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Identification of rare Epstein-Barr virus infected memory B cells and plasma cells in non-monomorphic post-transplant lymphoproliferative disorders and the signature of viral signaling

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Background and Objectives. In early and polymorphic post-transplant lymphoproliferative disorders (PTLD) Epstein-Barr virus (EBV), through its latency proteins, drives the proliferation of B lymphocytes, a process which in immunocompetent individuals leads to the establishment of latently infected memory B cells.

Design and Methods. We analyzed 11 cases, which included early and polymorphic PTLD, and 12 controls for latency of EBV infection and their antigenic profile.

Results. We identified a minority of terminally differentiated EBER$^+$ IRTA1$^+$ memory B cells and EBER$^+$ CD138$^+$ PRDM1$^+$ plasma cells in these samples. These elements were identified both in PTLD and in tumor-free tonsils from post-transplant patients but not in EBV$^-$ control tonsils. The expression of EBV latency proteins is heterogeneous, and is associated with activation of the NF-κB pathway. EBV signaling (through EBNA2, LMP1 and LMP2A) and NF-κB activation correlated with upregulation of target proteins: cMYC, JunB, CCL22, TRAF1 and IRF4. EBV-infected lymphocytes in early and polymorphic PTLDs represent a mixture of latencies II, III and, in at least 1/3 of infected cells, of latency 0.

Interpretation and conclusions. EBV infection correlates with NF-κB activation, with EBV-dependent cell signaling, and lastly, with the presence of EBV-infected plasma cells and memory cells.

Key words: post-transplant lymphoproliferative disorder, Epstein-Barr virus, viral latency, NF-κB signaling, plasma cell, memory B cell.

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Epstein-Barr virus (EBV)-associated post-transplant lymphoproliferative disorders (PTLD)[1] are a significant cause of mortality and morbidity in patients who have undergone heterologous organ transplantation. In these cases, EBV can not be properly controlled by the host and *de novo* infection or re-activation of a previous infection leads to lymphoproliferative disease.[2] Morphological and molecular analysis shows a spectrum of entities, ranging from *early* cases (plasmacytic hyperplasia, infectious mononucleosis-like PTLD) to polymorphic PTLD, and to aggressive post-transplant lymphomas (monomorphic PTLD), these last behaving similarly to *de novo* lymphomas in untransplanted patients.[1]

Cases of PTLD in all categories, but more often *early* cases, may regress after reduction of the immunosuppression. This suggests that, irrespective of the composition of the clone, some cases behave more like benign reactive proliferations, not unlike infectious mononucleosis,[2] and are still responsive to immune modulation. Molecular analysis of early and polymorphic PTLD cases does indeed show clonal EBV infection in poly-, oligo- or monoclonal populations (by Ig VH analysis), no alterations of major proto-oncogenes (*BCL1, BCL2, RAS, cMYC, p53*) and mutation of *BCL6* in a fraction of the cases.[3,4] In contrast, monomorphic PTLD are monoclonal according VH analysis and have a higher frequency of multiple genetic lesions.[3,4] Thus, the accumulation of molecular lesions in PTLD parallels clinical progression and the acquisition of frankly malignant features in previously benign, EBV-driven unchecked growth.

The molecular mechanism by which EBV enters and transforms B cells has been extensively studied.[5,6] The first viral gene to be transcribed after infection, EBNA2, is a potent transactivator of LMP1 and LMP2A, and numerous cellular genes, including cMYC.[7,8] LMP1, functionally similar to CD40, acts as a constitutively activated receptor[9] and can activate NF-κB signaling and downstream genes: the anti-apoptotic bfl-1 gene,[10] chemokines such as CCL17 and CCL22,[11] JunB, IRF-4, TRAF1, and BCL2L1 (BCL-XL).[12] *In vitro* infected B cells and *in vivo* naïve B cells display the *growth* latency program,[13] also defined as *latency III* status,[2] in which EBNA2, LMP1, LMP2A and other viral genes are expressed and sustain the

indefinite growth of infected B cells. To escape elimination by cytotoxic T cells and establish long-term latent infection, EBV+ cells switch to the *default* program[6,14] or *latency II*:[2] EBNA2 is silenced and LMP1, LMP2A and EBNA1 are expressed. This program allows EBV+ B cells to enter and exploit the B-cell differentiation pathway, where LMP1 and LMP2A may counteract a possible negative selection due to lack of CD154 or B-cell receptor (BCR) stimulation.[15] Latency I, defined by expression of EBER and EBNA1, has been documented only in Burkitt's lymphoma and primary effusion lymphoma.[5] EBNA1 is a DNA-binding protein, which is necessary for the maintanance of viral episome. EBER are EBV-encoded regulatory short RNA, ubiquitously expressed in any type of latency. EBV-infected lymphocytes may enter the lytic cycle by expressing BZLF1,[16] resulting in production of new virions. Laichalk *et al*. demonstrated that, in normal tonsils, BZLF1 expression could be detected only in small number of plasma cells,[16] suggesting that latently infected lymphocytes differentiating into plasma cells undergo viral replication. Latent infection in the majority of cells results in permanent lodging of EBV in a phenotypically defined *marginal zone* or *memory* B-cell compartment with a more restricted latency program (LMP2A and EBER only,[13,17] *latency 0*).[5,13,17] From there, B cells will recirculate and seed the peripheral lymphoid system. The majority of monomorphic PTLD and a fraction of polyPTLD are believed to originate in or have transited through the germinal center (GC).[18,19] Early and polymorphic PTLD are, however, valuable models for understanding of biology of EBV-associated diseases, given the fact that they harbor fewer additional genetic aberrations,[4,20] consistent with an earlier phase of lymphomagenesis. In order to characterize the phenotypic changes and the signaling pathways associated with PTLD, we studied the dynamics of latency changes in early and polymorphic PTLD and the expression profiles of proteins, regulated by EBV latency –associated factors.

