectively positive for CCL17 and CCL22. CCR4$^+$ cells and forkhead box protein 3$^+$ regulatory T cells were abundantly present, and the majority of forkhead box protein 3$^+$ cells were CCR4$^+$. Collectively, our data show production of CCL17 and CCL22 by LMP1$^+$ large-sized tumor cells and accumulation of CCR4-expressing cells including regulatory T cells in ALPD. (*Cancer Sci* 2008; 99: 296–302)

Epstein–Barr virus (EBV) is a ubiquitous human γ-herpes virus that is closely associated with a number of human malignancies such as endemic Burkitt's lymphoma in sub-Saharan Africa, nasopharyngeal carcinoma in southern China, approximately 50% of classical Hodgkin's lymphoma (cHL), EBV-related post-transplantation lymphoproliferative disorders (PTLD), and similar EBV$^+$ B-cell lymphomas in patients with AIDS.[1] Age-related EBV$^+$ B-cell lymphoproliferative disorder (ALPD) is a disease entity recently proposed by Oyama *et al*. in Japan who carried out a large-scale re-survey of cases diagnosed as diffuse large B-cell lymphoma (DLBCL) for the presence of EBV, cell lineage markers, histological features, and patients' backgrounds.[2] ALPD has emerged as a group of EBV$^+$ lymphoma (22/403) typically seen in elderly patients.[2] Thus, an age-associated decline in host immunity against EBV might be partly responsible for the development of ALPD. In the follow-up study, Oyama *et al*. has further shown that ALPD is associated with more aggressive clinical features and parameters than EBV$^-$ DLBCL.[3] Recently, Park *et al*. in Korea also carried out a large-scale retrospective study on DLBCL and similarly identified a group of EBV$^+$ lymphoma (34/380) that is significantly associated with an age of >60 years and poor prognosis.[4] It remains to be seen if similar age-related EBV$^+$ B-cell lymphomas are also frequent in other parts of the world.

Histologically, two possible subtypes were originally described in ALPD.[2] The polymorphic lymphoproliferative disorder subtype shows a broad range of B cell maturation from immunoblasts to plasma cells with an extensive inflammatory background, whereas the large cell lymphoma subtype is characterized by a more diffuse proliferation of large lymphoid cells. However, these two subtypes are in fact continuous with variable levels of mixed features.[2] Furthermore, the subsequent study has found no significant differences in any clinical characteristics or overall prognosis between these two possible subtypes.[3] Another frequent histological feature in ALPD is an abundant reactive cellular infiltration in the tumor background.[2] This feature is quite reminiscent of cHL, which is also frequently associated with EBV.[5,6] However, tumor cells of ALPD are clearly positive for the B-cell markers such as CD20 and/or CD79a,[2] whereas Hodgkin and Reed–Sternberg (H-RS) cells of cHL are typically negative for these markers.[5,6] It is now known that H-RS cells are derived from pre-apoptotic germinal center B cells,[7] and have a profound suppression in B cell-specific gene expression,[8] most probably to escape from apoptosis normally occurring with crippling mutations in immunoglobulin heavy chain genes. H-RS cells are also known to produce various chemokines likely to be responsible for the characteristic background cellular infiltration.[9–13] In particular, H-RS cells produce CCL17 (also called TARC) and CCL22 (also called MDC),[10,13] whose shared receptor CCR4 is known to be selectively expressed by Th2 cells and regulatory T cells.[14,15] Thus, CCL17 and CCL22 produced by H-RS cells are considered to play important roles in immune evasion by attracting Th2 cells and regulatory T cells.[16–19] Previously, we have shown that EBV-immortalized B cells produce various chemokines.[20] In particular, EBV-encoded latent membrane protein 1 (LMP1) selectively induces CCL17 and CCL22 through activation of nuclear factor κB (NF-κB).[20] As it is likely that ALPD tumor cells resemble EBV-immortalized B cells *in vitro*, we speculated that ALPD tumor cells also produce CCL17 and CCL22 through expression of LMP1. Here we show that: (i) as reported for PTLD and AIDS-related lymphomas,[21] ALPD tumor cells are often heterogeneous in size in accordance

[5]To whom correspondence should be addressed. E-mail: o.yoshie@med.kindai.ac.jp
[6]These authors contributed equally to this work.

doi: 10.1111/j.1349-7006.2007.00687.x
© 2008 Japanese Cancer Association

Table 1. Summary of the cases used in the present study

Case	Disease	Age (years) /Sex	Biopsy site	EBERs	LMP1	EBNA2	CCL17/CCL22	CCR4	FOXP3
1	ALPD	72/F	Lymph node	+	+	–	+	+	+
2	ALPD	70/M	Gingiva	+	–	–	–	–	ND
3	ALPD	68 M	Stomach	+	+	+	+	+	+
4	ALPD	75/F	Spine	+	+	–	+	–	ND
5	ALPD	62/M	Lymph node	+	+	+	+	+	+
6	ALPD	66/M	Stomach	+	+	–	+	+	+
7	ALPD	79/M	Spleen	+	+	–	+	+	+
8	ALPD	49/M	Kidney	+	+	–	+	+	+
9	ALPD	63/F	Lung	+	–	+	–	–	ND
10	ALPD	70/F	Stomach	+	–	–	–	+	+
11	ALPD	50/M	Stomach	+	+	–	+	+	ND
12	ALPD	69/M	Lymph node	+	+	–	+	+	+
13	ALPD	69/M	Small intestine	+	+	–	+	+	+
14	PTLD	12/M	Pharynx	+	+	+	+	+	+
15	PTLD	54/M	Lymph node	+	ND	ND	+	–	–
16	PTLD	18/M	Lymph node	+	–	–	–	+	+
17	PTLD	64/M	Tonsil	+	+	+	+	+	+
18	PTLD	16/M	Lymph node	+	+	+	+	+	+
19	PTLD	48/M	Pharynx	+	ND	ND	+	+	+
20	cHL (MC)	24/M	Lymph node	+	+	–	+	ND	ND
21	cHL (MC)	70/M	Lymph node	+	+	–	+	ND	ND
22	cHL (MC)	82/M	Lymph node	+	+	–	+	ND	ND
23	cHL (MC)	54/M	Lymph node	–	–	–	+	ND	ND
24	cHL (NS)	28/F	Lymph node	–	–	–	+	ND	ND
25	cHL (NS)	25/M	Lymph node	–	–	–	+	ND	ND
26	cHL (NS)	66/M	Lymph node	–	–	–	+	ND	ND
27	cHL (NS)	24/M	Lymph node	–	–	–	+	ND	ND

