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Research paper

# The interaction of Epstein-Barr virus encoded transcription factor EBNA2 with multiple sclerosis risk loci is dependent on the risk genotype

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ABSTRACT

*Background:* Epstein-Barr virus (EBV) infection may be necessary for the development of Multiple sclerosis (MS). Earlier we had identified six MS risk loci that are co-located with binding sites for the EBV transcription factor Epstein-Barr Nuclear Antigen 2 (EBNA2) in EBV-infected B cells (lymphoblastoid cell lines – LCLs).
*Methods:* We used an allele-specific chromatin immunoprecipitation PCR assay to assess EBNA2 allelic preference. We treated LCLs with a peptide inhibitor of EBNA2 (EBNA2-TAT), reasoning that inhibiting EBNA2 function would alter gene expression at these loci if it was mediated by EBNA2.
*Findings:* We found that EBNA2 binding was dependent on the risk allele for five of these six MS risk loci ($p < 0{\cdot}05$). Treatment with EBNA2-TAT significantly altered the expression of TRAF3 ($p < 0{\cdot}05$), CD40 ($p < 0{\cdot}001$), CLECL1 ($p < 0{\cdot}0001$), TNFAIP8 ($p < 0{\cdot}001$) and TNFRSF1A ($p < 0{\cdot}001$).
*Interpretation:* These data suggest that EBNA2 can enhance or reduce expression of the gene depending on the risk allele, likely promoting EBV infection. This is consistent with the concept that these MS risk loci affect MS risk through altering the response to EBNA2. Together with the extensive data indicating a pathogenic role for EBV in MS, this study supports targeting EBV and EBNA2 to reduce their effect on MS pathogenesis.
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## 1. Introduction

Epstein-Barr virus (EBV), a gammaherpesvirus that establishes persistent infection in more than 90% of the global population, has been identified as a risk factor for developing multiple sclerosis (MS) [1]. Nearly all patients with MS are seropositive for EBV [2,3]. The risk for MS increases in people with high anti-EBV antibody titres [4] or a history of the EBV syndrome infectious mononucleosis (IM) [5]. Similarly, in a study of 1047 clinically isolated syndrome (CIS) cases, a condition which precedes MS, only one patient was seronegative for EBV antigens [6]. There is evidence that EBV interacts with both genetic and environmental risk factors for MS. For example, the risk of MS for those with the HLA-DRB1*1501 allele who are positive for EBV infection is 14-fold higher in combination than due to each factor independently [7]. EBV interaction with other environmental factors is suggested by the overlapping of genomic targets of the EBV viral transactivator protein, Epstein-Barr Nuclear Antigen 2 (EBNA2) with those of the vitamin D receptor [8]. Together these studies indicate that EBV is necessary, but insufficient on its own, to cause MS [1].

Host response to EBV infection could therefore contribute to differences in MS susceptibility, and genetic factors affecting this response could be those affecting MS risk. Hence a proportion of MS risk genes, genes that are proximal to MS risk single nucleotide polymorphisms (SNPs), may contribute to MS pathogenesis through their effect on EBV infection of B cells, particularly through the latency III phase of EBV infection [9,10]. Although >200 MS risk SNPs have been identified [11], and 47 of these have been implicated in EBV functions [9,10,12]. any crosstalk between these MS risk SNPs/genes and EBV that would affect the risk of developing MS has yet to be clearly defined.

EBNA2 is essential for maintaining the latency III growth phase of EBV and acts through regulating both viral and cellular genes [13,14]. EBNA2 mediates this regulation through interactions with human transcription factors, including RBP-J$\kappa$ and EBF1 [15,16]. While

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**Research in context**

*Evidence before this study*

EBV infection appears to be necessary for the development of MS. Chromatin immunoprecipitation data indicated that the EBV transcription factor EBNA2 bound at MS risk loci very much more often than would be expected by chance and that risk loci were associated with gene expression, potentially dependent on the risk allele for six risk loci. This suggests EBNA2 may drive the association of these loci with MS susceptibility.

*Added value of this study*

We confirmed that EBNA2 binding at five of these six loci was allele dependent, and that inhibition of EBNA2 binding affected expression, including altering the association of the alleles with expression.

*Implications of all the available evidence*

These data, together with previously published findings on EBV and EBNA2, support the concept that EBNA2 binding, and so EBV infection, affects MS susceptibility. EBNA2 and EBV are therefore potential therapeutic targets to ameliorate this disease.

EBNA2 regulation of cellular gene expression promotes EBV survival, this and its manipulation of the host genome is implicated in affecting risk of lymphomas and several autoimmune conditions, including MS. Harley and colleagues identified several autoimmune disorders including MS which have an over-representation of EBNA2 binding sites at disease risk loci in EBV-infected B cells, lymphoblastoid cell lines (LCLs) [17]. Using *in silico* data from heterozygotes, they identified many autoimmune-associated risk variants that are associated with altered EBNA2 binding to the human genome. They also provided evidence that EBNA2 affected the expression of the autoimmune disease risk gene CD44 in an allele dependent manner through such binding by an allele-specific qPCR method [17].

We previously found that 25% of MS risk loci (47 SNP-gene pairs) are associated with MS risk gene expression in LCLs [9]. That is, they are expression quantitative trait loci (eQTL): their gene expression is associated with the risk SNP genotype. Six of these SNPs were co-located with EBNA2 chromatin immunoprecipitation sequencing (ChIP-seq) binding peaks [9]. EBNA2 expression levels also correlated with the expression of these risk genes, usually dependent on risk genotype [9]. These six SNPs are associated with the expression of seven MS risk genes, with expression associated with genotype differently in LCLs compared to other immune cells [9]. We therefore hypothesised that the risk effect of these loci on MS could be mediated by allelic differences in EBNA2 binding, which affect host response to EBV and MS susceptibility.

To address this hypothesis, we aimed to answer two key questions (Fig. 1). Firstly, does EBNA2 bind in an allele-dependent manner at these six MS risk loci? Secondly, does EBNA2 regulate gene expression of these MS risk genes dependent on the risk allele? An allele-specific ChIP assay was devised to determine whether EBNA2 binds to MS risk loci in an allele-dependent manner. To test whether EBNA2 may regulate gene expression of these seven MS risk genes (from six risk loci) in LCLs, we used a specific EBNA2 inhibitory peptide, EBNA2-TAT, as previously described [18], and compared gene expression across genotypes with and without EBNA2 inhibition. EBNA2-TAT consists of a ten amino acid peptide from the RBP-J$\kappa$ interaction domain of EBNA2 fused with the protein transduction domain of HIV-1 TAT. The EBNA2-TAT peptide has been demonstrated to effectively inhibit EBNA2/RBP-J$\kappa$ interaction and reduce transcription of EBNA2 target genes [18].

This work demonstrates, for the first time, that EBNA2 binding to these MS risk loci is allele-dependent, affecting gene regulation and potentially the EBV phenotype of LCLs. This is consistent with the concept that EBV contributes to MS risk through this specific mechanism, and consilient with other data (reviewed by Bar-Or et al [19].), supporting the targeting of EBV and EBNA2 for therapeutic benefit in MS.

