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Syk-Induced Phosphatidylinositol-3-Kinase Activation in Epstein-Barr Virus Post-Transplant Lymphoproliferative Disorder

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Abstract

Post-transplant lymphoproliferative disorder (PTLD)-associated Epstein Barr virus (EBV)+ B-cell lymphomas are serious complications of solid organ and bone marrow transplantation. The EBV protein LMP2a, a B-cell receptor (BCR) mimic, provides survival signals to virally-infected cells through Syk tyrosine kinase. Therefore, we explored whether Syk inhibition is a viable therapeutic strategy for EBV-associated PTLD. We have shown that R406, the active metabolite of the Syk inhibitor fostamatinib, induces apoptosis and cell cycle arrest while decreasing downstream phosphatidylinositol-3'-kinase (PI3K)/Akt signaling in EBV+ B-cell lymphoma PTLD lines *in vitro*. However, Syk inhibition did not inhibit or delay the *in vivo* growth of solid tumors established from EBV-infected B-cell lines. Instead, we observed tumor growth in adjacent inguinal lymph nodes exclusively in fostamatinib-treated animals. In contrast, direct inhibition of PI3K/Akt significantly reduced tumor burden in a xenogeneic mouse model of PTLD without evidence of tumor growth in adjacent inguinal lymph nodes. Taken together, our data indicate that Syk activates PI3K/Akt signaling which is required for survival of EBV+ B-cell lymphomas. PI3K/Akt signaling may be a promising therapeutic target for PTLD, and other EBV-associated malignancies.

Keywords

Epstein-Barr Virus; Post-Transplant Lymphoproliferative Disorder; PTLD; PI-3Kinase / Akt Pathway

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INTRODUCTION

EBV is a ubiquitous, B-cell lymphotropic human γ -herpesvirus (1). In immunocompetent individuals infection is controlled by a vigorous cytotoxic T lymphocyte (CTL) response. However, EBV can transform normal B-cells and promote lymphomagenesis when the CTL response is debilitated as in PTLT. PTLT-associated B-cell lymphomas can express a series of EBV latent genes, including six EBV nuclear antigens (EBNAs), both small RNAs (EBERs), and three latent membrane proteins, LMP1, LMP2a, LMP2b. In addition to participating in malignant transformation, these viral genes promote evasion of immune recognition, maintain viral latency, and hijack production of the autocrine growth factor, cellular IL-10 (2,3). Since over 90% of PTLT-associated lymphomas are EBV-positive (4), the virus itself and the mechanisms by which EBV drives lymphomagenesis are attractive targets for development of new therapeutics for PTLT.

LMP2a is a 54 kDa protein expressed in the membrane of EBV-infected B-cells and a functional, constitutively active mimic of the BCR (1,5). LMP2a provides constitutive, BCR-like signals primarily through recruitment and activation of Syk tyrosine kinase. Syk subsequently activates downstream signaling pathways including the PI3K/Akt and Erk mitogen-activated protein kinase (MAPK) pathways. Whether Syk or PI3K/Akt activation is required for EBV+ B-cell lymphomagenesis, tumor survival, and proliferation is unknown. Here, we used small molecule inhibitors of Syk and PI3K/Akt to probe whether Syk or PI3K/Akt activation is required for the survival and proliferation of EBV+ B-cell lymphomas. We also assessed the efficacy of Syk and PI3K/Akt inhibition on tumor burden in a xenotransplantation model of EBV-associated PTLT.

MATERIALS AND METHODS

Reagents

Fostamatinib is a soluble, orally-dosed pro-drug that is rapidly and extensively converted to the active metabolite R406. Both fostamatinib and R406 are Syk inhibitors. Fostamatinib-containing chow and the active component R406 were kindly provided by Rigel Pharmaceuticals and AstraZeneca. For cell culture studies, R406 (10 mM in dimethylsulfoxide (DMSO)) was aliquotted and stored at -80°C . The small molecule inhibitors of Erk (PD98059, 20 mM) and PI3K/Akt (LY294002, 20 mM) (Calbiochem) were diluted in DMSO (Sigma-Aldrich) to the indicated concentrations. The following antibodies were used: anti-Erk and anti-phospho-Erk (Tyr²⁰⁴) (Santa Cruz Biotechnology), anti-Akt, phospho-Akt (Ser⁴⁷³), B-cell linker protein (BLNK), and phospho-BLNK (Tyr⁹⁶) (Cell Signaling Technology), anti- β -actin (Sigma-Aldrich), horseradish peroxidase (HRP)-conjugated goat anti-rabbit and HRP-conjugated donkey anti-mouse antibodies (Jackson ImmunoResearch Labs, Inc).

Cell Lines

The EBV- Burkitt's lymphoma line Ramos was obtained from ATCC. Cell lines AB5, JB7, and ZD3 are spontaneously derived EBV+ B lymphoblastoid cell lines (SLCL) from peripheral blood (JB7, ZD3) or lymph nodes (AB5) of patients diagnosed with EBV+ PTLT

(2,3,6,7). The EBV- Burkitt's lymphoma line BL41 and the EBV-infected line BL41.B95 were provided by Dr. Elliot Kieff (Harvard Medical School). Cell lines were maintained in a 5% CO₂ humidified 37°C incubator in RPMI-1640 supplemented with 10% heat-inactivated fetal calf serum (Serum Source International), and 50 units/mL penicillin-streptomycin (Invitrogen).

