Regulation of MCL1, TSC1, and Cyclin D1 by Epstein-Barr Virus (EBV) Latent Membrane Protein 1 (LMP1)

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Abstract

Epstein-Barr Virus (EBV) is latent in 90% of the adult human population but can be deadly to those who are immunocompromised, such as in post-transplant proliferative disorder (PTLD), a potentially fatal complication to organ transplants. A complete understanding of the viral mechanisms of EBV and how it can lead to B cell lymphomas is necessary for the development of therapies. Here, we study how variation in the primary oncogene of EBV, latent membrane protein (LMP)1, can affect the expression of cellular proteins. LMP1 is a mimic of the B cell co-stimulatory membrane protein CD40 required for B cell differentiation and activation. Different genetic variants of LMP1 differentially regulate microRNA-193b (miR-193b), a post-transcriptional regulator known to target three target genes implicated in tumorigenesis: *MCL1* (MCL1), *TSC1* (TSC1), and *CCND1* (Cyclin D1). Here we study whether LMP1 regulates the expression of miR-193b targets MCL1, TSC1, and Cyclin D1 in B cells. To answer this question, we used a chimerically inducible model of LMP1 signaling using two LMP1 variants: B95.8 lab variant and a variant isolated from a patient with EBV+ PTLD (tumor variant). Previously, we showed that the B95.8 LMP1 but not tumor variant LMP1 regulates the expression of microRNA-193b (miR-193b). We hypothesized that after B95.8 LMP1 activation, expression of miR-193b target proteins would be decreased, but after activation of tumor variant LMP1, miR-193b target protein expression would not change. First, we used flow cytometry to analyze whether a NGFR.LMP1 model of LMP1 signaling was expressed and functional in EBV- BL41 B cells. Additionally, we determined the optimal conditions to detect MCL1, TSC1, and Cyclin D1 by Western blot. We were unable to detect Cyclin D1 by Western blot and were thus unable to include it in further analysis. After activation of LMP1 by crosslinking of NGRF.LMP1, we analyzed the expression of MCL1 and TSC1 with Western blots and quantified expressing using densitometry analysis. We found that after LMP1 activation, B cells transfected with B95.8 LMP1 decreased expression of TSC1 and significantly decreased expression of MCL1, but B cells transfected tumor variant LMP1 did not have altered expression of MCL1 or TSC1. These results indicate that genetic variation in EBV LMP1 leads to differential regulation of miR-193b target proteins MCL1 and TSC1.

Introduction

EBV Cause B Cell Malignancies

EBV, also known as human herpesvirus 4, is a γ-herpesvirus which is latently present in over 90% of the global adult population. EBV can be transmitted through saliva, and viral entry often occurs through oropharyngeal epithelia, followed by infection of naive B cells and persistence in memory B cells[1,2]. EBV is the etiological agent of infectious mononucleosis in adolescents and young adults, but in most carriers, it persists as a benign, lifelong infection. In some individuals, however, it has the potential to cause severe complications such as encephalitis, multiple sclerosis, Guillen-Barre Syndrome, and EBV associated cancer[3–7].

EBV is now associated with several B, T, NK, and epithelial cancers[8]. EBV was identified in Burkitt's Lymphoma in 1964 and became the first virus to be associated with cancer[9]. In 2010, EBV related cancers were estimated to cause 1.8% of all cancer deaths worldwide, which increased 14.6% between 1990 and 2010[10]. In people who are immunocompromised, EBV can cause lymphoproliferative diseases such as post-transplant proliferative disorder (PTLD), which can arise in people on immunosuppressive drugs after organ transplants[11]**.** As many as 20% of individuals receiving organ transplants can develop PTLD, depending on organ type[12]; prevalence of PTLD is particularly high in EBV- patients who receive EBV+ organ transplants[13]. Under conditions of immunosuppression, T cells are suppressed, which reduces modulation by T cells on B cell proliferation, creating an imbalance between

immunosuppression and immunosurveillance that would otherwise keep an EBV infection under control[13].

LMP1 is an Oncoprotein of EBV

Each of EBV's proteins and RNAs play a crucial role in EBV's ability to live in the host for their lifetime. EBV is a dsDNA virus with a 172kb genome encoding 80 proteins, 46 small untranslated RNAs[14] and 44 microRNAs (miRNAs)[15]. Nine EBV proteins are encoded by oncogenes, genes that have the potential to cause cancer: six nuclear antigens (EBNA) and three LMPs[16]. EBV infected cells are characterized by different latency patterns, 0, I, II, or III, and the lytic cycle which are defined by different patterns of expression of latent or lytic viral genes[17] (Figure 1). Latency 0 has no viral protein expression and exclusively involves expression of the non-coding RNAs – EBV encoded small-RNAs (EBERs) and BamHI A rightward transcripts (BARTs)[8,18,19]. The latency I pattern is defined by the expression of EBNA, EBERs, and BART microRNAs[19,20]. Type II latency is distinguished by the expression of EBNA1, LMP1, and LMP2[19]. Latency III is associated with lymphomas in immunodeficient individuals, including PTLD, lymphoblastoid cell lines (LCLs), and AIDs related lymphomas[21]. Latency III is characterized by the expression of EBV encoded proteins and several non-coding RNAs such as EBNA 1, 2, 3A, 3B, 3C, LP, LMP1, 2a, 2B, BART, and EBERs[22].

The LMPs integral membrane proteins co-opt host cell signaling[14]. LMP1 is the primary oncogene of EBV and a mimic of the cellular CD40[23], a co-stimulatory molecule on B cells, that when bound to its ligand CD40L on T cells, leads to differentiation and activation of B cells[24]. LMP1 is composed of a cytoplasmic amino-terminus, 6 transmembrane domains, and a carboxyterminal signaling domain located in the cytoplasm. LMP1 molecules cluster via their transmembrane domains and are thus constitutively active[14]. LMP1 signaling is initiated by the binding of its C terminus to signaling molecules such as Tumor necrosis factor receptor–associated factors (TRAFs) 1, 2, 3, 5, and Janus Kinase 3 (JAK3)[25,26]. LMP1 is critical for B cell transformation and signal transduction via the mitogen-activated protein kinase (MAPK), nuclear

