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Epstein-Barr virus (EBV) is a ubiquitous human herpesvirus that infects nearly 95% of the world's population. EBV is closely associated with many diseases, most commonly with infectious mononucleosis, though the virus is also linked to different types of cancer such as Burkitt's lymphoma, other non-Hodgkin's lymphomas, Hodgkin's lymphoma, gastric carcinoma, and nasopharyngeal carcinoma. Pesticides are globally used residentially and agriculturally and have been a topic of discussion with regard to their effects on human health. Chlorpyrifos, a neurotoxic acetylcholinesterase inhibitor, has been banned for residential use in the United States since 2000. However, it is still one of the most commonly used organophosphate pesticides for agriculture. Epidemiological case studies have investigated potential associations with individuals who have occupational organophosphate exposure (such as farmers) and certain types of cancer. However, many of these studies have inconclusive or conflicting results. An understudied area of investigation is interactions of ubiquitous human viruses and other environmental factors, such as pesticides, that may promote or further exacerbate adverse human health issues. It is equally important to understand if environmental factors affect virus-host interactions and by what cellular mechanisms. This dissertation investigates the effects of chlorpyrifos and its active metabolite, chlorpyrifos-oxon, on EBV-host interactions; specifically exploring the effects on EBV and B-lymphocyte replication. Findings suggest that chlorpyrifos and chlorpyrifos-oxon exposure produce low levels of oxidative stress and DNA damage to B-lymphocyte cells to initiate cellular signaling

cascades to induce cell cycle arrest. Additionally, we find that pesticide exposure affects EBV lytic replication and latency in addition to cellular targets that are involved with viral and host-cell replication and regulatory functions. Overall, our results show that the presence of EBV appears to have a protective response to cells under cytotoxic stress. Findings from this study will contribute to a better understanding of EBV-host biology and interactions in the presence of exogenous environmental factors that may be harmful to human health.

INVESTIGATION OF ENVIRONMENTAL VIRUS-HOST INTERACTIONS:

CHLORPYRIFOS EFFECTS ON EPSTEIN-BARR VIRUS

AND B-LYMPHOCYTE REPLICATION

by

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APPROVAL PAGE

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CHAPTER I

INTRODUCTION AND BACKGROUND

1. Introduction

Epstein-Barr virus (EBV) is a ubiquitous human herpesvirus that infects nearly 95% of the world's population.[1] EBV is most commonly linked with infectious mononucleosis, which is characterized by fatigue, swollen lymph nodes, fever, sore throat, body aches, and rash. The virus is also associated with different types of cancer such as Burkitt's lymphoma, other non-Hodgkin's lymphomas, Hodgkin's lymphoma, gastric carcinoma, and nasopharyngeal carcinoma.[1] Pesticide use is a worldwide practice residentially and agriculturally and has been a topic of discussion with regard to potential adverse effects to humans. Chlorpyrifos is a type of organophosphate pesticide that is neurotoxic to insects.[2–5] Although certain organophosphates are banned or heavily restricted for residential use, chlorpyrifos is still used (with restrictions) in agriculture/commercial farming.[4,6–12] Epidemiological case studies have suggested a connection between pesticide exposure (especially for farmers or other individuals who have occupational exposure) and certain types of cancer including non-Hodgkin's lymphoma.[7–9,13] In contrast, meta-analysis studies (using patient data, farmer/worker interviews, and blood and urine sample analysis), also report there is little to no direct connection with pesticide use and cancer formation.[6,10,14–19]

An understudied area is the investigation of viruses interacting with other environmental factors, such as pesticides, to further promote adverse human health issues. However, it is equally important to understand the basic research and science aspects of environmental factors effects on virus-host interactions.

2. Background

2.1 Epstein-Barr Virus

Epstein-Barr virus (EBV) is a well-studied, double stranded DNA (Baltimore class I), gamma human herpesvirus (HHV-4) that infects over 95% of the world's population.[1] EBV has important research implications and impact due to its global presence in human populations. EBV is primarily orally transmitted through the exchange of saliva (kissing, sharing food or drink, using the same utensils), but the virus can also be transmitted through blood transfusions, organ transplants, and sexual contact.[1] Initial infection of the virus typically occurs early in life with little (cold and flu like) to no symptoms, while if infected at adolescence or later, individuals may observe common symptoms of infectious mononucleosis.[20,21] Once infected with the virus, the individual is a carrier of the virus for life. Most commonly known to cause infectious mononucleosis, EBV is also widely associated with cancers such as Hodgkin's and non-Hodgkin's lymphoma, Burkitt's lymphoma (a type of non-Hodgkin's lymphoma), EBV-associated gastric carcinoma, and nasopharyngeal carcinoma.[1] Immunocompromised individuals are more prone to acquire these malignancies associated with EBV. It is well established that cancer and tumor formation from EBV infection involve the latency state of infected cells and the switch back to lytic replication.[22–25]

2.2 EBV Infection and Replication

EBV has two mechanisms of replication, lytic replication and latency. Lytic replication is the process of producing new virions in host epithelial and B-lymphocyte cells and is a key step in virus pathogenesis.[22–25] Once the host cell is initially infected with EBV (primary infection), the virus can either infect B-lymphocyte cells and enter latency and/or infect neighboring epithelial cells where the virus continues lytic replication.[1,26–29] EBV glycoproteins attach to host cell receptors or integrins and enter the host cell through glycoprotein mediated fusion (epithelial cells) or endocytosis (B-lymphocyte cells).[1] For B cells, EBV glycoprotein gp350 binds to the host receptor CR2/CD21 before being endocytosed.[1,29,30] Other important EBV glycoproteins necessary for infection include gB that mediates fusion of the virus to the B cell membrane, and the gHgL and gp42 complex that interacts with human leukocyte antigen (HLA) class II and aids in B cell infection.[28,30–33] EBV entry into epithelial cells on the other hand is still an area that researchers continue to explore. From what we know now, epithelial cells do not utilize endocytosis, but fusion for viral entry via BMRF2 and integrins for binding, then gHgL and gB for membrane fusion.[1,32,34] It is important to note that gp42 is an important glycoprotein that mediates and regulates cell type tropism for EBV.[1,30,31,33,34]

After attachment, the EBV viral capsid enters the cytoplasm and is transported to the nucleus via microtubules.[1] Once in the host cell nucleus, the EBV genome is released and lytic replication resumes.[1] Three types of viral genes and proteins are transcribed and translated during lytic replication: 1) immediate-early, 2) early, and 3) late.[1] Immediate-

early genes and gene products are transactivators that regulate and promote synthesis of EBV early genes and proteins which are associated with viral replication.[1,35–37] EBV late genes and proteins are involved with structural and survival characteristics of EBV, such as capsid formation and gene products that are involved with evasion of the host's immune system.[1,27,37,38]

Reactivation of the lytic cycle from latency in B cells is important for maintaining production of new virions and contributing to EBV pathogenesis.[1,39,40] Various factors can contribute to reactivation of lytic replication including DNA damage and chemical agents like sodium butyrate (NaB) and12-0-tetradecanoyl phorbol-13-acetate (TPA).[24] Production of new virions can advance infection of host B cells to become latent which can lead to carcinogenesis through the expression of latency genes and proteins.[1,39,40] Thus, the production of new EBV virions or the reactivation of lytic replication is necessary for the production of new latent cells.

2.3 EBV Latency and EBV Associated Cancers

After primary infection in epithelial cells, EBV can infect naïve B cells (cells that have not been exposed to antigens).[1] B cells infected with EBV transform to become memory, immortalized B cells where the EBV genome circularizes and the cells replicate as lymphoblastoid cell lines (LCLs).[1,41–44] B-lymphocyte cells infected with EBV that express genes for latent proteins may lead to cancers like non-Hodgkin's Lymphoma (such as Burkitt's lymphoma) and immunodeficiency dependent lymphomas.[1,23,45–47] EBV latency has three programs (I, II, and III) and within these programs different types of genes are expressed that are associated with the development of lymphomas or other

cancers.[1,23,46,48] The main types of latency gene products include: EBV nuclear antigens (EBNAs), EBV latent membrane proteins (LMPs), EBV-encoded small RNAs (EBERs), and miRNAs.[1] These are important for maintenance of the viral genome episome, replication, and enhancement of cell survival (EBNA-1)[49,50]; B cell transformation (EBNA- 3A/3C and LMP-1)[51]; oncogene, activator to oncogenic signaling pathway, and a tumor necrosis factor receptor (LMP1).[41,44,52] EBNA-1 is expressed in all latency programs, while other latency protein expression is dependent on the program and progression of certain EBV-associated diseases (Table 1).

Table 1. EBV-Associated Diseases and Latency Proteins Expressed. Adapted from Fields Virology.[1]

Disease	**EBV Genes/Proteins Expressed**	**Latency Program**
Burkitt's Lymphoma	EBNA-1	1
Gastric Carcinoma	EBNA-1	1
Hodgkin's Lymphoma	EBNA-1, LMP1, LMP2	2
Nasopharyngeal Carcinoma	EBNA-1, LMP1, LMP2	2
Post-transplant Lymphoproliferative Disease	EBNA-1, EBNA-2, EBNA-3, EBNA-LP, LMP1, LMP2	3

Low levels of latent viral proteins allow EBV to evade an immune response by the host. Latency of EBV mainly occurs in B-lymphocyte cells and can occur soon after infection or can occur after lytic replication.[1,38] EBV can still replicate while dormant, however, mechanistically, replication occurs through the host cells' cellular division rather than viral replication mechanisms.[1,27]

2.4 EBV-Host Interactions

It is well known that EBV and other herpesviruses utilize and hijack host mechanisms/signaling pathways for replication and survival.[38,53] EBV protein expression can either activate or disrupt certain cellular pathways/cascades or conversely the activity of a particular pathway might initiate EBV protein activity. Some examples of this include 1) EBV lytic replication is inhibited by the inactivation of mTORC1[54] and YY1 regulation,[24,55] 2) activation of EBV lytic proteins BZLF1 and BMRF1 induce activation of MAPKs like p38 and JNK[56] and the PI3K signaling cascade,[57] 3) ATM-mediated cell cycle signaling is induced with EBV lytic replication,[58] 4) latent EBV protein LMP1 disrupts NF-κB regulation to transform lymphocyte cells and to promote cell proliferation,[38,59] and 5) LMP2 activates the Ras/PI3K-AKT constitutively for B cell transformation.[60]

2.5 Neuronal and Immune Cell Acetylcholine and Acetylcholinesterase

Acetylcholine is a neurotransmitter that is typically found within the central nervous system at synapses. Choline acetyltransferase synthesizes acetylcholine from acetyl-CoA and choline at the presynaptic terminal of neuronal cells. Once released to the synaptic cleft, acetylcholine binds to either nicotinic (ligand-gated ion channels) or

muscarinic receptors (G-protein-coupled receptor/integral membrane protein) on the post-synaptic cell where acetylcholinesterase AChE breaks down acetylcholine (to choline and acetate) (Fig.1).[61] Choline can then reenter the presynaptic terminal to start synthesis with acetyl-CoA and choline acetyltransferase (Fig.1). AChE typically is stored in vesicles near acetylcholine receptors in the synaptic cleft (space between the presynaptic terminal and postsynaptic cell) of the cytoplasm of neurons.[62] Although acetylcholine is normally found in neuronal cells, it can also be found in a variety of mammalian immune cells, including human lymphocytes.[63–65] Like neuronal cells, lymphocyte nicotinic and muscarinic receptors are present and acetylcholine is synthesized also by choline acetyltransferase or carnitine acetyltransferase.[61,64,66–68] Studies have shown that only T-cells synthesize choline acetyltransferase, and not B cells; however, other studies have found that B cells do produce choline acetyltransferase.[62,68,69] It has also been established that both T and B cells express AChE mRNA.[64] Based on different studies investigating non-neuronal acetylcholine activity, it appears that in immune cells, like lymphocytes, acetylcholine receptors are found on the cell membranes where extracellular acetylcholine can bind to the receptor. From there, AChE can hydrolyze acetylcholine to acetate and choline, where choline and acetyl-CoA within the cell can form acetylcholine via an acetyltransferase where acetylcholine can be transferred to other cells.[63]

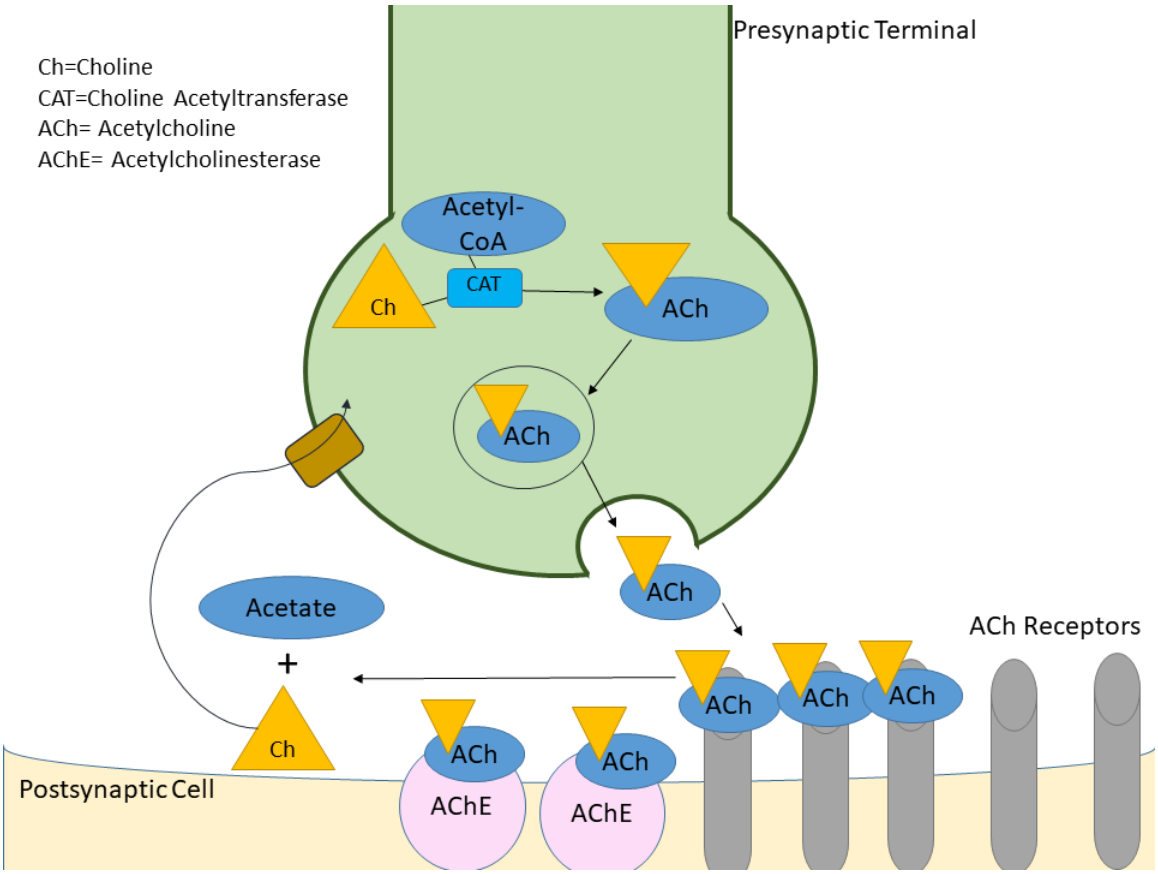

Figure 1. Overview of Synthesis and Regulation of Acetylcholine at Synapses. Acetylcholine is typically most associated at the synapses of neurons. Acetylcholine is synthesized in the presynaptic terminal by choline and acetyl-CoA with choline acetyltransferase (CAT or ChAT). Newly formed acetylcholine is packaged in vesicles and gets transferred to the synaptic cleft (space between the pre and postsynaptic cells) where it will bind to acetylcholine receptors (AChRs). Acetylcholinesterase (AChE) will then breakdown acetylcholine to choline and acetate. Choline will then get shuttled back into the presynaptic terminal to undergo acetylcholine synthesis again.

2.6 Organophosphates and Pesticide Use

Organophosphates (such as chlorpyrifos) are commonly used as pesticides for residential and agricultural use. Specifically, they act as neurotoxic insecticides and function as AChE inhibitors.[2,4,12,70–73] Organophosphates are irreversible inhibitors that prevents AChE from hydrolyzing acetylcholine. This in turn prevents the breakdown of acetylcholine (to choline and acetate) and results in acetylcholine accumulation causing overstimulation, thus creating issues with neurotransmission, and eventual neurotoxicity.[2,4,12,70–73]

Organophosphate poisoning can lead to sweating, muscle weakness, involuntary muscle movements, excessive production of saliva, and excessive constriction of the pupil.[71] More severe organophosphate poisoning can lead to extreme health conditions like loss of consciousness, failure of the respiratory system, convulsions, and in the some cases, death.[4,71,72]

The main route of absorption is through ingestion or inhalation, but organophosphates can also be absorbed dermally depending upon the organophosphate as they are lipophilic.[4] Many organophosphates are biotransformed to a more toxic form, such as oxons, though this form is more likely to degrade freely.[3,4,74,75] In humans, biotransformation occurs mainly in the liver through phase I and phase II reactions.[70,76,77] *In vivo*, organophosphates become transformed to their active metabolite through a variety of different process such as oxidation, desulfuration, hydroxylation, and other reactions not related to oxidation (Fig.2).[70,75,78,79] Detoxification of organophosphates occurs either through bond cleavage via hydrolysis, glutathione S-transferases (GST),

dearylation, and hydrolytic enzymes (Fig.2).[70,71,73,74,79,80] Other characteristics of organophosphates include being lipophilic, which allows easy absorption to target insects and also the ability to be easily stored in fat cells in humans, causing delayed neuropathy, and depending on the organophosphate, being prone to having additive effects when another organophosphate is present and absorbed.[4,70,72,81] Prolonged exposure to humans may potentially cause dysregulation in important cellular signaling pathways.

Chlorpyrifos (CPF) is an example of a commonly used organophosphate in agriculture. As of the early 2000s, there were 10 million pounds of CPF applied to crops each year and of those crops, corn used CPF the most at 5.5 million pounds of CPF.[11,82] Over the years, CPF and other organophosphates have been heavily regulated, banned, or phased out by the Environmental Protection Agency (EPA).[3,4,83] In the US, CPF is no longer allowed for residential use, but is allowed for agricultural use, and has undergone several reevaluations and human health risk assessments by the EPA,[83] with the most recent in 2016.[84]

CPF toxicity is caused through the irreversible binding of the active metabolite chlorpyrifos-oxon (CPO) to AChE.[85–87] CPO is the oxidized metabolite of CPF and is known to be more toxic than the parent compound.[87] CPF to CPO metabolism is cytochrome P450 (CYP450) mediated, more specifically CYP2B6 and CYP3A4.[74,88]

As mentioned with general organophosphate metabolism, CPF is detoxified by dearylation (CYP450 mediated) or hydrolysis to form the non-toxic 3, 5, 6-trichloro-2-pyridinol or TCP. The TCP metabolite is often used to detect CPF exposure in blood or urine samples (Fig.2).[74,85,87,89]

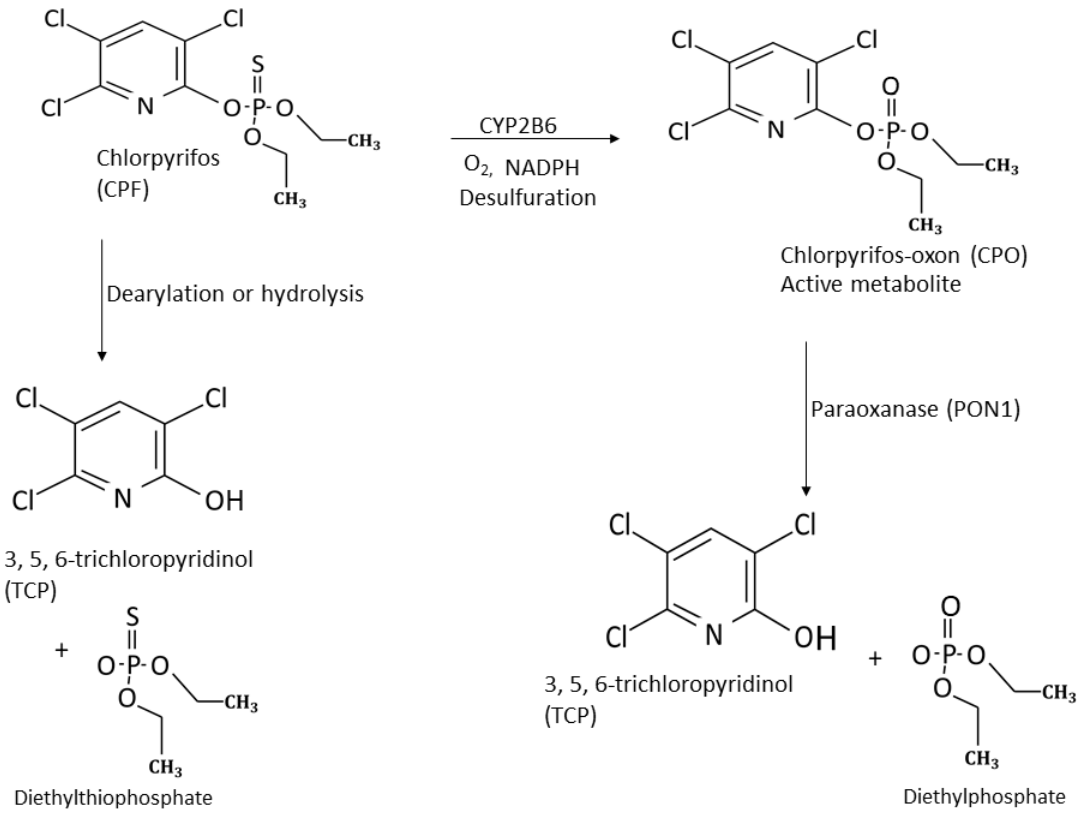

Figure 2. Biotransformation of Chlorpyrifos. Chlorpyrifos can undergo oxidative desulfuration to form its active metabolite, chlorpyrifos-oxon. Alternatively, Chlorpyrifos can also undergo a detoxifying step involving dearylation or hydrolysis to form the metabolites, 3,5,6-trichloropyridinol (TCP) and diethylthiophosphate. Chlorpyrifos-oxon also undergoes a detoxifying step via paraoxanase to form TCP and diethylphosphate.

