microRNA-155 target SHIP1 is regulated by Latent Membrane Protein 1 (LMP1) of Epstein-Barr Virus (EBV)

A Senior Thesis Presented to
The Faculty of the Department of Molecular Biology,
Colorado College

By
Carissa Sherman
Bachelor of Arts Degree in Molecular Biology

March 23rd, 2021

Dr. Olivia Hatton

Dr. Sara Hanson

Abstract

The number of deaths from cancers attributable to the Epstein-Barr Virus (EBV), such as EBV+ B cell lymphomas, is steadily increasing. Elucidation of the viral mechanisms that underlie the development of EBV+ B cell lymphomas could help to identify potential therapies. EBV is a ubiquitous herpesvirus that can alter the expression of host B cell genes, including microRNAs (miRs). miRs are post-transcription regulators that degrade mRNA or inhibit translation of target mRNA. The broad aim of this study was to elucidate how the primary oncogene of EBV – *LMP1* – regulates host B cell miRs. Our preliminary data indicate that LMP1 upregulates miR-155 via PI3K p110α. Moreover, the miR-155 target and tumor suppressor – SHIP1 – was not downregulated in EBV+ B cell lymphomas. To determine if LMP1 regulates expression of miR-155 target SHIP1 via PI3K p110α, we utilized EBV- B cell lymphoma lines that stably expressed chimeric NGFR.LMP1 molecules. NGFR.LMP1 has the extracellular transmembrane domains of nerve growth factor receptor (NGFR) and the C-terminus tail of LMP1 that contains its signaling domains. To ensure that the NGFR.LMP1 molecules were functional, we measured intracellular adhesion molecule (ICAM) and miR-155 levels after inducing LMP1 signaling by crosslinking NGFR.LMP1 molecules using mouse anti-NGFR and then goat anti-mouse antibodies. We observed a significant increase in both ICAM and miR-155, which supports that we have a functional model. We then activated LMP1 in the presence of a PI3K p110α inhibitor, BYL719, and measured expression of SHIP1 by Western blot. When LMP1 signaling is activated, SHIP1 expression significantly decreased, suggesting that LMP1 regulates SHIP1. Additionally, BYL719 treatment did not significantly rescue SHIP1 levels, which indicates that although LMP1 regulates SHIP1, LMP1 does not do so via activation of PI3K p110α. A future direction is to confirm these findings by knocking down PI3K p110α. Altogether, our data suggest LMP1 regulates expression of the miR-155 target SHIP1, but not via activation of PI3K p110α. Further study is needed to understand how SHIP1 is regulated. In sum, this mechanism suggests that miR-155 and SHIP1 may be potential therapeutic targets for EBV+ B cell lymphomas.

Introduction

Epstein-Barr Virus (EBV)

EBV is a highly infectious herpesvirus with more than 95% of the world's population seropositive (Saha & Robertson, 2011), meaning the virus is present in one's blood. EBV is transmitted primarily through saliva but can also spread through blood and organ transplants (Hoagland, 1955; Thorley-Lawson, 2015). In many cases, affected individuals are asymptomatic, because EBV remains "hidden," or rather latent, in host B cells for the duration of an individual's lifetime (Thorley-Lawson, 2015). Yet, EBV can result in infection or more serious diseases, such as various cancers.

EBV-Associated Malignancies

EBV was the first herpesvirus associated with human cancers (Epstein et al., 1964). EBV has contributed to EBV positive (EBV+) B cell lymphomas like diffuse large B cell lymphoma (DLBCL) (Li et al., 2020) and Post-Transplant Lymphoproliferative Disease (PTLD) (Martinez & Krams, 2017). 1.5% of human cancers are due to tumors resulting from EBV infection (Cao et al., 2021). Although the rate may appear low and EBV infection is normally benign, EBV remains a serious threat (Thorley-Lawson, 2015). Despite EBV appearing relatively safe, since there is no current vaccine and treatments are still in development, vulnerable individuals who are infected could be susceptible to fatal infectious mononucleosis, lymphoproliferative diseases and more (Andrei et al., 2019). Therefore, understanding the underlying viral mechanism of EBV that allows it to inhibit cell function, prevent apoptosis, and alter signaling pathways, will allow us to identify therapeutic targets and potential biomarkers to address the rising levels of EBV-associated malignancies.

Lifecycle of EBV

EBV essentially hijacks B cell biology. Broadly speaking, EBV enter via saliva and infect naïve B cells (Hoagland, 1955). EBV infection of B cells begin with the viral glycoprotein gp350 interacting with CD21, a receptor on the B cell surface (Nemerow et al., 1985; Shannon-Lowe & Rowe, 2011). Expression of EBV genes takeover the B-cell growth program to drive proliferation of transformed B cells (Andrei et al., 2019; Thorley-Lawson, 2015). Then, in the germinal center, infected B cells may differentiate. EBV can persist in memory B cells. In lytic replication, the virus is released from plasma cells to infect new, naïve B cells (Thorley-Lawson, 2015). To summarize, there are five key stages: 1) viral entry 2) infection 3) proliferation 4) differentiation and 5) persistence (Andrei et al., 2019).

Patterns of Latent Gene Expression

EBV-associated diseases and disorders can be categorized into various types based on the pattern of latent gene expression. There are four types of latency (0, I, II, III), and all are associated with expression of different EBV proteins and different malignancies (El-Sharkawy et al., 2018). In type III, a naïve B cell is initially infected, and EBV expresses all ten of its viral proteins that aid in its transformation and proliferation stages: EBV-encoded small RNAs (EBERS), EBV nuclear antigens (EBNAs), and Latent Membrane Proteins (LMPs) (Cameron et al., 2009; Godshalk et al., 2008). Type II occurs at the differentiation stage mentioned earlier, and EBERs, EBNA1, and LMP1 and LMP2 are expressed (El-Sharkaway et al., 2018). Type I has EBERs and EBNA1. EBNA1 enables the viral genome to divide with the cellular genome (Andrei et al., 2019). Finally, in type 0, EBV is maintained in memory B cells, allowing them to persist in the host and to go unrecognized by the immune system (Andrei et al., 2019); only EBERs are transcribed (El-Sharkawy et al., 2018).

Although EBV can shift between these various latency types, type III is the most immunogenic (El-Sharkawy et al., 2018). Type III can convert primary B cells into lymphoblastoid cell lines (LCLs) *in vitro* and lymphoproliferative diseases *in vivo* (Thorley Lawson, 2015). In other words, type III can contribute to various cancerous states via expression of EBV's viral oncoproteins, in particular the Latent Membrane Protein 1 (LMP1) (Gires et al., 1997).

