Selective Induction of Th2-Attracting Chemokines CCL17 and CCL22 in Human B Cells by Latent Membrane Protein 1 of Epstein-Barr Virus

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Chemokines are likely to play important roles in the pathophysiology of diseases associated with Epstein-Barr virus (EBV). Here, we have analyzed the repertoire of chemokines expressed by EBV-infected B cells. EBV infection of B cells induced expression of TARC/CCL17 and MDC/CCL22, which are known to attract Th2 cells and regulatory T cells via CCR4, and also upregulated constitutive expression of MIP-1 α /CCL3, MIP-1 β /CCL4, and RANTES/CCL5, which are known to attract Th1 cells and cytotoxic T cells via CCR5. Accordingly, EBV-immortalized B cells secreted these chemokines, especially CCL3, CCL4, and CCL22, in large quantities. EBV infection or stable expression of LMP1 also induced CCL17 and CCL22 in a B-cell line, BJAB. The inhibitors of the TRAF/NF- κ B pathway (BAY11-7082) and the p38/ATF2 pathway (SB202190) selectively suppressed the expression of CCL17 and CCL22 in EBV-immortalized B cells and BJAB-LMP1. Consistently, transient-transfection assays using CCL22 promoter-reporter constructs demonstrated that two NF- κ B sites and a single AP-1 site were involved in the activation of the CCL22 promoter by LMP1. Finally, serum CCL22 levels were significantly elevated in infectious mononucleosis. Collectively, LMP1 induces CCL17 and CCL22 in EBV-infected B cells via activation of NF- κ B and probably ATF2. Production of CCL17 and CCL22, which attract Th2 and regulatory T cells, may help EBV-infected B cells evade immune surveillance by Th1 cells. However, the concomitant production of CCL3, CCL4, and CCL5 by EBV-infected B cells may eventually attract Th1 cells and cytotoxic T cells, leading to elimination of EBV-infected B cells at latency III and to selection of those with limited expression of latent genes.

Epstein-Barr virus (EBV) is a ubiquitous gammaherpesvirus that infects all human populations, with the great majority of adults having antibodies to the virus (23). Primary infection by EBV is usually subclinical in early childhood but often causes infectious mononucleosis (IM) in teenagers and young adults. IM is characterized by massive lymphocytosis consisting of EBV-infected B cells in the earlier phase and EBV-specific cytotoxic CD8⁺ T cells (atypical lymphocytes) in the later phase. Following primary infection, EBV establishes a lifelong latent infection in B cells and is intermittently secreted into saliva. EBV is also closely associated with the occurrence of various human malignancies, such as endemic Burkitt's lymphoma (BL), nasopharyngeal carcinoma in southern China, a substantial fraction of Hodgkin's lymphoma, and opportunistic B-cell lymphomas in severely immunocompromised patients (23). In vitro, the oncogenic property of EBV is demonstrated by its efficient transformation of primary B cells into continuously growing lymphoblastoid cells (23). Based on the expression pattern of viral latency-associated genes, there are three major types of latency in EBV infection (23). In latency I, the EBV-encoded latent-protein expression is mostly restricted to EBNA1. This type of latency is typically seen in BL (7). In latency III, on the other hand, the expression of viral proteins

includes all six nuclear proteins (EBNA1 to -6) and three membrane proteins (LMP1, LMP2A, and LMP2B). This type of latency occurs in EBV-immortalized B cells in vitro, EBV-infected B cells in IM patients, and EBV-associated opportunistic lymphomas (3, 21). The intermediate latency II (EBNA1⁺, LMP1⁺, LMP2A⁺, and LMP2B⁺) is typically seen in nasopharyngeal carcinoma and EBV-associated Hodgkin's disease (6, 35). Furthermore, Joseph et al. and Thorley-Lawson have recently demonstrated several forms of latent EBV infection in healthy carriers (21, 41). In the tonsil, EBV-infected naive B cells express all known latent proteins, while EBV-infected memory B cells express a more restricted pattern of latent proteins (EBNA1⁺, LMP1⁺, and LMP2A⁺). In the peripheral blood, on the other hand, circulating EBV-infected memory B cells express no latent proteins, with the possible exception of LMP2A (21, 41). These different types of latency, which are closely related to the viral strategy for persistence and evasion of host immunosurveillance, are also likely to affect the gene expression of EBV-infected B cells differently.

Chemokines are a large group of structurally related cytokines that induce directed migration of various types of leukocytes through interactions with a group of seven transmembrane G protein-coupled receptors (45). In humans, >40 chemokines and 18 functional chemokine receptors have been identified. Based on the arrangement of the conserved cysteine residues in the N-terminal region, chemokines are classified into four subfamilies: CC, CXC, C, and CX3C. Recently, based

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on the classification of these four subfamilies, the systematic nomenclature system of the chemokine ligands has been formulated (47). A large number of studies have shown that migration and tissue microenvironmental localization of lymphocyte classes and subsets are finely regulated through the expression of a specific set of chemokine receptors in accordance with their differentiation pathways and maturation stages (45). Previously, the profile of chemokine receptor expression on EBV-immortalized B cells was studied, and it was demonstrated that, compared to normal B cells, EBV-immortalized human B cells express CCR6 and CCR10 at high levels and CXCR4 and CXCR5 at low levels (29). It was further shown that EBNA2 upregulates CCR6, while EBNA2, as well as LMP1, downregulates CXCR4 (29). On the other hand, the expression of CCR10, which is not observed in circulating B cells, or the downregulation of CXCR5 in EBV-immortalized B cells was not directly induced by EBV infection and thus was probably due to the plasmablast-like differentiation stage of EBV-immortalized B cells (29). Indeed, we and others have recently shown that plasma cells selectively express CCR10 and downregulate CXCR5 (25, 30). The ligands of CCR6 and CCR10, LARC/CCL20 and MEC/CCL28, respectively, are known to be expressed by epithelial cells in certain mucosal tissues, such as salivary glands and the tonsil (34, 40, 44), while CXCR5 promotes the migration of B cells into the B-cell follicles by responding to BLC/CXCL13 that is selectively expressed by follicular dendritic cells (13, 27, 45). Thus, our findings may provide a mechanistic explanation for the previous histological observation that EBV-infected B cells in IM tend to avoid the germinal center and preferentially accumulate under the epithelium of certain mucosal tissues (2, 31).

