Tumorigenesis and Neoplastic Progression

Expression of the Epstein-Barr Virus-Encoded Epstein-Barr Virus Nuclear Antigen 1 in Hodgkin's Lymphoma Cells Mediates Up-Regulation of CCL20 and the Migration of Regulatory T Cells

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In ~50% of patients with Hodgkin's lymphoma (HL), the Epstein-Barr virus (EBV), an oncogenic herpesvirus, is present in tumor cells. After microarray profiling of both HL tumors and cell lines, we found that EBV infection increased the expression of the chemokine CCL20 in both primary Hodgkin and Reed-Sternberg cells and Hodgkin and Reed-Sternberg cell-derived cell lines. Additionally, this up-regulation could be mediated by the EBV nuclear antigen 1 protein. The higher levels of CCL20 in the supernatants of EBV-infected HL cell lines increased the migration of CD4⁺ lymphocytes that expressed FOXP3, a marker of regulatory T cells (Tregs), which are specialized CD4⁺ T cells that inhibit effector CD4⁺ and CD8⁺ T cells. In HL, an increased number of Tregs is associated with the loss of EBV-specific immu-

nity. Our results identify a mechanism by which EBV can recruit Tregs to the microenvironment of HL by inducing the expression of CCL20 and, by doing so, prevent immune responses against the virus-infected tumor population. Further investigation of how EBV recruits and modifies Tregs will contribute not only to our understanding of the pathogenesis of virus-associated tumors but also to the development of therapeutic strategies designed to manipulate Treg activity. (Am J Pathol 2008, 173:195–204; DOI: 10.2353/ajpath.2008.070845)

The Epstein-Barr virus (EBV) is associated with the development of several human tumors, including Hodgkin's lymphoma (HL) and EBV-positive undifferentiated nasopharyngeal carcinoma (NPC).¹ In HL, the malignant Hodgkin's and Reed-Sternberg (HRS) cells constitute only a minority of the total tumor mass, and are surrounded by variable proportions of nonmalignant reactive cells. In approximately one-half of HL, EBV can be detected in HRS cells, where the virus expresses a limited subset of genes; these include the Epstein-Barr nuclear antigen-1 (EBNA1) and the latent membrane proteins, LMP1 and LMP2.² Although EBV-specific cytotoxic T cells (CTLs) can be detected in HL and NPC and have been shown to kill LMP1- and LMP2-expressing cells *in vitro*, they are unable to eliminate EBV-infected tumor cells *in vivo*.^{3–5} This failure may be because of increased recruitment of regulatory T cells

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(Tregs), specialized CD4⁺ T cells that control the activation of autoaggressive CD4⁺ and CD8⁺ T cells, and thereby prevent autoimmunity.^{6,7}

Tregs are elevated in the peripheral blood of HL patients compared to healthy controls, and in those patients with active disease compared to those in remission.^{8,9} Their numbers are also increased in HL tumor tissues where they are found close to HRS cells.^{9–11} In HL, an increased number of Tregs is associated with the loss of LMP1- and LMP2-specific immunity, whereas depletion of Tregs from peripheral blood mononuclear cells (PBMCs) enhances EBV-specific immunity.⁹ The recruitment of Tregs to tumors is poorly understood but might involve chemokines; it has recently been shown that naïve and memory Treg subsets, distinguished by their surface expression of CCR4 and CCR6, show strong chemotactic responses to their corresponding chemokines, CCL22 and CCL20.^{12,13}

Understanding how EBV disables the CTL response is critical to the development of adoptive T-cell therapies that target the virus in HL and in other tumors.^{14,15} We show here that EBNA1 up-regulates the expression CCL20 in HL cells and that this leads to an increased chemotaxis of Tregs. Our data suggest that EBNA1 expression might enable the escape of EBV-infected HL cells from the virus-specific CTL response.

Materials and Methods

This study received ethical approval from the South Birmingham Research Ethics Committee (LREC no. 0844), and from the Karolinska Institute Research Ethics Committee North (approval number 01-004).

