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An Epstein-Barr Virus MicroRNA Blocks Interleukin-1 (IL-1) Signaling by Targeting IL-1 Receptor 1

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ABSTRACT Epstein-Barr virus (EBV) encodes >44 viral microRNAs (miRNAs) that are differentially expressed throughout infection, can be detected in Epstein-Barr virus (EBV)-positive tumors, and manipulate several biological processes, including cell proliferation, apoptosis, and immune responses. Here, we show that EBV BHRF1-2 miRNAs block NF-κB activation following treatment with proinflammatory cytokines, specifically interleukin-1β (IL-1β). Analysis of EBV PAR-CLIP miRNA targetome data sets combined with pathway analysis revealed multiple BHRF1-2 miRNA targets involved in interleukin signaling pathways. By further analyzing changes in cellular gene expression patterns, we identified the IL-1 receptor 1 (IL1R1) as a direct target of miR-BHRF1-2-5p. Targeting the IL1R1 3′ untranslated region (UTR) by EBV miR-BHRF1-2-5p was confirmed using 3′-UTR luciferase reporter assays and Western blot assays. Manipulation of EBV BHRF1-2 miRNA activity in latently infected B cells altered steady-state cytokine levels and disrupted IL-1β responsiveness. These studies demonstrate functionally relevant BHRF1-2 miRNA interactions during EBV infection, which is an important step in understanding their roles in pathogenesis.

IMPORTANCE IL-1 signaling plays an important role in inflammation and early activation of host innate immune responses following virus infection. Here, we demonstrate that a viral miRNA downregulates the IL-1 receptor 1 during EBV infection, which consequently alters the responsiveness of cells to IL-1 stimuli and changes the cytokine expression levels within infected cell populations. We postulate that this viral miRNA activity not only disrupts IL-1 autocrine and paracrine signaling loops that can alert effector cells to sites of infection but also provides a survival advantage by dampening excessive inflammation that may be detrimental to the infected cell.

KEYWORDS Epstein-Barr virus, RNA interference, cytokines, herpesviruses, interleukins, microRNA

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Epstein-Barr virus (EBV) is a ubiquitous human gammaherpesvirus that persists in over 90% of adults worldwide. The virus initially infects epithelial and B cells in the oral pharynx and is thought to establish latency in the memory B cell compartment (1). Primary infection is normally asymptomatic or results in infectious mononucleosis that resolves after several weeks. Throughout this process, immune surveillance mechanisms are activated that control lifelong infection (2, 3). Immunosuppressed patients, such as those with HIV coinfection, primary immune deficiency, or undergoing organ transplant, have increased risk of developing EBV-associated lymphoproliferative diseases such as posttransplant lymphoproliferative disorder (PTLD) and B or T cell lymphomas (4). Epithelial tumors, such as nasopharyngeal and gastric carcinomas, are also linked to uncontrolled EBV infection (5).

Critical to persistent infection, EBV employs multiple, adept strategies to reduce recognition and avoid clearance by the immune system. Several viral proteins expressed during lytic infection manipulate innate or adaptive antiviral effector functions:

the EBV exonuclease BGLF5 induces degradation of multiple host mRNAs, including Toll-like receptor 2 (TLR2) and TLR9 that sense infection (3, 6); BPLF1, an EBV deubiquitinase, antagonizes TLR signaling and NF-κB activation by deubiquitinating TRAF6 (tumor necrosis factor receptor-associated factor 6) (7, 8); BCRF1 (vIL-10) mimics the anti-inflammatory effects of cellular interleukin-10 (IL-10) and dampens $CD4^+$ T cell responses (9); and BNLF2a blocks TAP (transporter associated with antigen presentation), reducing surface display of viral antigens and recognition of infected cells by $CD8^+$ T cells (10). During latency, viral gene expression is highly restricted, limiting viral antigenicity. The EBV episomal maintenance protein EBNA1 (EBV nuclear antigen 1), expressed in all latently infected cells, contains Gly/Ala repeats that slow peptide processing and diminish presentation by major histocompatibility class I molecules, effectively lowering cytotoxic T cell recognition (11). Recently, it has been observed that gammaherpesvirus immune evasion strategies are further abetted by virally encoded microRNAs (miRNAs).

miRNAs are ~22-nucleotide (nt), small noncoding RNAs that posttranscriptionally inhibit gene expression. Many cellular miRNAs participate in the fine-tuning of immunological circuits (12). EBV encodes 25 viral precursor miRNAs (pre-miRNAs), clustered in two regions of the genome (13). Viral miRNAs arising from the 22 BART pre-miRNAs are variably expressed in all cell types infected with EBV (14). The EBV *BHRF1* locus encodes miR-BHRF1-1-5p, miR-BHRF1-2-5p, miR-BHRF1-2-3p, and miR-BHRF1-3, which are detectable predominantly in B cells (13, 15). Like most cellular miRNAs, EBV miRNAs arise from primary miRNA transcripts which are processed into ~60 nt pre-miRNAs by nuclear Drosha, exported, and cleaved by Dicer in the cytoplasm to liberate ~22-bp miRNA:miRNA* duplexes. One duplex strand is incorporated into the RNA-induced silencing complex (RISC), minimally consisting of an Argonaute protein (i.e., Ago2) and the mature miRNA, and guides RISC to complementary sites on target RNAs – predominantly in 3′ untranslated regions (UTRs), inducing posttranscriptional silencing.

Several studies indicate that EBV miRNAs, particularly the BHRF1 miRNAs, have key roles in the EBV life cycle during the early stages of infection. BHRF1 miRNAs are detectable within 2 days postinfection (dpi) of $CD19^+$ B cells *in vitro*, levels strongly peak by ~3 dpi, and remain high throughout the first 7 to 10 days as B cells progress to immortalized lymphoblastoid cell lines (LCLs) (15, 16). *In vitro*, mutational inactivation of all or individual BHRF1 miRNAs limits LCL outgrowth; in B cell transformation assays, BHRF1 mutant viruses exhibit 20- to 30-fold reduced LCL outgrowth compared to wild-type viruses (17–19). LCLs established with these mutant viruses exhibit impaired S-phase entry and accumulate in G_0/G_1 but do not appear to undergo spontaneous apoptosis (17). Unlike other gammaherpesvirus miRNA knockouts, EBV BHRF1 miRNA mutant viruses do not appear to spontaneously reactivate *in vitro* (17–19). *In vivo*, following acute infection of humanized mice, deletion of all three BHRF1 miRNAs triggers significant delays in systemic viremia (20), which may be a consequence of impaired B cell activation, proliferation, and/or differentiation in the absence of these miRNAs. BHRF1 miRNA targets involved in these processes are not yet characterized. In addition, mice infected with BHRF1 miRNA knockout virus exhibit reduced peripheral $CD8^+$ T cell expansion compared to wild-type virus, suggesting a role for the miRNAs in inducing appropriate adaptive immune responses (21). Very recent studies demonstrate that multiple EBV BART and BHRF1 miRNAs can limit both $CD4^+$ and $CD8^+$ T cell responses early after *de novo* infection of B cells (21, 22).

EBV miRNAs downregulate several host factors involved in the development of antiviral immune responses. EBV miR-BHRF1-3 targets CXCL11, a chemoattractant for activated T cells (23). NLRP3, a component of the inflammasome complex, plays a role in the cytoplasmic maturation of interleukin-1β (IL-1β) and IL-18, key players in the inflammatory response. EBV miR-BART15-3p targets the NLRP3 3′ UTR, consequently attenuating cytokine production (24). Another EBV miRNA, miR-BART6-3p, may regulate IL-6 signaling since miRNA inhibition increases levels of IL-6 receptor chains (p80 and p130) (25). Ectopic EBV miR-BART20 or miR-BART8 expression downregulates luciferase activity from reporters containing either the interferon gamma (IFN-γ) or STAT1 3′ UTRs

(26), respectively, and binding sites for miR-BART20-5p have been reported in the TBX21/T-bet 3′ UTR, encoding a transcriptional regulator of gamma interferon (IFN-γ) and IL-2 (27). High-throughput RISC immunoprecipitation studies have also captured promyelocytic leukemia (PML) body components, C-type lectins, and natural killer (NK) cell ligands as targets of EBV BART miRNAs (28–31).

Other herpesvirus miRNAs target multiple components of NF-κB pathways and can manipulate cytokine responses by inhibiting Toll-like receptor (TLR) and IL-1 signal transduction (32–36). Cytokine activation of the IL-1 receptor engages the adaptor protein MYD88 (myeloid differentiation primary response protein 88), followed by recruitment of interleukin-1 receptor (IL-1R)-associated kinases, IRAK1 and IRAK4, and induction of NF-κB and mitogen-activated protein kinase (MAPK) pathways (37). Kaposi's sarcoma-associated herpesvirus (KSHV) miRNAs target two critical components of these pathways, MYD88 and IRAK1, reducing NF-κB-mediated production of inflammatory cytokines (32). Very recently, we demonstrated that two viral miRNAs encoded by a nonhuman primate gammaherpesvirus related to KSHV can also block IL-1 signaling (34).

