JVI Accepted Manuscript Posted Online 2 September 2015
J. Virol. doi:10.1128/JVI.01873-15

Epstein-Barr viruses deficient in EBER RNAs give higher LMP2 RNA expression in lymphoblastoid cell lines and efficiently establish persistent infection in humanized mice

Goran Gregorovic[1], Elizabeth A. Boulden[1], Rachel Bosshard[1], Claudio Elgueta Karstegl[1], Rebecca Skalsky[2,4], Bryan R. Cullen[2], Cornelia Gujer[3], Patrick Rämer[3], Christian Münz[3], Paul J. Farrell[1*]

[1]Section of Virology, Imperial College, Norfolk Place, London W2 1PG, UK

[2]Department of Molecular Genetics and Microbiology and Center for Virology, Duke University Medical Center, Durham, North Carolina 27710, USA

[3]Viral Immunobiology, Institute for Experimental Immunology, University of Zürich, Switzerland

[4]Current address: Vaccine and Gene Therapy Institute, Oregon Health and Science University, Beaverton, Oregon, 97006, USA

Running title: Epstein-Barr virus EBER RNAs

Word count abstract 75, text 1000

*Corresponding author, email p.farrell@imperial.ac.uk

ABSTRACT

Functions of EBER RNAs were tested in lymphoblastoid cell lines containing EBER mutants of Epstein-Barr Virus (EBV). Binding of EBER1 to RPL22 was confirmed. Deletion of EBER1 or EBER2 correlated with increased cytoplasmic EBV LMP2 RNA and with small effects on specific cellular miRNA levels but protein levels of LMP1 and LMP2A were not affected. Wild type and EBER deletion EBV had approximately equal ability to infect immunodeficient mice reconstituted with a human haematopoietic system.

Epstein-Barr virus (EBV) encoded RNAs (EBERs) are abundant viral non-coding RNAs in EBV transformed lymphoblastoid cell lines (LCLs). We previously identified cell genes whose expression in EBV LCLs correlates with deletion of EBER1 or EBER2 (1); here we use LCLs to test various mechanisms that have been proposed for EBER function.

Binding of EBER1 to RPL22 is confirmed in LCLs but no effect of EBER1 was detected on p53 protein level.

EBER1 can bind to ribosomal protein L22 (RPL22) (2) and studies on RPL22 knockout mice (3, 4) showed a p53 dependent defect in pro B cells (4). The effect is thought to involve RPL22 binding to p53 mRNA, reducing translation of p53 (5). Possibly EBER1 could modulate this pathway, although EBV infects mature B cells, in which no RPL22 phenotype was reported (4). We confirmed binding of RPL22 to endogenous EBER1 in extracts of an LCL (Fig 1A). The p53 protein level was determined by western blotting in LCLs and the response to cisplatin treatment, which stabilizes p53, was also tested. There was no significant difference in the p53 levels or responses between the cells with or without EBER1 expression (Fig 1B) so EBER1 does not appear to affect p53 expression in LCLs.

Effect of EBERs on cellular miRNAs

To determine whether the EBERs might affect cellular miRNAs, we used LCLs containing wild type (wt) B95-8 BAC EBV, EBER1 or EBER2 deletion mutants, or revertant viruses. Total RNA was isolated from each LCL and small RNAs were used to generate Illumina sequencing libraries (6). Appropriate expression of the EBER1 and EBER2 RNAs was confirmed by northern blotting (Fig 2A).

Sequencing detected miRNAs from all 8 EBV B95-8 pre-miRNAs and 491 mature human cellular miRNAs. Sequence reads are available at NCBI BioProject PRJNA287267. The general pattern of viral and cellular miRNA expression was comparable to that observed previously in EBV B95-8 LCLs (6); miR-155, miR-146a/b, and miRNAs encoded within the miR-17/92 cluster were highly abundant and the EBV miRNAs constituted ~12% of the population (Table S1). As in prior studies (6-8), we found no evidence for production of discrete miRNA-like products from the EBER RNAs.

