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"EBV and KSHV provide anti-apoptotic advantages to Primary Effusion Lymphoma (PEL)"

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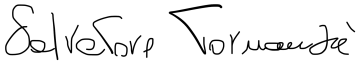

Academic year: 2023/24

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°C: Celsius degree

AIDS: Acquired immunodeficiency syndrome

Apaf-1: Apoptotic protease activating factor 1

ATP: Adenosine triphosphate

B-CLLs: B-cell Chronic Lymphocytic Leukemias

BAK: Bcl-2 homologous antagonist/killer

BARTs: BAMHI-A rightforward transcripts

BAX: Bcl-2 associated X protein

BCBL-1: Body Cavity Based Lymphoma-1

BCL2A1: BCL2-related protein A1

BCR: B-cell antigen receptor complex

Bfl-1: Bcl-2 related gene expressed in fetal liver

BH: BCL-2 homology regions

BID: Protein BH3 interacting-domain death agonist

BL: Burkitt Lymphoma

Cas9: CRISPR-associated protein 9

cDNA: Complementary DNA

CO_2: Carbon dioxide

Cp: C promoter

CRISPR: Clustered regularly interspaced short palindromic repeats

CRISPR/CasRx: Clustered regularly interspaced short palindromic repeats / RfxCas13d

CRISPRi: CRISPR interference

Ct: Cycle threshold

Cyto C: Cytochrome C

D10F: DMEM 10% FBS

dCas9: Deactivated CRISPR-associated protein 9

DISC: Death-inducing signaling complex

DLBCL: Diffuse Large B-cell lymphoma

DMEM: Dulbecco's modified Eagle's medium

DMSO: Dimethyl sulfoxide

DNA: Deoxyribonucleic acid

dnEBNA1: Dominant negative EBNA1

EBERs: EBV-encoded small RNAs

EBNA-LP: EBN nuclear antigen-leader protein

EBNA1: EBV nuclear antigen 1

EBNA2: EBV nuclear antigen 2

EBNA3A: EBV nuclear antigen 3A

EBNA3B: EBV nuclear antigen 3B

EBNA3C: EBV nuclear antigen 3C

EBV: Epstein-Barr Virus

ECL: Enhanced chemiluminescence

EDTA: Ethylenediaminetetraacetic acid

EGTA: Ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid

FAM: Fluorescein amidite

FBS: Fetal bovine serum

g**:** Gravity

GC: Gastric Cancer

H&E: Hematoxylin eosin

H3K9: Histone H3 lysine 9

HHV4: Human Herrpesvirus-4

HHV8: Human Herpesvirus-8

HL: Hodgkin lymphoma (

IABkFQ: Iowa Black FQ quencher

IC_{50}: Concentration required to inhibit half of the maximum biological response of the drug