Latent Membrane Protein 1 (LMP1)

As mentioned, when EBV-infected B cells are differentiating, it is characterized as type II, and when EBV-infected B cells are proliferating, it is type III (Andrei et al., 2019); LMP1 is expressed in both type II and type III (El-Sharkawy et al., 2018). LMP1 is the primary oncogene of EBV, as LMP1 is needed for B-cell transformation *in vitro* and oncogenesis *in vivo* (Cao et al., 2021; Gires et al., 1997). LMP1 is a transmembrane protein with six domains and a long C-terminal tail with two activation regions (Kay et al., 1993; Mainou et al., 2007). LMP1 acts like CD40, which triggers survival signals to B cells to prevent apoptosis, allowing the virus to persist in host memory B cells (Rastelli et al., 2008b). LMP1 has significant effects on cellular signaling pathways and growth, and thereby, has an important role in transforming B cells (Kaye et al., 1993; El-Sharkawy et al., 2018). Specifically, LMP1 activates p38 (Eliopoulos et al., 1999b), NF-κB (Huen et al., 1995), and PI3K/Akt (Lambert and Martinez, 2007) pathways via its C-terminal activating regions 1 and 2 (CTAR1 and CTAR2, respectively). CTAR1 of LMP1 regulates the PI3K/Akt pathway (Sang et al., 2019). This pathway is constitutively expressed in EBV+ B cell lymphoma lines (Sang et al., 2019). Due to the several functions of this pathway, including growth, metabolism, differentiation and even B cell survival (Nunes et al., 2019), it is unsurprising that EBV activates the PI3K/Akt pathway as a mechanism of oncogenesis (Sang et al., 2019). Altogether, LMP1 can alter host pathways as well as regulate expression of host microRNAs (miRs) as a means of tumorigenesis.

Regulation of Gene Expression by microRNAs (miRs)

miRs are post-transcription regulators that are small in length (ranging from 22 to 90 nucleotides), and they target the 3'-untranslated region of messenger RNAs (mRNAs) to either prevent translation or to degrade mRNAs (Mahesh & Biswas, 2019). Due to their importance in regulating gene expression, miRs are inherently involved in numerous cellular processes, including development, differentiation, and cell signaling (Mahesh & Biswas, 2019). They can also act as regulators of B-cell development and differentiation (Musilova & Mraz, 2015).

Aberrant expression of miRs can contribute to dysregulation of gene expression, potentially leading to various diseases, like cancers (Abd-Aziz et al., 2020; Musilova & Mraz, 2015). For instance, miR-155 was the first miR to have been shown to have an increase in expression in cancers (Eis et al., 2005). Additionally, miR-155 is the most frequently upregulated miR in numerous cancers (Linnstaedt et al., 2010; Wood et al., 2018). Increased levels of miR-155 have also been observed in EBV-associated malignancies (including B cell lymphomas), specifically during type III, but not type I latency (Linnstaedt et al., 2010; Wood et al., 2018; Yin et al., 2008).

miR-155 and regulation by LMP1

miR-155 is encoded by the miR-155 host gene, *MIR155HG*, also known as the B-cell Integration Cluster (*BIC*) gene (Eis et al., 2005). *BIC* RNA transcripts are the precursor to miR-155, and B cell receptor activation induces *BIC* promoting the encoding of miR-155 (Eis et al., 2005). Significantly, miR-155 can also be classified as an oncogenic miR (oncomiR) because it is constitutively overexpressed and it represses the translation of tumor suppressor genes to promote tumorigenesis (Abd-Aziz et al., 2020).

LMP1 – as well as other EBV viral genes – have been shown to increase levels of miR-155 to promote B cell survival (Rahadiani et al., 2008; Wood et al., 2018). This upregulation of

miR-155 is in part caused by LMP1 activating the transcription factor nuclear factor-kappa B (NF-κB) (Gatto et al., 2008), which has binding sites in the *BIC* gene. The activation of NF-κB and other signaling pathways, such as PI3K/Akt, increases the level of *BIC* RNA, which increases the levels of miR-155, and in turn, a downregulation of its targets. In addition, the CTAR1 of LMP1 activates Interferon regulatory factors (IRF), IRF4 specifically, which also induces *BIC*, upregulating miR-155 in EBV-transformed cells (Wang et al., 2011). Overall, the dysregulation of miR-155 by LMP1 contributes to lymphomagenesis.

SHIP1: A Target of miR-155

Src homology 2 (SH2) domain-containing inositol 5' phosphatase 1 (SHIP1) – encoded by inositol polyphosphate-5-phosphatase D, *INPP5D* – is a known target of miR-155 and can act as a tumor suppressor (O'Connell et al., 2009). Additionally, SHIP1 is largely and constitutively expressed in the hematopoietic system and has an important role in immune cell activation. Further, SHIP1 is a negative regulator of the PI3K/Akt pathway (O'Connell et al., 2009; Pauls & Marshal, 2017). SHIP1 dephosphorylates the PI3K product, phosphatidylinositol-3-4-5-trisphophate [$PI(3,4,5)P_3$] to produce phosphatidylinositol -3,4-bisphosphate [$PI(3,4)P_2$]. $PI(3,4)P_2$ inhibits Akt. Notably, high levels of $PI(3,4)P_2$ have been linked to tumorigenicity and has been observed in leukemia cells (Brooks et al., 2010). SHIP1 also contributes to oncogenesis because it can prevent apoptosis of transformed cells thereby enhancing survival of cancerous cells (Pauls & Marshall, 2017). Therefore, SHIP1 is a critical molecule in the PI3K/Akt pathway, and when normally regulated, SHIP1 can be a useful tumor suppressor (Pauls & Marshall, 2017).

Examining if EBV's LMP1 Regulates Expression of miR-155 target SHIP1 via PI3K p110α

Hatton et al. discovered a novel pathway for induction of miR-155 via PI3K p110α activation (2019). PI3K p110α is a catalytic isoform of PI3K. Of the various isoforms of PI3K and

their specific inhibitors, PI3K p110α and its inhibitor BYL719, most efficiently inhibited induction of miR-155 by LMP1 in B cells (Hatton et al., 2019). NF-κB and p38, and their specific inhibitors Bay11-7082 and SB203580, respectively were also able to reduce miR-155 induction by LMP1, but these pathways have been extensively studied, whereas PI3K p110α is novel, and therefore provides a prime area of study (Hatton et al., 2019).