## 2. Methods

### 2.1. Ethics

Blood was collected by femoral phlebotomy from healthy individuals (numbers as described in the Results section for each experiment) after each gave informed consent (Westmead Hospital Human Research Ethics Committee Approval 1425). All participants reported being in good general health.

### 2.2. LCL generation and culture

Ficoll-Paque Plus (VWR International, Radnor, PA, USA) was used to isolate peripheral blood mononuclear cells (PBMCs) from collected blood as previously described [20]. The generation of LCLs was carried out as previously described [9]. Briefly, PBMCs were incubated for 1 hr at 37°C with supernatant from the EBV B95.8 cell line, after which the cells were suspended in RPMI-1640 (Lonza, Basel, Switzerland) containing 10% fetal bovine serum (FBS, Sigma Aldrich, St Louis, MO, USA), 2 mM L-glutamine (Life Technologies), and 2 $\mu$g per ml cyclosporin A (Sigma Aldrich). The cells were plated at densities of $2.5 \times 10^6$ or $5 \times 10^6$ cells per well in 48-well plates. The media was supplemented weekly until the cells were expanded into a 25 $cm^2$ flask. LCLs were used then cryopreserved in 10% DMSO (MP Biomedical, Irvine, CA, USA) 50% FBS and RPMI-1640. The LCLs undergo routine mycoplasma testing.

### 2.3. LCL Proliferation

Proliferation assays were carried out using alamarBlue (Thermo Fisher) as per the manufacturer's instructions. Measurement of fluorescence was read with excitation at 560 nm and emission at 590 nm using a SpectraMax iD5 plate reader, 3 hr after the addition of alamarBlue reagent at a volume equivalent to 10% of the total volume.

### 2.4. MS risk genotyping

For genotyping, DNA was extracted from whole blood samples using Qiagen QIAamp DNA Blood Mini Kit (Qiagen). DNA samples were genotyped for MS associated SNPs (listed in Supplementary Table 1) using Taqman Assays (Supplementary Table 2) and Taqman Genotyping Master Mix (Thermo Fisher) according to the manufacturer's instructions.

### 2.5. RNA extraction and cDNA synthesis

Total RNA was isolated from LCLs using a Bioline Isolate II RNA Mini Kit (Meridian Bioscience, Memphis, TN, USA) according to the manufacturer's instructions. After treatments or incubation periods, cells were washed in DPBS and resuspended in 100 $\mu$L RLY Buffer provided in the kit. The samples were snap-frozen and stored at -80$^0$ C until required. Samples were thawed on ice and 100 $\mu$L of RLY Buffer and 2 $\mu$l of TCEP was added to samples and vortexed vigorously. The remaining steps for RNA extraction followed the manufacturer's instructions. RNA was checked for quality and quantified using a NanoDrop 2000 Spectrophotometer (Thermo Fisher). cDNA

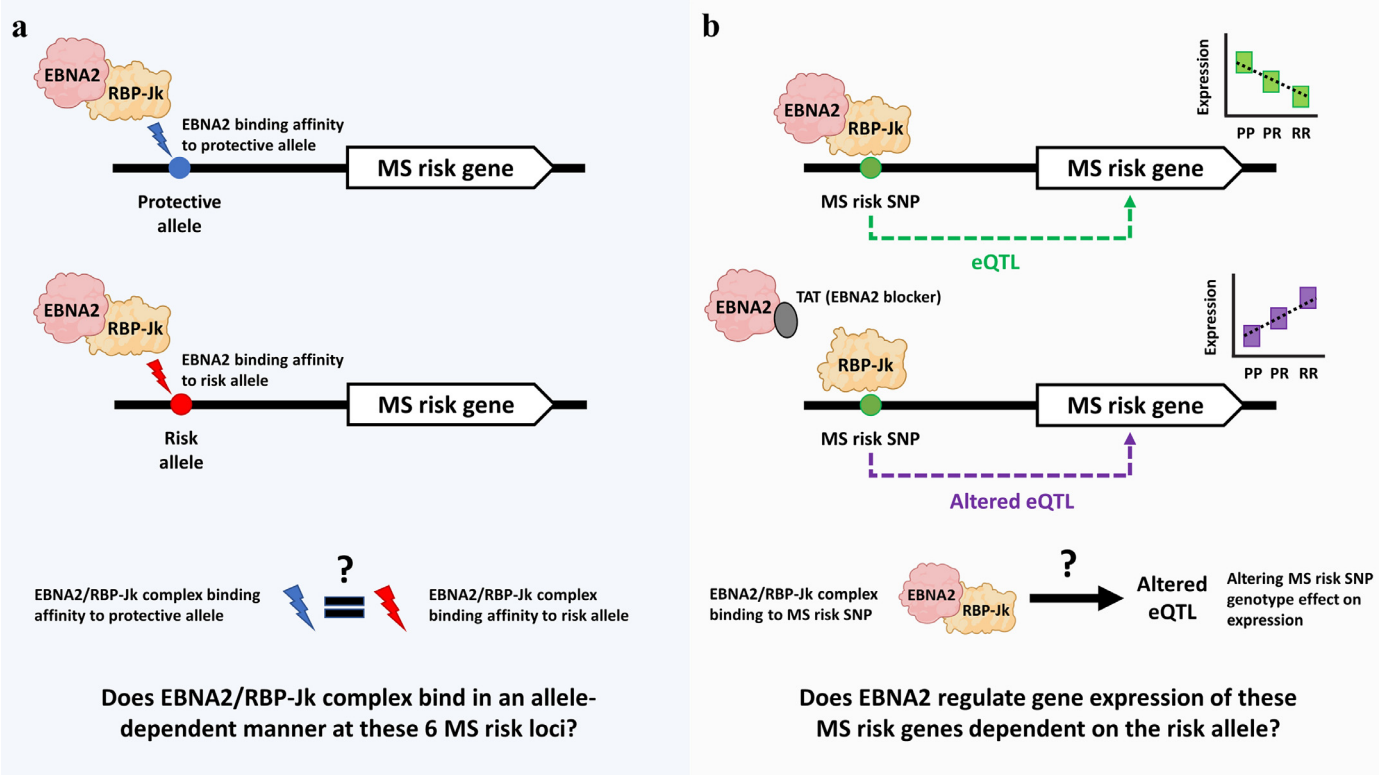


**Fig. 1. Study overview.** Six MS risk loci associated with MS risk gene expression in LCLs were also sites enriched for EBNA2 binding [9]. (a) We hypothesised that EBNA2 affects the regulation of associated genes through preferential binding at risk or protective alleles of their respective loci – tested by allele-specific chromatin immunoprecipitation PCR. (b) This preferential binding could alter the effect of risk alleles on gene expression (affecting the eQTL). This was tested by assaying gene expression on EBNA2 inhibition with EBNA2-TAT (grey). If so, this would provide direct molecular evidence that the basis for the association of the risk allele with MS could be due to its altering host susceptibility to EBV, enhancing the effect of EBV on MS pathogenesis. EBNA2-TAT functions by preventing EBNA2 binding to RBP-J$\kappa$. PP, PR and RR denote homozygous for the protective allele, heterozygous and homozygous for the risk allele, respectively. Figure partially created with BioRender.com.

was synthesised using qScript cDNA SuperMix (Quanta Biosciences, Beverly, MA, USA) according to the manufacturer's instructions.