Apoptosis and Cell Cycle Analysis

Cells ($0.5\text{--}1 \times 10^6$ cells/mL) were plated in serial dilutions of small molecule inhibitors R406, LY294002, or PD98059 (0–10 μM), or equivalent amounts of vehicle (DMSO, 0–1:1000). Drug and media were replenished after 48h of a total of 96h in culture at 37°C. Cell cycle analysis using propidium iodide was as previously described (4,7). The percentage of apoptotic cells was determined using Annexin V-enhanced GFP (EGFP) apoptosis detection kits (BioVision) per manufacturer's instructions. Data was analyzed on a FACScan flow cytometer (BD Biosciences).

Western Blotting

Cells (2×10^6 cells/mL) were cultured with small molecule inhibitors or DMSO vehicle. Tumor tissue was harvested during necropsy. After manual dissociation of tumor, single cell suspensions were collected after passing through a 70 μM cell strainer (BD Biosciences). Cells were harvested and washed with PBS containing 1 mM sodium orthovanadate. Lysate preparation and western blots were performed as previously described (8).

Phosflow for phospho-BLNK

BCR crosslinking was initiated by addition of F(ab)₂ fragments of anti-IgM (10 $\mu\text{g/mL}$), in the presence of R406 or vehicle. Fixation and permeabilization of cells was performed as described (9). Cells were stained with anti-phospho-BLNK-phycoerythrin (PE) (BD Pharmingen) before collection on a FACScan (BD Biosciences).

In Vivo Xenogeneic Model of PTLD

Six-week-old, male non-obese diabetic (NOD) severe combined immunodeficiency disease (SCID), NOD.CB17-*Prkdc^{scid}/J* mice were injected subcutaneously (s.c.) over the right flank with $5\text{--}10 \times 10^6$ EBV+ SLCL on day 0. For Syk inhibition studies, mice were placed on control chow or fostamatinib-containing chow the day prior to tumor cell injection and maintained on chow for the duration of the experiment. For PI3K/Akt inhibition studies, mice were injected intraperitoneally (i.p.) with 50 mg/kg LY294002 or DMSO vehicle 3 times weekly. Tumors were measured in two perpendicular dimensions along the plane of the body every 2–3 days using Vernier calipers. Tumor volume was calculated as described previously (7). The slope of the growth curves yielded the tumor growth rates (s) and was calculated by $s = \ln(v(t1)/v(t2))/(t2-t1)$, where $v(t1)$ and $v(t2)$ are the tumor volumes at timepoint 1 and 2, respectively ($t1$ and $t2$). Tumor doubling times were calculated as $\ln(2)/s$. At sacrifice, gross examination of the animal was performed to assess for tumor. All experimental procedures were performed in accordance with a Stanford Institutional Animal Care and Use Committee approved protocol.

Histology of Tumor Tissue

Tumor tissue was harvested at sacrifice, fixed in 10% neutral buffered formalin, and embedded in paraffin. Serial sections of 4 μm were cut from individual paraffin blocks, deparaffinized in xylene, and hydrated in a series of graded alcohol solutions. Sections were stained with hematoxylin and eosin (H&E) and subjugated to immunohistochemistry or labeled by *in situ* hybridization for EBV RNA using automated immunohistologic instruments (Ventana XT, Ventana Medical Systems). Primary antibodies to human CD20 (L26, 1:1000, Dako) and Ki-67 (rabbit polyclonal, 1:1000, Dako) were used. *In situ* hybridization for EBV was carried out using the EBER inform small RNA probe cocktail and developed using ISH iVIEW nitroblue tetrazolium (Ventana Medical Systems).

RESULTS

Syk Inhibition Induces Apoptosis of EBV+ SLCL *in vitro*

BCR crosslinking activates Syk, which can be measured by phosphorylation of the Syk substrate BLNK (10,11). In Ramos B lymphoma cells, BCR crosslinking induces robust phosphorylation of BLNK, which was ablated by addition of the Syk inhibitor R406 (Figure 1A). Additionally, R406 significantly reduces constitutive Syk signaling in EBV+ cell lines derived from patients with PTLT, termed SLCL (12). Therefore, R406 inhibits Syk activation.

Previously, we showed that inhibition of Syk diminishes the proliferation of EBV+ SLCL (12). While proliferation of 5 of 6 EBV+ SLCL was sensitive to R406 at submicromolar concentrations, proliferation of the JB7 EBV+ SLCL was more resistant to Syk inhibition (12). To determine whether the decrease in proliferation was due to cell cycle arrest or apoptosis, SLCL were cultured in the presence of R406 and cell cycle analyses was performed by propidium iodide staining. R406 induced apoptosis (Figures 1B and S1) and cell cycle arrest (Figure 1C) in 5 of 6 of the EBV+ SLCL, as shown for the representative line AB5. Notably, R406 was efficacious at inducing both apoptosis and cell cycle arrest at submicromolar concentrations, with exception of the JB7 cell line. These data indicate that R406 can inhibit cellular growth of most EBV+ SLCL through induction of both cell cycle arrest and apoptosis.