2.7 Cell Cycle Regulation and Cancer

The cell cycle is an important cellular mechanism necessary for chromosome replication and segregation. This process includes mitosis and interphase (G1, S, and G2 phases). During G1 phase, the cell awaits a signal to initiate entry into S phase, and prepares for DNA replication. S phase is the actual step where DNA is replicated, and during G2 phase the cell is preparing for the newly synthesized DNA to undergo mitosis. During mitosis the chromosomes, as well as all other cellular components, are segregated; this stage is immediately followed by cytokinesis.

The cell cycle is regulated and controlled by many different regulatory proteins, however cyclin dependent kinases (CDKs) are key in driving cell cycle progression (and also arrest).[90,91] There are nine known CDKs, of which five are necessary and activated during G1 (CDK2, 4, and 6), S (CDK2) or G2 (CDK1) phase.[90,92,93] Cyclins binding to CDKs are also necessary for cell cycle progression and regulation, especially for transitions and entry from one phase to another (such as G1 to S transition or G2 to mitosis transition).[90] Although there are at least sixteen types of cyclins, only cyclins A, B, D, E, and H are seen in cell cycle activity.[90] Notably, cyclin D is active during G1, cyclin E promotes the G1 to S transition and is active during S phase (along with cyclin A), and cyclin B regulates early M phase events.[93,94] Phosphorylation is also a major regulator for CDKs, with activating and inhibitory phosphorylation events. When the phosphatase Cdc25 dephosphorylates CDK at an inhibitory site, the CDK becomes active; conversely persistent phosphorylation of CDK due to either deactivated Cdc25 or

by the active kinases Wee1 or Myt1, results loss of CDK activity and thus cell cycle arrest.[90,91,95–101]

In order to regulate and maintain proper cell cycle progression, cell cycle checkpoints are present to ensure DNA is properly replicating and to prevent damaged cells from progressing through the cell cycle.[91,97,102] During these checkpoints, if the cell detects DNA damage or issues with DNA replication, it will induce cell cycle arrest to allow time to repair or to prepare for programmed cell death (apoptosis).[95–97,100,103] Cell cycle arrest can be initiated by the activation of tumor suppressor proteins such as p53 or by CDK inhibitors such as p21.[90,95,99] In the presence of DNA damage, a cascade of events involving the DNA damage response (DDR) signaling pathway is activated to activate ataxia-telangiectasia-mutated (ATM) and ataxia-telangiectasia-mutated and rad3 related (ATR) protein kinases.[100,104–107] Activation of ATM and ATR result in phosphorylation of either p53 or p21 or of Chk1 or Chk2 (checkpoint control proteins). Phosphorylation of p53 and p21 results in cell cycle arrest at the G1/S transition.[90,91,96,100,104,107] Phosphorylation of Chk1 via ATR results in phosphorylation (deactivation) of Cdc25C and subsequent phosphorylation/deactivation of CDK1 (thus a G2/M transition cell cycle arrest), while phosphorylation of Chk2 via ATM results in phosphorylation (deactivation) of Cdc25A which in turn phosphorylates/deactivates CDK2 (thus a G1/S transition cell cycle arrest).[90,91,93,96,100,104,107–109]

Cancer is the result of uncontrolled cell proliferation.[90] Exogenous factors can alter regular cell cycle function or can cause mutations to proto-oncogenes or tumor suppressor genes so that proto-oncogenes promote tumor growth versus healthy cell

proliferation, while mutated tumor suppressor genes will not stop cell cycle progression when needed.[99,102,110] Cycle dysregulation can also involve mutations or issues with CDKs, cyclins, other CDK substrates, as well as other cell cycle checkpoint proteins like Chk1 and Chk2.[99,100,111]

This dissertation project described the investigation of the effects of chlorpyrifos and its active metabolite chlorpyrifos-oxon on EBV-host/cell interactions in B-lymphocyte cells, with specific focus on EBV and B-lymphocyte replication. Our initial conceptual model for our hypothesis was that CPF and CPO irreversibly binds to B-lymphocyte AChE to inhibit the breakdown of acetylcholine and therefore accumulation of acetylcholine outside of the cell and depletion inside the cell (Fig. 3A). We also hypothesized that the presence of chlorpyrifos would affect both EBV and B-lymphocyte replication by organophosphate induced cytotoxicity. Additionally, we hypothesized that the presence of Epstein-Barr virus and the pesticide chlorpyrifos (and the active metabolite chlorpyrifos-oxon) would interact and initiate a combined effect on three different B-lymphocyte cell lines, two with the virus and one without the virus. That is, we thought cells infected with the virus would have a greater and different response in comparison to the cells without the virus (Fig. 3B). However, after analysis of our results, we now believe the interaction between the host cell and the presence of EBV and chlorpyrifos was more so a potentiation relationship/effect. With a potentiation effect, we saw that presence of EBV showed a protective response when cells were under organophosphate influenced stressors in comparison to EBV-negative cells. This effect would be beneficial to EBV or for potential disease progression where normal cellular

responses are delayed when cells were exposed to high concentrations of the organophosphate. In contrast, this effect could be a potentially dangerous disadvantage for host cells and human health given the same delayed in cellular response (e.g. cell cycle arrest at higher concentrations of CPF or CPO).

The results from this basic research approach will provide further insight about environmental virus-host interactions at the cellular level and how these effects can alter important cellular signaling pathways and mechanisms which in turn can impact human health. Overall, we found it fascinating that the presence or absence of EBV made a difference in how cellular mechanisms and pathways in B-lymphocytes reacted in the presence of CPF or CPO.

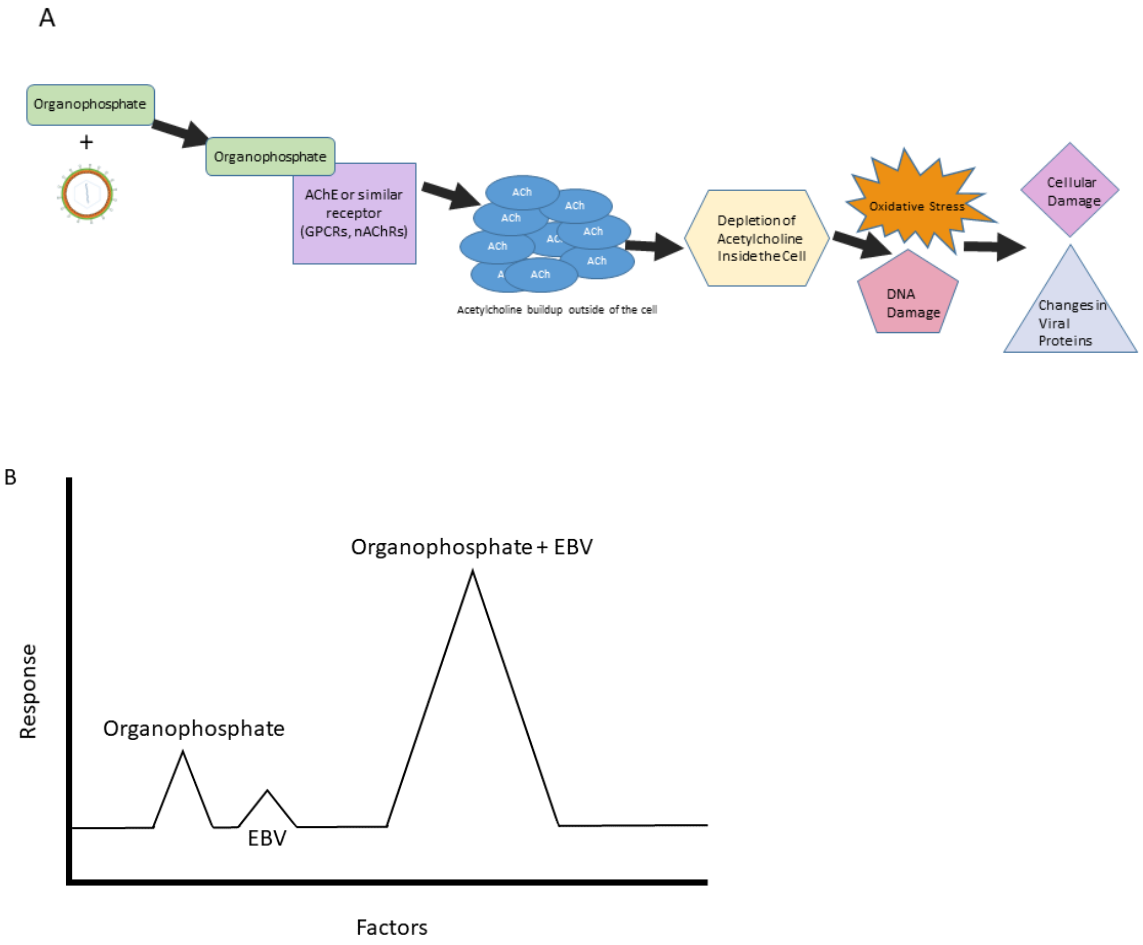

Figure 3. Initial Conceptual Models for the Effects of EBV Infection and Organophosphate Exposure. (A) Presence of organophosphates may cause acetylcholine buildup on the outside of B-lymphocyte cells and thus cause cellular damage and problems with cellular regulation. (B) Both EBV and organophosphates can cause independent health concerns, but the combination of both may cause a synergistic reaction that promotes human health issues.

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CHAPTER II

ORGANOPHOSPHATE EXPOSURE CAUSES VIRAL AND CELLULAR DYSREGULATION IN B-LYMPHOCYTE CELLS

This Chapter is coauthored by Katelyn Miller and Amy Adamson

1. Introduction

Epstein-Barr virus (EBV) is one of the most prevalent human herpesviruses, infecting more than 90% of the adult population.[1] The virus is known to be associated with a variety of human health issues including infectious mononucleosis, Burkitt's lymphoma, Hodgkin's and non-Hodgkin's lymphoma, and nasopharyngeal carcinoma and cancer.[1] Although EBV is a well- studied, ubiquitous virus, there is still a knowledge gap in virus-environmental interactions in relation to human health. An example of an understudied virus-environmental interaction is the potential relationship between EBV, pesticides, and cancer formation.

Organophosphate pesticides are commonly used worldwide, both in an agricultural and residential context (though most organophosphates have been banned, phased out, or restricted for residential use in the U.S. starting by the year 2006).[3,4,11] According to the Environmental Protection Agency (EPA), some organophosphates are moderately toxic and non-carcinogenic.[3,4] Organophosphates act as neurotoxins, targeting and inhibiting acetylcholine from binding to acetylcholinesterase.[3,4,12] This inhibition

prevents the breakdown of acetylcholine to choline and acetate, and thus leads to acetylcholine accumulation, hyperstimulation of acetylcholine receptors, and eventual neurotoxicity.[5] Organophosphates, in particular chlorpyrifos (CPF), have mainly been of concern for young children due to the pesticide's ability to cause developmental issues.[112–115] Recently, it has been suggested that the EPA ban CPF for agricultural use because of these developmental problems. Other studies have investigated how organohalogens, organochlorines, and organophosphates are toxic or contribute to developmental issues in environmental ecosystems like fresh water aquatic systems (from agriculture runoff) and animals (including farm livestock, birds, fishes, and amphibians).[75,116,117] Other commonly used pesticides like organohalogens and organochlorines have been previously studied in relation to antibody titers of EBV antigens and risk of non-Hodgkin's lymphoma. These studies concluded that blood samples with increased titer levels of EBV early antigen IgG have increased risk for hairy cell leukemia and other forms of non-Hodgkin's lymphoma with the presence of organochlorines and organohalogens.[118–120] Here, we are interested in how pesticides affect ubiquitous viruses like EBV and how the pesticide-virus interactions can contribute to human health at the cellular and molecular level.

Although some moderately-toxic organophosphate pesticides are considered non-carcinogenic, there have been several international meta-analyses that have investigated the relationship between lymphomas (Hodgkin and non-Hodgkin) and occupational exposure to pesticides. Many of these case studies focus around individuals who closely work with pesticides (insecticides, herbicides, fungicides) like farmers, field workers, and

their families.[6,8,14–16,121–124] Some studies analyzed data relating to individuals who have lymphomas and compared them to occupational exposure to a variety of different pesticides including organophosphates,[6,7] while other studies collected urine samples [13,124,125] or blood samples and looked for residual waste products of the organophosphates.[126] These studies found mixed results from their analyses for linking pesticide exposure and lymphoma formation; studies either cited a weak[122] or moderate correlation and stated that further studies needed to be conducted to factor in other variables.[6,8,123] With such inconclusive results, it is critical to investigate, outside of case study data, how two environmental factors, both potentially linked to cancer and other adverse health issues, interact with each other and contribute to human disease.

Here we examine how both the virus and host cells are affected when EBV infected B cells are exposed to organophosphate pesticides chlorpyrifos (CPF) and its active metabolite chlorpyrifos-oxon (CPO). Specifically, we investigated essential cellular mechanisms, including cell viability and the cell cycle, to determine if and how basic cellular biology was altered in the presence of an organophosphate, comparing EBV-positive (EBV+) and EBV-negative cells (EBV-). We used concentrations of CPF and CPO that represent the high end of environmental exposure (100-125 μM) as well as concentrations that exceed those environmental levels (150-200 μM).[127] As EBV requires host cellular machinery/mechanisms to be maintained in cells and to propagate, we also investigated how EBV biology itself was altered at the protein level, when EBV+ cells were exposed to organophosphates.

Only one other similar study has been undertaken to our knowledge. A study by Zhou et al. presented evidence that CPF caused oxidative stress in Raji cells [EBV+, Burkitt's lymphoma (BL)]. This was the only cell line used in their study and they report that CPF exposure caused latent EBV to reactivate when cells were exposed to CPF.[128] Within our study however we were not able to reproduce reactivation of EBV in EBV+, BL cells or other EBV lymphoblastoid cell lines upon any CPF exposure.

Here, we postulate that the organophosphate, CPF and CPO (active metabolite) bind to acetylcholine receptors on B cells and thus also irreversibly bind to AChE. The CPF and CPO presence and binding to AChE would then result in the accumulation of acetylcholine outside the cell, and thus prevent sufficient amounts of choline, and acetate inside the cell for B cell function and survival (Fig. 4A). We hypothesize that the combination of EBV infection and organophosphate exposure results in a synergistic effect that affects human health at the cellular level (Fig. 4B).

Our investigation of how organophosphates affected EBV+ and EBV- cells showed that cell viability decreased as the concentration of CPF, but not CPO, increased [EBV+, non-BL and EBV-, BL]. Additionally, we observed that both CPF and CPO induced a G1/S transition cell cycle arrest in EBV+ and EBV- cells. We also concluded that proteins necessary for EBV replication and proteins known to regulate EBV replication were affected with CPF and CPO exposure. Most interestingly though, we found that in almost all of our results, EBV- cells were overall more sensitive to the organophosphate exposure and elicited responses at lower concentrations in comparison to EBV+ cells.

That is, EBV- cells responded appropriately with CPF and CPO activity, while EBV+ cells had a delayed response in cellular activity, presumably, protected by EBV.

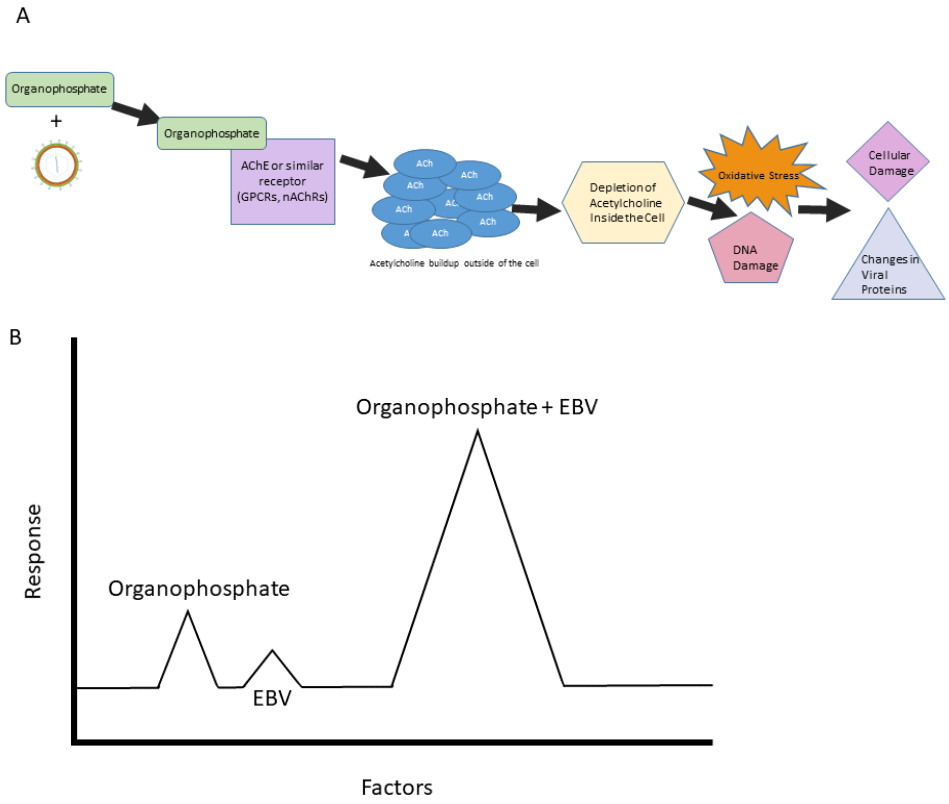

Figure 4. Initial Conceptual Models for the Effects of EBV Infection and Organophosphate Exposure. (A) Presence of organophosphates may cause acetylcholine buildup on the outside of B-lymphocyte cells and thus cause cellular damage and problems with cellular regulation. (B) Both EBV and organophosphates can cause independent health concerns, but the combination of both may cause a synergistic reaction that promotes human health issues.

2. Materials and Methods

2.1. Cell Culture

IM9 and Raji are immortalized, human derived B-lymphocyte cell lines that are infected with EBV. IM9 cells are non-cancerous, derived from an individual with infectious mononucleosis [EBV+, non-Burkitt's lymphoma (BL)] (ATCC), while Raji cells are cancerous, derived from an individual with Burkitt's lymphoma (EBV+, BL) (ATCC). Ramos cells are a B-lymphocyte cell line derived from an individual with Burkitt's lymphoma, but not infected with EBV (EBV-, BL) (ATCC). Cells were maintained at 37ºC and 5% CO_2 with RPMI-1640 media containing 10% fetal bovine serum, plus penicillin, streptomycin, and fungicide.

2.2. Organophosphate Pesticides

Organophosphates used in this experiment include CPF and CPO (the active metabolite). CPF and CPO (Chem Service Inc., West Chester, PA) were dissolved in DMSO for a final stock concentration of 100 mM.

2.3. Treatment Conditions

Cells were either left untreated, were treated with vehicle (DMSO, Sigma Aldrich), or were treated with organophosphate (ranging from 0-300 μM). Treatments were incubated for 24 hr prior to assays. EBV+ cells were induced into the lytic cycle with 20 ng/mL 12-0-tetradecanoyl phorbol-13-acetate (TPA) and 3 mM sodium butyrate (NaB).

2.4. Cell Viability

Cell viability was assayed with the Guava ViaCount reagent (Millipore Sigma, USA) according to manufacturer's protocol. Prepared samples were analyzed using the Guava easyCyte flow cytometer and the ViaCount program. Cell count was also carried out by the ViaCount program.

2.5. Cell Cycle

Cell cycle stages were determined with Guava Cell Cycle reagent (Millipore Sigma, USA) according to manufacturer's instructions. Samples were incubated in the dark at room temperature for 30 minutes prior to analysis using the Cell Cycle program on the Guava easyCyte flow cytometer.

2.6. Western Blot

Protein samples were lysed with ELB lysis buffer (0.25M NaCl, 0.1% NP4O, 50 mM HEPES pH 7.5, 5 mM EDTA, and protease/phosphatase inhibitors) and 20-40 µg were run on a 10% SDS-PAGE gel at 200V and transferred to an Immobilon membrane (Millipore) overnight at 100 mA.