In this study, we wanted to determine if LMP1 regulates expression of miR-155 targets via activation of PI3K p110α. In our proposed mechanism, LMP1 activates PI3K p110α, which in turn upregulates miR-155 (Fig. 1). With an upregulation of miR-155, we would expect a downregulation of its targets, FOXO3a, SHIP1, and PI3K p85α (Fig.1, Left to Right), with a specific interest on SHIP1 in this paper. Notably, previous data from Hatton et al showed that SHIP1 levels were not significantly altered in EBV+ B cell lymphomas (2019). This indicated that EBV does not regulate SHIP1 in the same way that it regulates FOXO3a or PI3K p85α. Despite this discrepancy, we predicted that LMP1 would likely downregulate SHIP1 for two reasons: first, miR-155 is highly elevated upon LMP1 induction, and second, miR-155 targets SHIP1 (O'Connell et al., 2009). When a small molecular inhibitor, BYL719 is present, it should inhibit PI3K p110α. Therefore, we would expect expression levels of miR-155 targets, including SHIP1, to be restored if the proposed mechanism is accurate.

Here, we showed that a chimeric nerve growth factor receptor (NGFR) and LMP1 chimeric molecule (NGFR.LMP1) is functional in EBV negative (EBV -) BL41 cells. Further, we showed that SHIP1 is significantly downregulated when LMP1 is activated but not via PI3K p110α. This study provides valuable insight into how LMP1 regulates host cell miRs. By elucidating part of EBV's viral mechanism, we were able to identify that miR-155 and its target SHIP1 could provide viable biomarkers and therapeutic targets.

Results

NGFR.LMP1 is a Functional Model for Viral LMP1

The six transmembrane domains of endogenous, viral LMP1 induce oligomerization of LMP1 molecules, and this clustering of LMP1 molecules enables constitutive signaling (Harris-Arnold et al., 2015; Lambert and Martinez, 2007). To model endogenous LMP1, we utilized EBV- BL41 cell lines that stably expressed chimeric NGFR.LMP1 molecules (Fig. 2). NGFR.LMP1 has the extracellular transmembrane domains of nerve growth factor receptor (NGFR) and the c-terminus tail of LMP1 that contains its signaling domains (Harris-Arnold et al., 2015; Lambert and Martinez, 2007). LMP1 signaling is activated when NGFR.LMP1 is crosslinked by mouse anti-NGFR, and then goat anti-mouse antibodies (Fig.2). Crosslinking mimics the clustering of molecules as is observed with endogenous LMP1 thereby giving us the ability to control and effectively measure LMP1 signaling. Significantly, NGFR.LMP1 is comparable to endogenous LMP1 activity (Hatton et al., 2012). This model is essential to addressing our research question: Does LMP1 regulate miR-155 target SHIP1 via activation of PI3K p110α?

We utilized EBV- BL41 cells as this allows us to control LMP1 signaling, and endogenous LMP1 is not expressed, which ensures that the LMP1 activity that we are measuring is coming from our chimeric system. Although NGFR.LMP1 has been used in other cell lines, these lines were provided by Dr. Elliot Kieff (Harvard Medical School) and allows us to address our research question. To determine functionality of chimeric NGFR.LMP1 molecules in EBV- BL41 cells (as illustrated in Fig. 2), we measured expression levels of intercellular adhesion molecule 1 (ICAM) and miR-155 via qPCR following activation by crosslinking. For NGFR.LMP1 to be functional, we would expect an increase in both ICAM (Huen et al., 1995) and miR-155 (Rahadiani et al., 2008; Wood et al., 2018) because that is observed in cells expressing endogenous viral LMP1. When NGFR.LMP1 molecules on EBV- BL41 cells were

crosslinked, there was a significant, 68.1-fold increase in ICAM expression compared to uncrosslinked cells (Fig. 3A). Additionally, there was a 7.34-fold increase in miR-155 levels when NGFR.LMP1-expressing cells were crosslinked compared to uncrosslinked cells (Fig. 3B). Because of the significant increase in both ICAM and miR-155 levels upon crosslinking, this indicates that we have an inducible, functional model of LMP1.

EBV LMP1 Reduces Expression of microRNA-155 Target, SHIP1

LMP1 has been shown to increase levels of miR-155 (Hatton et al., 2019; Rahadiani et al., 2008; Wood et al., 2018), and O'Connell et al showed that miR-155 targets SHIP1 (2009). Therefore, we reasoned that SHIP1 levels were likely to decrease upon LMP1 induction of miR-155, despite previous data showing that SHIP1 levels were not decreased in EBV+ B cell lymphomas (Hatton et al., 2019) To see if LMP1 regulates miR-155 target SHIP1, we crosslinked NGFR.LMP1 to induce LMP1 signaling and measured expression of SHIP1 and actin. SHIP1 expression levels decreased significantly by 46.1% when LMP1 was activated (in the absence of BYL719) (Fig. 4B). This suggests that LMP1 does regulate SHIP1 via miR-155.

Activation of PI3K p110α by LMP1 leads to an upregulation of miR-155 (Hatton et al., 2019). Since the data collected showed that SHIP1 is regulated by LMP1 induction, we then wanted to determine if this is through activation of PI3K p110α. To ensure that we are directly regulating miR-155 via PI3K p110α and measuring the effects on miR-155 expression, we used BYL719 (BYL), a small molecular inhibitor specific to PI3K p110α. We used BYL because BYL had significantly decreased miR-155 levels following LMP1 induction in B cells (Hatton et al., 2019). Thus, BYL can allow us to control expression of PI3K p110α. To see if LMP1 regulates SHIP1 via activation of PI3K p110α, we crosslinked NGFR.LMP1 in the presence of BYL, and again similarly examined SHIP1 and actin expression via Western blot. If LMP1 altered SHIP1 levels through activation of PI3K p110α, we would expect BYL to rescue SHIP1 expression levels. When BYL was present, the levels of SHIP1 were unchanging (Fig.4B) This indicates

that LMP1 does not regulate miR-155 target SHIP1 via activation of PI3K 110α. Collectively, our data show that LMP1 significantly reduces SHIP1 expression, but not through activation of PI3K p110α.

Discussion

This study is of relevance because the number of deaths attributable to EBV-associated malignancies is steadily increasing, and this study allows us to better understand the viral mechanisms that allow EBV to persist and initiate oncogenesis. The central aim of this study was to determine if LMP1 regulates expression of miR-155 target SHIP1 via activation of PI3K p110α. SHIP1 has an important role in regulating PI3K/Akt pathway. Dysregulation of this pathway can promote survival and growth of B cell malignancies (Brooks et al., 2010; O'Connell et al., 2009; Pauls & Marshall, 2017). Elevated levels of miR-155 lead to downregulation of SHIP1, which can contribute to prolonged Akt activation (Tili et al., 2013). In short, understanding miR-155, as well as how SHIP1 is regulated, may provide additional insight into the maintenance and development of EBV, establishing viable therapeutic targets.

