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Original Article

Epstein-Barr virus-positive diffuse large B-cell lymphoma of the elderly expresses EBNA3A with conserved CD8+ T-cell epitopes

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Abstract: Post-transplantation lymphoproliferative disorders (PTLD) arise in the immunosuppressed and are frequently Epstein-Barr virus (EBV) associated. The most common PTLD histological sub-type is diffuse large B-cell lymphoma (EBV+DLBCL-PTLD). Restoration of EBV-specific T-cell immunity can induce EBV+DLBCL-PTLD regression. The most frequent B-cell lymphoma in the immunocompetent is also DLBCL. 'EBV-positive DLBCL of the elderly' (EBV+DLBCL) is a rare but well-recognized DLBCL entity that occurs in the overtly immunocompetent, that has an adverse outcome relative to EBV-negative DLBCL. Unlike PTLD (which is classified as viral latency III), literature suggests EBV+DLBCL is typically latency II, i.e. expression is limited to the immuno-subdominant EBNA1, LMP1 and LMP2 EBV-proteins. If correct, this would be a major impediment for T-cell immunotherapeutic strategies. Unexpectedly we observed EBV+DLBCL-PTLD and EBV+DLBCL both shared features consistent with type III EBV-latency, including expression of the immuno-dominant EBNA3A protein. Extensive analysis showed frequent polymorphisms in EBNA1 and LMP1 functionally defined CD8+ T-cell epitope encoding regions, whereas EBNA3A polymorphisms were very rare making this an attractive immunotherapy target. As with EBV+DLBCL-PTLD, the antigen presenting machinery within lymphomatous nodes was intact. EBV+DLBCL express EBNA3A suggesting it is amenable to immunotherapeutic strategies.

Keywords: Epstein-Barr virus, diffuse large B-cell lymphoma, EBNA3A, T-cell, epitope, immunotherapy, post-transplantation lymphoproliferative disorder

Introduction

Immunosuppression is the most frequently recognized predisposing factor to lymphoma. The importance of defective immunosurveillance in malignancy is most marked in cells with strong antigenic potential including those that have undergone viral induction. Consistent with this observation, is the archetypal immunosuppression associated lymphoma: post-transplantation lymphoproliferative disorder (PTLD). Relative to the immunocompetent patient, the risk of lymphoma after transplantation is increased by the order of 30-times [1, 2]. PTLD is frequently associated with Epstein-Barr virus (EBV) positivity within the malignant B-cells. PTLD is sub-divided into poly and monomorphic histological subtypes. It is believed to have a latency gene expression program (latency III) analogous to *in-vitro* EBV-transformed lymphoblastoid cell-lines (LCL), in which all EBV-latent antigens are expressed including the immuno-dominant EBNA3A [3].

The most frequent histological sub-type of PTLD is diffuse large B-cell lymphoma (EBV+DLBCL-

PTLD). Similarly, the most frequent B-cell lymphoma histology in the immunocompetent is DLBCL, representing 30% of all non-Hodgkin lymphoma. DLBCL is a biologically and clinically heterogeneous aggressive lymphoma. The World Health Organization (WHO) recently classified a provisional new sub-type of DLBCL arising in immunocompetent patients that is EBV-associated, termed 'EBV-positive DLBCL of the elderly' (EBV+DLBCL) [4, 5]. It is defined as occurring in those over 50 years of age without a predisposing immune deficiency [4]. Cases are consistently c-myc rearrangement negative [6]. In Korea, Turkey, Spain and Japan the rates of EBV+DLBCL are approximately 5-10% [7-10]. The proportion rises with age (~30% in the >90 years) [8]. With the numbers of elderly predicted to markedly rise in many developed countries, EBV+DLBCL is likely to be an increasing health burden. EBV-encoded RNA *in situ hybridization* (EBER-ISH) on diagnostic DLBCL biopsies is not routine in many centres and data on frequency is limited and conflicting [9, 11, 12]. However a consistent finding from large series is that EBV+DLBCL is associated with reduced response to front-line chemotherapy therapy and reduced survival relative to EBV-negative DLBCL [7]. PTLD arises in patients given iatrogenic immunosuppression to prevent rejection of their transplanted organ. Antigen presentation in PTLD remains intact and defective immunosurveillance is strongly implicated in pathogenesis [2]. Furthermore restoration of EBV-specific T-cell immunity by cellular immunotherapy can result in long-term regression [13]. The WHO postulate that EBV+DLBCL is related to deterioration in immunity as part of the ageing process [4]. If correct, this would also implicate defective immuno-surveillance and suggests that EBV+DLBCL could potentially be treated by novel immune-based strategies including adoptive immunotherapy. There is a well-defined hierarchy of EBV-specific T-cell immunity against EBV-latent antigens in which EBV-nuclear antigen (EBNA)-1 and Latent Membrane Proteins (LMP)-1 and 2 elicit sub-dominant responses relative to EBNA2/3A/3B and 3C. Furthermore, variations in functionally defined EBV T-cell epitopes will influence whether EBV-specific T-cells generated by immunotherapy can recognize the viral strains expressed in the lymphoma. Therefore the prerequisite data necessary before such approaches can be considered includes EBV+DLBCL's viral protein expression, the lymphomas antigen presentation capability and the polymorphisms present within antigenic determinants. This study aims to demonstrate whether this new and highly aggressive lymphoma entity has characteristics amenable to T-cell based immunotherapies.

Materials and methods

Patient samples

All patients with EBV+DLBCL were aged over 50 years (mean 67 years, range 59-90). For EBV+DLBCL-PTLD, average age was 39 years (range 17-58). Patients were chosen solely on the availability of tissue. The majority of tissues were formalin-fixed paraffin-embedded (FFPE). DLBCL and EBV+DLBCL-PTLD samples were obtained from the Princess Alexandra Hospital, Brisbane, the Austin Hospital, Melbourne and the Department of Hematology and Oncology, Campus Virchow-Klinikum, Charité Universitätsmedizin Berlin. Three frozen tissues (two EBV+DLBCL and one EBV+DLBCL-PTLD) were supplied by the Australasian Leukaemia Lymphoma Group Tissue Bank. Cases were evaluated by a lymphoma pathologist (O.M.) as positive for EBV-encoded-RNA *in-situ-hybridization* (EBER-ISH) with a positive EBER-ISH reaction defined as ≥20% of nuclei positivity of examined cells [7]. EBV-tissue positivity was in all cases confirmed by real-time PCR for EBER-DNA (**Table 1A**). All patients were Human Immunodeficiency Virus (HIV) negative and (for EBV+DLBCL) not on immunosuppression medication. Transformed lymphoma and follicular lymphoma grade 3B were excluded. The study was approved by the relevant Human Research Ethics Committees and conducted in accordance with the Declaration of Helsinki. The patients reflected the ethnicity of the respective Hospital catchment areas. One was of Pacific Islander extraction (DL67); the others were Caucasian.