Kb: kilobase

KRAB: Krüppel associated box

KS: Kaposi's Sarcoma

KSHV: Kaposi Sarcoma-Associated Herpes Virus

LANA1: Latency-associated nuclear antigen 1

LANA2: Latency-associated nuclear antigen 2

LCLs: Lymphoblastoid cell lines

LMP1: Latent membrane protein 1

LMP2A: Latent membrane protein 2A

LMP2B: Latent membrane protein 2B

mA: Milliampere

MCD: Multicentric Castleman's Disease

MCL-1: Myeloid Leukemia Cell Differentiation

min: Minute

miRNAs: MicroRNA

ml: Milliliter

mM: Millimolar

MMLV: Murine leukemia viruses

NaCl: Sodium chloride

NF-kB: Nuclear factor-kappaB

NK: Natural killer

NPC: Nasopharyngeal Carcinoma

ORF: Open reading frame

oriLyt: Origin of lytic replication

oriP: Origin of plasmid replication

PBS: Phosphate buffer saline

PEG: Polyethylene Glycol – 8000

PEL-LCs: PEL-like cells

PEL: Primary Effusion Lymphoma

PTLD: Post-transplant lymphoproliferative disease

Qp: Q promoter

R10F: RPMI 10% FBS

rcf: Relative centrifugal field

RFP: Red fluorescent protein

RHO: Rhodopsin

RIPA: Radioimmunoprecipitation assay buffer

RLUs: Relative lights units

RNA: Ribonucleic acid

RPM: Revolutions per minute

RPMI: Roswell Park Memorial Institute 1640 medium

RT-qPCR: Quantitative reverse transcription polymerase chain reaction

RT: Reverse transcription

SDO: Sodium deoxycholate

SDS-PAGE: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis

SDS: Sodium Dodecyl Sulfate

sgRNA: Single guide RNA

shRNA: Small hairpin RNA

siRNA: Short interfering RNA

TAM: Carboxytetramethylrhodamine

tBID: Truncated BID

TBS: Tris-buffered saline

TBST: Tris-buffered saline (TBS) with 0.1% Tween 20

Tris: Tris(hydroxymethyl)aminomethane

TSS: Transcription start site

vFLIP: Viral Fas-associated protein with death domain-like interleukin-1β-converting enzyme/caspase-8-inhibitory protein

VSV: Vesicular stomatitis virus glycoprotein

Wp: W promoter

µg: Microgram

µl: Microliter

Abstract

This study investigated similarities between primary effusion lymphomas (PELs) and PEL-like cells (PEL-LCs). PEL-LCs represent an *in vitro* transformation of peripheral B cells by both KSHV and EBV, presenting an opportunity for a detailed mechanistic analysis of the viral and cellular genes that orchestrate the initial stages of the progression toward PEL. The study revealed a crucial dependency of both dually (EBV and KSHV) and KSHV-only infected PELs on the antiapoptotic MCL-1 protein for their survival, aligning with previously reported findings. Notably, inhibiting MCL-1 with a small molecule inhibitor led to cell death in PELs but not in PEL-LCs. Further scrutiny through mRNA and immunoblotting analyses uncovered that PEL-LCs transcribe MCL-1 mRNA without detectable translation into proteins, prompting an exploration into the identity of the antiapoptotic protein supporting the survival of PEL-LCs. The findings identified BCL2A1 as the antiapoptotic protein that is upregulated in PEL-LCs compared to PELs and underscore the potential significance of BCL2A1 in PEL pathogenesis, with PELs showing enhanced survival with BCL2A1 ectopic-expression following the inhibition of MCL-1.

This study identifies BCL2A1 as a potential mediator of the resistance of PEL-LCs and implicates two EBV genes, EBNA3A and LMP1, in regulating BCL2A1 expression, providing a survival advantage to PEL-LCs. Experiments with CRISPRi-mediated knockdown of MCL-1 or BCL2A1, in PELs and PEL-LCs respectively, did not result in significant downregulation compared to the rhodopsin control, raising challenges in establishing a control for an unequivocal interpretation. The complex interplay between EBV genes and BCL2A1 regulation adds layers of complexity to the apoptotic regulatory network in PEL. This study sets the foundation for further investigations into the dual infection of KSHV and EBV in PEL, shedding light on the distinct contributions of each virus to tumor survival and proliferation.

CHAPTER I.

Background and Introduction

Infectious agents are responsible for around 2.2 million cancer cases annually [1], contributing to roughly 15% of all human cancers worldwide [1-4]. The majority of these malignancies are induced by tumor viruses. One such oncogenic virus is the Kaposi Sarcoma-Associated Herpes Virus (KSHV), also known as Human Herpesvirus-8 (HHV8), possessing a double-stranded DNA genome of approximately 138 kb [Figure I.1] [5]. Initially isolated from Kaposi's Sarcoma (KS) lesions, a soft tissue tumor affecting endothelial and lymphatic cells, in a patient with acquired immunodeficiency syndrome (AIDS) [1], KSHV has been linked to other conditions, such as Primary Effusion Lymphoma (PEL), a lymphoma affecting body cavities, and Multicentric Castleman's Disease (MCD), a non-malignant lymphoproliferative disorder [6-9]. Similar to other herpesviruses, KSHV has two distinct phases in its life cycle: a lytic phase characterized by the regulated expression of immediate-early, early, and late viral genes, resulting in the production of infectious virions, and a latent phase where a multicopy plasmid persists in host cells, accompanied by the expression of a small subset of viral genes. In KS, PEL and MCD, KSHV primarily exists in its latent form within proliferating cells, with only a variable subset of tumor cells supporting lytic replication [10-14].