### 2.6. Gene expression

For the detection of MS risk genes, cDNA was used to detect expression of MS risk genes by real time PCR using predesigned TaqMan gene expression assays (Life Technologies, Carlsbad, CA, USA) and TaqMan Universal Master Mix II, with UNG, according to the manufacturer's instructions. Gene expression was measured using the $2^{-\Delta\Delta CT}$ method as previously described [21], using RPL30 as the reference gene. The probes are listed in Supplementary Table 3.

### 2.7. Viral gene expression

For the detection of expression of LMP1 expression, 3 $\mu$L of diluted cDNA used with SYBR primer sets and Takara SYBR Pre-Mix Master Mix (Takara Bio, Kusatsu, Shiga, Japan). Forward and reverse primers were used at a final concentration of 0·2 $\mu$M with 6 $\mu$L of SYBR mix. RPL30 was used as the reference gene. Primer sequences are listed in Supplementary Table 4.

### 2.8. Chromatin immunoprecipitation (ChIP)

$30 \times 10^6$ cells were prepared for shearing using the TruChIP Chromatin Shearing Kit according to the manufacturer's instructions (Covaris, Sydney, NSW, Australia) with some modifications. An additional crosslinking step of EBNA2 to its transcriptional complexes was added prior to the fixation step as described by the manufacturer [22]. Disuccinimidyl glutarate (DSG, Thermo Fisher Scientific, Waltham, MA, USA) was used to fix protein-protein interactions. DSG was freshly prepared with DMSO to provide a 200mM solution and added to cells for a final concentration of 2mM. The samples were rotated for 5 minutes at room temperature (RT). After washing, formaldehyde was added to a final concentration of 1% for an additional 1 minute with agitation at RT. Following this, 87 $\mu$L of Quenching Buffer E was added and placed on rocker at RT for 5mins, after which all steps were completed according to the TruChIP manufacturer's guidance for "high cell" quantity. An E220 Evolution Ultrasonicator (Covaris) was used to shear the chromatin with the conditions recommended by the manufacturer for target size of 200-700bp (5% duty cycle, 140 peak power, and 200 bursts per cycle) for 12 min. Sheared chromatin was precleared with 45 $\mu$L of Protein G Agarose at 4°C for 45 min. Cleared sonicate was incubated with 10 $\mu$g anti-EBNA2 (U.S. Biological, Salem, MA, USA, Cat. No. 376310) or the same volume of isotype control antibody overnight, after which 20 $\mu$litres of a 50% slurry of Protein G PLUS Agarose (Santa Cruz, Dallas, TX, USA, Cat. No. sc-2002) was added and incubated at $4^0$ C with rotation for 1 hr. The beads were then washed sequentially with 1mL volumes of wash buffers, each time rotated for 5 mins at $4^0$ C, followed by a spin at 1000 x g at $4^0$ C, except where noted. In order, the buffers used were: low salt buffer, high salt buffer, lithium chloride buffer (3 min, no rotation, kept on ice), TE buffer (two washes) (Buffer details, Supplementary Table 5). Then 250 $\mu$L of elution buffer (TE with 1% SDS) was added to the beads and they were incubated for 20 min at $65^0$ C, with regular gentle vortexing. Samples were then spun at 14,000 x g and the supernatant was removed. DNA was extracted using the QiaQuick PCR purification kit as per the manufacturer's instructions (Qiagen, Hilden, Germany). The samples were eluted in 60 $\mu$L buffer EB and stored at -$20^0$ C prior to ChIP

PCR. The anti-EBNA2 antibody used in this work has been previously validated for use in chIP studies.

### 2.9. Allele-specific ChIP-qPCR

An allele-specific PCR-based technique was applied to assess differential EBNA2 enrichment at heterozygous alleles, as previously described [23]. Two primer sets were needed for each locus, with the same forward primer for each set. For reverse primers, the sequences are the reverse complement of the sequence which follows the variant in the DNA. The last base in the primer is specific to the allele. The primer sets for each SNP were tested with verified genotyped DNA. The primers are listed in Supplementary Table 6.

To quantify allele specific protein binding we performed qPCR using Takara SYBR Pre-Mix Master Mix as outlined above. Standard curves were created for each primer set using genomic DNA heterozygous for the SNP. The $C_t$ values of the ChIP DNA were then used to extrapolate the relative DNA quantity from the standard curve.

### 2.10. EBV DNA copy number measurement

For detection of EBV DNA copy number, cells were removed from culture after the treatment periods, washed with Dulbecco's phosphate buffered saline (DPBS), and the pellets stored at -80$^0$ C. DNA was extracted from cells using a QIAamp DNA Blood Mini Kit (Qiagen) according to the manufacturer's instructions. Samples were eluted with 60 $\mu$L elution buffer.

EBV DNA copy number was detected by Quantitative PCR using a previously described primer set and probe for an EBV genome-specific repeat region to detect EBV DNA copy number [24]. Real-time PCR was performed on a Bio-Rad CFX384 qPCR System (Bio-Rad Laboratories, Hercules, CA, USA) using the same conditions as previously described [24]. A primer set for the single copy gene TTR was used as a reference to account for any DNA concentration variation between samples (Supplementary Table 7). The relative DNA copy number was calculated as the with the $2^{-\Delta\Delta CT}$ method as previously described [21].

### 2.11. eQTL data for B cells and LCLs

The eQTL data for these SNP:gene pairs in B cells (n=91) and LCLs (n=147) were obtained from DICE [25] and GTEx [26] datasets, respectively. DICE is a publicly available database providing gene expression, eQTLs and epigenomic data for 15 immune cell types (https://dice-database.org). GTEx portal is a publicly available resource providing the eQTL and RNA-seq based gene expression data for 54 different tissues derived from nearly 1000 healthy donors (www.gtexportal.org). The direction of eQTLs was adjusted by the risk allele effect on gene expression relative to protective allele.

### 2.12. Statistics

Statistical analyses were performed using GraphPad Prism version 8 unless otherwise stated. For normally distributed data, comparison of the mean between the two groups was done using a parametric test; either a paired two-tailed t-test; or an unpaired two-tailed t-test. For normally distributed data, comparison of the mean between two groups was done using a parametric test, for paired samples a paired t-test (two-tailed), for unpaired samples, an unpaired t-test (two-tailed). For data that was not normally distributed, a non-parametric test was performed, a Wilcoxon matched-pairs signed rank test for paired samples, and a Mann-Whitney U test for unpaired samples. The data for the allele-specific ChIP-PCR was not normally distributed, and therefore a Wilcoxon matched-pairs signed rank test was performed to compare the relative EBNA2 binding between the risk and protective alleles among heterozygous samples. For the gene expression analysis of the MS risk genes, the data was not normally distributed, therefore a Wilcoxon matched-pairs signed rank test was performed on paired samples and a Mann-Whitney U test was performed on unpaired samples. The data for LCL proliferation was normally distributed and therefore a paired t-test (two-tailed) was performed to compare the untreated group with EBNA2-TAT-treated groups.

