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Epstein-Barr virus is a human herpes virus that in conjunction with Malaria is responsible for endemic Burkitt's lymphoma, a B-cell cancer. The main distinguishing characteristic of Burkitt's lymphoma is a constitutively active c-Myc protein. The transcription factor c-Myc is considered to be a proto-oncogene, which is responsible for cell proliferation and differentiation. The activation of c-Myc leads to the production of cyclin D1 and Cdk4, which promote the G1 to S transition of the cell cycle. The activation of c-Myc is dependent on the Ras/ERK pathway, which can be activated by extracellular signals in the form of cytokines. Interleukin-10 is a cytokine that is produced by Burkitt's lymphoma cells and may act as an autocrine growth factor for the cancer. It may be possible that the Ras/ERK pathway can be activated by interleukin-10 in Epstein-Barr virus infected B-cells. This may lead to the phosphorylation of c-Myc and thus the promotion of the cell cycle and mitotic events in Epstein-Barr infected cells. A further understanding of the role of interleukin-10, the Ras/ERK pathway and c-Myc activation may lead to novel therapeutic interventions for Burkitt's lymphoma pathogenesis in Epstein-Barr virus infected B cells. This study was accomplished by treating Burkitt's lymphoma cells, Epstein-Barr virus infected non-Burkitt's lymphoma cells, and non-infected, non-Burkitt's lymphoma cells with interleukin-10 and assessing the effects of interleukin-10 on the Ras/ERK

pathway, c-Myc activation and Cyclin D1 production. Phosphorylation of ERK, total c-Myc and Cylclin D1 levels were significantly increased ($p<0.05$) in Epstein-Barr virus infected cells, where as IL-10 treatment decreased the viability of B cells lacking an Epstein-Barr infection. Ultimately leading to the conclusion, that IL-10 increases proliferation of Epstein-Barr virus infected B-lymphocytes.

INTERLEUKIN-10 PROMOTES CELL PROLIFERATION IN EPSTEIN-BARR INFECTED B CELLS THROUGH ACTIVATION OF RAS/ERK AND PHOSPHORYLATION OF C-MYC

by

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APPROVAL PAGE

This thesis written by JANSEN BARRETT SMITH has been approved by the following committee of the Faculty of The Graduate School at The University of North Carolina at Greensboro

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CHAPTER I

INTRODUCTION

Epstein-Barr virus, or EBV, is a herpes virus with a double stranded DNA genome that will infect more than 90% of people worldwide (God & Haque, 2010). Under normal circumstances, although the virus remains latent in lymphocytes, most infections are cleared. However in some cases over-proliferation of B-lymphocytes occurs and malignancies form (God & Haque, 2010). The exact molecular pathway for this over-proliferation is not known and greatly differs between different forms of lymphoma that are EBV related. One such EBV related lymphatic malignancy is Burkitt's Lymphoma (BL). Although not all cases of Burkitt's Lymphoma are EBV positive, the largest percentage of endemic (occurring in a specific geographical region) Burkitt's Lymphoma, or eBL, are associated with EBV. Among eBL, 95% of cases are EBV associated; also 5-15% of sporadic BL and 40% of HIV associated BL are EBV associated (Mangani et al., 2012).

BL is among the most aggressive cancers known to affect humans and it is believed that the association of EBV plays a role in this aggressive cell growth. The exact role of EBV is not known, but most cases of BL have an associated over-expression of the oncogene c-Myc. C-Myc is a transcription factor that is known to promote the cell cycle, as well as proliferation and differentiation (Mangani et al., 2012). As a transcription factor c-Myc is known to be a helix/loop/helix/leucine zipper protein that is activated by mitogenic factors under normal circumstances (God & Haque, 2010).

Along with association of EBV with BL, it is also believed that eBL has an association with Malaria infection. Malaria infection by *Plasmodium falciparum* has been found to be associated with eBL (God & Haque, 2010).

It is possible that reactivation of EBV replication by the severity of both Malaria and HIV infections could be responsible for over-proliferation of B-cells and the eventual development of BL. However the question remains as to which molecular pathways and mitogens associated with EBV infection and co-infection with HIV or Malaria are actively involved in B-cell proliferation.

It is possible that Interleukin-10 (a cytokine) acts as a mitogen to activate the Ras/ERK pathway that promotes c-Myc activity in EBV infected cells. Interleukin-10 is known to be associated with EBV, HIV and Malaria, as well as play a role in growth of BL (Masood et al., 1995).

Ras/ERK Pathway:

Among the most well known pathways that stimulate and regulate proliferation, differentiation and cell survival is the Ras/ERK pathway (Mendoza et al., 2011). This pathway results from activation of Receptor Tyrosine Kinases by extracellular signals, specifically mitogens, or growth factors (Mendoza et al., 2011). It is possible that the Ras/ERK pathway is responsible for growth of EBV infected cells, as well as for the activation of c-Myc in EBV associated BL.

The Ras/ERK pathway is considered to be a mitogen activated protein kinase (MAPK) pathway; in this case Extracellular signal-regulated kinase (ERK) is the MAPK. The initial activation of Ras, and then the ultimate MAPK cascade is dependent on many factors. Signals for activation can be integrated from Receptor Tyrosine Kinases (RTKs) and can be in a variety of forms such as growth factors, chemokines, hormones and phorbol esters (Mendoza et al., 2011). Another possible source for activation of this pathway could be cytokines such as Interleukins.

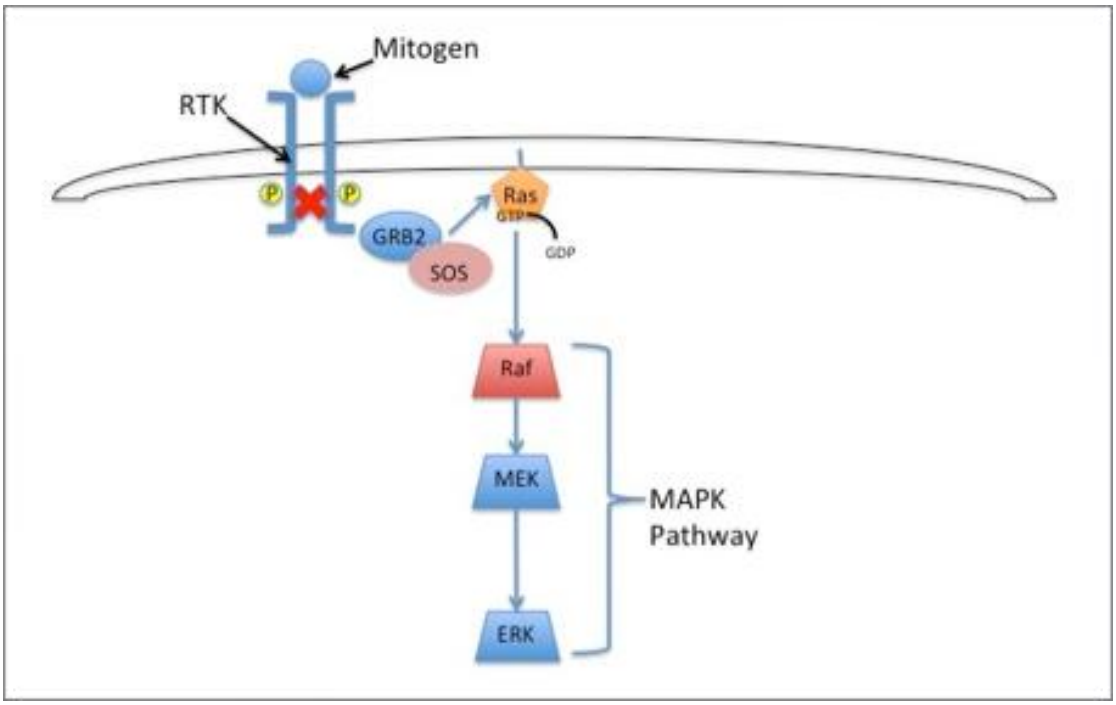

Figure 1. The Ras/ERK Pathway
Activation of the Ras pathway by a mitogen at an RTK that leads to activation of the ERK MAPK cascade