EBV-Infected Cell Lines and Clinical Samples

The HL cell lines used were all initially derived from pleural effusions; KM-H2 from a 37-year-old male with mixed cellularity HL, L591 from a 31-year-old female with nodular sclerosing HL, and L428 from a 37-year-old female with nodular sclerosing HL. EBV-negative KM-H2 cells were infected with Akata-derived recombinant EBV and cultured in 1 mg/ml of G418 as previously described.¹⁶ Control KM-H2 cells were generated by electroporation with a vector containing a neomycin resistance gene (pzpLNSNeo) and selected in the presence of G418. After serial dilution of EBV-positive L591 cells, EBV-negative clones were generated as previously described; the EBV-negative L591 SD3 clone was used in these studies.¹⁶

Snap-frozen biopsies from 23 patients with a histological diagnosis of nodular sclerosis (NS) HL were obtained from the Children's Cancer and Leukemia Group, and from the Karolinska Institute, Stockholm, Sweden. RNA was isolated from cryostat sections using the Qiagen RNeasy micro kit (Qiagen, Crawley, UK), and quantified using a Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, DE). EBV status was determined using *in situ* hybridization for the detection of EBER expression as previously described, using a corresponding paraffin wax tissue block from the same patient.¹⁷ Paraf-

fin-embedded HL tissues were obtained from the Queen Elizabeth Hospital, Birmingham, UK, and Russells Hall Hospital, Dudley, UK, and NPC samples from the Tung Shin Hospital, Kuala Lumpur, Malaysia.

Microarray Analysis

The transcriptional profile of EBV-positive and EBV-negative tumors was compared with that of purified germinal center (GC) B cells. Gene expression was measured on HG Focus GeneChips (Affymetrix, High Wycombe, UK) (13 of 23 tumors) and HG133 Plus 2.0 GeneChips (Affymetrix) (10 of 23 tumors) using standard Affymetrix protocols. Scanned images of microarray chips were analyzed using GCOS (GeneChip Operating Software) from Affymetrix with the default settings except that the target signal was set to 100. Probe sets present on both the HG Focus and HG133 Plus 2.0 arrays were selected for further analysis. Except where specified, relative gene expression values were calculated using the robust multichip average method¹⁸ and differentially expressed genes were identified using rank products¹⁹ with a false-positive cut-off value of 10%. We also used the results of two other microarray analyses in this study; the transcriptional profile of CD10-positive GC B cells, and transcriptional differences between EBV-positive and EBV-negative L591 and KM-H2 cells, are reported elsewhere (M. Vockerodt et al, manuscript submitted).^{16,20}

CCL20 Enzyme-Linked Immunosorbent Assay (ELISA)

HL lines (1×10^7 cells) were grown in RPMI (Invitrogen, Paisley, UK) containing 10% fetal calf serum (FCS) (Invitrogen) for 72 hours, and the conditioned media collected after centrifugation at $700 \times g$ for 5 minutes at 4°C. CCL20 protein in these media was quantified using the human CCL20/MIP3- α Quantikine ELISA kit (R&D Systems Europe Ltd., Abingdon UK) according to the manufacturer's instructions.

Microdissection and RNA Amplification

In addition, to the microarray experiments already described, gene expression analysis was performed on eight microdissected HL tumors and a tonsil exhibiting follicular hyperplasia using the PALM laser microbeam system (P.A.L.M. Microlaser Technologies GmbH, Bernried, Germany). Frozen sections were stained with hematoxylin using an RNase-free protocol. Between 150 and 200 HRS cells were isolated from each of the HL cases. GCs were isolated from a tonsil removed because of follicular hyperplasia. After RNA extraction, three rounds of linear T7-based mRNA amplification were performed using the ExpressArt TR system²¹ (AmpTec, Hamburg, Germany) according to the manufacturer's instructions. In the final *in vitro* transcription reaction, the RNA was biotinylated for analysis on Affymetrix GeneChip arrays.

The resulting yields of amplified RNA were between 30 to 40 μ g, derived from <10 ng of input total RNA.