### 2.13. Role of funding source

The funding sources did not influence design, data collection, data analyses, interpretation, or writing of this report.

## 3. Results

### 3.1. EBNA2 binds chromatin at MS risk loci dependent on risk allele

We undertook ChIP for EBNA2 in 33 samples, analysing LCLs by an allele-specific ChIP-PCR method. As both alleles are available for binding of the transcription factor, by comparing the binding at both alleles within a sample heterozygous for a given SNP, these samples provide a natural internally matched control, with improved control for batch effects and technical variability, since the only variable that has changed is the allele. Of the six MS risk SNPs (all previously identified as eQTLs in LCLs, with evidence implicating EBNA2 binding) investigated, EBNA2 was found to bind dependent on MS risk allele at five loci (Fig. 2, $p < 0{\cdot}05$). It bound preferentially to the risk allele of two loci, TRAF3/RCOR1 (rs1258869, $p < 0{\cdot}05$) and CD40 (rs1883832, $p < 0{\cdot}05$); and the protective allele of three loci, TNFAIP8 locus (rs32658, $p < 0{\cdot}001$), TNFRSF1A locus (rs180069, $p < 0{\cdot}05$) and TBX6 (rs3809627, $p < 0{\cdot}05$) (two-tailed Wilcoxon matched-pairs signed rank test performed for all comparisons).

### 3.2. EBNA2-TAT inhibits the EBV latency III growth program

We next investigated EBNA2-TAT effects on the LCL EBV latency III growth program since we previously reported LCL proliferation is associated with expression of several MS risk genes [10]. We performed a dose-response experiment to find the optimal concentration of EBNA2-TAT that inhibits EBNA2 transcriptional function and affects MS risk genes without excess cytotoxicity. We treated LCLs with increasing concentrations of EBNA2-TAT over 24 hr. LCL proliferation was reduced significantly by all concentrations tested with 50$\mu$M reducing LCL proliferation by 40% (Fig. 3a, $p < 0{\cdot}01$), 100$\mu$M by approximately 50% ($p < 0{\cdot}001$), and 200$\mu$M by over 50% ($p < 0{\cdot}001$) (two-tailed paired t-test). The cell morphology was also affected by increasing concentrations of EBNA2-TAT (Fig. 3b). Expression of the EBNA2 regulated EBV gene LMP1 was reduced significantly by EBNA2-TAT at 50$\mu$M at 24 hr (Fig. 3c, $p < 0{\cdot}05$). The reduction in LMP1 was greater at 24 hr than at 48 hr (Supplementary Fig. 1) (two-tailed paired t-test). Taken together, this indicates significant disruption of the EBV latency III growth program by EBNA2-TAT 50 $\mu$M at 24 hr, and therefore these treatment conditions were chosen for the subsequent experiments. The cellular uptake of EBNA2-TAT and its specific binding to the RBP-J$\kappa$ site on EBNA2 has been previously confirmed through fluorescein labelling and *in vitro* GST affinity assay, respectively [18]. We confirmed the previously shown finding [18] that treatment with a mutant peptide of EBNA2-TAT (with a 2 amino acid substitution) had no inhibitory effects on EBNA2 function, no effects on cell viability or growth or have any effect cell morphology (Supplementary Fig. 3) indicating that inhibition of the EBNA2 interaction with RBP-J$\kappa$ is responsible for the effects of the EBNA2-TAT.

