Analysis of Epstein–Barr virus and cellular gene expression during the early phases of Epstein–Barr virus lytic induction

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INTRODUCTION

Epstein–Barr virus (EBV) infection is ubiquitous and targets B lymphocytes and oropharyngeal epithelial cells. Primary EBV infection occurs during childhood, and the virus establishes a lifelong latent infection in the memory B-cell pool (Babcock et al., 1998). Viral reactivation has been associated with a number of malignancies, including Burkitt’s lymphoma (BL), nasopharyngeal carcinoma, Hodgkin’s lymphoma, gastric carcinoma and in immunosuppressed individuals, AIDS-associated lymphomas and post-transplant lymphoproliferative disease (PTLD) (Crawford, 2001).

The EBV genome is a large circular dsDNA episome that encodes approximately 80 ORFs. Around 70 EBV lytic RNAs and 12 latent RNAs are transcribed (Young & Rickinson, 2004). In addition, highly abundant RNA transcripts are transcribed from the BamHI A region of the genome, known as BamHI A rightward transcripts (BARTs), including RPMS1, A73, BART and BARF0, with proposed roles in maintenance of latency (Brooks et al., 1993; Smith et al., 2000; van Beek et al., 2003). Additionally, two highly abundant non-coding RNAs (EBV-encoded small RNAs, EBERs) are expressed in all forms of latency (Minarovits et al., 1992). Approximately 40 small non-coding RNAs known as microRNAs have also been discovered with potential roles in antisense regulation (Forte & Luftig, 2011).

EBV can also transform normal resting B cells in vitro, driving them into immortalized lymphoblastoid cell lines (LCLs) (Küppers, 2003). In LCLs, a limited set of EBV latent transcripts are expressed, including six EBV nuclear antigens (EBNA-1, -2, -3A, -3B, -3C and -LP), three latent membrane proteins (LMP-1, LMP-2A and LMP-2B), two small non-coding RNAs (EBER-1 and -2) and 25 pre-miRNAs (Young & Rickinson, 2004; Forte & Luftig, 2011). This expression profile is defined as latency III and is necessary for driving the indefinite proliferation of primary B cells and maintaining the viral genome (Forte & Luftig, 2011). EBV reactivation in LCLs can be artificially induced using 12-O-tetradecanoyl-13-phorbol ester and sodium butyrate.

Real-time PCR identified several highly induced (>90-fold) EBV lytic genes over a 48 h time course during the lytic induction phase. Latent genes were induced at low levels during this phase. The cellular response to lytic viral replication is poorly understood. Whole genome microarray analysis identified 113 cellular genes regulated twofold or more by EBV, including 63 upregulated and 46 downregulated genes, over a 24 h time course post-induction. The most upregulated gene was CHI3L1, a chitinase-3-like 1 protein (18.1-fold; P<0.0084), and the most downregulated gene was TYMS, a thymidylate synthetase (–7.6-fold). Gene Ontology enrichment analysis using MetaCore software revealed cell cycle (core), cell cycle (role of anaphase-promoting complex) in cell cycle regulation) and lymphatic diseases as the most significantly represented biological network processes, canonical pathways and disease biomarkers, respectively. Chemotaxis, DNA damage and inflammation (IL-4 signalling) together with lymphoproliferative disorders and non-Hodgkin’s lymphoma were significantly represented biological processes and disease biomarkers.

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Abbreviations: aRNA, antisense RNA; ATCC, American Tissue Culture Collection; BL, Burkitt’s lymphoma; EBER, EBV-encoded small RNA; EBV, Epstein–Barr virus; GO, Gene Ontology; IE, immediate early; LCL, lymphoblastoid cell line; LPD, lymphoproliferative disorder; NaB, sodium butyrate; PTLD, post-transplant lymphoproliferative disease; RT-PCR, reverse transcriptase PCR; SLR, signal log ratio; TPA, 12-O-tetradecanoyl-13-phorbol ester.

Six supplementary tables are available with the online Supplementary Material.
various agents, including phorbol esters such as 12-O-tetradecanoyl-13-phorbol ester (TPA); histone deacetylases (sodium butyrate, NaB); calcium ionophores; transforming growth factor beta (TGF-β); transfection using BZLF1; infection with EBV strain P3HR1 and immunoglobulin cross-linking in the Akata cell line (Fields et al., 2007). TPA has been widely used with NaB to induce lytic reactivation in the Raji cell line and provides a reproducible and inexpensive model to investigate EBV gene expression during the lytic induction phase.