2.7. Immunoblotting

Blots were blocked with 0.25% milk block solution (0.25% milk, 1x PBS, and 0.1% Tween-20). Primary antibodies used include α Tubulin (1:500; Santa Cruz Biotechnology), β-Actin (C4) (1:500; Santa Cruz Biotechnology), EBV ZEBRA (BZ1) (1:500; Santa Cruz Biotechnology), and EBV EBNA-1 (1EB12) (1:500; Santa Cruz Biotechnology) overnight at 4ºC. Secondary antibodies used include goat-anti-mouse IgG (H+L) and goat anti-rabbit IgG (H+L) (1:5000) (Jackson ImmunoResearch Laboratories,

Inc) for 10 minutes at room temperature. After each primary and secondary antibody incubation, blots were washed four times with Western wash solution (1x PBS, 0.1% Tween-20) using the SNAP i.d. 2.0 Protein Detection System (Millipore). Imaging and quantification were done using the C-DiGit Western Blot Scanner (LiCOR).

2.8. Statistical Analysis

A two-tailed student T-test was used to establish statistical significance with p-values <0.05 as significant for Western blots. A one-way ANOVA with a Dunnett's multiple comparison test was used for analysis when comparing treatments to the control when the standard deviations were equal while a Dunnett's T3 multiple comparison's test was used for unequal variance (with a Welch's one-way ANOVA). Statistical analysis and graphs were made using PRISM's Graphpad using the mean of all the trials and SEM.

3. Results

3.1 Chlorpyrifos, but not Chlorpyrifos-Oxon, Exposure Negatively Affects Cell Viability of B-Lymphocyte Cells

To determine if chlorpyrifos (CPF) treatment was toxic to cells, EBV+, BL and EBV+, non-BL cells were exposed to 14 different concentrations (10, 20, 40, 50, 70, 80, 100, 125, 150, 175, 200, 250, 275, and 300 µM) of CPF or to the DMSO vehicle for 24 hr and were assessed for cell viability (Fig. 5). EBV+, BL cell viability was statistically unaffected by CPF treatments relative to the DMSO control (93% viable). Treatments that ranged from 10-125 µM were 88%-94% viable. From 150 µM to 300 µM, there was a trend of decreased cell viability with increasing CPF concentration (going from 78% to

down to 49% viable) (Fig. 5A). EBV+, non-BL cell viability was more greatly affected by the CPF exposure with three of the treatment doses (175, 275, and 300 µM) and showed decreased cell viability with statistical significance in comparison to the DMSO control (91%viable) (Fig 4B). EBV-, BL cells were exposed to 100, 125, 150, 175, and 200 µM CPF, and showed statistically significant decreased viability at 125 and 175 µM in comparison to the DMSO control (Fig. 5C). Notably, the EBV+, BL and EBV+, non-BL cells remained viable at concentrations of CPF that were toxic to the EBV- cell line (125 µM).

Concentrations of the active metabolite of CPF, chlorpyrifos-oxon (CPO) used in subsequent experiments were based on results from this cell viability assay using CPF (Table 2). CPO was also tested for its effects upon cell viability on all three cell lines. Interestingly, this compound caused no statistically significant changes in cell viability (Fig. 5).

To investigate why CPF caused changes in cell viability and not CPO, we also looked at overall cell concentration (cells/mL) for each cell line and treatment (Fig. 6). CPF treated cells showed decreased total number of cells with increased CPF concentration, a similar trend to CPF cell viability results (Fig. 6A-C). CPO treated cells however showed that EBV+, BL cells increased in cell concentration in comparison to the DMSO control, EBV+, non-BL cells varied, but overall had a trend of slightly decreased cell concentrations, and EBV-, BL cells showed dramatic decrease of cell concentration at higher concentrations in comparison to the DMSO control (Fig. 6D-F).

Overall these results showed that EBV-, BL cells were more sensitive in assessing CPF exposure and cell viability. The results also showed that CPF affected B-lymphocyte cell viability more than CPO. Our results that looked at cell concentration provided evidence that CPF affects cell proliferation in a concentration dependent manner for all three cell lines. CPO treated cells showed that cell proliferation was not affected in EBV+, BL, but only slightly decreased cell viability in EBV+, non-BL, however; EBV-, BL was the most affected showing that CPO exposure decreased cell proliferation.

Table 2. Organophosphate Type and Concentrations

Organophosphate	**Concentration Used (μM)**
Chlorpyrifos	10, 50, 100, and 300
Chlorpyrifos-oxon	100, 125,150, 175, and 200

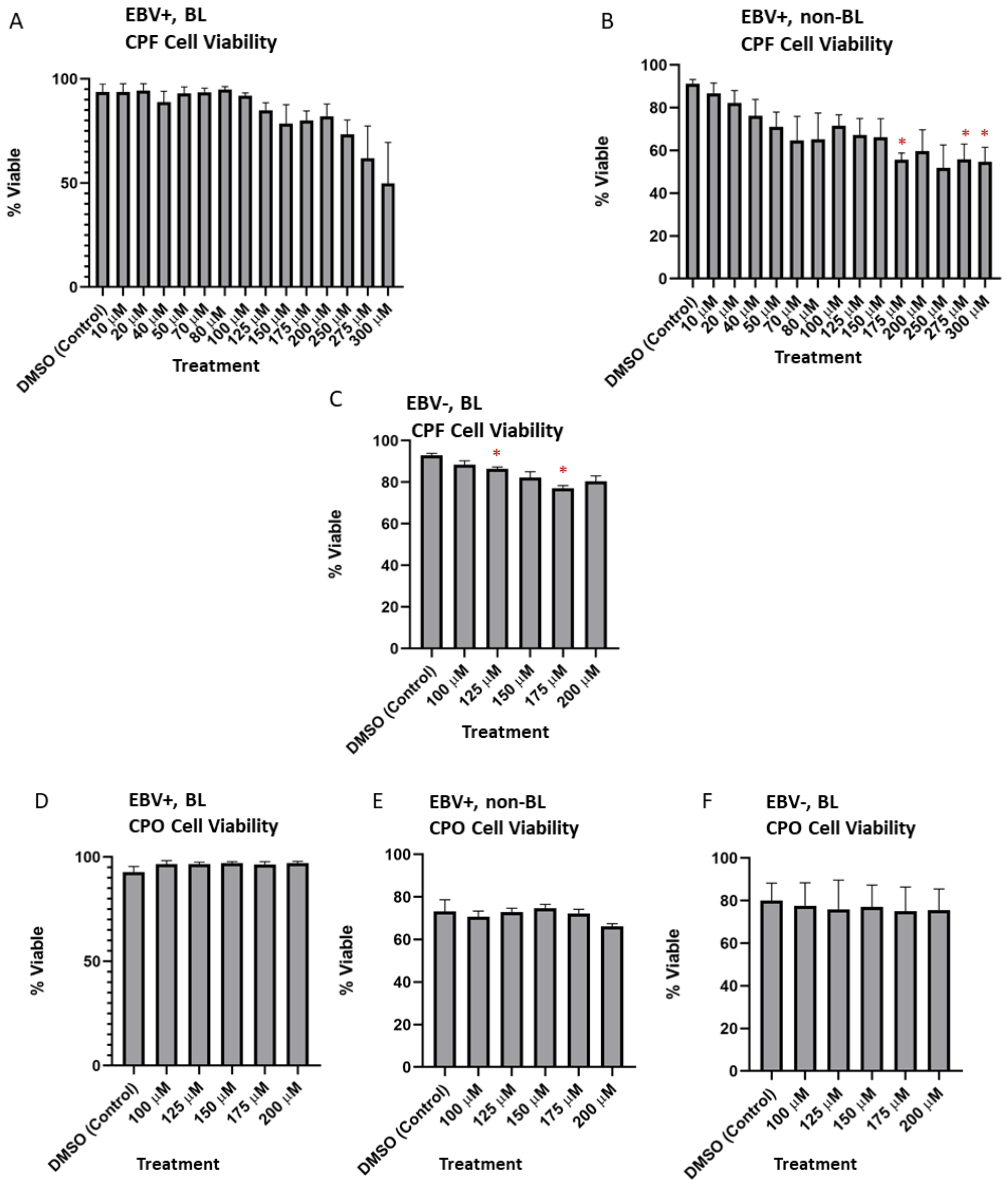

Figure 5. CPF Decreased Cell Viability while CPO showed no Change in Cell Viability in B Cells. (A) EBV+, BL, (B) EBV+, non-BL were treated with CPF (0-300 µM) for 24 hours, while (C) EBV-, BL were treated 0-200 µM of CPF. To compare to the parent compound, (D) EBV+, BL, (E) EBV+, non-BL, and (F) EBV-, BL cells were exposed to CPO for 24 hours at 100, 125,150, 175, and 200 µM. ViaCount reagent (EMDMillipore) and flow cytometry was used to measure cell viability of host cells when exposed to the insecticide. * indicates p-value <0.05, CPF=Chlorpyrifos, CPO=Chlorpyrifos-oxon, n= 4 for EBV+, BL for CPF treatments, n=5 for EBV+, non-BL CPF treatments, and EBV-, BL CPF and all CPO treatments experiments were n=3.

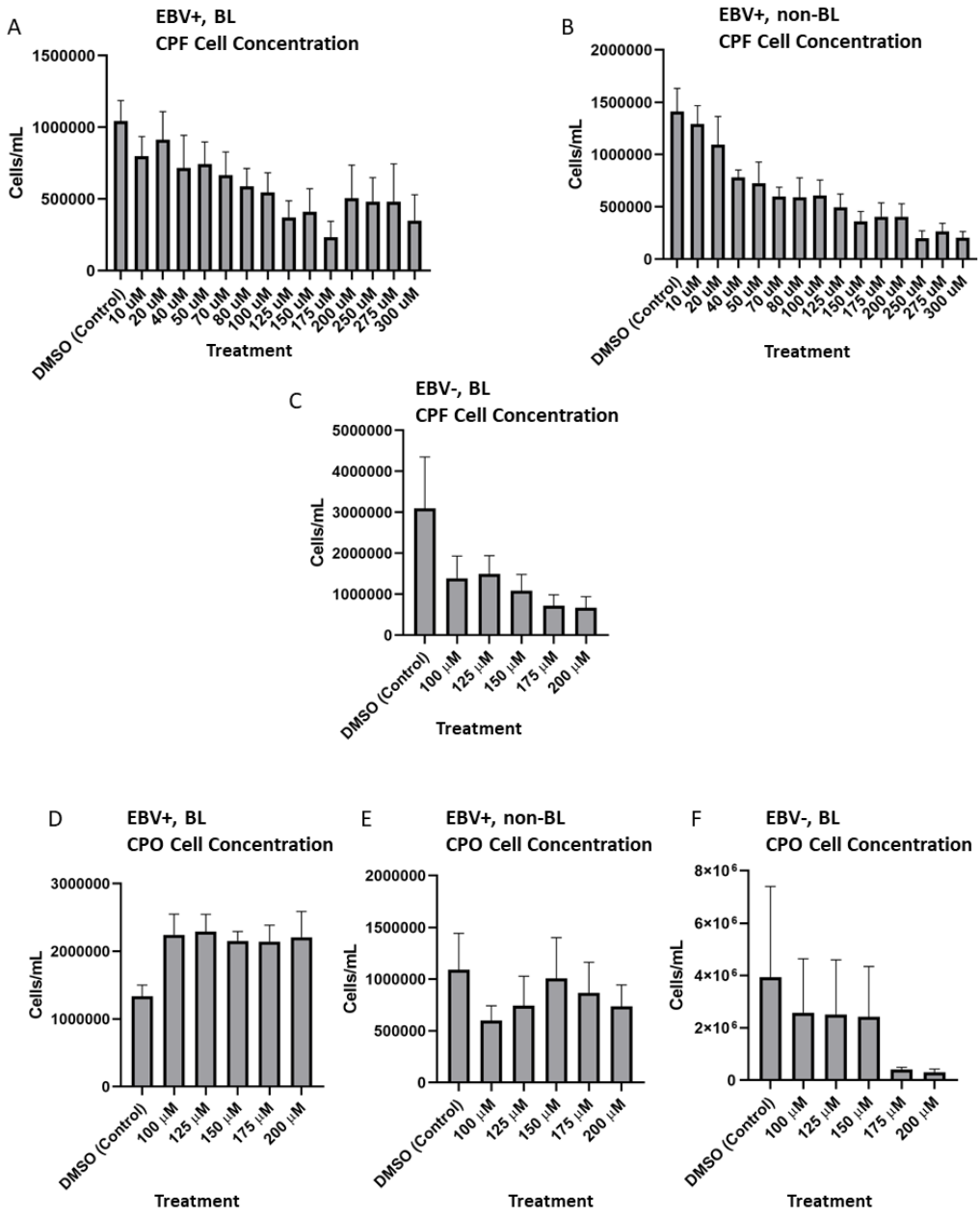

Figure 6. Increasing Concentrations of CPF Decreased Total Cell Concentration while Increasing Concentrations of CPO Affected Total Cell Concentrations in EBV- Cells. (A) EBV+, BL, (B) EBV+, non-BL, and (C) EBV-, BL cell lines were exposed to CPF for 24 hours. While (D) EBV+, BL, (E) EBV+, non-BL, and (F) EBV-, BL cell lines were exposed to CPO for 24 hours. Cells were analyzed via flow cytometry to determine the total cell concentration of each sample. * indicate p-values < 0.05 when compared to the vehicle control group (DMSO).

3.2 Organophosphates Alter Cell Cycle Regulation of B Cells

To determine if CPF exposure could affect cell cycle progression from one phase to another (G1, S, G2, and back into G1), EBV+, BL, EBV+, non-BL, and EBV-, BL cells were treated with four different concentrations of CPF (10, 50, 100, and 300 µM) or the DMSO vehicle for 24 hr and were assayed for cell cycle phases (G1, S, or G2) (Fig. 7). All cell lines exposed to CPF at all treatment doses did not show statistically significant changes in the percentage of cells in G1 phase (Fig. 7). EBV+, BL cells had a statistically significant decrease of the percentage of cells in S phase at 300 µM (9.95%) in comparison to the DMSO control (18.78%) (Fig. 7A). EBV+, non-BL and EBV-negative, BL cells did not have any statistically significant changes in the percentage of cells in S phase (Fig. 7B and 7C). All three cell lines showed decreased percentage of cells in G2 phase with statistical significance in comparison to the DMSO control (Fig.7): EBV-+, BL (DMSO: 17.18%, 300 µM: 8.25%), EBV+, non-BL (DMSO: 25.65%, 300 µM: 13.60%), and EBV-, BL (DMSO: 20.48%, 300 µM: 8.75%).

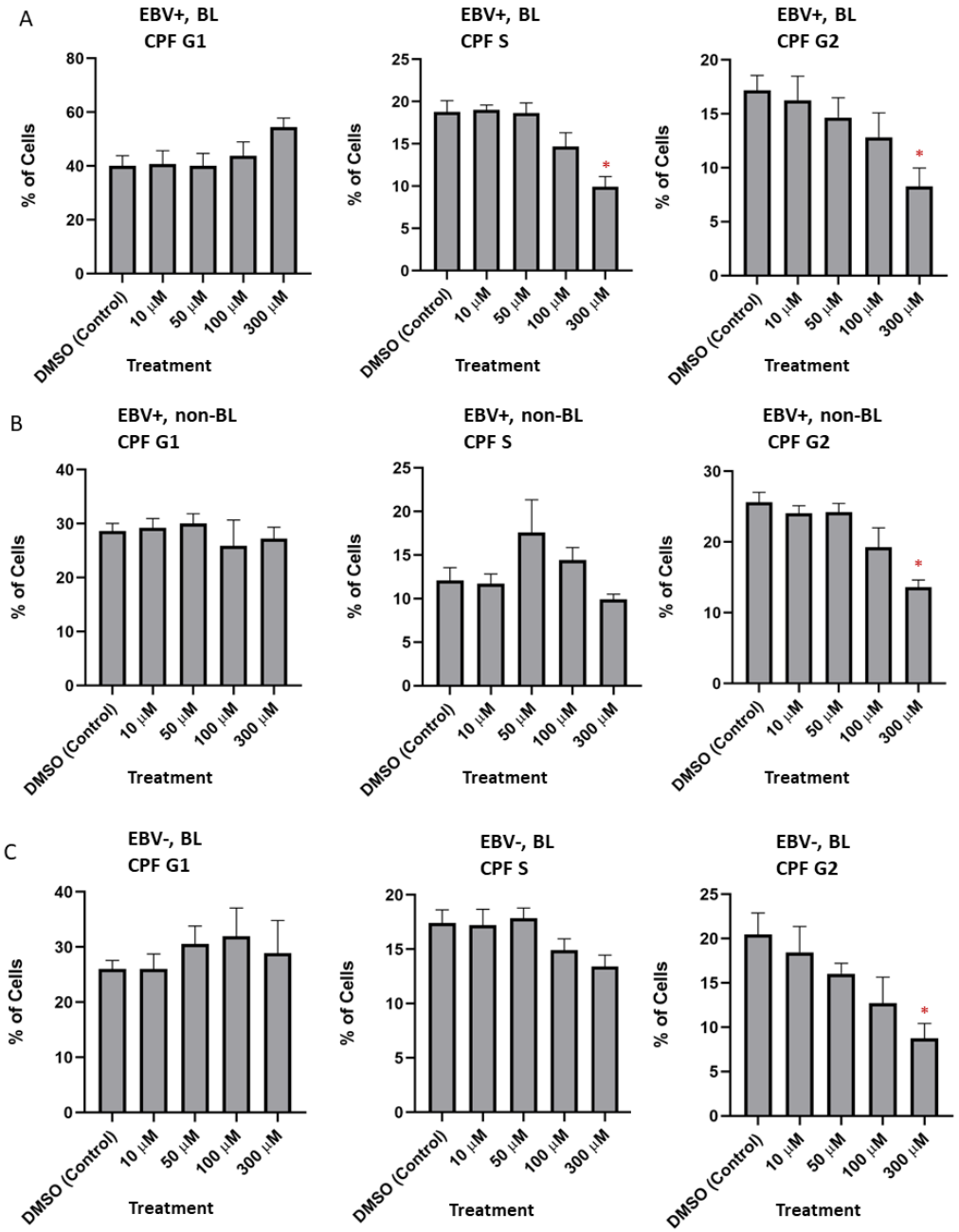

Figure 7. High Concentrations of CPF Induced Cell Cycle Arrest in B Cells. (A) EBV+, BL, (B) EBV+, non-BL, and (C) EBV-, BL cell lines were exposed to CPF for 24 hours. Cells were analyzed via flow cytometry to determine the percentage of cells in G1, S, or G2 phase of the cell cycle. * indicate p-values < 0.05 when compared to the vehicle control group (DMSO). n = 4.

Since the active metabolite is typically more toxic than the parent compound, we wanted to see if the potentially more toxic metabolite would cause cell cycle arrest at lower concentrations. We exposed CPO to all three cell lines at five different concentrations (100, 125, 150, 175, and 200 µM). In EBV+, BL cells exposed to the CPO, the average percentage of cells in G1 phase increased with concentration in comparison to the DMSO control (42.6%): 100 µM (48.96%), 125 µM (53.4%), 150 µM (56.33%), 175 µM (58.53%), and 200 µM (56.26%) (Fig 8A). The mean percentage of exposed cells in S phase of the cell cycle overall decreased with increased concentrations of the CPO when compared to the DMSO control (13.8%): 100 µM (15.86%), 125 µM (11.5%), 150 µM (9.6%), 175 µM (7.63%) and 200 µM (7.43%), with statistical significance at 175 and 200 µM (Fig. 8A). The average percentage of cells in G2 phase also decreased with increased concentrations compared to the control (22.53%): 100 µM (13.8%), 125 µM (13.43%), 150 µM (13.8%), 175 µM (14.6%) and 200 µM (17%), with statistical significance at 125 µM (Fig. 8A). For EBV+, non-BL cells, the mean percentage of cells in G1 phase in comparison to the DMSO control (14.28%) were as follows: 100 µM (22.05%), 125 µM (24.38%), 150 µM (28.20%), 175 µM (26.43%), and 200 µM (25%) (Fig. 8B). The average percentage of EBV+, non-BL cells in S phase in comparison to the control (5.62%) were as follows: 100 µM (5.1%), 125 µM (6.55%), 150 µM (9.35%), 175 µM (8.72%), and 200 µM (10.95%), with statistical significance at 150 µM and 200 µM (Fig. 8B). The mean percentage of EBV+, non-BL cells in G2 phase initially increased at 100 µM (40.05%), 125 µM (38.90%), and 150 µM (34.90%) and then decreased at 175 µM (24.08%) and 200 µM (23.08%) compared to the DMSO

control (32.30%) (Fig.8B). The average percentage of EBV-, BL cells at G1 phase increased at all concentrations compared to the control (23.43%): 100 µM (29.63%), 125 µM (31.73%), 150 µM (32.63%), 175 µM (34.70%), and 200 µM (36.17%) with statistical significance at 100 µM, 175 µM, and 200 µM (Fig. 8C). The percentage of cells in S phase did not show any statistical significance throughout treatments in comparison to the DMSO control (19.20%): 100 µM (18.97%), 125 µM (20.13%), 150 µM (19.67%), 175 µM (16.63%), and 200 µM (18.23%) (Fig. 8C). Similarly, the average percentage of EBV-, BL cells in G2 phase did not show any statistically significant differences when treatments were compared to the DMSO control (23.63%): 100 µM (21.83%), 125 µM (21.07%), 150 µM (20.53%), 175 µM (19.90 %), and 200 µM (16.90 %) (Fig. 8C).