+, positive; –, negative; ALPD, age-related Epstein–Barr virus-positive lymphoproliferative disorder; CCL17/CCL22, chemokines CCL17 and CCL22; CCR4, chemokine receptor CCR4; cHL, classical Hodgkin's lymphoma; DLBCL, diffuse large B-cell lymphoma; EBER, Epstein–Barr virs-encoded RNA; EBNA2, Epstein–Barr virus nuclear antigen 2; F, female; FOXP3, forkhead box protein 3; MC, mixed cellularity; ND, not done; NS, nodular sclerosis; LMP1, latent membrane protein 1; M, male; PTLD, post-transplantation lymphoproliferative disorder.

with variable expression of EBV latent genes at the single cell level; (ii) large-sized tumor cells expressing LMP1 selectively produce CCL17 and CCL22; (iii) infiltrating cells expressing CCR4 are abundantly present in the cellular background of ALPD; and (iv) forkhead box protein 3 (FOXP3)-expressing regulatory T cells,[22] present in the background, are mostly expressing CCR4. Thus, a very similar mechanism involving CCL17 and CCL22 might be operative for immune evasion in ALPD as in cHL.

Materials and Methods

Tissue samples. All clinical cases were diagnosed at the Aichi Cancer Center hospital (Nagoya, Japan) and its affiliated hospitals between 1997 and 2004 (Table 1). Only archival paraffin-embedded tissues were used in the present study. This study was approved by the local ethical committees.

***In situ* hybridization.** EBV-encoded RNA (EBER) was detected by *in situ* hybridization as described previously.[2] Briefly, a hybridization kit with a cocktail of fluorescein-isothiocyanate-labeled EBER oligonucleotides (one oligonucleotide corresponding to EBER1 and one to EBER2, both 30 bases long) (DAKO Japan, Kyoto, Japan) was used. Hybridization products were detected with mouse anti-fluorescein-isothiocyanate (DAKO Japan) and Vectastain ABC kit (Vector Laboratories, Burlingame, CA). Sections pretreated with RNase A or DNase I were used for negative controls, and EBER$^+$ cHL specimens were used for positive controls.

Immunohistochemistry. The following antibodies were purchased from commercial sources: anti-LMP1 (CS1-4, mouse immunoglobulin [Ig]G1; DAKO Japan); anti-EBV nuclear antigen 2 (EBNA2) (PE2, mouse IgG1; DAKO Japan), goat anti-TARC/CCL17 (R&D Systems, Minneapolis, MN); rabbit anti-MDC/CCL22 (PeproTech EC, London, UK); rabbit anti-NF-κB p65 (IBL, Gunma, Japan); anti-CD3 (PS1, mouse IgG2a; Novocastra Laboratories, Newcastle, UK); anti-CD4 (1F6, mouse IgG1; Novocastra Laboratories); anti-CD8 (1A5, mouse IgG1; Novocastra Laboratories); anti-CD45RO (UCHL1, mouse IgG2a; DAKO Japan); anti-CD69 (CH11, mouse IgG1; Novocastra Laboratories); anti-FOXP3 (236 A/E7, mouse IgG1; Abcam, Cambridge, UK); mouse IgG isotypes (DAKO Japan); goat IgG (IBL); and rabbit IgG (DAKO Japan). A mouse anti-CCR4 (KM2160, mouse IgG1) was kindly provided by Kyowa Hakko (Tokyo, Japan). Immunohistochemical staining was carried out as described previously.[9] In brief, tissue sections were treated at 121°C for 15 min in Target Retrieval Solution (S1699; DAKO Japan) or 1 mmol/L ethylenediaminetetraacetic acid/10 mmol/L Tris buffer, pH 9.0. Endogenous biotin was blocked using an Avidin/Biotin Blocking kit (DAKO Japan). Tissue sections were then incubated at 4°C overnight with each antibody. After washing, the sections were incubated with appropriate biotin-labeled secondary antibodies: biotin-labeled horse antimouse IgG; biotin-labeled rabbit antigoat IgG; or biotin-labeled goat antirabbit IgG (all from Vector Laboratories). After washing, sections were treated with Vectastain ABC/HRP kit (Vector Laboratories) according to the manufacturer's instructions. Peroxidase enzymatic development was carried out using 3,3′-diaminobenzidine (Liquid DAB Substrate Chromogen System; DAKO Japan), resulting in dark brown products in positive cells. Sections were counterstained with Gill's hematoxylin (Polysciences, Warrington, PA). After that, sections were dehydrated and mounted in Nonaqueous Mounting Medium (Muto Pure Chemicals, Tokyo, Japan).