Once a signal is encountered, cross phosphorylation of tyrosine residues occurs on the RTKs, and ultimately the activation of Ras, a GTPase (McKay & Morrison, 2007). After phosphorylation the RTK activates an adaptor protein, GRB2 (Sigal, 1988). GRB2 then associates with a guanine nucleotide exchange factor (GEF), SOS, which allows for the exchange of GDP for GTP by Ras (Mendoza et al., 2011). Figure 1 depicts the interaction of RTK, GRB2, SOS, Ras, and the ultimate activation of the MAPK cascade activated by a mitogenic ligand. MAPK pathways follow a specific sequence of cascading phosphorylation, in the form of MAPK kinase kinase (MAPKKK), then MAPK Kinase (MAPKK), and finally

MAPK. At first, a GTPase, in this case Ras, is activated by an extracellular signal, which leads to the activation of a GTPase-regulated kinase, Raf, then phosphorylation of an intermediate kinase, MEK1/2, and finally phosphorylation of ERK1/2. Then ERK 1/2 leads to the activation of various transcription factors (Mendoza et al., 2011).

ERK1/2 is a serine/threonine protein kinase return can phosphorylate residues of transcription factors (Crews, Alessandrini, & Erikson, 1991). Once phosphorylated by MEK1/2, ERK1/2 is a critical aspect of cell proliferation (Rozengurt, 2007). This had led to the belief that ERK plays a major role in oncogenesis in many malignancies (Mendoza et al., 2011). Without the activation of ERK, a cell cannot progress through the cell cycle. The phosphorylation of serine and threonine residues of transcription factors are required for cells to proceed through G1 of the cell cycle (Meloche & Pouysségur, 2007). Depending on which residues of transcription factors are phosphorylated by ERK, the cell cycle may or may not progress.

Phosphorylated residues on c-Myc determine cell cycle arrest and progression (Chang et al., 2003). Depending on which residue is phosphorylated, c-Myc will either hetero-dimerize with Max or Mad, two other transcription factors (Figure 2). If dimerization with Max occurs, the cell cycle progresses, while dimerization with Mad results in inhibition of cell survival and proliferation genes (Mendoza et al., 2011).

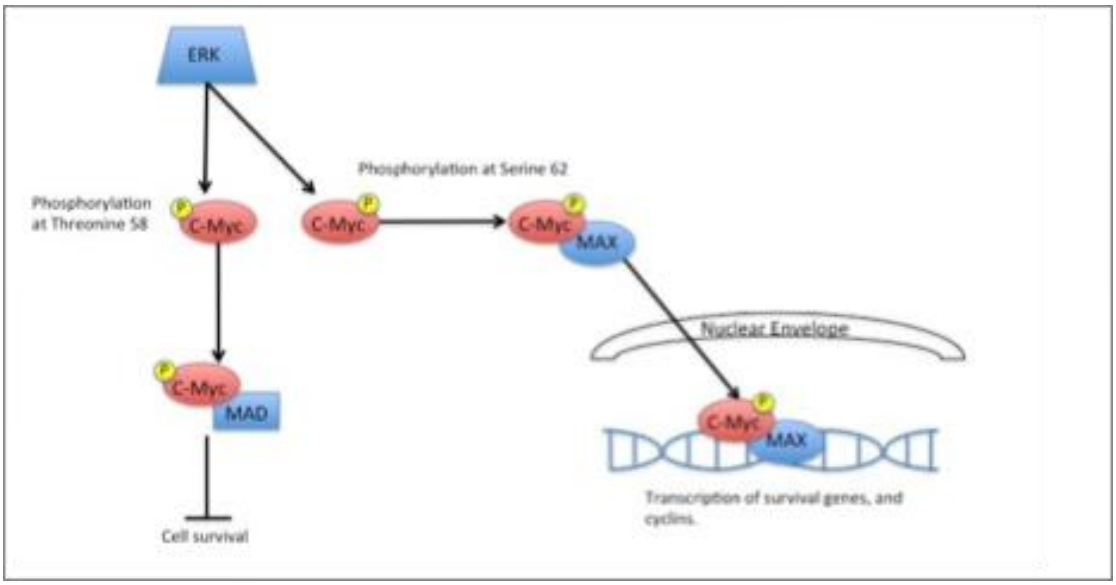

Figure 2. Phosphorylation of c-Myc
Phosphorylation of c-Myc at residue Threonine 58 leads to inhibition of cell survival. Where as, phosphorylation at residue Serine 62 leads to transcription of survival genes, specifically cyclins

C-Myc:

C-Myc, a transcription factor, activates transcription of key genes within cells that regulate cell proliferation or apoptosis depending on the amount of expression of c-Myc at times within the cell (Chang et al., 2003). Depending on the amount of c-Myc expression, a cell can undergo proliferation and differentiation, or in the case of de-regulated c-Myc, as in cancer cells, continuous proliferation in response to pro-growth signals (Dang, 1999). A normal amount of c-Myc expression regulates controlled proliferation and the cell cycle (Nasi et al., 2001).

The progression of the cell cycle can be directly activated by c-Myc. In particular, c-Myc has direct transcriptional activation affects on cyclin D1, D2, and

Cdk4 (Chang et al., 2003). Cyclin D1 is especially important for the activation of the cell cycle and mitotic events and is in fact required for the progression of the cell through the G1 phase of the cell cycle. Also Cdk4 is responsible for the phosphorylation and activation of cyclin D1 (Day et al., 2009). C-Myc is not absolutely required for the expression of these cyclins and Cdk, or cyclin dependent kinase; it can however act as a promoting agent for cyclin and Cdk transcription (Chang et al., 2003). This means that c-Myc activation can determine cell growth and mitotic events within the cell.

Activation of c-Myc occurs by phosphorylation by ERK1/2 (Figure 2). The phosphorylation can take place at either Threonine 58 (T58) residues or Serine 62 residues (S62). Depending on the phosphorylated state of these residues, c-Myc is either degraded by proteolysis (T58) or activated for hetero-dimerization (S62) (Wang et al., 2011). Once phosphorylated at the S62 residue, c-Myc can then hetero-dimerize with Max (Wang et al., 2011). Studies in yeast have shown that without this dimerization, c-Myc alone cannot bind DNA and thus cannot promote transcription (Amati et al., 1993b).

However, Max can also act as an inhibitor for the cell cycle and promote apoptosis if Max/Max dimerization occurs; these Max/Max dimers can and will bind to the same DNA sequences as c-Myc/Max dimers (Amati et al., 1993a).

C-Myc in non-transformed cells will only be expressed in response to mitogenic stimuli, but can be constitutively expressed in transformed cells and

therefore does not require mitogenic activity (Luscher & Eisenman, 1990a, 1990b). Such a constitutive expression occurs in BL (Mangani et al., 2012).