Syk Inhibition Does Not Affect *in vivo* Tumor Burden or Tumor Formation

We next asked if Syk inhibition affects tumor formation or tumor burden of EBV+ SLCL *in vivo*. We previously reported that injection of EBV+ PTLT lines into the flank of immunodeficient SCID mice yields tumors that are phenotypically and histologically indistinguishable from primary PTLT tumors (7). We used this xenogeneic model of PTLT in NOD.SCID mice to determine whether the Syk inhibitor fostamatinib, the prodrug of R406, can inhibit tumor growth of B-cell lymphoma lines *in vivo*.

NOD.SCID mice were given control chow or fostamatinib-containing chow beginning the day prior to tumor inoculation. Control mice ($n=5$) injected with the EBV+ SLCL AB5 (*in vitro* R406 $\text{IC}_{50} = 0.625 \mu\text{M}$) (12) developed palpable solid tumors in the third week after inoculation (Figure 2A and S2). On day 33 post-injection, the average tumor volume in

control animals was $1344 \pm 550 \text{ mm}^3$ (mean $\pm$ SD). Fostamatinib-treated mice injected with AB5 ($n=6$) also developed solid tumors by the third week post-injection (Figure 2A and S2). On day 33 post-injection, the average tumor volume in the fostamatinib-treated mice was $1649 \pm 590 \text{ mm}^3$, and was not significantly different ($p=0.4$) than control. Indeed, tumors in the control group grew at a rate of $0.18 \text{ mm}^3/\text{day}$ with a doubling time of 3.77 days, while tumors in the treated group grew at a rate of $0.2 \text{ mm}^3/\text{day}$ with a doubling time of 3.5 days, for days 28–37. Two additional cohorts of NOD.SCID mice injected with the AB5 cells (5×10^6) were monitored and no differences in time tumor formation or in tumor burden were observed between control ($n=7$) and fostamatinib-treated ($n=9$) groups (data not shown). Furthermore, no change in time to tumor formation or tumor burden was observed in fostamatinib-treated mice that were injected with another EBV+ SLCL, ZD3 (*in vitro* R406 $\text{IC}_{50} = 0.156 \mu\text{M}$) (12) (data not shown). These data suggest that the formation or growth of solid tumors established from EBV+ SLCL was not affected by fostamatinib.

Serum and tissue samples from mice were analyzed to confirm the presence of active metabolite R406 in the blood and tumor, respectively. R406 was detected in the serum (Figure 2B) and tumor site (data not shown) of animals receiving fostamatinib chow at levels above the reported IC_{50} values (12). To confirm R406 activity at the tumor site of treated animals, tumors were harvested at sacrifice from control and fostamatinib-treated animals and tumor lysates were analyzed for the presence of phosphorylated BLNK (pBLNK) by western blot. Indeed, we observed a reduction in pBLNK in fostamatinib-treated tumors, compared to control tumors (Figure 2C). These data suggest that Syk signaling was indeed impaired in fostamatinib-treated animals.

Histological Profiles of Control and Treated Tumors

We compared the histologic features of tumors from control and fostamatinib-treated mice. The EBV+ SLCL AB5 grown in NOD.SCID mice show a diffuse proliferation of large pleomorphic cells morphologically comparable to human diffuse B-cell lymphoma (Figure 3A). Like the tumors from control mice, the tumors from fostamatinib-treated mice were comprised of diffuse sheets of atypical, large lymphoid cells that infiltrate normal structures, such as skeletal muscle. There was minimal cellular infiltrate into tumor sites of both control and treated tumors. Nearly 100% of the tumor tissue stained positive for human CD20 and the EBV small RNA, EBER (Figure 3A), indicating the tumors are of human B-cell origin and EBV+. Both control and treated tumors displayed abundant mitotic activity and karyorrhexis, and displayed foci of necrosis. We detected no significant difference in the amount of necrosis assessed in H&E stained sections of tumors from control and fostamatinib-treated mice (Figure 3B) ($p=0.19$), nor was there a difference in the number of Ki-67+ proliferating cells (Figure 3A, 3C) ($p=0.13$). These findings indicate that Syk inhibition does not affect the morphology or proliferative characteristics of EBV+ B-cell lymphomas *in vivo*.