Here, we evaluated the role of EBV miRNAs in IL-1 signaling and found that the expression of the two EBV BHRF1-2 miRNAs inhibits IL-1β responsiveness. After the identification of multiple BHRF1-2 miRNA targets related to interleukin signaling, we demonstrate that miR-BHRF1-2-5p, in particular, directly targets the IL-1 receptor 1 (IL1R1) 3′ UTR and downregulates IL1R1 at both the RNA and protein levels. Consequently, ectopic expression of the BHRF1-2 miRNAs in B cells induces changes in steady-state cytokine levels while loss of miR-BHRF1-2-5p activity in latently infected cells amplifies IL-1 signaling events and proinflammatory cytokine expression.

RESULTS

IL-1 signaling is disrupted by EBV BHRF1-2 miRNAs. Previous studies have demonstrated that multiple herpesvirus miRNAs manipulate inflammatory cytokine signaling pathways (3, 13, 32, 34, 36). In screening the EBV miRNAs for potential involvement in cytokine signaling, we observed that expression of the BHRF1-2 miRNAs in 293T cells blocked activation of an NF-κB luciferase reporter upon stimulation with IL-1β (Fig. 1A). To investigate this further, we tested increasing doses of IL-1β. In control cells, luciferase activity increased 3-fold over mock-treated cells following IL-1β treatment, while the presence of the BHRF1-2 miRNAs blocked NF-κB activation (Fig. 1C). We also introduced the BHRF1-2 miRNAs into BJAB cells stably expressing an NF-κB luciferase reporter. Similar to 293T cells, BHRF1-2 miRNA expression abrogated IL-1β activation of the reporter (Fig. 1D), suggesting that the BHRF1-2 miRNAs disrupt IL-1 signal transduction.

To determine where in the IL-1 pathway this block might be occurring, we monitored IκBα levels in 293T-IκBαLuc cells. In these cells, the cytoplasmic NF-κB inhibitor IκBα is fused to photinus luciferase (38, 39). Induction of canonical NF-κB signaling (i.e., via inflammatory cytokine stimuli) leads to degradation of the IκBα fusion protein and thus results in reduced luciferase activity. Dose-dependent knockdown of IκBα-photinus luciferase was observed in control cells upon stimulation with increasing amounts of IL-1β; however, the presence of the BHRF1-2 miRNAs blocked knockdown (Fig. 1E). Next, 293T-IκBαLuc cells were transfected with increasing amounts of pcDNA3-miR-BHRF1-2 and then treated for 3 h with IL-1β. Cells were treated in parallel with a second proinflammatory cytokine, tumor necrosis factor alpha (TNF-α), to determine whether the negative effects of the BHRF1-2 miRNAs were broadly related to cytokine-mediated NF-κB activation or specific to IL-1 signaling. In control cells, treatment with either cytokine reduced IκBα luciferase activity >60%, as expected (Fig. 1F). The BHRF1-2 miRNAs had no effect on the IκBα reporter following TNF-α stimulation; however, in the presence of IL-1β, the BHRF1-2 miRNAs blocked IκBα degradation in a dose-dependent manner, which is consistent with inhibition of NF-κB activation.

To determine whether the BHRF1-2 miRNAs also inhibited IL-1β signaling in the context of latent EBV infection, NF-κB reporters were introduced into EBV B95-8 LCLs

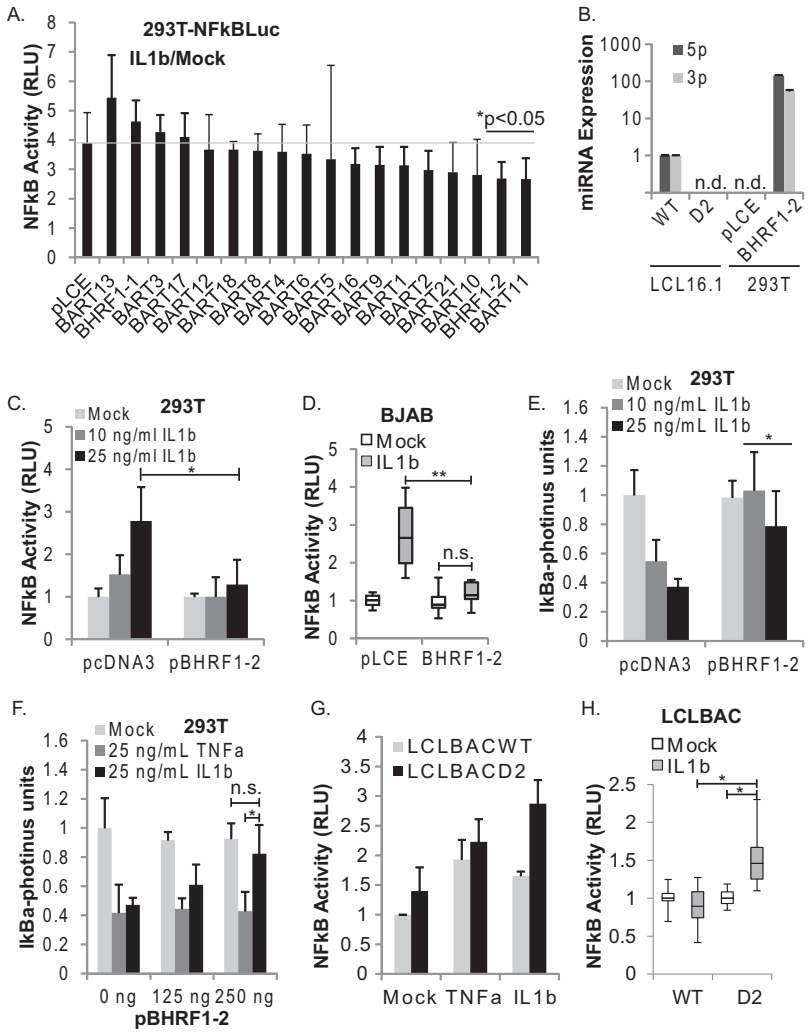

FIG 1 EBV BHRF1-2 miRNAs inhibit IL-1β-mediated NF-κB activity. (A) Functional screen of EBV miRNAs reveals BHRF1-2 and BART11 miRNAs inhibit NF-κB activation by IL-1β. 293T-NF-κBLuc cells were transfected with individual pLCE-based EBV miRNA expression vectors. At 44 h posttransfection, the cells were stimulated with 25 ng/ml IL-1β for 4 h and then lysed and assayed for dual luciferase activity. Reported are the averages of two independent experiments performed in triplicates. *, $P < 0.05$ (Student *t* test). (B) Expression of EBV miR-BHRF1-2-5p and miR-BHRF1-2-3p in 293T cells. 293T cells were transfected with pLCE or pLCE-BHRF1-2, and the total RNA was harvested at 48 h posttransfection. miRNA expression was assayed by stem-loop qRT-PCR (normalized to miR-16) and is reported relative to levels in EBV B9-8 wild-type (WT) or BHRF1-2 miRNA KO (D2) LCLs. (C) 293T-NF-κBLuc cells were transfected with 250 ng of pcDNA3 or pBHRF1-2 as indicated. At 44 h posttransfection, the cells were stimulated with increasing amounts of IL-1β for 4 h. Reported are averages and the SD of two independent experiments performed in quadruplicate. *, $P < 0.05$ (Student *t* test). (D) BJAB-NF-κBLuc cells were transduced with pLCE or pLCE-BHRF1-2. At 5 or 7 days postransduction, the cells were plated in 96-well black-well plates at 3×10^4 cells/well, treated with 25 ng/ml IL-1β for 18 h, and then lysed and assayed for dual luciferase activity. Box-and-whisker plots represent averages of two independent experiments with five replicates ($n = 10$). n.s., not significant. **, $P < 0.01$ (Student *t* test). (E) 293T-IκBαLuc cells were transfected with 250 ng of pcDNA3 or pBHRF1-2 as indicated. At 44 h posttransfection, the cells were stimulated with increasing amounts of IL-1β for 4 h and then lysed and assayed for luciferase activity. Reported are averages and the SD of two independent experiments performed in triplicates. *, $P < 0.01$ (Student *t* test) compared to empty-vector control cells. (F) 293T-IκBαLuc cells were transfected with increasing amounts of pBHRF1-2 as indicated. At 44 h posttransfection, the cells were stimulated with TNF-α or IL-1β for 4 h and then lysed and assayed for luciferase activity. Reported are averages and the SD of two independent experiments performed in triplicates. n.s., not significant. *, $P < 0.01$ (Student *t* test) compared to empty-vector control cells. (G) EBV B95-8 wild-type (LCLBACWT) or BHRF1-2 miRNA KO (LCLBACD2) LCLs were cotransduced with a NF-κB reporter (pL-NF-κB-Fluc) and a *Renilla* luciferase internal control (pL-RSV-Rluc). At 72 h postransduction, the cells were plated in fresh medium and stimulated with 100 ng/ml TNF-α or IL-1β for 3 h. Reported are averages of two experiments. (H) LCLBACWT or BHRF1-2 miRNA KO (LCLBACD2) cells were electroporated with 2.7 μg of pNF-kB-Fluc and 300 ng of pL-RSV-RLuc. At 24 h posttransfection, the cells were plated in 96-well plates at 1.2×10^5 cells per well and then treated for 18 h with 25 ng/ml IL-1β. Box-and-whisker plots represent averages of five independent experiments performed in quadruplicates ($n = 20$). *, $P < 0.01$ (Student *t* test). RLU, relative light units.

generated with either wild-type or BHRF1-2 miRNA knockout (KO) recombinant viruses (LCLBACWT and LCLBACD2, respectively) (18, 29, 40). Transduced LCLs were treated with TNF-α or IL-1β and monitored for NF-κB activity (Fig. 1G). We also transfected LCLs with an NF-κB reporter and then treated the cells with IL-1β (Fig. 1H). Notably, in LCLs lacking the BHRF1-2 miRNAs compared to wild-type LCLs, elevated NF-κB activity was observed after IL-1β stimulation, indicating that BHRF1-2 miRNAs control IL-1β responsiveness during latent EBV infection (Fig. 1G and H). No differences were observed with TNF-α treatment (Fig. 1G). Taken together, these results demonstrate that the BHRF1-2 miRNAs specifically disrupt IL-1β signaling events and that the block occurs upstream of IκBα degradation.