Taken together, these data indicated that the parent compound induced cell cycle arrest at the G1/S transition at high concentrations (300 µM). Whereas the oxon form, CPO, had the ability to cause cell cycle arrest at the G1/S transition in EBV- cells at lower concentrations (starting at 100 µM) in comparison to EBV+ cells. Interestingly, there was increased entry into S phase within EBV+, non-BL cells that were not yet cancerous, at specific doses (150 µM and 200 µM). This suggested that the active metabolite of CPF can potentiate with EBV to promote cell cycle progression under conditions that would normally halt the cell cycle and/or kill the exposed cells. Overall these results showed, again, that EBV-, BL cells were more sensitive and induced proper cellular response (cell cycle arrest in the presence of cytotoxic stressors) at lower concentrations in comparison to EBV+ cell lines that responded at higher concentrations.

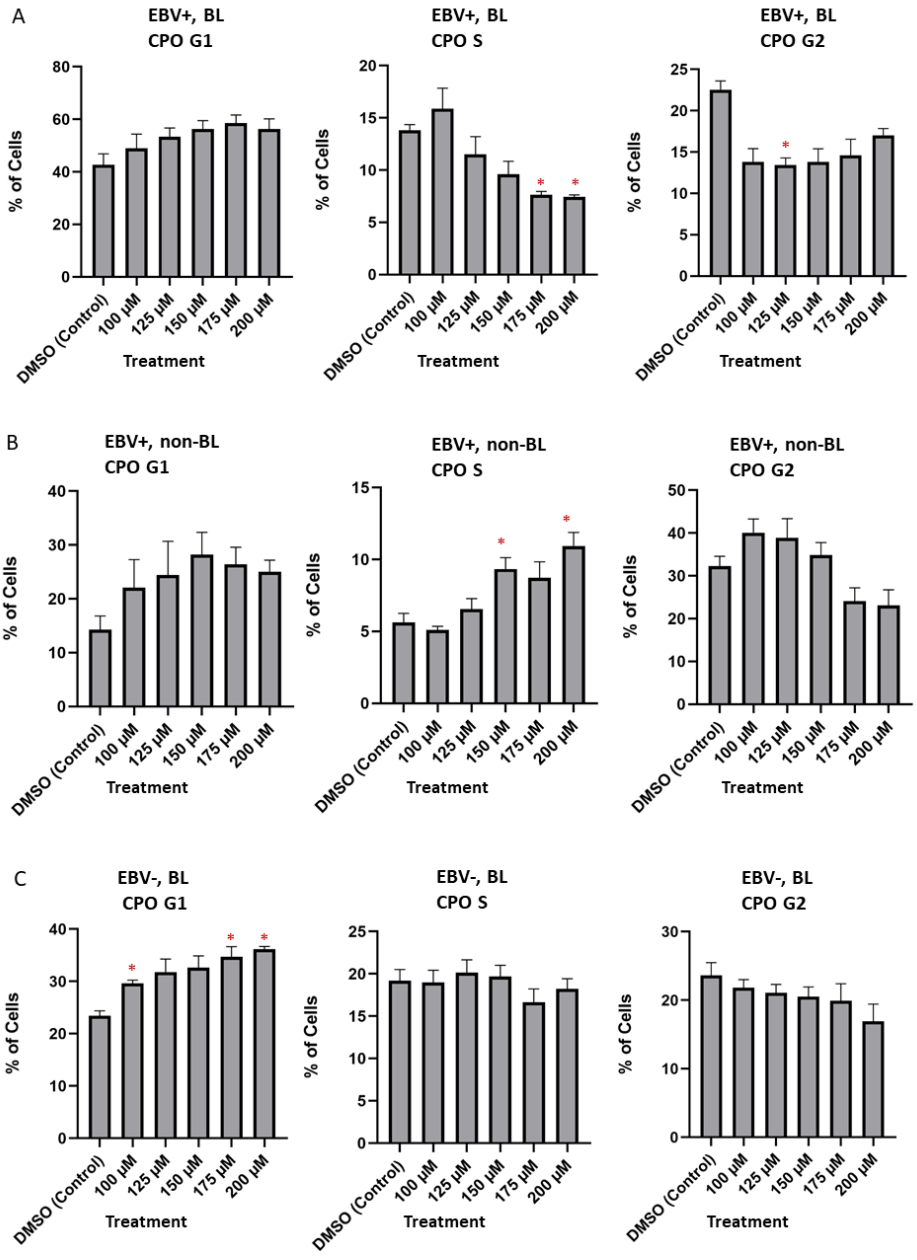

Figure 8. CPO Exposure Induced Cell Cycle Arrest to B Cells in a Dose Dependent Manner. (A) EBV+, BL, (B) EBV+, non-BL, and (C) EBV-, BL cell lines were exposed to CPO for 24 hours. Cells were analyzed via flow cytometry to determine the percentage of cells in G1, S, or G2 phase of the cell cycle. * indicate p-values < 0.05 when compared to the vehicle control group (DMSO). EBV+, BL and EBV-, BL: n=3, EBV+, non-BL: n=4.

3.3 Chlorpyrifos-Oxon Exposure Alters Expression of Important Viral and Cellular Proteins

To understand viral changes that may be occurring in EBV+ cells at a dose of organophosphate that did not cause major effects, cells were exposed (for 24 hours) to 100 µM of CPF or CPO in the presence of TPA/NaB, a chemical inducer of the EBV lytic life cycle. The 100 µM concentration of CPF or CPO was chosen since both EBV+ cell lines showed decreased cell viability, without killing cells when exposed to CPF during preliminary analysis; the same concentration was used for CPO since the active metabolite is typically more toxic than the parent compound. BZLF1 is an important EBV protein that acts as a transactivator for EBV early genes (necessary for viral replication). EBV+, BL cells showed decreased expression of the viral protein BZLF1 when compared to cells only exposed to TPA/NaB: the effect of the oxon form was the most dramatic (Fig. 9A). We found neither CPF nor CPO were able to induce lytic replication on their own, however (Fig. 9A). These results refute the results by Zhao et al, who claimed that CPF treatment of EBV+, BL cells readily triggered lytic replication[128].

Under the same conditions, EBV+, non-BL cells showed increased expression of BZLF1 when exposed to CPF, but showed decreased expression of BZLF1 when cells were exposed to CPO (Fig. 9B). Similarly, neither CPF nor CPO alone were able to induce lytic replication without TPA/NaB treatment.

EBV+, BL and EBV+, non-BL cells were exposed to a dosage series of CPO and Western blots were probed for the EBV latent protein EBNA1. EBNA1 is important for EBV replication during latency, host survival, and transcription for other latent genes.[1,26]

EBV+, BL cells did not show any expression changes of EBNA1 (Fig. 10A). While EBV+, non-BL cells, however, showed EBNA1 expression increased with statistical significance as compared to the DMSO control at 125 μM and 175 μM (Fig. 10B).

Overall, these results indicate that a dose of CPO that caused no visible cellular effects (in terms of cell viability and cell cycle) was able to have dramatic effects upon EBV gene expression. Such changes to viral gene expression may be the underlying causes for the subsequent cellular changes seen at higher doses of CPO.

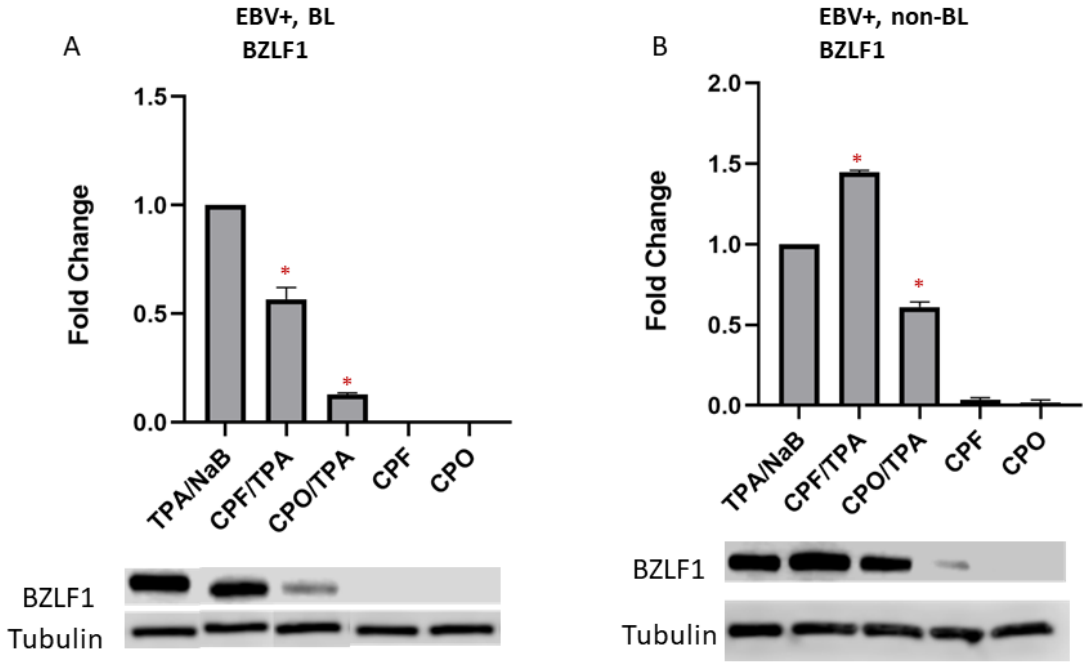

Figure 9. CPF and CPO Exposure Altered EBV BZLF1 Expression. (A)EBV+, BL cells treated with 100 µM CPF or CPO when the virus was active showed decreased expression in the viral protein BZLF1. (B) EBV+, non-BL cells treated with 100 µM CPF showed increased expression of BZLF1, while CPO showed decreased expression of BZLF1 when the virus was active. Induction of lytic replication was through chemical induction using TPA/NaB. Cells were treated with CPF or CPO and chemically induced for 24 hours. TPA/NaB= 12-O-Tetradecanoylphorbol-13-acetate and sodium butyrate, CPF= chlorpyrifos and CPO = chlorpyrifos-oxon. n=3, * indicate p-value <0.05.

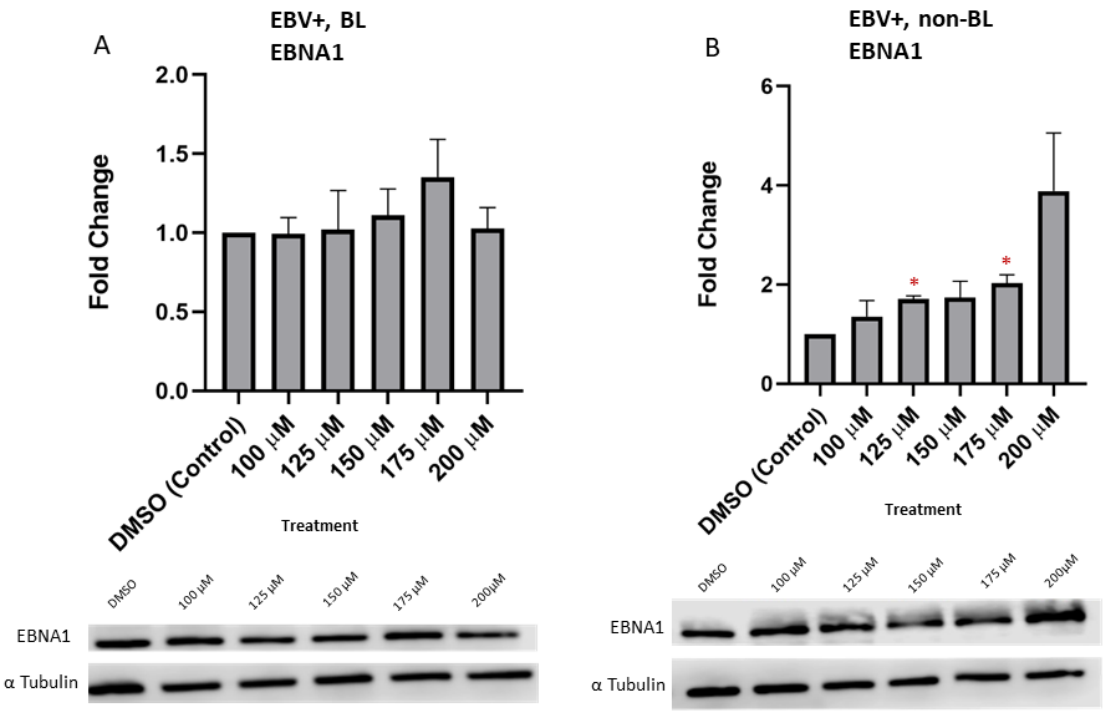

Figure 10. CPO Exposure Increased Expression of Latent EBV Protein EBNA1 in EBV+, non-BL Cells, but not EBV+, BL Cells. (A) EBV+, BL cells and (B) EBV+, non-BL (B) were treated with CPO for 24 hours. EBNA1 was standardized to the loading control (α-Tubulin) and treatments were compared to the DMSO control. n=3, * indicate p-value <0.05.

As viral protein levels were altered under CPO treatment conditions, we wanted to examine how CPO affected cellular protein levels, especially proteins that are also associated with and regulate EBV replication. One important regulator of protein translation is p70S6K, a kinase that is phosphorylated by mTORC1. Phospho-p70S6K in turn phosphorylates a variety of targets, most notably the ribosomal subunit S6; such activation is essential for protein translation.[129] EBV+, BL, EBV+, non-BL, and EBV-, BL cells were exposed to increasing doses of CPO (100, 125, 150, 175, and 200 μM) and Western blots were probed for phosphorylated p70S6K. EBV+, BL cells showed no changes in phosopho-p70S6K levels until the 200 μM dose, which exhibited a statistically significant decrease (Fig.11A). In contrast, EBV+, non-BL cells showed a statistically significant decrease of phospho-p70S6K at the lowest concentration of CPO (100 μM), but as the CPO dose increased, so did the phospho-p70S6K levels (Fig. 11B). EBV-, BL cells showed no change of phospho-p70S6K levels until the 200 μM dose, which exhibited a statistically significant increase (Fig. 11C). We did not probe for total p70S6K in this study. Interestingly, these results showed that EBV+ cells decreased in phosphorylated p-70S6K while EBV- cells increased in phosphorylated p-70S6K. These results also provided further evidence that presence of EBV alters how B-lymphocytes function. We were also curious to investigate if latency regulation was affected by CPO exposure so we investigated the cellular protein Ying-Yang1 (YY1).

YY1 is an essential, ubiquitous transcriptional activator and repressor that acts through DNA promotor tethering.[130,131] This is an important protein to investigate as it regulates essential cellular mechanisms like the cell cycle (cell proliferation and

apoptosis) and response to genotoxic stimuli,[55] as well as viral regulation of EBV latency (driving the virus to remain latent).[55,130,132] EBV+, BL, EBV+, non-BL, and EBV-, BL cells exposed to CPO (100, 125, 150, 175, and 200 μM) for 24 hours were probed for the cellular protein YY1. EBV+, BL cells did not show any YY1 expression changes (Fig. 12A). EBV+, non-BL cells only showed decreased YY1 expression with statistical significance at 200 μM (Fig. 12B). In contrast, EBV-, BL cells showed a statistically significant increase of YY1 expression at 125 μM of CPO (Fig. 12C). Overall, though, YY1 expression did not change drastically with increasing concentration of CPO, regardless of cell line. Presence and expression of YY1 would indicate a drive to keep the EBV latent replication.

For the two cellular proteins we assessed, we found that phospho-p70S6K showed a general trend for decreased levels within both EBV+ cell lines, while increased expression in the EBV- cell line. This correlated with our findings that CPO decreased EBV BZLF1 expression; we know from previous work that EBV lytic replication and BZLF1 levels were dependent upon mTORC1 activity, and specifically phospho-p70S6K levels (unpublished). This indicated that CPO exposure may cause mTOR pathway inhibition in an EBV specific manner. We also found that YY1 levels did not change in EBV+, BL cells, but decreased in EBV+, non-BL cells at the highest dose, and increased in EBV-negative, BL cells at lower doses. This indicated that YY1 not only appears to be unaffected by CPO exposure, but also may have influenced lytic EBV to shift to latency or to remain latent in the presence of CPO.

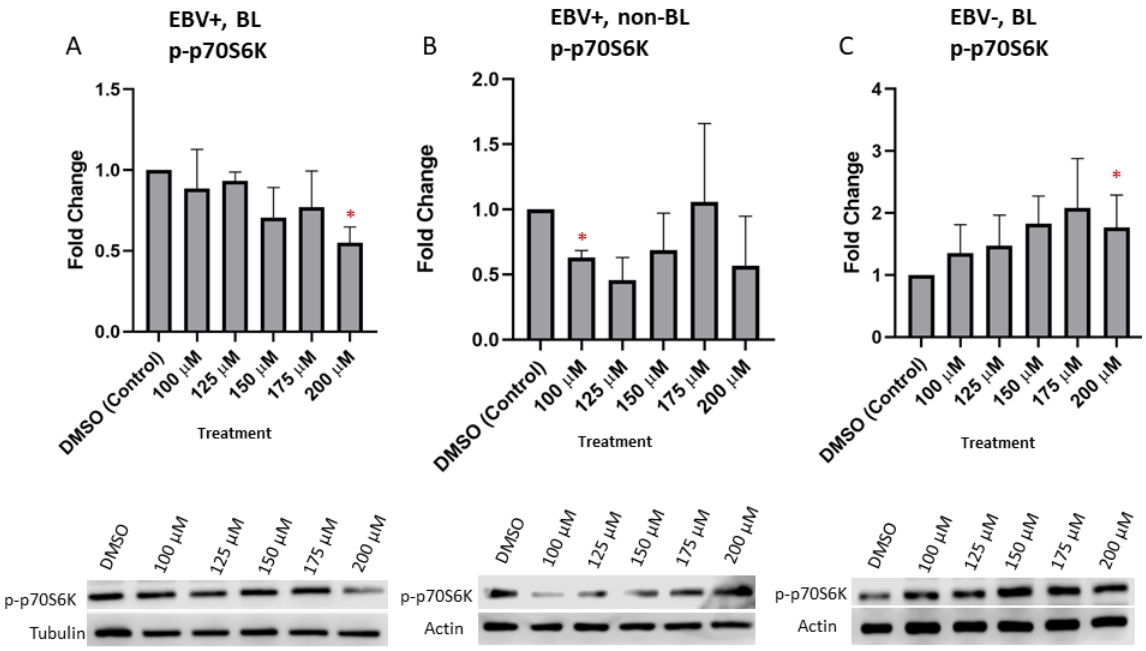

Figure 11. CPO Exposure Decreased Expression of p-p70S6K in EBV-Positive Cells, but Not EBV-Negative Cells. (A) EBV+, BL, (B) EBV+, non-BL, and (C) EBV-, BL cells were treated with CPO for 24 hours and assessed for the expression of phosphorylated p70S6K via Western Blot. Treatments were compared to the DMSO control and standardized to a loading control (actin). n=3, * indicate p-value <0.05.

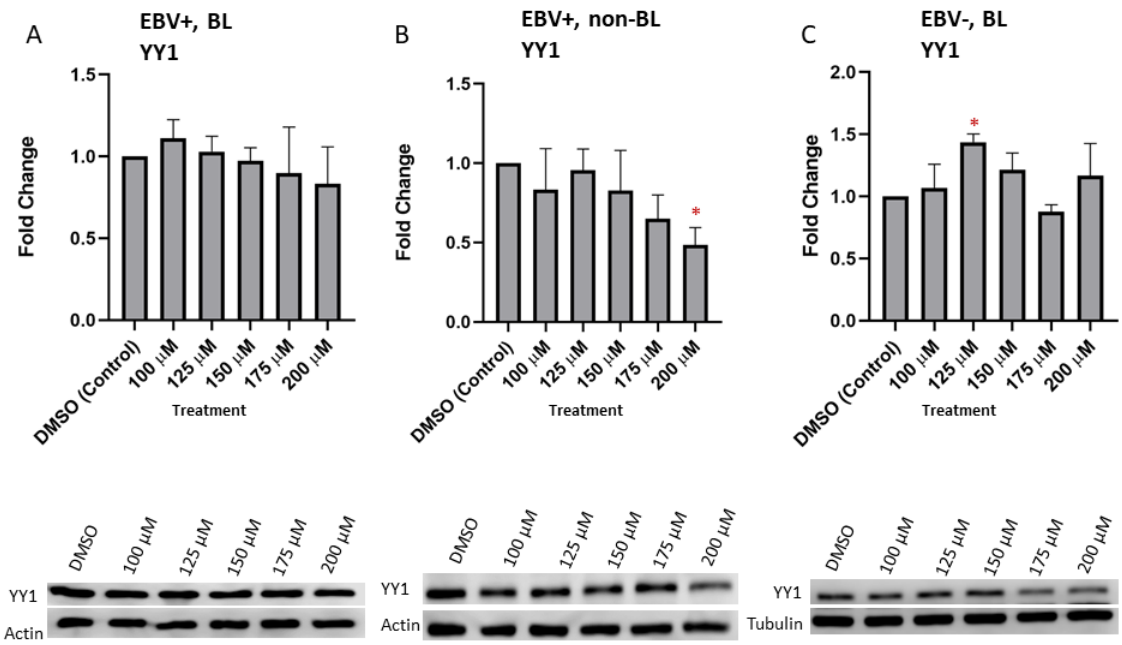

Figure 12. YY1 Expression in B Cells was not Greatly Affected by CPO Exposure. (A) EBV+, BL, (B) EBV+, non-BL, and (C) EBV-, BL cells were treated with CPO for 24 hours and assessed for YY1 expression via Western Blot. Treatments were compared to the DMSO control and standardized to a loading control (actin or tubulin). n=3, * indicate p-value <0.05.