Since its discovery, c-Myc has been associated with viruses. Initially c-Myc was discovered to be a homologue of v-Myc, a protein found in Avian Myelocytomatosis virus (Vennstrom et al., 1982). This linkage of c-Myc and unregulated activity remains with EBV infected BL. In EBV infected cells the addition of exogenous c-Myc from BL causes oncogenic activity and down regulates endogenous c-Myc activity (Lombardi et al., 1987). Also, a translocated chromosome 8 and 14 are required to cause oncogenic activity of c-Myc in BL. This translocation occurs in an immunoglobulin locus and leads to the constitutive activity of c-Myc and uncontrolled cell proliferation (Erikson et al., 1983). This un-regulated activity of c-Myc is considered to be a hallmark of BL pathogenesis, but it is not known what role if any EBV actually plays in this activity, or whether c-Myc activity can be up regulated in non-BL cell lines that are infected with EBV (Mangani et al., 2012). It is possible that mitogenic activity could activate Ras/ERK and lead to the dimerization of c-Myc and Max in EBV infected cells and promote cell proliferation. The mitogenic activity could be enacted by an interleukin such as IL-10.

Interleukin-10 Association with Burkitt's Lymphoma, EBV, Malaria and HIV:

Since its discovery by Dennis Burkitt in 1962, BL has been associated with Malaria infection. It has been detailed that in areas that are more prone to climatic changes, such as increased rainfall or are associated with greater Malarial risk, cases of BL are elevated (Kafuko & Burkitt, 1970). EBV was first discovered, by Michael Epstein and Yvonne Barr, in BL tissue culture and then discovered to be the infectious agent of infectious mononucleosis (Kafuko & Burkitt, 1970). More recently, supporting evidence has arisen that further suggests a relationship between EBV, Malaria and HIV in Africa (Rainey et al., 2007). Among HIV patients and BL patients, it was found that antibodies for EBV were present in most infected individuals, also it was noted that individuals resided in mosquito-borne disease areas that were common for Malaria (Rainey et al., 2007).

There is also a probability that IL-10 plays a role in BL pathogenesis. It has been noted that individuals with active Malaria infections secrete large quantities of IL-10, of around 2000pg/mL, when serum samples have been collected (Imai et al., 2011). More recently it has been noted that IL-10 is secreted in large amounts, and acts as an autocrine growth factor, from B-cell lines derived from BL patients (Benjamin et al., 1992). Also lines derived from patients with B-cell lymphomas and AIDS are now known to also secrete large quantities of IL-10 and the IL-10 consequently acts as a growth factor for these cells (Masood et al., 1995). Most recently it has been shown that RNA derivatives from EBV encode

IL-10 and can thus support growth of BL, specifically when associated with Latent Membrane Protein 1 (LMP1) a protein encoded for by the EBV genome (Kitagawa et al., 2000). EBV-encoded RNAs or EBERs caused increased IL-10 production in BL cells due to the encoding of an IL-10 homologue (Kitagawa et al., 2000). The evidence that IL-10 is associated with EBV and may account for growth in BL remains to be investigated. It is probable that the IL-10 produced by EBV infection plays a role not only in BL growth but also can promote proliferation in non-BL cells infected with EBV. This may be due to the activation of Ras/ERK and phosphorylation of c-Myc.

Aims of Study:

An understanding of the interactions of interleukin-10 and its effects on the Ras/ERK pathway activation of c-Myc and the cell cycle, within EBV infected cells, could ultimately lead to novel therapeutic targets for treatment and prevention of BL. In order to accomplish this several cellular interactions must be addressed. First, it must be considered that Interleukin-10 is a promoting factor for c-Myc, in EBV infected cells; secondly, that Ras/ERK pathway leads to the activation of c-Myc; and finally, that c-Myc activation leads to the production of Cyclin D1.

Interleukin-10 as a Promoting Factor for c-Myc:

Since the Ras/ERK pathway activates c-Myc, and IL-10 is considered to be an autocrine growth factor in BL (Benjamin et al. 1992), is it possible that IL-10 could ultimately lead to the activation of c-Myc within EBV infected cells. If this is true then IL-10 is also a growth factor for EBV infected cells. Treating cells with IL-10 and performing Western Blot analysis tested for total c-Myc present.

Ras/ERK Activation of c-Myc:

If c-Myc is activated in EBV infected cells by IL-10, then it is probable that the activation of c-Myc is due to the activation of the Ras/ERK pathway. If the Ras/ERK pathway is an integral part of c-Myc activation of EBV infected cells, then it can be said that phosphorylation by ERK leads to the activation of c-Myc. Also, depending on the phosphorylated state of c-Myc it can be determined if IL-10 and the Ras/ERK pathway promotes cell cycle progression in EBV infected cells. IL-10 treated cells were tested for the phosphorylation state of ERK, as well as for c-Myc phosphorylation; this was ascertained using Western Blot and a comparison of the amount of phosphorylated ERK to total amount of ERK was determined. In addition, total versus phosphorylated c-Myc was accounted for, using immunoprecipitation and Western Blot.

Cyclin Promotion by c-Myc:

In order to determine the state of the cell cycle within EBV infected cells due to IL-10 treatment, cyclin D1 production must be accounted for. If after treatment of IL-10, the Ras/ERK pathway is activated, c-Myc is phosphorylated, and cyclinD1 is transcribed then it is probable that IL-10 is in fact a growth factor for EBV infected cells. Therefore Western Blot analysis was performed in order to obtain the total amount of cyclin D1 present in the cells treated with IL-10.

If IL-10 is a mitogenic agent for B cells then novel targets within the Ras/ERK pathway could be explored for therapeutic measures and the state of the cell cycle in EBV infected cells could be arrested. The cessation of the cell cycle in EBV infected cells by targeting components of the Ras/ERK pathway could lead to better treatments and prevention of BL.

CHAPTER II

MATERIALS AND METHODS

Cell Culture and IL-10 Treatment:

Cells were grown in culture and treated with IL-10 or left untreated. Three cell lines were used: Raji, a BL line, IM9, an EBV positive non-BL line, and 8226, a non-EBV and non-BL line. The cells were grown in RPMI-1640 (Thermo Scientific) medium, with 10% fetal bovine serum (Atlanta Biological), and penicillin, streptomycin and fungicide (GIBCO). Cell culture was maintained at 37 degrees Celsius, with 5% CO_2. Before experiments all cells were serum starved by centrifuging stock cell lines at 3000 rpm, then removing the RPMI containing 10% fetal bovine serum and replacing the serum containing media with media devoid of fetal bovine serum. Cells were then serum starved for 24 hours before again centrifuging the cells removing the medium and replacing the medium with RPMI containing 10% fetal bovine serum. This was done to ensure cell cycle synchronization at G0. Cell counts were not completed. Cells were then plated with 1 mL of stock cells solution and 5 mL of full medium in 60mm tissue culture plates. After plating, treatment of the cells was completed. Plates of treated cells from each line received 0, 0.1 nM or 0.2 nM of IL-10 (Cell Signaling Tech.), for 24 hours, as well as for 48 hours for Cyclin D experiments.

Protein Extraction:

After treatment, cells were harvested. This was done by centrifugation at 3500 rpm for 10 minutes. Cellular pellets from centrifugation were incubated with lysis buffer (0.25 M NaCl, 0.1% NP40, 50 mM HEPES pH 7.0, 5 mM EDTA, and protease/phosphatase inhibitors (Thermo Scientific)). Once incubated with lysis buffer the cells were freeze/thawed for three cycles each consisting of 10 minutes. After three cycles of freezing and thawing the extracts were micro-centrifuged at 12000 rpm at 4 degrees Celsius for 10 minutes. The supernatant containing protein extracts was then collected and prepared for SDS-PAGE.