Design and Methods

Patients and cell line

Surgical specimens were obtained from the files of the Department of Pathology, Columbia University Medical Center, New York, after informed consent and/or exemption from it was obtained, according to CU and federal provisions. PTLD cases and controls are listed in Table 1. A tissue microarray was constructed using a Beecher Instruments microarrayer (Silver Spring, MD, USA) as previously described in detail.[21]

In situ hybridization

EBV status was assessed by using *in situ* hybridization for EBV-encoded small RNA (EBER; Novocastra, Newcastle-Upon-Tyne, England). For double *in situ* hybridization and immunohistochemistry (*see below*), the hybridization was done first, then the slides were briefly boiled in antigen retrieval solution and immunohistochemical analyses performed for antibodies surviving the *in situ* hybridization procedure. The *in situ* hybridization chromagen products survive boiling.

Table 1. Clinical characteristics of post-transplant patients with PTLD and control cases.

Case#	Diagnosis	Biopsy site	Age	Sex	Organ transplanted	Months after transplant
#1	IM-like PTLD	Tonsil and Adenoids	4	M	Liver	26
#2	polyPTLD	Lymph node	7	M	Heart	>36
#3	polyPTLD	Small bowel	10	M	Kidney	NA
#4	polyPTLD	Lymph node	26	F	Kidney	8
#5	polyPTLD	Lymph node	19	M	Heart	48
#6	polyPTLD	Tonsil and Adenoids	7	F	Heart	30
#7	polyPTLD	Palate	7	F	Heart	63
#8	polyPTLD	Cecum	16	M	Heart	NA
#9	polyPTLD	Lymph node	3	F	Liver	35
#10	monoPTLD	Lymph node	10	M	Heart	>72
#11	monoPTLD	Tonsil and Adenoids	18	F	Kidney	>48
#12-16	controlT&A*	Tonsil and Adenoids	2-32	1M,4F	none	na
#17-23	pTxT&A*	Tonsil and Adenoids	2-8	3M,4F	Heart, Liver	19-64
	control tissues*	various	na	na	none	na

**controlT&A: non transplant Tonsil and Adenoids; pTxT&A: post-transplant Tonsil and Adenoids; normal spleen (2), lymph nodes (14), thymus (1), CLL (1), mantle cell NHL (1), marginal zone NHL (4), follicular lymphoma (1), Non- Hodgkin lymphoma (7), three of which EBV+ DLCL in HIV.*

Immunohistochemistry

The antibodies, listed in Table 2, and the double immunohistochemistry and immunofluorescence techniques used have been previously published.[22,23] Briefly, antigen retrieval was performed on deparaffinized slides in 1mM EDTA pH.8, then a first single indirect immunohistochemical reaction was performed with either alkaline phosphatase- (AP) or biotinylated-secondary antibodies, followed by avidin-HRP and NBT/BCIP or AEC color development, respectively. The slides were then briefly boiled in 1 mM EDTA antigen retrieval solution pH 8, a second indirect immunohistochemical stain was applied with a non-cross-reacting reagent, and the slides were finally developed with a contrasting color.

Immunofluorescence

Double immunofluorescence was performed with primary antibodies raised in different species or of different isotypes, counterstained with fluorochrome-conjugated, species- or isotype-specific secondary antibodies.[22] Tyramide signal amplification of LMP1 and

Table 2. Antibodies and probes.