Immunohistochemistry

Four- μ m paraffin wax sections from classic HL were cut onto charged slides (Surgipath, Peterborough, UK) and heated for 1 hour at 60°C. Sections were deparaffinized, rehydrated, and treated in 0.3% H₂O₂. Antigens were retrieved using the agitated low-temperature epitope retrieval technique, as previously described.²² After a brief wash in water, sections were placed onto a Sequenza (Shandon, UK) and washed in Tris-buffered saline, pH 7.6. Primary antibodies to CCL20 (1:100, AF360; R&D Systems) or FOXP3 (1:100, Ab2481-100; Abcam, Cambridge, UK), were applied for 1 hour. Sections were then washed in Tris-buffered saline/Tween and incubated in rabbit anti-goat antibody (Z0454; DAKO, Glostrup, Denmark) at 1/200 for 15 minutes. The DAKO ChemMate EnVision kit (K5007, DAKO) was applied for 30 minutes and visualization completed with Vector NovaRED (SK-4800; Vector Laboratories, Burlingame, CA) or diaminobenzidine. Immunohistochemistry for LMP1 was performed as previously described.²² Tonsil sections were used as a positive control tissue for the CCL20 and FOXP3 antibodies and for LMP-1 a previously identified LMP-1-positive HL section was used. Negative controls involved replacing the primary antibody with the normal serum from the respective species, ie, goat for CCL20 and FOXP3 or mouse for LMP1. Lymphocytes staining positively for FOXP3 were counted in 10 high-power fields and expressed as a percentage of the total number of lymphocytes. When quantifying the intensity of CCL20 staining, tumor cells were graded as either CCL20^{neg} (where CCL20 could not be detected) or CCL20^{pos} (where staining was observed in the HRS cells). A total of 89 cases were stained for CCL20, of these 71 cases were available for FOXP3 analysis.

PBMC Chemotaxis Assay

PBMCs, freshly isolated using Lymphoprep (Nycomed, Oxford, UK), were resuspended in RPMI containing 10% FCS to a final concentration of 5×10^6 /ml. The stimulus for chemotaxis was 600 μ l of concentrated conditioned medium from L591, L591 SD3 cells, and RPMI with 10% FCS. The conditioned media were concentrated 20-fold using Centricon Plus 20 (5000 NMWL) concentrators (Fisher Ltd., Loughborough, UK). Conditioned media were preincubated for 15 minutes at 37°C in the presence or absence of blocking anti-CCL20 antibody (MAB360, R&D Systems). PBMCs (5×10^5) were added to the transwell culture inserts (no. 3421, 5.0- μ m transwell polycarbonate membrane; Corning, Birmingham UK) and incubated for 4 hours at 37°C in 5% CO₂. Migrated cells in the lower chamber were counted and the chemotactic index expressed as the ratio of cells that migrated in the presence of conditioned medium compared to those that migrated in the presence of RPMI plus 10% FCS alone.

Flow Cytometry

Aliquots of total PBMCs before transwell analysis, and aliquots of migrated and nonmigrated PBMCs were washed and resuspended in cold phosphate-buffered saline (PBS), and then stained with monoclonal antibodies (CD4-FITC, CD25-Pcy5, or Pcy5 isotype; all Beckman Coulter, High Wycombe, UK), for 20 minutes at 4°C. For FOXP3 analysis, cells were washed, fixed, and permeabilized using the FOXP3 kit (eBiosciences) before a brief incubation in rat serum, and then FOXP3-PE antibody (clone PCH101, eBioscience, San Diego, CA) or isotype control (rat IgG2a PE). Cells were washed in cold PBS and analyzed by flow cytometry within 1 hour (Beckman Coulter EPICS XL-MCL).

Quantitative Polymerase Chain Reaction (Q-PCR)

Each Q-PCR reaction contained: TaqMan Universal PCR Mastermix, TaqMan gene expression assay primer, and probe mix for either the target gene of interest or β -2-microglobulin (B2M) and cDNA. cDNA was prepared using AMV reverse transcriptase (Roche, Burgess Hill, UK) in conjunction with an anchored oligo-dT primer. Each sample was analyzed in triplicate. Q-PCR was performed on an ABI 7500 Fast real-time PCR system according to the manufacturer's instructions (Applied Biosystems, Foster City, CA). Data were analyzed using 7500 Fast System SDS software 1.3.1 (Applied Biosystems), this uses the 2- $\Delta\Delta$ CT method for quantifying expression relative to the B2M housekeeping control. The 2- $\Delta\Delta$ CT value of L428 HL cells, was set to a relative quantity (RQ) value of 1, and all other samples expressed as ratio of this.