Sixteen miRNAs (p-value < 0.01 and -2 > logFC > 2) had expression levels altered in response to the deletion of EBER1, while eleven miRNAs were changed in response to EBER2 deletion (Table S2). There were significant differences in the levels of miR-340-3p, miR-340-5p, miR-190a-3p, miR-190a-5p, and miR-33a-5p in EBER1-deleted LCLs compared to wt LCLs (Fig. 2B-D, Table S2). Only small changes in miRNA expression levels (such as miR-199a) were observed in EBER2-deleted LCLs (Fig. 2D, Table S2). Both the -3p and -5p miRNAs for miR-190 and miR-340 were affected by the deletion of EBER1, indicating that the promoters driving expression of the primary miRNA transcripts are affected (Fig. 2B), consistent with a recent report on miR-190 (9).

Additional Taqman quantitative RT-PCR assays on RNA from independently established LCLs supported expression of both miR-340-5p and -3p correlating with EBER1 expression (Fig 2E) but the fold change was lower in this assay. Since the levels of these miRNAs are all quite low in LCLs (Fig 2B, Table S1), we conclude that it is unlikely that the main function of EBERs in LCLs is to alter levels of cellular miRNAs but the changes in miRNA expression may contribute to the effects we reported previously on cellular mRNA levels in LCLs (1).

Deletion of EBER1 or EBER2 correlates with raised level of LMP2 RNAs in LCLs

Recently EBER2 was shown to bind PAX5 and to promote PAX5 binding to the terminal repeat region of the EBV genome (10). Knockdown of EBER2 by siRNA caused a 50% increase in LMP1 and LMP2 RNA levels (10). Using qPCR with the same primers on cytoplasmic RNA, we did not observe any significant difference in the level of LMP1 RNA between the EBER2 deletion LCLs or wild type EBV LCLs but LMP2A and LMP2B RNA levels were 2-3 fold higher when either EBER1 or EBER2 was deleted (Fig 3A,B), particularly when the cDNA synthesis was primed with oligo-dT (Fig 3B). LMP1 and LMP2A protein levels are quite variable in LCLs, shown in Fig 3C, and there was no apparent correlation with EBER expression. Our results thus provide some support for EBER2 tending to reduce the level of LMP2 RNA (10) but the specific binding of EBER2 to PAX5 and association with the terminal repeat may not mediate the LMP2 RNA effect we observed since there was an increase in LMP2 RNA when either EBER1 or EBER2 was deleted. The focus of the previous study on EBER2 meant that an effect of EBER1 deletion on LMP2 RNA levels was not tested in those experiments (10).

Infection of mice with a human haematopoietic system with EBV lacking EBER genes

Using the EBER mutant EBV strains, we tested whether EBERs affect establishment of EBV infection in NSG mice with a humanized haematopoietic system (11). In three separate experiments involving a total of 44 correctly reconstituted mice, there was no significant difference in the frequency of infection detected in the blood or the spleen with deletion of either or both EBER genes from the virus genome. Spleen and blood viral loads are shown in Fig 4A.

Journal of Virology

Analyzing parameters of cell mediated immune responses towards the viral infections, we observed a tendency towards more pronounced $CD8^+$ T cell expansion in the infection with EBER1 deficient viruses (Fig 4B). However, this tendency was primarily observed in one experiment and was not present in infections with an EBV virus deficient in both EBERs.

Most studies have focussed on cell intrinsic functions for EBERs but they have also been reported to be released from cells (12) and in exosomes (13), potentially affecting immune or inflammatory responses (M Pegtel, personal communication). Most likely, physiological phenotypes for the EBERs will be revealed by in vivo infection challenged by normal immune responses. We have now shown that deletion of EBERs does not prevent infection of humanized mice, making this type of analysis possible.