Syk Inhibition Results in Lymphoma Growth in Inguinal Lymph Nodes Adjacent to the Primary Tumor Site

We observed that fostamatinib-treated animals displayed tumor growth in inguinal lymph nodes adjacent to the site of tumor inoculation, whereas the majority of control animals did

not (Figure 3D and Table 1). Histological analysis of these adjacent inguinal lymph nodes revealed that they were composed of human CD20+, EBER+ tumor cells. There was no difference in necrosis or proliferation in these structures compared to the primary tumor site (data not shown). Interestingly, the EBV+ SLCL JB7 that was resistant to R406 *in vitro*, was highly metastatic *in vivo* in both control and fostamatinib-treated mice. In control mice, JB7 tumors metastasized primarily to the liver and abdomen. Fostamatinib-treated, JB7-injected animals displayed fewer abdominal metastases but qualitatively larger adjacent inguinal lymph nodes than control animals (Table 1). Tumor growth at distant sites was minimal in animals injected with the other cell lines. Specifically, four enlarged axillary lymph nodes and one liver metastasis were observed in the fostamatinib-treated mice injected with the SLCL AB5, and one enlarged axillary lymph node was observed in both the control and fostamatinib-treated mice injected with the SLCL ZD3. Tumor cells within the adjacent inguinal lymph nodes were detected more frequently in fostamatinib-treated animals than control animals inoculated with either R406-sensitive (AB5, $p<0.0001$ and ZD3, $p=0.05$) or R406-resistant (BL41, $p=0.06$ and BL41.B95, $p=0.06$) cells (Table 1). In prior studies we showed that BL41 and BL41.B95 are resistant to effects of Syk inhibition by R406 *in vitro* (12). Enlarged inguinal lymph nodes with CD20+ EBER+ tumor cells were found in both the EBV- line BL41, and its EBV-infected counterpart BL41.B95 (Table 1). These data suggest that Syk inhibition is associated with lymphoma localization in the lymph node adjacent to the primary tumor, regardless of *in vitro* sensitivity to R406 or the status of EBV infection.

R406 Inhibits PI3K/Akt Survival Signaling

Treatment of the EBV+ SLCL AB5 with R406 reduced the phosphorylation of both Akt and Erk *in vitro* (Figure 4A, right panel). We next asked whether either of these pathways participate in survival of EBV+ PTLD-derived B-cell lymphomas. EBV+ SLCL were cultured in the presence of the PI3K/Akt inhibitor LY294002 or the Erk MAPK inhibitor PD98059 and apoptosis was analyzed by Annexin V-EGFP staining. PI3K/Akt inhibition induced a time-dependent increase in apoptosis, while Erk inhibition had no effect on apoptosis (Figure 4B). These data suggest that while both PI3K/Akt and Erk MAPK are activated downstream of Syk, PI3K/Akt, but not Erk MAPK, contributes to survival of EBV + SLCL.

PI3K/Akt Inhibition Reduces Tumor Burden *in vivo*

We next asked if PI3K/Akt inhibition affects tumor burden in mice inoculated with EBV+ SLCL. In the first experiment, NOD.SCID mice were injected with 5×10^6 cells of the EBV+ SLCL AB5 on day 0. Treatment with either 50 mg/kg LY294002 ($n=6$) or an equivalent amount of vehicle ($n=6$) began before any evidence of palpable tumor and continued three times weekly throughout the study. We observed tumor formation in 4 of 6 control animals (Figure S3). In contrast, tumor formation was detected in only 1 of 6 animals treated with the PI3K/Akt inhibitor LY294002 (Figure S3), and that tumor was the smallest of all tumors identified in vehicle or treated animals at the time of necropsy.

To test whether PI3K/Akt inhibition could affect already established tumors, animals were inoculated with 5×10^6 cells of the EBV+ SLCL AB5 on day 0. The day after the formation

of palpable tumors, treatment with either 50 mg/kg LY294002 ($n=9$) or an equivalent amount of vehicle ($n=6$) was initiated and continued three times weekly throughout the experiment. By 21 days post-injection, the average tumor volume was significantly different between LY294002-treated and vehicle-treated mice ($77 \pm 101 \text{ mm}^3$ versus $322 \pm 105 \text{ mm}^3$, $p=0.0006$) (Figure 5A). At the time of sacrifice, the average tumor volume was significantly smaller in the LY294002-treated group ($171 \pm 219 \text{ mm}^3$ versus $998 \pm 373 \text{ mm}^3$, $p=0.0001$). Tumors in the control group grew at a rate of $0.14 \text{ mm}^3/\text{day}$ with a doubling time of 4.9 days, while tumors in the treated group grew at a rate of $0.1 \text{ mm}^3/\text{day}$ with a doubling time of 6.9 days, for days 21–29. The majority of the LY294002-treated animals displayed tumors that grew to a small size, and then either plateaued in growth or disappeared completely (Figure 5B). The two LY294002-treated tumors not fitting this pattern were of comparable size to the smallest control tumors (Figure 5B and Figure S4). These data indicate that PI3K/Akt inhibition attenuates the growth of solid tumors established from EBV+ B-cell lines from patients with PTLTD and is not associated with lymphoma growth in adjacent inguinal lymph nodes.

DISCUSSION

Syk inhibitors targeting tonic BCR signals have been pursued in the treatment of B-cell lymphomas. Unlike other B-cell lymphomas, a tonic, BCR-like signal is delivered to EBV+ B-cell lymphomas through Syk activation by self-oligomerization of the viral protein LMP2a. Treatment options for EBV-associated PTLTD B-cell lymphomas are limited and typically involve reduction of immunosuppression, which is often ineffective and puts the transplanted organ in jeopardy of rejection. Consequently, there is a need for new, more effective therapeutics that target the lymphoma without endangering graft survival.