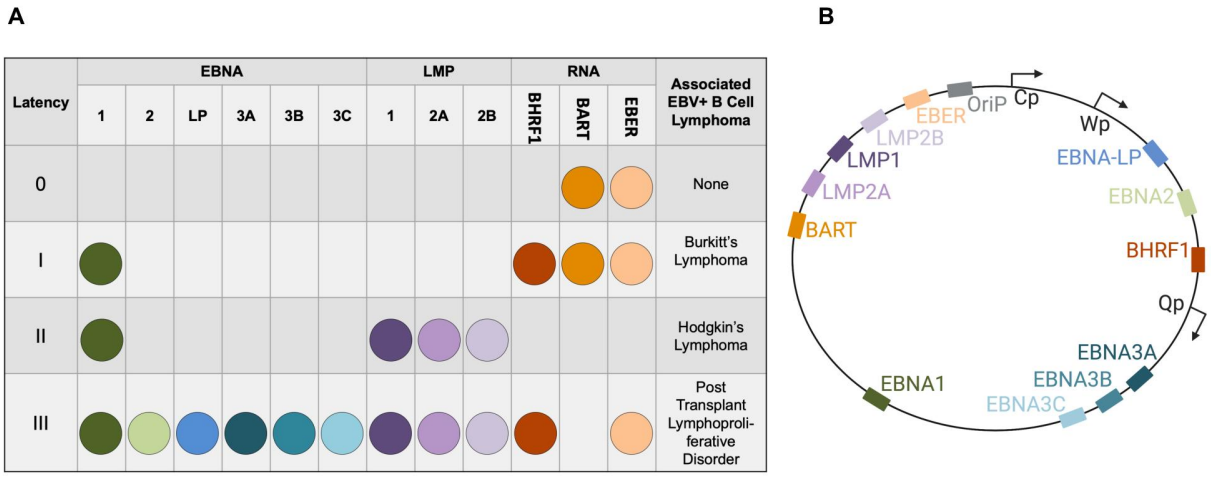

Figure 1: Patterns of EBV Gene Expression in EBV+ B Cell Lymphomas. (a) Expression of EBV nuclear antigens (EBNAs), latent membrane proteins (LMPs), BamHI-A region rightward transcript (BART) microRNAs (miRs), BamHI fragment H rightward open reading frame 1 (BHRF1), which exists as both a miR and a protein, and EBV-encoded small RNAs (EBERs) in the four stages of EBV latency. (b) Map of the EBV latency genes, miRs, EBERs, promoters (Cp, Wp, and Qp), and the origin of replication (OriP) in the EBV genome. Modified from Price and Lufgit, 2015[68]. Created using BioRender.com.

factor kappa-light-chain-enhancer of activated B cells (NF-kB), phosphoinositide-3-kinase–protein kinase B (PI3-K)/AKT, and interferon regulatory factor 7 (IRF7) pathways[26]. LMP1 exists in a lab strain (B95.8) form originally derived from a patient with infectious mononucleosis[27] and naturally occurring tumor variant forms[28,29]. A number of EBV+ lymphoma specific EBV variants have been identified[30–32]. EBV was isolated from patients with EBV+ lymphomas including PTLD, nasopharyngeal carcinoma, and Burkitt's lymphoma. LMP1 sequence variation compared to B95.8 LMP1 revealed mutations, deletions, and amino acid repeats in the carboxyl terminus of LMP1[28,29]. Notably, these variants function differently from lab-strain LMP1. Compared to the lab-strain, LMP1 variants were shown to increase motility, PI3K-Akt signaling, and upregulate markers of cell cycle progression in B and epithelial cells[31]. Much of the research on EBV has been done using B95.8 LMP1, so the effects of genetic diversity in EBV strains on B cells has not been well studied, however, recent studies suggests that natural variation in EBV proteins can affect its carcinogenicity[33].

EBV miRs Are Also Implicated in Tumorigenesis

LMP1 can regulate the expression of miRs[34–36], and different strains of LMP1 can differentially regulate those miRs[34]. miRs are short RNAs, around 22 nucleotides in length, that post-transcriptionally regulate gene expression. miR is transcribed from DNA and post-transcriptionally processed so it can leave the nucleus. Once in the cytoplasm, miRs associate with an RNA induced silencing complex (RISC) protein and acts as a guide RNA. This guide RNA can bind to complementary messenger RNA (mRNA), often but not exclusively in the 3' untranslated region, and induces transcriptional silencing by altering the EIF4F complex, which leads to the m7G cap of the mRNA being removed by the de-capping complex via recruitment of poly(A)-deadenylases. Decapped mRNA will then undergo degradation by exonucleases[37]. Since their discovery[38], miRs have been implicated in many biological processes, such as transplant tolerance[39], cellular metabolism[40], apoptotic regulation[41], and proliferation[42]. EBV not only encodes for 44 viral miR[15], but it is also capable of regulating host cell miRs.

miR-193b and its Targets MCL1, TSC1, and Cyclin D1

microRNA-193b-3p (miR-193b) is regulated by LMP1[34]. miR-193b is important in cell proliferation, metastasis, invasion, migration[43]. Additionally, miR-193b has been implicated in tumorigenesis of liposarcoma[44]. Three confirmed targets of miR-193b are *MCL1*[45], *TSC1*[46], and *CCND1*[47]. MCL1, the protein product of *MCL1*, is a BCL2 family protein. BCL2 family proteins are comprised of pro-survival and pro-apoptotic proteins. These proteins are important for activation of the intrinsic pathway of apoptosis, one of the 3 apoptotic pathways. Imbalances in these pro-apoptotic and pro-survival proteins can lead to cell death[48]. MCL1 is an antiapoptotic member of the BCL2 family that quickly degrades in response to stress stimuli. MCL1 is essential for cell survival and is dysregulated in many cancers[49]. TSC1/hamartin, the product of *TSC1*, forms a complex with TSC2/tuberin which initiates its GTPase activity to activate the G protein Ras homologue enriched in brain (Rheb), an activator of the mTOR signaling pathway[50,51]. The mTOR pathway can detect availability of nutrients, hypoxia, and stimulation by growth factors, allowing it to regulate cell cycle progression, cell growth, nutrient uptake, transcription, and translation[50]. Cyclins and cyclin dependent kinases drive cell division and proliferation by regulating the G_1/S-phase transition[52]. Cyclin D1, the protein product of *CCND1*, is one of these cyclin proteins and is one of the most dysregulated proteins found in cancers[53]. Cyclin D1 moves between the nucleus and the cytoplasm and effects a number of cell functions including DNA damage response, senescence, chromosome duplication and stability, autophagy, metabolism, migration, mitochondrial respiration, and immune surveillance[53,54]. These three proteins are all implicated in oncogenesis due to their ability to allow for unregulated growth and survival when dysregulated.