Double immunofluorescent staining. Tissue sections were heated in Target Retrieval Solution (DAKO Japan) with microwaves for 5 min three times and blocked with 10% normal rabbit serum or 10% normal goat serum for 30 min at room temperature. After

© 2008 Japanese Cancer Association

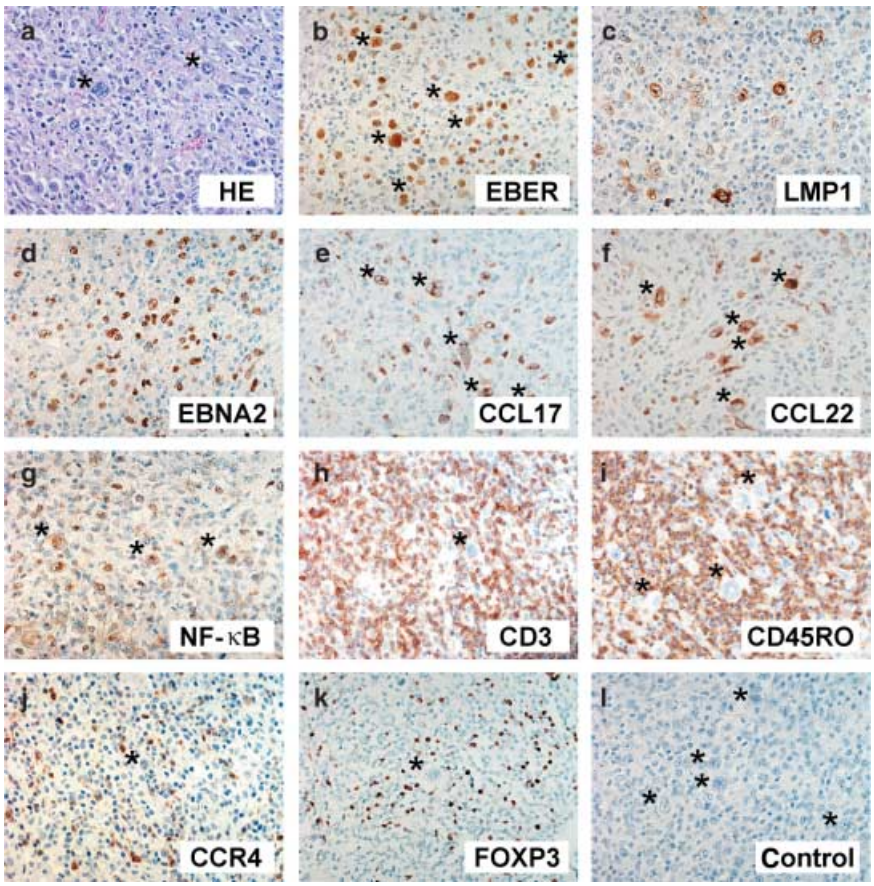

Fig. 1. *In situ* hybridization and immunohistochemistry of age-related Epstein–Barr virus-positive lymphoproliferative disorder (ALPD). (a) Hematoxilin–eosin (HE); (b) Epstein–Barr virus-encoded RNA (EBER; *in situ* hybridization); (c) latent membrane protein 1 (LMP1); (d) Epstein–Barr virus nuclear antigen 2 (EBNA2); (e) chemokine CCL17; (f) chemokine CCL22; (g) nuclear factor (NF)-κB; (h) CD3; (i) CD45RO; (j) chemokine receptor CCR4; (k) forkhead box protein 3 (FOXP3); (l) an isotype control. *Large-sized tumor cells. Representative results from 13 ALPD cases are shown (as detailed in Table 1). (a, c, e, f, k) case #8; (b, d, g) case #5; (h–j, l) case #13 (original magnification: ×400).

washing, tissue sections were incubated with goat anti-TARC/CCL17 (R&D Systems) or rabbit anti-MDC/CCL22 (Peprotech) at 4°C overnight. Normal goat IgG or normal rabbit IgG was used as the negative control. After washing, tissue sections were incubated with Alexa Fluor 488-labeled rabbit antigoat IgG (Invitrogen, Carlsbad, CA) or Alexa Fluor 488-labeled goat antirabbit IgG (Invitrogen) for 1 h at room temperature. After washing, tissue sections were incubated with mouse monoclonal anti-LMP1 (DAKO Japan) at 4°C overnight. Normal mouse IgG1 was used as the negative control. After washing, tissue sections were incubated with Alexa Fluor 546-labeled rabbit antimouse IgG (Invitrogen) or Alexa Fluor 546-labeled goat antimouse IgG (Invitrogen) for 1 h at room temperature. After washing and mounting, single- and double-color fluorescence images were taken by a BZ-8000 fluorescence microscope (Keyence, Osaka, Japan).

Double immunoenzyme staining. Double immunoenzyme staining was carried out as described previously.[23] In brief, sections treated at 121°C for 15 min in Target Retrieval Solution (DAKO Japan) were incubated at 4°C overnight with anti-CD4 (1F6), anti-CD8 (1A5), anti-CD69 (CH11), anti-CCR4 (KM2160), goat anti-CCR5 (Capralogics, Hardwick, MA) or anti-chemokine receptor CXCR3 (1C6, mouse IgG1; BD Biosciences). Isotype-matched mouse IgG (DAKO Japan) or normal goat IgG (IBL) was used as the negative control. After washing, sections were incubated with biotin-labeled horse antimouse IgG or rabbit antigoat IgG (Vector Laboratories). After washing, sections were treated with Vectastain ABC/HRP kit (Vector Laboratories) according to the manufacturer's instructions. Peroxidase enzymatic development was carried out using 3,3′-diaminobenzidine, resulting in dark brown products in positive cells. After washing, sections were again treated at 121°C for 20 min in Target Retrieval Solution (S1699; DAKO Japan) and incubated with mouse monoclonal anti-FOXP3 at 4°C overnight. After washing, sections were incubated with biotin-labeled horse antimouse IgG (Vector Laboratories). After further washing, sections were treated with Vectastain ABC/HRP kit (Vector Laboratories). Peroxidase enzymatic development was carried out using Vector-SG (Vector Laboratories), resulting in blue/gray products in positive cells. After that, sections were dehydrated and mounted in Nonaqueous Mounting Medium (Muto Pure Chemicals). For each slide, >200 cells were counted to determine the frequency of positive cells.