Using R406, the active metabolite of the Syk inhibitor fostamatinib, we provide evidence for the requirement of Syk activation in PI3K/Akt and Erk MAPK signaling, and in survival of EBV+ B-cell lymphomas *in vitro*. We also establish that PI3K/Akt signaling, but not Erk MAPK signaling, is a critical survival pathway for PTLTD-derived EBV+ B-cell lymphomas. Given that Syk activation is required for survival *in vitro*, we expected to observe a similar benefit *in vivo*. However, Syk inhibition had no effect on primary tumor burden in a xenogeneic model of PTLTD. Instead, Syk inhibition resulted in tumor growth in inguinal lymph nodes adjacent to the site of tumor inoculation. Importantly, PI3K/Akt signaling is a key outcome of Syk activation in PTLTD, because PI3K/Akt inhibition is efficacious in reducing tumor burden without tumor growth in adjacent lymph nodes.

Deregulated PI3K/Akt signaling is characteristic of a myriad of human cancers as a result of mutations in members of the PI3K/Akt pathway, including the PI3K catalytic subunit p110 α or Akt. PI3K/Akt deregulation also results from mutation, amplification, or overexpression of the PI3K-activating receptor tyrosine kinases (RTKs), such as epithelial growth factor receptor (EGFR) or human epidermal growth factor receptor 2 (HER2) (13). Intriguingly, preliminary data from phase I clinical trials of PI3K or Akt inhibitors in tumors displaying a mutation in the gene encoding the PI3K p110 α subunit (*PIK3CA*), including endometrial and colorectal cancers, show few responses but rather the establishment of tumor stasis, or stable disease (14). In our model of EBV+ B-cell lymphomas, we observed both tumor stasis

and tumor regression in the majority of animals treated with the PI3K inhibitor LY294002. To our knowledge, EBV+ PTLD lymphomas do not contain mutations in either PI3K or Akt. Instead, PI3K/Akt activation is driven by the EBV latent membrane proteins, LMP2a and LMP1, and more closely resembles PI3K-activation by receptor tyrosine kinases (RTKs). Therapies targeting PI3K-activating RTKs, however, such as herceptin for HER2-positive breast cancers and gefitinib or erlotinib for EGFR signaling in non-small cell lung carcinoma, pancreatic cancer, and glioblastoma have been very efficacious in the clinic. These therapies provide some of the strongest evidence for the concept of oncogene addiction, the observation that despite the complexity of cancerous cells, their growth is often impaired by inactivation of a single oncogene (15). Given the apoptosis we observe *in vitro* and the tumor regression we identified in our xenogenic model of PTLD with PI3K/Akt inhibition, our data suggest that EBV hijacks and re-wires cellular signaling pathways through LMP2a and LMP1 to create a state of oncogenic addiction to PI3K/Akt in PTLD-derived EBV+ B-cell lymphomas. It will be of interest in the future to test whether oncogenic addiction to PI3K/Akt signaling is operating in EBV+ B-cell lymphomas.

The Syk inhibitor R406 induced both apoptosis and cell cycle arrest in a panel of PTLD-derived EBV+ B-cell lines *in vitro*. However, we observed no significant effect of Syk inhibition on time to tumor formation or tumor burden in a xenogenic model of PTLD, despite the presence of active drug in the serum and at the tumor site of treated animals. Syk inhibition by fostamatinib or the Syk inhibitor curcumin was efficacious in murine models of other non-PTLD B-cell lymphomas including diffuse large B-cell lymphoma (DLBCL) (16), B-cell non-Hodgkin's lymphoma (NHL) (17) and chronic lymphocytic leukemia (CLL) (18). In these studies, murine tumor cell lines were administered to immunocompetent, syngeneic animals in a disseminated fashion – by i.p. injection for CLL, and by i.v. injection for DLBCL and B-cell NHL. Thus, several key differences between those models and our model could account for the disparate findings. These include the varied types of lymphomas studied, the use of syngeneic murine cell lines in immune competent mice versus our use of human cell lines in immunodeficient mice, and the use of disseminated tumor models versus our use of a solid tumor model.

These studies are the first to report B-cell lymphoma growth in lymph nodes adjacent to the primary tumor site after Syk inhibition. This occurred regardless of *in vitro* sensitivity to R406 or the status of EBV infection of the cell lines tested. Plausible explanations for this observation include a role for Syk in regulating the peripheralization of lymphoma cells or homing to local lymph nodes, an off-target effect of the inhibitor fostamatinib, or a feature of the xenogenic model of PTLD we utilized. Interestingly, in phase I/II trials with fostamatinib, a transient increase in circulating lymphocytes was observed in all patients with small lymphocytic leukemia (SLL) or chronic lymphocytic leukemia (CLL); this increase exceeded 50% of baseline circulating lymphocytes in 9 out of 13 of patients (19). While we did not test for a similar peripheralization of lymphoma cells, the paucity of metastases at distant sites suggests that Syk inhibition predominantly affects lymph node homing. We did not observe the accumulation of tumor cells in adjacent lymph nodes in animals treated with the PI3K/Akt inhibitor LY294002. Given that Syk also activates a variety of other signaling pathways, including Erk MAPK, Jun N-terminal kinase (JNK),