Ectopic Expression of EBNA1 in Cell Lines

L428 and KM-H2 cells were transfected with EBNA1 expression plasmid or empty vector (pSG5 alone) by nucleofection using the cell line Nucleofector Kit T (VCA-1002; Amaxa GmbH, Cologne, Germany) for the Nucleofector device (AAD-1001, Amaxa GmbH), according to the manufacturers' protocol. HONE-1 or Ad/AH NPC cells (2×10^5) were plated in six-well plates for 24 hours before transfection. One μ g of EBNA1 plasmid or pSG5 empty vector was precomplexed with Plus Reagent and mixed with Lipofectamine (10964-013, Invitrogen) in serum-free Opti-MEM media (11058-021, Invitrogen). The DNA-Plus-Lipofectamine complexes were added to the cells and incubated for 3 hours at 37°C after which medium containing serum was added to make a final serum concentration of 10%. Forty-eight hours after transfection HL and NPC cells were harvested for RNA extraction.

Statistical Analysis

Statistical analysis was performed in Microsoft Excel (Microsoft Corp, Redmond, WA) using either a two-tailed Student's *t*-test assuming the two samples displayed unequal variance or a χ^2 test.

Results

Identification of EBV-Regulated Genes in Primary NS HL and in HL Cell Lines

The transcriptional profile of 23 whole primary NS HL tumors was compared with that of purified GC B cells, the presumptive progenitor of HRS cells. Probe sets ($n = 1160$) (1133 named genes) were significantly up-regulated and 601 probe sets (586 named genes) significantly down-regulated in NS tumors compared with GC B cells (Supplemental Tables 1 and 2 at <http://ajp.amjpathol.org>). Next, we analyzed how expression of these NS-associated genes varied with EBV status using 7 EBV-positive and 12 EBV-negative tumors. One hundred forty-six probe sets (142 named genes) were significantly up-regulated, and 164 probe sets (162 named genes) were significantly down-regulated in EBV-positive NS tumors compared with EBV-negative NS tumors (Supplemental Table 3 at <http://ajp.amjpathol.org>). Next, to identify which of these gene expression changes arose within the tumor cells rather than within the nonmalignant infiltrate of HL, we compared the expression of these presumptive EBV-associated genes with those previously identified in a comparison of gene expression before and after the loss of EBV from the latency III-expressing NS HL cell line, L591.²⁰ Ten were up-regulated and one down-regulated in the presence of EBV in both primary NS tumors and in L591 cells (Table 1). These genes included autotaxin (ENPP2), which we have previously shown to be up-regulated by EBV infection of HL cells.¹⁶ The remaining nine EBV-induced genes included four chemokines (CXCL9, CXCL10, CCL22, and CCL20). Quantitative PCR (Q-PCR) confirmed the up-regulation of CXCL9, CXCL10, and CCL22 in EBV-positive L591 cells compared with EBV-negative L591-SD3 cells, and in HL tissue compared with GC B cells (Supplemental Figure 1, A and B, at <http://ajp.amjpathol.org>).

Up-Regulation of CCL20 by EBV in Primary Tumors and Cell Lines

CCL20 was of particular interest because of its reported role in the chemotaxis of Treg.¹³ Q-PCR confirmed that CCL20