ACKNOWLEDGEMENTS

We thank David Wiest for helpful discussion and Richard Longnecker for advice on LMP2A antibodies. This work was supported by grants to PJF from Leukaemia and Lymphoma Research (11012), and by NIH grant R01-AI067968 to BRC. RLS was supported in part by NIH grant T32-CA90111. CM grant support was from Cancer Research Switzerland (KFS-3234-08-2013), Worldwide Cancer Research (14-1033), $KFSP^{MS}$ and $KFSP^{HHLD}$ of the University of Zurich, the Baugarten Foundation, the Sobek Foundation, Fondation Acteria, the Swiss Vaccine Research Institute and the Swiss National Science Foundation (310030_143979 and CRSII3_136241).

REFERENCES

1. **Gregorovic G, Bosshard R, Karstegl CE, White RE, Pattle S, Chiang AK, Dittrich-Breiholz O, Kracht M, Russ R, Farrell PJ.** 2011. Cellular gene expression that

correlates with EBER expression in Epstein-Barr Virus-infected lymphoblastoid cell lines. J Virol **85:**3535-3545.

2. **Toczyski DP, Matera AG, Ward DC, Steitz JA.** 1994. The Epstein-Barr virus (EBV) small RNA EBER1 binds and relocalizes ribosomal protein L22 in EBV-infected human B lymphocytes. Proc Natl Acad Sci U S A **91:**3463-3467.

3. **Anderson SJ, Lauritsen JPH, Hartman MG, Foushee AMD, Lefebvre JM, Shinton SA, Gerhardt B, Hardy RR, Oravecz T, Wiest DL.** 2007. Ablation of Ribosomal Protein L22 Selectively Impairs [alpha][beta] T Cell Development by Activation of a p53-Dependent Checkpoint. Immunity **26:**759-772.

4. **Fahl SP, Harris B, Coffey F, Wiest DL.** 2015. Rpl22 Loss Impairs the Development of B Lymphocytes by Activating a p53-Dependent Checkpoint. J Immunol **194:**200-209.

5. **Rashkovan M, Vadnais C, Ross J, Gigoux M, Suh WK, Gu W, Kosan C, Moroy T.** 2014. Miz-1 regulates translation of Trp53 via ribosomal protein L22 in cells undergoing V(D)J recombination. Proc Natl Acad Sci U S A **111:**E5411-5419.

6. **Skalsky RL, Corcoran DL, Gottwein E, Frank CL, Kang D, Hafner M, Nusbaum JD, Feederle R, Delecluse HJ, Luftig MA, Tuschl T, Ohler U, Cullen BR.** 2012. The viral and cellular microRNA targetome in lymphoblastoid cell lines. PLoS Pathog **8:**e1002484.

7. **Chen SJ, Chen GH, Chen YH, Liu CY, Chang KP, Chang YS, Chen HC.** 2010. Characterization of Epstein-Barr virus miRNAome in nasopharyngeal carcinoma by deep sequencing. PLoS One **5:**e12745.

8. **Skalsky RL, Kang D, Linnstaedt SD, Cullen BR.** 2014. Evolutionary conservation of primate lymphocryptovirus microRNA targets. J Virol **88:**1617-1635.

9. **Cramer EM, Shao Y, Wang Y, Yuan Y.** 2014. miR-190 is upregulated in Epstein-Barr Virus type I latency and modulates cellular mRNAs involved in cell survival and viral reactivation. Virology **464-465:**184-195.

10. **Lee N, Moss WN, Yario TA, Steitz JA.** 2015. EBV noncoding RNA binds nascent RNA to drive host PAX5 to viral DNA. Cell **160:**607-618.

11. **Gujer C, Chatterjee B, Landtwing V, Raykova A, McHugh D, Munz C.** 2015. Animal models of Epstein Barr virus infection. Curr Opin Virol **13:**6-10.

12. **Iwakiri D, Zhou L, Samanta M, Matsumoto M, Ebihara T, Seya T, Imai S, Fujieda M, Kawa K, Takada K.** 2009. Epstein-Barr virus (EBV)-encoded small RNA is released from EBV-infected cells and activates signaling from Toll-like receptor 3. J Exp Med **206:**2091-2099.