Defining high-confidence BHRF1-2 miRNA targets from PAR-CLIP data sets. A few targets for miR-BHRF1-2-5p and miR-BHRF1-2-3p have been previously described (29, 38), but few have been investigated in detail or linked to phenotypes (17, 18, 19, 20, 41). To identify targets of BHRF1-2 miRNAs that could be involved in IL-1 signaling, we analyzed Ago PAR-CLIP (photoactivatable ribonucleoside-enhanced cross-linking and immunoprecipitation) data sets from EBV B95-8 LCLs, wild-type EBV LCLs, and rLCV-infected rhesus macaque LCLs that express BHRF1-2 miRNAs or homologs. In order to obtain a high-confidence set of 3′-UTR targets for the two BHRF1-2 miRNAs, we also analyzed Ago PAR-CLIP data sets from B cells lacking the BHRF1-2 miRNAs: BHRF1-2 miRNA KO LCLs (LCLBACD2), KSHV+/EBV-negative BC3 primary effusion lymphoma (PEL) cells, and KSHV+/EBV+ BC1 PEL cells that express BART miRNAs but not the BHRF1 miRNAs (Fig. 2A) (29, 30, 38, 40). Any RISC-interaction sites that overlapped with the BHRF1-2 miRNA-negative PAR-CLIP libraries were eliminated from the final data set (Fig. 2A). In total, 225 high-confidence 3′-UTR targets fulfilled our stringent criteria ($>=$ 7mer seed match to miR-BHRF1-2-5p or miR-BHRF1-2-3p).

Next, we performed *in silico* pathway analysis using the curated pathway database Reactome (http://www.reactome.org) to ask if the 225 targets were enriched for specific cellular processes. Based on the total number of target genes mapping to each pathway, the top two reactome pathways were signal transduction (48 entities; R-HSA-162582) and immune system (40 entities; R-HSA-168256). Intriguingly, 18 targets (highlighted in Fig. 2A) were associated with signaling by interleukins (R-HSA-449147) ($P < 0.01$), including two strong candidates that could be responsible for the BHRF1-2 miRNA block in IL-1 responses: (i) IL1R1 and (ii) the TLR signaling adaptor MYD88. We also compared our list of 225 BHRF1-2 miRNA targets to a published list of 118 proteins identified through a published small interfering RNA screen that are involved in IL-1β-mediated NF-κB activation (39). Only two genes overlapped: RNF11 (ring finger protein 11, a mediator of ubiquitin ligases) and, notably, IL1R1.

From the list of target genes related to interleukin signaling, we chose three targets with seed matches to miR-BHRF1-2-5p (IL1R1, BIRC3, and SOS1) and two targets with seed matches to miR-BHRF1-2-3p (MYD88 and PLCG1) to experimentally validate. 3′ UTRs were cloned downstream of renilla luciferase in the dual reporter vector psi-Check2 and cotransfected with increasing amounts of the EBV BHRF1-2 miRNA expression vector into HEK293T cells. The presence of the BHRF1-2 miRNAs resulted in >20% dose-dependent knockdown of luciferase activity for reporters containing the 3′ UTR of PLCG1, SOS1, BIRC3, or IL1R1 (Fig. 2B). Surprisingly, the MYD88 3′-UTR reporter was not responsive to BHRF1-2 miRNA activity. Mutation of individual BHRF1-2 miRNA seed match sites using site-directed mutagenesis fully rescued miRNA-mediated luciferase knockdown for IL1R1, PLCG1, and BIRC3 (Fig. 2C), confirming the binding sites. Figure 2D illustrates the PAR-CLIP identified miR-BHRF1-2-5p interaction sites captured in the BIRC3 and IL1R1 3′ UTRs. We also mutated the MYD88 PAR-CLIP'ed site to determine whether this might significantly increase reporter activity, but BHRF1-2 miRNAs had no effect, leading us to conclude that MYD88 is not a direct target for these miRNAs.

IL1R1 and TYW3 transcripts are upregulated in the absence of the BHRF1-2 miRNAs. miRNAs often induce degradation of their target RNAs leading to inverse correlations between miRNA levels and steady-state target transcripts (42). Previous

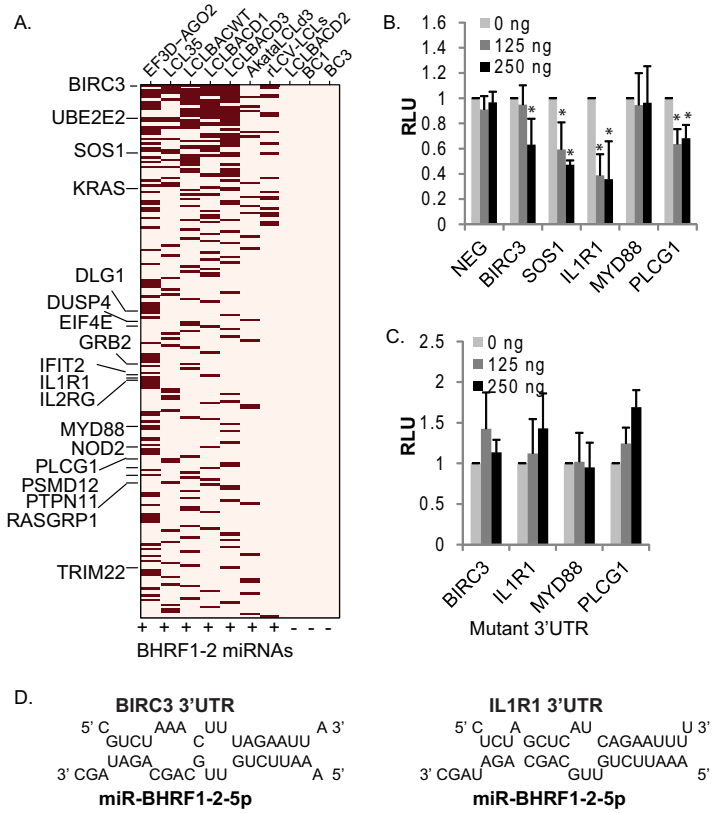

FIG 2 Targets of the EBV BHRF1-2 miRNAs are involved in interleukin signaling. (A) Analysis of BHRF1-2 miRNA interaction sites captured in PAR-CLIP data sets. Data sets are from EBV$^+$ LCLs, rhesus LCLs, and KSHV$^+$ PELs. BHRF1-2 miRNA expression is indicated for each cell line. A total of 225 PAR-CLIP'ed, cellular 3′-UTR interaction sites were identified that exhibited ≥7mer canonical seed matches to either miR-BHRF1-2-5p or miR-BHRF1-2-3p. Red boxes indicate presence of the interaction site within each PAR-CLIP sample. The 18 genes highlighted on the left were identified by pathway analysis through the reactome database and are involved in signaling by interleukins (R-HSA-449147). (B and C) 3′-UTR reporter assays confirm BHRF1-2 miRNA target interactions. Select 3′ UTRs were cloned downstream of *Renilla* in the psiCheck2 dual reporter luciferase vector. For panel C, PAR-CLIP identified seed match sites for the BHRF1-2 miRNAs were mutated using site-directed mutagenesis. 293T cells were cotransfected with 20 ng of 3′-UTR reporter and pcDNA3 or pBHRF1-2 as indicated. Lysates were harvested 48 to 72 h posttransfection and analyzed for dual luciferase activity. Reported are the averages of at least three independent experiments performed in triplicates. *, $P < 0.01$ (Student *t* test).

transcriptome sequencing (RNA-Seq) experiments on established EBV B95-8 LCLs lacking individual BHRF1 miRNAs indicate that for the BHRF1-2 and BHRF1-3 miRNAs, in particular, the steady-state levels of target cellular mRNAs are impacted by loss of the viral miRNA (40). To investigate whether BHRF1-2 miRNA targets are altered at the RNA level, we compared the published gene expression profiles of wild-type EBV B95-8 LCLs (LCLBACWT) and BHRF1-2 miRNA KO LCLs (LCLBACD2). A total of 25,788 cellular genes were identified between the two RNA-Seq data sets; based on the TPM (transcript per million) values, 1,042 genes were upregulated in LCLs in the absence of the BHRF1-2 miRNAs ($\log_2$ fold change [log2FC] ≥ 1.5) (Fig. 3A).