KSHV's closest human relative is Epstein-Barr Virus (EBV), formerly known as Human Herrpesvirus-4 (HHV4), which belongs to the herpesvirus family with a double-stranded DNA genome of around 165kb [Figure I.2] [15]. Notably, EBV holds the distinction of being the first human tumor virus, discovered in 1964. Over more than 55 years of research, compelling evidence has shown that EBV is responsible for the development of tumors. Nowadays, we know EBV is associated with both lymphoid tumors, including Burkitt Lymphoma (BL), Diffuse Large B-cell lymphoma (DLBCL), post-transplant lymphoproliferative disease (PTLD), NK/T lymphomas, and Hodgkin lymphoma (HL), and epithelial tumors, such as Nasopharyngeal Carcinoma (NPC) and Gastric Cancer (GC) [16]. In EBV-associated tumors, the virus establishes

a latent infection marked by the restricted expression of specific viral latent genes. The distinct expression patterns of these latent genes give rise to three unique latency programs, each associated with a particular tumor type [Figure I.3] [17]. The Latency III pattern involves the expression of EBV-encoded small RNAs (EBERs) and BAMHI-A rightforward transcripts (BARTs) along with all EBV latent proteins, including the six nuclear antigens (EBNA1, EBNA2, EBNA3A, EBNA3B, EBNA3C, and EBNA-LP) and three latent membrane proteins (LMP1, LMP2A, and LMP2B). This pattern is characteristic of lymphoblastoid cell lines (LCLs) and PTLD. The Latency II pattern includes the expression of EBERs, BARTs transcripts, EBNA1 protein, and the latent membrane proteins (LMP1, LMP2A, and LMP2B). It is associated with HL and NPC. Finally, the Latency I pattern is found in BL and GC and is characterized by the expression of both transcripts, EBERs and BARTs, and the EBNA1 protein. While B cells can support any of these three types of latent infection, non-B cells typically exhibit either a Latency I or Latency II type of infection [18]. While EBV infects approximately 90% of the global population [19], KSHV infects a smaller proportion of people with the highest seroprevalence found in Sub-Saharan Africa [20].

Primary Effusion Lymphoma (PEL)

PEL is an exceptionally aggressive type of non-Hodgkin's lymphoma, predominantly observed in immunocompromised individuals living with Human Immunodeficiency virus (HIV) and patients receiving organ transplants [21]. Non-Hodgkin lymphoma may arise in lymph nodes anywhere in the body, whereas Hodgkin lymphoma typically begins in the upper body, such as the neck, chest or armpits. PEL typically manifests as serous effusions without detectable masses and predominantly develops within body cavities, most commonly in

pleural, pericardial and peritoneal cavities. Typically, only a single body cavity is involved [22, 23]. Histologically, lymphoma cells usually exhibit CD45 expression, but they do not display pan-B-cell markers including CD19, CD20, and CD79a (B-cell antigen receptor complex-associated protein alpha chain, BCR) [21, 24]. Structural changes in the MYC gene, an oncogene affecting proliferation or apoptosis [25], are absent, yet MYC protein regulation is disrupted owing to the influence of latent proteins encoded by HHV8 [26]. Unfortunately, the prognosis for patients is poor, as the median survival period post-diagnosis is approximately six months [21, 27]. Rare cases have shown positive responses to chemotherapy and/or immune modulation [28]. Additionally, it is believed that PELs arise at advanced stages of B-cell differentiation or activation [21].

PELs are causally associated with KSHV and 80% of them are also co-infected with EBV [7, 8, 21,24, 27, 29] and gene expression profiling of AIDS-related PEL shows a distinct profile, with features of both plasma cells and EBV-transformed lymphoblastoid cell lines [30]. The maintenance of KSHV and EBV genomes as extrachromosomal plasmids in PEL indicates that both viruses contribute to tumor phenotypes, as these viral genomes are lost from actively dividing cells unless they confer specific advantages to their host [31-33]. PEL, therefore, represents a unique tumor type, being the sole known example of a tumor dependent on the presence of two oncogenic viruses.