The extracts were subjected to a Bradford protein assay test using a spectrophotometer (Eppendorf) in order to obtain protein amounts in ug/ul.

SDS-PAGE:

The extracts were then separated using SDS-PAGE. 20ug of the extracts were placed into 2x protein loading buffer (125 mM Tris pH 6.8, 20% glycerol, 10% Beta-mercaptoethanol, 4% SDS, .025% Bromophenol blue) and loaded into a 10% polyacrylamide gel. The gel was then subjected to electrophoresis in 1x SDS running buffer solution (25 mM Tris, 192 mM glycine, 0.1% SDS).

Western Blot Analysis:

After electrophoresis gels were placed into transfer cassettes (Bio-Rad) along with Immobilon (EMD Millipore) paper and the proteins were transferred. The cassettes were placed into a transfer buffer consisting of 1x electroblotting buffer (0.25 mM Tris, 1.92 M glycine, pH 8.3) plus 20% methanol. Gels were then transferred overnight at 100 mAmps, after which the Immobilon blot was placed in a 5% non-fat milk, 1 x PBS and 0.1% Tween-20 blocking solution and incubated at 4 degrees Celsius for one hour.

Immunostaining:

After blocking, the blot was placed into a 1:1000 dilution of appropriate rabbit or mouse primary antibody and 5% non-fat milk, 1 X PBS and 0.1% Tween-20 blocking solution. A rabbit monoclonal anti-ERK (Cell Signaling Tech.) and rabbit monoclonal anti-Phospho-ERK antibodies (Cell Signaling Tech.) were used for the ERK experiments. A rabbit monoclonal anti-c-Myc antibody (Cell Signaling Tech.) was used for Western-Blot. For the immunoprecipitation the same anti-c-Myc antibody was used, but mouse monoclonal anti-Phospho-Serine (Santa Cruz Biotech.) and anti-Phospho-Threonine (Santa Cruz Biotech.) antibodies were also used. Finally, for the Western Blot of cyclin D1, a mouse monoclonal anti-cyclin D1 antibody (Santa Cruz Biotech.) was used. This was then incubated at four degrees Celsius overnight. After incubation the blot was washed three times, at 10 minutes each, in a 1 X PBS and 0.1% Tween-20 wash

solution. Following washing, the blot was placed in a 1:5000 goat anti-rabbit, or anti-mouse, secondary antibody-conjugated to HRP (Both from DSHB) in 5% non-fat milk, 1 X PBS and 0.1% Tween-20 blocking solution and incubated at room temperature for one hour. The blot was then washed 3 times more in washing solution. At this point ECL developing agent (BioExpress) was added to the blot. Each Blot was also probed with a mouse monoclonal anti-tubulin antibody (DSHB) to ensure equal loading of protein extracts.

Image Developing and Protein Band Quantification:

The developed blot was imaged using a Bio-Rad ChemiDoc™ XRS+ System, and ChemiDoc Image Lab™ image acquisition and analysis software. After imaging, protein bands were quantified using Bio-Rad QuanityOne™ software.

Immunoprecipitation of c-Myc:

Immunoprecipitation of c-Myc was done for all cell types. This was accomplished by first growing cell culture of previously serum starved cells, in 100mm tissue culture plates. Three plates were grown for each cell type,

consisting of 5mL serum starved cells in 5mL of RPMI for a total of 10mL per plate. One plate was not treated, while one was treated with 0.1nM IL-10 and the last plate received 0.2nM IL-10. All plates were incubated for 24 hours.

At this point the cells were harvested and pellets were placed in 250uL c-Myc Binding Buffer (20mM Tris pH 7.5, 50mM NaCl, 0.5% Triton X-100, 0.5% deoxycholic acid, 0.5% SDS, and 1mM EDTA, and protease/phosphatase inhibitors (Thermo Scientific)). The cells were then sonicated, with Fisher Scientific sonicator for 10 seconds, and centrifuged at 4 degrees Celsius for 10 minutes, after centrifugation the supernatant protein levels were measured using a Bradford protein assay test (Eppendorf). Then amounts equal to 100ug of total protein were added to 250uL of Binding Buffer and anti-c-Myc monoclonal rabbit antibody (Cell Signaling Tech.). These were then incubated at 4 degrees, under constant rotation, for one hour. Upon completion of the one-hour incubation, 40uL of Protein AIG Agarose beads (50 % slurry) (Santa Cruz Biotech.) was added to each sample and again incubated in the same manner as mentioned above for another hour.

At the end of this hour the samples were centrifuged and the supernatant removed; the remaining pellets were then washed 3 times with the Binding Buffer. At this time 20uL of 2X protein dye was added to the samples and SDS-PAGE and then Western Blotting were completed. Three Western Blots were performed for each cell line and then one of each was probed for total c-Myc

(Cell Signaling Tech.), phospho-threonine (Santa Cruz Biotech.), and phospho-serine (Santa Cruz Biotech). The amounts of phospho-threonine and phospho-serine were then compared to the total amount of c-Myc present.

Cell Viability:

Cell viability counts for both treated and untreated cell lines were done using a Guava easyCyte™ flow cytometer. Centrifuged cells were washed with 1X PBS and placed into GuavaViaCount solution and loaded into the flow cytometer. Cell counts were obtained using Guava easyCyte™ 6-2L software.

Microscopy and Imaging:

Cells were observed for phenotypic changes using an inverted Olympus® light microscope. Morphological differences such as, cell shape and size, were recorded by obtaining images of treated and untreated cell lines at 200x magnification.

Repetition and Statistics:

All experiments were done in triplicate and results from protein band quantification were analyzed using a student t-test, using Microsoft® Excel.

CHAPTER III

RESULTS

Viability of EBV+ Cells is not Affected by IL-10 but non-EBV Cells are Less Viable:

In order to determine if IL-10 has an effect on the overall viability of B cells, cells were grown in culture with either 0, 0.1 nM, or 0.2 nM IL-10 for 24 hours. The cells were then harvested and incubated with GuavaViaCount solution. Viability was assessed using the Flow Cytometer to determine if the cell nucleus was intact