Next, BHRF1-2 miRNA target genes (≥7mer canonical seed match, 3′-UTR sites only) were extracted from six published LCL PAR-CLIP data sets (EF3D-Ago2, LCL35, LCLBACWT, LCLBACD1, LCLBACD3, and AkataLCLd3) (29, 38), eliminating any sites that were captured in BHRF1-2 miRNA-negative PAR-CLIP data sets (BC3, BC1, and LCLBACD2) (30, 40), and compared to the list of 25,778 detected genes. Of the 225 PAR-CLIP-identified BHRF1-2 miRNA 3′-UTR targets, eight target genes were moderately upregulated (real FC > 1.5; log2FC > 0.6) in LCLBACD2 compared to LCLBACWT (Fig. 3B). Intriguingly, these genes included IL1R1 (log2FC = 2.57), TYW3 (tRNA-YW synthesizing protein 3 homolog, log2FC = 1.71), and MYD88 (log2FC = 1.08).

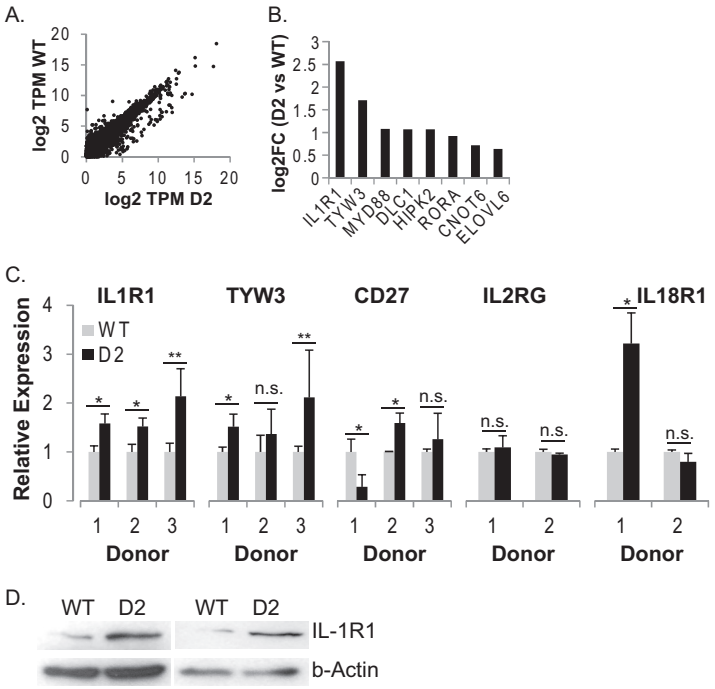

FIG 3 IL1R1 is upregulated in the absence of the BHRF1-2 miRNAs. (A) RNA-Seq analysis of LCLBACWT (WT) and LCLBACD2 (D2) reveals upregulated cellular transcripts in LCLs lacking the BHRF1-2 miRNAs. TPM, transcripts per million mapped. (B) Eight upregulated genes in LCLBACD2 (compared to WT) overlap PAR-CLIP-identified BHRF1-2 miRNA targets. Shown are log2FC values relative to LCLBACWT. (C) qRT-PCR analysis of IL1R1, TYW3, IL2RG, CD27, and IL18R1 transcripts in donor-matched LCL pairs generated with wild-type EBV B95-8 (WT) or BHRF1-2 miRNA KO (D2) viruses. Values are normalized to GAPDH and are reported relative to WT LCLs for each pair. Reported are average expression values with the SD from at least three independent RT experiments; qPCR was performed in duplicate. For IL1R1, $n = 5$ experiments for donors 1 and 2 and $n = 4$ experiments for donor 3. n.s., not significant. *, $P < 0.01$; **, $P < 0.05$ (Student t test). (D) Western blot analysis of IL-1R1 and β-actin in LCLBACWT and LCLBACD2 (donor 1). Shown are blots from two independent experiments.

To confirm expression differences and determine whether genes were reproducibly changed in BHRF1-2 miRNA KO LCLs, we measured the levels of IL1R1 and TYW3 in three donor-matched LCLs: LCLBACD2 compared to LCLBACWT (donor 1, ca. 10 to 12 weeks), LCL-D2 compared to LCL-WT (donor 2, ca. 8 to 10 weeks), and LCL16-D2 compared to LCL16-WT (donor 3, ca. 4 to 6 weeks postinfection). These LCLs represent both long-term established cultures, as well as shorter-term cultures. By quantitative reverse transcription-PCR (RT-PCR) analysis, IL1R1 and TYW3 transcript levels inversely correlated with the presence of the BHRF1-2 miRNAs and were upregulated between 1.5- to 2-fold in all three BHRF1-2 KO LCLs (Fig. 3C). We also measured the levels of three other cytokine receptor genes—IL2RG (contains one miR-BHRF1-2-5p binding site; Fig. 2) (29), CD27 (member of the TNF-α superfamily; no PAR-CLIP BHRF1-2 miRNA target sites), and IL18R1 (another member of the IL-1 receptor family that is reportedly upregulated by EBNA2 [43]; no PAR-CLIP BHRF1-2 miRNA target sites)—but did not observe any reproducible differences in expression patterns, as detected for IL1R1 and TYW3 (Fig. 3C).

To determine whether changes in IL1R1 transcript levels were reflected at the protein level, we then performed Western blot analysis of lysates from LCLBACWT and LCLBACD2 (donor 1). In two separate experiments, IL1R1 protein levels were upregulated in LCLs lacking the BHRF1-2 miRNAs compared to wild-type control cells (Fig. 3D). These results are consistent with the increase in IL1R1 RNA (Fig. 3C) and likely explain the observed increase in response to IL-1β when BHRF1-2 miRNAs are absent (Fig. 1H).

The IL-1 receptor is regulated at both the RNA and the protein level by BHRF1-2 miRNAs. To demonstrate that IL1R1 and TYW3 were indeed regulated at the RNA level,

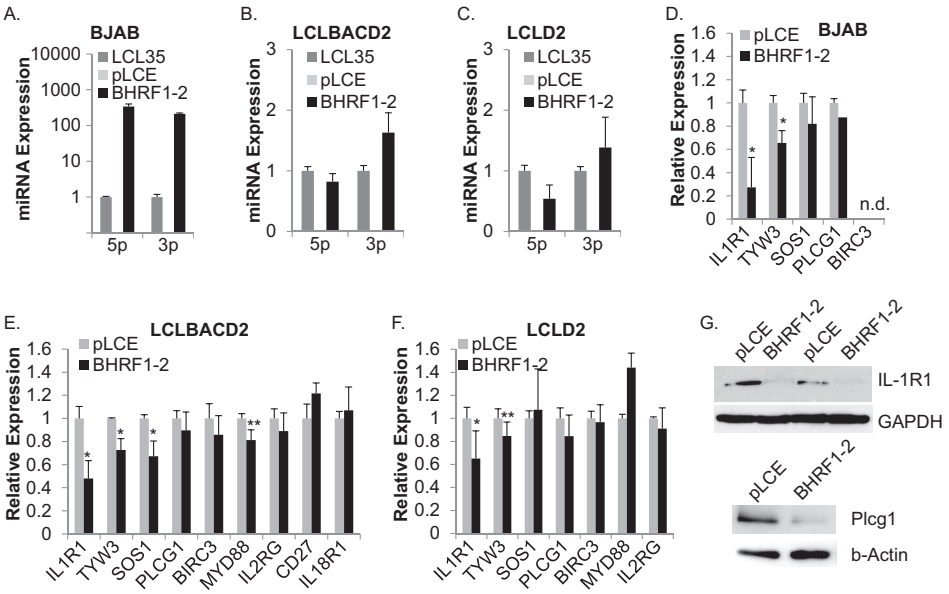

FIG 4 Ectopic BHRF1-2 miRNA expression reduces IL1R1 levels. (A) qRT-PCR analysis of BHRF1-2 miRNA expression levels in BJAB cells stably transduced with pLCE (control vector) or pLCE-BHRF1-2 ($n = 2$ in duplicate). (B and C) qRT-PCR analysis of BHRF1-2 miRNA expression levels in BHRF1-2 miRNA KO LCLs (LCLBACD2 or LCLD2) stably transduced with pLCE (control vector) or pLCE-BHRF1-2 ($n = 3$ in duplicate for LCLBACD2; $n = 4$ in duplicate for LCLD2). For panels A to C, miR-BHRF1-2-5p and miR-BHRF1-2-3p expression values are normalized to snoRNA U6 or miR-16 and reported relative to levels in EBV B95-8 LCL35. (D to F) qRT-PCR analysis of IL1R1 transcript levels and other cellular genes in BJAB cells and BHRF1-2 KO LCLs (LCLBACD2 and LCL-D2) ectopically expressing BHRF1-2 miRNAs. Values are normalized to GAPDH and reported relative to control cells (pLCE). Average expression values and standard deviations were calculated from three to five independent experiments. *, $P < 0.005$; **, $P < 0.05$ (Student t test). (G) Western blot analysis of IL1R1 or PLCγ1 (Plcg1) in transduced BJAB cells. GAPDH or β-actin levels are shown as loading controls.