13. **Ahmed W, Philip PS, Tariq S, Khan G.** 2014. Epstein-Barr virus-encoded small RNAs (EBERs) are present in fractions related to exosomes released by EBV-transformed cells. PLoS One **9:**e99163.

14. **Friedlander MR, Mackowiak SD, Li N, Chen W, Rajewsky N.** 2012. miRDeep2 accurately identifies known and hundreds of novel microRNA genes in seven animal clades. Nucleic Acids Res **40:**37-52.

15. **Robinson MD, McCarthy DJ, Smyth GK.** 2010. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics **26:**139-140.

16. **Robinson MD, Oshlack A.** 2010. A scaling normalization method for differential expression analysis of RNA-seq data. Genome Biol **11:**R25.

17. **Schmittgen TD, Livak KJ.** 2008. Analyzing real-time PCR data by the comparative C(T) method. Nat Protoc **3:**1101-1108.

18. **Antsiferova O, Muller A, Ramer PC, Chijioke O, Chatterjee B, Raykova A, Planas R, Sospedra M, Shumilov A, Tsai MH, Delecluse HJ, Munz C.** 2014. Adoptive transfer of EBV specific CD8+ T cell clones can transiently control EBV infection in humanized mice. PLoS Pathog **10:**e1004333.

FIGURE LEGENDS

Figure 1

A) Flag tagged RPL22 or negative control protein (C, Flag tagged Schlafen) were expressed by Neon transfection in an LCL containing B95-8 BAC EBV. Cell extracts were incubated with M2 anti-FLAG antibody (Sigma-Aldrich) bound to protein G sepharose beads. RNA was extracted from the beads (IP) and the unbound supernatant (Sup) and tested for EBER RNA by northern blotting

B) Three independent ΔEBER1 LCLs (light grey bars) or wt EBV LCLs (black bars) were treated with 20ug/ml cisplatin (C) in triplicate or untreated (U) as a control. For each sample, 2ml of cell suspension (at 2.5 x 10^5 viable cells/ml, determined by trypan blue exclusion) was set up per well in 6 well plates, with or without cisplatin as indicated. The number of viable cells (excluding trypan blue) was quantified after 16h and p53 protein was detected by western blotting of cell extracts made at the same time point. DO1 antibody (Santa Cruz) was used for detection of p53 by western blotting. Actin was used as loading control on the western blots.

Figure 2

A) EBER1 and EBER2 expression in the wt and EBER-deleted LCLs used for deep sequencing analysis was confirmed by northern blotting (1). 0.5 ug of total RNA was used per well.

B) Normalized miRNA reads counts for miR-190a and miR-340 in EBER1-deleted (delE1) LCLs compared to wild-type (wt) and EBER1 revertant (E1R) LCLs. "A" and "B" denote the two individual LCLs analysed for each condition. About 10 million sequencing reads were obtained for each LCL library. Reads were processed as described previously (6) using scripts from the fastx toolkit (http://hannonlab.cshl.edu/fastx_toolkit/). To determine miRNAs, reads were aligned to the human (HG19) and EBV B95-8 genomes and annotated according to mirbase v21 (http://www.mirbase.org/). To determine miRNA expression levels, miRNA read counts were obtained using the quantifier module of miRDeep2 (14) (Table S1). To identify differentially expressed (DE) miRNAs, miRNA read counts were analysed by edgeR using trimmed mean of M-values (TMM) for normalisation (15, 16). A minimum of 40 reads per miRNA in at least one of the ten LCL libraries was required to be included in the analysis. DE miRNAs with a LogFC p value < 0.05 are reported in Table S2.

C) Normalized miRNA read counts for miR-33a in EBER1-deleted LCLs.

D) Summary of fold changes (logFC) of select cellular miRNAs showing significant changes correlating with EBER1 or EBER2 expression as determined by deep sequencing and edgeR analysis.