Characterization of LMP1 Regulation of miR-193b Targets

Recently, the Hatton lab has demonstrated that LMP1 differentially regulates miR-193b expression in cells expressing B95.8 and tumor variant LMP1 (Figure 2). LMP1 activation leads to an 8 fold increase in miR-193b expression while tumor variant B cell lines have almost no increase in miR-193b expression[34]. In this study, we use a chimerically inducible LMP1 molecule – NGFR.LMP1 – stably transfected in EBV- B cell lines to analyze whether activation of LMP1 regulates the expression of three miR-193b target proteins: MCL1, TSC1, and Cyclin D1 (Figure 3). We hypothesized that when LMP1 is activated, expression of miR-193b targets MCL1, TSC1, and Cyclin D1 would be downregulated in cells expressing B95.8 LMP1 but not those expressing tumor variant LMP1 because complementary targets of miRs get degraded.

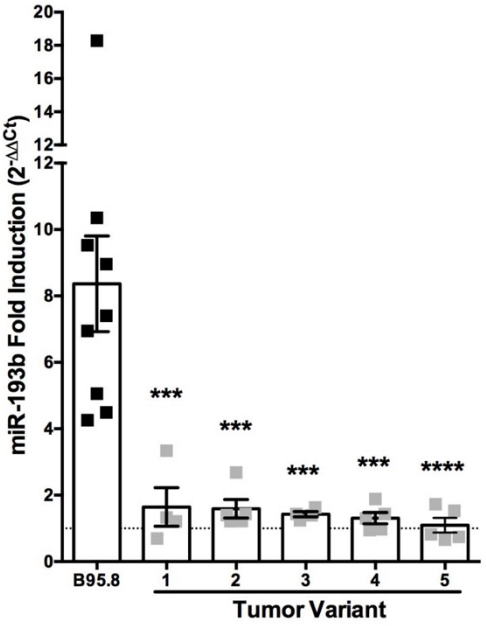

Figure 2. B95.8 cells differentially regulate miR-193b expression than Tumor Variant B Cells. Preliminary data from Hatton et al 2019[34] of miR-193b fold increase after NGFR.LMP1 crosslinking signaling in different B cell lines expressing LMP1 variants from the B95.8 lab strain of EBV or LMP1 variants isolated form patients with EBV+ PTLD. All samples were normalized to an endogenous control. We compare B cell lines expressing NGFR.LMP1 constructs contain either B95.8 LMP1 or Tumor Variant #2. ***$p \leq 0.001$ by one-way ANOVA comparing to miR-193b expression in cells transfected with B95.8 NGFR.LMP1 to cells transfected with tumor variant NGFR.LMP1.

Methods

Cell lines

Burkitt's Lymphoma cells lines (BL41) that do not express endogenous LMP1 but do express nerve growth factor receptor (NGFR).LMP1 were the parental line for cell lines expressing B95.8 or tumor variant NGFR.LMP1[34]. The tumor variant NGFR.LMP1 construct was obtained from AB5 Burkitt's Lymphoma cell line. Cell lines were cultured in complete RPMI (RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum and 50 units/ml penicillin/streptomycin; ThermoFisher Scientific, Waltham, MA, USA) and kept at 37°C with 5% CO_2. 700 µg/ml geneticin (Sigma- Aldrich, St. Louis, MO, USA) was added to the growth media for cells to select for cells containing the NGFR.LMP1 expression vector.

Cross-linking of NGFR.LMP1 to activate LMP1 Signaling

Cells were spun at 1250 rpm for 5 minutes at room temperature, decanted, and were resuspended to 1 x10^6 cells/mL using cRPMI. LMP1 signaling was activated by adding unconjugated mouse anti-NGFR 0.5 µg/10^6 cells, (BioLegend, San Diego, CA, USA) and incubating the sample for 30 min at room temperature. Goat anti-mouse $F(ab)_2$ 2 µg/10^6 cells, (Jackson ImmunoResearch, West Grove, PA, USA) was then added, and samples were incubated at 37°C and 5% CO_2 overnight.

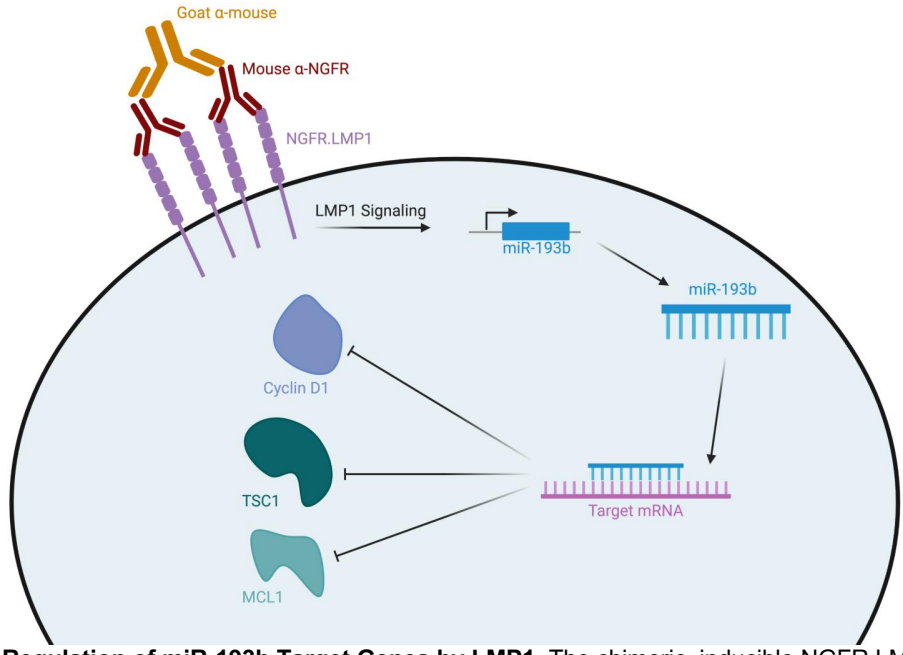

Figure 3. Model for Regulation of miR-193b Target Genes by LMP1. The chimeric, inducible NGFR.LMP1 transgene is used to activate signaling indistinguishable from endogenous viral LMP1. Crosslinking NGFR.LMP1 with the indicated antibodies activates the signaling which upregulates miR-193b expression. miR-193b targets mRNA encoding for Cyclin D1, TSC1, and MCL1 proteins by forming a complex with the RNA induced silencing complex (RISC) and targeting complementary mRNA for degradation RISC. Created using BioRender.com

Staining for NGFR and ICAM and Flow Cytometry Analysis

After cross-linking, aliquots of cells expressing B95.8 or tumor variant were spun at 1250 rpm for 5 minutes at room temperature and decanted. To wash cells, cold FACS buffer (PBS, 1% BSA, 0.02% sodium azide) was added, and samples were spun at 1250 rpm for 5 min at room temperature and decanted. 5.6 mg whole molecule Chrompure Mouse IgG (Jackson Cat # 015-000-003) was added to crosslinked samples, and cells were incubated on ice for 20 minutes. Cells were washed with cold FACS buffer as previously indicated. 0.5 µg isotype staining antibody (Biolegend Cat#400114, Clone MOPC-21), 0.5 µg NGFR staining antibody (BioLegend Cat#345106, Clone ME20.4), or 0.5 µg ICAM staining antibody (Biolegend Cat#353106, Clone HA58) were added samples. Samples were incubated in the dark on ice for 30 min. Cells were washed with cold FACS buffer as previously indicated. Samples were resuspended in 250 µL of FACS buffer then assayed using Millipore Guava easyCyte5 and analyzed using InCyte.