4. Discussion and Conclusion

Studying virus-host interactions is important to discover crucial details about cellular and viral responses and how they affect diseases related to viral infection. The relationship between virus-host interactions and how they are affected by exogenous environmental factors, such as pesticides, remains unclear. This study investigated whether the presence of EBV infection and organophosphate pesticides (CPF and its active metabolite CPO) created a potentiated effect that would cause changes to the virus and to host cells, thus contributing to a disease (such as cancer) phenotype more so than either factor alone. We found that a globally used insecticide, CPF, and its active metabolite CPO, affected B-lymphocyte cell viability, cell proliferation, cell cycle progression, and EBV protein expression.

In terms of cell viability, EBV- cells were most sensitive to CPF at lower doses, whereas EBV+ cells experienced little to no change in cell viability at these lower doses. EBV+, non-BL cells were the most sensitive to CPF at higher doses. Interestingly, CPO exposure at all doses had little effect on both EBV+ and EBV- cells. In fact, EBV+, BL cell line experienced higher cell viability percentages (though not statistically significant) in comparison to the EBV-, BL cell line. With cell proliferation (cell concentration, cells/mL), we saw that with CPF exposure, both EBV+ and EBV- cell lines experienced decreased cell concentrations with increasing concentrations of CPF. However, with CPO exposure, EBV+ cell lines overall did not show any decreased cell concentrations whereas EBV- cells showed a drastic decrease in cell concentration. The differences of results between CPF and CPO related cell viability could be explained if CPF has a

different molecular target to cause cell death more readily in comparison to cells exposed to CPO. These results provided us with 1) a series of pesticide doses to use in subsequent experiments that were not just toxic to cells, and 2) the knowledge of other cellular changes (for example, changes to the cell cycle stages) would not be artifacts of toxicity. Together, we saw that presence of EBV made the cells more resistant or protected when exposed to the organophosphate.

For the cell cycle, we found that CPF exposure likely induced a G1/S transition cell cycle arrest at high concentrations (300 μM). The active metabolite, however, told a different story. CPO at the lowest dose (100 μM) was capable of eliciting a significant shift of cells into G1 phase in EBV-, BL cells, an expected response for cells exposed to a potentially damaging agent. EBV+ cells did not experience a significant shift of cells into G1, however, overall EBV+, BL and EBV+, non-BL both followed the trend of a G1/S transition arrest. These results suggest that the presence of the virus in these cells allowed for the continuation of DNA replication and mitosis despite exposure to a potentially damaging agent. If cytotoxic damage was occurring in these cells, it would likely be propagated. Again, it is interesting to note that EBV-, BL cells were once again more sensitive to CPO and triggered cell cycle arrest at lower concentrations.

To examine the effects the organophosphate upon EBV biology, we monitored the expression of a lytic and latent EBV protein. We found that CPO caused decreased expression of the EBV protein BZLF1, an important transcription factor necessary for lytic viral replication. In contrast to a previous study by a different laboratory,[128] we did not observe lytic reactivation when EBV infected cells were exposed to just the

organophosphate for 24 hours at 100 μM, nor 4 hours at 50, 100, 150, and 200μM (data not shown). The expression of EBNA1, an important protein for viral replication during latent EBV infection and for infected cell survival,[1] was increased when EBV+ cells were exposed to CPO, specifically in EBV+, non-BL cells. The response we observed, that CPO decreased BZLF1 expression and increased EBNA1 expression, suggests that the pesticide drives cells towards a more latent state. Furthermore, increased EBNA1 levels have been recently positively correlated to an increased incidence of lymphoma.[133] Altogether, it appears that CPO causes EBV protein expression changes that both increase cell survival and promote cell cycle progression.

P70S6K is downstream of the mTORC1 pathway within the PI3K/Akt signaling pathway and plays a major role in translation.[134,135] When active, mTOR directly phosphorylates p70S6K, and this kinase then phosphorylates downstream targets to promote protein translation. Studies have shown that inhibitors of the mTOR pathway (such as rapamycin) directly affect phosphorylation of p70S6K expression.[54,136,137] Interestingly, when we examined phospho-p70S6K after 24 hours of CPO exposure, we noticed a trend of decreased levels of this protein in EBV+, BL (at 200 μM) and EBV+, non-BL cells (at 100 μM), while increased expression in EBV-, BL cells. As EBV utilizes the host mTOR pathway to translate its viral proteins during lytic replication, and actually promotes phosphorylation and activation of p70S6K (unpublished data), the fact that CPO led to decreased levels of phospho-p70S6K may at least partially explain why BZLF1 exhibited a decreased expression under CPO conditions. Results further provided evidence that the presence or absence of EBV affected how host cells responded.

YY1, another cellular protein that regulates EBV, is an essential protein that acts as both a transcriptional activator and repressor for both humans and viruses.[130] For EBV, YY1 has been shown to act as a negative regulator for latent to lytic replication switches; YY1 represses BZLF1 transcription, leaving the virus to remain latent (inactive).[130,132,138] Our study showed that BZLF1 was not expressed in the absence of TPA/NaB and the presence of CPF or CPO. Consistent expression of YY1 throughout most of the treatments (with the exception of decreased expression of YY1 at 200 μM in (EBV+, non-BL cells) may have played a role as to why BZLF1 was not expressed in the absence of TPA/NaB and why EBNA1 did not deplete with increasing concentrations of CPO. It was likely that YY1 was unaltered during CPO exposure and continued to repress BZLF1 production and keep EBV in the latent phase.

Other studies have shown the cytotoxic and genotoxic effects of organophosphate exposure on different types of cells and tissues.[139–141] These studies have also stated that the mechanisms and pathways involved with these effects are not fully understood. There are older studies that investigated pesticide exposure effects on EBV titers.[118–120] However, current literature have focused on other environmental factors, like environmental pollutants and lifestyle choices, and how it relates to EBV infection and associated diseases like nasopharyngeal carcinoma, gastric cancer, and multiple sclerosis.[142–146]

We acknowledge that our study only investigated thoroughly one organophosphate and its active metabolite. Other organophosphates and combination treatments are necessary to fully understand the effects of pesticides on virus-host

interactions. The concentrations we chose for CPF and CPO exposure are relatively high in comparison to environmental exposure levels, however; the lower end of our range (100-125 μM) falls within reasonable and realistic environmental levels for humans.[127]

Overall our study found that cells exposed to CPF and CPO exhibited a potentiated effect (as opposed to our initially hypothesized synergistic effect) depending if EBV was present or not. This effect appeared to have contributed to the survival and propagation of pesticide-exposed, EBV+ cells. EBV+ cells were only affected at higher doses of CPF or CPO, whereas, the EBV-, BL cells responded to CPF or CPO exposure more readily at lower concentrations. This protective or delayed cellular response when EBV+ cells are exposed to the organophosphate could potentially promote health issues, such as lymphoma formation, associated with EBV infection. Our revised model to explain EBV and pesticide relationship can be seen in Fig. 13.

To further understand the effects of organophosphate exposure on viral and host replication, we need to understand the affected mechanisms or pathways from the presence of the pesticide. We suspect that DNA damage and oxidative stress may play a role in our results. We hypothesize that the organophosphate is binding to acetylcholinesterase receptors on B lymphocyte cells, creating irreversible inhibition when bound to AChE to then cause deficient levels of acetylcholine inside the cell. This deficiency may lead to DNA damage, oxidative stress, and other factors related to issues with replication, since we know that acetylcholine is necessary for B cell survival and overall function.[66,147]

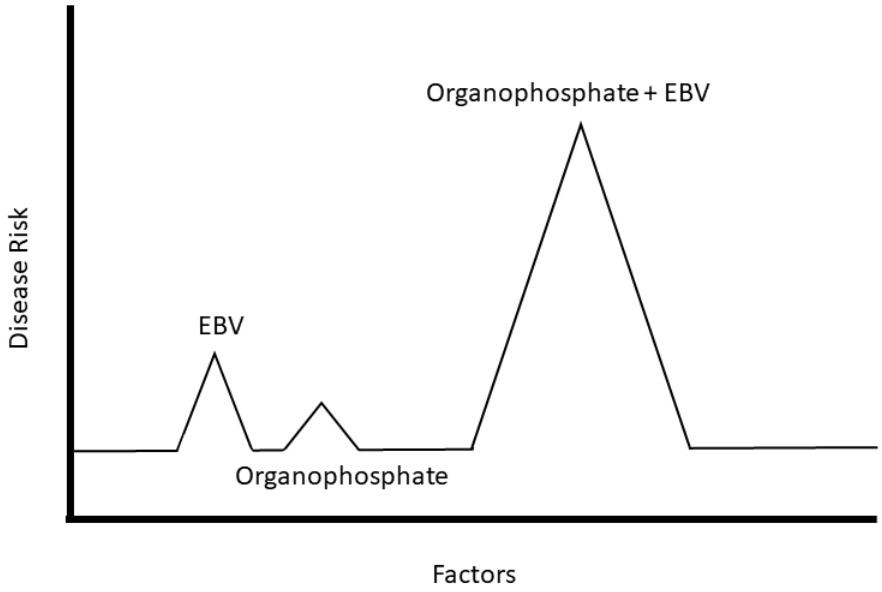

Figure 13. Revised Model for the Effects of Environmental Factors.

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CHAPTER III

EFFECTS OF OXIDATIVE STRESS AND DNA DAMAGE CONTRIBUTIONS TO ABNORMAL REGULATION IN EBV INFECTED B-LYMPHOCYTES

This chapter is coauthored by Katelyn Miller and Amy Adamson.

1. Introduction

Epstein-Barr virus (EBV) is one of the most common human herpesviruses, infecting more than 90% of adults worldwide. EBV is a known oncovirus associated with cancers like Burkitt's lymphoma and other non-Hodgkin's lymphomas, gastric cancer, and nasopharyngeal carcinoma.[1] Several studies have investigated how EBV-associated diseases arise in the presence of environmental factors, for example, endemic Burkitt's lymphoma and malaria, and nasopharyngeal carcinoma and lifestyle factors like smoking and diet.[143,145,146,148] However, less commonly-studied areas are environmental virus-host interactions. For example, the effects moderately-toxic pesticides, like the organophosphate chlorpyrifos, have on the cellular mechanisms that EBV hijacks. One interesting and conceivable cellular pathway that the pesticide may be able to affect is oxidative stress and DNA-damage induced cell cycle arrest.

When there is an imbalance between the production and capacity of antioxidants and the production of reactive oxygen species (ROS), like free radicals (hydroxyl and superoxide radicals) and hydrogen peroxide, oxidative stress occurs.[104,149–151] The

inability for cells to combat the free radical imbalance through antioxidant production or radical scavenging enzymes can lead to DNA, protein, and lipid damage.[104,149–151] Damage to macromolecules can therefore lead to major cellular issues like carcinogenesis, genotoxicity, and cytotoxicity. Oxidative stress is thus a factor that can contribute to the DNA damage response (DDR) and downstream consequences to cell cycle regulation, such as cell cycle checkpoints.[104,151–153] In order to understand how chlorpyrifos can disturb the cell cycle, we first have to understand normal cell cycle function.

In unperturbed cell cycle activity, unphosphorylated Cdc25s (Cdc25A and Cdc25C for example) have the responsibility of dephosphorylating cyclin dependent kinases (CDKs) via phosphatase activity.[91] Removing the phosphate group from a CDK allows the cell cycle to progress.[91] In the presence of ssDNA or stalled replication forks, ATR activates and in turn phosphorylates/activates Chk1at Ser317 and subsequently Ser345.[91,96,106,154] Activated Chk1 can then phosphorylate Cdc25A or Cdc25C phosphatases.[91,96,99,100,106] This phosphorylation of Cdc25A induces ubiquitin-mediated proteasome degradation of Ccd25A and thus inhibits activity/dephosphorylation of CDK1.[91,96,97] Phosphorylation of Cdc25C by Chk1 causes inactivation and thus prevents dephosphorylation of CDK1, causing cell cycle arrest.[91,96,97] Chk2 is activated through double stranded DNA breaks and subsequent ATM activation.[95,155] Phosphorylated Chk2 will similarly phosphorylate/inactivate Cdc25A or Cdc25C, but will inhibit CDK2 activity.[95,155] Inhibition of CDK1 activity results in G2/M transition cell cycle arrest,

while CDK2 inhibition results in G1 cell cycle arrest.[100,108,155–158] A summarized diagram of normal cell cycle can be seen in Figure 14.

Our previous study (Chapter 2) revealed that the moderately toxic insecticide chlorpyrifos (CPF) caused decreased cell viability for EBV+, non-BL cells and EBV-, BL cells, but not EBV+, BL cells. In contrast, its active metabolite chlorpyrifos-oxon (CPO) did not cause any changes in cell viability to any of the three cell lines used. We also observed overall cell concentrations were affected in a concentration dependent manner in all three cell lines for CPF. However, only EBV-, BL cells showed a noticeable cell concentration change when exposed to CPO (decrease total cell concentration with increasing CPO concentration). Our results also showed that CPF exposure at a high concentration (300 μM) led to likely G1/S transition cell cycle arrest for EBV+ and EBV- cells. Overall, CPO exposure at lower concentrations caused cell cycle arrest at the G1/S transition for EBV+ and EBV- cells. However, EBV-, BL cells were clearly more sensitive and were more readily entering cell cycle arrest at lower concentrations in comparison to EBV+, BL and EBV+, non-BL cells.

To better understand our previous results and possible driving mechanisms of inducing cell cycle arrest, we decided to investigate oxidative stress, DNA damage, and the cell cycle checkpoints 1 and 2, in relation to CPO, CPF, and EBV interactions. Our results showed that CPO induced oxidative stress and affected EBV+ cells more so than EBV- cells. We also found that EBV+ and EBV- cells showed weak presence of DNA damage caused by either high doses of CPF and CPO. Interestingly, when we investigated how the cell cycle checkpoint proteins were affected by CPO exposure, we

found that cell cycle checkpoint and DDR related proteins were still activated despite the overall low presence of DNA damage. EBV+ cells overall showed decreased levels of phosphorylated Chk1 (pChk1) at high doses and increased levels of phosphorylated Chk2 (pChk2). Meanwhile, EBV- cells showed little change of pChk1 and pChk2 levels at all CPO exposure doses. Taken together with previous flow cytometry cell cycle data, it appears that CPO was able to induce cell cycle arrest at the G1/S transition in all cell lines. Results from this study also provided further evidence that the presence or absence of EBV affects how B-lymphocytes will function in the presence of cytotoxic agents.

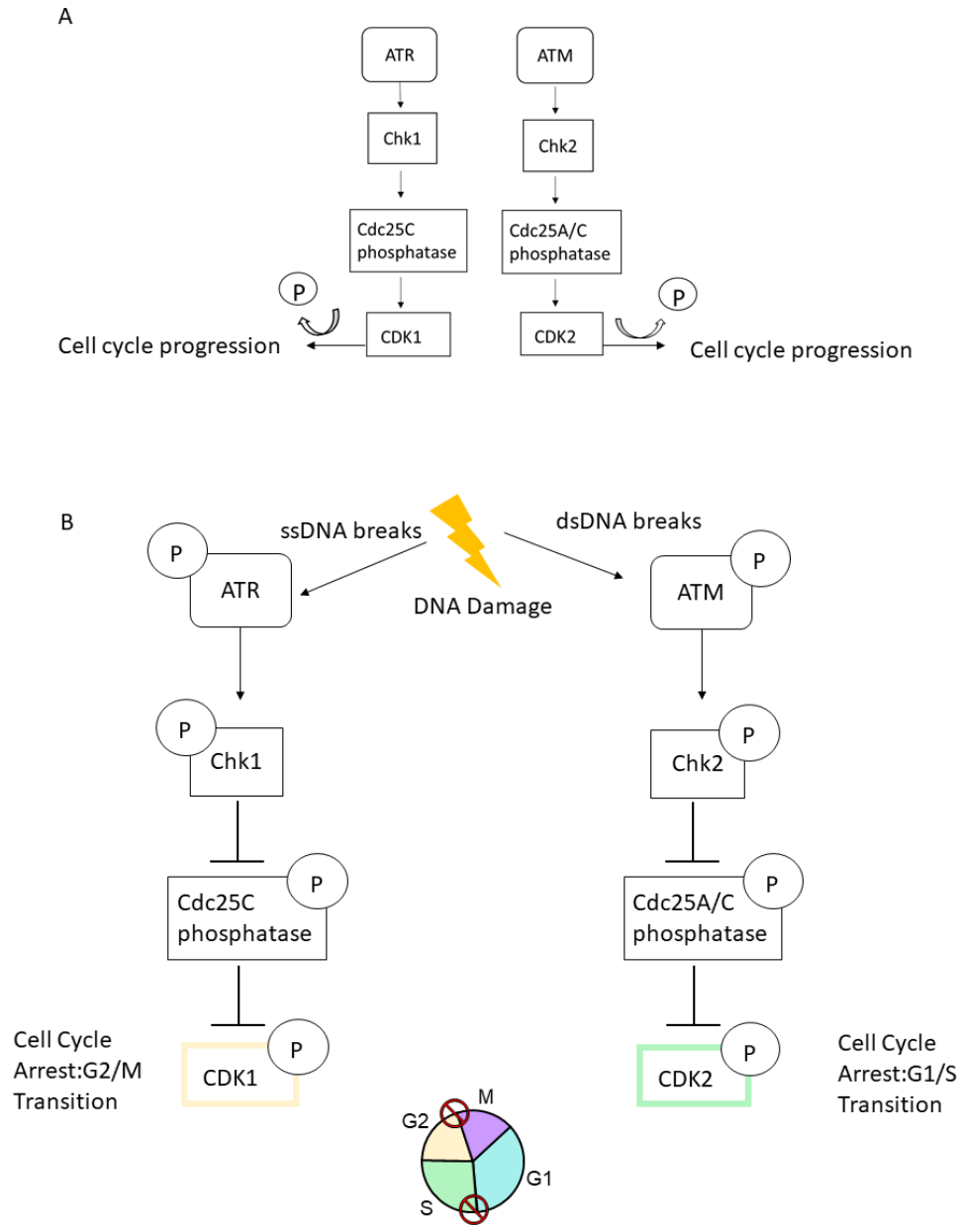

Figure 14. Summary of Unperturbed Cell Cycle and DNA Damage Induced Cell Cycle Arrest. (A) Illustrates unperturbed cell cycle progression while (B) illustrates cell cycle arrest induced by ssDNA or dsDNA breaks.

2. Materials and Methods

2.1 Cell Culture

Two EBV+, B-lymphocyte, immortalized cell lines, Raji and IM9, and one EBV-, immortalized cell line, Ramos, were used in this study. Raji cells were derived from an individual who had EBV+ Burkitt's lymphoma (EBV+, BL), IM9 cells were derived from an individual with EBV+ mononucleosis (EBV+, non-BL), and Ramos cells were derived from an individual with Burkitt's lymphoma but without EBV infection (EBV-, BL). All cell lines were purchased from ATCC, and cultured at the following conditions: 37ºC at 5% CO_2, and maintained in RMPI-1640 medium with 10% fetal bovine serum, with streptomycin, penicillin, and fungicide.

2.2 Organophosphate Pesticide

This study mainly used the active metabolite of chlorpyrifos (CPF), chlorpyrifos-oxon (CPO) (Chem Service Inc., West Chester, PA). CPF and CPO were prepped with DMSO for a stock solution at 100 mM and stored at -20 ºC.

2.3 Organophosphate Treatments

Cells were treated with DMSO for the vehicle control or at five concentrations (100, 125, 150, 175, 200 µM) of CPO for 24 hours. The TUNEL assay also included 200 µM of CPF.

2.4 General Oxidative Stress/CM-H2DCFDA

To test general for general oxidative stress, the CM-H2DCFDA kit (Invitrogen/ThermoScientific) was used according to manufacturer's protocol. This kit used 2,7-dichlorofluorescein diacetate or DCFDA to measure ROS activity like hydroxyl

and peroxyl anions. Once the dye diffused into the cell, DCFDA underwent deacetylatation and oxidation by ROS to form 2,7-dichlorofluorescein or DCF. Therefore, if the organophosphate exposed to the cells generated oxidative stress, the ROS would oxidize the cells to produce DCF and make the samples fluoresce. Samples were analyzed via Guava easyCyte flow cytometer (Millipore Sigma, USA) with InCyte software.

2.5 DNA Damage/TUNEL Assay

The presence of DNA damage was assayed using the APO-DIRECT™ kit (BD Biosciences) per manufacturer's protocol using the Incyte software of the Guava easyCyte flow cytometer (Millipore Sigma, USA). EBV+, BL and EBV-, BL cells were tested with DMSO, 100, 150, 200 µM of CPO and 200 µM of CPF. Positive and negative control cells included in the kit were also used to establish presence or absence of DNA damage. After cells were treated and for 24 hours, cells were fixed in 1% paraformaldehyde (dissolved in 1x PBS) and stored in ice cold 70% ethanol before staining with FITC-dUTP (a fluorescent label) and propidium iodide. Presence of DNA breaks (both single stranded and double stranded breaks) at the 3'-OH termini were labeled with FITC-dUTP. Cells were also stained with propidium iodide to indicate intact DNA. Therefore, FITC-dTUP bound to DNA breaks fluoresced when assayed via flow cytometry.