To further explore the potential roles of the chemokine system in the EBV-associated diseases, the present study was initiated to elucidate the repertoire of chemokines produced by EBV-immortalized B cells. We have found that upon EBV infection, B cells are induced to express TARC/CCL17 and MDC/CCL22, which are known to preferentially attract Th2 cells and regulatory T cells via CCR4 (18, 20, 45). EBV infection also induces B cells to upregulate their constitutive expression of MIP-1 α /CCL3, MIP-1 β /CCL4, and RANTES/CCL5, which are known to attract Th1 cells and activated cytotoxic T cells via CCR5 (32, 45). We have further shown that LMP1 induces the expression of TARC/CCL17 and MDC/CCL22 in B cells via the activation of NF- κ B and probably ATF2. Selective induction of Th2-attracting chemokines in B cells by LMP1 may be advantageous for EBV-infected cells to suppress host Th1 immune responses. However, the concomitant upregulation of CCR5 ligand chemokines by EBV-infected B cells, which is likely to be the phenotype of activated B cells (4), may eventually attract Th1 cells and cytotoxic T cells (19, 32, 45), leading to elimination of B cells carrying EBV at latency III and selection of less immunogenic EBV-infected memory B cells (41).

MATERIALS AND METHODS

Cells and reagents. BCL-NU, BCL-SH, BCL-SM, and BCL-TOS are EBV-immortalized human B-cell lines generated from peripheral blood mononuclear cells (PBMC) from healthy adult donors (29). AKATA, Daudi, Jijoye, and Raji are EBV-positive BL lines. BJAB is a human EBV-negative B-cell line. BJAB and BJAB infected with the B95-8 strain of EBV were kindly provided by E.

Kieff. The pZioNeoSV(x)I expression vectors for EBNA2 and LMP1 were also provided by E. Kieff. BJAB clones stably expressing EBNA2 or LMP1 were described previously (29). The mouse L1.2 pre-B-cell line was kindly provided by E. Butcher. L1.2 cells stably expressing CCR4 (L-CCR4) and CCR5 (L-CCR5) were described previously (33). All of the cell lines were maintained in RPMI 1640 supplemented with 10% fetal bovine serum and antibiotics. CD19⁺ B cells were purified from PBMC by positive selection with magnetic cell sorting (Miltenyi Biotec Bergish Gladbach, Germany) after being labeled with anti-CD19 microbeads. The purity of CD19⁺ B cells was >95%, as revealed by flow cytometry after they were stained with fluorescein isothiocyanate-labeled anti-CD19 (Beckman Coulter, San Jose, Calif.). CD19⁺ B cells were infected with the B95-8 strain of EBV as described previously (29). In some experiments, the cells were treated for 12 h with various signaling inhibitors: PD98059, SB202190, JNK inhibitor, JAK3 inhibitor, AG490, and BAY11-7082. All of the inhibitors were purchased from Calbiochem (La Jolla, Calif.). Recombinant human chemokines were all purchased from R&D Systems (Minneapolis, Minn.).

RT-PCR analysis. Reverse transcription (RT)-PCR analysis was carried out as described previously (29). In brief, total RNA was prepared from cells by using Trizol reagent (GIBCO-BRL, Gaithersburg, Md.). The RNA was further purified by using RNeasy (Qiagen, Hilden, Germany). Total RNA (1 μ g) was reverse transcribed using oligo(dT)₁₈ primer and SuperScript II reverse transcriptase (GIBCO-BRL). The resulting first-strand DNA (equivalent to 20 ng of total RNA) and original total RNA (20 ng) were amplified in a final volume of 20 μ l containing 10 pmol of each primer and 1 U of Ex-Taq polymerase (Takara Shuzo, Kyoto, Japan). The amplification conditions were denaturation at 94°C for 30 s (5 min for the first cycle), annealing at 60°C for 30 s, and extension at 72°C for 30 s (5 min for the last cycle) for 33 cycles for all chemokine receptors and 27 cycles for GAPDH (glyceraldehyde-3-phosphate dehydrogenase). The amplification products (10 μ l each) were separated by electrophoresis on 2% agarose and stained with ethidium bromide. The primers used were 5'-GTGG CTCTCTTGGCAGCCTTCCTGAT-3' (+) and 5'-TCTCCACAACCCCTGTC ACCCAGTTT-3' (-) for IL-8/CXCL8; 5'-TGCAAGCCAAATTTGTCCACG TGTG-3' (+) and 5'-GCAGCTGATTGGTGACCATCATTTGG-3' (-) for IP-10/CXCL10; 5'-CATGCTGGTGAGCAAGCAGTTTGAA-3' (+) and 5'-CACTTCTGTGGGGTGTGGGGACAAG-3' (-) for MIG/CXCL9; 5'-CCC TCTGTGAGATCCGCTCTTTGGCGT-3' (+) and 5'-TCTGATTGCAACCTG AACCCCTGCTG-3' (-) for SDF-1 α /CXCL12; 5'-TGATCGAATTCAAATC TTGCCCGTG-3' (+) and 5'-AAGCTTGAGTTTGCCCCATCAGTCC-3' (-) for BLC/CXCL13; 5'-ACTACACGAGGTTCAGTCC-3' (+) and 5'-C TTTGTCCGAGACAGTGATC-3' (-) for CXCL16; 5'-GCCCGGTGTCAT CTTCCTAACCAAGC-3' (+) and 5'-AGGGGACAGGGGAACCTCTCAGAG CAA-3' (-) for MIP-1 α /CCL3; 5'-TGCTGCTTTTCTACACCGCGAGGA A-3' (+) and 5'-AGAAGGGACAGGAAGTGGCGAGAGGA-3' (-) for MIP-1 β /CCL4; 5'-CAAAGTGAAGCTCGCAGCTGCGCTC-3' (+) and 5'-AGCA CAGATCTCCTTGGCCACAATGG-3' (-) for MCP-1/CCL2; 5'-ACCACCTC TCACGCCAAAGCTCACAC-3' (+) and 5'-CGGCACAGATATCTTGGCC AGTTTG-3' (-) for eotaxin/CCL11; 5'-AGGACAGAGCATGGCTCGCCTA CAGA-3' (+) and 5'-TAATGGCAGGGAGGTAGGGCTCTGA-3' (-) for MDC/CCL22; 5'-ACTGCTCCAGGGATGCCATCGTTTTT-3' (+) and 5'-AC AAGGGGATGGGATCTCCCTCACTG-3' (-) for TARC/CCL17; 5'-CCCCG TGCCACATCAAGGAGTATTT-3' (+) and 5'-CGTCCAGCTGGGGAA GGTTTTTGTA-3' (-) for RANTES/CCL5; 5'-TACTCCACCTCTGCGCGG AATCAGAA-3' (+) and 5'-GTGAAACCTCCAACCCAGCAAGGTT-3' (-) for LARC/CCL20; 5'-CCAGCCTCACATCACTCACACCTTTGC-3' (+) and 5'-TGTTGTGAACACTACAGCAGGCACCC-3' (-) for ELC/CCL19; 5'-AACCAAGCTTAGGCTGCTCCATCCCA-3' (+) and 5'-TATGGCCCTTTA GGGTCTGTGTGACCG-3' (-) for SLC/CCL21; 5'-AAGGCCCAAGCAGCA CCAGAAGACAT-3' (+) and 5'-ATGTGGTTTCCAGAGCCCAATGG A-3' (-) for I-309/CCL1; 5'-CCAAGGTGTCTTTGAGGACTGCTGCC-3' (+) and 5'-GGGAGACATTCTTGTGCTGTGCTG-3' (-) for TECK/CCL25; 5'-CTACAGCAGCATTCCTACTGCTG-3' (+) and 5'-ATGGAGCTTTCTCTCT TGGTG-3' (-) for ILC/CCL27; 5'-AGAAGCCATACTTCCATTGC-3' (+) and 5'-AGCTTGCACTTTCATCCACTG-3' (-) for MEC/CCL28; 5'-ACACC ATCAGGAAGGCTCCTTGAGA-3' (+) and 5'-AGTGAATGAGCTGGC TGGTGAGA-3' (-) for lymphotactin/XCL1; 5'-GCCATGTTCACCTACC AGAGCCTCCA-3' (+) and 5'-TGGAAGGTGGAGAATGGTCAAGGCT G-3' (-) for fractalkine/CX3CL1; 5'-ATTTTGTCACCTCTGTGCACAACC-3' (+) and 5'-TGGTTCTGGACTATCTGGATCAT-3' (-) for EBNA2; 5'-CAT CGTTATGAGTGACTGGACTGG-3' (+) and 5'-AGAGCAATATGAGCA GGATGAGG-3' (-) for LMP1; and 5'-GCCAAGGTCATCCATGACAACCTT TGG-3' (+) and 5'-GCCTGCTTACCACCTTCTTGATGTC-3' (-) for GAPDH.