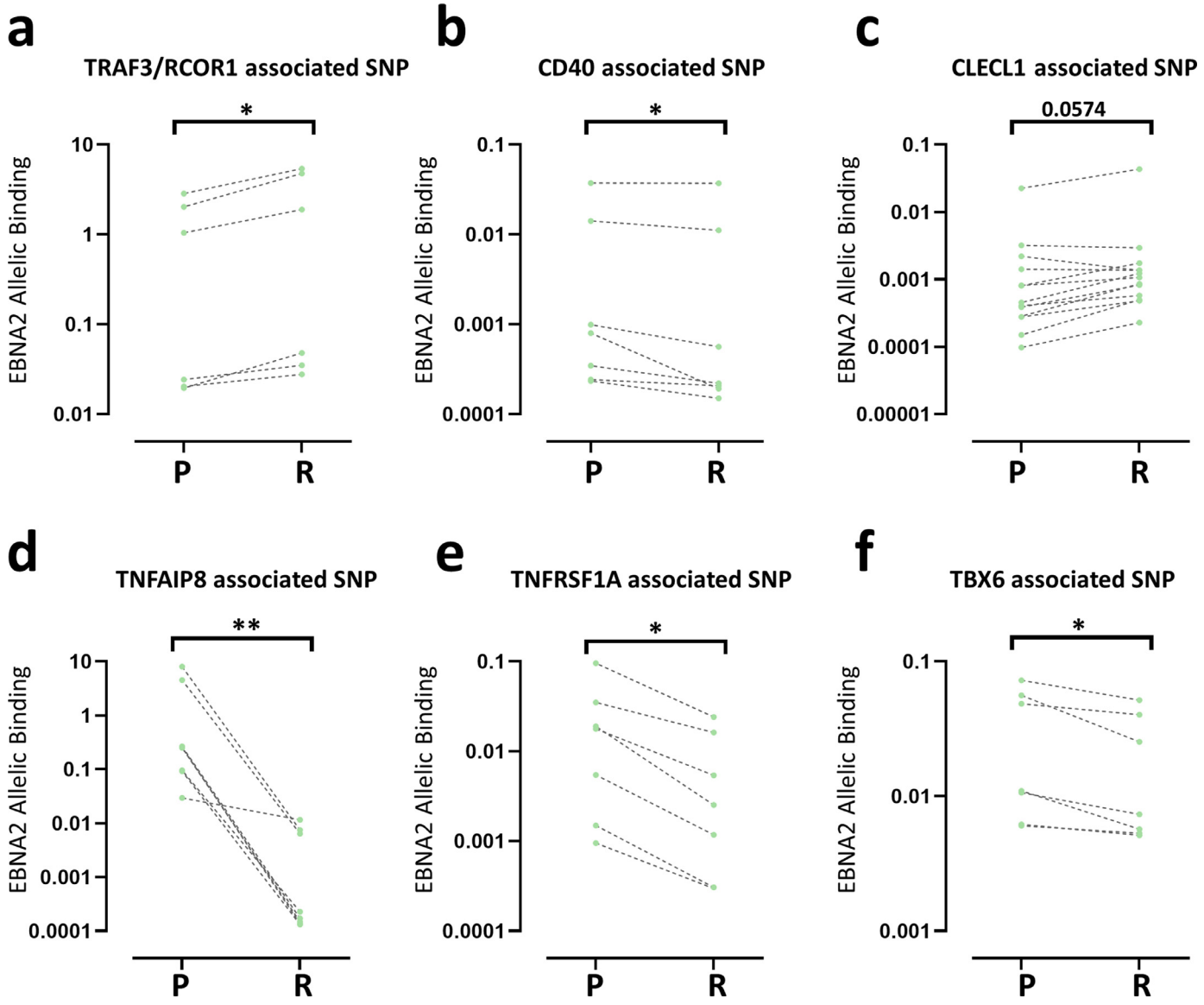


**Fig. 2. EBNA2 differential allelic binding at MS risk loci in heterozygous samples.** Allelic binding determined by allele-specific ChIP-PCR for six MS risk loci. (a) EBNA2 binds preferentially to the MS risk allele at the TRAF3/RCOR1 associated SNP rs1258869 (n = 6), (b) CD40 associated SNP rs1883832 (n = 7), and (c) CLECL1 associated SNP rs7977720 (n = 11). Binding of EBNA2 in LCLs is preferential towards the protective allele for (d) TNFAIP8 associated SNP rs32658 (n = 7), (e) TNFRSF1A associated SNP rs1800693 (n = 7), and (f) TBX6 associated SNP rs3809627 (n = 6). P denotes MS protective allele, R denotes MS risk allele according to the NHGRI-EBI GWAS Catalog. * < 0·05, ** <0·01). PCR performed in duplicate. n represents the biological replicates available that are heterozygous for the SNP. Wilcoxon matched-pairs signed rank test performed (two-tailed).

### *3.3. Inhibition of EBNA2 alters MS risk gene expression in LCLs*

We next treated our cohort of 35 LCLs with the optimum treatment of EBNA2-TAT (50 $\mu$M for 24 hr). Of the seven MS-associated genes tested, gene expression of five was significantly affected by EBNA2 inhibition (Fig. 4), TRAF3 ($p < 0{\cdot}05$), CD40 ($p < 0{\cdot}001$), CLECL1 ($p < 0{\cdot}0001$), TNFAIP8 ($p < 0{\cdot}001$), and TNFRSF1A ($p < 0{\cdot}001$) (two-tailed Wilcoxon matched-pairs signed rank test). Expression of two genes was not affected by EBNA2-TAT treatment: TBX6 and RCOR1. The reduced risk gene expression on EBNA2 inhibition for CD40, CLECL1 and TNFAIP8 suggests this transcription factor increases expression of these risk genes in LCLs. Conversely, these data suggest EBNA2 inhibits expression of TNFSF1A and TRAF3 in LCLs.

### *3.4. The effect of EBNA2 inhibition and the six MS risk loci on EBV DNA Copy number in LCLs*

EBV DNA copy number is a stable phenotype in LCLs [24]. We previously demonstrated that several MS risk SNPs and genes are associated with EBV DNA copy number [10]. We assessed the EBV DNA copy number for untreated and EBNA2-TAT treated LCLs (n=35). The TNFAIP8 locus demonstrated a genotype effect on EBV DNA copy number in untreated LCLs (Supplementary Fig. 5, $p < 0{\cdot}05$, Mann-Whitney U test), and this genotype effect was ablated upon EBNA2-TAT treatment.

### *3.5. Is the EBNA2 effect on gene expression at MS risk loci dependent on genotype?*

Since EBNA2 was demonstrated to bind one allele more than the other, we expected that EBNA2 would alter the allelic association with expression compared to that found in B cells. This was true for TRAF3, CD40, TNFAIP8, TNFRSF1A, TBX6 and RCOR1, but not CLECL1 (Fig. 5a). EBNA2 bound preferentially to the risk allele for TRAF3, and that allele had lower expression. It bound to the protective allele for CD40, TNFAIP8 and TNFRSF1A; and in each case that allele had relatively lower expression. However, from the effect of EBNA2-TAT