Antibody/ Probes	Source	Species	Clone or serum	Isotype	Dilution
EBNA2	DakoCytomation	mouse	PE2	G1	1:200
EBV LMP1	DakoCytomation	mouse	CS1-4	G1	1:200
EBV LMP2A	Niedobitek G	rat	4E11, 15F9	G	1:50
EBER in situ hybridization	Novocastra				as directed
MUM 1	Falini B., U.Perugia	mouse	2H9	G1	1:50
MUM1/IRF-4	SCBT	goat	(sc-6059)	G	1 µg/mL
c-Rel (NF-kB)	Oncogene Research	rabbit	Ab-1	G	1:2000
CCL22 (MDC)	PeproTech	rabbit		G	0.5 µg/mL
TRAF-1	SCBT	mouse	H3	G1	1 µg/mL
JunB	SCBT	mouse	C-11	G1	1 µg/mL
BclX	LabVision	mouse	2H12	G2a	0.5 µg/mL
cMYC	SCBT	rabbit	(sc-764)	G	1 µg/mL
CD30	DakoCytomation	mouse	Ber-H2	G1	1 µg/mL
Ki-67	Gerdes J, MIB, Germany	mouse	MIB 1	G1	1:50
BCL6	SCBT	rabbit	(sc-858)	G	0.1 µg/mL
PRDM1/ BLIMP-1	KL Calame, C.U. NY USA	mouse	3H2E8	G1	1:30
IRTA1	Cattoretti G	rabbit		G	0.5 µg
PAX5	SCBT	goat	(sc1974)	G	1 µg/mL
PAX5	BD	mouse	24	G1	1 µg/mL
CD10	Novocastra	mouse	56C6	G1	1:50
CD20	DakoCytomation	mouse	L26	G2a	0.5 µg/mL
CD23	Novocastra	mouse	1B12	G1	1:100
CD79a	LabVision	rabbit	SP18	G	1:200
CD138	Serotec	mouse	B-B4	G1	1:200
CD3e	DakoCytomation	rabbit		G	0.5 µg/mL
CD4	Novocastra	mouse	CD4-1F6	G1	1:50
CD8	Novocastra	mouse	CD8-4B11	G2a	1:50
CD57	BD	mouse	HNK-1 (Leu7)	M	0.5 µg/mL
TIA-1	Beckman Coulter	mouse	2G9A10F5	G1	1:50
negative control	Sigma	rabbit, mouse		G	1 µg/mL

EBNA2 staining was performed according to the manufacturer's instructions (Perkin-Elmer, Wellesley, MA, USA) on avidin-HRP-counterstained immunostains, followed by Tyramide-FITC. The slides were counterstained with DAPI and mounted (Molecular Probes, Eugene, OR, USA). Gray scale images were taken and mounted using Adobe Photoshop software.

Polymerase chain reaction analysis of immunoglobulin heavy chain gene rearrangement

DNA was extracted from paraffin embedded tissue using the Qiamp kit (Qiagen, Valencia, CA, USA), amplified using previously published primers,[24] and subjected to heteroduplex analysis and electrophoresis on a precast 4-20% polyacrylamide gel (Invitrogen, Carlsbad, CA, USA). A sharp band which persisted following heteroduplexing was considered evidence of clonal immunoglobulin heavy chain gene rearrangement.

Results

The clinical and pathological features of the post-transplant and control cases are listed in Table 1.

Expression of EBNA2, LMP1, LMP2A and EBER

Lesions were classified according to the WHO classification system.[1] Five samples of tonsils and adenoids (*tonsils*) from non-transplant patients had no EBER$^+$ cells or too few to be evaluable by double-staining experiments; Tonsils from transplant patients without PTLD (pTxTonsils) had rare EBER$^+$ cells/high power field. IM-like, polymorphic and monomorphic PTLD had progressively increasing numbers of EBER$^+$ cells, reaching nearly 100% of cells (Table 3 and Figure 1A). EBER$^+$ cells ranged morphologically from small lymphocytes to immunoblasts and Reed-Sternberg-like cells.

In pTxTonsils with reactive follicular hyperplasia, EBER$^+$ cells were localized predominantly in subepithelial and interfollicular areas with occasional cells being found in the mantle zone. In IM-like, polymorphic and monomorphic PTLD the distribution of EBER$^+$ cells was diffuse. The EBER$^+$ cells matched the frequency and distribution of lesional cells in each sample. Five out of seven pTxTonsils, which contained small number of EBER$^+$ cells, had no detectable expression of EBNA2, LMP1 or LMP2A (Figure 1B).

In early (IM-like) and polymorphic PTLPD, up to 90% of cells were EBER$^+$, but only a subset of EBER$^+$ cells expressed EBNA2 and/or LMP1 (see Table 3). Comparing the staining for LMP1 and EBNA2 with findings of EBER *in situ* hybridization showed that >70% of the infected B cells (defined by Pax5 [*not shown*] or CD79a expression), lacked latency II and III viral proteins, as found in six out of seven polyPTLD cases (Figure 1A). Staining for LMP2A revealed positive cells exclusively in polymorphic PTLD: scattered positive cells were identified in five out of eight cases. BZLF1 staining revealed rare scattered positive cells in three out of eight polymorphic PTLD. No other cases expressed lytic protein. BZLF1+ cells were negative for CD20, CD138 and IRTA1 (*data not shown*) in PTLD and positive for CD20 in the EBV$^+$ Burkitt control cell line. Thus, early and polymorphic PTLD represent a mixture of EBV-infected lymphocytes with variable expression of latency factors, predominantly in latency I or 5.