Quantitative real-time PCR was carried out using the TaqMan assay and 7700

Sequence Detection System (Applied Biosystems, Foster City, Calif.). The conditions for PCR were 50°C for 2 min, 95°C for 10 min, and then 50 cycles of 95°C for 15 s (denaturation) and 60°C for 1 min (annealing-extension). The primers and fluorogenic probes for MDC/CCL22, TARC/CCL17, I-309/CCL1, and GAPDH were from the TaqMan kit. Other primers used were as follows: 5'-C ACAGAATTTTCATAGCTGACTACTTTTGA-3' (+) and 5'-GCTTCTCTTTGG TTAGGAAGATGAC-3' (-) for MIP-1 α /CCL3; 5'-CAGCGCTCTCAGCACCC AA-3' (+) and 5'-TTCCTCGCGGTGTAAGAAAAG-3' (-) for MIP-1 β / CCL4; 5'-GCATCTGCCTCCCCATATTC-3' (+) and 5'-AGTGGGCGGGCA ATGTAG-3' (-) for RANTES/CCL5; and 5'-CTGGCTGCTGTGCATCCTTC A-3' (+) and 5'-TTTTCTTGGCAGCTTGCACTT-3' (-) for MEC/CCL28. Also, other probes used were as follows: 5'-ACGAGCAGCCAGTGCTCCAA GCC-3' (+) for MIP-1 α /CCL3; 5'-CTCAGACCTCCACCGCTGC-3' (+) for MIP-1 β /CCL4; 5'-TCGGACACCACCCCTGCTGCT-3' (+) for RAN- TES/CCL5; and 5'-CAGCCCGCACACCATACTGTTAAGCAGT-3' (+) for MEC/CCL28. The probes were labeled with the fluorescent reporter dye 6-FAM at the 5' end. Quantification of chemokine expression was obtained using se- quence detector system software (Applied Biosystems).

ELISA. Sandwich-type enzyme-linked immunosorbent assays (ELISAs) for MDC/CCL22 and TARC/CCL17 with a detection limit of 15 and 0.6 pg/ml, respectively, were described previously (16). Measurements of I-309/CCL1, MIP-1 α /CCL3, MIP-1 β /CCL4, RANTES/CCL5, and MEC/CCL28 were conducted using ELISA kits purchased from R&D Systems. For standardization of the assay, serially diluted recombinant chemokines were always included in each ELISA plate. Serum samples were obtained from healthy control donors ($n = 9$) and patients with IM ($n = 9$).

Chemotaxis assay. Chemotaxis assays were carried out using murine L1.2 cells stably expressing human CCR4 and CCR5 as described previously (29). In brief, 2.5×10^5 cells suspended in 25 μ l of phenol red-free RPMI 1640 containing 1 mg of bovine serum albumin (Sigma)/ml and 20 mM HEPES, pH 7.4, were applied to the upper wells of a CHEMOTX chemotaxis chamber with 5- μ m pore size (Neuro Probe, Gaithersburg, Md.). Test samples were preincubated with or without anti-TARC/CCL17 monoclonal antibody (54026.11) (R&D Systems), anti-MDC/CCL22 monoclonal antibody (57226.11) (R&D Systems), polyclonal anti-MIP-1 α /CCL3 (R&D Systems), polyclonal anti-MIP-1 β /CCL4 (R&D Sys- tems), or polyclonal anti-RANTES/CCL5 (R&D Systems) for 30 min at 0°C and then applied to the lower wells in a volume of 30 μ l. After 2 h at 37°C, cells that had migrated into lower wells were lysed with 0.1% Triton X-100 and measured by using PicoGreen double-stranded DNA quantitation reagent (Molecular Probes, Eugene, Oreg.). Cell migration was expressed as a percentage of input cells. All assays were done in triplicate.