Primary infection with KSHV is not well characterized, partly due to its lower infection rate. While cells of endothelial and fibroblast origin can be efficiently infected with KSHV *in vitro*, efforts to infect lymphoblastic cell lines have been unsuccessful [34-37]. Even when lymphoblastic cells can be infected, KSHV has not been observed to induce transformation. Notably, in these studies, neither EBV-negative nor EBV-positive cell lines could be infected. In contrast, EBV is capable of infecting and transforming peripheral B cells *in vitro,* promoting

their long-term proliferation [38, 39]. Recently, Faure and colleagues demonstrated that EBV facilitates the infection of peripheral B cells with KSHV, with optimal efficiency achieved within 24 hours of EBV infection. They characterized the dually infected proliferating cells, which are transformed and capable of maintaining both KSHV and EBV for years in culture [40]. The *in vitro* transformation of peripheral B cells by both KSHV and EBV presents an opportunity for a detailed mechanistic analysis of the viral and cellular genes that orchestrate the initial stages of the progression towards PEL.

EBV and KSHV can contribute to the survival of their associated tumors

While it is widely accepted that the genesis and sustained viability of PELs is primarily propelled by the latent genes of KSHV, an intriguing observation emerged from Mack and colleagues. When they attempted to evict EBV using a dominant negative EBNA1 (dnEBNA1), which competes and inhibits the binding of EBNA1, a viral protein essential for EBV's plasmid replication, from dually infected PELs, the cells failed to grow in colony-forming assay under limiting dilution supporting the notion that EBV is necessary to sustain proliferation [41]. However, the idea that PEL is caused by KSHV finds reinforcement in observations showing that the inhibition of latent KSHV genes leads to either the death or growth arrest of the PEL cells [42, 43]. Furthermore, efforts to establish PEL cell lines lacking latent KSHV genomes have not been successful [44], underscoring that the latent genomes provide survival factors to the PELs. In accordance with these findings, it is worth noting that the latent genes of KSHV have all demonstrated oncogenic properties. This subset of genes, located within an approximately 10kb locus referred to as the latency locus, includes a latency-associated nuclear antigen (LANA1) encoded by Orf73, a viral cyclin D homolog (v-Cyclin) encoded by

Orf72, a viral Fas-associated protein with death domain-like interleukin-1β-converting enzyme/caspase-8-inhibitory protein (vFLIP) encoded by Orf71, Kaposin proteins and all the currently known microRNA (miRNAs) of KSHV. Genes in this subset are the only ones expressed in latently infected cells of KS while latently infected PEL cells may also express an additional gene LANA2 (v-IRF3/ORF K10.5), which is strictly expressed during the lytic phase in KS [45].

Understanding the significance of dual infection and the distinct roles each virus plays in the tumorigenesis of this lymphoma has presented a complex challenge. Several latent KSHV genes have been identified as direct inhibitors of apoptosis in PEL cells. Apoptosis is a highly regulated process that plays a crucial role in eliminating unnecessary, damaged or infected cells throughout an organism's lifespan, thereby ensuring cellular homeostasis. This regulation hinges on a delicate equilibrium between proapoptotic and antiapoptotic regulators of apoptotic pathways, orchestrating the survival of cells in various tissues. When this balance is disrupted, as seen in diseases like cancer, where antiapoptotic proteins are overexpressed, it confers certain cells a selective survival advantage that fosters malignancy [46]. Apoptosis can be initiated via two main pathways: the extrinsic pathway, involving the activation of death receptors, and the cell-intrinsic pathway, triggered by various forms of cellular stress. In the cell-intrinsic pathway, signals for apoptosis converge on mitochondria leading to the release of cytochrome C (Cyto C) into the cytosol. This, in turn, activates caspases, ultimately resulting in cell death [47]. Central to this intrinsic apoptotic pathway are the Bcl-2 family members, which play an essential role in regulating the release of Cyto C from mitochondria, as an initiator of cell death [Figure I.4].