2.6 Western Blot and Immunoblotting

Samples exposed to CPO at 100, 125, 175, and 200 µM were lysed with 0.25M NaCl, 0.1% NP4O, 50 mM HEPES pH 7.5, 5 mM EDTA , and protease/phosphatase

inhibitors (ELB lysis buffer). Samples were stored at -80 ºC. 20-40 µg were electrophoresed on a 10% SDS-PAGE gel (at 200V) and transferred to an Immobilon membrane (Millipore) at 100 mA, overnight.

After transferring proteins to the membrane, blots were blocked with 0.25% milk block solution (0.25% milk, 0.1% Tween-20, and 1x PBS). Primary antibodies and concentrations used to analyze proteins included: Total ATR (C1) (1:500, Santa Cruz Biotechnology), phospho-ATR (Thr68) (1:1000, Cell Signaling), phospho-Chk1(Ser317)(D12H3)XP (1:1000; Cell Signaling), phospho-Chk2 (Thr68)(C13C1) (1:1000; Cell Signaling), total chk1 (1:500; Santa Cruz Biotechnology), β-Actin (C4) (1:500; Santa Cruz Biotechnology), and α Tubulin (1:500; Santa Cruz Biotechnology). Goat-anti-mouse IgG (H+L) and goat-anti-rabbit IgG (H+L) (Jackson ImmunoResearch Laboratories, Inc) were used as secondary antibodies at 1:5000 dilution for 10-20 minutes at room temperature. Blots were washed with wash solution (1x PBS, 0.1% Tween-20) four times after each primary and secondary antibody incubation. The SNAP i.d. 2.0 Protein Detection System (Millipore) was used to perform the blot washes. WesternBright ECL (Advansta), a horseradish peroxidase (HRP) substrate, was used for chemiluminescent detection (incubated at room temperature for 5-10 minutes) before imaging and quantifying the blot on the C-DiGit Western Blot Scanner (LiCOR).

Phosphorylated and total ATR and Chk1 levels were assessed separately via Western blot, but the phospho and total levels were also assessed by comparing the ratios to each other. For each trial, the treatment values from the Western blots were normalized

to the loading control. The trials were then averaged and the ratio was calculated (phosphorylated levels/total levels).

2.7 Statistical Analysis

CM-H2DCFDA and immunoblotting were conducted with n=3 while the TUNEL assay was conducted with a sample size of n=4. CM-H2DCFDA and TUNEL assay used either a one-way ANOVA or a Welch's one-way ANOVA and Dunnett's multiple comparison or Dunnett's T3 multiple comparison tests, depending on equal variances, to compare treatment groups to the DMSO control. Western blots were analyzed using a two-tailed, two-sample with unequal variance, student T-test to compare each treatment group to the DMSO control. Statistical significance was measured using $p<0.05$. Graphs represent both the average and individual data points from each trial. PRISM Graphpad was used for statistical analysis and to generate graphs. Only the averages of the Phospho/Total ratios for ATR and Chk1 protein levels were graphed.

3. Results

3.1 Chlorpyrifos-Oxon Induced Oxidative Stress in EBV-Positive B Cells but not EBV-Negative B Cells

Due to our previous work that indicated cell cycle dysregulation, presence of cytotoxicity at high concentrations, and altered expression of important regulatory proteins occurred in CPF and CPO treated B cells, (Chapter 2), it was of interest to investigate what factors contributed to these findings. EBV+, BL, EBV+, non-BL, and EBV-, BL cells were treated with CPO (100, 125, 150, 175, and 200 μM) for 24 hours and assayed for the presence of oxidative stress using a CM-H2DCFDA kit which detects

reactive oxygen species (ROS) within cells. EBV+, BL cells exhibited an increased level of ROS at 100 µM (77.71%), 125 µM (78.33%), 150 µM (78.81%), 175 µM (85.83%), and 200 µM (87.34%) in comparison to the DMSO control (61.81%), with statistical significance at 175 and 200 µM (Fig. 15A). EBV+, non-BL cells showed no statistically significant increase of ROS when cells treated with CPO were compared to the DMSO control (67.37%): 100 µM (79.11%), 125 µM (83.13%), 150 µM (77.42%), 175 µM (82.96%), and 200 µM (83.53%), although there was a general trend of increased ROS presence with increased CPO dosage (Fig. 15B). EBV-, BL cells interestingly showed no statistical changes in ROS when treated samples were compared to the DMSO control (62.43%): 100 µM (66.80%), 125 µM (67.09%), 150 µM (65.41%), 175 µM (62.58%), and 200 µM (60.02%) (Fig. 15C).

These results suggest that CPO exposure contributed to oxidative stress in EBV+ cells, but not in EBV- cells. This indicates that the presence of both EBV and the pesticide can interact to cause a greater level of cell damage versus the presence of pesticide only.

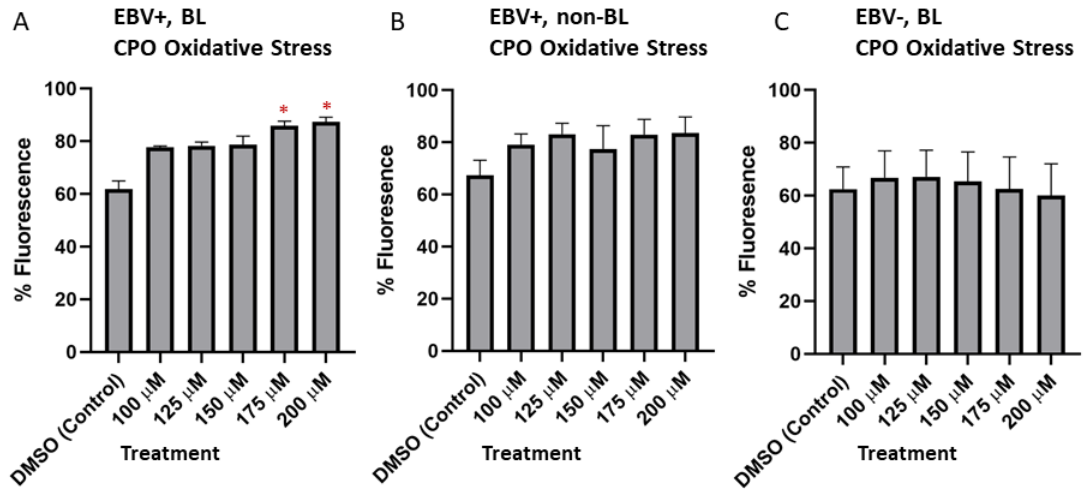

Figure 15. CPO Exposure caused Oxidative Stress in EBV-Positive Cells, but not EBV-Negative Cells. (A) EBV+, BL, (B) EBV+, non-BL, and (C) EBV-, BL cells were treated with CPO for 24 hours and assessed for the presence of oxidative stress. Treatments were compared to the DMSO control. n=3, * indicate p-value <0.05.

3.2 EBV-Positive and Negative Cells Exhibited Low Levels of DNA Damage when Exposed to Chlorpyrifos-Oxon

To further investigate factors that contributed to changes in B cell biology when exposed to CPF and CPO, we assayed for the presence of DNA damage with the TUNEL assay. This assay tests for the presence of damaged DNA associated with both ssDNA and dsDNA breaks. EBV+, BL and EBV-, BL cells were employed in this experiment and treated with 100, 150, or 200 µM of CPO or 200 µM of CPF. The percentage of FITC-dUTP that associated with DNA was measured in order to identify the presence of DNA damage in these cells. EBV+, BL cells did not show any statistically-significant differences in this assay when cells were treated with CPO or CPF when compared to the DMSO control (3.88%): CPO-100 µM (3.92%), 150 µM (4.55%), 200 µM (7.66%), and

CPF 200 µM (8.32%) (Fig.16A). However, there was a general trend of increasing DNA damage with higher CPO or CPF doses (Fig.16A). EBV-, BL cells also showed a trend of increasing of FITC-dUTP incorporation with increasing concentrations when compared to the DMSO control (3.97%): CPO-100 µM (2.40%), 150 µM (7.98%), 200 µM (11.91%), and CPF 200 µM (24.21%), with statistical significance at 200 µM of CPF (Fig. 16B). These results suggest that CPO exposure triggers a minor amount of DNA damage in B cells. Additionally, we saw that EBV-, BL cells appeared to be more sensitive to the increasing CPO concentrations and the CPF exposure when compared to EBV+, BL cells.

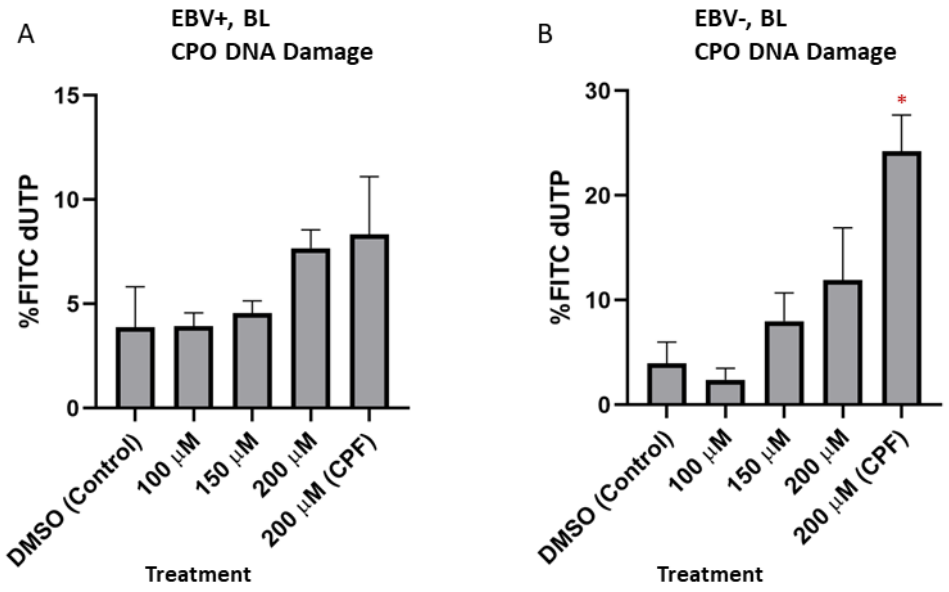

Figure 16. CPO Exposure Contributed DNA Damage to B Cells. (A) EBV+, BL and (B) EBV-, BL cells were treated with CPO or 200 μM CPF for 24 hours and assessed for the presence of DNA damage via the TUNEL assay. Treatments were compared to the DMSO control. n=4, * indicate p-value <0.05.

3.3 CPO Exposure Caused Misexpression of Proteins Associated with the DNA Damage Response

ATM and ATR (Ataxia-telangiectasia mutated/ATM-and Rad3-Related) are important kinases in the DDR pathway that phosphorylate specific proteins in the presence of DNA damage (ssDNA and dsDNA breaks). Activated ATR (and ATM) allow for phosphorylation and activation of cell cycle checkpoint proteins (such as Chk1 and Chk2), which in turn initiate a cascade of phosphorylation events that lead to cell cycle arrest. An ATR response is activated in the presence of ssDNA and replication fork stalls, versus ATM's activation through DNA double-stranded breaks.

We investigated, via Western blot, how CPO exposure affected phosphorylated and total ATR protein levels in EBV+, BL and EBV-, BL to further investigate if the presence or absence of EBV affected cellular function. EBV+, BL cells exposed to CPO showed a general decrease in phosphorylated ATR (phospho-ATR), with statistical significance at 175 µM (Fig. 17A). EBV-, BL cells showed a similar trend with a decrease in phospho-ATR levels, with statistical significance at 100, 125, and 150 µM of CPO when compared to the DMSO control (Fig. 17B). EBV+, BL cells showed a statistically-significant decrease of total ATR at 200 µM (Fig. 18). EBV-, BL cells did not show a statistically-significant decrease of total ATR, however there was a trend of decreased levels starting at 175 µM (Fig. 18). When comparing the phosphorylated levels to total levels, the EBV+, BL cell line showed a trend of decreased ratio with increasing concentrations of CPO, while the EBV-, BL cell line showed the inverse trend (Fig. 19). This suggested that CPO exposure caused a decrease in ATR production in EBV+, BL cells, whereas EBV-, BL cells appeared to have an initial decreased production of ATR then increased production with higher concentrations. Overall these results suggested that although still phosphorylated and active, ATR activity decreased in the presence of CPO. EBV-, BL cells particularly showed this decreased activity at lower concentrations in comparison to EBV+, BL cells.

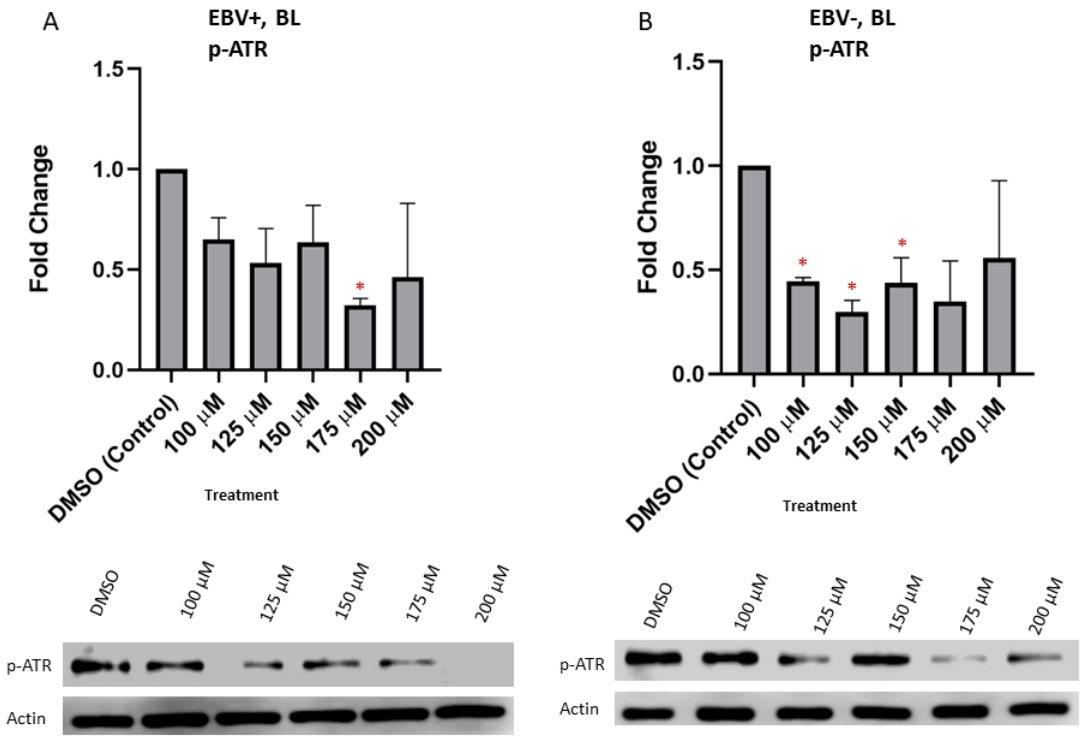

Figure 17. CPO Exposure Decreased Phosphorylated Levels of ATR in B Cells. Raji (A) EBV+, BL and (B) EBV-, BL cells were treated with CPO for 24 hours and assessed for the expression of phospho-ATR via Western Blot. Treatments were compared to the DMSO control. n=3, * indicate p-value <0.05.

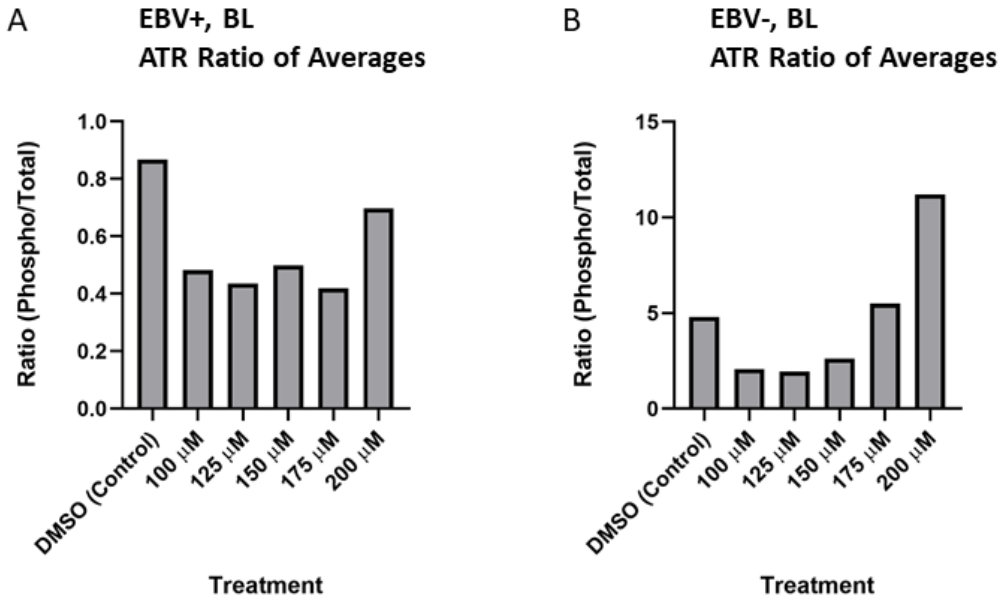

Figure 18. CPO Exposure Decreased Total Levels of ATR in B Cells. (A) EBV+, BL and (B) EBV-, BL cells were treated with CPO for 24 hours and assessed for the expression of total ATR via Western Blot. Treatments were compared to the DMSO control. n=3, * indicate p-value <0.05.

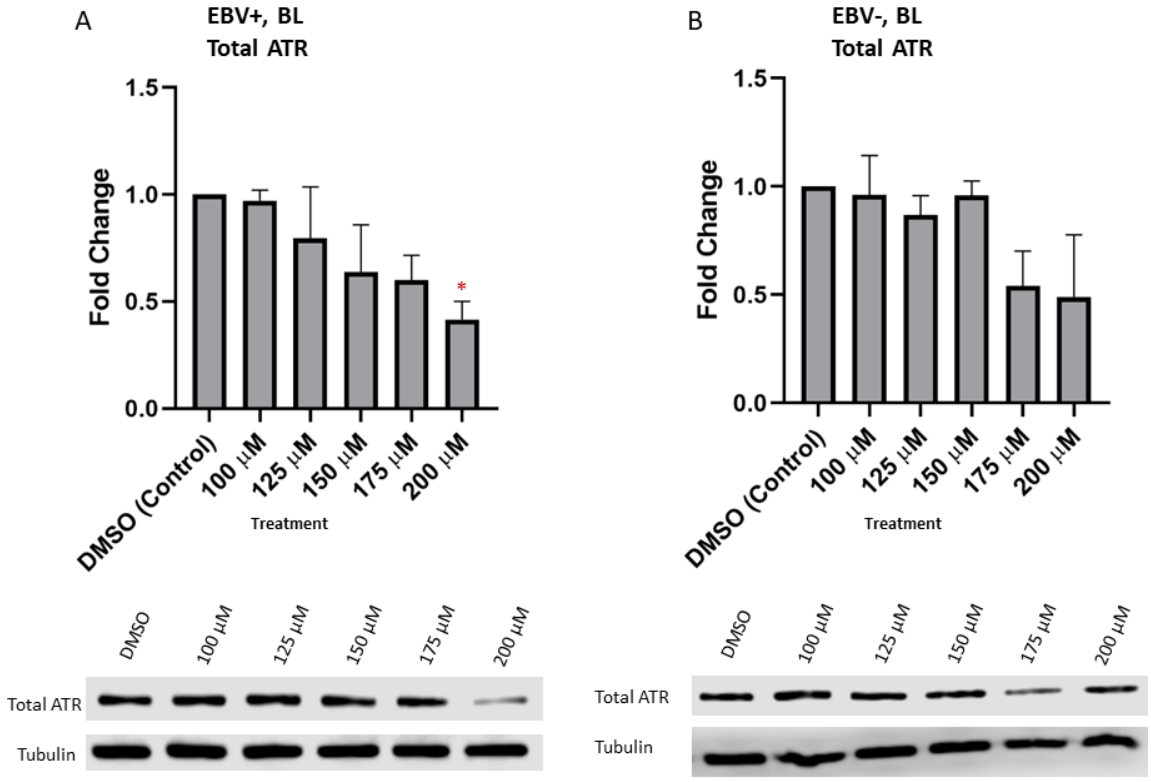

Figure 19. Phosphorylated ATR and Total ATR Ratios. (A) EBV+, BL and (B) EBV-, BL cells were treated with CPO for 24 hours. The phosphorylated and total levels of ATR were compared.