we stably expressed the BHRF1-2 miRNAs in EBV-negative BJAB cells. To examine levels in the context of latent EBV infection, we also introduced the miRNAs into BHRF1-2 miRNA KO LCLs. miRNA qRT-PCR analysis confirmed that both miR-BHRF1-2-5p and miR-BHRF1-2-3p were expressed at high levels in BJAB cells (Fig. 4A) and restored to near-physiological levels (relative to LCL35, a wild-type EBV B95-8 LCL) in the two mutant LCLs (Fig. 4B and C). In BJAB cells, stable BHRF1-2 miRNA expression significantly reduced the levels of both IL1R1 and TYW3 transcripts but not other validated BHRF1-2 miRNA targets (Fig. 4D). Notably, significant decreases in IL1R1 and TYW3 levels also occurred in the transduced LCLs, confirming that these targets are responsive at the RNA level in EBV-infected cells (Fig. 4E and F). Although both mutant LCLs exhibited reduced IL1R1 and TYW3 levels upon introduction of the BHRF1-2 miRNAs, greater knockdown was observed in LCLBACD2, which is likely due to the higher levels of BHRF1-2 miRNAs in these cells (Fig. 4B). Of note, SOS1 was also significantly diminished in LCLBACD2 expressing BHRF1-2 miRNAs, suggesting that this transcript may also be regulated at the RNA level (Fig. 4E).

We next examined IL1R1 and PLCγ1 protein levels in BJAB cells. Compared to control cells, stable expression of the BHRF1-2 miRNAs in BJAB cells substantially reduced expression of the IL1R1 to nearly undetectable levels (Fig. 4G). These data are consistent with the potent downregulation of IL1R1 transcript levels upon BHRF1-2 miRNA expression (Fig. 4D) and suggest that BHRF1-2 miRNAs may induce cleavage and/or degradation of the IL1R1 transcript during EBV infection. PLCγ1 levels were also reduced in BJAB cells upon ectopic expression of the BHRF1-2 miRNAs (Fig. 4G), confirming PLCG1 as a target.

BHRF1-2 miRNA activities impact steady-state cytokine expression. IL1R1 (also called CD121a) belongs to the IL-1 family of cell surface cytokine receptors that bind prototypical IL-1 inflammatory cytokines (IL-1α and IL-1β) to initiate a signaling cascade

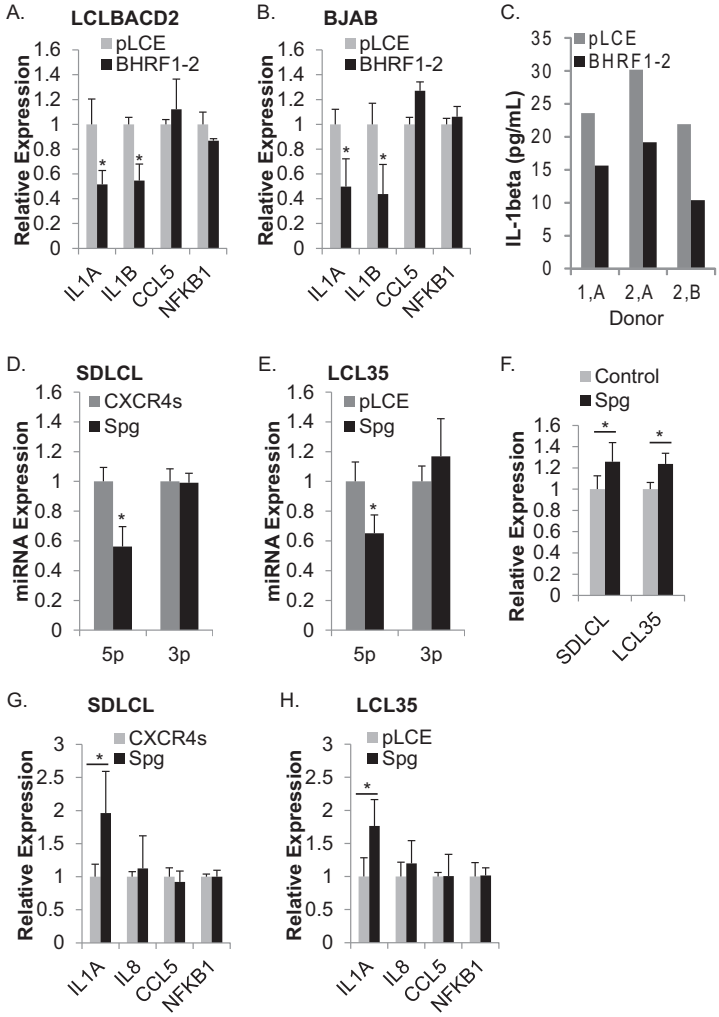

FIG 5 BHRF1-2 miRNA activity regulates steady-state IL-1 expression. (A and B) Ectopic BHRF1-2 miRNA expression dampens cytokine expression. Total RNA was harvested from BHRF1-2 KO LCLs (LCLBACD2) (A) or EBV-negative BJAB cells (B) transduced with pLCE or pLCE-BHRF1-2. NF-κB-responsive cytokine genes were measured by TaqMan qRT-PCR. Values are normalized to GAPDH as an internal control and reported relative to cells transduced with pLCE control vector. Shown are the averages of four independent experiments for LCLBACD2 and three independent experiments for BJAB, performed in duplicate. *, $P < 0.005$ (Student *t* test). (C) Secreted IL-1β is reduced in the presence of BHRF1-2 miRNAs. BHRF1-2 KO LCLs transduced with pLCE or pLCE-BHRF1-2 were plated at 10^6 cells/ml, and cell culture supernatants were harvested after 24 h. IL-1β levels were measured by ELISA. Donor 1, LCLBACD2; donor 2, LCLD2. (D and E) Sponge inhibition of miR-BHRF1-2-5p increases cytokine expression. BHRF1-2 miRNA expression was analyzed by qRT-PCR in two EBV B95-8 wild-type LCLs (SDLCL and LCL35) transduced with the miR-BHRF1-2-5p sponge (Spg). 5p and 3p indicate miR-BHRF1-2-5p and miR-BHRF1-2-3p. Values were normalized to U6 and are reported relative to miRNA expression levels in cells transduced with control vector (CXCR4s or pLCE). (F to H) qRT-PCR analysis of IL1R1 and cytokine transcripts in miR-BHRF1-2-5p sponged LCLs. Values were normalized to GAPDH and are reported relative to cells transduced with control vector (pLCE-CXCR4s or pLCE). For panels D to H, reported are the averages and the SD of at least three independent experiments ($n = 4$ for IL1A) performed in duplicate. *, $P < 0.005$ (Student *t* test).

that results in the activation of inflammatory responses, including NF-κB-mediated transcription of nascent IL1A and IL1B (37). Since EBV BHRF1-2 miRNAs regulate multiple targets in the interleukin signaling pathway, in addition to IL1R1 (Fig. 2), we hypothesized that proinflammatory cytokine expression might be regulated through these viral miRNAs. To test this, we initially monitored steady-state levels of IL-1 cytokines. Basal IL1A and IL1B transcript levels were reduced ~2-fold in LCLBACD2 or BJAB cells expressing the BHRF1-2 miRNAs, whereas other NF-κB-responsive transcripts (RANTES [CCL5] and NFKB1) were unaffected (Fig. 5A and B).

To determine whether BHRF1-2 miRNA-dependent changes in basal IL-1 transcript levels were reflected at the protein level, we performed enzyme-linked immunosorbent assays (ELISAs) to quantify secreted IL-1β in LCL culture supernatants. Constitutive inflammasome activation and caspase 1-mediated cleavage of pro-IL-1β to mature, active IL-1β has been observed in latently infected B cells (44). Previous reports also indicate wild-type LCLs secrete very low or near undetectable levels of IL-1 cytokines and that these cytokines may even be encapsulated within exosomes (44). Interestingly, using ELISA we were able to detect low levels of secreted IL-1β from unstimulated cells lacking the BHRF1-2 miRNAs (Fig. 5C). Ectopic expression of the BHRF1-2 miRNAs in either LCLBACD2 (donor 1) or LCL-D2 (donor 2) reproducibly reduced secreted IL-1β levels by ~50% compared to control vector (Fig. 5C), consistent with observed miRNA-dependent changes at the RNA level.