Results

Relationship between EBV latent gene expression and cell morphology in ALPD tumor cells. Previously, Brink *et al*. examined expression of EBV latent genes in tumor cells from PTLD and AIDS-related lymphomas.[21] They showed that the graded expression of EBV latent genes at the single cell level was closely associated with cellular morphology. Thus, smaller tumor cells were positive for EBNA2 but negative for LMP1, whereas larger tumor cells, sometimes resembling H-RS cells of cHL, were positive for LMP1 but negative for EBNA2, and intermediate ones were positive for both LMP1 and EBNA2.[21] Furthermore, a major fraction of small tumor cells were only positive for EBNA1 and EBERs.[21] We therefore first examined expression of EBV latent genes and cellular morphology in ALPD ($n = 13$) together with PTLD ($n = 6$) and cHL ($n = 8$) (Table 1). In ALPD, 10 out of 13 cases were clearly positive for LMP1, whereas only three cases were found to be positive for EBNA2. Histologically, ALPD is often highly polymorphic in appearance (Fig. 1a), and EBER$^+$ tumor cells constitute only a minor population admixed with

doi: 10.1111/j.1349-7006.2007.00687.x
© 2008 Japanese Cancer Association

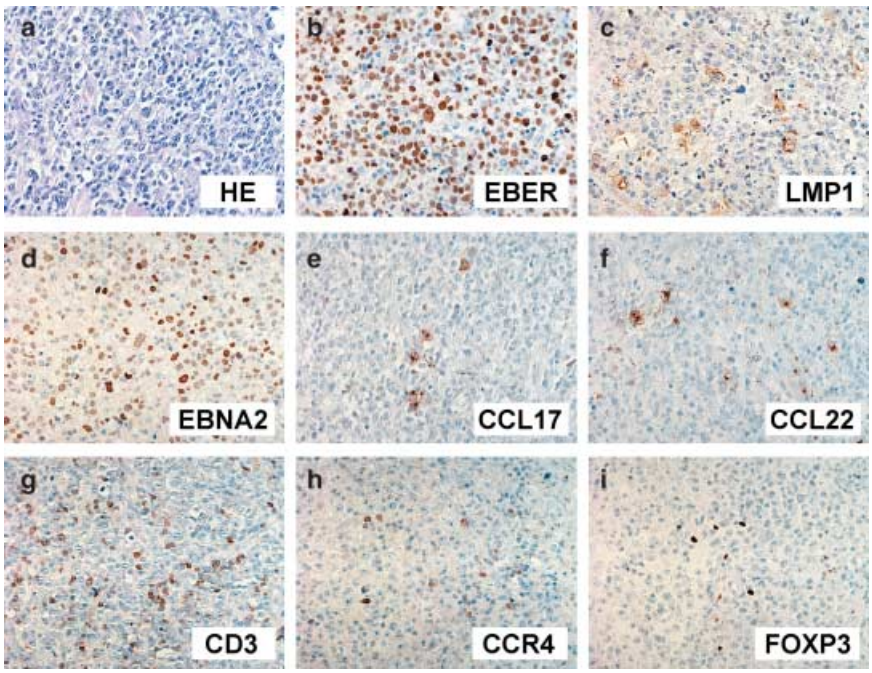

Fig. 2. *In situ* hybridization and immunohistochemistry of post-transplantation lymphoproliferative disorder (PTLD). (a) Hematoxylin–eosin (HE); (b) Epstein–Barr virus-encoded RNA (EBER; *in situ* hybridization); (c) latent membrane protein 1 (LMP1); (d) Epstein–Barr virus nuclear antigen 2 (EBNA2); (e) chemokine CCL17; (f) chemokine CCL22; (g) CD3; (h) chemokine receptor CCR4; (i) forkhead box protein 3 FOXP3. Representative results from six PTLD cases are shown (as detailed in Table 1). (a–g) case #4; (h, i) case #1 (original magnification: ×400).

abundant infiltrating cells (Fig. 1b). The cellular sizes of EBER$^+$ tumor cells were often quite heterogeneous, including large-sized RS-like cells. Tumor cells expressing LMP1 were typically large in size (Fig. 1c), whereas those expressing EBNA2 were small to intermediate (Fig. 1d). Tumor cells expressing only EBERs were small. However, PTLD tumors appeared more homogeneous (Fig. 2a) and contained more numerous EBER$^+$ tumor cells (Fig. 2b). Again LMP1 was expressed by large-sized tumor cells (Fig. 2c), whereas tumor cells expressing EBNA2 were smaller in size (Fig. 2d). Furthermore, tumor cells only positive for EBERs were small as reported previously.[21] Thus, at the single cell level, ALPD tumor cells were heterogeneous in terms of the expression of EBV latent genes together with the accompanying differences in the cellular morphology as reported for PTLD and AIDS-associated lymphomas.[21] However, H-RS cells even from EBV$^+$ cHL cases were typically more homogeneous in size and appearance as shown by EBERs (Fig. 3a) and LMP1 staining (Fig. 3b).

Immunohistochemistry for CCL17 and CCL22. In ALPD, CCL17 and CCL22 were found to be selectively expressed by RS-like large-sized tumor cells that obviously corresponded to those expressing LMP1 (Fig. 1e,f). Such RS-like cells were also strongly positive for nuclear staining of NF-κB, supporting the activation of NF-κB (Fig. 1g). By counting >100 RS-like large-sized tumor cells in sections from six cases (#1, 3, 5, 8, 12, 13), we found that 97 ± 3% (mean ± SD, $n = 6$) were positive for LMP1, 96 ± 3% were positive for CCL22, and 91 ± 7% were positive for NF-κB. Similarly, by counting >100 RS-like large-sized tumor cells in sections from five cases (#3, 6, 8, 12, 13), 89 ± 6% (mean ± SD, $n = 5$) were positive for CCL17. We also carried out double staining of LMP1 and CCL17 or CCL22. As shown in Fig. 4, the signals of LMP1 and CCL17 or CCL22 were mostly overlapping in RS-like large-sized tumor cells in all eight cases examined. Similarly, large-sized tumor cells were selectively positive for CCL17 and CCL22 in PTLD (Fig. 2e,f). Furthermore, ALPD and PTLD cases not expressing LMP1 were found to be negative for these chemokines (Table 1). These results are highly consistent with our previous findings that LMP1 induces CCL17 and CCL22 through activation of NF-κB in EBV-immortalized B cells.[20] H-RS cells in all cHL cases ($n = 8$) were consistently positive for