Transfection and luciferase assay. To generate the promoter-reporter con- structs, the promoter regions of human TARC/CCL17 and MDC/CCL22 genes were cloned from the genomic DNA of normal human PBMC by using PCR and inserted into *XhoI-HindIII* sites in pGL3-luciferase (Promega, Madison, Wis.). Deletions and site-directed mutations were also done by PCR. The expression vector pcDNA3.1-(His)₆-p65 encoding the NF- κ B subunit p65 with additional hexahistidines and anti-Xpress epitopes (Invitrogen, Carlsbad, Calif.) was kindly provided by T. Okamoto (Nagoya City University, Nagoya, Japan) (11). Cells (5×10^6) in 250 μ l of growth medium were mixed with 10 μ g of each plasmid plus 3 μ g of pSV- β -galactosidase and transfected by electroporation at 230 V and 960 μ F using a Bio-Rad GenePulser II electroporator. After 27 to 36 h, luciferase activity was determined by using a Luciferase Assay kit (Promega). Luciferase activity was normalized by β -galactosidase activity, which served as an internal control for transfection efficiency.

RESULTS

Expression of various chemokine genes by EBV-immortal- ized B cells. Using RT-PCR, we first analyzed the expression profile of chemokine genes in EBV-immortalized B cells in comparison with that of fresh peripheral-blood CD19⁺ B cells and BL-derived B-cell lines. Figure 1 shows the highlight of the results. While peripheral-blood B cells hardly expressed TARC/CCL17, MDC/CCL22, or I-309/CCL1, EBV-immortal- ized B cells consistently expressed these chemokines. While peripheral-blood B cells weakly expressed MEC/CCL28, MIP-1 α /CCL3, MIP-1 β /CCL4, and RANTES/CCL5, EBV-immor- talized B cells consistently upregulated the expression of these chemokines. While peripheral-blood B cells hardly expressed

FIG. 1. RT-PCR analysis of expression of chemokine genes in EBV-immortalized B cells. Total RNA was prepared from PBMC treated with phytohemagglutinin (PHA) for 3 days (positive control), freshly isolated CD19⁺ peripheral-blood B cells, EBV-immortalized B-cell lines (BCLs) (LCL), and EBV-positive (+) BL cell lines (AKATA, Daudi, Jijoye, and Raji) (BL). RT-PCR analysis was carried out for a total of 22 chemokines (see Materials and Methods), ENBA2, LMP1, and GAPDH. For details, see Materials and Methods. The highlights of representative results from three independent experi- ments are shown.

IL-8/CXCL8 or LARC/CCL20, EBV-immortalized B cells ex- pressed these chemokines at low levels. On the other hand, neither peripheral-blood B cells nor EBV-immortalized B cells expressed MIG/CXCL9, IP-10/CXCL10, or I-TAC/CXCL11. BL cell lines consistently expressed only MIP-1 β . However, Raji cells also expressed TARC/CCL17 and MDC/CCL22 at high levels. Notably, Raji was the only BL cell line that ex- pressed EBNA2 and LMP1 at levels similar to those of EBV- immortalized B cells.

Secretion of chemokine proteins by EBV-immortalized B cells. We next examined the secretion of TARC/CCL17, MDC/ CCL22, I-309/CCL1, MEC/CCL28, MIP-1 α /CCL3, MIP-1 β / CCL4, and RANTES/CCL5 by EBV-immortalized B cells in comparison with peripheral blood B cells and two BL cell lines, Daudi and Raji. As shown in Fig. 2, while peripheral-blood CD19⁺ B cells secreted MDC/CCL22, MIP-1 α /CCL3, and MIP-1 β /CCL4 at very low levels, EBV-immortalized B cells

FIG. 2. Secretion of chemokines by EBV-immortalized B cells. Freshly isolated CD19⁺ peripheral-blood B cells from three donors, three EBV-immortalized B-cell lines (BCLs), and two BL cell lines (Daudi and Raji) were seeded in six-well plates at 10⁶/well and cultured for 3 days. TARC/CCL17, MDC/CCL22, I-309/CCL1, MEC/CCL28, MIP-1α/CCL3, MIP-1β/CCL4, and RANTES/CCL5 contents in the culture supernatants were measured by ELISA. For details, see Materials and Methods. All assays were done in triplicate. Each bar represents the mean plus the standard error of the mean from three separate experiments.

secreted these chemokines in very large quantities. EBV-immortalized B cells also secreted TARC/CCL17 and RANTES/CCL5 at substantial levels and I-309/CCL1 and MEC/CCL28 at low levels. Notably, Raji cells, which expressed EBNA2 and LMP-1 at levels similar to those of EBV-immortalized B cells (Fig. 1), also secreted TARC/CCL17, MDC/CCL22, and MIP-1β/CCL4 at levels comparable to those of EBV-immortalized B cells. On the other hand, Daudi cells, which did not express EBNA2 or LMP1 (Fig. 1), secreted hardly any of these chemokines except for MIP-1β/CCL4 at a level similar to that of fresh peripheral-blood B cells. These results were highly consistent with those of RT-PCR shown in Fig. 1. However, there were also notable discrepancies in the levels of mRNA expression and actual protein secretion in chemokines such as MEC/CCL28, MIP-1α/CCL3, and MIP-1β/CCL4. Such discrepancies, also reported for the production of RANTES/CCL5

protein by T cells (39), are likely to be due in part to their posttranscriptional regulation.