Cell-lines

The following were used: Burkitt's lymphoma (BL) cell-lines without EBV: BJAB and Ramos; lymphoblastoid cell-line (LCL) DeMo (EBV+ B95-8); and T2 (transporter-associated protein [TAP1 and TAP2] deficient lymphoid cells).

In situ hybridization and immunohistochemistry

EBER -1 and -2 oligonucleotide probes (Leica Microsystems, UK) for EBER-ISH and immunohistochemistry for LMP1 (Dako, Germany), were performed as previously described [14]. EB-

Table 1. Oligonucleotide primers and probes

1A. Oligonucleotide primer/probe combinations for EBV gDNA real-time PCR

Gene	Primer name	Sequence 5'-3'	Co-ordinate (AJ507799.2)
Alb*	Alb-F1	GCTGTCATCTCTTGTGGGCTGT	19583-19604
	Alb-R1	AAACTCATGGGAGCTGCTGGTT	19723-19702
	Probe	CCTGTCATGCCCACACAAATCTCTCC	19640-19665
EBER	EBER-F1	AAACCTCAGGACCTACGCTGC	6622-6642
	EBER-R1	AGACACCGTCCTCACCAC	6726-6709
	Probe	TAGAGGTTTTGCTAGGGAGGAGACGTG	6645-6671

**Genbank accession number: NG_009291.1*

1B. Oligonucleotide primers for EBV gene expression profile and HLA class I, TAP1, TAP2 expressions by real-time RT-PCR

Transcript	Primer name	Sequence 5'-3'	Co-ordinate (AJ507799.2)
EBNA2	Y2/YH-T1F1	GCTTAGCCAGTAACCCAGCACT	35702-35711/36098-36109
	YH-R2	TGCTTAGAAGGTTGTTGGCATG	36181-36160
EBNA3A	BLRF3/BERF1-F1	GGCTACGCGCATCGACACA	80287-80293/80382-80393
	BERF1-R2	TGTACATCTCGGTATTTGAAATCTGGG	80445-80419
LMP1	LMP1-F2	TTGTATACTCCTACTGATGATCACCCTCC	168768-168749/168670-168678
	LMP1-R2	ACAATGCCTGTCCGTGCAAATTCC	168623-168646
LMP2A	LMP2A-F1	ATACGAAGAAGCGGGCAGAGG	166435-166455
	LMP2A/B-R1	GAGGTAGGGCGCAACAATTACAGG	100-77
LMP2B	LMP2B-F1	GGGAGGCCGTGCTTTAGGGG	169422-169441
	LMP2A/B-R1	GAGGTAGGGCGCAACAATTACAGG	100-77
BART	BART-F1	AGATGCCCTCCAGGTCAAAGA	156884-156904
	BART-R1	ATCCAGTGTCCCTCGTTTGG	158669-158650
HLA class I*	HLA-A-F1	AGGAGGAAGAGCTCAGATAGAAAA	997-1020
	HLA-A-R1	AAGCTGTGAGGGACACATCA	1087-1068
TAP1**	TAP1-F1	TCTCACCATAGCCAGTGCAG	1034-1053
	TAP1-R1	GTGGCCCATGGTGTTGTTAT	1100-1080
TAP2***	TAP2-F1	TTCTCCTTTGGCAGCTCACT	717-736
	TAP2-R1	ATCCGCAAGTTGATTCGAGA	796-777
B2M****	B2M-F1	TCACCCCCACTGAAAAAGATG	329-349
	B2M-R1	ATGATGCTGCTTACATGTCTCG	430-409

*GenBank accesion number: *NM_002116.5,**NM_000593.5,***NM_000544.3,****NM_004048.2*

NA3A (ab16126, Abcam, UK) was used at a dilution of 1/1000, with a ready-to-use Goat Horse Radish Peroxidase secondary antibody. Human Leucocyte Antigen (HLA) class I, TAP1 and TAP2 (Abcam, UK ab70328, ab60112, ab60113 respectively) were performed as per manufacturer's instructions.

Nucleic acid extraction

RecoverAll Total Nucleic Acid Isolation Kit (Applied Biosystems, TX, USA) were employed for simultaneous DNA and total RNA extractions from FFPE tissue sections as manufacturer's instructions. For cell-lines and frozen tissues, DNA and RNA was extracted by QIAamp DNA blood mini-kit (QIAGEN, Hilden, Germany) and *mir*Vana RNA isolation kit (Applied Biosystems, TX, USA) respectively as manufacturer's instructions. To remove contaminated DNA in total RNA, the TURBO DNA-*free* Kit (Applied Biosystems, USA) was used for all RNA samples. Both