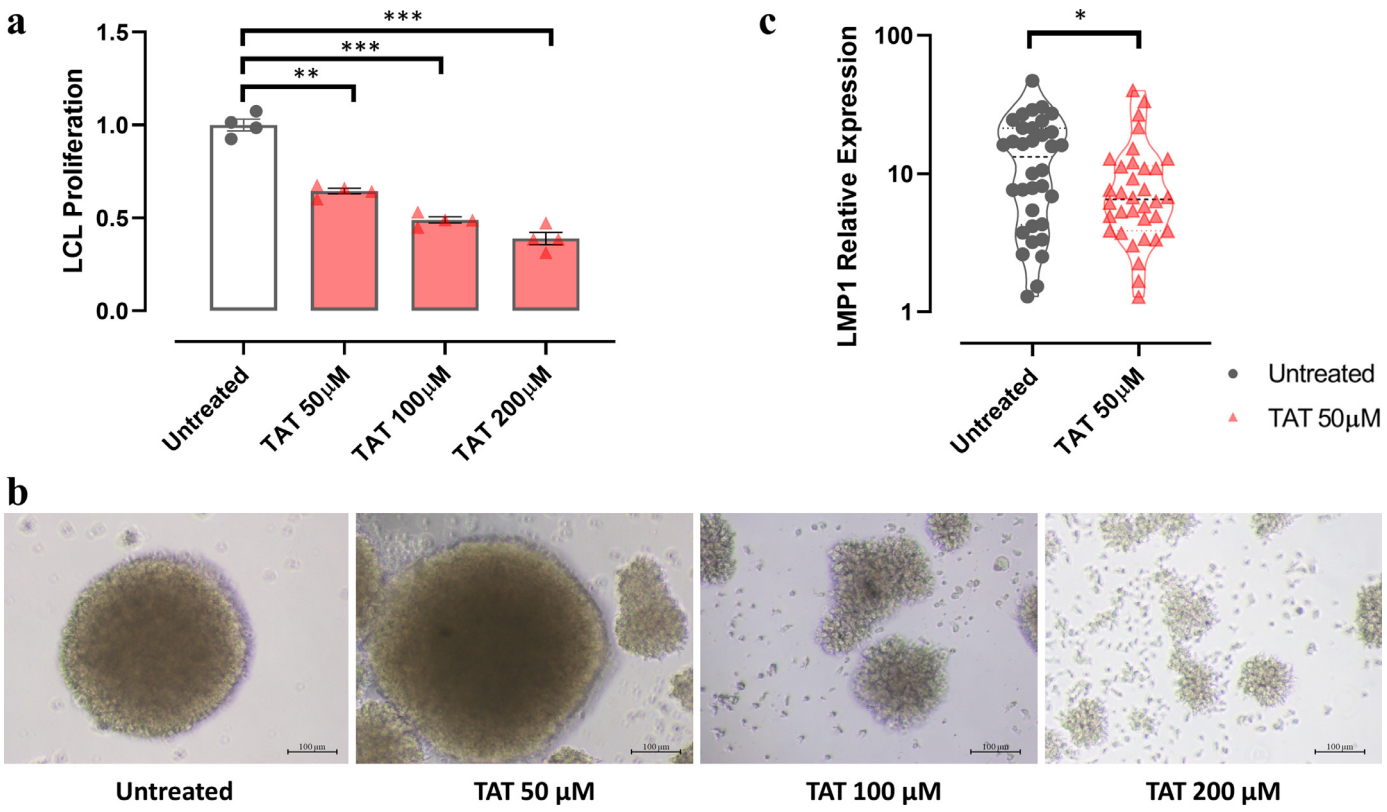


**Fig. 3. EBNA2-TAT effects on latency III growth program.** (a) LCL cell proliferation assay following 24 hr treatment with EBNA2 inhibitor EBNA2-TAT (n = 4); assay performed in triplicate. (b) Effect of EBNA2-TAT treatment on LCL colony growth and morphology. Untreated LCLs grow in large colonies but increasing concentrations of EBNA2-TAT inhibit this by blocking EBNA2 functions. (c) EBNA2-TAT interferes with the LCL latency III growth program through interference with EBNA2 regulation of EBV gene LMP1 (n = 34); PCR performed in triplicate. * < 0·05, ** <0·01, *** < 0·001. Paired t-test (two-tailed) performed for LCL proliferation. Wilcoxon matched-pairs signed rank test (two-tailed) performed for LMP1 expression. Gene Expression relative to RPL30 expression. TAT indicates EBNA2-TAT.

inhibition on expression, we can deduce that EBNA2 increases the expression of CD40, CLECL1, and TNFAIP8; and decreases the expression of TRAF3 and TNFRSF1A. This suggests that while altering gene expression in one direction, it can alter the relative allelic expression in the same direction (TRAF3, TNFRSF1A) or in the alternate direction (CD40, TNFAIP8). The effect on expression due to EBNA2 preferential binding is the effect that would be expected to confer increased immune evasion if the EBNA2 binding is the basis for the increased MS risk.

### *3.6. Does the EBNA2 interaction with MS risk alleles increase host immune evasion?*

EBNA2 interacts with MS risk loci, dependent on genotype (Fig. 2). The direction of effect on risk gene expression can be inferred from the alteration in the relative allelic expression in LCLs compared to uninfected B cells (Fig. 5a, Supplementary Table 8). Each of these MS risk genes have putative functions in the EBV latency III program proximal to LMP1 and LMP2 signalling [9]. On the basis of the inferred EBNA2 effect on expression and risk allele preference, we have postulated the effect of the EBNA2/risk gene interaction on the program, and so evasion of the host immune response (Fig. 5b). [9,27–31] The effect of EBNA2/risk gene interaction is to increase gene expression (CLECL1, TNFAIP8, TNFRSF1A) or decrease expression (TRAF3, CD40, RCOR1), as described in Fig. 5b. In each case, there are plausible explanations for an enhanced EBNA2 function on the latency III infection cycle through its regulation of these risk genes.

## 4. Discussion

In this study we sought to determine if EBNA2 regulates MS risk gene transcription in EBV infected B cells through an allelic imbalance in binding at MS risk loci. We had earlier identified six risk loci which were eQTLs in LCLs, and for which we had preliminary ChIP-seq evidence of binding by EBNA2 at the risk SNP itself [9]. We confirm that EBNA2 binds at least five of the six MS risk loci with allelic imbalance, and that inhibition of EBNA2 alters expression of five of these genes. The association of the risk alleles with expression in LCLs compared to that seen in uninfected B cells or blood cells was altered by this allelic preference. These data therefore provide evidence for EBNA2 interacting directly with these risk loci, affecting their expression and so function in these EBV infected cells. These genes all function on proximal pathways important in EBV immortalisation [9], and so likely affect the survival of EBV infected B cells in the host. This is consistent with a role for these risk alleles in altering MS susceptibility and/or progression through altering EBV susceptibility to its host. This work, confirms the allelic imbalance in EBNA2 binding identified in ChIP-seq studies, using much larger sample numbers, and indicates the consequence of that interaction from binding inhibitor studies and investigations of eQTL changes.

The risk SNP could alter the binding of the EBNA2 transcription factor complexes in a number of ways: directly altering the stochiochemistry of the DNA:transcription factor protein complex; altered comparative binding to competing transcription factor complexes; and/or altering epigenetic factors such as methylation and chromatin states. In each case, the altered EBNA2 allelic preference would be associated with the risk locus in a way that could drive altered disease risk due to the genetic effect of the locus.

Despite the increasing evidence implicating EBNA2 in MS in recent years [8,9,17]. the extent to which EBNA2 drives the association of SNPs with expression at MS risk loci in LCLs, which in turn affects EBV evasion of host immune responses and MS susceptibility, has remained unknown. Such evidence would support investigations