The focus of our work was to investigate EBV and host gene expression during the early stages of lytic induction in Raji cells. By using real-time reverse transcriptase PCR (RT-PCR) and whole-genome arrays, we hoped to gain further insights into the gene expression patterns at this stage of the virus’ life cycle and identify potential markers of EBV-related disease. The Raji cell line was chosen because it maintains a stable copy number of EBV episomes and because lytic replication cannot occur, to minimize potential effects of late gene expression during the induction time course experiments used to investigate virus/host interactions (Nutter et al., 1987; Lu et al., 2010). Furthermore, the Raji cell line exhibits latency type III, similar to that seen in PTLD (Brink et al., 1997).

We report the identification of several highly expressed EBV and cellular genes that could act as early markers of EBV-related disease in whole blood samples from transplant recipients. Preliminary experiments were carried out using the data from these experiments to confirm the expression patterns found and to determine their clinical utility in routine diagnostic assays.

METHODS

Cell lines. All cell lines were obtained from the American Tissue Culture Collection (ATCC). The EBV-positive BL cell line Raji (ATCC CCL-86) containing approximately 50 copies of the EBV genome and Ramos (ATCC CCL-1596), an EBV-negative BL cell line, were used in this study.

Cell culture. All cells were maintained in RPMI 1640 medium (Sigma) supplemented with 2 mM L-glutamine, 10% heat-inactivated foetal calf serum (Sigma-Aldrich) and 100 IU ml⁻¹ penicillin and maintained in a 5% CO₂ incubator at 37 °C. Cell lines were maintained in 75 cm² tissue culture flasks (VWR International) at a density of 3.5×10⁶ cells ml⁻¹. Cells were split every 2 to 3 days at dilutions of 1:3. Cell viability was assessed using trypan blue (Sigma-Aldrich) exclusion.

Induction of the EBV lytic cycle. Raji and Ramos cells were reseeded/seeded 1 day (at densities of 5×10⁶ cells ml⁻¹) before lytic induction. After 24 h, cells (at a density of 1×10⁸ cells ml⁻¹) were treated with TPA (30 ng ml⁻¹) (Sigma-Aldrich) and NaB/n-butyrate (2 mM) (Sigma-Aldrich) and incubated for 48 h. At 0, 2, 6, 24 and 48 h post-induction, the cells were harvested, resuspended in lysis buffer containing β-mercaptoethanol and stored at −80 °C until RNA extraction. Cultures of uninduced Raji and Ramos cells were also set up as a control for lytic induction.

Extraction of RNA. Total cellular RNA was extracted from approximately 1×10⁶ cells using QIAmp RNeasy Mini Kits (Qiagen) according to the manufacturer’s instructions. The absence of residual DNA was shown by RT-PCR and by using a minus RTase control (i.e. samples that were not reverse transcribed). The concentration of total RNA eluate was quantified at 260/280 nm using a NanoDrop ND-8000 spectrophotometer (LabTech International) and stored at −80 °C.

RT-PCR. Total RNA (2 μg) was reverse transcribed into cDNA using RETROscript kits (Ambion) according to the manufacturer’s instructions and stored at −20 °C prior to real-time PCR.

Real-time PCR. Expression levels of EBV genes were determined by real-time RT-PCR using SYBR Green-based detection (Bio-Rad) with a RotorGene instrument (Qiagen). Eighty-eight genes (2 immediate early (IE), 33 early, 42 late and 11 latent) were analysed. Eighty-two of the 88 primers, including the housekeeping gene β-actin (ACTB), were described previously by van Beek et al. (2003) and Pan et al. (2005). Three of the 82 primers (Table 1) were redesigned for LMP-2A (previously LMP-2), BSMLF1 (previously SM, BMLF1) and BKRF2 (previously gp25) using the Invitrogen OligoPerfect Designer software (Invitrogen) for newly available GenBank sequences of the EBV-1 genome (accession no. NC_007605) found at the National Centre for Biotechnology Information (NCBI). In addition, PCR primers were designed for a further seven genes/transcripts (A37/RPMs1, BDLF3.5, BRRF1A, BGLF3.5, LF1, LF2 and LF3) that became available in GenBank NCBI (Table 1). The sensitivities and specificities of all PCRs were confirmed using B95-8 genomic DNA and DNA and RNA extracted from both Raji and Ramos (EBV negative) cell lines by real-time PCR/RT-PCR and analytical agarose gel electrophoresis (data not shown). The expression levels of all genes were determined for each of the time points using the 2⁻ΔΔCt method (Livak & Schmittgen, 2001). The single-copy housekeeping gene β-actin (ACTB) was used as the reference gene. The fold induction level of each gene was determined by dividing the relative expression levels for the induced by the relative expression levels for the uninduced condition. Real-time PCR was performed in triplicate for each gene. The s0 of triplicate values was determined for each gene target using Prism. Real-time PCR was performed using the following programme: 95 °C for 10 min (hold); 50 cycles of 95 °C for 10 s (denaturation), 57 °C for 20 s (annealing) and 72 °C for 20 s (extension); final extension: 72 °C for 1 min (hold); and melt-curve analysis: ramp from 72 to 99 °C and rising by 1 °C pre-melt to 5 °C.