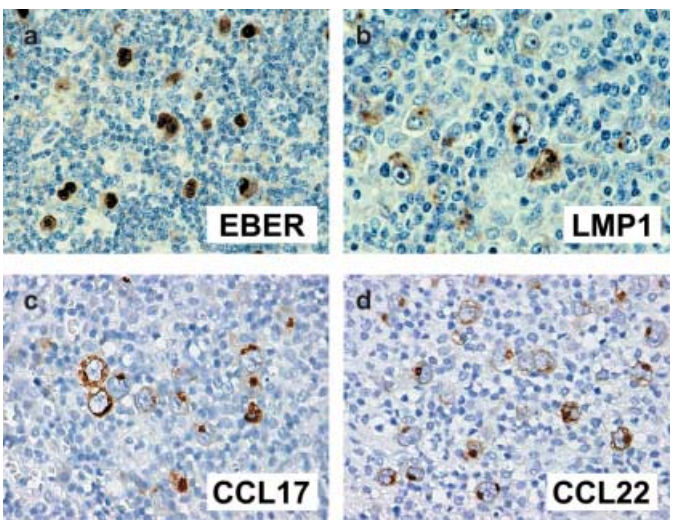

Fig. 3. *In situ* hybridization and immunohistochemistry of classical Hodgkin's lymphoma (cHL). (a) Epstein–Barr virus-encoded RNA (EBER; *in situ* hybridization); (b) latent membrane protein 1 (LMP1); (c) chemokine CCL17; (d) chemokine CCL22. Representative results from eight cHL cases are shown (as detailed in Table 1). (a, b) case #21; (c, d) case #23 (original magnification: ×400).

CCL17 (Fig. 3c) and CCL22 (Fig. 3d) irrespective of EBV association, as reported previously.[10,13]

Infiltration of CCR4$^+$ cells and FOXP3$^+$ cells in ALPD. Large numbers of CD3$^+$ T cells were almost homogeneously infiltrating in the background of ALPD (Fig. 1h). Furthermore, the majority of infiltrating cells in ALPD were CD45RO$^+$ (Fig. 1i). Thus, the majority of infiltrating cells in ALPD were memory/effector T cells. In accordance with the production of CCL17 and CCL22 by large-sized tumor cells, cells expressing their shared receptor CCR4[15] were also abundantly present in the background of ALPD (Fig. 1j). CCR4$^+$ cells could be Th2 cells or regulatory T cells.[14,15] Indeed, cells expressing FOXP3[22] were also present

© 2008 Japanese Cancer Association

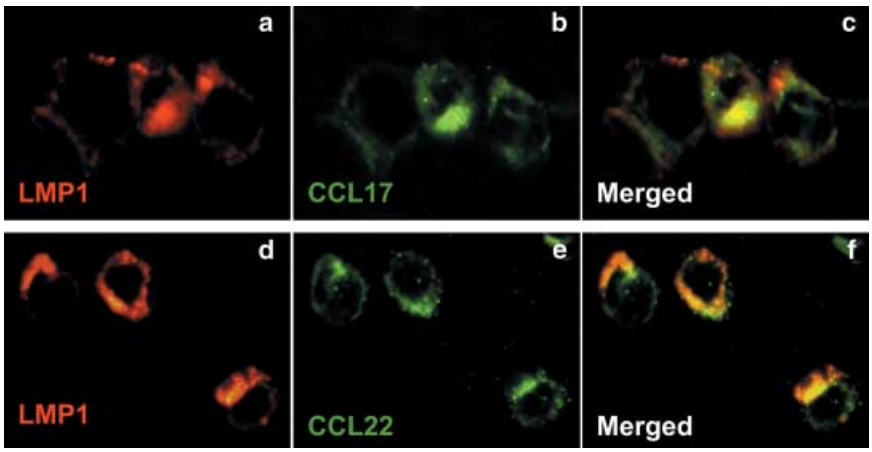

Fig. 4. Double immunofluorescent staining of latent membrane protein 1 (LMP1) and chemokines CCL17 or CCL22. (a) LMP1; (b) CCL17; (c) LMP1 and CCL17; (d) LMP1; (e) CCL22; (f) LMP1 and CCL22. Representative results from eight age-related Epstein–Barr virus-positive lymphoproliferative disorder cases are shown (case #1, 3, 5, 6, 7, 8, 12, 13 as detailed in Table 1). (a–f): case #8 (original magnification: ×400).