expression was higher in HL tissue than in GC B cells; and that it was higher in EBV-positive tumors than in EBV-negative tumors (Figure 1C, left). Q-PCR also showed increased CCL20 expression in EBV-positive L591 cells compared to EBV-negative L591-SD3 cells (Figure 1A, top); and ELISA revealed higher levels of CCL20 protein in the supernatant of EBV-positive L591 cells (Figure 1B, top). EBV infection of KM-H2 cells also led to the up-regulation of CCL20 mRNA and protein in the supernatant (Figure 1, A and B; bottom). Two further experiments were used to study the expression of CCL20 in primary HRS cells and its relationship to EBV status. The first, a microarray analysis of eight microdissected HL tumors, revealed higher levels of CCL20 expression in HRS cells than in either purified GC B cells or microdissected GC cells (Figure 1C, right); this up-regulation was more marked in the EBV-positive samples. The second, an immunohistochemical analysis of primary tumors, showed that CCL20 expression was more common in the EBV-positive cases (Figure 2 and Table 2) than in those that were EBV-negative (79% versus 13%; χ^2 31.721; $P = 0.000$). In CCL20-positive cases >75% of tumor cells were stained. A minority of cells in the infiltrate, which included some neutrophils, also stained positive for CCL20. However, these cells were far less numerous than the positively stained HRS cells. The frequency of FOXP3-positive cells in these tumors did not vary significantly with either EBV status ($P = 0.15$) or CCL20 status ($P = 0.75$).

Up-Regulation of CCL20 by EBV Infection of HL Cells Can Be Mediated by EBNA1

LMP1 has previously been shown to up-regulate CCL20 in BL cells.³¹ However, LMP1 is not expressed in EBV-positive KM-H2 cells and could not therefore account for the up-regulation of CCL20 in these cells. The EBV-encoded EBNA1 is one of only several virus proteins expressed in both EBV-positive L591 cells and EBV-positive KM-H2 cells.¹⁶ Figure 3 shows that EBNA1 expression in EBV-negative L428 and KM-H2 HL cells up-regulated CCL20 expression. Ectopic expression of other individual EBV

Table 1. Genes Concordantly Differentially Expressed in EBV⁺ NS HL versus EBV⁻ NS HL and in EBV⁺ L591 Cells Compared with EBV⁻ L591 SD3 Cells

Accession number	Gene symbol	Description	Mean fold change EBV ⁺ versus EBV ⁻ tumors	Mean fold change L591 versus L591 SD3	Reported in HL*
D13720	<i>ITK</i>	IL-2-inducible T-cell kinase	1.67	2.02	No
NM_005345	<i>HSPA1A/1B</i>	Heat shock 70-kDa protein 1A/1B	1.66	1.64	Yes ²³
L35594	<i>ENPP2</i>	Autotaxin	1.57	3.10	Yes ¹⁶
NM_004591	<i>CCL20 (MIP-3α)</i>	Chemokine (C-C motif) ligand 20	1.49	2.40	No
NM_002416	<i>CXCL9 (MIG)</i>	Chemokine (C-X-C motif) ligand 9	1.46	2.14	Yes ^{24,25}
BC001638	<i>ASCL1</i>	Achaete-scute complex-like 1	1.44	1.94	No
NM_001565	<i>CXCL10 (IP-10)</i>	Chemokine (C-X-C motif) ligand 10	1.43	9.74	Yes ^{25,26}
NM_004010	<i>DMD</i>	Dystrophin (muscular dystrophy, Duchenne and Becker types)	1.36	2.55	Yes ²⁷
NM_001877	<i>CR2 (CD21)</i>	Complement component (3d/Epstein Barr virus) receptor 2	1.27	4.24	Yes ^{28,29}
NM_002990	<i>CCL22 (MDC)</i>	Chemokine (C-C motif) ligand 22	1.24	10.74	Yes ³⁰
NM_014333	<i>IGSF4 (TSLC1)</i>	Immunoglobulin superfamily member 4	-1.73	-1.60	No