For the early time points (0, 2 and 6 h), induction levels were below two-fold or not detectable (data not shown); subsequent analyses were carried out on samples from 24 and 48 h inductions.

Array hybridization (cDNA synthesis and antisense RNA labeling). In order to determine highly differentially expressed cellular genes during the EBV lytic induction phase, replicates of both Raji and Ramos cell lines induced and uninduced for the lytic cycle were generated. Total RNA extracts from the cell lines were used to generate transcription data using the Affymetrix Human Genome U133 Plus 2.0 GeneChip™. EBV-negative Ramos cells served as a control for EBV specificity, whereas the uninduced cells served as a control for the induction. Total RNA was extracted from duplicate cell cultures of induced and uninduced Raji and Ramos cells at the 24 h time point and pooled for microarray analysis. The 24 h time point was chosen because the EBV expression data demonstrated lytic induction and the focus of the investigation was on the earliest stages of gene expression. Pools of total RNA were quantified using a NanoDrop ND-8000™ spectrophotometer (LabTech International), and the quality of RNA was assessed on RNA 6000 Nano assay chips with the Agilent RNA LabChip Kit and using an Agilent 2100 Bioanlyser (Agilent).

Preparation of biotin-labelled antisense RNA. T7 in vitro transcription was carried out to produce biotin-labelled antisense RNA (aRNA) using the MessageAmp Premier RNA amplification kit (Ambion), according to manufacturer’s instructions.
Microarray data analysis. The raw signal intensity data (CEL file) was captured from the scanner and analysed using the microarray suite 5.0 (MAS 5.0) algorithm (Affymetrix Suite v5.0) built into the Affymetrix GeneChip operating software. Data processing was performed using the MAS 5.0 algorithm to include background correction, expression summary and normalization, by global scaling performed across all gene chips at a signal of 5.0. Following data pre-processing, the data was log₂ transformed. To determine whether genes were highly differentially expressed and regulated between the induced and uninduced conditions, a pairwise comparison was performed, and genes with a greater than twofold change across the gene chips were extracted/filtered. The MAS 5.0 algorithm was used to filter data by (i) detection call, (ii) change call and (iii) signal change. The bias for the top 250 most highly differentially expressed probe sets in the data sets was filtered as follows: (i) remove all absent probe sets on both arrays, leaving only present probe sets; (ii) remove all probe sets not changing between the comparison, leaving only increased or decreased probe sets and (iii) remove all probe sets changing below twofold, leaving probe sets changing by twofold or more. Additional analysis steps were performed to distinguish between genes with a high signal log ratio (SLR) but low signal value, leaving 250 most highly differentially expressed and regulated probe sets. Genes with altered expression in response to lytic induction were mapped to Gene Ontology (GO) using MetaCore algorithm to determine biological, canonical and biomarker processes.

Real-time RT-PCR validation of microarray data. For validation of the microarray results, cDNA was generated from the pooled total RNAs (1 µg) of induced and uninduced samples (Raji) using the high capacity reverse transcription kit (Applied Biosystems), according to the manufacturer’s instructions. Microarray data were validated by real-time PCR for 22 cellular genes showing altered expression in response to lytic induction using TaqMan probe-based detection (Applied Biosystems) on the RotorGene instrument (Qiagen). TaqMan gene expression assays for each target gene were obtained from Applied Biosystems and tested in triplicate on cDNA template (1:2 dilution). Quantification of gene expression was determined using the 2^(-ΔΔCt) method (Livak & Schmittgen, 2001). The single-copy housekeeping gene β-actin (ACTB) was used as the normalization control. The fold change was determined by dividing the relative expression level for each gene in the induced by the uninduced condition, and the SD was determined.