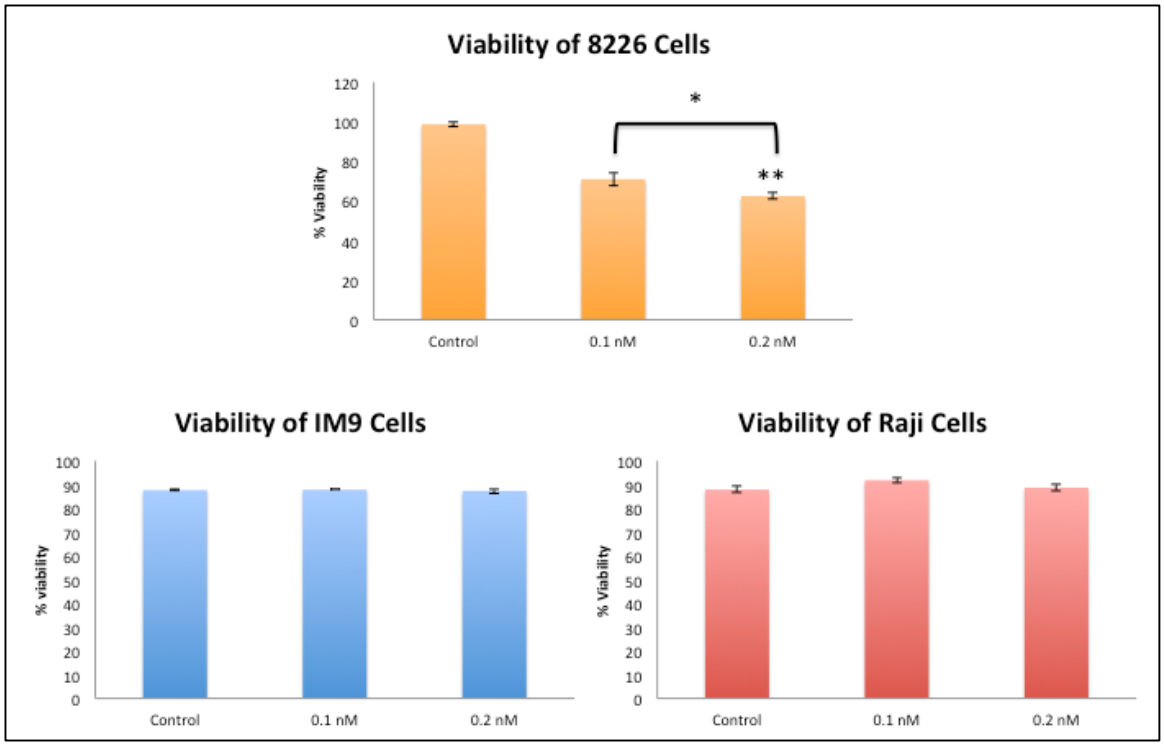

Figure 3. Overall viability of B cells in response to IL-10
*p<0.05 when compared to the control
**p<0.05 when compared to 0.1 nM of IL-10

The overall viability of cells treated with either 0.1 nM or 0.2 nM IL-10 was not affected in EBV-positive cells, but was decreased in the non-EBV cell line, 8226 (Figure 3). The decrease in viability for 8226 cells was significant ($p<0.05$) between the control and both the 0.1 nM treatment group and 0.2 nM treatment group. Additionally a significant decrease ($p<0.05$) in viability was found between the 0.1 nM group and the 0.2 nM group.

After Treatment with IL-10 EBV+ Cells Change Morphologically:

To assess whether IL-10 effects the appearance of B cells, cells were treated with 0, 0.1 nM, or 0.2 nM IL-10 and grown in culture for 24 hours. After treatment with IL-10 cells were observed under the microscope and changes in cell shape and size were noted for each cell line (Figure 4).

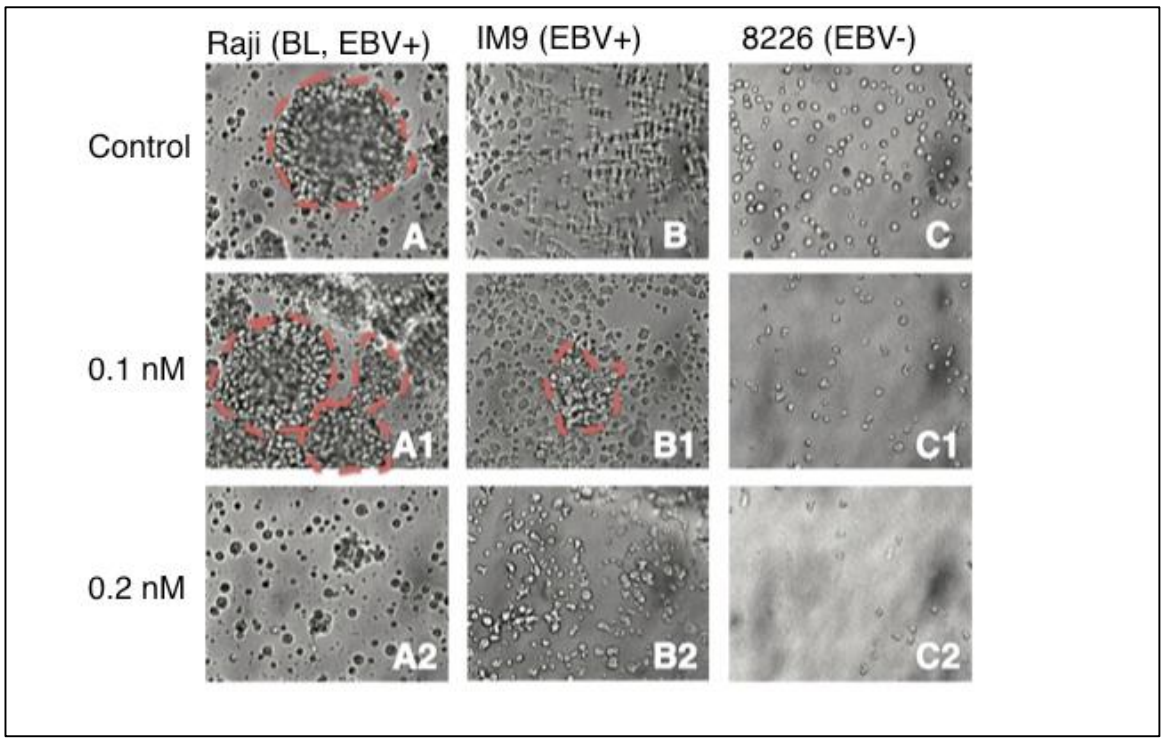

Figure 4. Images showing changes between cell lines before and after IL-10 treatment. Obtained using an inverted microscope (200x).

When IM9 (EBV+) cells were treated with 0.1 nM IL-10 (B1) the cells began to aggregate together and form masses of cells that adhered together much like the observed control state of the Raji cells (BL, EBV+) (A). The masses formed by Raji cells increased in size with 0.1 nM IL-10 treatment as well (A2). IM9 cells

also took on a more rounded and somewhat smaller appearance; similar to the untreated Raji cells. With 0.2 nM IL-10 treatment both IM9 and Raji (A2 and B2) cells decreased in number and had less formation of masses.

It can also be noted that 8226 (EBV-) cells began to undergo what appeared to be apoptosis, or possibly necrosis, after treatment with 0.1 nM IL-10 (C1) and were mostly dead leaving only cellular debris after 0.2 nM treatment (C2).

Phosphorylation of ERK Increases with IL-10 Treatment in EBV+ Cells:

To quantify the amount of phosphorylation of ERK in response to IL-10, cells were again grown for 24 hours under the same treatment conditions as mentioned above. After 24 hours proteins were extracted and SDS-PAGE and Western Blot were performed for each cell line (Figure 5). Proteins were quantified and the results were placed into an Excel sheet for statistical analysis.