1C. Oligonucleotide primers for PCR amplification and sequence analysis

Gene	Primer name	Sequence 5'-3'	Co-ordinate (AJ507799.2)
EBNA1	E1C-F1	AAAAAGGAGGGTGGTTTGGA	97040-97059
	E1C-R1	CATTCCAAAGGGGAGACGAC	97290-97271
LMP1	LMP1C-F4	TGACATGGTAATGCCTAGAAGTAAA	167671-167695
	LMP1C-R3	CTGGAGGTGGTCCTGACAAT	168129-168110
EBNA2A	EBNA2A-F	AACTTCAACCCACACCATCA	36925-36944
	EBNA2A-R	TTCTGGACTATCTGGATCAT	37040-37021
EBNA2B*	EBNA2B-F	TACTCTTCCTCAACCCAGAA	36749-36768
	EBNA2B-R	GGTGGTAGACTTAGTTGATG	36868-36849
EBNA3A	E3A-F6	ATGTATGCCATGGCCATTC	92736-92754
	E3A-R6	TCCTCCCAGATTTTCGTGAG	93127-93108
	E3A-F7	TCGCCAGTGGTTGTATGTTC	93008-93027
	E3A-R7	TTTCACCGGTAGCACCTTC	93378-93360
	E3A-F3	ACGGCACAGGCTTGGAAT	93279-93296
	E3A-R8	GTTGGGGGTTCTGGGACTT	93673-93655
	E3A-F8	ACCAGAGGTCCCACAAAGC	93605-93623
	E3A-R3	ACAGGGACGGGTTCTACTGG	93949-93930

*Note: *GenBank accession number: DQ279927.1. EBNA2A and EBNA2B primers are for PCR amplification of EBV types 1 and 2, respectively.*

the quality and quantity of purified DNA and RNA were measured by a NanoDrop ND-100 spectrophotometer (Wilmington, DE, USA). All genomic DNA and total RNA were store at -20^{0}C and -80^{0}C, respectively.

Real-time comparative quantification PCR

Primers to identify EBV gene / expression (Sigma Aldrich, Victoria, Australia) were designed for PCR / real-time reverse-transcription (RT) PCR respectively, as shown in **Table 1B**. Primers were designed to pick up the transcripts in a spliced adjunction to eliminate the amplification of DNA remaining in the total RNA. Product sizes were between 60-100bp to ensure suitability for the amplification of RNA extracted from FFPE sections. β2M was used as the internal control for each sample. The DeMo (B95-8 EBV) LCL is used as a calibrator for all studied genes. Real-time RT-PCR was performed in 20 µl of reaction using SYBR Green PCR Master Mix (Applied Biosystems, UK) with 5µl diluted cDNA and the thermal cycle was as: 37^{0}C x 10 minutes; 95^{0}C x 10 minutes; 40 cycles: 95^{0}C x 15 seconds, 60^{0}C x 45 seconds running by the Rotor-Gene 3000. The data were analysed using Rotor-Gene 6.0 and Microsoft Excel 2007 and the amplified products were also checked by the separation in 2.5% agarose gel with ethidium bromide staining and visualized under UV light. The heat-map was generated by Genesis software v1.0. Primers for HLA-A, TAP1 and TAP2 for real-time RT-PCR are shown in **Table 1B**.

CD8+ T-cell epitope sequencing for EBNA1, LMP1 and EBNA3A and phylogenetic analysis

To enable CD8+ T-cell epitope sequencing and phylogenetic analysis, selected DNA regions were chosen for PCR amplification and sequence analysis [15, 16]. The primers (**Table 1C**) were designed using Primer3 software and were synthesized by Sigma-Aldrich, Victoria, Australia. A nested PCR was done with the same primers in PCR1 or using inner primers for the samples that were negative in the first round PCR. All PCR products were purified using QIAquick Gel Extraction Kit (QIAGEN, Germany). The sequence analysis was performed using the BigDye 3.1 sequencing protocol (Applied Biosystems, USA). Briefly, the BigDye reaction was performed containing 1.2µl of BigDye terminator v3.1, 3.6 µl of 5x sequencing buffer, 1ng template DNA per 100bp and 3.2 pmol primer in a total 12µl amplification reaction. The thermal cycles included an initial denature 94^{0}C x 2 minutes and then 35 cycles: 94^{0}C x 10 seconds, 50^{0}C x 5 seconds and 60^{0}C x 2 minutes. DNA was precipitated with 72µl of 70% isopropanol at room temperature x 15 minutes and centrifuged at 13,000rpm x 30 minutes. The DNA was dried for one hour at room tempera-

Table 2. Summary of EBNA1 sub-types and LMP1 variants detected in EBV+DLBCL-PTLD and EBV+DLBCL

			EBNA1 SUB-TYPES				LMP1 VARIANTS						
No	Samp.		P-ala	P-ala-v2	P-thr	V-val-v3	B95-8	Ch1	Ch 2	AL	NC	Med-	Med+
1	PDL3	EBV+DLBCL-PTLD	+#	-	-	-	+	-	-	-	-	-	-
2	PDL4		-	-	+	-	-	-	-	-	-	+	-
3	PDL5		-	-	+	-	ND						
4	PDL6		-	-	+	-	-	+	-	-	-	-	-
5	PDL7		+#	-	-	-	+	-	-	-	-	-	-
6	PDL8		-	+	+	-	-	-	-	-	+	+	-
7	PDL9		+	-	-	-	+	-	-	-	-	-	-
8	DL4	EBV+DLBCL	-	-	+	-	-	+	-	-	-	-	-
9	DL14		+#	-	-	-	ND						
10	DL26		-	-	+	-	-	-	-	-	-	+	-
11	DL29		-	-	+	-	-	+	-	-	-	-	-
12	DL44		-	+	-	-	-	-	-	-	+	-	-
13	DL67		-	-	-	+	-	-	+	-	-	-	-
14	DL82		-	-	+	-	-	+	-	-	-	-	-

Note: EBNA1 and LMP1 were sub-typed as previously described.[15, 16] "+" the sub-type detected, and "-" is undetected. V-val-v3 is a sub-type of V-val (P529Q), due to two sub-types V-val-v1 and V-val-v2. ND: not done. PDL: EBV+DLBCL-PTLD; DL: EBV+DLBCL. #Some minor changes compared with P-ala: PDL3 and PLD7: E483D; DL14: E500K.

ture and diluted in 20μl formamide. The sequence analysis was run by *ABI* 3100 *automated DNA sequencer* (*Applied Biosystems*, CA, USA). Data was analysed with the FinchTV 1.4 DNA sequence analysis program (http://en.biosoft.net/dna/FinchTV.html). The phylogenetic trees were drawn as previously outlined [16] from amino acid sequences using ClustalW2 (www.ebi.ac.uk/Tools/clustalw2) for EBV+DLBCL and EBV+DLBCL-PTLD and for three EBV strains from GenBank: B95-8 (V01555), AG876 (DQ279927.1) and GD1 (AY961628.3).