protein kinase C (PKC), and nuclear factor of activated T cells (NFAT) (20–22), it is possible that another downstream pathway is involved in the accumulation of tumor cells in adjacent lymph nodes. With regard to off-target effects of fostamatinib and its active component R406, the kinases FLT3, c-Kit, Lck, JAK1, JAK3, and VEGFR2 have been reported to be inhibited at doses relevant to our study (23). For example, given that VEGFR2 signaling can regulate angiogenesis, tumor growth in the adjacent lymph node in fostamatinib-treated animals may reflect tumor migration from a hypoxic environment. Whether these molecules play a role in dissemination of EBV+ B-cell lymphomas is unknown.

Activation of PI3K/Akt signaling is not entirely Syk-dependent; RTKs, G-protein coupled receptors, integrins, and cytokine receptors can all activate the PI3K/Akt pathway independent of Syk. Therefore, it is possible we did not observe a reduction in tumor burden in fostamatinib-treated animals because PI3K/Akt signaling was concurrently activated by a Syk-independent mechanism. For example, cell-to-cell contact, cytokines, or other growth factors may provide cues that override or augment the Syk survival signal in EBV+ SLCL *in vivo* that are not observed *in vitro*. Indeed, the efficacy of Syk inhibition in CLL is attributed to attenuation of both tonic B-cell signals as well as Syk-driven signals stimulated in CLL by stromal cell contact (24). In a broad sense, this may imply that efficacy of Syk inhibition depends on its ability to dampen signaling by a variety of oncogenic pathways, including PI3K/Akt.

Taken together, our data indicate that PI3K/AKT signaling is a critical outcome of Syk activation that drives EBV+ B-cell lymphoma survival. These findings also indicate the possibility that PI3K/AKT oncogenic addiction may be exploited as a therapeutic target for PTLD, and potentially other EBV-associated malignancies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

PTLD	Post-Transplant Lymphoproliferative Disorder
EBV	Epstein-Barr Virus
BCR	B-cell receptor
PI3K	Phosphatidylinositol-3'-kinase
CTL	cytotoxic T lymphocyte
EBNA	EBV nuclear antigen

EBERs	EBV small RNAs
LMP	latent membrane protein
MAPK	mitogen-activated protein kinase
DMSO	dimethylsulfoxide
BLNK	B-cell linker protein
HRP	horseradish peroxidase
SLCL	spontaneously derived B lymphoblastoid cell lines
EGFP	enhanced GFP
PE	phycoerythrin
NOD	non-obese diabetic
SCID	severe combined immunodeficiency disease
s.c	subcutaneously
i.p	intraperitoneally
H&E	hematoxylin and eosin
pBLNK	phosphorylated BLNK
RTK	receptor tyrosine kinase
EGFR	epithelial growth factor receptor
HER2	human epidermal growth factor receptor 2
DLBCL	diffuse large B cell lymphoma
NHL	non-Hodgkin's lymphoma
CLL	chronic lymphocytic leukemia
SLL	small lymphocytic leukemia
JNK	Jun N-terminal kinase
PKC	protein kinase C
NFAT	nuclear factor of activated T cells

References

1. Young LS, Rickinson AB. Epstein-Barr virus: 40 years on. *Nat Rev Cancer* (2004). 2004 Oct; 4(10): 757–68. [PubMed: 15510157]
2. Snow AL, Martinez OM. Epstein-Barr virus: evasive maneuvers in the development of PTL. *Am J Transplant*. 2007 Feb; 7(2):271–7. [PubMed: 17229074]
3. Beatty PR, Krams SM, Martinez OM. Involvement of IL-10 in the autonomous growth of EBV-transformed B cell lines. *J Immunol*. 1997 May 1; 158(9):4045–51. [PubMed: 9126962]
4. Gottschalk S, Rooney CM, Heslop HE. Post-transplant lymphoproliferative disorders. *Annu Rev Med* (2005). 2005; 56:29–44. [PubMed: 15660500]