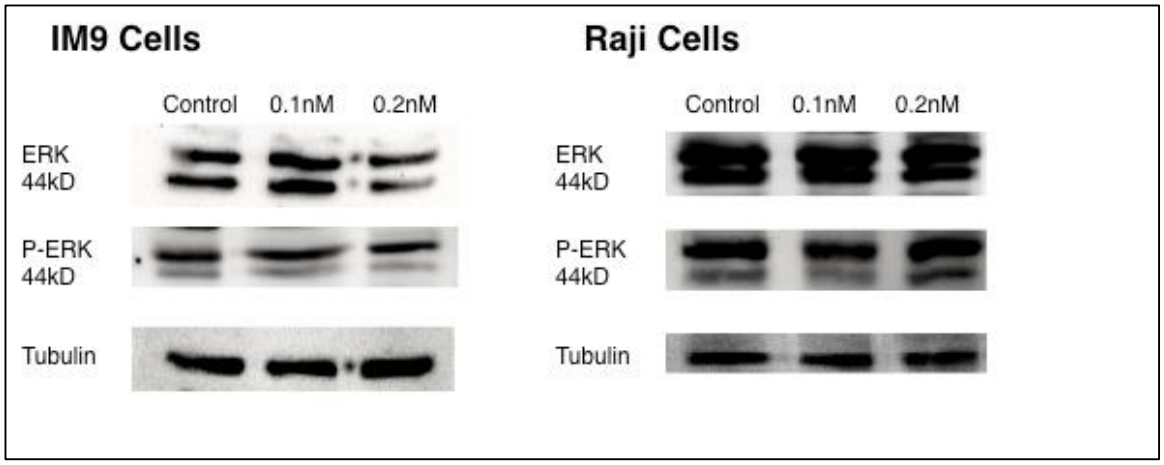

Figure 5. Western Blots of the phosphorylation state of ERK in EBV + B cells.

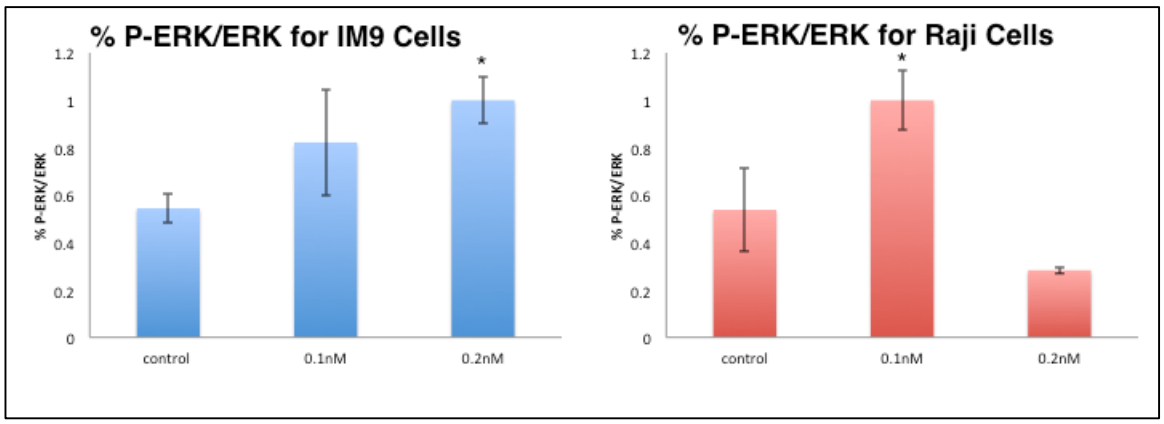

Figure 6. The percent of phosphorylation of ERK in relation to total ERK for EBV + B cells. *p<0.05 when compared to the control group.

When considering the levels of expression of ERK in the treated cells and obtaining percentages of phosphorylation of ERK, it was observed that a significant increase in the ratio of P-ERK/ERK occurs in both EBV+ cell lines. The increase was significant with $p<0.05$ in the 0.2 nM IL-10 treatment in IM9 cells after 24 hours, and the increase was significant with $p<0.05$ in the 0.1 nM IL-10 treatment group for Raji cells after 24 hours (Figure 6). Due to an overall

lack of protein the levels of expression in EBV- (8226) cells were not obtained. This is most likely due to decreased viability of these cells after IL-10 treatment. This was demonstrated by the possible apoptotic activity observed under the microscope and a decreased viability (Figure 3 and 4).

C-Myc Expression Levels Increase in EBV+ Cells but not in BL:

To determine if IL-10 causes a change in expression of c-Myc with in B cells, all three cell lines were again treated and grown in culture for 24 hours. After 24 hours the cells were harvested and SDS-PAGE and Western Blot were performed (Figure 7). The levels of c-Myc were not obtained in 8226 cells due to an overall lack of protein derived from these cells. Following the quantification of protein levels the data were placed into a spreadsheet and statistical analysis was performed (Figure 8).

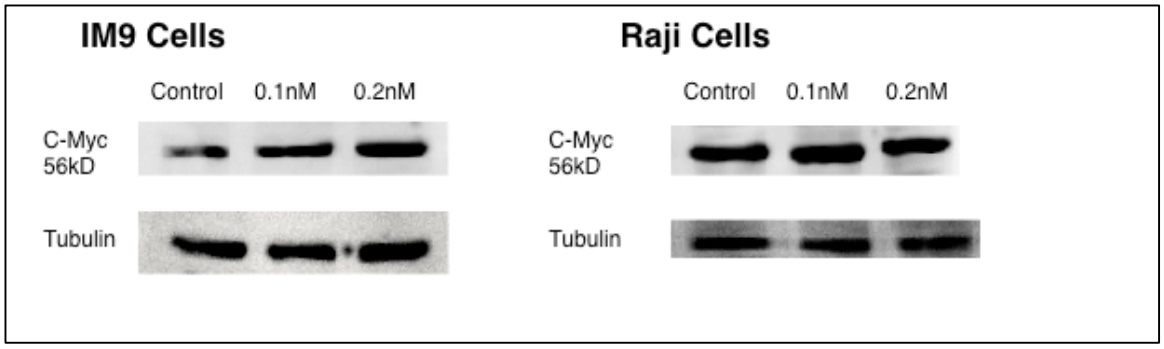

Figure 7. Levels of total c-Myc expression for EBV + B Cells.

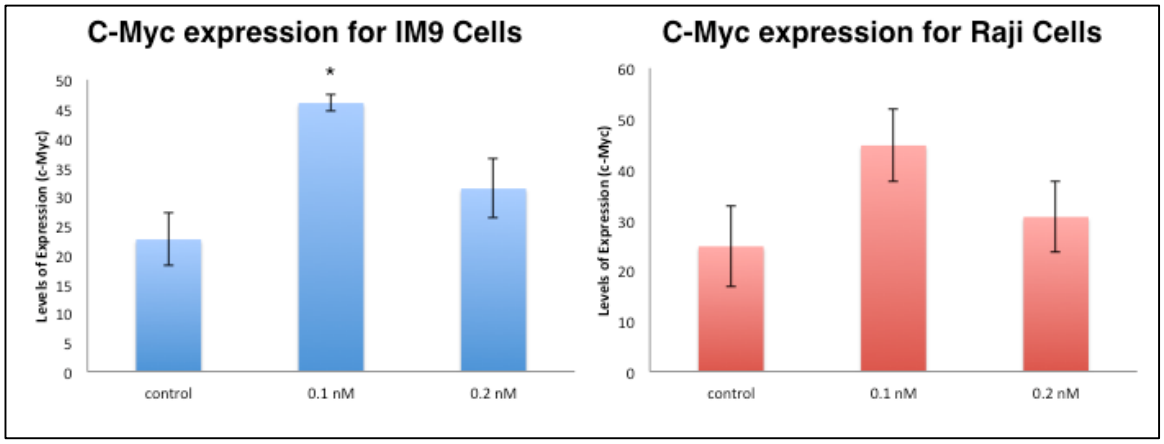

Figure 8. Total levels of transcription factor c-Myc for EBV + B cells.
*$p<0.05$ when compare to the control.