Statistical analysis. Statistical analysis was performed in GraphPad Prism version 5. Graphs were created in Prism. The fold induction/fold change and SD were determined in Prism. \( P<0.05 \) was considered statistically significant.

RESULTS

Validation of Raji EBV genome deletions in the lytic induction phase

EBV deletions were confirmed by real-time RT-PCR and agarose gel analyses. Of the 88 EBV genes analysed, four genes (BALF1, BARF1, BZLF2 and EBNA-3C) are deleted from the Raji EBV genome, and one, BALK2, was truncated (Hatfull et al., 1988). However, a single amplification product and a 10.64- and 7.47-fold induction of the BALF2 gene were found for the 24 and 48 h time points, respectively (data not shown).

Identification of highly induced EBV early and genes in the lytic induction phase

Real-time PCR using SBYR Green-based detection was used to analyse the expression of 88 EBV genes during the lytic induction phase and identify highly induced genes that might be important in the development of EBV-related disease. For this, gene expression levels were measured at 0, 2, 6, 24 and 48 h time points using cDNA derived from total RNA isolated from TPA/NaB-induced and uninduced Raji and Ramos cell lines.

Table 1. In-house oligonucleotide primer sequences

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Sequence</th>
<th>Amplicon size (bp)</th>
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<tr>
<td>A73/RPMS1</td>
<td>5'AGACACTCGATATCGAAGGCGCAG-3'</td>
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</tr>
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<td></td>
<td>5'TCACCTTGGCTGTCAGTCGAGC-3'</td>
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<td>BDLF3.5</td>
<td>5'TCAGAGACCTCCGCTCAAA-3'</td>
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</tr>
<tr>
<td></td>
<td>5'CCGATGCTGAGAAAGGCCAG-3'</td>
<td></td>
</tr>
<tr>
<td>BFRF1A</td>
<td>5'GGTCTCAGCGTATGATCCGAGC-3'</td>
<td>155</td>
</tr>
<tr>
<td></td>
<td>5'TTATCAGCTACGCGCTGAA-3'</td>
<td></td>
</tr>
<tr>
<td>BGLF3.5</td>
<td>5'CCGGCTCTAGCTGTTGATG-3'</td>
<td>223</td>
</tr>
<tr>
<td></td>
<td>5'AGAGGCTCGTGAAGAACAGGAGC-3'</td>
<td></td>
</tr>
<tr>
<td>BRF2</td>
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<tr>
<td></td>
<td>5'GGTCTGCTGCTGATCAGGGG-3'</td>
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<tr>
<td>BSMLF1</td>
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<tr>
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<td>5'CTCGGCGGTTAGGAAGGGGAGG-3'</td>
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<td>LF1</td>
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<tr>
<td>LMP-2A</td>
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<td></td>
<td>5'ATCGAGGAGGCGGCGGAC-3'</td>
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For the early time points (0, 2 and 6 h), induction levels were below twofold (data not shown). Because of the relatively small changes in expression levels in these early time points, subsequent analyses were carried out only on samples from the 24 and 48 h inductions. Induction levels of the EBV IE/early genes, apart from the four deletions described previously, are shown in Tables S1 and S2 (available in the online Supplementary Material). The IE gene BZLF1 was induced 19- and 156-fold above uninduced levels, at 24 and 48 h, respectively (Table S1). The IE gene BRLF1 was induced 3-fold at 24 h up to 60-fold at 48 h. The mean of the variation in induction level was low for both BZLF1 and BRLF1. Of the 33 early genes, 16/33 (48.5%) were induced over 10-fold at 24 h post-induction, whereas 25/33 (75.8%) were induced over 10-fold at 48 h post-induction (Table S2). With the exception of the two deletions discussed previously (BALF1 and BARF1), expression was detected in all genes (Table S2). This included the truncation in BALF2, induced at 10- and 7-fold at 24 h and 48 h, respectively. At the 24 h time point, 3/33 (9.09%) early genes (BcRF1, BGLF4 and BXLF1) were induced over 100-fold (104-, 114- and 153-fold, respectively), two (BcRF1 and BGLF4) of which had maximal induction levels at 24 h. At the 48 h time point, 8/33 (24.24%) genes (BDLF4, BFLF2, BFRF1, BFRF2, BILF1, BMRF1, BORF2 and BXLF1) were induced above 100-fold, two (BFRF2 and BMRF1) of which were also induced over 1000-fold from 24 to 48 h.