How is SHIP1 regulated?

We discovered that LMP1 significantly downregulated SHIP1, but since there was not a significant rescue upon inhibition of PI3K p110α (Fig. 4), indicated that SHIP1 is not regulated by activation of PI3K p110α.This finding suggests that SHIP1 is regulated by other mechanisms. For example, since LMP1 regulates SHIP1, but not EBV itself (Hatton et al., 2019) indicates that LMP1 and other EBV latent genes may regulate SHIP1. In addition, there are other pathways that LMP1 regulates miR-155 levels by, which in turn, could regulate levels of SHIP1. Finally, there are mechanisms that are independent of miR-155 that could regulate SHIP1. Any or a combination of the aforementioned ways could regulate SHIP1.

Alternative EBV latent genes

Since EBV does not regulate SHIP1, but LMP1 does, suggests that SHIP1 is likely modulated by other latent, viral genes in EBV+ malignancies (Hatton et al., 2019). The EBV proteins in type III (EBERs, EBNAs, LMPs) provide a future area of study since they also regulate miR-155 expression (Wood et al., 2018), and miR-155 in turn regulates SHIP1. In particular, EBV Nuclear Antigen 2 (EBNA2) would be of interest. EBNA2 in conjunction with another protein (RBPJ) has been shown to activate a miR-155 enhancer (Wood et al., 2018). Notably, EBNA2 also upregulates LMP1 (Wood et al., 2018). Further examination of EBNA2 could illuminate more about the regulation of SHIP1 because EBNA2 activity leads to a direct and indirect upregulation of miR-155, which consequently downregulates SHIP1. In sum, further study into EBNA2 and other EBV proteins could reveal how SHIP1 is being regulated.

miR-155-dependent pathways

In this study, we examined LMP1 activation of PI3K p110α. However, LMP1 regulates other pathways that also modulate miR-155, which targets SHIP1. For example, we could also explore pathways that involve NF-κB, IRF4, and p38, all of which interact with miR-155, which in turn regulates SHIP1 (Fig.5).

Further study into IRF4 could be fruitful. IRF4 has been shown to lead to a decrease in SHIP1 levels in EBV- B cells (Wang et al., 2011). This is likely because IRF4 also regulates *MIR155HG*, or *BIC*, transcription, leading to an upregulation of miR-155. Intriguingly, EBNA2 can also activate transcription of IRF4 (Wood et al., 2018). This provides further support that examining EBNA2 may help us to understand how SHIP1 is regulated.

Another pathway for additional study is LMP1—NF-κB—miR-155. LMP1's CTAR2 has been shown to activate NF-κB (Huen et al., 1995). NF-κB upregulates miR-155 by inducing *BIC*, and miR-155 targets SHIP1. Interestingly, SHIP1 negatively regulates calcium signaling, which

in turn, indirectly, negatively regulates NF-κB (Pauls & Marshall, 2017). Perhaps SHIP1 is involved in a negative feedback loop with NF-κB (Fig. 5). By SHIP1 decreasing NF-κB, this would result in a general decrease in miR-155 levels, allowing for SHIP1 to increase. If NF-κB is inhibited with Bay11-7082, which has been shown to decrease miR-155 levels (Hatton et al., 2019), we would predict that that SHIP1 levels may be rescued. Therefore, one future direction could be replicating our experiment with inhibitors specific to NF-κB, such as Bay11-7082 (Hatton et al., 2019). SHIP1 levels would still be decreased upon NGFR.LMP1 crosslinking, and inhibition of NF-κB may lead to an increase in SHIP1 levels. In other words, SHIP1 could be regulated by LMP1 activating NF-κB, rather than PI3K p110α. In short, SHIP1 could be regulated by LMP1 regulating miR-155 via activation of other host signaling pathways, such as IRF4 and NF-κB and similar logic could be applied to p38.

miR-155-independent mechanisms

It is established that SHIP1 is regulated by miR-155 (O'Connell et al., 2009). In this study, we had explored SHIP1 regulation by LMP1 and miR-155. However, since SHIP1 levels were not rescued upon inhibition of PI3K p110α, we can explore miR-155 independent, regulatory mechanisms. For instance, studies have shown that SHIP1 is also regulated by ubiquitination (Pauls & Marshall, 2017; Ruschmann et al., 2010) (Fig.6).

Ruschmann et al. suggested that SHIP1 is phosphorylated by Src family kinases, possibly by c-Cbl and then degraded by the proteasome (2010). Additionally, Hatton et al. were the first to show that Fyn and Syk—both of which are c-Cbl kinases—are needed to initiate PI3K/Akt pathway by LMP1 in B cells (2012). Hatton et al. also demonstrated that there was an increase in Akt phosphorylation when Src family kinases were phosphorylated (2012). This is relevant because it aligns with SHIP1 being phosphorylated by Src kinases, ubiquitinated and degraded. Thus, SHIP1 levels would be decreased allowing for Akt to be more active, promoting survival of cancerous cells (Fig.6). Together, these studies hint that SHIP1 may be degraded by

Src family kinases (Fyn and Syk) that are activated by LMP1 rather than miR-155 (Fig.6). Significantly, this is consistent with the lack of rescue when BYL was present.

However, if SHIP1 is regulated independent of miR-155, we would still need to ensure that SHIP1 is being regulated by ubiquitination. We can conduct immunoprecipitations and Western blots with antibodies specific to phosphorylation of SHIP1 and ubiquitin (Ruschmann et al., 2010). Alternatively, the Hatton lab was also trying to optimize conditions to efficiently knockdown P13K p110α using small interfering RNA (siRNA)(data not shown). In addition to validating our findings, we could also knockdown miR-155 and measure the effects of SHIP1. If SHIP1 is being regulated by phosphorylation and ubiquitination, then we would not expect a significant effect on SHIP1 levels when miR-155 is knocked down. Thus, this would support that SHIP1 is regulated independently of miR-155.