5. Caldwell RG, Wilson JB, Anderson SJ, Longnecker R. Epstein-Barr virus LMP2A drives B cell development and survival in the absence of normal B cell receptor signals. *Immunity*. 1998 Sep; 9(3):405–11. [PubMed: 9768760]
6. Snow AL, Chen LJ, Nepomuceno RR, Krams SM, Esquivel CO, Martinez OM. Resistance to Fas-mediated apoptosis in EBV-infected B cell lymphomas is due to defects in the proximal Fas signaling pathway. *J Immunol*. 2001 Nov 1; 167(9):5404–11. [PubMed: 11673559]
7. Nepomuceno RR, Balatoni CE, Natkunam Y, Snow AL, Krams SM, Martinez OM. Rapamycin inhibits the interleukin 10 signal transduction pathway and the growth of Epstein Barr virus B-cell lymphomas. *Cancer Res*. 2003 Aug 1; 63(15):4472–80. [PubMed: 12907620]
8. Lambert SL, Martinez OM. Latent membrane protein 1 of EBV activates phosphatidylinositol 3-kinase to induce production of IL-10. *J Immunol*. 2007 Dec 15; 179(12):8225–34. [PubMed: 18056366]
9. Irish JM, Czerwinski DK, Nolan GP, Levy R. Kinetics of B cell receptor signaling in human B cell subsets mapped by phosphospecific flow cytometry. *J Immunol*. 2006 Aug 1; 177(3):1581–9. [PubMed: 16849466]
10. Fu C, Turck CW, Kurosaki T, Chan AC. BLNK: a central linker protein in B cell activation. *Immunity* (1998). 1998 Jul; 9(1):93–103. [PubMed: 9697839]
11. Wienands J, Schweikert J, Wollscheid B, Jumaa H, Nielsen PJ, Reth M. SLP-65: a new signaling component in B lymphocytes which requires expression of the antigen receptor for phosphorylation. *J Exp Med* (1998). 1998 Aug 17; 188(4):791–5. [PubMed: 9705962]
12. Hatton O, Phillips LK, Vaysberg M, Hurwich J, Krams SM, Martinez OM. Syk Activation of Phosphatidylinositol 3-Kinase/Akt Prevents HtrA2-dependent Loss of X-linked Inhibitor of Apoptosis Protein (XIAP) to Promote Survival of Epstein-Barr Virus+ (EBV+) B Cell Lymphomas. *J Biol Chem*. 2011 Oct 28; 286(43):37368–78. [PubMed: 21908615]
13. Yuan TL, Cantley LC. PI3K pathway alterations in cancer: variations on a theme. *Oncogene*. 2008 Sep 18; 27(41):5497–510. [PubMed: 18794884]
14. Courtney KD, Corcoran RB, Engelman JA. The PI3K pathway as drug target in human cancer. *J Clin Oncol*. 2010 Feb 20; 28(6):1075–83. [PubMed: 20085938]
15. Weinstein IB, Joe A. Oncogene addiction. *Cancer Res*. 2008 May 1; 68(9):3077–80. discussion 3080. [PubMed: 18451130]
16. Gururajan M, Dasu T, Shahidain S, Jennings CD, Robertson DA, Rangnekar VM, et al. Spleen tyrosine kinase (Syk) a novel target of curcumin is required for B lymphoma growth. *J Immunol* (2006). 2007 Jan 1; 178(1):111–21. [PubMed: 17182546]
17. Young RM, Hardy IR, Clarke RL, Lundy N, Pine P, Turner BC, et al. Mouse models of non-Hodgkin lymphoma reveal Syk as an important therapeutic target. *Blood* (2008). 2009 Mar 12; 113(11):2508–16. [PubMed: 18981293]
18. Suljagic M, Longo PG, Bennardo S, Perlas E, Leone G, Laurenti L, et al. The Syk inhibitor fostamatinib disodium (R788) inhibits tumor growth in the Eμ-TCL1 transgenic mouse model of CLL by blocking antigen-dependent B-cell receptor signaling. *Blood*. 2010 Dec 2; 116(23):4894–905. [PubMed: 20716772]
19. Friedberg JW, Sharman J, Sweetenham J, Johnston PB, Vose JM, Lacasce A, et al. Inhibition of Syk with fostamatinib disodium has significant clinical activity in non-Hodgkin lymphoma and chronic lymphocytic leukemia. *Blood* (2009). 2010 Apr 1; 115(13):2578–85. [PubMed: 19965662]
20. Gobessi S, Laurenti L, Longo PG, Carsetti L, Berto V, Sica S, et al. Inhibition of constitutive and BCR-induced Syk activation downregulates Mcl-1 and induces apoptosis in chronic lymphocytic leukemia B cells. *Leukemia* (2008). 2009 Apr; 23(4):686–97. [PubMed: 19092849]
21. Buchner M, Fuchs S, Prinz G, Pfeifer D, Bartholomé K, Burger M, et al. Spleen tyrosine kinase is overexpressed and represents a potential therapeutic target in chronic lymphocytic leukemia. *Cancer Res*. 2009 Jul 1; 69(13):5424–32. [PubMed: 19549911]
22. Mócsai A, Ruland J, Tybulewicz VLJ. The SYK tyrosine kinase: a crucial player in diverse biological functions. *Nat Rev Immunol*. 2010 Jun; 10(6):387–402. [PubMed: 20467426]
23. Braselmann S, Taylor V, Zhao H, Wang S, Sylvain C, Baluom M, et al. R406, an orally available spleen tyrosine kinase inhibitor blocks fc receptor signaling and reduces immune complex-

- mediated inflammation. *J Pharmacol Exp Ther* (2006). 2006 Dec; 319(3):998–1008. [PubMed: 16946104]
24. Buchner M, Baer C, Prinz G, Dierks C, Burger M, Zenz T, et al. Spleen tyrosine kinase inhibition prevents chemokine- and integrin-mediated stromal protective effects in chronic lymphocytic leukemia. *Blood* (2010). 2010 Jun 3; 115(22):4497–506. [PubMed: 20335218]