Detection of late lytic gene expression in the lytic induction phase

Induction was detected for late lytic gene expression, including three highly induced genes (BdRF1, BXL2 and BOLF1) with induction levels over 1000-fold (Table S3).

Low-level induction of EBV latent genes in the lytic induction phase

Fold changes were also analysed for 11 latent genes, with the exception of the deletion EBNA-3C (Table S4). At the 24 h time point, the fold change for the latent genes was below 2, with the exception of A273/RPMS1 and BARF6, which were induced to 2- and 11-fold, respectively. At the 48 h time point, five genes were induced below 2-fold, and the remaining were induced below 10-fold, with the exception of EBNA-3A, which was induced over 15-fold.

Alteration of cellular gene expression in the lytic induction phase

Highly differentially expressed and regulated genes were identified in response to lytic reactivation by using a fold change of 2 as an arbitrary threshold and a P-value of <0.0001. A total of 6517 and 5437 probe sets were identified for the two Raji replicates. A Venn diagram combining the Raji cells showed that 3884 probe sets were commonly modulated between the replicates (Fig. 1).

Identification of the most highly differentially expressed cellular genes

Owing to the high signal intensities represented by the data set, a sum x SLR formula was applied to the data to extract probe sets with high SLR but low signal values that represent overall low signals. The 250 most highly differentially expressed and regulated probe sets were identified and ranked (Table S6). Of these probe sets, 191 (76.4%) were commonly modulated in both biological replicates.

Identification of probe sets regulated specifically by EBV infection

In order to identify which of the 191 highly differentially expressed probe sets were expressed specifically by EBV replication and not in response to lytic cycle reagents, the overlap of the 191 probe sets with the 199 most highly differentially expressed probe sets from TPA/NaB-induced EBV-negative Ramos cells was assessed. The overlap identified 58 probe sets commonly modulated in both induced Raji and Ramos cells which left 133 probe sets representing 113 genes regulated in Raji cells alone, as shown in Table S6.

Of the 113 genes regulated specifically by EBV lytic infection, 64 genes were upregulated (up to 18.1-fold), and the remaining 49 genes were downregulated (up to –7.6-fold) at 24 h post-induction and one gene, heterogeneous nuclear...
Validation of microarray data using real-time RT-PCR

To assess the validity of the microarray data, TaqMan probe-based detection was used to determine the fold changes of a selected subset of 22 cellular genes. These were selected based on the following: (i) not regulated by TPA/NaB, (ii) previously demonstrated to be involved in EBV infection, (iii) highly regulated and with functions associated with infections and (iv) identified in significantly represented pathways in GO analysis. The fold changes and regulation of expression were similar between the microarray and real-time PCR (Table S5), indicating that the microarray results were an accurate reflection of the transcript levels in the cells. Thirteen (59.1%) of these candidates (CHI3L1, TIMP-1, CXCL9, CCL5, ULBP2, ISG15, ANXA2, RASA1, CCL22, CXCL10, CCL3, CD52 and VIM) were upregulated, including CHI3L1, which was the most upregulated gene (18.1-fold; P<0.0084). The remaining nine (40.9%) genes (IGJ, HDGF1, ANP32E, PCNA, LRMP, MAD2L1, CDC2, HMGB1 and RGS13) were downregulated.

Identification of functional groups enriched in the EBV lytic induction phase

Highly differentially expressed and regulated cellular genes are represented by a variety of biological functions as shown in Table S6, including anti-apoptosis, cell cycle and DNA synthesis. To determine on a global scale what functional groups were regulated by EBV during the lytic induction in the microarray data, GO analysis was performed using enrichment analysis GO in MetaCore™ for biological processes, canonical pathways and disease biomarkers. Histograms representing the 10 most highly representative pathways/networks/diseases are shown in Fig. 2(a–c). Of the canonical pathways, ‘cell cycle’ (role of anaphase-promoting complex in cell cycle regulation) was the most highly representative pathway and the most relevant for induction of the lytic induction phase (Fig. 2a). ‘Cell cycle’ (core) was also the most highly representative network in the data set for biological processes (Fig. 2b). In addition, highly significant enrichments were seen for ‘mitosis’ and ‘M-phase’, ‘chromosome separation’, ‘chromosome condensation’ and ‘cytoskeleton’ in cell cycle canonical pathways and chemotaxis, DNA damage and inflammation (IL-4 signalling) in biological processes (Fig. 2b). Of the disease biomarkers, lymphatic diseases were the most highly representative, with lymphoproliferative disorders and non-Hodgkin’s lymphoma significantly represented (Fig. 2c).