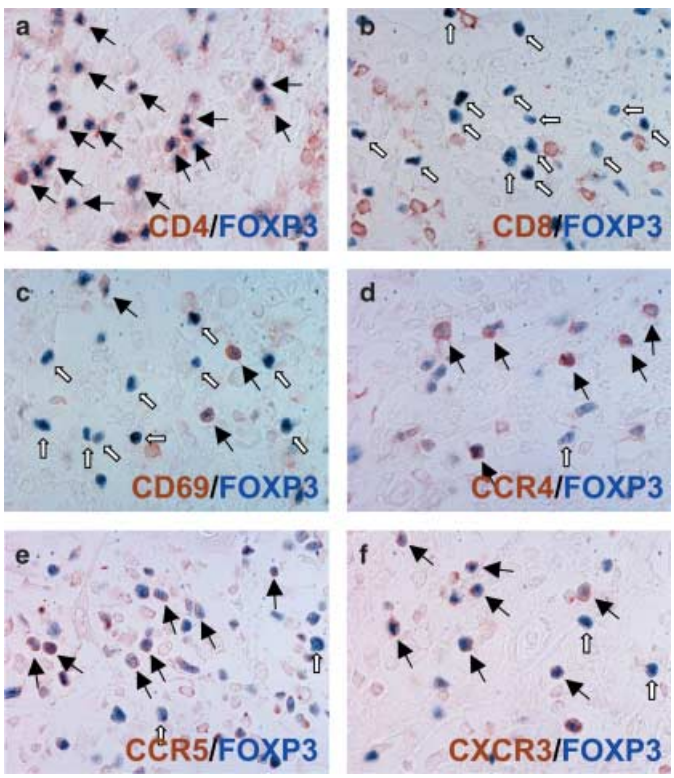

Fig. 5. Double immunoenzyme staining of forkhead box protein 3 (FOXP3) with surface markers/chemokine receptors in age-related Epstein–Barr virus-positive lymphoproliferative disorder (ALPD). (a) CD4 and FOXP3; (b) CD8 and FOXP3; (c) CD69 and FOXP3; (e) chemokine receptor CCR4 and FOXP3; (e) chemokine receptor CCR5 and FOXP3; (f) chemokine receptor CXCR3 and FOXP3. Arrows indicate FOXP3$^+$ cells that are also positive for CD4 (a), CD69 (c), CCR4 (d), CCR5 (e), or CXCR3 (f). Open arrows indicate FOXP3$^+$ cells that are negative for CD8 (b), CD69 (c), CCR4 (d), CCR5 (e), or CXCR3 (f). Representative results from three ALPD cases (case #6, 12, 13, as detailed in Table 1) are shown. (a–d) case #6 (original magnification: ×400).

in ALPD (Fig. 1k). In contrast, infiltrating CD3$^+$ T cells were far less frequent in PTLD (Fig. 2g), and only very few CCR4$^+$ cells (Fig. 2h) and FOXP3$^+$ cells (Fig. 2i) were present in the background.

Expression of various chemokine receptors by FOXP3$^+$ cells. We further analyzed FOXP3$^+$ cells in three ALPD cases (#6, 12, 13) for co-expression of CD4 and CCR4. By double staining with CD4 (Fig. 5a) or CD8 (Fig. 5b), essentially all FOXP3$^+$ (>95%) were confirmed to be CD4$^+$ T cells. To exclude activated T cells expressing CD69 from authentic CD69$^-$ regulatory T cells,[24] CD69 and FOXP3 were also double-stained. This confirmed that the majority of FOXP3$^+$ cells (66 ± 6% mean ± SD; $n = 3$) were negative for CD69 (Fig. 5c), representing the authentic regulatory T cells.[24] By double staining with CCR4 (Fig. 5d), the majority of FOXP3$^+$ cells (80 ± 3% mean ± SD; $n = 3$) were confirmed to be CCR4$^+$.

Recent studies have shown that regulatory T cells can express various chemokine receptors other than CCR4.[25–27] Therefore, we further examined co-expression of FOXP3 and CCR5 or CXCR3 in the same three ALPD tumor tissues. Indeed, we observed that the majority of FOXP3$^+$ cells were also positive for CCR5 (68 ± 8%) and CXCR3 (77 ± 6% mean ± SD; $n = 3$). Therefore, given the high frequencies of FOXP3$^+$ cells expressing CCR4, CCR5 or CXCR3, it is likely that a large fraction of regulatory T cells infiltrating ALPD tumor tissues co-expressed two or three of these chemokine receptors.

Discussion

EBV$^+$ B-cell lymphoproliferative disorders are known to occur in patients with immunodeficiency conditions.[1] ALPD is a newly proposed member of EBV$^+$ B-cell lymphoproliferative disorders.[2] However, ALPD patients have no apparent predisposing immunodeficiency conditions except for relatively advanced age.[2] Thus, an age-associated decline in cellular immunity to EBV might be partly responsible for its development. Histologically, ALPD often shows similarity to cHL, also frequently associated with EBV.[6] Thus, the distinction between ALPD and EBV$^+$ cHL might be sometimes fuzzy. However, ALPD can be differentiated from cHL in a number of ways[2,5,6]: (i) ALPD is typically associated with advanced age, whereas cHL is usually a disease of young people; (ii) ALPD often involves extranodal sites, whereas cHL is primarily a nodal disease; (iii) ALPD tumor cells are 100% EBV$^+$, whereas EBV association is less than 50% in cHL; (iv) ALPD tumor cells are clearly positive for the B-cell markers such as CD20 and/or CD79a, whereas H-RS cells are typically negative for such B-cell markers; (v) ALPD tumor cells are negative for CD15 and variably positive for CD30, whereas H-RS cells are typically positive for CD15 and CD30; and, as shown in the present study, (vi) ALPD tumor cells are often highly polymorphic in association with differential expression of EBV latent genes at the single cell level, whereas H-RS cells are typically quite homogeneous in appearance and large in size. Collectively, ALPD is substantially different from cHL and is more similar to PTLD and AIDS-related EBV$^+$ lymphomas.[28,29] However, in contrast to PTLD and other opportunistic EBV$^+$ B cell lymphomas in immunologically compromised patients, ALPD typically occurs in elderly patients without any apparent

doi: 10.1111/j.1349-7006.2007.00687.x
© 2008 Japanese Cancer Association

predisposing immunodeficiency conditions.[2] Thus, ALPD might be potentially misdiagnosed as EBV$^+$ cHL. The so-called atypical EBV$^+$ cHL in elderly patients, with often poor prognosis,[30,31] might thus contain substantial cases of ALPD. Future studies involving micromanipulation of single tumor cells will be necessary to definitively determine the relationship between ALPD and EBV$^+$ cHL.