Current Approaches for treatment of EBV

Because of the overexpression of miR-155 and its consequential downregulation of its targets and tumor suppressors, like SHIP1 (O'Connell et al., 2009), addressing miR-155 activity is a viable direction to treating EBV+ malignancies. miR-155 has the potential to act as a diagnostic and prognostic biomarker. Diagnostic biomarkers provide an indication of the presence of diseases and potential stages, and prognostic markers suggest a potential outcome. Unfortunately, high levels of miR-155 often indicate a poor prognosis (Due et al., 2016). However, miR-therapies are becoming more widely studied. There are two broad types of miR-therapies: ones that restore miR activity and ones to inhibit miR activity—this depends on the function and targets of the miRs of interest. In this instance, the goal would be to inhibit miR-155, so that SHIP1 levels could be restored. Alas, there are several challenges to miR-therapies. For instance, finding effective delivery systems and vectors. An additional challenge is that there could be imperfect matching, allowing the miRs to have off-target effects (Abd-Aziz et al., 2020). Overall, with further study into miR-155, and by having a better understanding of

EBV's viral mechanism, we can work towards identifying therapeutic targets and restoring levels of miR-155 targets, like SHIP1.

Current SHIP1 therapies work to either restore or mimic SHIP1 function. For example, Fuhler et al. showed that SHIP enzymatic activators were able to mimic tumor-suppressor function (2012), and SHIP1 inhibitors may have less off-target effects. In addition, Brooks et al. showed that the SHIP1 inhibitor—α-aminocholestane—has been shown to trigger apoptosis of blood cancer cells (2010). Further, an inhibitor specific to Akt, MK-2206, could be a placeholder for SHIP1 function; this inhibitor has been proposed to be an effective treatment for EBV+ PTLD (Sang et al., 2019). Altogether, this study and further examination into SHIP1 could advance the therapies for EBV-associated malignancies.

Conclusion

Those who are immunocompromised or immunosuppressed are vulnerable to EBV malignancies, like B cell lymphomas. Due to the high infectious rate of EBV, and its elusive viral mechanisms, it is vital that we work towards identifying therapeutic targets. In this paper, we found that LMP1 significantly downregulated SHIP1, a known target of miR-155 and a negative regulator of the PI3K/Akt pathway (Fig.4) (O'Connell et al., 2009). Further studies into the regulation of SHIP1 would help us to better understand EBV's viral mechanism. Overall, this study adds to the possible targets and treatments for EBV-associated malignancies, which are steadily increasing.

Materials and Methods

Cell Lines and Sub-culturing

EBV negative (EBV-) Burkitt's lymphoma line BL41 cells were used (Dr. Elliott Kieff, Harvard Medical School). BL41 did not express endogenous LMP1 but expressed a LMP1 and nerve growth factor receptor (NGFR) chimeric molecules, NGFR.LMP1 (Lambert and Martinez, 2007). To count cells with a hemocytometer, 0.4% Trypan Blue was added (to identify dead cells) to aliquoted BL41 cells in a 1:4 dilution. Cells were resuspended with complete RPMI (cRPMI) to 0.5×10^6 cells/mL every 2 to 3 days. The cRPMI was made with RPMI 1640 media, 10% heat-inactivated fetal bovine serum (FBS), and 50 units/ml of penicillin /streptomycin (ThermoFisher Scientific). To select cells expressing NGFR.LMP1 constructs, 700 µg/mL geneticin (Sigma Aldrich) was added. Cells were incubated at 37°C at 5% CO_2.

Functionality of NGFR.LMP1 Crosslinking measured with qPCR

6 million BL41 cells expressing NGFR.LMP1 were acquired and spun at 1250 rpm for 5 min at room temperature (RT). Cells were resuspended with cRPMI without the selection reagent, geneticin. To activate LMP1 signaling, cells were crosslinked (X-linked) by first adding Mouse Anti-Human NGFR 0.5 µg/10^6 cells (BioLegend), then after a 30 min incubation at RT, Goat Anti-Mouse IgG 4 µg/10^6 cells (Jackson ImmunoResearch). Cells were incubated at 37°C at 5% CO_2 overnight for 16 hours.

To measure functionality of NGFR.LMP1, cells were lysed, RNA was isolated, cDNA was generated, and qPCR was performed. X-linked, BL41 cells were spun 5 min at 1250 rpm at RT, then resuspended in PBS. Total RNA for ICAM and GAPDH was isolated using the PureLink RNA Mini Kit (ThermoFisher), and total RNA for miR-155 and U47 was isolated using the MiRVana miRNA Isolation Kit (ThermoFisher/LifeTech/ Ambion), both as indicated by the manufacturer's instructions. Samples were quantified by Nanodrop One and stored at –80°C

until use. cDNA was generated using the iScript Advanced cDNA Synthesis Kit (BioRad) for ICAM and GAPDH, and the TaqMan miRNA Reverse Transcriptase Kit (ThermoFisher Scientific) for miR-155 and U47, per manufacturer's instructions. Quantitative polymerase chain reaction (qPCR) was used to measure the relative expression of ICAM, GAPDH, miR-155 and U47 using the TaqMan miRNA Assays (ThermoFisher Scientific). Targets were amplified with TaqMan Universal Master Mix II, No AmpErase UNG (2X) (ThermoFisher Scientific) by CFX Connect Real-Time PCR Detection System (BioRad). Relative expressions were normalized to endogenous controls (ΔCt) and then to unactivated samples (ΔΔCt). Fold induction ($2^{-\Delta\Delta Ct}$) is shown. GAPDH was the endogenous control for ICAM. U47 was the endogenous control for miR-155.

Inhibition of Signaling Pathways, Lysate Generation & Quantification

To examine the signaling pathways used by LMP1 to regulate host miR-155 (and its targets), BYL719 (BYL), a PI3K p110α inhibitor was used, 10 μM (SelleckChem, Hatton et al., 2019). BL41 cells were collected and spun at 1250 rpm for 5 min to pellet. Cells were cultured with BYL in DMSO stock or equivalent amounts of DMSO to a final 2×10^6 cells/mL. Following a 30 min incubation at RT, anti-NGFR (0.5 mg/10^6 cells) was added and gently mixed. After an additional 30 min incubation, goat anti-mouse IgG (2mg/10^6 cells) was added. Samples then incubated overnight for 16 hours at 37°C, 5% CO_2.