Lysate Generation and Pierce 660 nm Protein Assay

3 x10^6 cells were acquired, crosslinked as described above, and spun at 1250 rpm for 5 minutes then supernatant was aspirated. The cell pellet was washed with 1mM sodium orthovanadate (OV) (New England BioLabs, Ipswitch, MA, USA) in phosphate buffered saline (PBS) then spun at 1250 rpm for 5 minutes. Cells were lysed with Phospholysis Buffer stock (PLB) (50 mM of 0.5M Tris pH 7.4, 1% of 10% NP-40, 0.5% of 5% DOC, 150 mM of 5M NaCl, and 0.5 mM of 0.5M EDTA) supplemented with 1X Halt Phosphatase & Protease Inhibitor (ThermoFisher Scientific) and 1mM OV. After vortexing, samples were incubated for 30 minutes on ice and vortexed after 15 minutes of incubation. Samples were spun for 15 min at 13000 rpm in cold centrifuge. A 1:2 dilution of the lysate was made with PLB

plus inhibitors. Lysate concentration was quantified using Pierce 660 nm protein assay kit (ThermoFisher Scientific), per manufacturer's instructions, using a NanoDrop ONE[55].

Western Blotting

Cells were crosslinked, and lysates were generated as described above. After equalizing concentrations, samples were prepared in 1X Lamelli Smaple Buffer (BioRad, Hercules, CA, USA) and stored at −20°C until SDS-PAGE. Samples were thawed, quick spun, then denatured in dry bath for 5 minutes at 95°C. 30 µg of sample were loaded and run on an 4-20% tris-glycine gel in 1X tris buffered saline with tween (TBST) at 80 volts for 30 min then 120 volts until the dye front reaches the end of the gel. Samples were then transferred to a 0.2 µm nitrocellulose membrane by running in 1X TBST for 60 minutes at 20 volts at room temperature.

Membranes were washed distilled water. Stained with a 1:10 dilution of Red Alert to visualize proteins on membrane, then rinsed using distilled water. Membranes were blocked in 5% milk/ 1X TBST while rocking at room temperature for 50-60 minutes. Membranes were then probed in a 1:1000 or 1:250 solution of primary antibody in 5% milk/ 1X TBST. The primary antibodies used were from Cell Signaling Technology (Danvers, MA, USA): MCL1 (#4572), Cyclin D1 (#55506), TSC1 (#6935), and HRP conjugated β-actin (#12620). For detection of HRP conjugated β-actin, HPR conjugated secondary antibodies (#7076) were used. Samples were kept at 40°C until needed.

Blots were visualized using a ThermoFisher iBright FL1000 Imaging System. Densitometry analysis was performed using ImageJ normalizing to actin. Statistical significance was quantified using paired, two tailed t-test.

Results

B95.8 and Tumor Variant B cells expression functional NGFR.LMP1

The BL41 B cells expressing NGFR.LMP1, where LMP1 sequences are derived from B95.8 or tumor variant sequences, but not endogenous LMP1 provides a chimerically inducible LMP1 system that was used in this study. Self-aggregation of endogenous LMP1 leads to chimeric activation, thus it's expression cannot be controlled; this model system allows us to control activation of LMP1 signaling. The cross-linking of NGFR.LMP1 by mouse α-NGFR and goat α-mouse antibodies allows for activation of LMP1 signaling that is indistinguishable from wild-type LMP1 signaling[56] (Figure 3). To confirm that the BL41 cell lines stably transfected with expression vectors for B95.8 NGFR.LMP1 or tumor variant NGFR.LMP1 expressed B95.8 or tumor variant NGFR.LMP1, we examined NGFR expression in these stably transfected BL41 cell lines by flow cytometry. In cell lines containing the B95.8 NGFR.LMP1 construct, the median fluorescence of staining with phycoerythrin (PE)-conjugated NGFR antibodies was 2393.9 relative fluorescence units (RFU) compared to PE-conjugated isotype control antibodies, negative control antibodies used to measure non-specific binding (median 5.6 RFU, Figure 4A). In cell lines containing untreated tumor variant NGFR.LMP1, the median fluorescence of staining with was 17.5 RFU compared to 3686.3 RFU for untreated tumor variant NGFR.LMP1 and 9.9 for crosslinked tumor variant NGFR.LMP1 (Figure 4C). Overall, these data indicate that the B95.8 and tumor variant cell lines express NGFR.LMP1.

To confirm the chimerically inducible model of LMP1 is functional in B95.8 and tumor variant cell lines, we crosslinked NGFR.LMP1-expressing cells and examined ICAM (CD54) expression by flow cytometry. ICAM is a protein downstream of B95.8 and tumor variant NGFR.LMP1 and is known to be upregulated by LMP1[57]. As expected, cross-linking of B95.8 NGFR.LMP1

increased ICAM expression 5-fold (Figure 4B). Similarly, cross-linking of tumor variant NGFR.LMP1 increased ICAM expression 3-fold (Figure 4D). These data indicate that LMP1 signaling is functional by B95.8 and tumor variant NGFR.LMP1.