It is now known that H-RS cells produce various chemokines that are likely to account for the characteristic accumulation of background infiltrating cells in cHL.[9–13] In particular, CCL17 and CCL22 are the ligands of CCR4 that are known to be selectively expressed by Th2 cells and regulatory T cells.[14,15] Thus, the production of CCL17 and CCL22 by H-RS cells is considered to be advantageous for immune evasion of tumor cells by attracting Th2 cells and regulatory T cells through CCR4.[13,19] Indeed, the abundant presence of cells expressing CCR4 and FOXP3$^+$ regulatory T cells has been indicated in cHL.[16,17] Furthermore, the majority of FOXP3$^+$ regulatory T cells in cHL were shown to express CCR4.[18] Previously, we have shown that EBV-immortalized B cells produce various chemokines including LMP1-inducible CCL17 and CCL22.[20] In the present study, we have shown that LMP1-expressing large-sized tumor cells in ALPD are selectively positive for CCL17 and CCL22. Moreover, CCR4$^+$ cells and FOXP3$^+$ regulatory T cells are abundantly present in the background of ALPD. We have further shown that the majority of FOXP3$^+$ cells are CCR4$^+$. Collectively, a very similar mechanism involving CCL17 and CCL22 might be operative for immune evasion in ALPD as in cHL. However, we also observed FOXP3$^+$ cells in one ALPD case (#10) in which tumor cells were not expressing LMP1 and therefore were also negative for CCL17 and CCL22. Recent studies have shown that regulatory T cells can express various chemokine receptors other than CCR4.[25–27] Indeed, we observed that a large fraction of FOXP3$^+$ cells in three ALPD tumor tissues (cases #6, 12, 13) also expressed CCR5 ($68 \pm 8\%$) or CXCR3 ($77 \pm 6\%$) as well as CCR4 ($80 \pm 3\%$). Therefore, it is possible that the CCL17/CCL22–CCR4 axis is not the only chemokine system involved in the accumulation of FOXP3$^+$ regulatory T cells in ALPD.

NF-κB has been shown to be constitutively active in H-RS cells of cHL and to be essential for their survival and tumorigenicity.[32] Given that NF-κB is the transcriptional master regulator of immune responses and induces expression of various cellular genes, including chemokines and cytokines,[33] it is likely that the constitutive activation of NF-κB is also responsible for the production of chemokines by H-RS cells of cHL. In the case of ALPD tumor cells, EBV-encoded oncogenic protein LMP1 might activate NF-κB.[34] We have indeed shown that LMP1-expressing large-sized tumor cells in ALPD are the main producers of CCL17 and CCL22, and are mostly positive for the nuclear staining of NF-κB (Fig. 1). Thus, the LMP1-mediated activation of NF-κB is likely to be important for production of CCL17 and CCL22 as well as tumor cell growth and survival in ALPD.

In conclusion, LMP1-expressing large-sized tumor cells in ALPD selectively produce CCL17 and CCL22 and might attract cells expressing CCR4, including FOXP3$^+$ regulatory T cells in ALPD. LMP1-expressing tumor cells in PTLD also produce CCL17 and CCL22, but the extent of cellular infiltration is far less than that in ALPD. This is most probably because PTLD develops in patients with severe iatrogenic impairment of lymphocytes, whereas patients with ALPD still retain relatively normal lymphocyte functions. Thus, like cHL, ALPD could provide a useful model to understand interactions between tumor cells and host immune cells. As CCR4-expressing cells constitute only a fraction of infiltrating CD3$^+$ T cells (Fig. 1), other chemokine–chemokine receptor axes are also likely to be involved in the background accumulation of reactive infiltrating cells including FOXP3$^+$ regulatory T cells in ALPD.

Acknowledgments

This work was supported by: a Grant-in-Aid for Young Scientist (B) from the Ministry of Education, Culture, Sports, Science and Technology, Japan (to T.N.); Solution-Oriented Research for Science and Technology from Japan Science and Technology Corporation (to O.Y.); and the High-Tech Research Center Project for Private Universities, matching fund subsidy from the Ministry of Education, Culture, Sports, Science and Technology of Japan, 2002–09 (to O.Y.). We thank Dr Chiho Ohbayashi (Kobe University Hospital), Sakae Murakami (Chukyo Hospital Nagoya) and Hisashi Tateyama (Kasugai Municipal Hospital) for providing valuable samples. We also thank Namie Sakiyama for her excellent technical assistance.