Evaluation of cell cycle checkpoint activity was also of interest to us to understand if and how the DDR pathway played a role in CPO-exposed B cells. Chk1and Chk2 are not only are involved with cell cycle regulation, but also play roles in the regulation of DDR. We assed total and phosphorylated levels of Chk1 and Chk2 in EBV+, BL, EBV+, non-BL, and EBV-, BL cells when exposed to CPO via Western blot. Chk1 and Chk2 are activated (phosphorylated) by single-stranded DNA breaks/ATR activation, or double stranded DNA breaks/activation of ATM, respectively[100,159–161]. Compared to the DMSO control, EBV+, BL cells exposed to 175 µM and 200 µM of

CPO showed statistically-significant decrease in phospho-Chk1 levels (Fig. 20A). This correlated with decreased levels of total Chk1 protein in EBV+, BL cells, with statistical significance at 175 µM (Fig. 21A). Similarly, EBV+, non-BL cells exposed to CPO at 150 µM, 175 µM, and 200 µM showed statistically-significant decreases of phospho-Chk1, compared to the DMSO control (Fig. 20B). This also correlated with the decreased levels of total Chk1, with statistical significance for all treatments when compared to the DMSO control (Fig. 21B). Although EBV-, BL cells did not show statistically significant changes in phospho-Chk1 (Fig. 20C), total Chk1 levels did show a statistically significant decrease at 200 µM (Fig. 21C). When comparing the phospho/total ratios for Chk1, EBV+, BL cells showed decreased ratios at higher concentrations (175 and 200 µM), EBV+, non-BL showed increased and decreased ratios throughout different concentrations, and EBV-, BL cells showed increased ratios with increasing concentrations at 175 and 200 µM (Fig. 22). Ratio results suggested that CPO exposure caused overall decreased production of Chk1 in EBV+, BL whereas EBV-, BL cells showed increased production of Chk1 at high concentrations. Since we saw changing levels of Chk1, we were also interested to see if Chk2 levels showed a similar trend and to see if the ATM/Chk2 side of DDR was also activated.

Here, we only probed Western blots for phosphorylated Chk2. EBV+, BL cells exposed to CPO showed statistically-significant increases of phospho-Chk2 levels at 100 µM, 125 µM, and 200 µM when compared to the DMSO control (Fig. 23A). Similarly, EBV+, non-BL cells showed increased levels of phospho-Chk2 with statistical significance at 125 µM CPO (Fig. 23B); EBV-, BL cells did not show any statistically-

significant difference in phospho-Chk2 levels at any dose of CPO, although there was a general trend of increasing phospho-Chk2 with increasing dose (Fig. 23C). We did not look at total levels of Chk2, though it would be of interest to us to see if total levels increased, decreased, or stayed the same so we could compare the phospho/total ratios.

In the presence of dsDNA damage, we would expect to see increased levels of phospho-Chk2 (an inhibitor of Cdc25A) which would then lead to decreased CDK2 activity, decreased cell entry into S phase, and thus eventually a G1 arrest. In turn, we would expect decreased levels of phospho-Chk1 (an inhibitor of Cdc25C plus an activator of wee1), and activated CDK1, which would allow for G2 to M transition. For EBV+, BL cells, we see significantly decreased phospho-Chk1 at higher doses of CPO along with increased levels of phospho-Chk2 at lower doses, aligning with a cell cycle arrest at G1 phase. With EBV+, non-BL cells, we see significantly decreased phospho-Chk1 at lower doses of the CPO along with increased levels of phospho-Chk2 at slightly higher doses, supporting a scenario where there is a more likely progression into S phase, along with transition into M phase. In contrast, EBV-, BL cells showed little change in phospho-Chk1 and –Chk2 levels. Taken together, the presence of the virus may predispose B cells to cell cycle deregulation in the presence of the pesticide, leading to cell cycle abnormalities. Once more, we saw evidence that the presence of EBV changed how B-lymphocytes were functioning in the presence of the organophosphate.

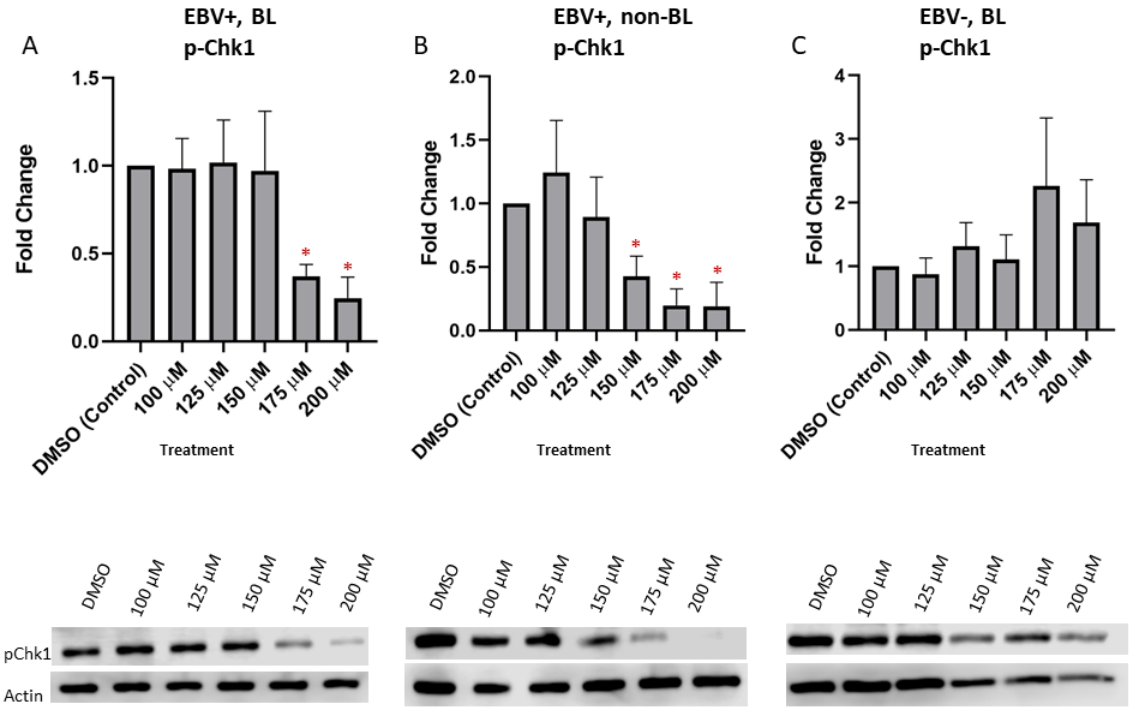

Figure 20. CPO Exposure Decreased Phosphorylated Chk1in B Cells. (A) EBV+, BL, (B) EBV+, non-BL, and (C) EBV-, BL cells were treated with CPO for 24 hours. P-Chk1was standardized to the loading control (β Actin) and treatments were compared to the DMSO control. n=3, * indicate p-value <0.05.

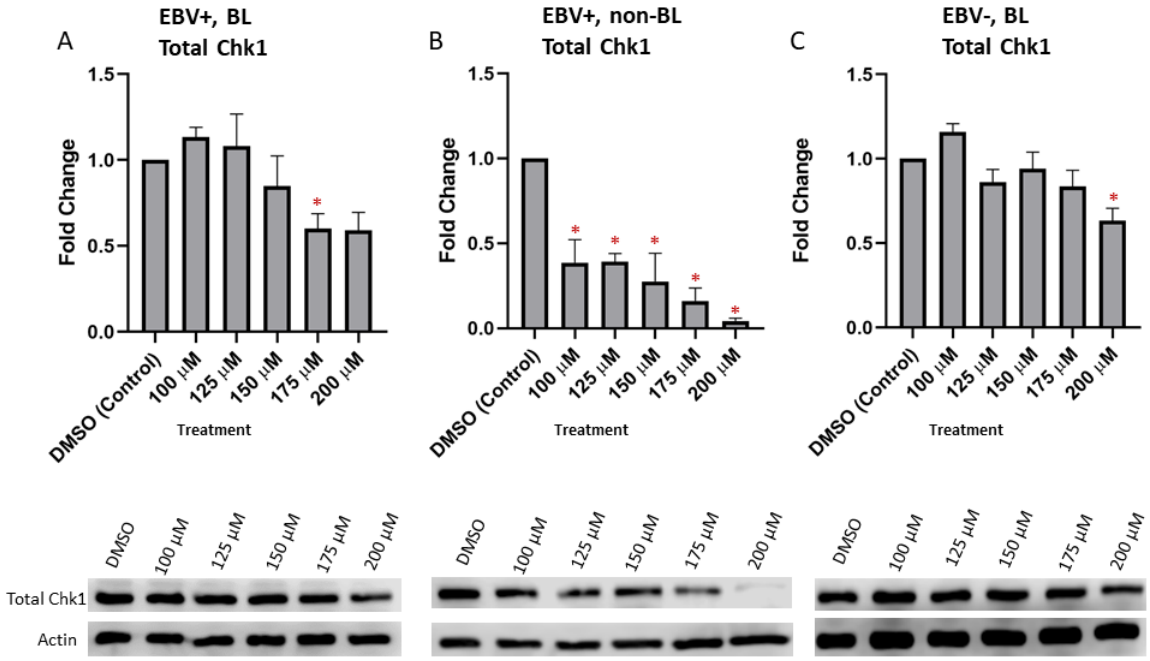

Figure 21. CPO Exposure Decreased Total Chk1 Protein Levels. (A) EBV+, BL, (B) EBV+, non-BL, and (C) EBV-, BL cells were treated with CPO for 24 hours. Total Chk1was standardized to the loading control (β Actin) and treatments were compared to the DMSO control. n=3, * indicate p-value <0.05.

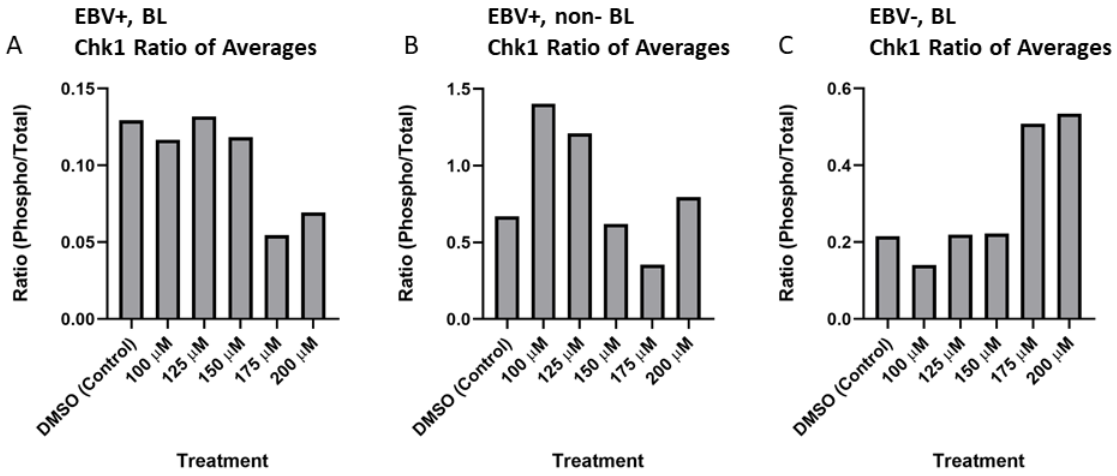

Figure 22. Phosphorylated and Total Chk1 Ratios. (A) EBV+, BL and (B) EBV-, BL cells were treated with CPO for 24 hours. The phosphorylated and total levels of Chk1 were compared.

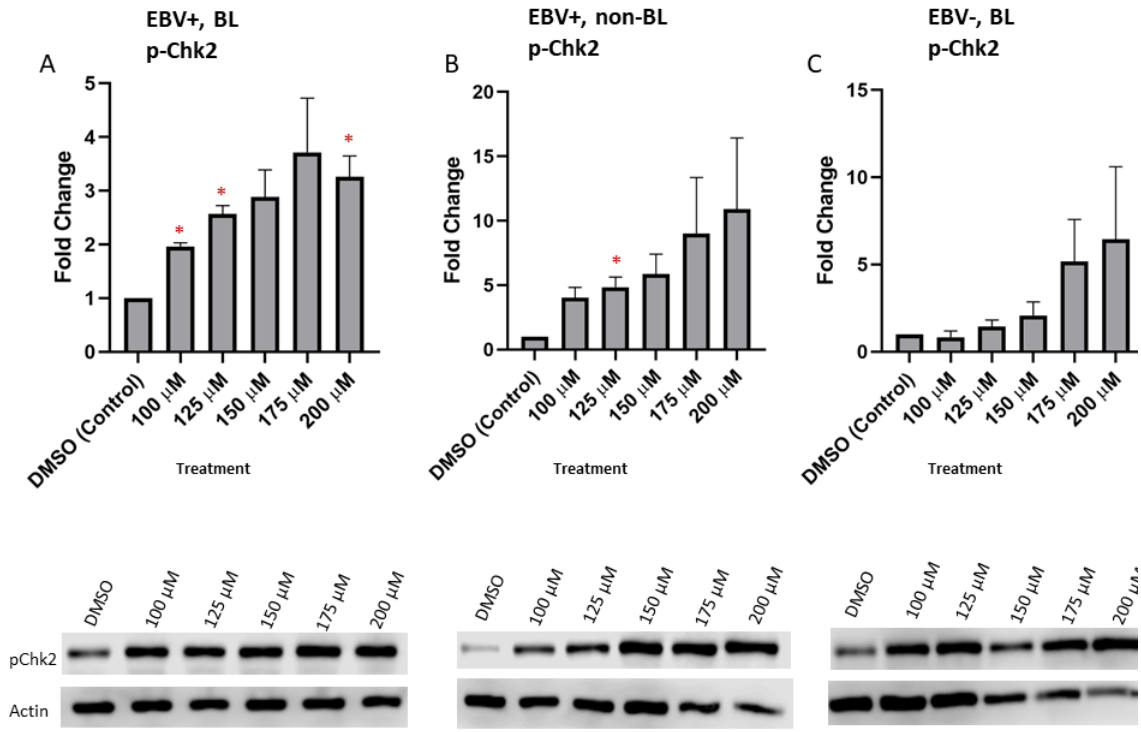

Figure 23. CPO Exposure Increased Phosphorylated Chk2 in B Cells. (A) EBV+, BL, (B) EBV+, non-BL, and (C) EBV-, BL cells were treated with CPO for 24 hours. P-Chk2 was standardized to the loading control (β Actin) and treatments were compared to the DMSO control. n=3, * indicate p-value <0.05.

4. Discussion and Conclusions

Previous work (Chapter 2), showed CPF exposure of EBV+ and EBV- B-lymphocyte cells decreased cell viability, caused dysregulation of the cell cycle, altered levels of important EBV replication proteins (BZLF1 and EBNA-1), and caused changes to levels of important cellular proteins like p70S6K and YY1. These results were particularly interesting since there appeared to be some level of viral effect, where cells that were EBV- showed a more sensitive response to CPO exposure in comparison to EBV+ cells. That is, EBV appeared to have a protective effect on the CPO exposed host

cell. Therefore, we wanted to further explore what contributed to some of the effects that we observed. We hypothesized that oxidative stress and the DNA damage response signaling pathway could be contributing factors to the effects we observed in Chapter 2. We used H2-CMDCFDA (for general oxidative stress), TUNEL assay, and Western blot data to assess this hypothesis and to better understand how CPO affects EBV-host/cell interactions.

Studies have shown that CPF and CPO exposure can induce oxidative stress in different tissues (spleen, kidney, brain, and liver of rats)[162] and cell types (neuroblastoma, blood, and oligodendrocyte progenitor in humans, erythrocytes and neurons in mice).[2,89,127,163–167] Our results showed that only EBV+, BL cells showed a statistically-significant increased presence of oxidative stress at high concentrations of CPO. Even though EBV+, non-BL cells exposed to CPO did not show any statistically significant changes in oxidative stress, the results still showed a trend of increasing oxidative stress at high concentration of CPO exposure. EBV-, BL cells on the other hand, did not show any significant changes to oxidative stress. Together, this indicated that the presence of both EBV and CPO may have elicited a potentiated effect with each other to increase the production of reactive oxygen species and eventual oxidative stress. Additionally, results indicated, regardless of statistical significance, that all treatments exhibited some level of oxidative stress.

Literature also supports that in rat and human peripheral lymphocytes, organophosphate (including CPF) exposure induced DNA damage.[168,169] With the exception of one treatment in EBV-, BL cells (200 μM of CPF) we did not see

statistically-significant changes in DNA damage; however, both EBV+, BL and EBV-, BL cells did show that CPO and CPF exposure resulted in some DNA damage. This lower level of damage could be explained by the fact that B-lymphocyte cells are not the targets for CPF or CPO and thus, the response that B cells have to CPO exposure, in comparison to neurons, is not as robust. We also did not expect to observe great amounts of DNA damage as our data indicated CPO caused few changes in cell viability (Chapter 2). Additionally, it was interesting to compare oxidative stress and DNA damage results since EBV+, BL cells showed evidence of increasing oxidative stress, but not DNA damage while EBV-, BL cells showed no change of oxidative stress, but most were responsive to DNA damage. It was likely that DNA damage was not the main mechanism that was affected by oxidative stress, even though we have shown evidence that the DDR pathway is in some way activated. Lipid peroxidation, which can be also be triggered by oxidative stress, may have played a role in the effects that we saw in Chapter 2. Also it is likely that a combination of oxidative stress, DNA damage, and lipid peroxidation were contributing factors to cell viability, cell proliferation, cell cycle related changes when B-lymphocytes were exposed to CPF or CPO. Regardless, it was still interesting to see that with the little DNA damage produced by CPF and CPO, EBV+ and EBV- cells showed activation of proteins associated with the DDR pathway.

The presence of ssDNA or dsDNA breaks via oxidative stress is known to trigger the DDR signaling pathway by activating ATM (Ataxia-telangiectasia mutated) or ATR (ATM-and Rad3-Related).[100,104,152,154] As our results showed the presence of oxidative stress and DNA damage (though not statistically significant), we investigated if the DDR

pathway was activated. We found EBV+, BL and EBV-, BL cells showed a trend of decreasing (but yet still activated) phospho-ATR and total ATR levels with higher CPO concentrations. This suggested that at lower CPO doses, oxidative stress associated DNA-damage functions through ATR (due to the presence of ssDNA). In contrast, at higher CPO doses, DDR may be activated via ATM (due to dsDNA breaks). Given our results, we can further explain and understand our results from Chapter 2.

In our previous study (Chapter 2), we observed changes in cell cycle regulation when cells were exposed to CPF and CPO, suggesting that the presence of this organophosphate may play a role in cell cycle progression. Zhao et al. also found similar results when they exposed EBV+, BL cells to CPF for 4 hours.[128] In our study, with additional cell lines, concentrations, and the addition of the active metabolite (CPO), we observed statistically significant data points supporting a model where CPO altered cell cycle regulation to induce a G1/S transition arrest. Interestingly, when we investigated the protein levels of cell cycle checkpoint regulators (Chk1 and Chk2), we observed a statistically-significant decrease in phospho-Chk1 and increase in phospho-Chk2 levels. We observed that phospho levels of Chk1 and Chk2 in EBV+ cells were affected by the CPO more so than the EBV- cells, suggesting the presence of the virus influenced cellular behavior when exposed to CPO. We also observed that decreased phospho and total Chk1 levels corresponded to decreased phospho- and total ATR levels. Taken together we believe it is possible that both ATR and ATM pathways were likely activated (dsDNA breaks can form ssDNA residuals),[98,109] but the increased Chk2 levels suggest

the ATM pathway was favored. Overall, these results provided further support that CPF and CPO exposure induced cell cycle arrest at the G1/S transition in EBV+ and EBV- cells.

When we take the cell cycle, oxidative stress, DNA damage, and the cell cycle checkpoint protein data together, we can better understand how CPO interacts with EBV and B-lymphocytes in relation to oxidative DDR mechanisms. In EBV+, BL cells, we believe that at lower concentrations, CPO produced low levels of oxidative stress to induce small amounts of DNA damage to trigger the ATR response, as we observed increased levels of phospho-Chk1. However, at higher concentrations (175 and 200 μM of CPO), we believe there was a switch from ATR activation to ATM activation. Although we did not have ATM protein level-data, there was a dramatic decrease of phospho-Chk1 levels at 175 and 200 μM of CPO exposure, an increase of p-Chk2 levels as CPO exposure increased, and a decrease in phospho-ATR levels. This agrees with our cell cycle data where EBV+, BL cells appeared to have a G1/S transition arrest; the activation of ATM and Chk2, and the subsequent deactivation of Cdc25A/C and CDK2 result in a G1/S transition arrest. Given only having oxidative stress, Chk1, and Chk2 data, we can speculate that EBV+, non-BL cells behave similarly to EBV+, BL cells resulting in ATM and Chk2-mediated DDR. However, this does not necessarily correspond with our cell cycle data from Chapter 2 since increasing CPO exposure did not result in cell cycle arrest at the G1/S transition; the number of cells actually increased as CPO doses increased. Though it's possible that there was a cell cycle arrest at the S/G2 transition since we also saw a decrease of cells in G2 phase with CPO exposure. In

comparison, we believe EBV-, BL cells experienced enough oxidative stress, despite seeing little to no change of oxidative stress (perhaps inducing some other type) with increasing CPO doses, to trigger a mainly ATM mediated DNA-damage response since we saw an overall trend of increased levels of phospho-Chk2. Again it is important to note that Chk1 and Chk2 can both be active since double strand DNA breaks can form single strand break intermediates,[98,109] as we saw in the EBV-, BL cells.

Our study further investigated how virus-host/cell interactions are affected when a potentially toxic, exogenous environmental factor is introduced into a system. Our findings showed that the presence of increasing CPO doses varied in exhibiting oxidative damage and genotoxic stress, depending on whether the B-lymphocyte cell line was infected with EBV or not. We also found that EBV+, BL cells potentially showed an ATR-mediated DNA-damage response at lower concentrations of CPO, then switched to mainly an ATM-mediated DNA-damage response and cell cycle arrest. EBV+, non-BL cells demonstrated a more interesting behavior where cells exhibited similar patterns seen in EBV+, BL cells, but did not result in evident cell cycle arrest at the G1/S transition. The EBV-, BL cells appeared to be acting through an ATM-mediated response despite not having an overabundance of oxidative stress and genotoxic stress. Similar to our previous study, it appears that EBV+ cells respond differently when compared to EBV- cells. This suggests that presence of EBV plays a role in B-lymphocyte regulation and response mechanisms in relation to potentially dangerous exogenous environmental factors.