Among latent KSHV genes, v-FLIP and LANA2 were found to be required for survival in PEL cells. V-FLIP prevents death receptor-mediated apoptosis by inhibiting caspase activation

[48,49]. Additionally, V-FLIP has been shown to activate nuclear factor-kappaB (NF-kB) and when v-FLIP is depleted using short interfering RNA (siRNA), it results in the induction of apoptosis in PEL cells [42]. LANA2, another documented anti-apoptotic KSHV gene expressed during latency, interacts with p53 and hinders p53-mediated apoptosis [50, 51]. Furthermore, when LANA2 expression is partially reduced using siRNA, it leads to decreased proliferation of PEL cells and an increase in caspase 3/7 activity [52]. LANA1 and KSHV miR-K10a have also been shown to inhibit apoptosis [53-56]. However, the direct contribution of LANA1's antiapoptotic role to PEL remains uncertain. Notably, when expression of LANA1 is inhibited using small hairpin RNA (shRNA) in PEL cells, it results in a gradual decrease of KSHV genomes, accompanied by growth inhibition or apoptosis, which varies depending on the PEL cell lines used [43]. It's plausible that the observed effect on apoptosis may stem from the loss of the other viral genes due to the reduction in KSHV genomes.

The contributions of EBV to the maintenance of PELs likely mirror its role in canonical BLs. As mentioned earlier, newly EBV-infected B cells, lymphoblastoid cell lines (LCLs), and certain EBV-positive lymphoproliferative disorders exhibit type III latency of EBV [57, 58]. BLs and dually infected PELs, on the other hand, display a restricted type I latency of EBV [59-62]. Numerous EBV genes, including those expressed in PEL cells, have been implicated in having anti-apoptotic functions. EBNA1 has been shown to prevent apoptosis in EBV-positive cells by destabilizing tumor suppressor p53 protein (reviewed in [63]). However, the significance of these antiapoptotic functions in the context of EBV-positive tumors is not clear since the majority of EBV-positive tumors maintain an intact p53 pathway (reviewed in [63]). Additionally, EBV-positive cells are known to undergo apoptosis mediated by p53 upon the induction of DNA damage [64]. A more convincing antiapoptotic gene of EBV expressed in PELs is the cluster of BART miRNAs. Canonical B cells die by apoptosis upon the forced eviction

of EBV genomes from them, but the introduction of BART miRNAs alone can rescue EBV-negative cells from apoptosis by inhibiting the pro-apoptotic caspase 3 transcript [65]. Within the BHRF1 locus, three miRNAs and one protein are encoded. The specific functions of the miRNAs within this locus are not well understood, but the protein encoded by this locus is a viral homolog of the cellular anti-apoptotic gene and proto-oncogene BCL2 [66]. Nevertheless, this protein is not expressed in a majority of BL cells and is only variably expressed in PELs, which casts uncertainty on its potential role in PEL maintenance.

As mentioned earlier, the in vitro transformation of peripheral B cells by both KSHV and EBV, as demonstrated by Faure et colleagues, provides a unique opportunity for a detailed mechanistic analysis of the viral and cellular genes orchestrating the initial stages of progression towards PEL. These newly EBV-KSHV infected B cells, referred to as PEL-like cells (PEL-LCs), undergo transformation and retain both KSHV and EBV in culture. PEL-LCs exhibit type III latency of EBV, whereas PELs typically exhibit type I latency. Most viral genes expressed in latency III are known to promote survival and proliferation. EBV promotes the proliferation of infected B cells directly through LMP1, which mimics CD40 and also supports the survival of BCR-negative B cells via signaling mediated by LMP2A [67]. In experiments conducted by Pratt and colleagues, the introduction of LMP1 into EBV-positive and EBV-negative cell lines showed a direct correlation with increased levels of the antiapoptotic protein BCL2A1 and LMP1 [68]. Conversely, when two EBV-negative epithelial cell lines were transfected with increasing amounts of a vector encoding LMP1, *Bcl2a1* was not detected by RT-qPCR [68]. Price and collogues employed both wild-type (WT) EBV and a strain of EBV lacking EBNA3A (ΔEBNA3A) to infect and transform B cells (LCLs). Cells infected with ΔEBNA3A exhibited sensitivity to ABT-737, an inhibitor for antiapoptotic proteins BCL-2, BCL-xL, and BCL-w, in contrast to WT EBV-infected cells, a phenotype rescued when they expressed

ectopically EBNA3A [69]. EBNA3A and 3C, expressed in some malignancies, downregulate the expression of proapoptotic tumor suppressor Bim coordinately and can confer resistance to cytotoxic drugs including nocodozole, cisplatin and roscovitine [70]. A truncated form of EBNA-LP, found in some tumor lines, has been shown to protect cells against apoptosis induced by verotoxin 1 or staurosporine [71].