Cells were harvested and spun for 5 min at 1250 rpm at RT. Cells were washed with PBS + 1 mM sodium orthovanadate (OV). Cells were spun again under same conditions. Phospholysis Buffer and inhibitors (PLB + Inhib) was added. PLB was made from Phospholysis Buffer Stock: 50 mM Tris pH 7.4: 1% NP-40: 0.5% DOC: 150 mM NaCl: 0.5 mM EDTA+1X Halt Phosphatase and Protease Inhibitor + 1 mM OV. Samples then incubated on ice for 30 min and vortexed every 15 min. After incubation, they were spun 15 min at 13000 rpm. Supernatants were transferred to new tubes and Lamelli sample buffer was added to 1X final. To quantify

lysates, 1:2 dilutions of lysates to PLB + inhibitors were made. Pierce 660 nm Assay Reagent was added and mixed. After incubation for 5 min at RT, lysate concentration was measured with NanoDrop One. To equalize lysate concentration to 1-2 mg/mL among the lysates, 1X Lamelli sample buffer in PLB+inhib was added. Lysates were stored at –20°C until use.

Separation by SDS-Page and Western Blot

Lysates were thawed, vortexed, and incubated at 95°C for 5 min. After spinning in microcentrifuge for 10 sec, samples (20µg/lane) were separated by SDS-PAGE on Novex WedgeWell Bix-Tris 4-20% gels. The gels ran at 80V for 30 min in 1X Tris-SDS Glycine running buffer (ThermoFisher) and then switched to 100V and ran for about 30 min.

Samples were then transferred to PVDF membranes. Sponges were soaked with 1X Transfer Buffer [(25 mM Tris, 192 mM glycine, 20% methanol (pH 8.3)]. The transfer ran for 12V for 1 hour. Blots were then blocked with 5% milk in 1X Tris-Buffered Saline, 0.1% Tween (TBST) for 1 hour at RT. Primary antibodies for SHIP1 and actin (Cell Signaling Technologies) were used for various blots. For SHIP1 a 1:250 antibody dilatation and 5% milk in 1X TBST was added to the membrane. The membrane was then rocked overnight at 4°C. The membrane was washed with 1X TBST for a total of 3 washes at 5 min each. A secondary antibody dilution of anti-rabbit HRP was added to the membrane, which then incubated with rocking for 60 min at RT. After again washing with 1X TBST, blots were prepared with SuperSignal West Pico PLUS Chemiluminescent Substrate (ThermoFisher Scientific) and imaged with ThermoFisher iBright FL1000 Imagine System at Colorado College. To detect β-actin, a dilution of actin-HRP with 5% milk in 1 X TBST (1:1000) was added to blot and rocked overnight at 4°C. The blot was then imaged the same way as described before. Densitometry analysis was then performed using ImageJ.

Acknowledgements

I am thankful for Dr. Olivia Hatton, who has provided endless support regarding academics, lab, graduate school and beyond. I am also thankful for her guidance in writing this thesis, and for her showing me all her drafts of various manuscripts to show that writing takes many, many revisions. I am also thankful for Dr. Sara Hanson, who has provided me with the opportunity to have a holistic outlook on science through her courses. Dr. Hanson pushes me to think creatively and has emphasized the importance of scientific communication. I am also thankful for my fellow lab mates, Madison Alexander, Melanie Mandell, Madeline Smith, and Madeline Stesney. I am also appreciative of the encouragement from my family. Finally, I am grateful to have been a part of the Noyce Fellowship that enabled me to be a part of this research through its generous funding from the National Science Foundation.

Figures

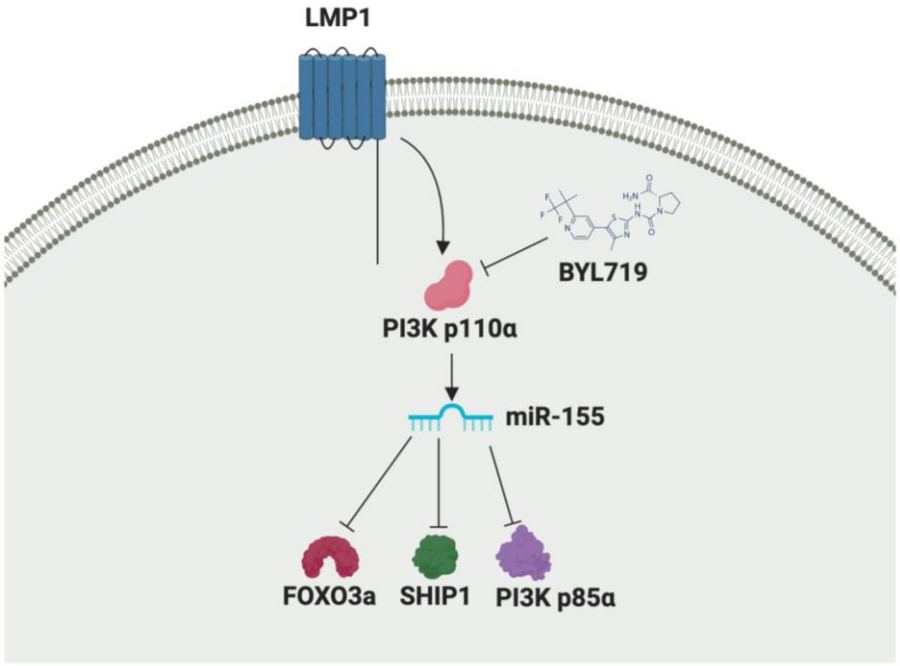

Figure 1. **Proposed Mechanism for Regulation of miR-155 targets by LMP1**

LMP1 has six transmembrane domains and a long C-terminus tail that has two activating regions (CTARs), not illustrated. LMP1's CTAR1 activate PI3K p110α (Lambert and Martinez, 2007), which upregulates miR-155. miR-155 further downregulates its targets, FOXO3a, SHIP1, and PI3K p85α. To verify our proposed mechanism, we can utilize a small molecular inhibitor of PI3K p110α, BYL719, which we expect will restore expression of miR-155 targets upon LMP1 signaling. Image made with BioRender by Hatton lab.