Results

EBV+DLBCL has a latency profile similar to EBV+DLBCL-PTLD

To further characterize the EBV latency profile, we performed real-time RT-PCR on a series of seven EBV+DLBCL and seven EBV+DLBCL-PTLD cases (**Table 3**). In agreement with prior work in EBV-positive cancers, expression was not identical in all samples [17-19]. There was no discernable differences between EBV+DLBCL and EBV+DLBCL-PTLD. In most cases the majority of EBV latent antigens were expressed, consistent with type III latency. EBNA3A was strongly expressed (defined as equivalent or above DeMo LCL) in 12/14 cases, whereas EBNA2 was strongly expressed in only 4/14, consistent with the low detection rate of EBNA2 by immunohistochemistry by others [4, 8]. The EBNA2 region

Table 3. EBV gene expression profiles in EBV+DLBCL (DL4, 26, 29, 44, 67, 122, 82) and EBV+DLBCL-PTLD (PDL3-9), using DeMo (B95-8) LCL as a calibrator for all genes. The EBV-negative lines (Ramos and BJAB-) and EBV-negative DLBCL-PTLD primary samples (PDL1 and PDL2) were negative controls. β2M expression was used as an internal control for each gene and every duplicate experiment. Assays were repeated in two independent experiments.

	EBER	BART	LMP1	LMP2A	LMP2B	EBNA2	EBNA3A
DEMO LCL	+	+	+	+	+	+	+
RAMO	-	-	-	-	-	-	-
BJAB-	-	-	-	-	-	-	-
PDL1	-	-	-	-	-	-	-
PDL2	-	-	-	-	-	-	-
PDL3	+	+	+	+	-	-	+
PDL4	+	+	+	-	+	+	+
PDL5	+	+	+	+	+	+	+
PDL6	+	+	-	-	+	-	-
PDL7	+	+	+	+	+	-	-
PDL8	+	+	+	-	-	-	-
PDL9	+	+	+	-	-	-	-
DL4	+	-	-	-	-	-	+
DL26	+	+	+	-	-	-	+
DL29	+	+	-	-	+	-	+
DL44	+	+	-	-	-	+	+
DL67	+	+	-	+	-	-	+
DL122	+	+	-	+	-	-	+
DL82	+	-	+	-	-	-	-

we amplified (to distinguish EBV types 1 and 2) spanned this deleted region. Since DNA from this region could be amplified in all cases, the low EBNA2 / high EBNA3A gene expression pattern we observed in EBV+DLBCL was not due to the EBNA2 gene deletion. Protein expression was tested by immunohistochemistry in nine DLBCL cases with either high or low viral latent gene expression. LMP1 and EBNA3 protein was detectable in all cases with high gene expression, and not in those with low expression (**Figure 1**).

EBV+DLBCL has similar antigen processing machinery expression to EBV+DLBCL-PTLD

EBV+DLBCL-PTLD and LCL are known to be capable of presenting endogenous antigens for recognition by T-cells. Using immunohistochemistry (IHC), we compared EBV+DLBCL, EBV+DLBCL-PTLD and LCL for expression of the components of antigen processing machinery: MHC class I, TAP-1 and TAP-2. All were consistently present, with similar results obtained on both frozen and paraffin-wax sections. Control TAP deficient cell lines were not stainable for TAP-1 and TAP-2. To accurately quantify and compare the components required for antigen presentation, we performed real-time RT-PCR for MHC class I, TAP1 and TAP2 gene expression on EBV+DLBCL, EBV+DLBCL-PTLD, and EBV-negative DLBCL tissues, each relative to the LCL line DeMo. This line was chosen as it is known to be capable of presenting endogenous EBV antigens and is regularly used as a positive target cell control in functional T-cell assays. Levels were equivalent between samples for each of six EBV+DLBCL, seven EBV+DLBCL-PTLD, and seven EBV-negative DLBCL (P= NS) (**Figure 2**).

Polymorphisms in EBV regions encoding for CD8+ T-cell epitopes are infrequent for EBNA3A but common for EBNA1 and LMP1

There are 2 major types of EBV (types 1 and 2) distinguished by polymorphisms in EBNA2 [20]. All fourteen EBV+DLBCL and EBV+DLBCL-PTLD cases were type 1 (data not shown). Using direct sequence analysis, the gene variations of