Based on RNA hybrid predictions (45), the ~2-kbp IL1R1 3′ UTR contains two strong seed match sites (≥7mer1A) for miR-BHRF1-2-5p and a single site (nt 2 to 7 match) for miR-BHRF1-2-3p. LCL PAR-CLIP studies captured a single binding site for miR-BHRF1-2-5p, but no other EBV miRNAs, in the IL1R1 3′ UTR (29, 38), and mutation of this site alleviated luciferase knockdown (Fig. 2), suggesting that miR-BHRF1-2-5p is the major player in regulating IL1R1. Both the 5p and 3p miRNAs are expressed from the BHRF1-2 miRNA expression vector, which confounds observed phenotypes. Thus, to test whether the 5p miRNA was specifically responsible for changes in cytokine levels, we inhibited activity of endogenous miR-BHRF1-2-5p in two wild-type EBV B95-8 LCLs using a green fluorescent protein (GFP)-expressing lentiviral vector-based miRNA sponge (29, 46, 47). This sponge contains nine tandem imperfect binding sites for miR-BHRF1-2-5p embedded in the GFP 3′ UTR; high GFP expression is an indicator of increased miRNA silencing. LCLs were sorted using fluorescence-activated cell sorting for high GFP expression following stable transduction, and total RNA was collected after 8 to 10 days of recovery in culture to analyze miRNA knockdown by qRT-PCR. The levels of miR-BHRF1-2-5p decreased at least 50% in both sponged LCLs compared to LCLs transduced with a control vector, confirming sponge activity (Fig. 5D and E). As expected, IL1R1 RNA levels were upregulated upon inhibition of miR-BHRF1-2-5p (Fig. 5F). We measured the basal cytokine and chemokine levels in these cells, testing IL1A, as well as IL-8, a chemokine reported to be responsive to IL-1β stimulation (37). Importantly, compared to control LCLs, LCLs inhibited for miR-BHRF1-2-5p activity consistently expressed ~2-fold more IL1A RNA (Fig. 5G and H), supporting a role for this miRNA in the regulation of proinflammatory cytokine expression.

Silencing of IL1R1 phenocopies miR-BHRF1-2-5p activity. We next sought to determine whether perturbation by RNA interference (RNAi) of any of the individual validated targets could phenocopy the effects of miR-BHRF1-2-5p. miR-30-based shRNAs were generated against IL1R1, SOS1, PLCG1, and BIRC3 and introduced into HEK293T cells. Knockdown of each transcript was confirmed by qRT-PCR (Fig. 6A). Notably, inhibition of IL1R1, but not other transcripts, significantly decreased steady-state IL1A levels (Fig. 6B). IL1B was not detectable in unstimulated cells (not shown). We then measured NF-κB activation in response to IL-1β, testing the IL1R1 shRNA alongside the BHRF1-2 miRNA expression vector. Both pLCE-BHRF1-2 and pLmCherry-shIL1R1 fully blocked NF-κB reporter activity (Fig. 6C), confirming that IL1R1 is the major miR-BHRF1-2-5p target involved in the disruption of IL-1 signaling.

miR-BHRF1-2-5p regulates IL-1 responsiveness during EBV infection. Finally, to validate the functional consequences of miR-BHRF1-2-5p silencing in the context of EBV infection, we stimulated miR-BHRF1-2-5p-sponged or control LCLs with IL-1β and examined IL-1-responsive gene expression patterns by qRT-PCR (Fig. 7A and B). Compared to mock-treated cells, IL-1β treatment induced the expression of multiple inflammatory cytokines, including IL-1A, IL-1B, IL-6, and IL-8. As anticipated, inhibition of miR-BHRF1-2-5p activity significantly amplified the response to IL-1β stimulation, evidenced by the fact that levels of IL-1A, IL-1B, and IL-6 were at least 2-fold above the induction levels observed in control sponge cells (Fig. 7A and B). Although IL-1A, IL-1B,

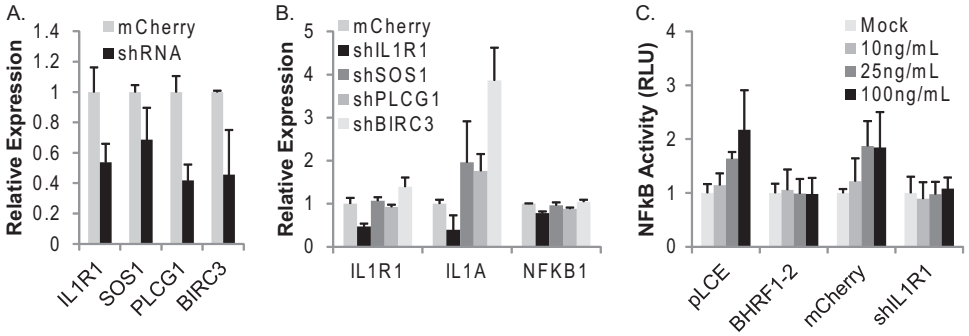

FIG 6 Inhibition of IL1R1 phenocopies miR-BHRF1-2-5p activity. (A) 293T cells were transduced with pLmCherry-shRNAs, and total RNA was harvested at 72 h. Knockdown of individual target genes was assayed by qRT-PCR analysis. (B) Steady-state IL1A transcripts, assayed by qRT-PCR, are reduced by the IL1R1 shRNA in 293T cells. For panels A and B, the expression levels were normalized to GAPDH and are reported relative to control (mCherry) cells. Reported is the average and the SD of two independent experiments performed in duplicate. (C) 293T cells were transfected with 20 ng of NF-κB luciferase reporter, 20 ng of *Renilla* expression vector, and either 250 ng of control vector (pLCE or pLmCherry) or miRNA or shIL1R1 vector as indicated. At 44 h posttransfection, the cells were stimulated with increasing amounts of IL-1β for 4 h. Lysates were harvested and assayed for dual luciferase activity. Reported is the average and the SD of two independent experiments performed in triplicate.

and IL-6 were affected, inhibition of miR-BHRF1-2-5p did not significantly alter the upregulation of IL-8; this result could be due to a number of confounding factors, including the A/U-rich element (ARE) in the IL-8 3′ UTR that contributes to its stability (48). IL-6 has been previously suggested to be targeted by EBV miRNAs (specifically, miR-BART6-3p which is not present in EBV B95-8 LCLs [25]). It is important to note that none of the cytokine mRNAs assayed contain predicted miR-BHRF1-2-5p binding sites,

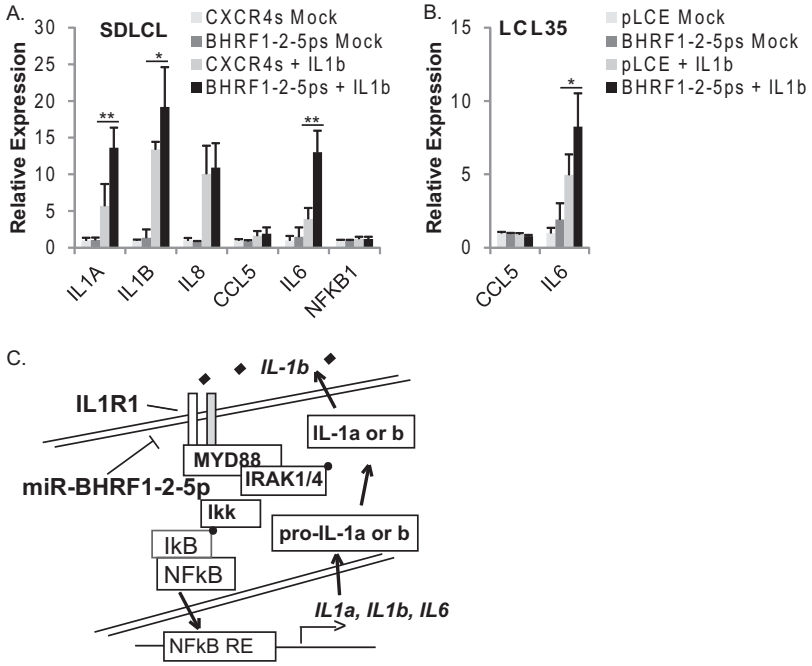

FIG 7 miR-BHRF1-2-5p regulates IL-1β responsiveness during latent EBV infection. (A and B) SDLCL or LCL35 cells stably transduced with either control vector (pLCE-CXCR4s or pLCE) or miR-BHRF1-2-5p sponge inhibitor (BHRF1-2-5ps) were plated in 12-well plates and treated for 18 h with IL-1β. Total RNA was harvested and assayed using qRT-PCR for expression of cytokine and chemokine transcripts. Shown are the averages of three independent experiments; PCR was performed in duplicate. *, $P < 0.05$; **, $P < 0.005$ (Student *t* test). (C) Schematic model depicting EBV miR-BHRF1-2-5p regulation of IL-1 signaling that occurs primarily through inhibition of the IL-1 receptor. By silencing IL1R1, the viral miRNA disrupts a positive loop that stimulates inflammatory cytokine expression in both infected and neighboring cells through autocrine and paracrine signals.

and thus the observed effects arise indirectly from suppression of miR-BHRF1-2-5p activity. Taken together, these experiments unequivocally demonstrate that miR-BHRF1-2-5p controls IL-1β signaling during latent EBV infection in B cells through direct silencing of IL1R1. This leads us to a model in which EBV miRNA activity disrupts the autocrine and/or paracrine signaling events mediated through the IL-1 receptor and consequently changes the cytokine milieu in the cellular environment (Fig. 7C).