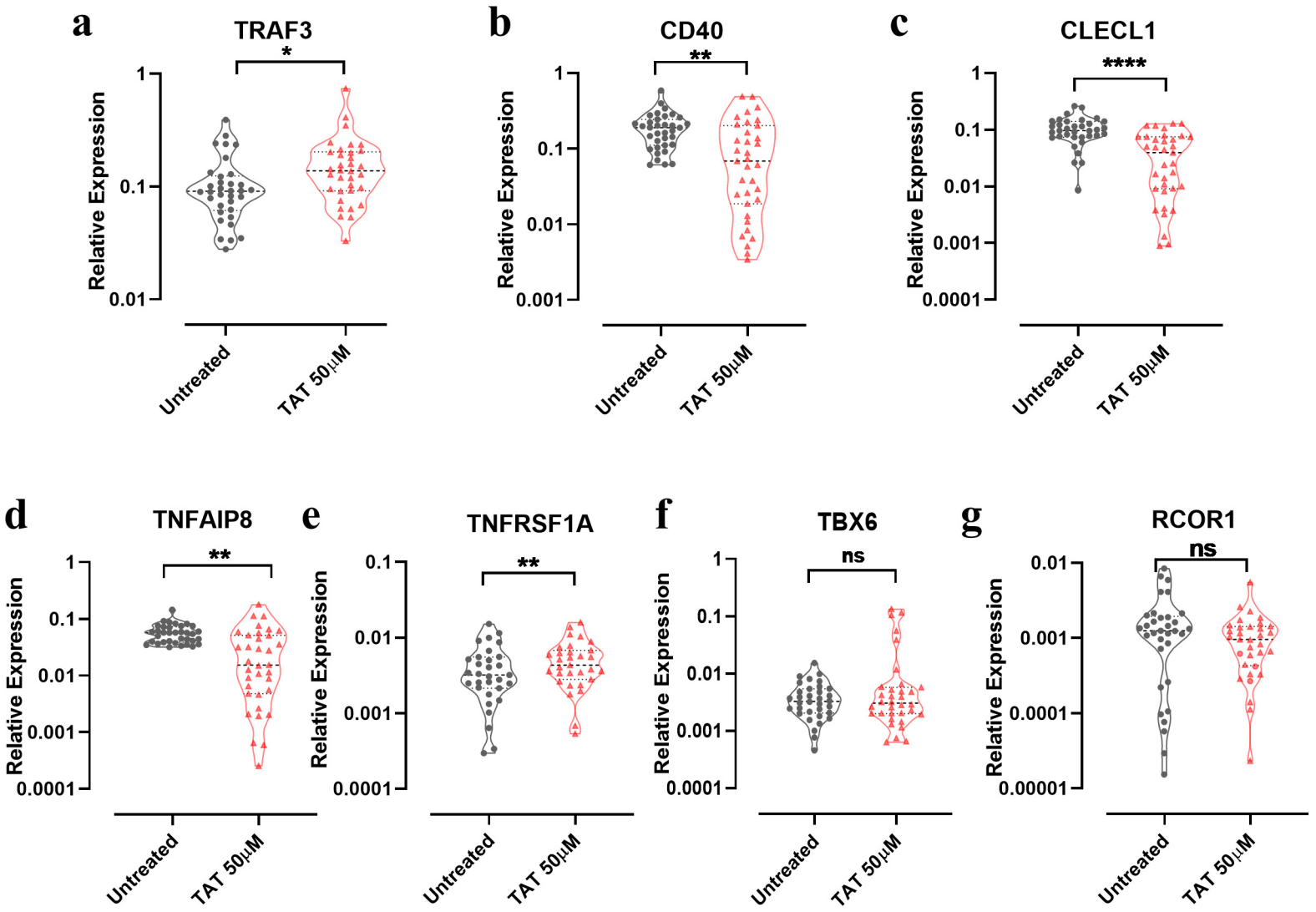


**Fig. 4. Effect of inhibition of EBNA2 function on the expression of seven EBNA2 targeted MS risk genes in LCLs.** Relative mRNA expression detected by real-time PCR following 24 hr treatment with EBNA2 inhibitor EBNA2-TAT (TAT) for (a) TRAF3, (b) CD40, (c) CLECL1, (d) TNFAIP8, (e) TNFSF1A, (f) TBX6, (g) RCOR1. ns: not significant, * < 0·05, ** < 0·01, *** < 0·001, **** < 0·0001. n = 35. PCR performed in duplicate. Expression relative to RPL30 expression. Wilcoxon matched-pairs signed rank test performed (two-tailed).

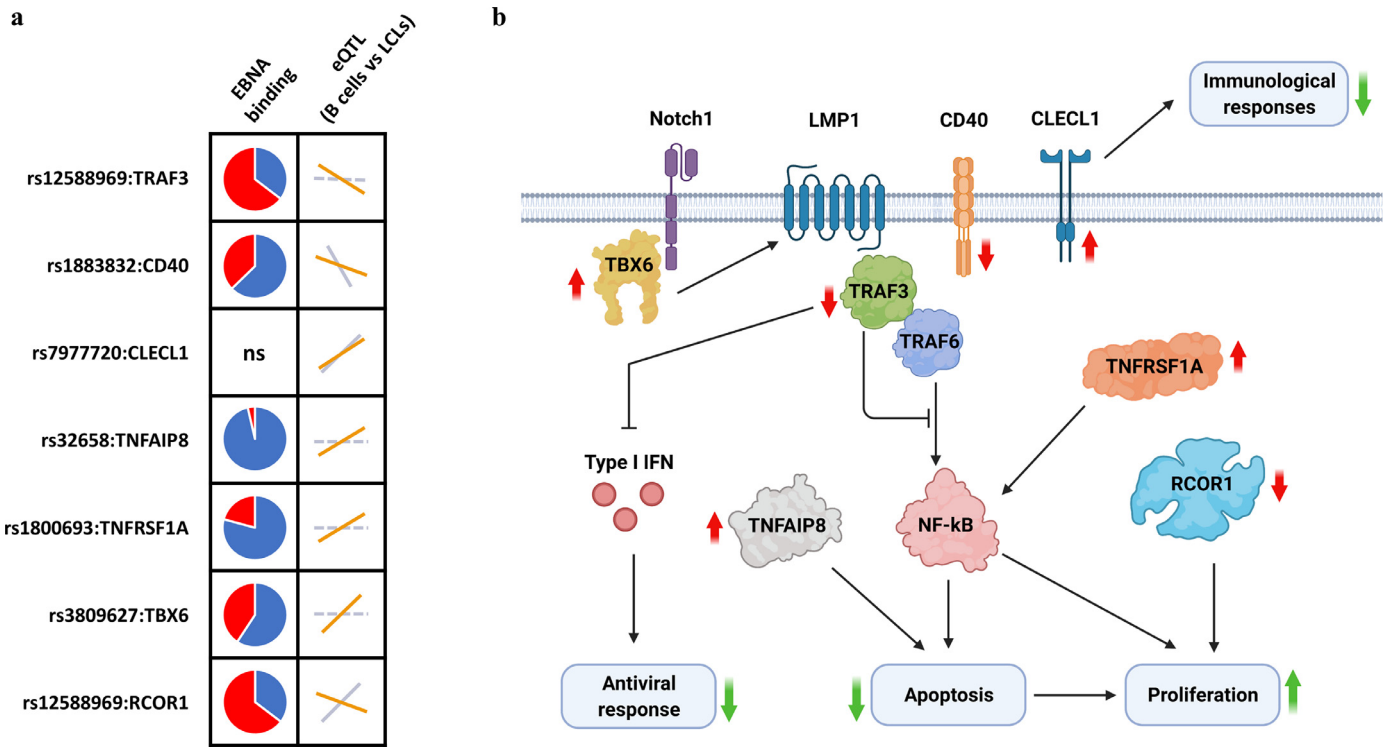


**Fig. 5. EBNA2 interaction with MS risk alleles may increase host immune evasion of EBV.** (a) Represents the EBNA2 binding allele preference (left column) and the MS risk SNP eQTL effect on gene expression (right column). Left column; blue and red indicates the EBNA2 affinity to protective and risk allele, respectively. Greater proportion of each colour in circles represents more affinity of EBNA2 to that allele. Right column represents the eQTL effect of SNP:genes in B cells and LCLs. The slope of orange and grey lines represents the direction and effect size of eQTL effect, (protective allele left, risk allele right). Solid grey and orange lines indicate a significant eQTL effect in B cells and LCLs, respectively. Dotted lines indicate non-significant eQTL effect. **(b)** Model of mechanisms by which EBNA2 interaction at MS risk loci might contribute to EBV latency III survival. The red arrows indicate the net effect on gene expression of EBNA2 binding of the risk allele. The overall effect of EBNA2 manipulation of these MS risk genes would be to reduce apoptosis and increase cell proliferation and energy production (green arrows), which enhance EBV latency III survival and could increase MS susceptibility. CLECL1 function is inferred based on knowledge of the role of related C type lectins in response to infection.[45] Further work is required to determine if the CLECL1 locus demonstrates an allelic imbalance in EBNA2 binding as the difference was not statistically significant in this study. Our data suggests higher expression increases immune evasion through the net function of this gene. Fig. partially created with BioRender.com.