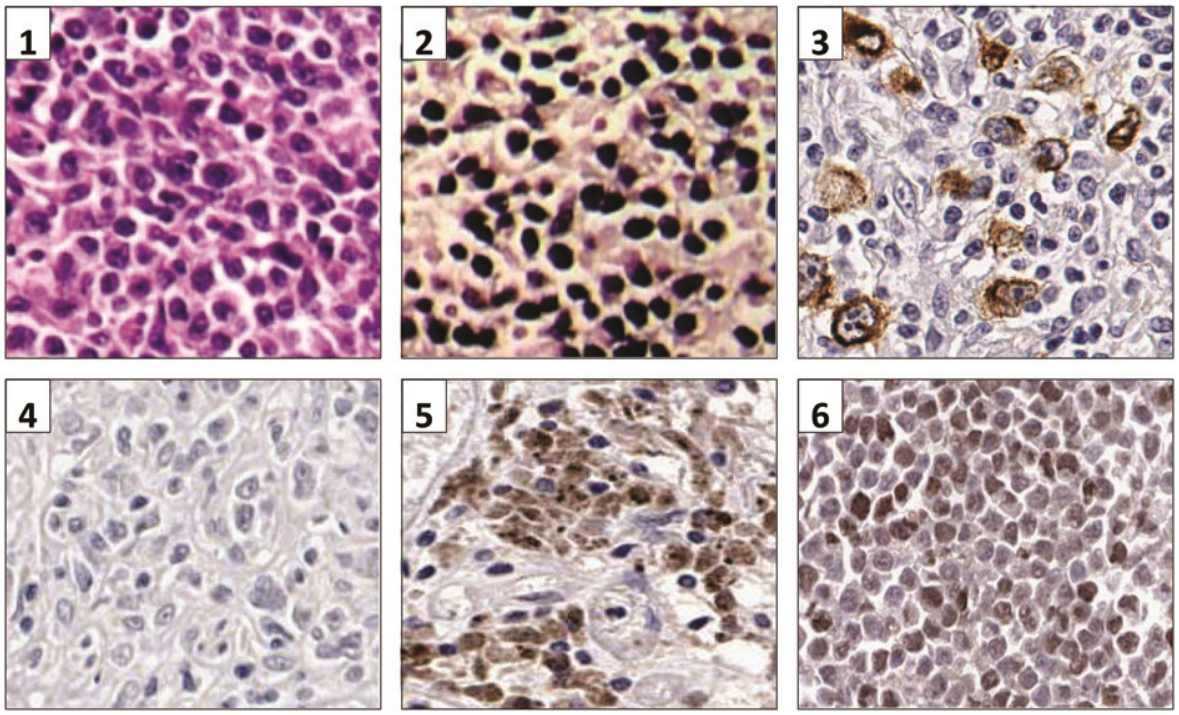

Figure 1. EBV latent gene and protein expression. 1. Panels 1 and 2: Haematoxylin and Eosin stain, and EBER-ISH respectively on DL67. Panel 3: LMP1 immunohistochemistry on DL26. Panels 4-6: EBNA3A immunohistochemistry on an EBV-negative DLBCL, an EBV+DLBCL-PTLD (PDL6) and on DeMo LCL respectively.

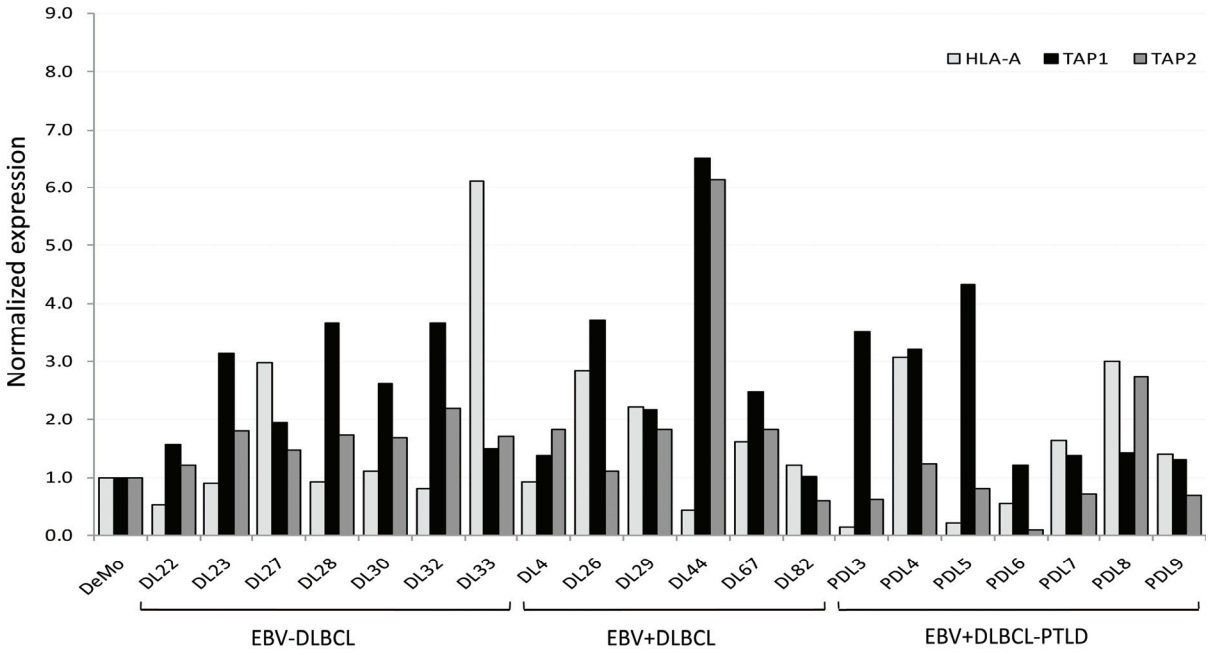

Figure 2. HLA class I, TAP1 and TAP2 gene expression in EBV+DLBCL is equivalent to EBV+DLBCL-PTLD. The expression of HLA-A, TAP1 and TAP2 was quantified by real-time RT-PCR. DeMo B95-8 LCL was used as a reference. All experiments were run in duplicate and repeated once. No significant differences were observed between groups.

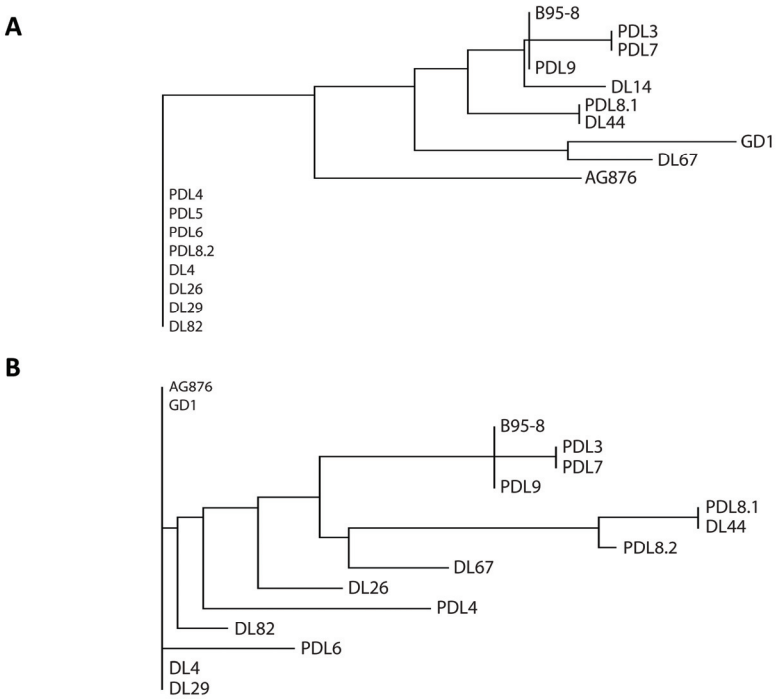

Figure 3. Phylogenetic Analysis of EBNA1 and LMP1 protein sequences from EBV+DLBCL and EBV+DLBCL-PTLD. The phylogeny of EBV strains can be inferred from EBNA1 and LMP1 strain variation analysis, relative to the prototype type 1 strain B95-8, the prototype type 2 strain AG876, and the type 1 strain GD1. With EBNA1 (**A**), the tree had 4 main branches. For LMP1 (**B**), a five branch pylogenetic tree was made. The first large cluster in EBNA1 is separated into 3 clusters in the LMP1 phylogram because of the 30bp deletion in the C-terminus (not present in B95-8). The first (PDL4 and DL26) samples has no 30bp deletion, the second (PDL6, DL4, DL29 and DL82) has the 30bp deletion and the third (AG876, GD1) contains the deletion. DL: EBV+DLBCL and PDL: EBV+DLBCL-PTLD.