Figure 1. CCL20 expression in HL cell lines and primary tumors. **A:** Q-PCR demonstrates the up-regulation of CCL20 mRNA in EBV-positive L591 HL cells, compared with EBV-negative L591-SD3 cells (**top**) and in KM-H2 cells infected with a recombinant EBV, compared with EBV-negative KM-H2 control cells. These differences were statistically significant ($P = 0.004$ for L591 and $P = 0.0002$ for KM-H2-EBV). All Q-PCR data are presented as the mean of three replicates. For comparability across all Q-PCR assays RQ values for CCL20 in L428 HL cells (data not shown) were normalized to a value of 1. **Asterisk** denotes P value of <0.05 . **B:** ELISA shows increased CCL20 protein in the supernatant of EBV-positive L591 and KM-H2 cells compared to their EBV-negative counterparts. **C: Left:** Q-PCR analysis of CCL20 expression in purified GC B cells (GCB I, GCB II), EBV-negative primary HL tumors (light gray bars) and EBV-positive primary HL tumors (dark gray bars). CCL20 expression was higher in HL tissue than in GC B cells; and generally in EBV-positive tumors than in EBV-negative tumors. **Right:** GCOS signal for CCL20 determined from the microarray analysis of HRS cells microdissected from five EBV-positive primary NS tumors (HLB, HLE, HLG, HLI, HLJ) (dark gray bars) compared to that from three EBV-negative tumors (HLM, HLL, HLH) (white bars), purified GC B cells (GC#1 to GC#3) (light gray bars), and microdissected GC cells (GC1) (black bar).

genes in HL cells (*LMP2A*, *LMP1*, *BamH1A* transcripts) did not up-regulate *CCL20* expression (data not shown).

We also observed the up-regulation of CCL20 after expression of EBNA1 in two NPC cell lines; HONE-1 and Ad/AH (Supplemental Figure 2 at <http://ajp.amjpathol.org>). Furthermore, almost all cases of primary EBV-positive undifferentiated NPC (24/25) showed strong staining for CCL20 in tumor cells (Supplemental Figure 2C at <http://ajp.amjpathol.org>); this was independent of LMP1 status (Table 3). In LMP1-positive cases $>90\%$ of tumor cells expressed LMP1. CCL20 expression was present in almost all tumor cells. Supplemental Figure 2 at <http://ajp.amjpathol.org> is representative of all cases. We con-

clude that EBNA1, but not LMP1, can up-regulate CCL20 expression in HL and NPC cells.

Increased Chemotaxis of PBMCs to Conditioned Media from EBV-Positive HL Cells Is Mediated by CCL20

We next studied whether the increased CCL20 secreted by EBV-infected HL cells could influence lymphocyte chemotaxis. Figure 4A shows that there was a threefold to fourfold increase in PMBC chemotaxis to conditioned medium from EBV-positive L591 cells compared to me-

Figure 2. CCL20 protein expression in primary HL. **A:** CCL20 expression in normal tonsil. GC B cells show very low or undetectable levels of CCL20 protein. In contrast, rare small lymphocytes (**white arrows**) scattered throughout the GC and within the mantle zone (MZ) were positive. **B and C:** Strong staining in HRS cells (**black arrows**) of primary EBV-positive HL. **D:** Absence of CCL20 expression in HRS cell (**black arrow**) of EBV-negative HL; small lymphocytes in the background are positive (**white arrow**).

dium from EBV-negative L591-SD3 cells (Student's *t*-test, $P = 0.0000$). This effect was abolished by a CCL20 blocking antibody, but not by an isotype control antibody. Similar results were obtained using conditioned medium from EBV-positive and EBV-negative KM-H2 cells (Figure 4B).

PBMCs Attracted to Conditioned Media from EBV-Positive HL Cells Are Enriched for Tregs

We performed a phenotypic analysis of the subpopulation of PBMCs that migrated to conditioned medium from EBV-positive L591 cells. To determine the frequency of CD4⁺FOXP3⁺ T cells, gates were set from cells stained with CD4 and the isotype control antibody. Figure 5A

shows that the migrated CD4-positive PBMCs contained significantly more FOXP3-positive cells than either than the starting population or the nonmigrated PBMCs (28.86% versus 4.14% and 4.22%, respectively; Student's *t*-test, $P = 0.026$). Most of the migrated CD4⁺FOXP3⁺ cells were also CD25⁺ (mean, 71.04%; data not shown). Figure 5B confirms the specificity of this effect because the increased migration of CD4⁺FOXP3⁺ cells was abolished by anti-CCL20 antibody. We conclude that the enhanced migration of CD4⁺FOXP3⁺ Tregs to conditioned medium from EBV-positive HL cells is a consequence of the increased expression of CCL20.