DISCUSSION

In the present study, we examined the role of EBV miRNAs in proinflammatory cytokine signaling, and show that EBV BHRF1-2 miRNAs block the IL-1 pathway, including NF-κB activation and IL-1-responsive gene expression. To query the BHRF1-2 miRNA targets involved in this phenotype, we performed bioinformatics and pathway analyses of published PAR-CLIP data sets and noted an overrepresentation of host targets related to interleukin signaling (Fig. 2). Most notably, IL1R1, encoding the major IL-1 receptor, is a direct target of miR-BHRF1-2-5p and is potently downregulated at both the RNA and protein levels in the presence of the viral miRNA (Fig. 4). Inversely, IL1R1 transcripts and protein are upregulated in latently infected cells lacking miR-BHRF1-2-5p activity (Fig. 3 and 5), underlining the importance of IL-1 receptor inhibition during EBV infection.

IL-1 cytokines, produced by monocytes, macrophages, and epithelial, endothelial, and other cell types in response to injury, are required for lymphocyte activation and play important roles in recruiting innate immune cells to elicit inflammatory reactions (reviewed in reference 37). *In vivo*, IL-1β, IL-18, and IL-12 can function together to trigger IFN-γ production, thus activating T cells, and driving Th1 and Th17 responses. It is worth noting that while this work was in preparation, Tagawa et al. showed that multiple EBV miRNAs, including the 3p BHRF1-2 miRNA, can target the 3′ UTR of IL12B and reduce $CD4^+$ T cell responses *in vitro* (22). IL-1α and IL-1β bind ubiquitously expressed IL-1R1 to induce multiple NF-κB and MAPK target genes, including expression of nascent IL-1A and IL-1B which are processed and signal in autocrine or paracrine fashions, thereby generating a positive-feedback loop that amplifies the response (49, 50) (Fig. 7). Using a transcriptional readout, we found that miR-BHRF1-2-5p activity alters the steady-state levels of IL-1A and IL-1B cytokines in EBV-infected cell populations, and when EBV-infected cells are challenged with exogenous IL-1β, the activity of this miRNA disrupts induction of additional cytokines (IL-1A, IL-1B, and IL-6) that have potential to act on neighboring cells (Fig. 6 and 7). Thus, miR-BHRF1-2-5p activity blocks amplification of the IL-1 signal.

In healthy individuals, the IL-1 signaling loop is normally countered by expression of antagonists such as the IL-1 receptor antagonist (IL-1Ra) or decoy receptors such as IL-1 receptor type 2 (IL1R2), which can terminate the effects (37, 49, 51). A combination of IL-1β and IL-6 gives rise to immunosuppressive regulatory B cells that produce anti-inflammatory IL-10, thus contributing to self-limiting reactions and guarding against chronic or excessive inflammation (52). Deficiencies in IL-1Ra have been observed in autoinflammatory diseases and genetic mutations, leading to imbalances in both activators (IL-1β) and antagonists, and have also been implicated in malignant transformation and tumorigenesis (51, 53, 54). In bacterial infection models, the lack of appropriate IL-1β expression leads to improper immune surveillance (55). Thus, disruption of IL-1 signaling by viral miRNAs may influence not only antiviral responses but may inadvertently contribute to virus-associated oncogenesis through multiple mechanisms.

Viruses utilize multiple strategies to manipulate cytokine expression and evade immune responses during infection. Virus manipulation of IL-1 signaling is common, and several DNA viruses encode gene products that target this pathway. Poxviruses produce soluble viral proteins (vIL-18BP) that bind to and sequester extracellular IL-18, preventing interactions with IL-1 family surface receptors (56). KSHV encodes miRNAs that downregulate IL-1/TLR signaling adaptor molecules, MYD88 and IRAK1, thereby dampening signals transmitted through IL-1 receptors (32). We examined MYD88 as a

potential target of miR-BHRF1-2-3p, but have ruled this out since the 3′-UTR reporter was unresponsive, and no changes were observed at the RNA level (Fig. 3) or protein level by Western blotting (not shown) upon BHRF1-2 miRNA expression. Of note, EBV infection induces cellular miRNAs, such as miR-146a and miR-155, which target several TLR-signaling adaptor molecules (57–59). miR-155 also targets the ETS family member SPI1 and CCAAT/enhancer binding protein β (C/EBPβ) (59, 60); both of these transcription factors recognize binding site motifs in the IL1B promoter, which also contains recognition motifs for NF-κB, AP-1, HIF-1a, and others. Hijacking of cellular miRNAs thus provides EBV with yet another mode for influencing IL-1-mediated signal transduction.

After IL1B transcription, pro-IL-1β accumulates in the cytosol until cleavage by inflammasome-associated caspase 1 into mature, biologically active IL-1β (50, 61). Viruses also target this part of the loop, altering secreted IL-1 levels. Human papillomavirus 16 E6 protein, for example, attenuates IL-1β by enhancing proteasome-dependent degradation of pro-IL-1β (62). For EBV, a BART miRNA, miR-BART15-3p, targets a component of the inflammasome (NLRP3), which could lead to dampened cytokine processing, including IL-1β maturation (24). Confounding this observation, in several different EBV-infected cells, the inflammasome has been reported to be constitutively active and compared to uninfected Ramos cells, latency III LCLs generate more cleaved IL-1β (44). Inflammasome activity is also altered by other viral proteins, including those expressed by influenza and baculoviruses, which leads to changes in IL-1β and IL-18 during infection (reviewed in reference 63). By ELISA, we did observe reduced supernatant levels of IL-1β when BHRF1-2 miRNAs were active (Fig. 5). However, since the IL-1B transcript levels were similarly reduced, and none of the 231 BHRF1-2 miRNA targets have functions attributed to inflammasome activity, it is unlikely that the BHRF1-2 miRNAs alter posttranscriptional IL-1β processing steps.

In addition to IL1R1, in this work, we provide experimental evidence that the EBV BHRF1-2 miRNAs target other host factors (BIRC3, PLCG1, and SOS1) which play ancillary roles in interleukin signaling. BIRC3 (baculoviral IAP repeat-containing protein 3) encodes the E3 ubiquitin ligase cIAP2 (cellular inhibitor of apoptosis protein 2) and can be induced by IL-1β. During TNF receptor signaling, this ligase is recruited by TRAF2 to ubiquitinate TAB proteins (64). The presence or absence of BHRF1-2 miRNAs did not obviously alter TNF-α-mediated IkBα degradation or NF-κB activation (although LCLs were not highly responsive to TNF-α treatment) (Fig. 1). Likely, BIRC3/miR-BHRF1-2-5p phenotypes are masked by other potent effects of the miRNA (such as through the other 230 targets of the BHRF1-2 miRNAs, Fig. 2). SOS1 (SOS Ras/Rac guanine nucleotide exchange factor 1) binds the signaling adaptor molecule Grb2 to promote signal internalization and activation of the GTPase Ras (65). Cross talk between cytokine priming and SOS1-mediated signal transduction has been observed in T cells (66). PLCG1, a target of miR-BHRF1-2-3p, encodes phospholipase Cγ1 which, among many things, participates in IL-1 induced Ca^{2+} signaling and MAPK activation (67). Using individual shRNAs against IL1R1, SOS1, BIRC3, or PLCG1, we determined that inhibition of IL1R1, specifically, could phenocopy BHRF1-2 miRNA activity by dampening steady-state IL1A levels and disrupting IL-1-mediated NF-κB activation (Fig. 6).

A scan of other PAR-CLIP'ed targets related to IL-1 signaling identified several candidates with 3′-UTR interaction sites for EBV miRNAs. These include CHUK (miR-BART15), TOLLIP (multiple BART miRNAs), SKP1 (miR-BART5), and the PELI1 3′ UTR, which was previously confirmed to interact with miR-BART2-5p (29, 30, 38). Notably, these components are not specific to IL-1 signaling and cross talk with other cytokine receptor and TLR pathways that lead to activation of NF-κB, MAPK, AP1, and other transcription factors, predicting that EBV miRNAs collectively suppress many extracellular inflammatory signals.

Dissecting functionally relevant miRNA interactions is a key part of understanding how EBV coopts host cells to facilitate lifelong infection. Our experiments show that in latently infected B cells, miR-BHRF1-2-5p actively inhibits IL-1 cytokine signaling through downregulation of the IL-1 receptor. We hypothesize that this activity not only prevents the production of new cytokines through autocrine and paracrine signaling

loops that can alert effector cells to sites of infection but also provides an additional survival advantage by dampening excessive signaling that could be detrimental to persistence of the infected cell. A challenge will be to ascertain when these BHRF1-2 miRNA interactions are most critical: during acute infection or at later stages. Future experiments, such as the use of CRISPR technology to inactivate individual miRNA binding sites or examination of miRNA activity *in vivo* in the presence of immune effector cells, should unveil additional information about the relevance of IL-1 regulation in specific aspects of the EBV life cycle.