the EBNA1- and LMP1-C terminus functional domains were used to further type EBV into sub-strains [16]. Consistent with previous studies of EBV-positive lymphomas [21, 22] in 13/14 cases only one viral sub-strain could be identified. In polymorphic analysis of wild-type sub-strains in biopsy samples, the EBNA1 P-ala and LMP1 B95-8 sub-strains type the same as the type 1 laboratory strain B95-8. In EBV+DLBCL and EBV+DLBCL-PTLD tissues respectively only 1/7 and 3/7 samples were EBNA1 P-ala, versus 0/6 and 3/6 with LMP1 B95-8 sub-strains (**Table 2**). By EBNA1 typing, the most frequent sub-type was P-thr (7/14). For LMP1, the most common was Ch1 (4/12). PBMC from PDL7, DL4 and DL82 was sequenced and the detected EBNA1 and LMP1 sub-types were the same with that in the tumor (data not shown). Put together these data indicate considerable genetic diversity within the viral strains present in the lymphoma.

We performed EBNA3A sequencing in five EBV+DLBCL and four EBV+DLBCL-PTLD tissues in regions associated with ten functionally identified $CD8^+$ T-cell epitopes presented by seven different (plus one currently unidentified) HLA class I alleles. This showed conservation of HLA class I epitopes in 85/90 epitopes tested (**Table 4**). Exceptions include DL67, a Pacific Islander who showed a type 1 EBNA2 by PCR but an HLA B*08 epitope (LLRGRAYGQ as opposed to FLRGRAYGL) identical to the type 2 prototype viral strain AG876. Extensive LMP1 and EBNA3A sequencing (data not shown) in this case showed the patient to be compatible to a previously reported viral sub-strain in which both type 1 and type 2 features are known to co-exist, that was originally identified in Papua New Guinea [23]. In all nine lymphoma cases the same HLA A*29 epitope (VSSDRGVAC as opposed to VFSDRGVAC) was identified. VSSDRGVAC is the most frequent variant observed in Australian Caucasians [24]. The HLA B*35.01 epitope YPLHEQHGMA was present in 5/9 cases. YPLHKQHGMA was observed in one EBV+DLBCL-PTLD case, and YTLHEQHGMA in three EBV+DLBCL cases.

No phylogenetic distinction between EBV strains present in EBV+DLBCL versus EBV+DLBCL-PTLD

Phylogenetic analysis was performed to assess for relationships between viral strains present in patient biopsy samples. We compared results between EBV+DLBCL and EBV+DLBCL-PTLD. Previous work has shown that the phylogeny of

Table 4. EBNA3A HLA class 1 epitope polymorphisms compared to B95-8, GD1 and AG876 strains.

	HLA Class I allele restriction									
Samples	A*24	A*29	A*30.02	B*07	B*07	B*08	*B*08	B*35.01	B*62	Unknown
B95-8	RYSIFFDY	VFSDGRVAC	AYSSWMYSY	RPPIFIRRL	VPA**P**AGPIV	QAKWRLQTL	FLRGRAYG**I**	YPLHEQH**G**MA	LEKARGSTY	HLAAQGMAY
GD1	RYSIFFDY	V**S**SDGRVAC	AYSSWMYSY	RPPIFIRRL	VPAPAGPIV	QAKWRLQTL	FLRGRAYG**L**	YPLHEQ**R**GMA	LEKARGSTY	HLAAQGMAY
PDL3	RYSIFFDY	V**S**SDGRVAC	AYSSWMYSY	RPPIFIRRL	VPAPAGPIV	QAKWRLQTL	FLRGRAYG**L**	YPLHEQHGMA	LEKARGSTY	HLAAQGMAY
PDL4	RYSIFFDY	V**S**SDGRVAC	AYSSWMYSY	RPPIFIRRL	VPAPAGPIV	QAKWRLQTL	FLRGRAYG**L**	YPLHEQHGMA	LEKARGSTY	HLAAQGMAY
PDL7	RYSIFFDY	V**S**SDGRVAC	AYSSWMYSY	RPPIFIRRL	VPAPAGPIV	QAKWRLQTL	FLRGRAYG**L**	YPLHEQHGMA	LEKARGSTY	HLAAQGMAY
PDL8	RYSIFFDY	V**S**SDGRVAC	AYSSWMYSY	RPPIFIRRL	VPAPAGPIV	QAKWRLQTL	FLRGRAYG**L**	YPLH**K**QHGMA	LEKARGSTY	HLAAQGMAY
DL26	RYSIFFDY	V**S**SDGRVAC	AYSSWMYSY	RPPIFIRRL	VPAPAGPIV	QAKWRLQTL	FLRGRAYG**L**	YPLHEQHGMA	LEKARGSTY	HLAAQGMAY
DL82	RYSIFFDY	V**S**SDGRVAC	AYSSWMYSY	RPPIFIRRL	VPAPAGPIV	QAKWRLQTL	FLRGRAYG**L**	YPLHEQHGMA	LEKARGSTY	HLAAQGMAY
DL29	RYSIFFDY	V**S**SDGRVAC	AYSSWMYSY	RPPIFIRRL	VPAPAGPIV	QAKWRLQTL	FLRGRAYG**L**	Y**T**LHEQHGMA	LEKARGSTY	HLAAQGMAY
DL44	RYSIFFDY	V**S**SDGRVAC	AYSSWMYSY	RPPIFIRRL	VPAPAGPIV	QAKWRLQTL	FLRGRAYG**L**	Y**T**LHEQHGMA	LEKARGSTY	HLAAQGMAY
DL67	RYSIFFDY	V**S**SDGRVAC	AYSSWMYSY	RPPIFIRRL	VPAPAGPIV	QAKWRLQTL	**LLRGRAYG**Q	Y**T**LHEQHGMA	LEKARGSTY	HLAAQGMAY
AG876	**C**YSIFFDY	V**P**K**D**GR**G**AC	AYSSWMYSY	RPPIF**L**RRL	VPA**L**AGPIV	Q**V**KWR**M**T**T**L	**L**LRGRAYG**Q**	YPLH**Q**QH**S**MA	L**A**KA**P**RR**T**Y	***H-------