When the total levels of c-Myc expression were obtained (Figure 8) in both IM9 and Raji cell lines, c-Myc expression significantly increased, with $p<0.05$, in IM9 cells within the 0.1 nM IL-10 treatment group. There was no significant change in expression in the Raji cell line.

Overall Phosphorylation of c-Myc Decreases with 0.1 nM IL-10 Treatment in EBV+ Cells:

To assess if any differences in phosphorylation of c-Myc occur in response to IL-10 immunoprecipitation of c-Myc protein was performed. Initially IM9 and Raji cells were grown in large amounts and treated with 0, 0.1 nM, or 0.2 nM IL-10. The cells were harvested and c-Myc was precipitated from the protein extracts. The precipitated protein was separated by SDS-PAGE and then Western Blots were performed. Western Blots were probed for total c-Myc, phospho-Serine, and phospho-Threonine (Figure 9). Due to the absence of c-Myc in 8226 cells an immunoprecipitation was not completed.

The levels of protein were then obtained. The amount of phosphorylation at each residue was compared to total amount of c-Myc present and percentages were obtained. The data were placed into a spreadsheet for statistical analysis (Figure 10).

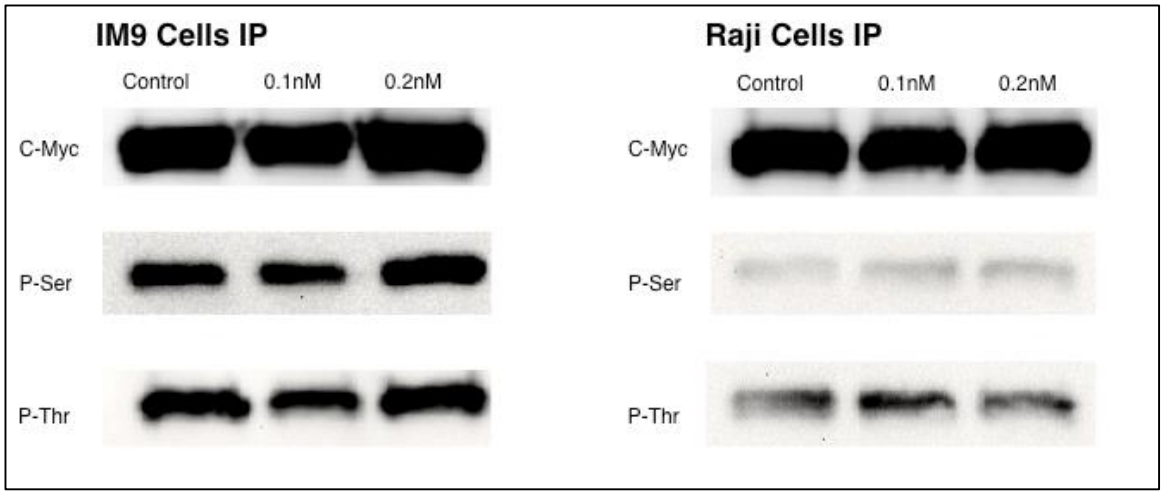

Figure 9. Immunoprecipitation showing the relative phosphorylation of c-Myc at Serine and Threonine residues.

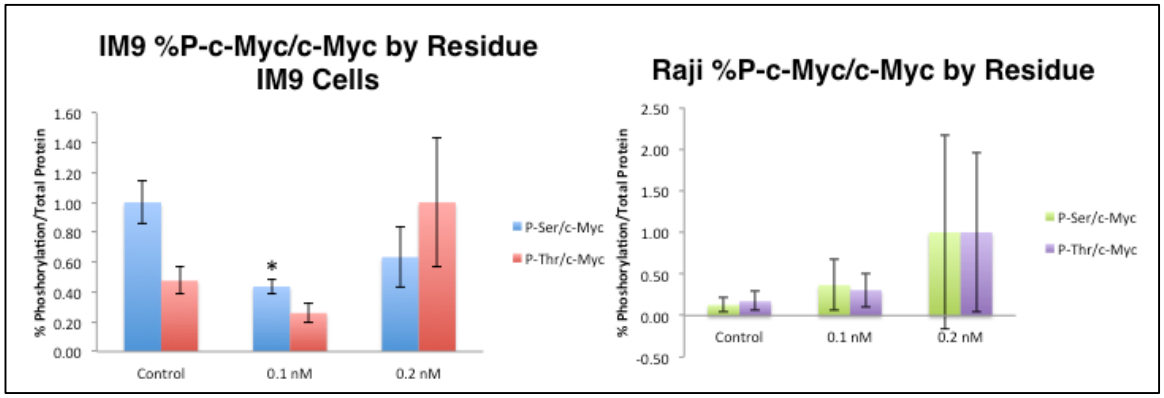

Figure 10. Immunoprecipitation of percent phosphorylation by residue for c-Myc.
*p< 0.05 when compared to the control.

When immunoprecipitation for c-Myc (Figure 9) was completed on Raji and IM9 cells it was found that in IM9 cells the levels of phosphorylation at the Serine 62 residue of c-Myc, in the 0.1 nM IL-10 group, decreased with $p<0.05$ (Figure 10) when compared to the control. There was not a significant change in Raji cells.

Cyclin D1 Expression Increases in EBV+ Cells with IL-10 Treatment:

To ascertain if IL-10 causes any significant effects to the levels of cyclin D1 expression cells were again treated and grown in culture. However, cells were grown for 48 hours instead of 24 hours due to cyclin D1 appearing later in the cell cycle. After treatment cells were harvested, proteins extracted and SDS-PAGE and Western Blot were performed (Figure 11). The data obtained from the Western Blot were then placed into a spreadsheet and statistical analysis performed.

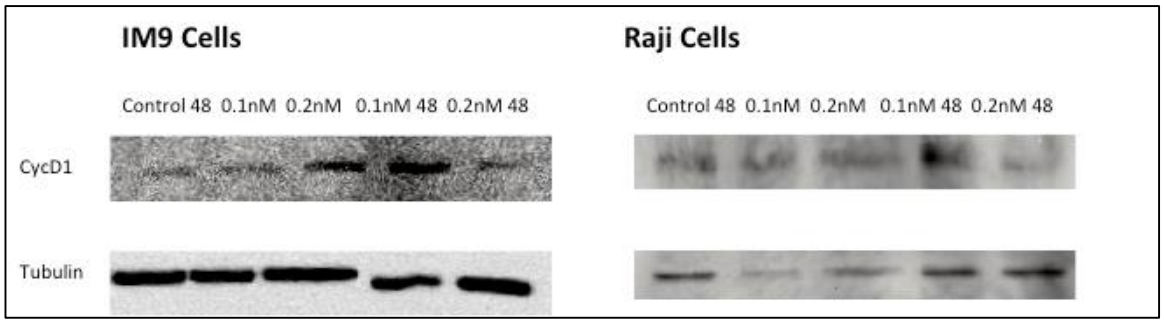

Figure 11. Levels of expression of cyclin D1 expression in EBV+ cells.