DISCUSSION

Latent genes predominate in EBV-related cancers and have been shown to have an important role in their pathogenesis (Crawford, 2001). Reactivation from latent EBV infection during immunosuppression is thought to contribute to the development of PTLD through an increase in viral replication (Hopwood et al., 2002). EBV lytic transcripts have been demonstrated alongside latent gene expression in PTLD tumour biopsies (Rea et al., 1994a; b; Brink et al., 1997). However, the exact role of the EBV lytic genes in oncogenesis is unclear (Cai et al., 2015). The aim of this study was to identify differentially expressed EBV and cellular genes during the early lytic phase in a model system of induction in order to develop novel host/pathogen diagnostic assays, suitable for routine laboratory use, to more accurately diagnose and stage EBV-related disease. Pooled duplicate biological replicates were analysed for the array; the same duplicates were used in the RT-PCR analysis for EBV gene expression, to maintain the biological connection between the host–virus expression patterns. Ideally, a minimum of three biological replicates should be used; however, since the arrays were being used as a preliminary method to identify gene targets that would be subsequently tested by real-time RT-PCRs, it was felt that two replicates would be sufficient.

Eight early EBV genes were more highly induced at the 24 h time point compared with the 48 h time point (see Table S2). Six of these genes are involved directly in viral DNA replication, with four (BALF5, BBLF2, BBLF3 and BBLF4) forming part of the replication initiation complex (Tsurumi, 2001). Six genes (BALF2, BALF3, BALF5, BBLF2, BBLF3 and BBLF4) also contain methylated sites in their promoters (Bergbauer et al., 2010). The EBV BZLF1 IE protein, Zta, binds preferentially to methylated sites within promoter regions in order to activate the lytic cycle cascade (Bergbauer et al., 2010), and the early, high-level expression of these six genes does accord with this important function. Notably, two genes (BcRF1 and BGLF4) were induced maximally over 100-fold. BcRF1 encodes a TATA box-binding-like protein essential for activation of late viral gene expression (Gruffat et al., 2012). BGLF4, a serine/threonine protein kinase, is localized in the virus replication compartment, where it acts by phosphorylating BMRF1, a DNA polymerase processivity factor, essential for lytic replication (Neuhierl & Delecluse, 2006). In addition, BFRF1, BFRF2, BMRF1 and BXLFI were also highly induced. Although not directly involved in lytic replication, BFRF1 plays a role in transporting mature virions across the nuclear membrane (Gonnella et al., 2005). BFRF2 encodes a viral tegument protein but appears to be present only at a transcript level (Concha et al., 2012), while BXLFI encodes a viral thymidine kinase; however, their exact roles are not fully understood.

Many EBV lytic mRNAs are overlapping in the EBV genome (Baer et al., 1984; de Jesus et al., 2003), sharing 3′-polyadenylation sites. As a result, primers in overlapping sequence
Fig. 2. Histogram showing the top 10 most significantly represented canonical pathways (a), biological processes (b) and disease biomarkers (c) in probe sets derived from Affymetrix microarray gene expression data, altered in response to EBV reactivation in TPA/NaB-induced Raji cells. Gene expression data was set at a threshold of 1.5 and \( P < 0.05 \) and subjected to enrichment analysis using the GeneGo MetaCore. Black bars represent data from replicate A; grey bars represent data from replicate B.
regions may amplify more than one transcript, and not all transcripts can be detected uniquely. For instance, the mRNAs of 21 early/late genes (BFLF2, BORF2, BMRF1, BMRF2, BSMFI1, BLRF2, BLLF2, BKRF4, BBRF2, BBLF1, BGLF5, BGRF1, BGLF2, BGLF1, BDLF3, BDLF2, BVRF1, BdRF1 and BNLF2a/BNLFL2b/LMP-1) are completely contained within longer transcripts, as illustrated in the EBV transcription map (http://www1.imperial.ac.uk/department/medicine/divisions/infectiousdiseases/viro/epsteinbarr/ebvmap/). Thus, measures of transcription may be a combination of overlapping mRNAs representing one or more genes (i.e. transcription units) and may be overestimated. This is likely to be the case for expression levels of BMRF1 (>1000-fold), which is contained within the transcription unit BarF1/BMRF1/BLRF1. In contrast, BGLF4 is the longer overlap of the transcription unit BBLF1/BGLF3/ BGLF4, and therefore, expression levels (>100-fold) of this transcript are likely to be accurate. Despite agarose gel analyses (not shown) that showed the correct amplicon size for each primer set, it is difficult to tease apart the expression of some individual genes. Nevertheless, the regulation patterns of the EBV transcription units and host responses are likely to reflect the natural history of the infective life cycle.