Underlined amino acids are different as compared to B95-8. Gray highlighted epitopes indicate differences among studied samples. There were 5/90 differences identified. *FLRGRAYGL is the functionally defined HLA B8 epitope. FLRGRAYGI is observed only in B95-8. **DL67 (a Pacific Islander) showed a type 1 EBNA2 by PCR but an HLA class I epitope identical to the type 2 EBV strain AG876. Extensive LMP1 and EBNA3A sequencing showed this case to be compatible to a previously reported viral sub-strain originally identified in Papua New Guinea [23] ***Amino acid deletion.

EBV viral strains can be inferred from EBNA1 and LMP1 strain variation analysis, relative to three strains that have had full-length sequencing: B95-8, AG876 and GD1. With EBNA1, the tree had two main branches. The first branch had EBV+DLBCL-PTLD's PDL4, PDL5, PDL6 and EBV+DLBCL's DL26, DL29 (**Figure 3A**). The other branch then sub-divided into two, one of which contained AG876 and the other containing B95-8 and (in a sub-branch GD1). The EBV+DLBCL-PTLD samples PDL3, PDL7 and PDL9 were closely clustered to B95-8. For LMP1, a three branch phylogenetic tree was made (**Figure 3B**). One branch contained AG876 and GD1, another DL4 and DL29, and the last the remaining samples and B95-8, which again was closely clustered to PDL3, PDL7 and PDL9. There was broad overlap with the EBNA1 tree. However, there were some differences due to the presence or absence of a 30bp deletion present in the LMP1 C-terminus (this deletion is not present in B95-8 but is present in AG876 and GD1). This resulted in samples that formed the first EBNA1 branch, not clustering together in the LMP1 phylogram. PDL4 and DL26 sample had no 30bp deletion, whereas PDL6, DL4 and DL82, DL29 contained the 30bp deletion. The presence of clusters containing both EBV+DLBCL and PTLD-DLBCL biopsy samples suggests no phylogenetic distinction between viral strains implicated in DLBCL arising in the immunosuppressed versus the overtly immunocompetent.

Discussion

EBV+DLBCL has a poor outcome relative to EBV -negative DLBCL, indicating that new approaches need to be investigated [7-9]. Results from cellular immunotherapy in other EBV-associated malignancies are sufficiently encouraging for this modality to be explored in the setting of EBV+DLBCL [13, 25, 26]. However, before these strategies are undertaken, there are a number of fundamental questions that should be addressed. These include EBV+DLBCL's viral latency profile, the polymorphisms present within relevant EBV T-cell epitopes, and its antigen presentation capability.

Previous studies suggest that EBV+DLBCL generally have a viral latency type II pattern, i.e. expression is limited to EBNA1, LMP1 and LMP2. Only a minority of cases showed the EBV-latency type III pattern seen in LCL in which a broad range of EBV-latent antigens including EBNA3A are expressed. The EBV-latency pattern is of critical importance for the rational design of EBV-specific cellular immunotherapies that target relevant tumor associated viral antigens. The reason for this is that in the hierarchy of EBV-latent antigen specific $CD8^+$ T-cell immunity, the dominant response is against EBNA2/3A/3B/3C, whereas EBNA1, LMP1 and LMP2 $CD8^+$ T-cells are usually sub-dominant [27, 28]. Thus if EBV+DLBCL has a restricted viral latency profile it will be a major impediment for cellular immunotherapy.

Studies of EBV-positive cell-lines have broadly identified several forms of virus latency. Although classifying EBV-latency types based on cell-line data is useful, data from primary tumor biopsies indicate that such categorization is overly simplistic. Firstly, real-time RT-PCR indicates that patterns of virus gene expression are variable between primary tissues of the same histology (inter-patient variability) [17-19]. Secondly, tissue immunohistochemistry demonstrates differences in expression between malignant cells within the same biopsy (intra-patient variability) [29]. Furthermore, inferences regarding viral latency are frequently based on the use of antibodies to LMP1 (expressed in >90% of EBV+DLBCL) and EBNA2 (expressed in 25-30% of EBV+DLBCL) [4, 8]. The rationale is that EBNA2 acts as a transcriptional regulator of EBV nuclear antigens, and hence positivity can be regarded as a surrogate marker of EBNA3A/3B/3C, and to indicate latency type III [8]. Prior work in EBV+DLBCL based on antibodies to LMP1 and EBNA2 suggest that EBV+DLBCL generally have a viral latency type II pattern, i.e. expression is limited to EBNA1, LMP1 and LMP2. Only a minority of cases showed the EBV-latency type III pattern seen in LCL in which a broad range of EBV-latent antigens including EBNA3A are expressed. However, data from primary tissues in other EBV-positive malignancies indicates that patterns of virus gene expression are more accurately identified by real-time RT-PCR [17]. However our gene expression data shows that the inferences from EBNA2 protein expression should be re-visited. Instead, EBV+DLBCL lymphomas have a variant type III pattern with EBNA2 down-regulated but the immuno-dominant EBNA3A expressed. The same expression profile was present in EBV+DLBCL-PTLD. To our knowledge our study is the first attempt to compare latency profiles in immuno-

suppressed / non-immunosuppressed EBV-positive DLBCL by real-time RT-PCR. Our data in indicate that the presence or absence of iatrogenic immunosuppression does not influence the EBV-viral latency pattern.