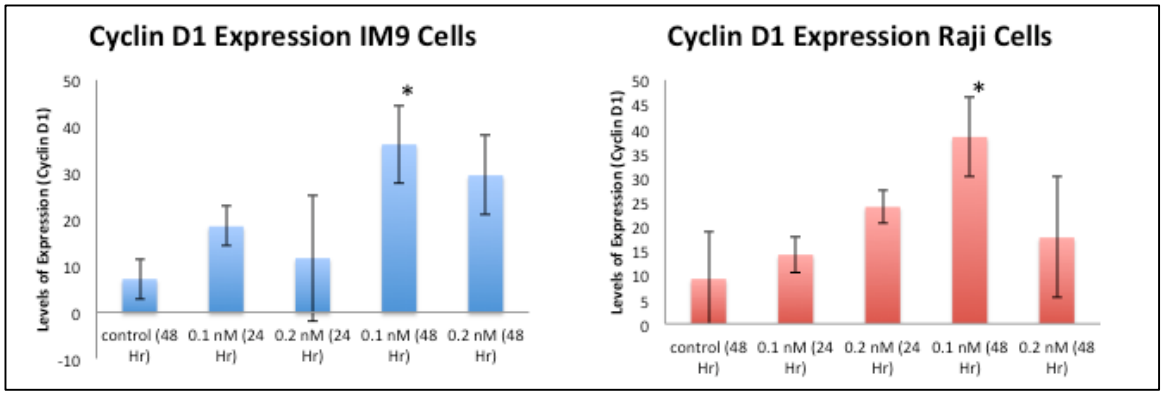

Figure 12. Cyclin D1 expression in EBV+ cells.
*p<0.05 when compared to the control.

When looking at cyclin D1 expression (Figure 11), it was found that in the 0.1 nM IL-10 treatment group cyclin D1 after 48 hours had a significant increase, with $p<0.05$ when compared to the control (Figure 12), in both IM9 and Raji cell lines. Again levels for the 8226 cell line were not obtained due to an overall lack of protein.

CHAPTER IV

DISCUSSION

Viability in non-EBV Cells is Decreased with IL-10 Treatment:

The results of the viability test that were performed on EBV – cells (8226) indicated that cells, which do not possess the virus, begin to die in response to IL-10, which is the normal response associated with B cells that are in no way transformed. However, both cell lines that were EBV + (Raji and IM9) exhibited little to no difference in viability in response to IL-10. This indicated that presence of the virus does in fact change the cell's response to IL-10, causing an abnormal response.

This response could be due to the IL-10 homologue encoded by LMP1 gene from the viral genome. The virus changes the cell's response so that, during latency viral products are not toxic to the cell. How this occurs remains unknown; by possibly examining the amount of LMP1 transcripts this could be further explained. Also, if LMP1 could be inhibited and then EBV + cells treated, it could be noted whether or not the cells become less viable in the presence of

IL-10. This would signify that LMP1 does increase the viability of cells in the presence of IL-10. This needs to be addressed in the future and could be done by utilizing qRT-PCR and examining the amount of LMP1 produced by EBV + cells.

EBV+ Cells Exhibit a Morphology Similar to BL in the Presence of Low Doses of IL-10:

The IM9 cells, when treated with 0.1 nM IL-10 began to appear similar to Raji cells. After treatment the cells aggregated and formed masses similar to the Raji control as well as the Raji treated 0.1 nM IL-10 group. In higher doses (0.2 nM IL-10) this change in morphology was reversed in IM9 cells and actually resulted in Raji cells exhibiting a more normal morphology.

The 8226 cells appear to be apoptotic with 0.1 nM IL-10 treatment and only cellular debris was present with 0.2 nM IL-10 treatment. This result aligns with the viability data that were also obtained from this cell line that showed a significant decrease in the number of viable cells when treated with IL-10.

The changes present in the IM9 cell line are probably likely due to the increased proliferation of cells. The increased proliferation could lead to a mass formation due to the number of cells being replicated during a decreased amount of time. With faster replication times cells have a higher probability of experiencing mutations and therefore are likely to become cancerous. This could be confirmed for the IM9 cells with further genetic screening.

IL-10 Up-regulates ERK and Proliferation in EBV Infected B Cells:

The results of the ERK and phospho-ERK experiments demonstrate that IL-10 plays a role in up-regulation of replication of EBV-infected B cells during a latent infection. Binding of IL-10 to its corresponding RTK increases the activity of the Ras/ERK pathway, phosphorylating ERK. When observing the changes in phosphorylation of ERK in relation to treatment in the IM9 cell line, the cells appear to have increased activity that is relatable to the increased activity seen in BL. The results from the IM9 cells correlate with the results from the known BL cell line Raji.

The results do not fully confirm that IL-10 is responsible for BL it merely suggests that IL-10 may play a significant role in the initial increase in cell proliferation associated with BL. Further research comparing the amount of mRNA transcripts for ERK could support this to a greater extent. If the transcripts are also elevated, an overall increase of ERK production by the cell could possibly be attributed to increased levels of IL-10. Also the amount of viral genes that are transcribed and whether or not viral proteins are elevated after IL-10 treatment could further relate the increase in ERK activity to the virus and not endogenous cellular activity.

The Total Amount of c-Myc is Increased by IL-10, but Phosphorylation Decreases:

The increased activity of ERK when cells were exposed to IL-10 could account for the increased presence of c-Myc, but does not explain the lack of phosphorylation of c-Myc associated with IL-10 treatment. It would be suspected that phosphorylation would increase due to increased kinase activity of ERK.

However, this is not the case; instead of an increase in phosphorylative activity there is a decrease in response to IL-10, especially in the 0.1 nM group. What can be said about this is that it resembles the response of the Raji cells to 0.1 nM treatment. This resemblance actually adds credibility to the thought that IL-10 plays a role in the development of BL, since the EBV + cells begin to resemble the intracellular activity that is associated with BL cells. This does not mean that c-Myc is constitutively active in the EBV + cells after treatment but it could point to the potential for constitutive activity to develop. Karyotyping and observing whether or not a chromosomal translocation has occurred, such as the translocation from chromosome 8 to 14 that is prevalent in BL could further assess this.

Cyclin D1 Increases in EBV + Cells after 48 Hours in Response to IL-10:

Cyclin D1 was found to be elevated in both IM9 and Raji cell lines after 48 hours in response to 0.1 nM IL-10. The increased activity of ERK combined with increased presence of c-Myc in turn translates to increased transcription of Cyclin D1 and progression of the cell cycle thus initiating replication.

The results indicates that IL-10 is in fact a mitogen for EBV + B cells. Since the Ras pathway is activated, ERK is phosphorylated, and c-Myc levels increase, it can be said that IL-10 follows the traditional cascade of an RTK receptor (Mendoza et al., 2011).

The mitogenic activity could possibly be a link between Malaria and BL. The increased presence of IL-10 during Malaria infection and a latent EBV infection could account for the initial stages of over-proliferation that accompany early BL pathogenesis. This is not the ultimate cause of BL but likely plays a role in its progression. To further investigate the effect of IL-10 on proliferation in the future transcript levels should be compared to protein levels after treatment, and inhibition studies should be completed to see if proliferation decreases when c-Myc is inhibited.

Viral genes and the exact role that they play in the increased proliferation associated with IL-10 should be assessed. The main viral gene responsible is more than likely LMP1 (Kitagawa et al., 2000).

Significance of Study:

The ultimate goal of this study was to determine if IL-10 could be considered an association between EBV and Malaria resulting in BL. The results of this study show that IL-10 does have the potential to act as a mitogen in EBV + B cells. This could ultimately lead to potential treatment targets for BL. More importantly this could lead to the development of preventative measures toward BL. Further understanding of this relationship is needed. But the results presented here are the beginnings of understanding a linkage between EBV and Malaria and the ultimate development of BL.

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