Expression of EBNA2 and LMP1-regulated target proteins

EBNA2 was detected in four cases, three of which were polyPTLD. cMYC is directly upregulated by EBNA2:[25] all EBNA2$^+$ cases were cMYC$^+$, and most of the cells co-expressed both antigens (Figure 2A1-A2, Table 3 and supplemental Figure 1). Four additional cases were EBNA2- cMYC$^+$ (Figure 2A3-A4). By double CD20-staining, all cMYC$^+$ cells were B cells (*not shown*).

Table 3. Immunophenotypic profile of PTLDs.

Case#	Diagnosis	Clonality	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
#1	IM-like	polyclonal	++++	–	+	–	–	+++	+	+	++	+	+	+	+	+	++	+	+	–	+	–	Foc Remnants
#2	polyPTLD	monoclonal	++++	–	+	–	–	+++	+	-	+	+	+	+	–	–	–	+	–	–	–	–	Remnants
#3	polyPTLD	monoclonal	+++	+++	++	+	–	+	+	+	+++	+	+	+++	+	++++	++	-	+	+	–	–	absent
#4	polyPTLD	monoclonal	++++	+++	++	+	+	+++	+++	+	+++	+	+	+++	+	++	+++	–	+	–	–	–	absent
#5	polyPTLD	monoclonal	++++	–	++	+	–	+++	+	++++	-	++++	++++	–	–	++	-	+	–	–	–	–	absent
#6	polyPTLD	polyclonal	++++	–	+	+	+	+++	+++	+	++	+	+	+++	+++	+++	+++	++	+++	+	+++	+++	absent
#7	polyPTLD	minor clonal band	+	++	+	na	–	na	+	+	+	++++	+	+	–	+	+++	–	–	–	–	+	Scatt.cells
#8	polyPTLD	polyclonal	++	–	+	-	–	na	+	+	+	+	–	na	++	+	na	na	na	–	+	–	absent
#9	polyPTLD°	minor clonal band	++++	–	+	+	+	+++	++	+	++	+	+	++	++	+++	+++	++++	++++	+	+	–	Foc Remnants
#10	monoPTLD	NA	+++	–	+++	na	–	+++	++++	++++	+	–	–	++++	-	++++	++++	-	+++	-	–	–	absent
#11	monoPTLD	monoclonal	+++	+++	++	–	–	+	++++	+	+++	–	–	+	+++	++++	++++	–	–	–	–	–	Scatt.cells
12-16	controlT&A		0/5	0/5	0/5	0/5	0/5	na	N°°	N	N	N	na	N	N	N	N	N	N	N	0/5	0/5	N
17-23	pTxT&A		5/7	0/7	0/7	0/7	0/7	na	N	N	N	N	na	N	N	N	N	N	N	N	1/7	4/7	N

1: EBER; 2: EBNA2; 3: LMP1*; 4: LMP2A*; 5: BZLF1; 6: EBER+ LMP1-EBNA2-; 7: JunB/CD20**; 8: JunB/LMP1; 9: TRAF-1/PAX-5; 10: cREL/CD20; 11: cREL/LMP1; 12: CCL22/Pax5; 13: MUM1/CD20; 14: CD30; 15: cMYC; 16: CD138; 17: PRDM1; 18: PRDM1+ CD20+; 19: CD138+EBER+; 20: IRTA1+EBER+; 21: CD23+ DRC. *EBNA2, LMP1, LMP2A was scored on an arbitrary 5 points scale (-, +, ++, +++, ++++). All other staining were scored according to the % of positive cells (nil: -, 1%-10%: +, 11%-20%: ++, 21%-50%: +++, >50%: ++++). **Staining scores for the first antigen in front of the slash is the numerator, the antigen after the slash is the denominator. °This case contained lambda restricted B cell population and a clone with a 47, XX, t(1;13) (p36;p21), + mar 1[cp18]. °°n=normal distribution, as observed in normal lymphoid tissue. Na: not available.*