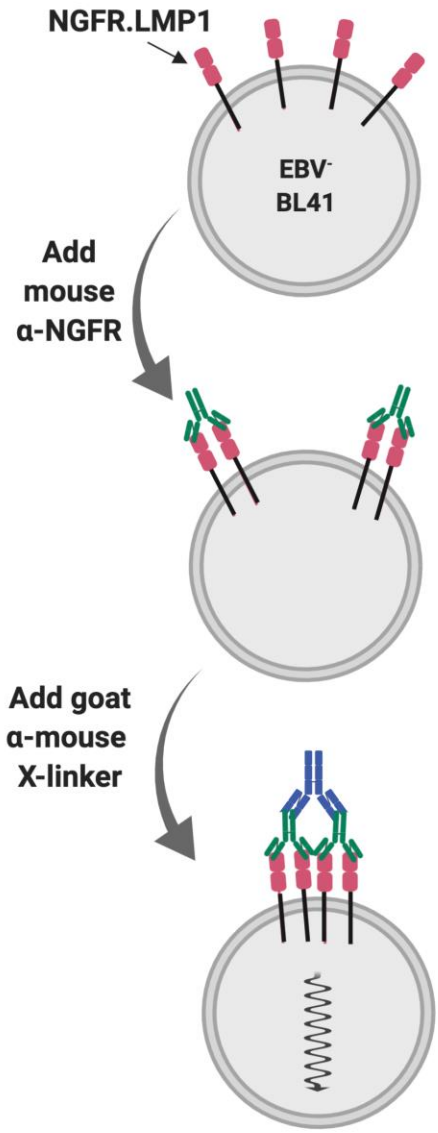

Figure 2. **NGFR. LMP1 as a model for endogenous LMP1**

LMP1 signaling is induced when the chimeric NGFR.LMP1, stably expressed in EBV- BL41 cells lines, is crosslinked (Xlink) by mouse anti-NGFR (mouse α-NGFR) and goat anti-mouse (goat α-mouse X-linker) antibodies. Made with BioRender (Hatton Lab, 2019).

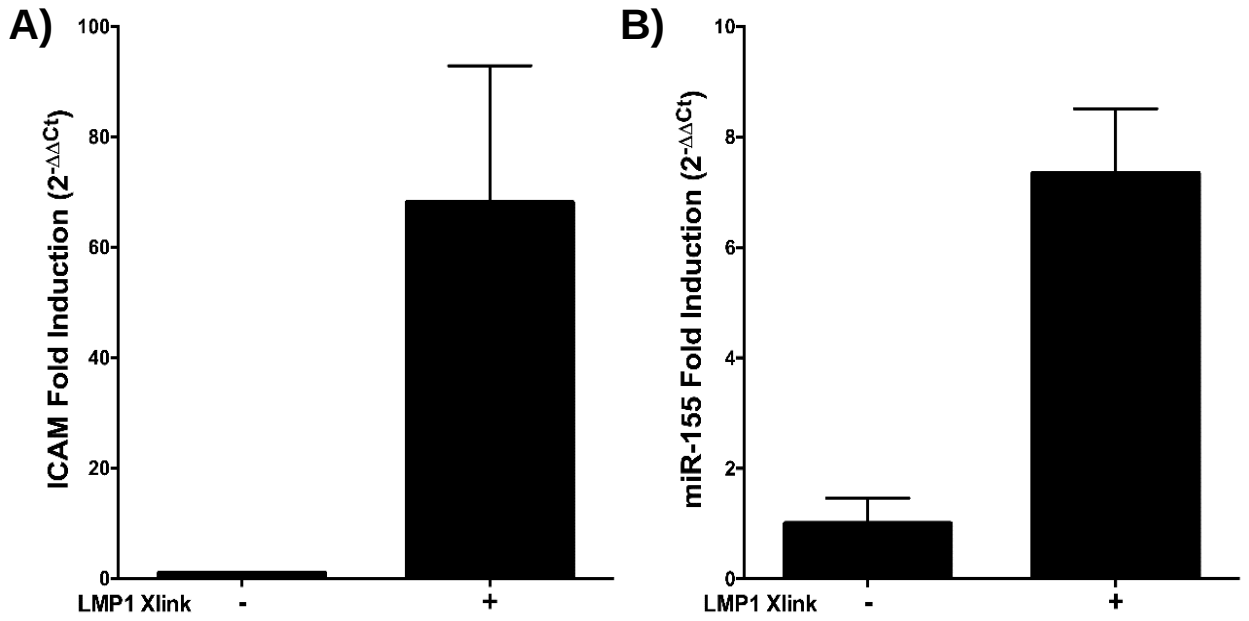

Figure 3. **NGFR. LMP1 is a model for viral LMP1 function**

(A, B) LMP1 was crosslinked (Xlink) with mouse anti-NGFR and goat anti-mouse antibodies as illustrated in Fig.3. Lysate generation, RNA isolation, cDNA generation, and qPCR were performed to measure ICAM and miR-155 levels and their endogenous controls, GAPDH and U47, respectively. Relative expressions were normalized to endogenous controls (ΔCt) and then to unactivated samples ($\Delta\Delta C_t$). Fold induction ($2^{-\Delta\Delta Ct}$) is shown. ICAM = intracellular adhesion molecule.

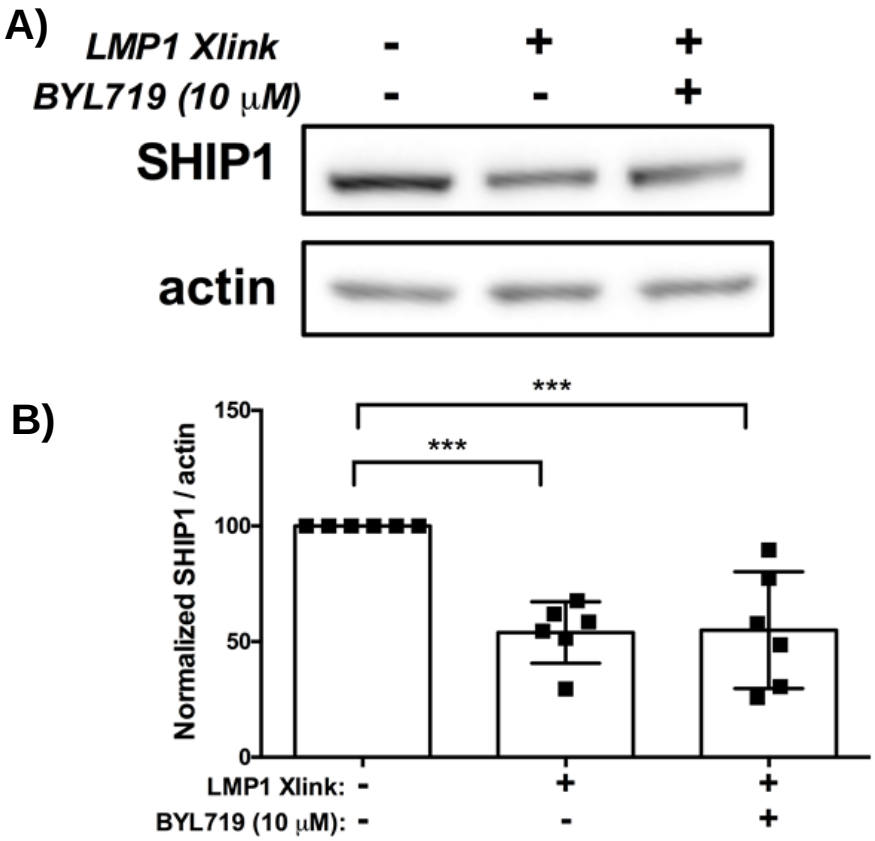

Figure 4. **LMP1 downregulates SHIP1 with no rescue upon PI3K p110α inhibition**

(A) LMP1 was activated in the presence of BYL719 as indicated above, and SHIP1 was detected by Western blot. Actin acts as a loading control. (B) Densitometry analysis with ImageJ. Expression of SHIP1 was normalized to actin. ***$p \leq 0.001$ by one-way ANOVA with post hoc multiple comparisons.