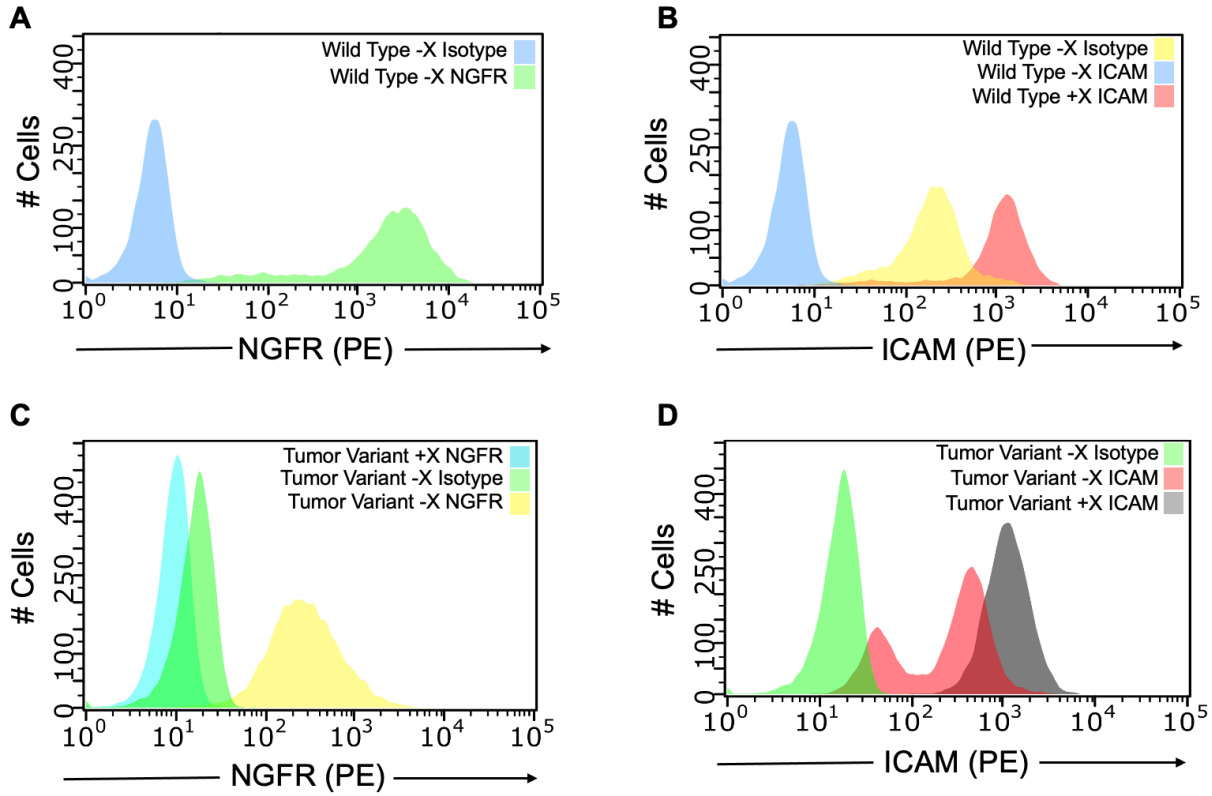

Figure 4. BL41 cell lines express functional B95.8 or tumor variant NGFR.LMP1. BL41 cells expressing either B95.8 or tumor variant NGFR.LMP1 were acquired. PE-conjugated mouse α-NGFR and goat α-mouse antibodies were used as depicted in Figure 3 to crosslink NGFR.LMP1 and activate LMP1 signaling (+X). Samples were assayed by flow cytometry using Millipore Guava easyCyte5 and analyzed using InCyte for expression of (**A**, **C**) NGFR, to indicate NGFR.LMP1 expression or (**B**, **D**) ICAM, to indicate functionality of NGFR.LMP1 to activate downstream pathways.

Detection of miR-193b target proteins MCL1, TSC1, and Cyclin D1

To use the chimerically inducible NGFR.LMP1 system to test whether LMP1 signaling regulates expression of MCL1, TSC1, and Cyclin D1, we needed to first determine optimal conditions to detect those proteins. To determine the optimal conditions for detecting miR193b target proteins MCL1, TSC1, and Cyclin D1 by Western blot, we tested a 1:250 and 1:1000 dilution of primary antibodies recognizing MCL1, TSC1, or Cyclin D1 in cells expressing B95.8 NGFR.LMP1, with actin as a loading control. TSC1 is approximately 150 kDa[58]; a band at approximately that molecular weight was detected as well as two non-specific bands at 30 kDa and 50 kDa. (Figure 5A). MCL1 is 40 kDa[59], and a band was detected at approximately that size with no non-specific binding. (Figure 5B). Cyclin D1 is 36 kDa[60]. No binding was seen when blotting for Cyclin D1, but actin was seen at 45 kDa[61], as expected, indicating successful loading of the sample (Figure 5). These data suggest that antibodies for MCL1 and TSC1 successfully detect these proteins at a 1:1000 dilution.