Finally, we repeated these experiments using HL cells stably expressing EBNA1 alone. Figure 6A shows that L428 cells stably expressing EBNA1 had higher levels of CCL20 in their supernatant than did controls cells (L428-GFP). Increased PBMC migration was also observed to conditioned medium from EBNA1-expressing L428 cells compared to control cells; this increase could be inhibited by pretreatment with anti-CCL20 antibody (Figure 6B). Furthermore, the proportion of CD4⁺ cells expressing FOXP3 was higher in those PBMCs that migrated to conditioned medium from EBNA1-expressing L428 cells

Table 2. CCL20 Expression in EBV-Positive and EBV-Negative Hodgkin's Lymphoma

CCL20 status	EBV-positive	EBV-negative
CCL20 ^{pos}	19	9
CCL20 ^{neg}	5	56
Total CCL20 ⁺	19/24	9/65

Figure 3. Up-regulation of CCL20 in HL cells is mediated by the EBV-encoded EBNA1. **A:** Q-PCR analysis of CCL20 mRNA expression in EBV-negative KM-H2 cells (**top**) and L428 cells (**bottom**) 48 hours after transient transfection with either EBNA1 expression vector or control empty vector. In both cell lines the ectopic expression of EBNA1 resulted in the up-regulation of CCL20 mRNA. These differences were statistically significant ($P = 0.013$ for KM-H2 and $P = 0.009$ for L428). The RQ value for CCL20 in the vector-only control was set at 1. **B:** RT-PCR confirms the expression of EBNA1 in the KM-H2 cells (**top**) and L428 cells (**bottom**) 48 hours after transfection with EBNA1 expression vector. EBV-positive L591 cells were used as a positive control for EBNA1 expression.

compared to those that migrated to conditioned medium from L428-GFP cells; this increased migration could be also be abolished by addition of anti-CCL20 antibody (Figure 6C). We conclude that HL cells expressing EBNA1 alone can induce the migration of Tregs.

Table 3. CCL20 Expression in LMP1-Positive and LMP1-Negative Nasopharyngeal Carcinoma

CCL20 status	LMP1-positive	LMP1-negative
CCL20 ^{pos}	10	15
CCL20 ^{neg}	0	0
Total CCL20 ^{pos}	100% (10/10)	100% (15/15)

Discussion

We report for the first time the contribution of EBV to the recruitment of Tregs, the predominant cell type within the T-cell population of HL. We show that the presence of EBV in HL cell lines and in primary tissues was associated with up-regulation of the chemokine, CCL20, and that this increased the chemotaxis of FOXP3-expressing Tregs. LMP1 has previously been shown to up-regulate CCL20 in BL cells.³¹ However, we have shown that EBNA1 alone is sufficient to induce the up-regulation of CCL20 in HL and NPC cells. This is an important observation because EBNA1 is unique insofar as it is expressed in all EBV-associated malignancies. Therefore, the up-regulation of CCL20 by EBNA1 could be important for the pathogenesis of EBV-associated tumors, such as NPC, which do not always express LMP1. In support of this proposition, we found that the expression of CCL20 in NPC was independent of LMP1 status. How EBNA1 affects the transcription of CCL20 is not known, but we

Figure 4. Enhanced migration of PBMCs toward conditioned medium from EBV-positive L591 HL cells. **A:** Increased migration of PBMCs to conditioned medium from EBV-positive L591 cells compared with that to conditioned medium from EBV-negative L591-SD3 cells or RPMI plus 10% FCS alone ($P < 0.0000$ and $P < 0.0000$, respectively). This enhanced migration could be inhibited by the preincubation of L591-conditioned medium with anti-CCL20 antibody (**left**) but remains unaffected in the presence of an isotype control antibody shown here as a separate experiment ($P = 0.0034$ compared to either RPMI or medium from L591-SD3 cells, **right**). **B:** Increased PBMC migration was also observed to conditioned medium from EBV-positive KM-H2 cells ($P < 0.0003$ compared to EBV-negative KM-H2 medium). This could be inhibited by pretreatment with anti-CCL20 antibody.