References

1 Thompson MP, Kurzrock R. Epstein–Barr virus and cancer. *Clin Cancer Res* 2004; **10**: 803–21.
2 Oyama T, Ichimura K, Suzuki R *et al*. Senile EBV$^+$ B-cell lymphoproliferative disorders: a clinicopathologic study of 22 patients. *Am J Surg Pathol* 2003; **27**: 16–26.
3 Oyama T, Yamamoto K, Asano N *et al*. Age-related EBV-associated B-cell lymphoproliferative disorders constitute a distinct clinicopathologic group: a study of 96 patients. *Clin Cancer Res* 2007; **13**: 5124–32.
4 Park S, Lee J, Ko YH *et al*. The impact of Epstein–Barr virus status on clinical outcome in diffuse large B-cell lymphoma. *Blood* 2007; **110**: 972–8.
5 Haluska FG, Brufsky AM, Canellos GP. The cellular biology of the Reed–Sternberg cell. *Blood* 1994; **84**: 1005–19.
6 Brauninger A, Schmitz R, Bechtel D, Renne C, Hansmann ML, Kuppers R. Molecular biology of Hodgkin's and Reed/Sternberg cells in Hodgkin's lymphoma. *Int J Cancer* 2006; **118**: 1853–61.
7 Kuppers R, Rajewsky K. The origin of Hodgkin and Reed/Sternberg cells in Hodgkin's disease. *Annu Rev Immunol* 1998; **16**: 471–93.
8 Schwering I, Brauninger A, Klein U *et al*. Loss of the B-lineage-specific gene expression program in Hodgkin and Reed–Sternberg cells of Hodgkin lymphoma. *Blood* 2003; **101**: 1505–12.
9 Hanamoto H, Nakayama T, Miyazato H *et al*. Expression of CCL28 by Reed–Sternberg cells defines a major subtype of classical Hodgkin's disease with frequent infiltration of eosinophils and/or plasma cells. *Am J Pathol* 2004; **164**: 997–1006.
10 Hedvat CV, Jaffe ES, Qin J *et al*. Macrophage-derived chemokine expression in classical Hodgkin's lymphoma: application of tissue microarrays. *Mod Pathol* 2001; **14**: 1270–6.
11 Maggio EM, Van Den Berg A, Visser L *et al*. Common and differential chemokine expression patterns in rs cells of NLP, EBV positive and negative classical Hodgkin lymphomas. *Int J Cancer* 2002; **99**: 665–72.
12 Teruya-Feldstein J, Jaffe ES, Burd PR, Kingma DW, Setsuda JE, Tosato G. Differential chemokine expression in tissues involved by Hodgkin's disease: direct correlation of eotaxin expression and tissue eosinophilia. *Blood* 1999; **93**: 2463–70.
13 van den Berg A, Visser L, Poppema S. High expression of the CC chemokine TARC in Reed–Sternberg cells. A possible explanation for the characteristic T-cell infiltratein Hodgkin's lymphoma. *Am J Pathol* 1999; **154**: 1685–91.
14 Iellem A, Mariani M, Lang R *et al*. Unique chemotactic response profile and specific expression of chemokine receptors CCR4 and CCR8 by CD4 (+) CD25 (+) regulatory T cells. *J Exp Med* 2001; **194**: 847–53.
15 Yoshie O, Imai T, Nomiyama H. Chemokines in immunity. *Adv Immunol* 2001; **78**: 57–110.
16 Marshall NA, Christie LE, Munro LR *et al*. Immunosuppressive regulatory T cells are abundant in the reactive lymphocytes of Hodgkin lymphoma. *Blood* 2004; **103**: 1755–62.
17 Alvaro T, Lejeune M, Salvado MT *et al*. Outcome in Hodgkin's lymphoma can be predicted from the presence of accompanying cytotoxic and regulatory T cells. *Clin Cancer Res* 2005; **11**: 1467–73.
18 Ishida T, Ishii T, Inagaki A *et al*. Specific recruitment of CC chemokine receptor 4-positive regulatory T cells in Hodgkin lymphoma fosters immune privilege. *Cancer Res* 2006; **66**: 5716–22.
19 Poppema S, van den Berg A. Interaction between host T cells and Reed–Sternberg cells in Hodgkin lymphomas. *Semin Cancer Biol* 2000; **10**: 345–50.
20 Nakayama T, Hieshima K, Nagakubo D *et al*. Selective induction of Th2-attracting chemokines CCL17 and CCL22 in human B cells by latent membrane protein 1 of Epstein–Barr virus. *J Virol* 2004; **78**: 1665–74.

© 2008 Japanese Cancer Association

21 Brink AA, Dukers DF, van den Brule AJ *et al*. Presence of Epstein–Barr virus latency type III at the single cell level in post-transplantation lymphoproliferative disorders and AIDS related lymphomas. *J Clin Pathol* 1997; **50**: 911–18.
22 Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. *Science* 2003; **299**: 1057–61.
23 Lan HY, Mu W, Nikolic-Paterson DJ, Atkins RC. A novel, simple, reliable, and sensitive method for multiple immunoenzyme staining: use of microwave oven heating to block antibody crossreactivity and retrieve antigens. *J Histochem Cytochem* 1995; **43**: 97–102.
24 Lim HW, Hillsamer P, Kim CH. Regulatory T cells can migrate to follicles upon T cell activation and suppress GC-Th cells and GC-Th cell-driven B cell responses. *J Clin Invest* 2004; **114**: 1640–9.
25 Wysocki CA, Jiang Q, Panoskaltsis-Mortari A *et al*. Critical role for CCR5 in the function of donor $CD4^+CD25^+$ regulatory T cells during acute graft-versus-host disease. *Blood* 2005; **106**: 3300–7.
26 Valmori D, Merlo A, Souleimanian NE, Hesdorffer CS, Ayyoub M. A peripheral circulating compartment of natural naive CD4 Tregs. *J Clin Invest* 2005; **115**: 1953–62.
27 Lim HW, Broxmeyer HE, Kim CH. Regulation of trafficking receptor expression in human forkhead box P3+ regulatory T cells. *J Immunol* 2006; **177**: 840–51.
28 Hopwood P, Crawford DH. The role of EBV in post-transplant malignancies: a review. *J Clin Pathol* 2000; **53**: 248–54.
29 Chetty R, Biddolph S, Gatter K. An immunohistochemical analysis of Reed–Sternberg-like cells in posttransplantation lymphoproliferative disorders: the possible pathogenetic relationship to Reed–Sternberg cells in Hodgkin's disease and Reed–Sternberg-like cells in non-Hodgkin's lymphomas and reactive conditions. *Hum Pathol* 1997; **28**: 493–8.
30 Glaser SL, Dorfman RF, Clarke CA. Expert review of the diagnosis and histologic classification of Hodgkin disease in a population-based cancer registry: interobserver reliability and impact on incidence and survival rates. *Cancer* 2001; **92**: 218–24.
31 Jarrett RF, Krajewski AS, Angus B *et al*. The Scotland and Newcastle epidemiological study of Hodgkin's disease: impact of histopathological review and EBV status on incidence estimates. *J Clin Pathol* 2003; **56**: 811–16.
32 Bargou RC, Emmerich F, Krappmann D *et al*. Constitutive nuclear factor-kappaB-RelA activation is required for proliferation and survival of Hodgkin's disease tumor cells. *J Clin Invest* 1997; **100**: 2961–9.
33 Baldwin AS Jr. The NF-kappa B and I kappa B proteins: new discoveries and insights. *Annu Rev Immunol* 1996; **14**: 649–83.
34 Kaye KM, Devergne O, Harada JN *et al*. Tumor necrosis factor receptor associated factor 2 is a mediator of NF-kappa B activation by latent infection membrane protein 1, the Epstein–Barr virus transforming protein. *Proc Natl Acad Sci USA* 1996; **93**: 11085–90.

doi: 10.1111/j.1349-7006.2007.00687.x
© 2008 Japanese Cancer Association