Figure 1. Effects of the Syk Inhibitor, R406, on BLNK activation, apoptosis, and cell cycle in B cell lymphomas
 (A) Phospho-flow staining for phospho-BLNK in Ramos cells, either untreated (shaded), after 16 min of BCR crosslinking (solid line), or after 16 min of BCR crosslinking in the presence of the Syk inhibitor R406 (dashed line). (B–C) Cell cycle analysis of EBV+ SLCL AB5 and JB7. Cells were treated for four days with the indicated amounts of R406 and cell cycle analyzed by propidium iodide. Data are shown as (B) the percentage of cells with sub-G₁ apoptotic DNA content and (C) G₁ arrest as defined by a ratio of the percentage of cells in G₁ versus the percentage of cells in S + G₂/M. AB5 is shown as the representative R406-sensitive EBV+ SLCL, while JB7 is the only R406-resistant EBV+ SLCL.

Figure 2. Effect of the R406 prodrug, fostamatinib, on *in vivo* tumor growth

NOD.SCID mice were injected with the EBV+ SLCL AB5 and fed control chow or fostamatinib-containing chow (Fosta Chow). (A) Average tumor volume (mm³) from mice given control chow ($n=5$, solid lines) or fostamatinib-containing chow ($n=6$, dashed lines) is shown. (B) HPLC measurements of the active component R406 in the serum of fostamatinib-treated animals at week 4. The concentration of R406 which results in 50% inhibition of proliferation in AB5 cells compared to untreated AB5 cells is indicated by the dashed line (AB5 IC₅₀, 0.625 μM) (12). (C) Western blots for pBLNK and total BLNK from lysates of control and fostamatinib-treated tumors. Results from two animals in each group are shown.

Figure 3. Histological profiles of tumors

(A) Hematoxylin and eosin (H&E), human CD20 (hCD20), EBV small RNA (EBER), and Ki-67 stains of representative tumors from control or fostamatinib-treated animals are shown in parallel for comparison. (B) Quantified percentage of necrotic cells from H&E stained sections. Each dot represents tumor from one animal. There was no statistical difference between the control and treated groups ($p=0.19$, student's t test). (C) Percentage of proliferating cells as determined by Ki-67 staining in tumor tissue sections. Each dot represents tumor from one animal. There was no statistical difference between the control and treated groups ($p=0.13$, student's t test). (D) Representative primary tumor sites following injection of the AB5 cell line of both control and fostamatinib-treated animals at time of sacrifice (T = tumor, LN = adjacent inguinal lymph node).

Figure 4. Downstream pathways of Syk activation and effects on EBV+ SLCL survival

(A) Western blot analysis of phospho-Akt (pAkt), total Akt, phospho-Erk (pErk), and total Erk in the EBV+ SLCL AB5. Cells were treated with the indicated amount of the Syk inhibitor R406 or DMSO vehicle for 30 minutes prior to preparation of cell lysates. (B) Effect of PI3K/Akt and Erk inhibition on apoptosis of EBV+ SLCL AB5. The number of apoptotic cells from DMSO (Vehicle)-, LY294002, and PD98059-treated EBV+ SLCL was assayed by AnnexinV staining and flow cytometry at the indicated time points. The fold-increase in apoptosis, as determined by the ratio of Annexin V-EGFP⁺ cells in the treated group versus the DMSO vehicle group at each time point, is shown. The EBV+ SLCL AB5 is shown as a representative cell line.

Figure 5. Effects of the PI3K/Akt inhibitor LY294002 on *in vivo* tumor growth

NOD.SCID mice were injected with the EBV+ SLCL AB5 on day 0 and treatment with DMSO vehicle or the PI3K/Akt inhibitor LY294002 was initiated on day 14, one day after formation of palpable tumors. (A) Average tumor volume (mm³) from mice treated with DMSO vehicle (*n*=6, solid lines) or LY294002 (*n*=9, dashed lines). The day of tumor formation (day 13) and the day that treatment began (day 14) are indicated. (B) Representative primary tumor sites of both control and LY294002-treated animals at time of sacrifice.

Table 1

Sites of Tumor Growth Away from Primary Injection

Tumor	R406 IC ₅₀ (μM)	EBV	Adjacent Lymph Node		Other Lymph Node		Abdomen		Lung		Liver	
			Ctrl	Fosta	Ctrl	Fosta	Ctrl	Fosta	Ctrl	Fosta	Ctrl	Fosta
AB5	0.625	+	0/12	14/15*	0/12	4/15	0/12	0/15	0/12	0/15	0/12	1/15
ZD3	0.156	+	2/15	8/15**	1/15	1/15	0/15	0/15	0/15	0/15	0/15	0/15
JB7	>10	+	8/10	6/6 ⁺	0/10	0/6	7/10	2/6	0/10	0/6	10/10	6/6
BL41	>10	–	0/6	4/6***	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6
BL41, B95	>10	+	0/6	4/6***	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6

* p<0.0001;

** p=0.05;

*** p=0.06 from Fisher's exact test;

⁺ lymph nodes qualitatively larger than control