Figure 5. Increased migration of regulatory T cells toward conditioned medium from EBV-positive L591 HL cells. **A:** Flow cytometric analysis reveals a significant increase in the proportion of CD4⁺ T cells expressing FOXP3 within the population of cells that migrated toward conditioned medium from EBV-positive L591 cells ($n = 6$, $P = 0.026$). **B:** Flow cytometry shows that the proportion of CD4⁺ cells expressing FOXP3 was higher in those PBMCs that migrated to conditioned medium from L591 EBV-positive cells (black bars) compared to those that migrated to conditioned medium from L591-SD3 EBV-negative cells (white bars). This increased migration could be abolished by addition of anti-CCL20 antibody (gray bars). Shown here are results for three separate donors.

have shown that EBNA1 can activate AP-1 signaling (J.D. O'Neil et al, manuscript submitted), and it has been reported that the CCL20 promoter has an AP-1 binding site.³² We have also demonstrated that EBV can up-regulate CCL22 (data not shown). Although LMP1 can induce CCL22 in EBV-infected B cells,³³ we observed that CCL22 expression was increased in EBV-positive cells that lack LMP1 expression. It remains to be established if EBNA1 can also up-regulate CCL22, and in doing so, stimulate the recruitment of naïve Tregs.

FOXP3 expression on T lymphocytes is mainly restricted to CD4⁺ cells and is a faithful marker of Tregs.³⁴ However, we showed that Treg numbers in primary HL, as defined by expression of the transcription factor, FOXP3, do not vary significantly with EBV status, nor with CCL20 status.¹¹ Thus, although CCL20 and CCL22 might contribute to the recruitment of FOXP3 Tregs in EBV-positive tumors, alternative mechanisms must exist to recruit these cells in the majority of EBV-negative tumors. It is also possible that distinct subsets of Tregs are differentially recruited to EBV-positive and EBV-negative tumors. For example, in one study, LAG-3-positive Tregs were more numerous in EBV-positive tumors.⁹ These apparently contradictory findings are consistent with the

Figure 6. Increased migration of regulatory T cells toward conditioned medium from EBNA1-expressing L428 cells. **A:** ELISA reveals increased CCL20 in the supernatant of EBNA1-expressing L428 cells. **B:** Increased PBMC migration was observed to conditioned medium from EBNA1-expressing L428 cells. This could be inhibited by pretreatment with anti-CCL20 antibody. **C:** Flow cytometry shows that the proportion of CD4⁺ cells expressing FOXP3 was higher in those PBMCs that migrated to conditioned medium from EBNA1-expressing L428 cells (black bars) compared to those that migrated to conditioned medium from EBNA1-negative control cells (L428-GFP, white bars). This increased migration could be abolished by addition of anti-CCL20 antibody (dark gray bars).

possibility that EBV preferentially recruits distinctive subsets of Tregs. CCL20 is overexpressed in pancreatic and hepatocellular carcinoma when it is associated with late stage and metastatic disease.^{35–37} CCL20 binding to CCR6 directly enhances cell growth and facilitates invasion.^{38,39} CCL20 and CCR6 were co-expressed in three of our five HL cell lines (data not shown) suggesting that the stimulation of CCR6 contributes to tumor growth and progression in HL.

In summary, we show that EBNA1, an essential viral maintenance protein, can stimulate the chemotaxis of Tregs by inducing expression of CCL20, which may in turn explain how EBV inhibits virus-specific CTL responses. Clearly, such a mechanism although important for the development of HL and NPC, could also be involved in the pathogenesis of other virus-associated malignancies, such as Burkitt's lymphoma and NK lymphomas. Furthermore, the recruitment of Tregs might also modify the host response during asymptomatic primary infection/infectious mononucleosis, and facilitate the establishment of latency and subsequent virus persistence.

A recent study by Marshall and colleagues⁴⁰ monitored T-cell responses both during acute infectious mononucleosis and throughout recovery. Although during acute disease the patients were able to mount a dominant Th1 effector cell response, the response during recovery switched to a predominantly regulatory T-cell response. Further investigation of how EBV recruits and modifies Tregs, will contribute not only to our understanding of the pathogenesis of virus-associated tumors but also to the development of therapeutic strategies designed to manipulate Treg activity.

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