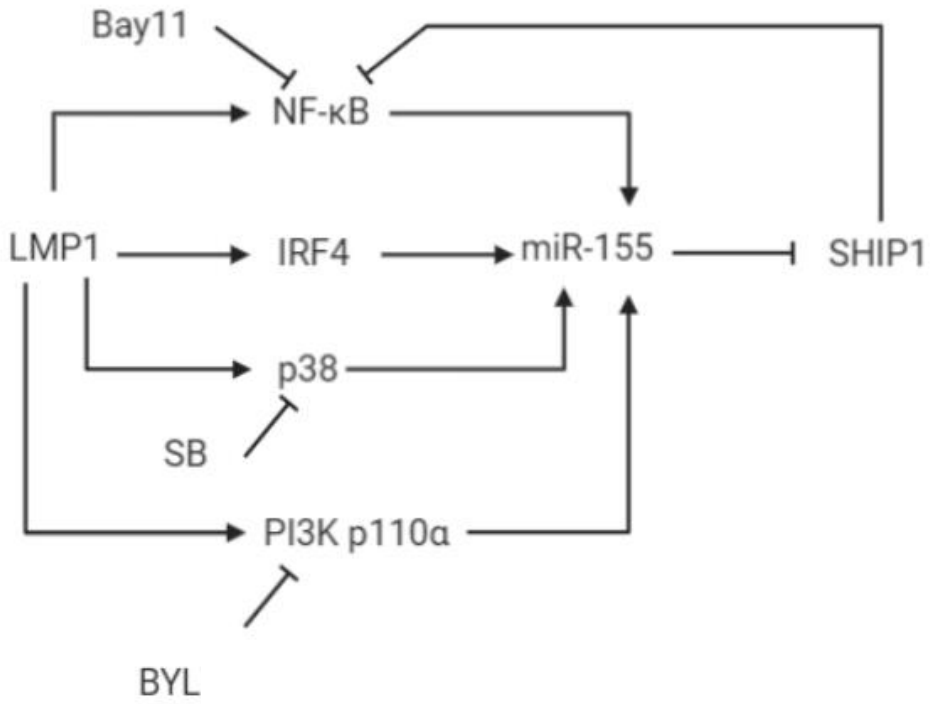

***Figure 5*. Regulation of SHIP1 via miR-155-dependent pathways that are activated by LMP1**

LMP1 induces various host signaling pathways, including NF-κB, IRF4, p38, and PI3K p110α, all of which contribute to an upregulation of miR-155 and in turn a downregulation of its targets, such as SHIP1 (others not included). SHIP1 downregulates NF-κB via negatively regulating calcium signaling. Corresponding inhibitors are as follows, BYL = BYL719; Bay11= Bay11-7082 and SB = SB203580. Created with BioRender.

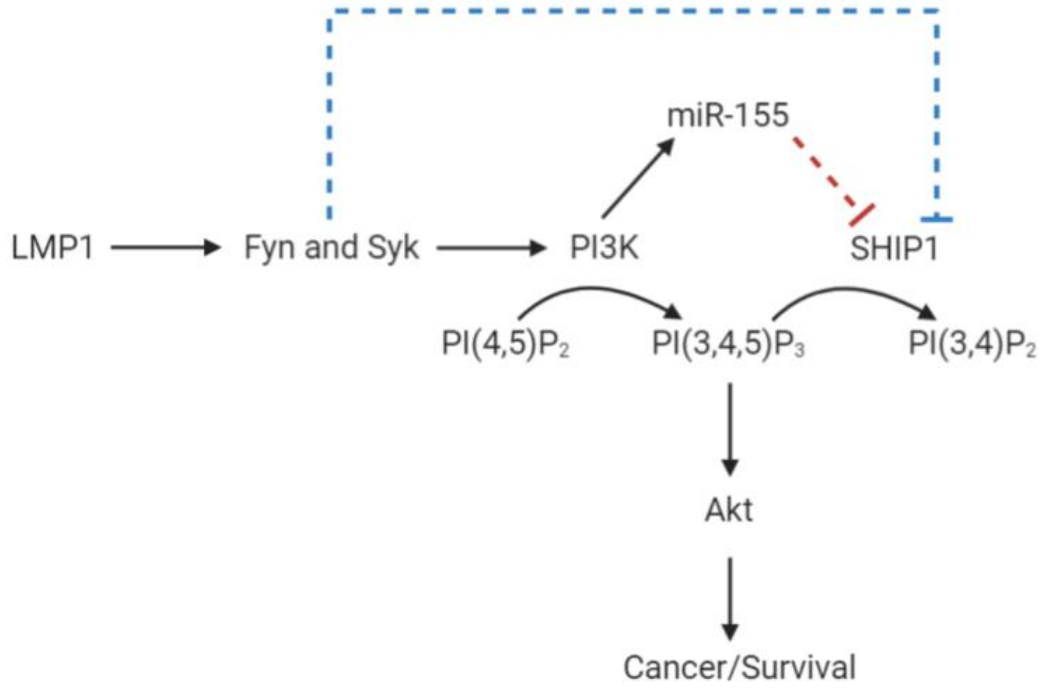

***Figure 6*. Potential regulation of SHIP1, independent of miR-155**

LMP1 activates the PI3K/Akt pathway via Fyn and Syk (Hatton et al., 2019). SHIP1 can be regulated by two pathways: miR-155 and ubiquitination. When SHIP1 is phosphorylated, possibly by Fyn and Syk, this marks SHIP1 for ubiquitination, which causes SHIP1 to be degraded via a proteosome (blue, dashed line). miR-155 also targets SHIP1 (red, dashed line). SHIP1 dephosphorylates the $PI(3,4,5)P_3$ to $PI(3,4)P_2$, which would downregulate Akt, since $PI(3,4,5)P_3$ activates Akt. Created with BioRender.

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