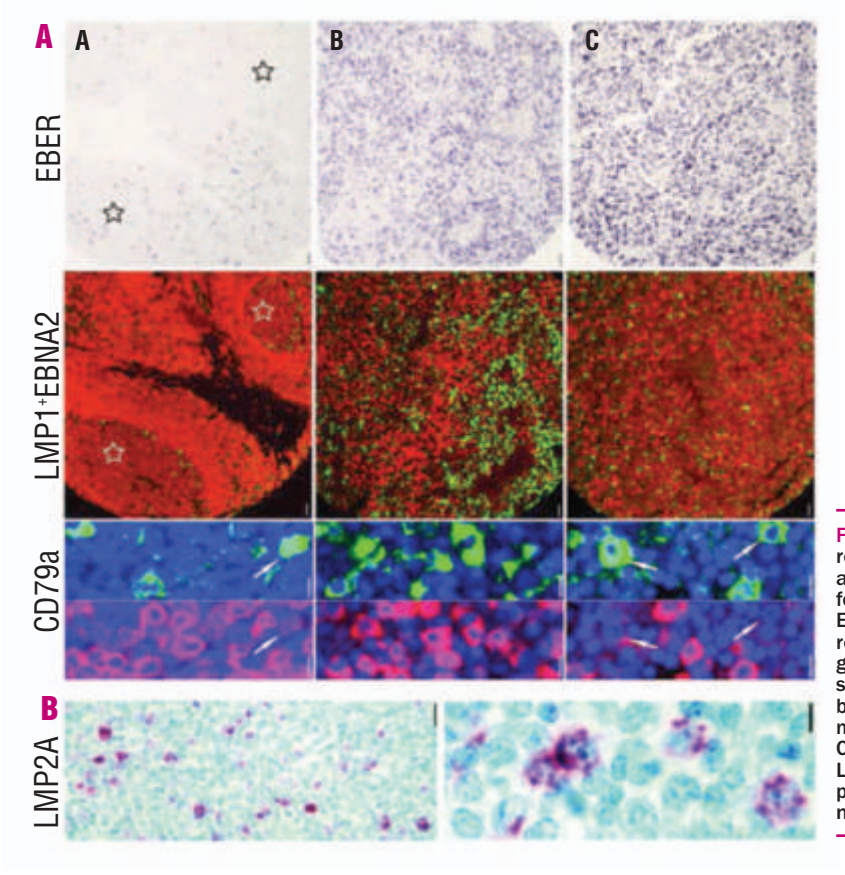

Figure 1. Latency pattern in PTLD. Three representative samples of pTxT&A (A), and polyPTLD (B,C) are shown, hybridized for EBER (black, top row) and for LMP1+ EBNA2 versus CD79a (low power middle row, high power bottom row; red CD79a, green LMP1+EBNA2. Light DAPI counterstain in blue). Note rare EBV+ cells at the border of two GC (stars) in histologically normal tonsil in (A). Arrows point to CD79a dim/negative infected B cells; LMP2A expression (D,E) in a case of polyPTLD. Scale bar: 40 μm upper and middle panels, 1 μm lower panels.