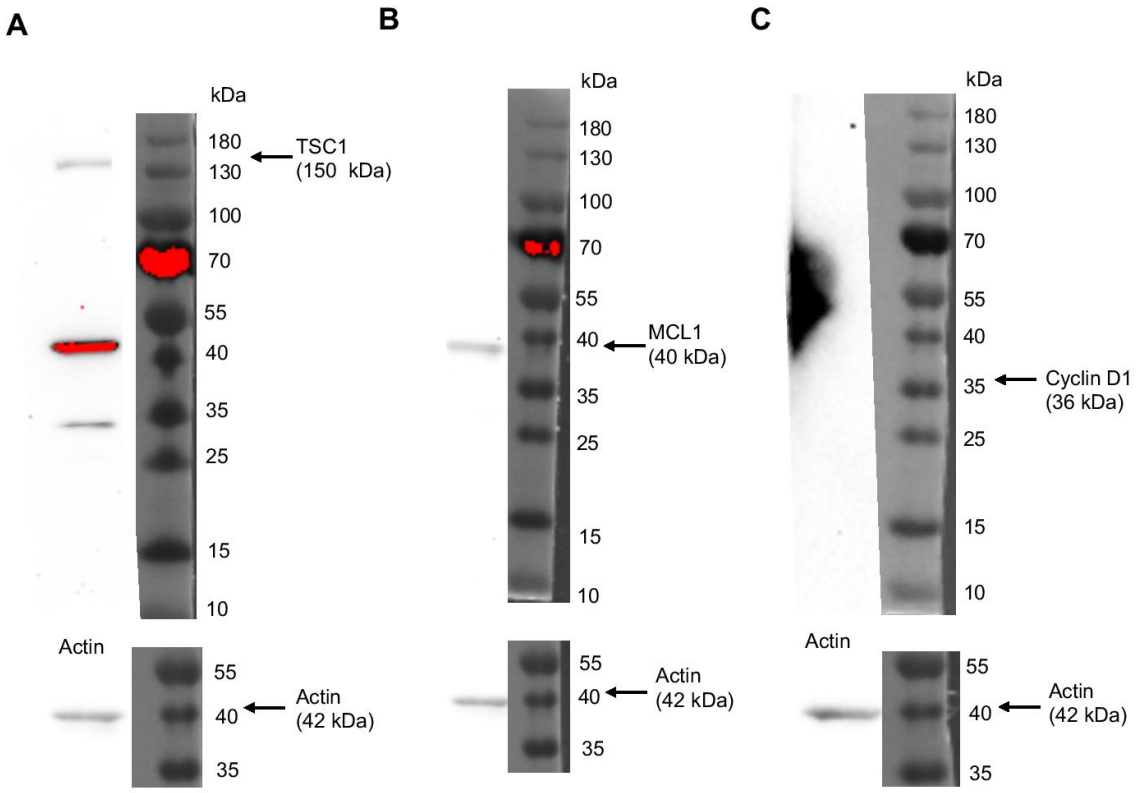

Figure 5. MCL1 and TSC1, but not Cyclin D1, can be detected by Western blot. Western blot analysis was used to detect miR-193b targets MCL1, TSC1, and Cyclin D1 in B95.8 cells. Blot was probed for actin (42 kDa) and (**A**) TSC1 (150 kDa), (**B**) MCL1 (40 kDa), or (**C**) Cyclin D1 (36 kDa) at 1:250 dilution compared to PageRuler Ladder. A 1:1000 dilution was performed, and similar results were achieved but are not shown here. Samples were visualized using the iBright Imager. Red spots on parts (**A** and **B**) are due to overexposure of the ladder or non-specific binding when exposing at a time which allowed visualizing the target protein.

LMP1 does not significantly regulate expression of TSC1 in B95.8 or tumor variant cells

B95.8, but not tumor variant, LMP1 is known to regulate miR-193b[34]. Thus, we hypothesized that miR-193b targets MCL1 and TSC1 would be downregulated after cross-linking in B cells expressing B95.8 LMP1 because miR-193b would be upregulated, but miR-193b targets would not be differentially regulated after cross-linking in cells expressing tumor variant LMP1 because miR-193b expression would stay the same. To determine whether activation of LMP1 alters expression of miR-193b target TSC1, we performed Western blots probing for TSC1 of B cells expressing B95.8 or tumor variant LMP1 with or without induction of NGFR.LMP1 cross-linking (Figure 6). Densitometry analysis by ImageJ of our n=4 repeats reveals that TSC1 expression is reduced after LMP1 cross-linking, though the results were not statistically significant ($p = 0.0571$, Figure 6A). We had hypothesized that there would be a statistically significant difference based on preliminary data from Hatton et al 2019 that LMP1 upregulates miR-193b expression in B95.8 cells[34]. Thus, these results suggest that there may be other mechanisms that regulate TSC1 in cells B95.8 NGFR.LMP1.

In tumor variant NGFR.LMP1, the densitometry analysis by ImageJ of our n=4 repeats reveals that TSC1 expression is minimally reduced after LMP1 cross-linking (Figure 6B). These are the expected results; we did not expect that miR-193b targets would be

downregulated because miR-193b expression is unchanged by tumor variant NGFR.LMP1 cross-linking[34].

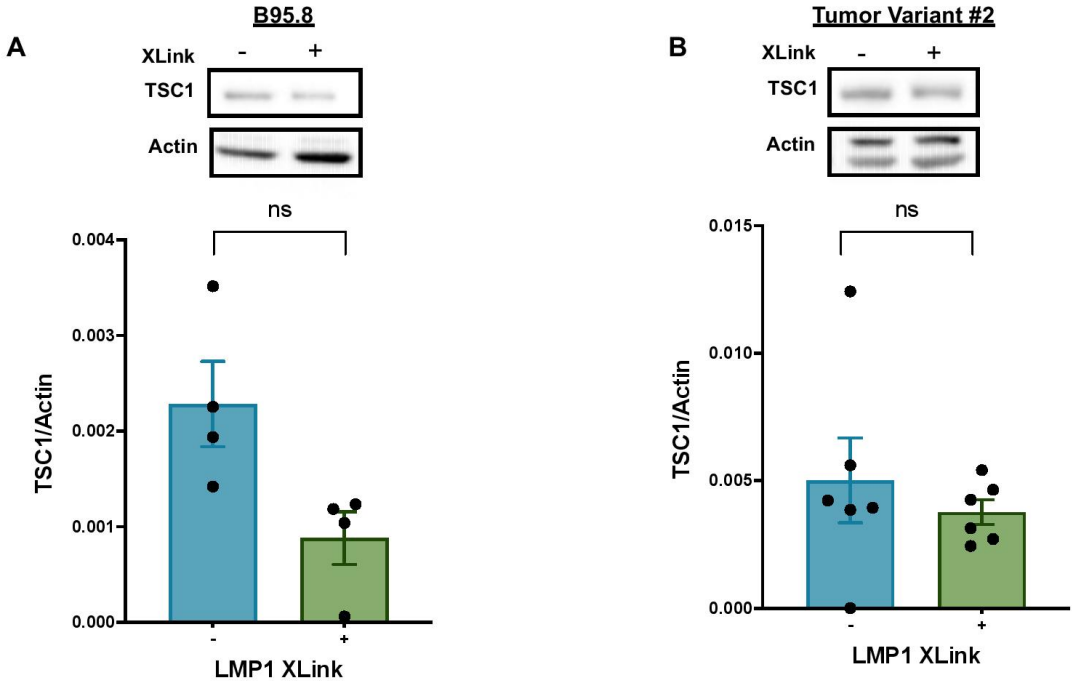

Figure 6. B95.8 LMP1 reduces the expression of miR-193b target TSC1 compared to tumor variant LMP1. 4.5 x 10^6 cells expressing (**A**) B95.8 and (**B**) tumor variant NGFR.LMP1 were treated as indicated prior to lysis. Lysates were quantified using Pierce 660 nm Protein assay, normalized to the same concentration, loaded and separated by molecular weight using SDS-PAGE, and transferred to a nitrocellulose membrane. Membranes were then probed for TSC1 or Actin as a control, using conditions identified in Figure 5, and imaged using the iBright. Blots shown are a replicate representative of n = 4 experiments. Densitometry analysis was performed using ImageJ and normalized to actin in cells where NGFR.LMP1 was (+) or was not (+) crosslinked (Xlink). Each point represents one experimental replicate. * $p<0.05$ by two tailed, paired t-test. Black dots show each replicate. Error bars represent the standard error of the mean (SEM).