Chemotactic activity of chemokines secreted by EBV-immortalized B cells. We further examined whether the culture supernatants of EBV-immortalized B cells were chemotactic for cells expressing CCR4, the receptor for TARC/CCL17 and MDC/CCL22, as well as for those expressing CCR5, the receptor for MIP-1α/CCL3, MIP-1β/CCL4, and RANTES/CCL5 (45). As shown in Fig. 3, the culture supernatants of EBV-immortalized B cells indeed induced vigorous migration of mouse L1.2 cells expressing CCR4 (Fig. 3A) or CCR5 (Fig. 3B). Furthermore, migration of CCR4-expressing L1.2 cells was effectively blocked by anti-MDC/CCL22 (Fig. 3A), while migration of CCR5-expressing L1.2 cells was partially blocked by anti-MIP-1α/CCL3 and by anti-MIP-1β/CCL4 and was completely blocked by a combination of these two antibodies

FIG. 3. Chemotactic activities of chemokines secreted by EBV-immortalized B cells. Culture supernatants of EBV-immortalized B cells were tested for chemotactic activity by using mouse L1.2 cells stably expressing human CCR4 or CCR5 (solid bars) or a control vector (open bars). (A) Chemotactic activity for L-CCR4. medium, medium only; sup (1/25), test culture supernatant diluted 1:25; sup (1/5), test culture supernatant diluted 1:5; sup, test culture supernatant (original); Ab, antibody; a-, anti-. (B) Chemotactic activity for L-CCR5. Cell migration was expressed as a percentage of input cells. Each bar represents the mean plus the standard error of the mean from three separate experiments.

(Fig. 3B). Thus, the chemokines secreted by EBV-immortalized B cells, mainly MDC/CCL22, MIP-1 α /CCL3, and MIP-1 β /CCL4 (Fig. 2), were biologically active.

Induction of chemokine gene expression in B cells upon EBV infection. To explore the role of EBV in the expression of TARC/CCL17, MDC/CCL22, I-309/CCL1, MEC/CCL28, MIP-1 α /CCL3, MIP-1 β /CCL4, and RANTES/CCL5 in EBV-immortalized B cells, we next carried out a time course study using purified CD19⁺ B cells freshly infected with the B95-8 strain of EBV. Figure 4A shows the expression time course of EBNA2 and LMP1 in infected B cells. As reported previously, the induction of EBNA2 was very rapid, while the full expression of LMP1 was seen only at later time points (36). Figure 4B shows the expression time course of the chemokine genes analyzed by quantitative real-time PCR. Upon EBV infection, the expression of TARC/CCL17 and MDC/CCL22 was induced and progressively upregulated during the period of 8 days, apparently in parallel with the expression of LMP1. In contrast, MIP-1 α /CCL3, MIP-1 β /CCL4, and RANTES/CCL5 were constitutively expressed by resting peripheral-blood B cells, quickly upregulated by day 2 postinfection, and maintained at similar levels thereafter. On the other hand, I-309/CCL1 or MEC/CCL28 was hardly induced even on day 8 postinfection.

Roles of EBNA2 and LMP1 in the expression of chemokine genes. To determine the roles of EBV latent genes in the expression of chemokine genes, we next examined their expression in BJAB cells (an EBV-negative human B-cell line), BJAB cells infected with the B95-8 strain of EBV (BJAB-EBV), and clones of BJAB cells stably transfected with an expression vector with or without EBNA2 or LMP1 (referred to below as BJAB-vector, BJAB-EBNA2, and BJAB-LMP1 [three clones each]). As shown in Fig. 5, EBV infection of BJAB strongly induced the gene expression of TARC/CCL17 and MDC/CCL22 but not that of I-309/CCL1 or MEC/CCL28. Furthermore, BJAB clones stably expressing LMP1, but not those expressing EBNA2, consistently expressed TARC/CCL17 and MDC/CCL22. On the other hand, EBV infection or stable expression of EBNA2 or LMP1 did not affect the constitutive expression of MIP-1 α /CCL3, MIP-1 β /CCL4, or RANTES/CCL5 in BJAB cells. Thus, LMP1 appeared to be mostly responsible for the induction of TARC/CCL17 and MDC/CCL22 in EBV-infected B cells. This was consistent with the selective expression of TARC/CCL17 and MDC/CCL22 in Raji cells that only expressed LMP1 among BL cell lines (Fig. 1).

Effects of various signaling inhibitors on the expression of chemokine genes. To understand the signaling pathways involved in the expression of chemokine genes in EBV-immortalized B cells, we next examined the effects of various signaling inhibitors. As shown in Fig. 6A, the expression of TARC/CCL17 and MDC/CCL22 was significantly suppressed by the inhibitor of the p38/ATF2 pathway (SB202190) and that of the TRAF/NF- κ B pathway (BAY11-7082), with the latter more effective. The expression of I-309/CCL1 and MEC/CCL28 was not affected by any of the inhibitors tested. The expression of MIP-1 α /CCL3 was significantly suppressed by the inhibitor of the MEK/ERK pathway (PD98059) and the JAK3 inhibitor. The expression of MIP-1 β /CCL4 was significantly suppressed by the MEK/ERK pathway (PD98059), JNK inhibitor, and

FIG. 4. Role of EBV infection in the expression of chemokine genes in CD19⁺ B cells. Total RNA was prepared from peripheral-blood CD19⁺ B cells; CD19⁺ B cells infected with EBV for 2 (2d), 4, and 8 days; and EBV-immortalized B cells (BCL-NU). (A) Expression time course of EBV latent genes. RT-PCR analysis was carried out for EBNA2, LMP1, and GAPDH. For details, see Materials and Methods. Representative results from three separate experiments are shown. (B) Expression time course of chemokine genes. Real-time RT-PCR was carried out for TARC/CCL17, MDC/CCL22, I-309/CCL1, MEC/CCL28, MIP-1 α /CCL3, MIP-1 β /CCL4, and RANTES/CCL5. For details, see Materials and Methods. Data are shown as the mean plus the standard error of the mean from three separate experiments.

JAK3 inhibitor. The expression of RANTES/CCL5 was significantly suppressed by the inhibitor of the MEK/ERK pathway (PD98059), the JNK inhibitor, the JAK3 inhibitor, and the inhibitor of the TRAF/NF- κ B pathway (BAY11-7082).