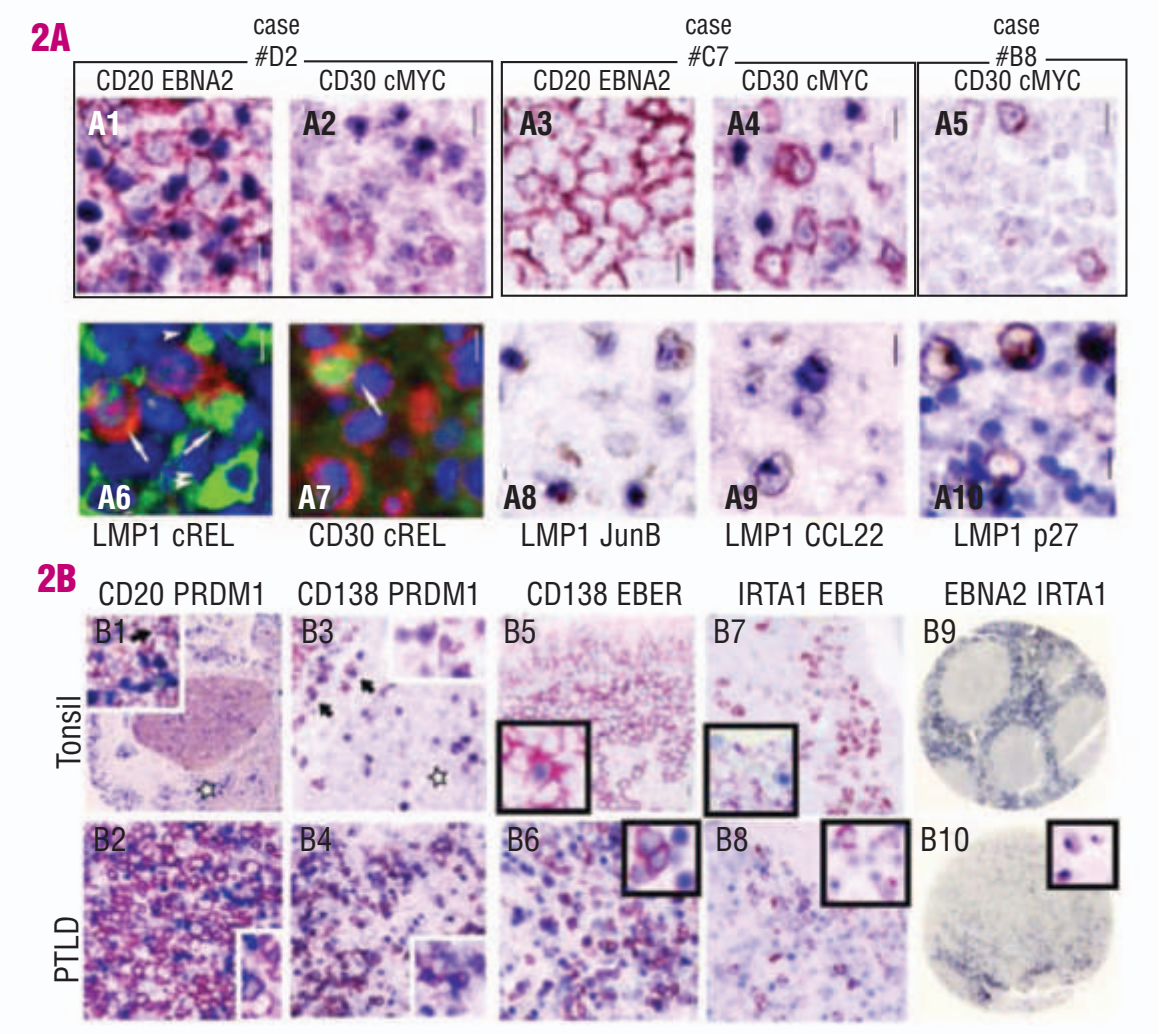

Figure 2A. EBV signaling and B-cell differentiation in normal tonsil (T) and PTLD. A. An EBNA2$^+$ PTLD coexpress EBNA2 (black) and CD20 (red) (A1), cMYC (black) and CD30 (red) (A2). Another PTLD is EBNA2-negative and expresses only cMYC (black) on CD20$^+$ (red, A3) CD30+ cells (red, A4). An EBNA2- cMYC- case is shown in with rare CD30$^+$ cells (red, A5). LMP1$^+$ (red) infected cells are heterogeneously positive for nuclear cRel (green, arrow A6) and surrounded by nuclear (arrowhead) or cytoplasmic (double arrowhead) positive cells, LMP1-negative. Nuclear cRel (green) is expressed in part of the CD30$^+$ (red) cells (A7). LMP1$^+$ cells (red) contain nuclear JunB (black, A8) and CCL22 (black, A9). LMP1 (red) and nuclear p27 (black) are mutually exclusive (A10). Scale bar = 1 μm. B. PRDM1+ lymphocytes (black) are distributed subepithelially, interfollicularly (asterisk) and inside a CD20$^+$ (red) germinal center (B1). In the inset PRDM1 is expressed in CD20- plasma cell precursors and in CD20$^+$ centrocytes (arrows). PRDM1$^+$ (black) CD20+ (red) or CD20- elements are seen in PTLD (B2). PRDM1$^+$ lymphocytes (black) coexpress CD138 (red) in the subepithelium (arrow and right inset), but are CD138- negative in the GC (asterisk and left inset (B3). PRDM1$^+$ lymphocytes (black) are heterogeneously CD138+ (red) in PTLD (B4). CD138+ plasma cells and epithelial cells (red) are EBER negative (B5). In the inset a rare EBER+ cell (blue) is stained by CD138 amidst negative plasma cells. Numerous CD138$^+$ plasmacytoid cells and plasma cells (red) are EBER+ (blue) in PTLD (B6). IRTA1+ subepithelial lymphocytes (red) are EBER- (blue) (B7). In the inset rare EBER+ cells (blue) are stained by IRTA1 amidst negative cells in a normal Post Txp Tonsil. Numerous IRTA1$^+$ (red) cells are EBER$^+$ (blue) in PTLD (B8). A low power image of a normal tonsil core with preserved immunoarchitecture, highlighted by interfollicular IRTA1$^+$ cells. (B9) Disrupted architecture in a tonsil core stained for IRTA1 (black). In the inset, a case is double-stained for EBNA-2 (golden) and IRTA1 (black) (B9, B10). Scale bar: B1, B9, B10: 40 μm; Other panels: 10 μm.

cMYC was heterogeneously positive on large CD30$^+$ blasts. The smaller, strongly EBNA2$^+$ cMYC$^+$ B cells were usually CD30- (Figure 2 A2,A4,A5).

We then determined the expression of a group of genes, regulated by LMP-1, based on transcriptional profiling and other *in vitro* studies: TRAF1, c-Rel, IRF4, CCL22, JUN-B, BCL2L1 (BCL-XL), CD10 and BCL6.[26,27] Each case was double-stained for LMP1 and for B-restricted markers (CD20, Pax5) in order to selectively evaluate the infected B cells. LMP1$^+$ cells in all PTLD cases contained variable proportions of cytoplasmic and nuclear c-Rel (N-cRel) (Figure 2A6). Notably, small LMP1-, N-cRel$^+$ cells were often observed. Double-staining for CD30 and c-Rel gave similar results (Figure 2A7). LMP1$^+$ cells were also JunB$^+$ and contained variable amounts of CCL22 (Figure 2 A8-A9). The frequency and distribution of Pax5$^+$ B cells were similar to those of LMP1$^+$ cells. CD79a was often weak or markedly reduced in LMP1$^+$ cells (Figure 1).