into whether inhibition of EBNA2 might be a useful approach for the treatment of MS. In support of that, it is important to address what the likely consequences of the EBNA2 regulation of these MS risk genes would be in EBV latency III. EBNA2 can increase [16] or decrease transcription [32,33]. It typically increases transcription of target genes by binding to corepressor complexes, such as through RBP-J$\kappa$ [34]. However, its interactions with other EBV factors (notably EBNA3, usually antagonistic to EBNA2, which itself forms complexes with the MS risk gene BATF [35,36] and human transcription factors (notably EBF1) could result in complex effects on expression, that may even be different between individuals, and dynamic through infected cells over time. Only about 80% of EBNA2 binding sites are also RBP-J$\kappa$ sites [37], the remainder would not be directly affected by the EBNA2-TAT inhibitor [18]. Since these six MS risk loci all bound EBNA2 and expression was affected by EBNA2-TAT inhibition of the EBNA2/RBP-J$\kappa$ interaction for five of them (not TBX6), it seems likely EBNA2/risk loci binding were all at colocalisation sites for the two transcription factors. From empirical evidence of effect on expression of the risk SNP in LCLs we can infer if the EBNA2 allele preference increases or decreases expression (Fig. 5).

In general, EBNA2 manipulation of the MS risk loci studied here can be plausibly linked to enhanced EBV survival, which could increase MS susceptibility (Fig. 5b). Immune control of infected B cells is both intracellular, through antiviral pathways such as the interferon response pathway; and extracellular, predominantly due to recognition of infected cells by CD8 T cells and NK cells [38,39]. Further, evasion of B cell checkpoints in clonal proliferation may be crucial to disease progress [19], and this may also be affected by the interaction between MS risk alleles, EBNA2 regulation, and host immune response. Notably, three of these risk loci (TNFRSF1A, TRAF3, TBX6) are amongst the most mutated in nasopharyngeal carcinoma tumours, another EBV associated disease [40]. Risk loci for CD40, TRAF3, TNFRSF1A and CLECL1 are associated with other EBV implicated autoimmune diseases, and associated with lymphocyte numbers (GWAS catalog [41], Supplementary Table 9).

Genes can have multiple functions, in part due to the balance of interactions with other genes in the infected cell. We had previously suggested that lower TRAF3 expression and lower CD40 expression promoted EBV infected B cell survival: less TRAF3 by less inhibition of the TRAF6 pathway/upregulation of the interferon response pathway [42], less CD40 would lead to less competition with LMP1, the viral homologue of CD40 [9]. This is consistent with the effect of allelic preference by EBNA2 identified here. RCOR1 (shared locus with TRAF3) is a corepressor that controls cell proliferation, so that the lower expression seen for the preferred EBNA2 allele may indicate less inhibition of proliferation of infected B cells. The transcription factor TBX6 is known to increase NOTCH1 signalling [30,31] which means it could be expected to upregulate LMP1 [43] and LMP2A [44], and the higher allelic expression on EBNA2 binding is consistent with increased NOTCH1 signalling being favourable for the virus. TNFAIP8 and TNFRSF1A both interact with NF-$\kappa$B signalling. TNFAIP8 is upregulated by NF-$\kappa$B [28] and can inhibit or promote apoptosis. TNFRSF1A interacts directly with the NF-$\kappa$B complex [29]. To be consistent with the concept that preferential EBNA2 binding promotes immune evasion for these risk genes, this suggests the direction of effect on the target pathways depends on interactions with other

regulators of these pathways. CLECL1 is a transmembrane C type lectin of largely unknown function. Related C type lectins play key roles in response to infection [45]. Our data suggests higher expression, or other EBNA2 transcriptional effects, increases immune evasion through the net function of this gene. EBV antibodies are elevated in MS associated with the MS risk allele HLA DRB1:1501 [46]. Specific testing of the effect of the risk loci investigated here on T/NK control, B cell phenotypic features, EBV viral load, and EBV antibodies may clarify the relative importance of these processes in host response to EBV and to disease risk.

For a number of reasons, EBNA2 regulation of these MS risk genes might not be the basis for their association with MS. The MS risk genes have other roles which could drive MS risk, especially in B cells, so that attributing the basis for the allelic associations with disease is challenging. Further, it is difficult to delineate the EBNA2 specific effects on gene transcription from that of other EBV and host factors. Also, the capture of the host B cell antigen proliferation pathways by EBNA2[37] likely involves interactions with some host polymorphisms that have little effect on EBV infection, although this is less likely where the SNPs are also associated with MS. The consequences of the allelic imbalance on binding are likely to extend beyond affecting expression levels, as splicing [47], timing of response, conditions used for testing response and inhibition, and interaction with other genes and genetic variants[48] are all likely to be important. Further, the statistical power to evaluate these effects requires much larger samples than were available for this study. Finally, as the MS risk alleles have been identified in Caucasian samples, and EBV strains are different in Africa and potentially Asia, our findings might not be representative of EBV effects on MS in these populations.

However, these studies do further implicate EBV and EBNA2 in MS pathogenesis. The empirical consequences of inhibiting EBNA2 need to be further evaluated in LCLs, and in the mouse model of EBV infection and MS [49]. EBNA2 drives EBV latency III, further supporting the role of this EBV stage in MS. However, the consequences in inhibiting this stage when disease has developed ('the horse has bolted') will be difficult to answer but may be investigated with mouse models. Finally, EBNA2 genomic targets overlap with those of the vitamin D receptor [8]. Vitamin D is implicated in MS pathogenesis, and many genes regulated by it are MS risk genes [50], implicating interaction between these two environmental factors, EBV and vitamin D, in MS aetiology.

In summary, these data are consistent with the concept that EBV contributes to MS risk through the interaction of EBNA2 with MS risk alleles and indicate that targeting of EBNA2 in MS could be a promising therapeutic strategy. EBNA2 could be targeted to alter MS risk gene expression, or more broadly, due to its essential role in latency III maintenance, could be used to deplete EBV-infected B cells. This work also provides further insights into poorly understood mechanisms through which environmental factors can interact with human genetic factors to alter human disease. In addition to EBV, similar approaches to more clearly understand allelic imbalance in MS could focus on other environmental factors such as the Vitamin D receptor interaction at MS risk loci.

## 5. Contributors

JTK, DRB, AA and GPP devised the experiments. SDS assisted in preparation and analysis of cell culture experiments, in addition to cell culture preparation for the ChIP samples. AA assisted in analysis of large datasets for B cells and LCLs in addition to preparation of Figs 1 and 5; the underlying data was verified by JTK. GPP assisted in data analysis. JTK conducted all other experiments and analyses, the underlying data was verified by AA. JTK and DRB wrote the manuscript. All authors read and approved the final manuscript.

## Declaration of Competing Interest

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## Data Sharing Statement

Deidentified data are available to other researchers for use in independent scientific research after a justified request (david.booth@sydney.edu.au).

## Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.ebiom.2021.103572.

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