LMP1 alters expression of MCL1 in B95.8 but not tumor variant cells

To determine whether activation of LMP1 alters expression of miR-193b target MCL1, we performed Western blots probing for MCL1 of B cells expressing B95.8 or tumor variant LMP1 with or without induction of NGFR.LMP1 cross-linking (Figure 7). Densitometry analysis by ImageJ of our n=4 repeats reveals that MCL1 expression is significantly reduced after LMP1 cross-linking ($p = 0.01456$, Figure 7A). These results are consistent with our hypothesis that LMP1 activation would lead to a decrease in MCL1 expression.

In tumor variant NGFR.LMP1, the densitometry analysis by ImageJ of our n=4 repeats reveals that MCL1 expression is minimally reduced after LMP1 cross-linking (Figure 7B). These are the expected results; we did not expect that miR-193b targets would be downregulated because miR-193b itself would not be upregulated.

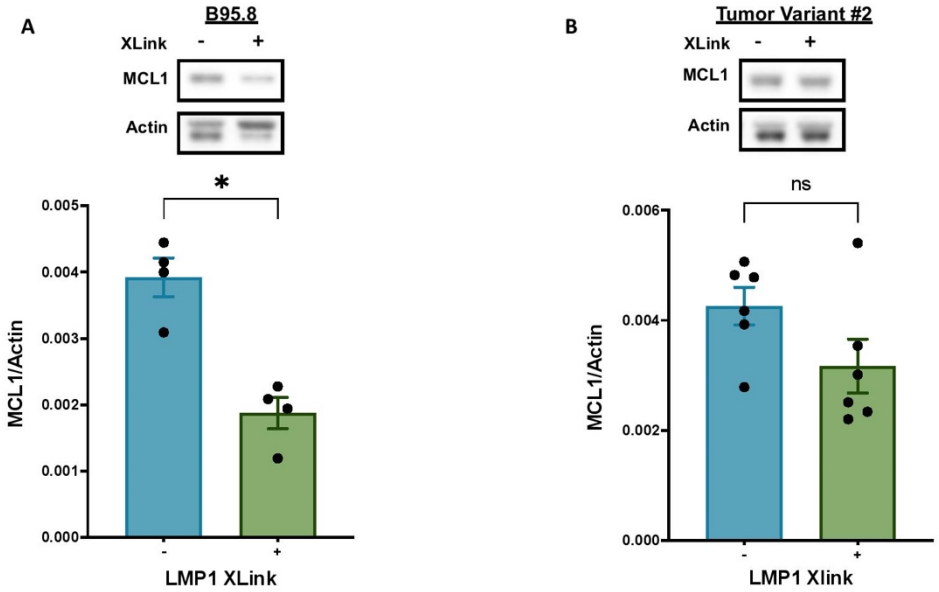

Figure 7. B95.8 LMP1 significantly regulates the expression of miR-193b target MCL1 compared to tumor variant LMP1. 4.5 x 10^6 cells expressing (**A**) B95.8 and (**B**) tumor variant NGFR.LMP1 were treated as indicated prior to lysis. Lysates were quantified using Pierce 660 nm Protein assay, normalized to the same concentration, loaded and separated by molecular weight using SDS-PAGE, and transferred to a nitrocellulose membrane. Membranes were then probed for MCL1 or Actin as a control, using conditions identified in Figure 5, and imaged using the iBright. Blots shown are a replicate representative of n = 4 experiments. Densitometry analysis was performed using ImageJ and normalized to actin in cells where NGFR.LMP1 was (+) or was not (+) crosslinked (Xlink). Each point represents one experimental replicate. * $p<0.05$ by two tailed, paired t-test. Black dots show each replicate. Error bars represent the standard error of the mean (SEM).

Discussion

B95.8 and tumor variant LMP1 differentially regulate miR-193b expression[34], so we sought to determine whether B95.8 and tumor variant LMP1 differentially regulate the protein products of miR-193b targets *MCL1*, *TSC1*, and *CCND1* using a chimeric, inducible NGFR.LMP1 constructs. We hypothesized that levels of these miR-193b target proteins would be decreased after cross-linking in BL41 cells stably expressing B95.8 NGFR.LMP1 because miR-193b would be increased; however, in BL41 cells stably expressing tumor variant NGFR.LMP1, we expected there to be no difference in target protein expression because miR-193b expression was not expected to change. Using flow cytometry, we found that B95.8 and tumor variant NGFR.LMP1 were expressed and functional in parental EBV- BL41 cell lines. We noted that less NGFR was detectible after cross-linking of tumor variant NGFR.LMP1 (Figure 4C), which we hypothesize is due to internalization of the receptor after cross-linking. These experiments allowed us to verify that we could use this model system to examine our main question of whether B95.8 and tumor variant LMP1 differentially regulate MCL1, TSC1, and Cyclin D1. We found that after cross-linking of B95.8 but not tumor variant LMP1, MCL1 expression was significantly decreased and TSC1 expression was also decreased, but not significantly.

We expected that levels of MCL1 would be decreased after cross-linking in BL41 cells with B85.9 NGFR.LMP1 but not for cells with tumor variant NGFR.LMP1. After determining optimal conditions to detect MCL1 by Western blot, we found that MCL1 levels were significantly decreased after cross-linking of B95.8 LMP1 but not tumor variant NGFR.LMP1. We plan to validate this finding using quantitative polymerase chain reaction (qPCR) to detect levels

of *MCL1* transcripts. Additionally, we plan to increase the number of replicates to n=6 to try to further assess statistical significance and confirm our original findings. Lastly, we plan to directly analyze whether LMP1 regulates MCL1 via miR-193b. We will use small interfering RNA (siRNA) to knockout miR-193b and analyze whether MCL1 levels change after cross-linking. If LMP1 regulates MCL1 via miR-193b, we expect that when miR-193b is knocked out, we would not see a significant difference in MCL1 expression after cross-linking for NGFR.LMP1.

We also plan to examine the functional consequence of differential regulation of MCL1 by B95.8 versus tumor variant LMP1. MCL1 is an antiapoptotic BCL2 family member. When levels of MCL1 are too low and there is an imbalance of pro and anti-apoptotic BCL2 family proteins, cells will undergo the intrinsic pathway of apoptosis, leading to cell death[48,49,62]. MCL1 has been noted to be upregulated by miR-193b downregulation in cancers such as in melanoma[45]. Although this is not the mechanisms of MCL1 regulation by miR-193b our data currently supports in EBV+ PTLD, ultimately having more MCL1 expressed could provide a protective role in cancer cell survival. MCL1 is an anti-apoptotic protein, so cells that do not express high enough levels of MCL1 will undergo cell death. For EBV to infect other cells, its current host cell must undergo the lytic cycle, resulting and lysis and the release of viral particles. Thus, for non-cancerous EBV infected cells expressing B95.8 LMP1, undergoing cell death leads to the release of viral particles and the infection of more cells. But, for cancer cells, avoiding cell death is important for its survival, and thus, it is not advantageous to downregulate MCL1, which would lead to cell death. We could test the importance of MCL1 on cancer cell survival by using *MCL1* knockout cells and analyzing their survival compared to cell expressing MCL1 using an assay to measure apoptosis, such as an annexin V positivity assay, which uses flow cytometry to measure annexin V bound to phosphatidylserine, a phospholipid on the outside of the cell during early stages of apoptosis. We would hypothesize that cells that do not express MCL1 would undergo apoptosis at a higher rate than those expressing MCL1.