E) Relative levels of miR-340-5p and miR-340-3p as determined by Taqman qRT-PCR assays in LCLs. The expression levels of miRNAs were normalized to RNU48. The values represent means ± SE of 6 independent samples per EBV type (5 in the case of EBER1 revertants). The wild-type expression levels were set to 1 for each miRNA, whereas the levels of other samples were expressed relative to the wild type. Each cDNA sample was analysed in triplicate.

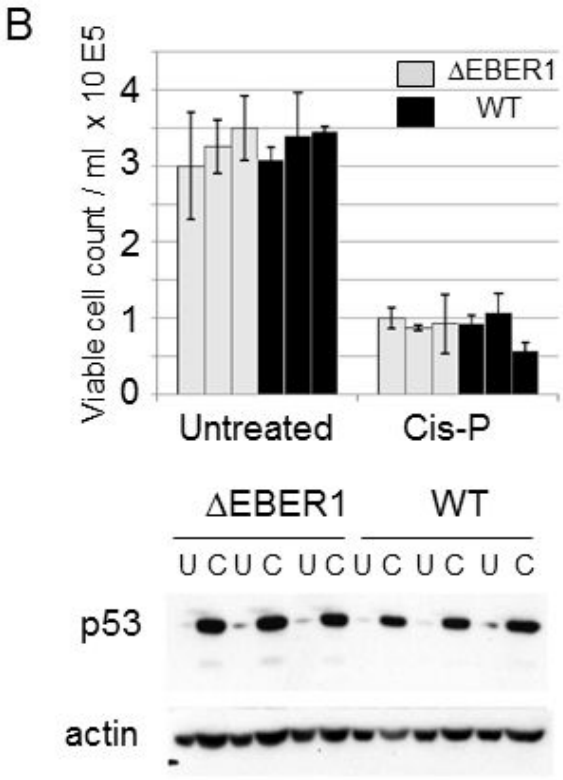
Figure 3

Cytoplasmic RNA from two LCLs each for EBV wild type (WT), deletion of EBER1 (ΔE1), revertant (E1R), deletion of EBER2 (ΔE2) or revertant (E2R) was used for cDNA synthesis using either (A) random primers or (B) oligo dT using the ProtoScript First Strand cDNA Synthesis Kit (New England BioLabs). Q-PCR with the same primers as in (10) was then used in duplicate assays to measure the levels of RNA for LMP1, LMP2A and LMP2B, using GAPDH as a reference. The 2-ΔΔCT method of comparative PCR (17) was used to analyse the results, expressed as fold change relative to the E1R LMP1 value. (C) RIPA lysates were prepared from LCLs and equal amounts of cell protein were analysed by western immunoblotting. Membranes were probed with: 1/1000 dilution of anti-LMP2A (Abcam, 14B7), 1/500 dilution of anti-LMP1 (DAKO, CS. 1-4), 1/5000 dilution of anti-β actin (SIGMA, AC-74. Secondary antibodies were horseradish peroxidase-conjugated sheep anti-mouse immunoglobulin (GE Healthcare) or horseradish peroxidase-conjugated rabbit anti-Rat immunoglobulin (Sigma). Bound immunocomplexes were detected by enhanced chemiluminescence (GE Healthcare).

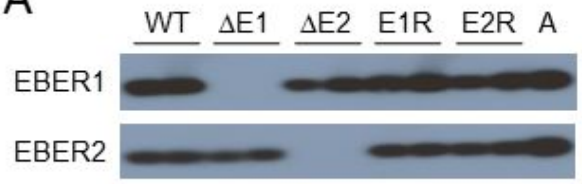
Figure 4

A) Newborn HLA-A*0201 transgenic NOD/LtSz-Scid IL2RΔnull (NSG-A2tg) mice were irradiated and injected intrahepatically with $CD34^+$ human hematopoietic progenitor cells as described previously (18). The reconstitution of human immune system components in the peripheral blood was analysed prior to the beginning of experiments (normally 12 weeks after engraftment). Groups of mice were infected with 10^5 infectious units of virus and monitored over a 4-8 week period in three experiments, each using different groups of reconstituted mice. EBV viral loads were quantified in spleen and whole blood four weeks after infection for wild-type (EBV wt), EBER1 deficient (EΔ1), EBER2 deficient (EΔ2), EBER1 and 2 deficient (EΔ12) and EBER2 revertant (E2rev) viruses.