To assess the proliferative status of lesional cells, double staining for LMP1 and p27 was performed and showed mutually exclusive staining in each case (Figure

2 A10) and in the latency III-type cell lines (*not shown*). As for N-cRel, a range of positivity for p27 was noted in LMP1-, CD20$^+$ cells. BCL2L1 (BCL-XL) staining was broadly present on normal and pathologic B-cell subsets (*not shown*) and was therefore not further evaluated. All of our PTLD were CD10$^-$; BCL6 expression was very weak in a subset of cells, never exceeding positivity of 15% of cells in any given category of PTLD (*data not shown*). In summary, PTLD show NF-κB activation and expression of proteins associated with EBNA2 and LMP1 signaling.

Terminal B-cell differentiation in PTLD

CD40 and BCR signaling are required for activation and differentiation of antigen-specific B cells. Having found evidence of EBNA2- and LMP1-associated signaling, we evaluated terminal differentiation in our samples using plasma cell and memory cell markers. PRDM1, a master regulator of plasma cell differentiation stained in control tonsils and pTx tonsils a subpopulation of B cells inside the GC and subepithelial plasma cells, as previously described.[22] The expression of PRDM1 in PTLD was variable and ranged from 5% in early lesions, to up to 50% in polymorphic lesions, correlating with CD138 expression (Table 3, Figure 2). Double staining for PRDM1 and CD3, CD20 and CD138 demonstrated that the overwhelming majority of PRDM1$^+$ cells are CD138$^+$ plasma cells, with a minority having a CD20$^+$/PRDM1$^+$/CD138$^-$ pre-plasma cell phenotype (Figure 2 B1,B2,B6,B7). No CD3/PRDM1 double positive cells were detected in our cases. LPM1 and PRDM1 staining was mutually exclusive (*not shown*).

To investigate the role of EBV in plasma cell differentiation we double stained for EBER and CD138. In early IM-like PTLD there were rare CD138/EBER double-positive cells. Three out of eight polymorphic PTLD cases had CD138/EBER double positive cells, including a case that was monoclonal according to VH analysis, had light chain restriction and bore clonal karyotypic abnormality (Fig.2B). In these three cases, EBER expression in plasma cells was relatively weak. EBER$^+$ plasma cells were not detected in five control tonsils, but were seen in one out of seven pTxTonsils (Figure 2 B3, B8).

FCRL4 (IRTA1) identifies a subset of memory B cells in tissue.[28] In all cases, IRTA1$^+$ cells were enriched for in interfollicular areas (Figure 2B). IRTA1$^+$ memory cells were also present in six out of eight polymorphic PTLD, with reduced numbers and with a disorganized pattern, compared to in normal tonsils (Figure 2 B9). Four out of seven pTxTonsils and two out of eight polymorphic PTLD cases contained IRTA1/EBER-positive cells. EBER expression in IRTA1$^+$ cells was weak or negligible (Figure 2 and Table 3). In three cases we detected EBNA-2$^+$/IRTA1$^+$ cells (Figure 2). A case of non-transplant related IM did show EBER$^+$ and EBNA2$^+$ IRTA1$^+$ cells (*not shown*). Double staining for LMP1 and IRTA1 in two cases did not reveal co-expression (*not shown*).

Discussion

Biological significance of EBV-infected plasma cells and memory B cells in PTLD

We have identified for the first time in polymorphic PTLD a small subset of EBV-infected terminally differentiated memory B cells and plasma cells. These cells morphologically and phenotypically resemble their uninfected counterpart, but contain the virus in a latent phase (see below). The reason why only the minority of plasma cells contain EBER is unclear. One possibility is low absolute sensitivity of chromogenic detection of EBER, although other infected lymphocytes demonstrate consistently stronger staining. Another possibility is that EBER-positive plasma cells selectively die, leaving the non-infected plasma cells in a majority. This possibility is not likely, since EBV induces plasma cell death through the induction of BZLF1 expression and the lytic cycle,[16] and we did not detect BZLF1 expression in CD138$^+$ plasma cells. We favor the hypothesis that, upon establishment of latency, EBER is downregulated in a substantial fraction of plasma cells and memory cells, possibly via methylation of the viral genome.[29] The leading candidate for such repression in plasma cells is PRDM1, known to recruit methyltransferases to genomic targets.[30] Other mechanisms may be at play in memory B cells, including a global reduction of RNA synthetic activity associated with quiescence. We cannot discriminate primary infection of naïve B cells from re-infection of terminally differentiated B cells, since, irrespectively of the putative origin, all differentiated cells consistently display a latent infection viral profile (*see below*). EBV$^+$ plasma cells had already been identified in samples from patients with infectious mononucleosis.[31]

Critical role of EBV-dependent NF-κB activation in shaping PTLD biology

Antigen-dependent stimulation of the antigen receptor in normal cells, together with engagement of CD40 and other co-stimulatory molecules, ultimately leads to NF-κB-mediated terminal B-cell differentiation as memory or secretory mature B cells.[26] EBV is thought to exploit this very same mechanism via surrogate molecules, such as LMP1 and LMP2A.[9,15,32]