Our data indicates considerable genetic diversity within the viral strains detected between lymphoma biopsies. The strains were associated with known polymorphisms in functionally identified EBNA1 and LMP1 $CD8^+$ T-cell epitopes. For example the HLA A*02 B95-8 defined 9mer LMP1 epitope (YLLEMLWRL) has between one and three amino acid differences from Ch1 (YFLEILWRL), Ch2 (YFLEILWRL), NC (YFLEILWRL) and Med- (YLLEILWRL) sub-strains, that were identified in 83% of biopsies tested. The Med- found in 3/12 cases does not appear to influence HLA peptide binding or recognition by YLLEMLWRL-specific CTL clones [30]. However Ch1, Ch2 and NC have changes in the anchoring position within the epitopes that do interfere with MHC-I binding and fail to elicit a CTL response in chromium release assays [30]. However these strains have no differences with another well-defined functionally confirmed HLA A*02 restricted epitope (YLQQNWWTL) [30, 31]. Outside of BL, EBNA1 sequence analysis in EBV-positive lymphoma samples is limited. We demonstrate considerable genetic diversity from the B95-8 strain, with 79% of biopsies harbouring a non-P-ala sub-strain. Previous work has shown that this sequence polymorphism greatly influences T-cell recognition [32]. Of note, sub-typing covered the amino acid residue 524 located in the EBNA1 epitope described by Bell *et al.* 2008 [32]. P-ala codes for Threonine (ACT) at the 524 position (T-cell epitope YNLRRGTAL) whereas sub-types P-thr or V-val contain Isoleucine (ATT) (coding for YNLRRGIAL) and the P-ala-v2 subtype encodes for Valine (GTT) (resulting in YNLRRGVAL). In our study, only 3/7 (DLBCL-PTLD) and 1/7 (EBV+DLBCL) biopsies had the P -ala sub-type. Polymorphism at this site has previously been shown to influence CD8+ T-cell recognition of an endogenously processed HLA B*08 binding EBNA1 epitope [32]. Polymorphism in the EBNA1 amino acid residue 524 site also influences the frequency of EBNA1-specific $CD8^+$ T-cells that can be generated *in-vitro* in pre-therapy blood from patients PTLD [33]. In Australia 24.7% are HLA B*08 and 46.5% type as HLA A*02 [34]. The high incidence of EBNA1 non-P-ala, LMP1 non-B95.8, and HLA B*08, HLA A*02 alleles in our population has implications for future vaccines, which will need to be designed to cater for EBNA1 and LMP1 strain variation in epitope encoding regions. Any future EBV vaccine that exclusively delivers the B95-8 sequence is likely to stimulate $CD8^+$ T-cells which in a significant proportion of individuals may have a limited capacity in recognizing the EBNA1 and LMP1 epitopes encoded by the EBV sub-strain within the tumor.

Our data in indicate that the presence or absence of iatrogenic immunosuppression does not influence the EBV-viral latency pattern. To further examine the relationship between EBV+DLBCL and EBV+DLBCL-PTLD, we typed viral strains so as to perform a phylogenetic analysis. The lack of phylogenetic distinction that we observed between the viral strains identified in EBV+DLBCL and EBV+DLBCL-PTLD biopsy samples further emphasize the similarities in viral biology between the two entities. Further work is required to establish the pathogenic links between EBV+DBLCL and EBV+DLBCL-PTLD.

Latency III is the expression profile used during infectious mononucleosis [35]. Normally, the cytotoxic T-lymphocyte (CTL) response targets the immuno-dominant EBNA2,3A,3B,3C latent proteins [27]. The CTL response eliminates EBV-latently infected cells before they develop into a pathogenic lymphoproliferation. This is exemplified in individuals who develop PTLD due to iatrogenic immunosuppression following organ transplantation. Selective immune impairment in EBV-positive HL suggests a similar role of immunopathogenesis in HL arising in immunocompetent vs immunodeficient [36, 37]. Reconstitution of EBV-specific CTL has been associated with objective clinical response in EBV-positive lymphomas arising in both overtly immunocompetent and immunosuppressed patients, and may be a potential strategy for EBV+DLBCL [13, 26, 38, 39]. Results to date suggest cellular immunotherapy is relatively low-risk and might be an attractive option for the older patient. Antigen processing and presentation is known to be intact in EBV-positive PTLD and in LCL. We observed that HLA class I, TAP1 and TAP2 in EBV+DLBCL expression were equivalent to EBV+DLBCL-PTLD (and EBV-negative DLBCL), and was greater than a control LCL in the majority of cases. In contrast to EBNA1 and LMP1, we observed extensive conservation of functionally defined CD8+ T-cell epi-

topes (85/90 or 94% of epitopes sequenced) encoded by the immuno-dominant EBNA3A, making this an attractive immunotherapy target. All nine patients exhibited the HLA A*29 epitope VSSDRGVAC (which is identical to GD1, but distinct to B95-8's VFSDRGVAC). This is the most frequent form of the epitope seen in Australian Caucasians and does not appear to influence immunogenicity [24]. The 5/90 epitope variations included an EBV+DLBCL case in which the HLA B*08 epitope LLRGRAYGQ (identical to the type 2 prototype viral strain AG876), and an EBV+DLBCL-PTLD case where the HLA B*35.01 epitope YPLHKQHGMA was found. These variants are known to be essentially unrecognized by FLRGRAYGL and YPLHEQHGMA specific CTL respectively [40]. There were also three EBV+DLBCL cases of YTLHEQHGMA. This epitope shows reduced recognition by YPLHEQHGMA specific CTL [41].

EBV+DLBCL have a highly adverse outcome to chemotherapy relative to EBV-negative DLBCL [7-10]. We demonstrate that EBV+DLBCL has many similarities to EBV+DLBCL-PTLD, including expression of EBNA3A, indicating that such an approach may be feasible in EBV-positive DLBCL arising in the overtly immunocompetent as well as in the context of PTLD. Further studies are required to assess the nature of EBV specific T-cell immunity in these patients. Strategies such as immunotherapies specifically targeting the virus within the malignant cell should be actively explored.

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