We also examined the effects of the signaling inhibitors on the LMP1-induced expression of TARC/CCL17 and MDC/CCL22 in BJAB (Fig. 6B). Consistent with the results from

FIG. 5. Roles of EBNA2 and LMP1 in the expression of chemokine genes. Total RNA was prepared from EBV-immortalized B-cell lines (BCL-NU and BCL-SH), BJAB, BJAB infected with EBV (BJAB-EBV), BJAB stably transfected with vector only (BJAB-vector; three clones), BJAB stably transfected with EBNA2 (BJAB-EBNA2; three clones), and BJAB stably transfected with LMP1 (BJAB-LMP1; three clones). RT-PCR analysis was carried out for TARC/CCL17, MDC/CCL22, I-309/CCL1, MEC/CCL28, MIP-1α/CCL3, MIP-1β/CCL4, RANTES/CCL5, EBNA2, LMP1, and GAPDH. For details, see Materials and Methods. Representative results from three independent experiments are shown.

EBV-immortalized B cells (Fig. 6A), the inhibitors of the p38/ATF2 pathway (SB202190) and the TRAF/NF-κB pathway (BAY11-7082) significantly suppressed the expression of TARC/CCL17 and MDC/CCL22 in BJAB cells induced by LMP1. On the other hand, the inhibitors of the MEK/ERK pathway (PD98059), the JNK/AP-1 pathway, the JAK3/STAT pathway, and the JAK2/STAT pathway (AG490) had no effect on the expression of MDC/CCL22 and TARC/CCL17 induced by LMP1.

Roles of NF-κB and AP-1 sites in the activation of the MDC/CCL22 promoter by LMP1. LMP1 is known to induce gene expression via activation of NF-κB, AP-1, ATF2, and STAT (10, 12, 22, 24). Figure 7A schematically depicts the potential transcriptional elements in the promoter regions of TARC/CCL17 and MDC/CCL22 as revealed by the TF-SEARCH program (14). For comparison, the promoter regions of I-309/CCL1, MEC/CCL28, MIP-1α/CCL3, MIP-1β/CCL4, and RANTES/CCL5 are also shown. Notably, the promoter regions of TARC/CCL17 (−819 to −22) and MDC/CCL22 (−722 to −11) selectively and commonly contain STAT, NF-κB, and AP-1 sites. To explore the roles of such potential elements in the induction of TARC/CCL17 and MDC/CCL22 by LMP1, we generated luciferase reporter constructs using the promoter regions of TARC/CCL17 and MDC/CCL22. By transient-transfection assays using BJAB-LMP1 and BJAB-vector as host cells, we examined promoter activation by LMP1. We were unable to detect any significant activation of the TARC/CCL17 promoter by LMP1 (data not shown). Thus, the TARC/CCL17 promoter region (−819 to −22) employed may not be sufficient for efficient activation by LMP1 in BJAB cells. On the other hand, we consistently detected ~4-fold activation of the MDC/CCL22 promoter by

FIG. 6. Effects of various signaling inhibitors on the expression of chemokine genes in EBV-immortalized B cells and BJAB-LMP1. EBV-immortalized B cells (A) or BJAB-LMP1 cells (B) were treated for 12 h with or without PD98059 (the inhibitor of the MEK/ERK pathway) at 30 μM, SB202190 (the inhibitor of the p38/ATF2 pathway) at 10 μM, JNK inhibitor (the inhibitor of the JNK/AP-1 pathway) at 30 μM, JAK3 inhibitor (the inhibitor of the JAK3/STAT pathway) at 30 μM, AG490 (the inhibitor of the JAK2/STAT pathway) at 20 μM, and BAY11-7082 (the inhibitor of the TRAF/NF-κB pathway) at 5 μM. The concentrations of the inhibitors used were carefully chosen as optimal based on preliminary experiments. Total RNA was prepared, and quantitative real-time RT-PCR was carried out for TARC/CCL17, MDC/CCL22, I-309/CCL1, MEC/CCL28, MIP-1α/CCL3, MIP-1β/CCL4, and RANTES/CCL5. For details, see Materials and Methods. The data are shown as means plus standard errors of the mean from three separate experiments.

LMP1. We therefore proceeded to examine the roles of various transcriptional elements in the MDC/CCL22 promoter region with a series of truncation and mutation constructs. As shown in Fig. 7B, while the deletion of the upstream sequence until −289, which is still upstream of the potential NF-κB sites, did not significantly affect promoter activation by LMP1, the

FIG. 7. Mechanism of transcriptional activation of MDC/CCL22 promoter by LMP1. (A) Schematic depiction of the potential transcriptional elements in the promoter regions of TARC/CCL17, MDC/CCL22, I-309/CCL1, MEC/CCL28, MIP-1 α /CCL3, MIP-1 β /CCL4, and RANTES/CCL5. The TFSEARCH program was used to localize potential regulatory elements. (B) Deletion analysis. BJAB-LMP1 and BJAB-vector were transfected with a series of the MDC/CCL22 promoter-luciferase constructs: pGL3, pGL3-MDC/CCL22(-722/-11), pGL3-MDC/CCL22(-553/-11), pGL3-MDC/CCL22(-476/-11), pGL3-MDC/CCL22(-289/-11), pGL3-MDC/CCL22(-114/-11), and pGL3-MDC/CCL22(-80/-11). (C) Mutation analysis. BJAB-LMP1 and BJAB-vector were transfected with the MDC/CCL22 promoter-luciferase constructs pGL3, pGL3-MDC/CCL22(-722/-11), pGL3-MDC/CCL22- Δ STAT, pGL3-MDC/CCL22- Δ NF- κ B (1), pGL3-MDC/CCL22- Δ NF- κ B (2), pGL3-MDC/CCL22- Δ NF- κ B (1) and (2), pGL3-MDC/CCL22- Δ AP-1, and pGL3-MDC/CCL22- Δ NF- κ B (1) and (2) + AP-1. After 27 to 36 h, relative luciferase activity was determined with a luminometer. Promoter activation by LMP1 was expressed as the induction (*n*-fold) of luciferase activity in BJAB-LMP1 versus that in BJAB-vector. Each bar represents the mean plus standard error of the mean from three separate experiments. (D) Transactivation of MDC/CCL22 promoter by overexpression of NF- κ B p65. BJAB cells were cotransfected with MDC/CCL22 promoter-luciferase constructs, namely, pGL3, pGL3-MDC/CCL22(-722/-11), pGL3-MDC/CCL22- Δ NF- κ B (1), pGL3-MDC/CCL22- Δ NF- κ B (2), and pGL3-MDC/CCL22- Δ NF- κ B (1) and (2), and an expression vector for (His)₆-p65 or a control vector. After 27 to 36 h, relative luciferase activity was determined with a luminometer. Promoter activation by p65 was expressed as the induction (*n*-fold) of luciferase activity in BJAB transfected with p65-vector versus that in BJAB transfected with control vector. Each bar represents the mean plus standard error of the mean from three separate experiments.