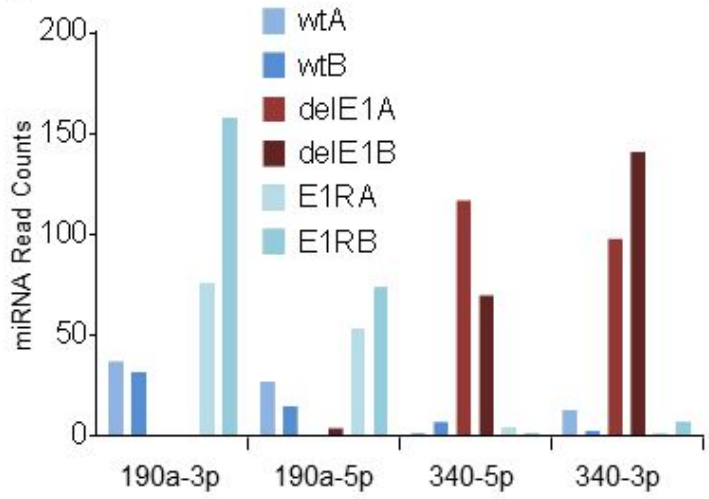
B) $CD8^+$ T cell expansion is slightly elevated in the absence of EBER1. $CD8^+$ to $CD4^+$ T cell ratio was assessed by flow cytometry after four weeks of infection with the same viruses as in A. The composition of blood and spleen samples from the humanized mice was analyzed using anti-human CD45 (HI30, Biolegend), anti-CD3 (UCHT1, Biolegend), anti-CD4 (RPA T4, Biolegend), anti-CD8 (SK1, Biolegend), anti-HLA-DR (L243, Biolegend), anti-CD45RO (UCHL1, BD Pharmingen) and anti-CD19 (HIB19, BD Pharmingen). Spleens were mechanically disrupted and filtered through a 70µm cell strainer. Erythrocytes were lysed in whole blood or in spleen suspensions using NH_4Cl. Cell suspensions were stained with the indicated antibodies for 15min, 4°C and washed. Statistical analysis for all mouse experiments used two tailed T-tests. A *P* value of < 0.05 was considered statistically significant.



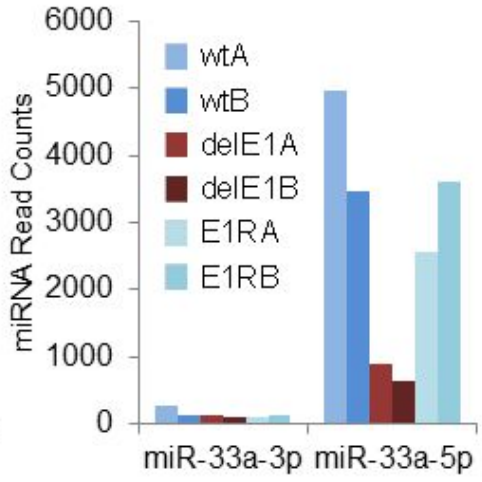
A



B



C



D

	EBER1	
miRNA	logFC	p-value
miR-340-5p	4.438	1.58E-12
miR-340-3p	4.818	5.07E-10
miR-190a-3p	-9.097	6.20E-10
miR-190a-5p	-3.825	1.43E-04
miR-33a-5p	-2.276	3.21E-05
	EBER2	
miRNA	logFC	p-value
miR-199a-3p	-2.324	6.53E-03

E