We also analyzed whether B95.8 or tumor variant LMP1 regulates TSC1. Like MCL1, we expected that levels of TSC1 would be decreased after cross linking in BL41 cells with B95.8 NGFR.LMP1 but not for cells with tumor variant NGFR.LMP1. After determining the optimal conditions to detect TSC1 by Western blot, we found that B95.8 LMP1 decreases expression of TSC1 after cross-linking, but not significantly, and tumor variant LMP1 does not decrease TSC1 expression after cross linking. We plan to increase the number of replicates to n=6 to try to further assess statistical significance. Additionally, we plan to validate these findings using qPCR to detect mRNA levels of *TSC1* instead of protein levels. If we find that B95.8 LMP1 significantly decreases TSC1 expression compared to tumor variant LMP1, we will analyze whether LMP1 regulates TSC1 via miR-193b. We will test this using siRNA to knockout miR-193b and analyze whether levels of TSC1 change after cross-linking of NGFR.LMP1. If LMP1 regulates TSC1 via miR-193b, we expect not to see a significant difference in TSC1 expression after cross-linking of NGFR.LMP1 when miR-193b is knocked out.

Additionally, we plan to examine the functional outcome of differential regulation of TSC1 by B95.8 versus tumor variant LMP1. TSC1 forms a complex with TSC2 to regulate the mTOR signaling pathway. The mTOR pathway is important for cell cycle progression, cell growth, transcription, translation, and nutrient uptake[50]. mTOR signaling can be initiated several ways, including by AKT, DNA damage, TNF, the MAPK pathway, and via insulin. Insulin initiated mTOR signaling is negatively regulated by the TSC1/ TSC2 complex[63] by targeting of mTORC1, an autophagy inhibitor which leads to cell death[46]. Dysregulation of the mTOR pathway is linked

to tumor development and tumorigenesis due to its role in cell cycle progression and proliferation by upregulating cell growth signals, apoptosis reduction by inhibition of glycogen synthase kinase-3, an activator of caspase 3, and tumor metabolism control by regulating proliferation based on nutrient availability[64,65]. Additionally, upregulation of TSC1 by miR-193b has been linked to development of neurodegenerative diseases such as Amyotrophic Lateral Sclerosis due to its inhibition of mTOR signaling and subsequent increase in apoptosis of motor neurons[46]. Although in EBV+ PTLD we see a lack of downregulation of TSC1, the net result is sufficient TSC1 available to fuel mTORC1 inhibition. As TSC1 is a regulator of the mTOR pathway, which allows cells to proliferate, differentiate, and undergo apoptosis in times of cellular stress, it is advantageous for tumor cells to have TSC1 present to increase tumor growth and survival. But, in EBV infected non-cancerous cells that express B95.8 LMP1, the downregulation of TSC1 could benefit the survival of the viral population because cell death and lysis is required for infection of new cells. We could test the importance of TSC1 in cell survival by using *TSC1* knockout cells and analyzing whether their growth, measuring tumor size and survival using an annexin V positivity assay, is different than cells expressing TSC1. We would hypothesize that cells that do not express TSC1 would undergo apoptosis at a higher rate than those expressing TSC1.

Finally, we analyzed whether Cyclin D1 is regulated by B95.8 or tumor variant LMP1. Before testing this, we tested for the optimal conditions of detecting Cyclin D1. We were unsuccessful at detecting Cyclin D1 at 1:250 and 1:1000 dilutions; there was background, indicating a potential technical error. Without a means of detecting Cyclin D1, we could not perform further analysis on its expression. For future analysis, we will continue to test for Cyclin D1 expression using different concentrations of the antibody or use a different antibody altogether. Additionally, we could use qPCR to detect *CCND1* mRNA to quantify expression.

Cyclin D1 is a driver of cell cycle progression from G_1 to S phase as well as a regulator of cell proliferation and differentiation[53]. In gastric cancer cells with a *CCND1* knockout, the target of miR-193b, cells had reduced proliferation[66]. Additionally, miR-193b was found to be downregulated in lung cancer, leading to an upregulation in Cyclin D1. Further, when miR-193b was upregulated, decreasing Cyclin D1 expression, less invasion and migration was observed[67]. Cyclin D1 is required for proliferation and differentiation, and cancer cells are highly proliferating, making Cyclin D1 a key driver in the growth of tumors. If upregulation of miR-193b by B95.8 LMP1 leads to a decrease in expression of Cyclin D1, then cells are less able to proliferate because Cyclin D1 is required for the transition from G_1 to S phase of division. Thus, tumor cells that express tumor variant LMP1 are able to continue proliferating because they have Cyclin D1 levels that are comparable to uninfected cells. We could test Cyclin D1's importance in cancer progression by inhibiting Cyclin D1 using *CCND1* knockout cells then analyzing whether the cells are able to proliferate and differentiate by measuring tumor size. We would hypothesize that cells that express Cyclin D1 proliferate at a higher rate than those not expressing Cyclin D1.

Together, our results indicate that LMP1 genetic diversity differentially regulates miR-193b target TSC1 and significantly regulates miR-193b target MCL1. Elucidating the functional differences in natural variants of EBV LMP1 and how they affect downstream proteins and miRs can provide a necessary first step in the development novel cancer treatments for EBV+ PTLD. This further understanding of the underlying mechanisms that lead to EBV+ PTLD, and specifically how LMP1 diversity can impact expression of proteins implicated in cancer progression, such as MCL1 and TSC1, is an important step in the development of these types of treatment. Our data indicates in non-cancerous EBV infected B cells expressing B95.8 LMP1, MCL1 and TSC1 are downregulated, but this does not occur in tumor cells

expressing tumor variant LMP1. Thus, our data suggests that targeting MCL1 and TSC1 in cancer cells could provide a novel therapy as it could reduce the levels of those proteins to that of healthy B cells.

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