FIG. 8. Elevated serum MDC/CCL22 and MEC/CCL28 contents in IM. Serum samples were obtained from healthy donors ($n = 9$) and patients with IM ($n = 9$). Serum TARC/CCL17, MDC/CCL22, I-309/CCL1, MEC/CCL28, MIP-1 α /CCL3, MIP-1 β /CCL4, and RANTES/CCL5 contents were measured by ELISA. All assays were done in duplicate. The horizontal lines indicate mean values. Statistical significance was determined by the unpaired Welch's t test. *, $P < 0.05$; **, $P < 0.01$.

deletion including the two NF- κ B sites (-114) greatly diminished induction by LMP1. Furthermore, as shown in Fig. 7C, the individual mutations at NF- κ B site 1, NF- κ B site 2, and the AP-1 site all partially reduced the activation of the MDC/CCL22 promoter by LMP1. Double mutations targeting the two NF- κ B sites further reduced the activation of the MDC/CCL22 promoter by LMP1. Furthermore, triple mutations targeting the two NF- κ B sites and the single AP-1 site completely abrogated the activation of the MDC/CCL22 promoter by LMP1. On the other hand, the mutation in the upstream STAT site did not affect the activation of the MDC/CCL22 promoter by LMP1. To further address the role of NF- κ B in the transcriptional activation of the MDC/CCL22 promoter, we co-transfected the full-length promoter-luciferase constructs with an expression vector of the p65 subunit of NF- κ B. As shown in Fig. 7D, overexpression of p65 indeed resulted in strong activation of the wild-type MDC/CCL22 promoter in BJAB cells. The individual mutations at NF- κ B site 1 and NF- κ B site 2 partially suppressed the activation of the MDC/CCL22 promoter by p65, while the double mutations targeting both of the NF- κ B sites completely suppressed the activation of the MDC/CCL22 promoter by p65. Collectively, the two NF- κ B sites play an essential role in the activation of the MDC/CCL22 promoter by LMP1. In addition, a single downstream AP-1 site is necessary for the full activation of the MDC/CCL22 promoter by LMP1.

Serum chemokine contents in healthy donors and IM patients. To explore the *in vivo* relevance of the present findings, we measured the TARC/CCL17, MDC/CCL22, I-309/CCL1, MEC/CCL28, MIP-1 α /CCL3, MIP-1 β /CCL4, and RANTES/CCL5 contents in serum samples from healthy adults and IM patients. As shown in Fig. 8, the serum MDC/CCL22 levels were dramatically elevated in IM patients. Notably, the levels of MEC/CCL28 were also significantly elevated in these patients. Even though statistically insignificant, the serum TARC/CCL17 and MIP-1 β /CCL4 levels were also elevated in some IM patients. On the other hand, the serum MIP-1 α /CCL3 and RANTES/CCL5 levels did not show such elevation in IM patients.

DISCUSSION

Certain herpesviruses and poxviruses are known to encode chemokine homologues, and 13 viral chemokine homologues have been identified (1, 28). Most viral chemokines are broad-spectrum chemokine receptor antagonists which are likely to inhibit cellular infiltrates in virus-infected tissues. Notably, however, vMIP-I, vMIP-II, and vMIP-III, encoded by Kaposi's sarcoma-related herpesvirus (KSHV)-human herpesvirus 8, are chemokine receptor agonists; vMIP-I and vMIP-II are the agonists for CCR8, while vMIP-III is the agonist for CCR4 (1, 28). Since CCR4 and CCR8 are known to be selectively expressed by Th2 cells and immunosuppressive regulatory T cells (18, 20, 45), tissues infected with KSHV may preferentially attract these types of T-cell subsets via vMIP-I, vMIP-II, and vMIP-III. Consistently, Sozzani et al. demonstrated that T cells infiltrating KSHV-infected tissues predominantly secreted Th2-type cytokines (37). Since Th1 cells play the major roles in host immune responses against virus-infected cells, selective accumulation of Th2 cells and local production of Th2-type cytokines in virus-infected tissues may be advantageous for the viruses by downregulating host antiviral Th1 responses. In keeping with this notion, several viruses also encode Th2-cytokine homologues and Th1-cytokine inhibitors; EBV encodes an interleukin-10 (IL-10) homologue, KSHV encodes vIL-6 and the gamma interferon inhibitor vIRF-1, and myxoma virus encodes the gamma interferon inhibitor M-T7 (1).

TARC/CCL17 and MDC/CCL22 are the natural ligands of CCR4, while I-309/CCL1 is the ligand of CCR8 (45). Here, we have demonstrated that EBV-immortalized B cells selectively express the genes for TARC/CCL17, MDC/CCL22, and I-309/CCL1 (Fig. 1) and secrete these chemokines, especially MDC/CCL22, in large quantities (Fig. 2). Furthermore, EBV infection induced peripheral-blood B cells to express TARC/CCL17 and MDC/CCL22 in parallel with the expression of LMP1 (Fig. 4). We further showed that (i) LMP1 induces the expression of TARC/CCL17 and MDC/CCL22 in BJAB cells (Fig. 5), (ii) the inhibitors of the TRAF/NF- κ B pathway and the p38/ATF2 pathway effectively suppress the expression of TARC/CCL17 and MDC/CCL22 in EBV-immortalized B cells and LMP1-transfected BJAB cells (Fig. 6), and (iii) two proximal NF- κ B

sites and a single AP-1 site in the promoter region of MDC/CCL22 are critically involved in the activation of the MDC/CCL22 promoter by LMP1 (Fig. 7). Collectively, LMP1, which is known to induce its target genes via signaling pathways involving TRAF/NF- κ B, JNK/AP-1, p38/ATF2, and JAK3/STAT (10, 12, 22, 24), is likely to induce the gene expression of TARC/CCL17 and MDC/CCL22 in EBV-infected B cells via activation of NF- κ B and most probably ATF2. Currently, LMP1 is known to induce the B-cell activation markers CD23 and CD40, adhesion molecules such as LFA-1 and ICAM-1, antiapoptotic molecules such as bcl-2 and A20, matrix metalloproteinase 9, and CD83 (9, 15, 17, 26, 43, 46). Thus, the list of cellular genes induced by LMP1 now includes the chemokines TARC/CCL17 and MDC/CCL22.