We identified NF-κB activation in LMP1$^+$ cells in all polyPTLD by tracing the nuclear translocation of c-Rel, a member of the NF-κB family. Nuclear relocation of c-Rel was previously validated to be proof of NF-κB activation in human lymphomas.[23,33,34] LMP1 expression was detected in cells that were IRTA1$^-$ and PRDM1$^-$ and, therefore, not terminally differentiated. In polymorphic

PTLD, abundant membranous LMP1 does not correspond to ubiquitous relocation of c-Rel. The reason for this is unclear. One possibility is that other NF-κB family members (p65, p50) may be activated first, followed by c-Rel. We could not reproducibly use other available anti NF-κB antibodies on our material, therefore this is a possibility that remains to be investigated.

N-cRel$^+$, LMP1$^-$ small lymphoid cells were also noted. This may be the evidence that other molecules besides LMP1 provide NF-κB signaling, such as CD154. It has been shown that CD40 ligation downregulates EBNA2 and LMP135. LMP1 and CD40 signaling both suppress entry into the viral lytic cycle and, together with LMP2A, contribute to progression toward a latent infection.[36] Finally, the heterogeneity in the association between LMP1 and N-cRel may reflect the short, transient, oscillating presence of c-Rel in the nucleus in physiologic conditions in normal cells[37] as opposed to in established lymphomas and cell lines.[23,33,34]

Consistently with the evidence of NF-κB-mediated signaling, a number of downstream up-or downregulated genes were found to be expressed or absent in the infected B cells: Jun-B, CCL22, TRAF1, IRF4 (upregulated) and CD10, BCL6[11,27,38] (downregulated). Our data are highly suggestive that ongoing, EBV-induced NF-κB signaling in a subset of infected B cells leads to these cells' terminal differentiation toward the memory or plasma cells and to the establishment of viral latency.

The EBV lytic cycle, associated with BLZF1 expression and plasma cell differentiation,[16] was observed in a very small proportion of cells in a few cases; these observations were inadequate to draw any conclusions, except for the rarity of the EBV lytic cycle in PTLD *in vivo*.

Dynamic range of EBV latencies in PTLD

In all EBV$^+$ cases tested, EBER$^+$ cells outnumbered the cells expressing EBNA2, LMP1 and LMP2A, suggesting that each case is an admixture of transitory latency stages. The phenomenon of broadening of viral latent gene expression during the consecutive passages of lymphoblastoid cell lines has been demonstrated *in vitro* by Gregory *et al*.[39]. A mixed pattern of viral gene expression has been also detected in IM.[31,40] This observation may imply that post-transplant EBV$^+$ patients harbor predominantly latently infected memory B cells of latency *0*, possibly because of ongoing NFκB signaling.

In a variable minority of cells, however, the *growth* transcriptional program of EBV is epitomized by cMYC expression. cMYC has a potent and pleiotropic growth-promoting effect[41] and is a direct activation target of EBNA2.[8] All our cases expressing EBNA2 also expressed cMYC, although some of our polymorphic PTLD cases expressed cMYC without EBNA2. The expression of cMYC in the EBNA2$^-$ cases is not likely to be due to mutations, since cMYC is largely unmutated in polymorphic PTLD.[4,20] Upregulation of cMYC may be due to cellular (as opposed to viral) factors, such as antigen-dependent signaling, post-transcriptional regulation or both.[42]

In addition, EBNA2 and LMP1 independently promote cell growth and replication, conditions in which entry into the cell cycle is favored by removal of inhibitory molecules such as p278. Indeed, we showed mutually exclusive expression of LMP1 and p27 in all cases of PTLD analyzed. None of our samples showed cytoplasmic sequestration of p27 or elevated cyclin D1 expression (*not shown*), both known mechanisms for bypassing p27-mediated growth arrest. We therefore conclude that polymorphic PTLD retain a normal regulation of p27.

Based on these findings, we conclude that in early and polymporphic PTLD lesions there is ongoing LMP1- and possibly LPM2A-mediated, EBV-dependent signaling, which recapitulates and mimics physiologic B-cell receptor signaling, leading to the terminal differentiation of infected B cells. Greater understanding of PTLD biology may lead to better therapeutic strategies for EBV-associated diseases including lymphomas.

RS prepared and scored the TMA, evaluated pathology, co-wrote the manuscript; KB provided gene expression profiling data on CD40L stimulation; GB co-wrote the manuscript, evaluated pathology; MM evaluated molecular analysis; GH generated essential reagents, co-wrote the manuscript; VVVM evaluated the genetic composition of cases; MB evaluated EBV infection on the TMA; GN evaluated pathology, provided essential reagents; BA evaluated pathology, co-wrote the manuscript; GC scored the TMA, evaluated pathology, co-wrote the manuscript.

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