MATERIALS AND METHODS

Cell culture, virus, and infections. Established LCLs and EBV-negative BJAB cells were maintained at 37°C in a 5% CO_2-humidified atmosphere in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and 1% penicillin, streptomycin, and L-glutamine (P/S/G). LCLBACWT and LCLBACD2 have been previously described (29, 40). Additional BHRF1-2 miRNA KO and wild-type control LCLs were generated as previously described (18). All human primary B lymphocytes used in this study were isolated from anonymous whole blood purchased from Research Blood Components. Briefly, 10^6 primary B cells were infected with EBV-BAC derived virus (multiplicity of infection = 10 Raji-GFP units) at 37°C in RPMI 1640 supplemented with 20% FBS, P/S/G, and 400 ng/ml cyclosporine. The medium was changed at 24 h postinfection, and half of the medium was replaced every 3 to 4 days until LCLs formed (~3 weeks postinfection) and were expanded. HEK293 EBV-BAC producer cells, a gift from the DeLecluse lab (German Cancer Research Center), were maintained in 100 μg/ml hygromycin B (Gibco) in high-glucose Dulbecco modified Eagle medium (DMEM) supplemented with 10% FBS and 1% P/S/G. To generate virus, HEK293 EBV-BAC producer cells were transfected with EBV BZLF1 and EBV gp110 expression plasmids as previously described (18), and the supernatant was harvested 48 to 96 h posttransfection.

HEK293T-IκBα cells, HEK293T-NF-κBLuc cells (38), and HEK293T cells were maintained in high-glucose DMEM supplemented with 10% FBS and 1% P/S/G. To prepare lentiviruses, HEK293T cells were plated in 15-cm plates in complete medium and transfected using polyethylenimine with 7.5 μg of pL-based lentivector, 5 μg of pDeltaR8.75, and 2.5 μg of pMD2G. The medium was changed to complete RPMI 1640 at between 8 and 16 h posttransfection. Lentiviral particles were harvested by sterile filtration of the supernatant using a 0.45-μm-pore size filter at 48 and 96 h posttransfection and used to transduce ca. 1×10^6 to 5×10^6 cells. LCLs were transduced with two or three rounds of lentivirus and monitored by fluorescence microscopy for transduction efficiency (ca. 50 to 65%) by GFP expression.

Plasmids. EBV miRNA expression vectors in pcDNA3.1 and pLCE were as previously described (29, 38, 68). To generate 3′-UTR luciferase reporters, 3′ UTRs were PCR amplified from LCL genomic DNA and cloned into the XhoI and NotI sites downstream of *Renilla* luciferase in the psiCheck2 dual luciferase reporter vector containing an expanded multiple cloning site (36). The psiCheck2-MYD88 3′-UTR reporter was a gift from J. Nelson's lab (VGTI; Oregon Health and Science University). When achievable, the entire 3′ UTR was cloned for a given target. For longer 3′ UTRs, a minimum of 1 kb containing the region predicted to be targeted by each miRNA was cloned. Mutant 3′-UTR reporters containing nucleotide changes in miRNA seed match sites as identified by PAR-CLIP were generated by Phusion *Taq* site-directed mutagenesis as described previously (69). The pL-CMV-eGFP-based miRNA sponge inhibitor for miR-BHRF1-2-5p was designed as previously described (29, 46, 47) and contains nine imperfect target sites for the miRNA in the GFP 3′ UTR; the control sponge (pLCE-CXCR4s) was as previously described (29, 46, 47). The miR-30-based shRNA constructs were designed and cloned into the XhoI/EcoRI sites of pL-CMV-mCherry vector using IL1R1.4634, BIRC3.4400, PLCG1.1980, and SOS1.1532 oligonucleotides as described earlier (70). All plasmids were confirmed by restriction enzyme digestion and sequencing. Oligonucleotide sequences are provided upon request.

Western blot analysis. Cells were lysed in NP-40 lysis buffer (50 mM HEPES [pH 7.5], 150 mM KCl, 2 mM EDTA, 1 mM NaF, 0.5% [vol/vol] NP-40, 0.5 mM dithiothreitol), and protein concentrations were determined using a BCA protein assay kit (Thermo Scientific). Then, 20 μg of total protein per lane was resolved on 10% Tris-glycine SDS-PAGE gels and transferred onto Immobilon polyvinylidene difluoride membranes. After blocking in 5% milk in Tris-buffered saline/Tween, blots were probed with primary antibodies to IL1R1/CD121a (PA5-28834; Thermo Fisher Scientific), PLCγ1 (2822; Cell Signaling Technology), β-actin (clone C4; sc-47778; Santa Cruz), or GAPDH (ab8245; Abcam) then probed with horseradish peroxidase-conjugated secondary antibodies (anti-rabbit IgG or anti-mouse IgG). Blots were developed using enhanced chemiluminescent substrate (Pierce) and exposed to film.

Luciferase reporter assays. NF-κB activity was assayed using either stable cell lines (HEK293T-NF-κBLuc or BJAB-NF-κBLuc cells), HEK293T cells, LCLs transiently transfected with pNF-kB-Fluc, or LCLs transduced with pL-NF-κB-Fluc and pL-RSV-renilla as an internal control. IκBα stability was assayed using HEK293T-IκBα-photinus luciferase cells as previously described (38, 39). HEK293T reporter cells were transfected with the indicated amounts of plasmid DNA using Lipofectamine2000 (Thermo Fisher) according to the manufacturer's instructions. LCLs (3×10^6 cells) were transfected with 3 μg of total plasmid DNA using an Amaxa Nucleofector II device (Lonza; program A-023) in 100 μl of Ingenio electroporation solution (Mirus). To activate the NF-κB or IκBα reporters, the cells were treated with the indicated amounts of recombinant human IL-1β (Thermo Fisher) for 4 to 6 h or 18 h as indicated prior to lysis. For 3′-UTR reporter assays, HEK293T cells plated in 96-well black-well plates were cotransfected with 20 ng of 3′-UTR reporter and 250 ng of control vector (pcDNA3.1) or with miRNA expression vector (pcDNA3.1-BHRF1-2) using Lipofectamine 2000 (Thermo Fisher) according to the manufacturer's instruc-

tions. At 48 to 72 h posttransfection, the cells were lysed in 1× passive lysis buffer (Promega). Lysates were assayed for luciferase activity using a dual luciferase reporter assay system (Promega) and a 96-well plate luminometer with dual injectors. All values are reported as relative light units (RLU) relative to firefly luciferase internal control and normalized to the empty vector.

Quantitative RT-PCR analysis. Total RNA was extracted using TRIzol (Thermo Fisher), DNase treated, and reversed transcribed using MultiScribe (Thermo Fisher) with random hexamers. Cellular genes were detected using SYBR green qPCR and oligonucleotides (available upon request) designed to amplify gene specific regions of ~200 bp or using TaqMan qPCR and the following assays: Hs02758991_g1 (GAPDH), Hs00174092_m1 (IL1A), Hs01555410_m1 (IL1B), Hs00985639_m1 (IL-6), Hs00174103_m1 (IL-8), Hs00174575_m1 (CCL5), Hs00355671_g1 (NFKB1), Hs01573837_g1 (MYD88), and Hs00991002_m1 (IL1R1). BHRF1-2 miRNAs were detected using TaqMan-based miRNA stem-loop qRT-PCR as described previously (36). miRNA levels are reported relative to U6 (assay 001973; Thermo Fisher). All PCRs were performed in technical replicates (duplicates or triplicates).

IL-1β ELISA. Transduced LCLs were plated at 10^6 cells/ml in fresh medium; supernatants were harvested 24 h later, filtered using 0.45-μm-pore size filter, and stored at −80°C. Secreted IL-1β was quantified by human IL-1β enzyme-linked immunosorbent assay (ELISA) kit (sensitivity range, 2 to 150 pg/ml; eBioscience) using 100 μl of supernatant, and standard curves were generated according to the manufacturer's instructions.

RNA-Seq and PAR-CLIP data sets. The paired-end, RNA-seq data sets for LCLBACWT (SRX273677) and LCLBACD2 (SRX273680), read mapping by Bowtie to the human genome (HG19) (71), and RSEM (RNA-Seq by Expectation Maximization) analyses (72) on the gene level to estimate read counts were completed as previously described (40). PAR-CLIP data sets for EBV BHRF1-2 miRNA-expressing LCLs (EF3DAGO2, LCL35, LCLBACWT, LCLBACD1, LCLBACD3, and Akata-LCLd3), rhesus macaque rLCV-infected LCLs, and EBV BHRF1-2 miRNA-negative B cells (BC3, BC1, and LCLBACD2) were as previously described (29, 30, 38, 40). Raw fastq files were preprocessed as previously described to remove any 5′ or 3′ adapter sequences and eliminate low-quality reads (29, 30). Reads ≥13 nt in length were aligned concurrently to the human genome (HG19) and either the EBV B95-8 genome (V01555.2) or the wild-type EBV1 genome (NC_007605.1) using Bowtie (71). Mapped reads were analyzed by PARalyzer as previously described (73) to define miRNA target interactions. Two rLCV LCL PAR-CLIP data sets (38) were merged and mapped to rheMac3 prior to PARalyzer analysis; homology to human was determined on the gene level and miRNA target interactions for the BHRF1-2 miRNA homologs, rLCV miR-rL1-2-5p and −3p, were extracted.

Statistical analyses. All data are reported as means with the standard deviations (SD). For PCR experiments, values are derived from at least three independent experiments with technical replicates. Statistical significance was determined by using a paired Student *t* test with Microsoft Excel 2010, and *P* values of <0.05 were considered significant.

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