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CHAPTER IV

DISCUSSION AND CONCLUSIONS

1. Overall Discussion and Conclusions

This dissertation investigated the organophosphate pesticide, chlorpyrifos (CPF) and its active metabolite chlorpyrifos-oxon (CPO), and its effects on B-lymphocyte cells infected with and without the ubiquitous Epstein-Barr virus (EBV). We were interested in whether or not CPF and CPO exposure altered virus-host interactions both at the viral and cellular level and what mechanisms were driving these effects. Using EBV+, BL, EBV+, non-BL, and EBV-, BL cells, we were able to see how the presence of both EBV and the organophosphate affected cellular and viral replication and the mechanisms that drove these processes. Visual result summaries of the dissertation for both EBV+, BL, EBV+, non-BL, and EBV-, BL can be seen in Figures 24 and 25.

In unperturbed cells, we can assume that most cellular processes are functioning normally and in the presence of potential danger, cellular mechanisms would activate to protect and repair the cell. We found that CPF induced cytotoxicity to decrease cell viability in EBV+, non-BL and EBV-, BL cells, but not EBV+, BL cells. Interestingly, none of the cell lines showed any significant changes in cell viability when cells were exposed to CPO. Overall, this suggested that CPF may produce more immediate cytotoxic effects on cells even though CPO, as the active metabolite, is more toxic. However, this could be explained by CPF and CPO having neuronal cells as their target

(CPF is a neurotoxin)[61,76,87] cell type in contrast to B-lymphocytes. Thus, the effects we see in our results may not be as robust as they would be if we used neuronal or brain cells. Alternatively, CPF and CPO may have different molecular targets when binding to acetylcholine receptors which may have changed the toxicity of the organophosphate. Although CPO may not be causing direct cytotoxic effects to cause cell death, the active metabolite may alter other cellular functions to cause defects or dysregulation in important pathways like the cell cycle since we did see CPF and CPO affect total cell concentrations with increasing concentrations of the pesticide.[88,170]

To investigate putative organophosphate-mediated irregularities in cellular mechanisms, we decided to examine the cell cycle to determine if CPF and CPO exposure induced cell cycle arrest or if exposure induced cells to progress through cell cycle phases. In unperturbed cells, we would expect the cell cycle to aid in cell proliferation of healthy cells and regulate and undergo proper defense and repair mechanisms (activation of tumor suppressor proteins, deactivation of oncoproteins, and cell cycle arrest to allow time for repair) in the presence of genotoxic or cytotoxic factors.[94,102,159] EBV+, BL, EBV+, non-BL, and EBV-, BL cells were exposed to CPF and CPO for 24 hours then assessed for cell cycle progression in G1, S, and G2 phases. We found that CPF exposure in all cell lines, at the highest dose (300 μM), showed decreased number of cells in G2 phase, which suggests that this high dose induced a G1/S transition arrest. CPO exposure on the other hand exhibited interesting results given that we observed statistically significant results at lower CPO concentrations. Overall, EBV+, BL cells appeared to experience a G1/S transition cell cycle arrest, as well as the EBV-,

BL cells, though the evidence of cell cycle arrest in EBV-, BL cells was more apparent at lower concentrations of CPO. EBV+, non-BL cells on the other hand, did not show clear evidence of any type of cell cycle arrest. In fact, at certain CPO doses, the number of cells entering S phase increased. One possible explanation would be that cell cycle checkpoints were altered to prohibit an arrest, or the cells were undergoing an S/G2 transition arrest since we did see decreased percentage of cells in G2. These results suggest EBV+ cells exhibit a delayed response in cell cycle arrest (seen in EBV+, BL cells) or a response that promotes cell cycle progression through S phase (as seen in EBV+, non-BL cells), because EBV-, BL cells showed evidence of cell cycle arrest at lower concentrations of CPO.

Given that we know CPF and CPO exposure caused changes in cell viability, cell concentration, and the cell cycle, it was of interest to us to investigate how viral replication was affected, considering EBV replication and survival requires hijacking and utilizing the host's cellular mechanisms.[1,38] EBV immediate-early protein BZLF1 is an important viral protein in lytic replication. BZLF1 acts as a required trans-activator for EBV early proteins so that viral replication can occur,[1,35,57] thus its presence is a marker for lytic replication. When EBV+, BL and EBV+, non-BL cells were exposed to 100 μM of CPF or CPO for 24 hours and chemically induced for lytic replication (using sodium butyrate and 12-O-tetradecanoylphorbol-13-acetate), BZLF1 levels decreased in EBV+, BL but increased in EBV+, non-BL cells, while both cell lines exhibited decreased BZLF1 levels with CPO exposure. We also examined the effects of CPO on EBV latent proteins as EBV is typically maintained in latency. Latent protein EBNA-1 is expressed

in all latency types and therefore is a marker for EBV latency. When EBV+, BL and EBV+, non-BL cells were exposed to different doses of CPO for 24 hours, the cells displayed decreased levels of EBNA-1. All together these results suggest that organophosphate exposure to EBV+ cells drives the virus towards latency if lytic replication is induced, or drives already latent cells to remain latent and not trigger lytic reactivation. This was further supported when we examined cellular proteins that are associated with the regulation of EBV replication such as YY1, which is a ubiquitous promotor and repressor protein, and p70S6K, a protein most notably associated with translation that is downstream of mTORC1.[129,171] YY1 is known to bind to EBV promoters to repress or keep the virus in latency[131]; CPO exposure did not decrease levels of YY1 in EBV+ cells which explains the decreased levels in BZLF1 and null change in EBNA-1 levels. Previous studies have indicated that EBV lytic replication relies heavily on mTORC1 activity[54,136]; our results showed that phospho-p70S6K levels decreased with CPO exposure. Decreased levels of activated p70S6K indicate that mTORC activity is being inhibited by CPO and therefore helps explain decreased levels of BZLF1. Although EBV-, BL were not related to regulation of EBV, it was interesting to see how the absence of the virus actually increased levels of phospho-p70S6K.

After observing CPO-induced changes in both EBV and B-lymphocyte replication, it was then of interest to investigate potential driving cellular mechanisms that contributed to these results. Past literature and studies have established organophosphates, and specifically chlorpyrifos, are able to induce oxidative stress and DNA damage in certain cell types.[2,89,128,168,170,172,173] However, most of these studies did

not specifically investigate how EBV+ B-lymphocyte cells influenced cellular signaling pathways and cellular regulatory mechanisms in relation to cytotoxic and genotoxic stressors. Therefore, we examined if B-lymphocytes exhibited organophosphate-induced oxidative stress and DNA damage, in addition to proteins associated with these stressors.

EBV+, BL and EBV+, non-BL cells exposed to CPO for 24 hours overall showed increased presence of oxidative stress, while EBV-, BL cells did not exhibit a decrease nor increase of oxidative stress with increasing concentrations of CPO. This suggests that EBV presence and CPO exposure together caused a greater cellular response in comparison to only pesticide exposure. Additionally, other types of stressors may be affecting EBV-, BL cells when exposed to CPO. Although not statistically significant, we also determined CPF and CPO produced low levels of DNA-damage in EBV+, BL and EBV-, BL cells, which may play a role in both viral and cellular changes pertaining to replication such as triggering the Ataxia telangiectasia mutated (ATM)/Ataxia telangiectasia Rad3 related (ATR) signaling cascade to induce cell cycle arrest. Although the organophosphate did not produce large amounts of DNA damage, results still suggest that CPF and CPO exposure were still able to act as cytotoxic and genotoxic stressors in B-lymphocyte cells despite not dramatically affecting cell viability. To further explore genotoxic stressors in relation to the cell cycle, we also explored how DDR and cell cycle checkpoints were affected with CPO exposure.

In the presence of cellular stressors like DNA-damage, ATM and ATR are typically activated to induce checkpoint proteins Chk1 and Chk2 activity to initiate a cascade of signaling proteins for cell cycle arrest.[95–97,100,104,149,155,160] In the absence of

DNA damage, Chk1 and Chk2 are not phosphorylated, which allows for Cdc25A/C to either remove a phosphate group from CDK1 or CDK2 which in turn allows for the cell cycle to progress.[90,91] In the presence of ssDNA or double strand DNA breaks, cells should respond by activating ATR (single stranded DNA breaks) or ATM (double stranded DNA breaks) and then phosphorylate Chk1 (via ATR activation) or Chk2 (via ATM activation).[98,100] Phosphorylation of Chk1 and Chk2 then results in the phosphorylation Cdc25A and Cdc25C; this phosphorylation of Cdc25s inhibits its activity and therefore will leave CDK1 (via ATR and Chk1) or CDK2 (via ATM and Chk2) phosphorylated and thus will cause cell cycle arrest.[91,93,95,96,101] We found that CPO exposure most likely induced an ATM-mediated response via double strand DNA breaks in EBV+, BL and EBV-, BL cells, though ATR-mediated DNA-damage was likely also present because double strand breaks can produce single strand DNA intermediates to trigger the ATR-signaling cascade.[98] This was supported by observing phosphorylated and total ATR levels decreasing, phosphorylated and total Chk1 levels decreasing, and phosphorylated Chk2 levels increasing when cells (especially EBV+, BL and EBV+, non-BL cells) were exposed to CPO.

Overall, this dissertation addressed our initial hypotheses that 1) CPF and CPO exposure would induce viral and host cell alterations related to replication, 2) EBV and CPF (and its active metabolite CPO) alter virus-host interactions to produce a combined effect, that is the presence of both EBV and CPF/CPO produces greater cellular responses in comparison to cells that are not infected with EBV, and 3) CPF and CPO exposure to B-lymphocyte cells induces cellular damage and irregularities in vital cellular

mechanisms (such as the cell cycle) that in turn can affect EBV and B-lymphocyte replication. Our results suggested that CPF and CPO exposure caused alterations in EBV and host cell replication mechanisms with a) changes in EBV replication protein levels, b) likely G1/S transition cell cycle arrest, and c) changes in total cell concentration in a pesticide concentration dependent manner. We concluded that CPO exposure to B-lymphocyte cells produced small amounts of oxidative stress and DNA damage and it is likely that other factors like lipid peroxidation may also play a role in our results. We speculate that this small amount of damage and stress was still enough to elicit a cellular response to trigger cell cycle arrest. We also speculate that CPO may have bound to non-target receptors in comparison to CPF, where CPF caused changes in cell viability while CPO did not. This would explain, in part, why CPF may have reacted differently with the host cells and virus in comparison to CPO. Lastly, we concluded that EBV and the organophosphate alter virus-host interactions more so in a potentiation effect in comparison to a combined effect, as initially hypothesized (Fig. 26). Our results suggested that the presence of EBV has a protective response in favor of the virus, where appropriate cellular responses are delayed until cells are exposed to higher concentrations of CPF or CPO. In contrast, cells without the virus (EBV-, BL) are readily responding to the organophosphate exposure at lower concentrations. We saw this effect in nearly all of our results.

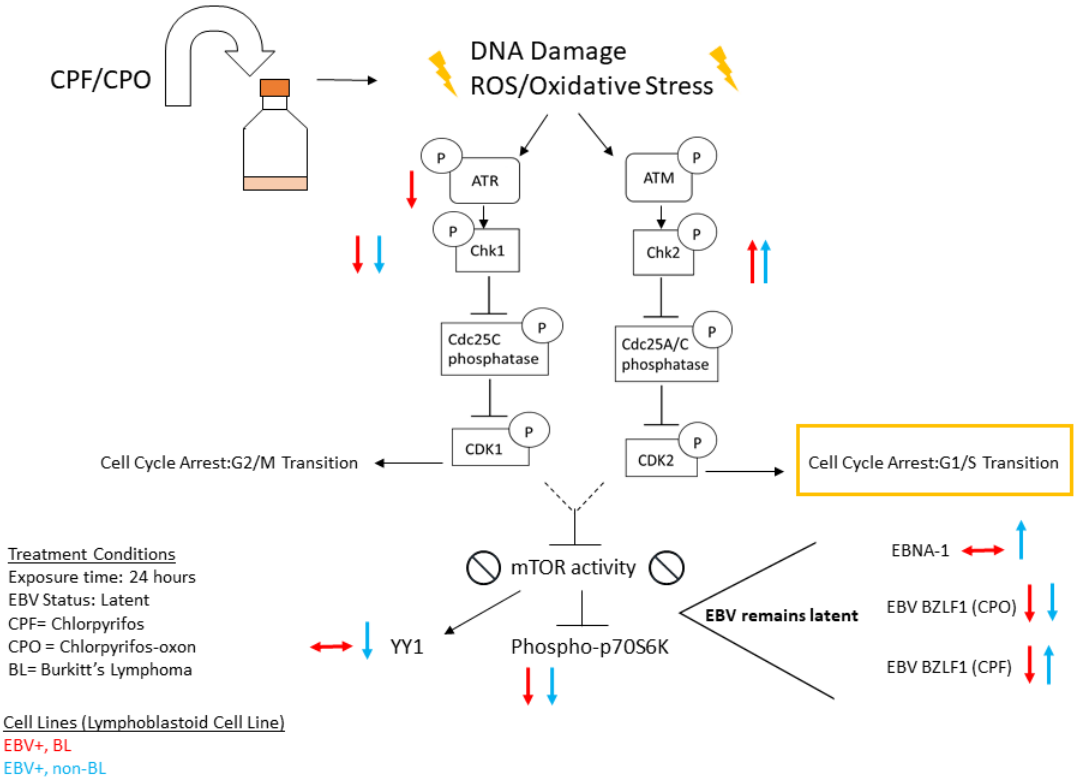

Figure 24. Summary of Results when EBV+, BL and EBV+, non-BL Cells were Exposed to CPF and CPO.

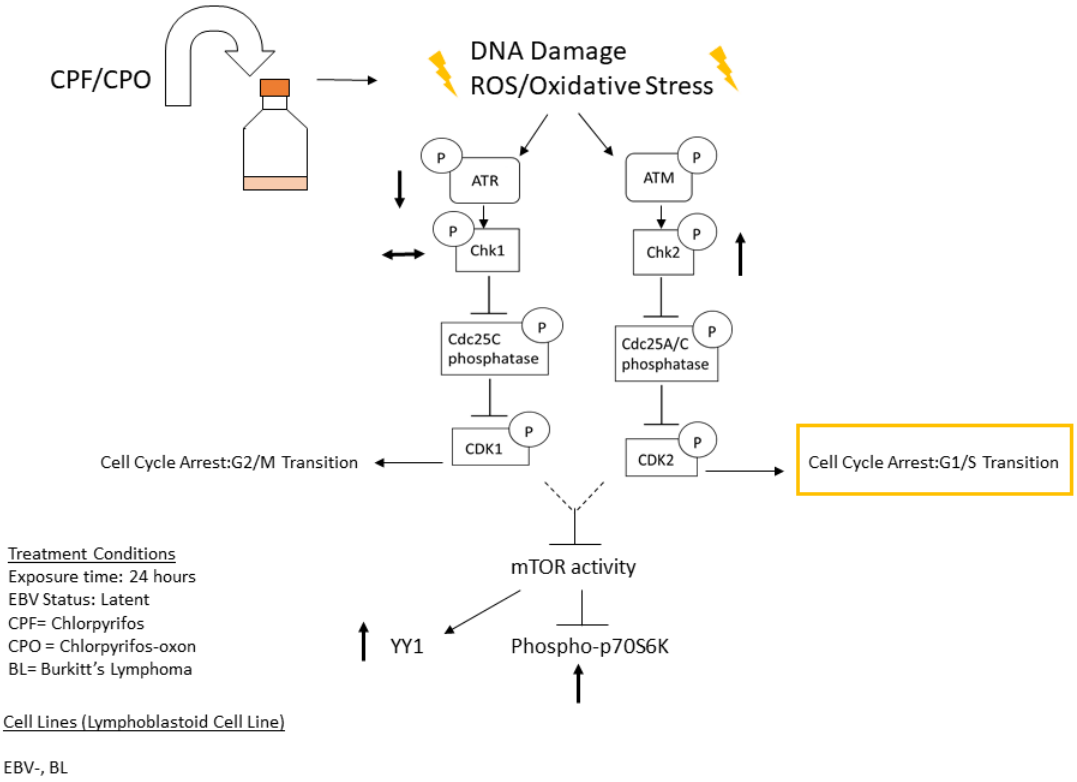

Figure 25. Summary of Results when EBV-, BL Cells were Exposed to CPF and CPO.

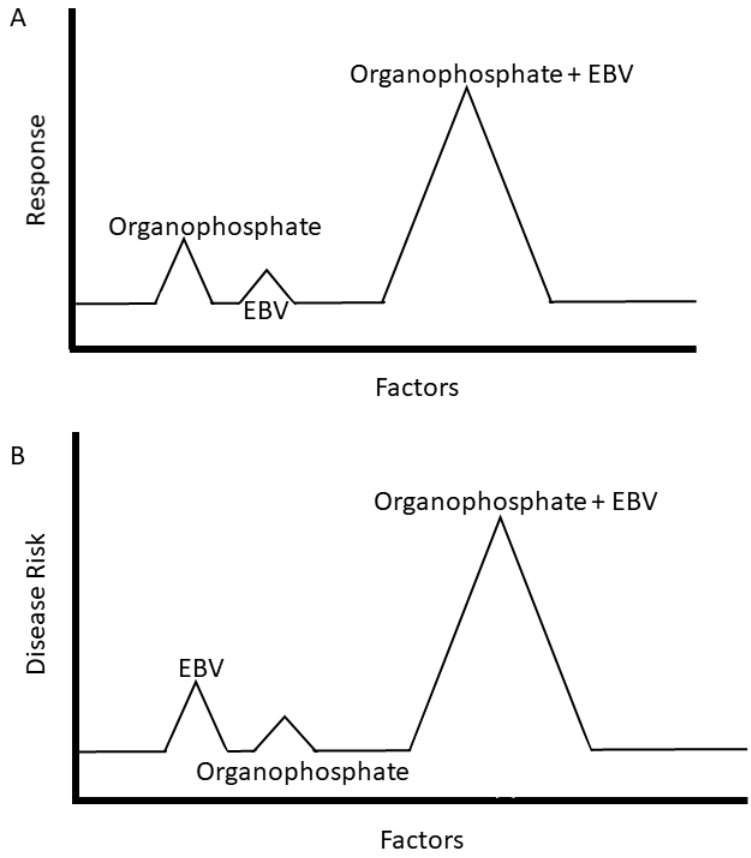

Figure 26. Revised Conceptual Model of EBV-Host Interactions and Organophosphate Exposure. (A) Initial model hypothesized a combined effect when EBV was present with the organophosphate. (B) Our revised model now illustrates the presence of EBV and organophosphates likely increases disease risk. Our results suggest the combination of these factors had a protective response for the virus and delayed cellular response when exposed to CPF or CPO.

2. Future Directions

To fully understand the effects of CPF and CPO on B-lymphocytes, a more in depth investigation of the relationship between acetylcholine and B-lymphocytes is needed. Although information on non-neuronal acetylcholine receptors are well described, acetylcholine receptors in B-lymphocytes is less defined in comparison to other immune cells like T cell and natural killer cells. One way to approach this is to first identify if acetylcholinesterase (the target for CPO) is present in B-lymphocytes. This can be done by probing for acetylcholinesterase via Western blot or by qRT-PCR to identify if transcripts are present in these cells. Similarly, it would be necessary to identify which type of acetylcholine receptors are present in B-lymphocytes, nicotinic or muscarinic receptors; this could also be done through Western blot or qRT-PCR. If acetylcholine receptors are detected, a colorimetric assay can detect levels of acetylcholine for cell suspensions, where hydrogen peroxide (the byproduct of oxidized choline from hydrolyzed acetylcholine) can be detected by the colorimetric probe. Knowing what type of acetylcholine receptor and if acetylcholinesterase is present in B cells would provide evidence that would determine if CPF and CPO have the same or different targets/receptors. Thus, may help explain differences in results between the parent compound and the active metabolite.

It would also be of interest to also investigate further aspects of the cell cycle and cell cycle arrest. Targets downstream of Chk1 and Chk2 like the cell cycle regulators CDKs and components that regulate CDKs (cyclins and CDK inhibitors) would be useful in determining if cell cycle arrest has taken place in the presence of organophosphate

exposure. Additionally, investigation of oncogenes, tumor suppressors and genes related to DNA repair would also provide evidence that the cell cycle is being affected during organophosphate exposure.

Lastly, it would be of great interest to further investigate this topic using an additional cell line to fully compare EBV+, non-BL results. EBV+, BL and EBV-, BL provides useful comparisons since both cell lines are both Burkitt's lymphoma, so an additional cell line, EBV-, non-BL would be beneficial. The addition of this cell line would help determine if EBV-, non-BL cells showed a more sensitive response to lower concentrations in comparison to EBV+, non-BL cells and if the presence of EBV and organophosphates allows the cells to be protective of the virus and thus delays regular cellular responses until higher concentrations. This would indicate that EBV presence does play a major role in certain disease pathogenesis as the virus would disrupt normal cellular functions.

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