An unexpected finding was the detection of late lytic gene expression, particularly so for BALF2 with fold increases of 10.64 and 7.47 at 24 and 48 h, respectively. This is surprising because it is known that the Raji EBV genome has two major deletions within the BamHI A region, resulting in three gene deletions, BALF1, BARF1 and BZLF2, and two truncations, BALF2 and EBNA-3C (Hatfull et al., 1988). As a consequence, the Raji EBV genome is incompetent for transformation, lytic DNA replication and crucially late lytic gene expression (i.e. an abortive lytic cycle). The mechanism of late lytic gene expression involves both viral and cellular gene expression (Gruffat et al., 2012), compared to a dependence on viral DNA synthesis only (Chevallier-Greco et al., 1986; Laux et al., 1988; Decausin et al., 1995). Possible explanations for the levels of late gene expression are the measurement of ‘read-through transcription’ from the early lytic genes, including some overlapping mRNAs, into downstream late regions and non-specific amplification of cellular mRNAs, not mature cytoplasmic mRNAs, as total cellular RNA was extracted.

In this study, whole-genome microarray analysis was undertaken to identify which cellular genes were regulated during the lytic induction phase. The analysis revealed that EBV lytic induction of Raji cells uniquely altered the expression of 113 cellular genes (more than twofold). The most upregulated was CHI3L1 (18.1-fold), a chitinase-3-like 1 protein, implicated in several cancers (Eurich et al., 2009). The most downregulated cellular gene was TYMS (−7.6-fold), a thymidylate synthetase and a key enzyme for pyrimidine biosynthesis and an essential component of the DNA synthesis pathway. Its expression is increased in several cancers, particularly in colorectal cancer (Teijpar et al., 2010). Many of the genes identified have been shown to be involved in chemotaxis, anti-apoptosis, immune response, DNA damage, cell proliferation and DNA synthesis. Several of the 113 genes that we identified were also regulated at 24 h after lytic induction in other studies (Cahir-McFarland et al., 2004; Yuan et al., 2006). These include DUSP5, EGR1, SQSTM1, OAS1, OASL and PLEC, with diverse biological functions involved in cell proliferation, autophagy, apoptosis and differentiation (Shin et al., 2013; Myung et al., 2014; Zhao et al., 2014); tumorigenesis (Parkhition et al., 2011) and type 1 interferon signalling in antiviral response (Kristiansen et al., 2011).

Some of the genes were upregulated (CCL3, CCL5, CCL22, CXCL9 and CXCL10) and previously shown to be regulated by LMP-1, EBNA-2 (Nakayama et al., 2004; Uchihara et al., 2005; Vockerodt et al., 2005) and EBNA-3A (Hertle et al., 2009). These genes encode chemokine proteins and are likely to be expressed in response to EBV infection. Additional genes (JUND, CCL5, Ili4I, PCNA and CD69) were identified that have previously been shown to be regulated by LMP-1 (Cahir-McFarland et al., 2004) and EBNA-2 in LCLs (Spender et al., 2006; Zhao et al., 2006; Lucchesi et al., 2008). Interestingly, some of the differentially expressed genes have previously been shown to be regulated more than twofold (P<0.05) in microarray analyses of monomorphic PTLD tumour biopsies; these include ANP32E, CDC2, CD52, HDGF1, HMGB1, LRMP, MAD2L1, PCNA and TIMP1. The products of these genes are involved in processes likely to be relevant to PTLD progression such as anti-apoptosis; (ANP32E) (Tsukamoto et al., 2008); cell growth and apoptosis; (CDC2, CD52, MAD2L1 and LRMP) (Kallakury et al., 1997; Manenti et al., 2006; Rodig et al., 2006; Guo et al., 2010b; Lee et al., 2010); apoptosis, cell growth, cell invasion and apoptosis; (HDGF1) (Lee et al., 2010); regulation of transcription/antitumour immunity; (HMGB1) (Liu et al., 2011); cell proliferation; (PCNA) (Malkas et al., 2006) and cell proliferation, apoptosis, angiogenesis and cellular signalling; (TIMP1) (Peng et al., 2011).