Even though the inhibitor of the p38/ATF2 pathway efficiently suppressed the expression of TARC/CCL17 and MDC/CCL22 in EBV-immortalized B cells and LMP1-transfected BJAB cells (Fig. 6), we were unable to find any potential ATF2 site in the TARC/CCL17 and MDC/CCL22 promoter regions (−819 to −22 and −722 to −11, respectively) (Fig. 7). In addition, a single AP-1 site in the proximal promoter region of MDC/CCL22 was necessary for the full activation of the MDC/CCL22 promoter (Fig. 7), even though the inhibitor of the JNK/AP-1 pathway did not suppress the expression of TARC/CCL17 and MDC/CCL22 in EBV-immortalized B cells and LMP1-transfected BJAB cells (Fig. 6). In this context, ATF2 was shown to activate the promoters of IL-8/CXCL8 and c-Jun via the AP-1 site (10, 42). Thus, ATF2 is likely to be involved in the promoter activation of TARC/CCL17 and MDC/CCL22 by targeting the AP-1 site.

Even though we were unable to directly demonstrate the activation of the TARC/CCL17 promoter (−819 to −22) by LMP1 under the present experimental conditions, we have also found potential NF- κ B and AP-1 sites in the TARC/CCL17 promoter region (−819 to −22) in an arrangement similar to that of MDC/CCL22 (Fig. 7). Thus, these NF- κ B and AP-1 sites may also be responsible for the induction of TARC/CCL17 by LMP1. On the other hand, I-309/CCL1 and MEC/CCL28 were not directly induced by EBV (Fig. 4 and 5), and their expression in EBV-immortalized B cells was not affected by various signaling inhibitors (Fig. 6). Thus, their expression may be due to the plasmablast-like stage of differentiation of EBV-immortalized B cells. Indeed, we have consistently observed the expression of I-309/CCL1 and MEC/CCL28 in human myeloma cell lines (data not shown). Since TARC/CCL17 and MDC/CCL22 are known to selectively attract Th2 cells and immunosuppressive regulatory T cells via CCR4 (18, 20, 45), EBV may induce host B cells to produce these chemokines to preferentially attract Th2 cells and immunosuppressive regulatory T cells. This may be advantageous for the survival of EBV-infected B cells by shifting local immune responses to Th2. In addition, Th2-type cytokines, such as IL-4, IL-5, IL-6, and IL-10, are also known to promote the growth of EBV-immortalized B cells (23).

The repertoire of chemokines produced by EBV-immortalized B cells is not, however, so straightforward. While resting peripheral-blood B cells constitutively express MIP-1 α /CCL3, MIP-1 β /CCL4, and RANTES/CCL5 (all CCR5 ligands), EBV-immortalized B cells further upregulate their expression (Fig. 1) and secrete these chemokines, especially MIP-1 α /CCL3 and

MIP-1 β /CCL4, in large quantities (Fig. 2). EBV infection of purified B cells also rapidly upregulates the expression of these chemokines (Fig. 4). However, it appears that EBV is not directly responsible for the upregulation of these chemokines (Fig. 5). Furthermore, in contrast to TARC/CCL17 and MDC/CCL22, their expression is mainly dependent on signaling pathways, such as the MEK/ERK and JAK3/STAT pathways (Fig. 6). Since LMP1 and LMP2A provide a surrogate T-cell help and a surrogate antigen receptor signal, respectively (5, 12, 41), EBV-immortalized B cells are phenotypically activated B cells. Thus, the upregulation of MIP-1 α /CCL3, MIP-1 β /CCL4, and RANTES/CCL5 in EBV-immortalized B cells may be related to the activated phenotype of B cells imposed by EBV infection. In agreement with this idea, previous studies have shown that the MEK/ERK and JAK3/STAT pathways are involved in signals for B-cell activation (8, 38), and activated B cells selectively produce MIP-1 α /CCL3 and MIP-1 β /CCL4 (4).

MIP-1 α /CCL3, MIP-1 β /CCL4, and RANTES/CCL5 produced by EBV-infected B cells are likely to attract Th1 cells and activated cytotoxic T cells via CCR5 (19, 32, 45). Thus, EBV-infected B cells may paradoxically promote their own elimination by the host immune system. However, this may be beneficial for the eventual survival of EBV by self-limiting its highly tumorigenic latency III infection and establishing persistent infection in a pool of memory B cells with a more restricted pattern of expression of latent proteins (41). Interestingly, however, Bystry et al. have recently demonstrated that activated B cells produce MIP-1 α /CCL3 and MIP-1 β /CCL4 and attract regulatory T cells in naive mice (4). Similarly, therefore, the production of MIP-1 α /CCL3, MIP-1 β /CCL4, and RANTES/CCL5 by EBV-infected B cells may initially attract regulatory T cells instead of Th1 cells and cytotoxic T cells in naive human hosts.

In conclusion, we have elucidated the expression profile of chemokines in EBV-immortalized B cells. In particular, EBV-immortalized B cells produce TARC/CCL17, MDC/CCL22, MIP-1 α /CCL3, MIP-1 β /CCL4, and RANTES/CCL5 in substantial-to-large quantities. The upregulation of TARC/CCL17 and MDC/CCL22 is mainly induced by LMP1, while that of MIP-1 α /CCL3, MIP-1 β /CCL4, and RANTES/CCL5 is likely to be due to the activated-B-cell phenotype of EBV-infected B cells. Consistently, the serum MDC/CCL22 levels were significantly elevated in IM patients (Fig. 8). The production of these chemokines by EBV-infected B cells may variably affect the virus-host relationship and may account for some features of IM and other EBV-associated diseases.

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