A number of cellular pathways are altered by EBV lytic induction in Raji cells. The GO analysis showed that the functions of genes differentially expressed in induced Raji cells ranged from cell cycle, chemotaxis, DNA damage and inflammation (IL-4 signalling). The cell cycle scored as the most significantly represented canonical pathway. Although EBV is known to use host cell components to take over pathways that control cell cycle checkpoints and DNA repair, in particular the dysregulation of the G1/S transition, the regulation of cellular genes during EBV lytic replication is not well understood (Guo et al., 2010a).

It is likely that interleukin signalling, chemotaxis and inflammation (IL-4 signalling) are regulated in response to virally induced chemokine genes. Viral factors are known to facilitate lytic replication by activating an S-phase-like environment (Kudoh et al., 2003; Chang et al., 2012). In our model, BZLF1 was upregulated 19.4-fold, and BGLF4 was upregulated 114.2-fold at 24 h. The BZLF1 product, Zta, is postulated to cause cell growth arrest at the G1/S boundary in EBV-permissive B95-8 cells (Kudoh et al., 2003), similar
to that seen in both cytomegalovirus infection (Salvant et al., 1998) and herpes simplex virus 1 infection (Kudoh et al., 2003). The BGLF4 product, a proline-dependent serine/threonine protein kinase and a member of the conserved herpes protein kinases, also interferes with DNA replication and S-phase progression by mimicking cellular Cdk activities (Chang et al., 2012). It was shown that BGLF4 prevents cells from entering G2/M phase by inducing highly condensed chromosomal structures, preventing cell cycle progression and facilitating viral DNA replication (Chang et al., 2012). In fact, chromosome separation and chromosome condensation were highly represented in cell cycle canonical pathways. The DNA damage response is also elicited during EBV lytic replication by activation of the ataxia telangiectasia-mutated (ATM) checkpoint signalling pathway and shown to be necessary for progression of virus replication (Kudoh et al., 2005).

Genes enriched for disease biomarkers were analysed to identify altered expression patterns during lytic reactivation. Despite the uncertain role of lytic gene expression in oncogenesis discussed previously, lymphatic diseases were the most highly represented. Lymphoproliferative disorders (LPDs) and non-Hodgkin lymphoma, both known EBV-associated lymphomas (Crawford, 2001) associated with the above cellular altered pathways, were significantly represented. These findings are supported by the work of Hong et al. (2005), who demonstrated the potential role of lytic gene expression in LPDs in a series of elegant experiments using BZLF1- and BRLF1-deleted virus mutants in SCID mice. It was shown that the lytic defective viruses were also defective in promoting EBV-mediated LPD. Levels of BZLF1 and BRLF1 were both upregulated in our experiments 19.38-fold and 156.00-fold, respectively, at 48 h post-induction (see Table S1). Restoration of lytic EBV gene expression enhanced LPD growth in SCID mice. It was also noted that expression of cellular IL-10 and vIL-10 (BCRF1) was reduced in lytic defective LCLs and increased when the deletions were restored. These results suggest that lytic gene expression may contribute to EBV-associated lymphoproliferative disease, potentially through induction of paracrine B-cell growth factors.

In summary, real-time PCR analysis of EBV gene expression identified several highly induced EBV genes, the majority of which were early genes. Microarray analysis of the lytic induction phase revealed 113 cellular genes regulated by EBV infection, 22 of which were validated by real-time PCR. The viral and cellular gene expression altered during the lytic induction phase could be regulated in vivo and might play a role in the development of EBV-associated malignancies and be exploited as potential diagnostic markers.

Based on fold change data and additional evidence from the literature, we are actively assessing a range of host and EBV targets derived from this work. We have collected sequential samples from post-transplant recipients over the past 12 months and are using real-time RT-PCR assays to analyse both host and EBV gene expression levels to investigate the development and early prediction of EBV-related disease in this setting. The host targets include VCAM1, IL-6, IL-7, IL-10, TERT and PHLD2A. The EBV targets include BZLF1, BFRF1, BALK1, BBLF4, EBNA2 and EBER1.

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