Given these observations, it is likely that EBV infection is a prerequisite for initiating and maintaining B cell proliferation, while KSHV contributes to later stages of the transformation process and/or the maintenance of PELs post-transformation. If EBV is a prerequisite for B cell transformation, then what triggers the transformation of EBV-negative PELs? One explanation for these lymphomas is the “hit-and-run” model, where EBV initially transforms B cells, but the tumor cells gradually become less dependent on EBV. There is evidence supporting this theory. Firstly, while KSHV is present in nearly every cell of all PELs, the presence of EBV varies between 50-90% of the cells (unpublished data) in dually positive PELs, suggesting that the cells within a PEL population exhibit varying degrees of dependence on EBV. Some may even become entirely independent of EBV’s contributions. Secondly, there is precedent for EBV-associated tumors to evolve to be less dependent or entirely independent of EBV. The extent of the tumor’s reliance on EBV is inversely correlated with the number of viral genes expressed in the tumors [32]. Some tumors with EBV’s latency type I, including PELs, such as canonical BLs show minimal defects in survival and growth when EBV genomes are evicted from them [32,72]. For instance, the Akata tumor cell lines, not only can survive upon forced eviction of EBV genomes in the absence of complementation with exogenous anti-apoptotic genes [72] but can even spontaneously lose EBV in culture [73]. Given that PELs also contain KSHV, which expresses a variety of potential oncogenic genes of its own, it is quite possible that some PELs evolve to be independent of EBV at some frequency.

Alternatively, it is also possible that the origin of EBV-negative PELs differs from the EBV-dependent origin of dually positive PELs.

Prelude to my studies

The presence of KSHV plasmids in nearly 100% of tumor cells suggests that they confer a selective advantage to the host cells. The advantages are likely related to cell proliferation and the prevention of apoptosis. This likelihood is consistent with the known properties of the latent genes encoded by KSHV, which have demonstrated both proliferative and antiapoptotic effects. While KSHV can efficiently infect endothelial and fibroblast cells *in vitro*, attempts to infect lymphoblastic cell lines have been unsuccessful. In contrast, EBV is capable of infecting and transforming peripheral B cells *in vitro*, promoting their long-term proliferation. EBV and KSHV co-transform about 1 – 2 % of primary B cells to yield PEL-LCs. This study aims to elucidate the specific contributions of each virus to the characteristics of PEL-LCs.

In Chapter III of my work, I investigated whether PELs and PEL-LCs share similar properties. My findings showed that both dually and KSHV-only infected PELs rely on the antiapoptotic Myeloid Leukemia Cell Differentiation (MCL-1) protein for their survival, in agreement with previous reports [74, 75]. Inhibiting MCL-1 with a small molecule inhibitor [76] results in cell death in PELs but not PEL-LCs. Furthermore, my mRNA and immunoblotting analyses revealed that PEL-LCs transcribe MCL-1 mRNA but do not translate it detectably into proteins. This raises the question of which antiapoptotic protein is responsible for the survival of PEL-LCs. Given that various EBV genes expressed in PEL-LCs are associated with anti-apoptotic functions, I speculate that one or more EBV proteins expressed in latency III play a vital role in their survival.

In Chapter IV, I explored the role of antiapoptotic proteins expressed in both PELs and PEL-LCs, as identified in Chapter III, in regulating the survival of the cells that express them. My findings indicate that PEL-LCs primarily rely on BCL2A1 for their survival. Moreover, I hypothesized that this antiapoptotic advantage could be conferred by the latent EBV proteins, LMP1 or EBNA3A, which might mirror one of the contributions of EBV during the initial steps of infection and transformation in PEL.

Figure I.1 *Expression levels of viral genes in PELs and PEL-LCs*

Genes highlighted in yellow are orthologs of host genes (*i.e.* they likely originated in the host genome, but the virus has adapted them for its own use). KSHV encodes 87 open reading frames (ORFs) and at least 17 microRNAs (purple boxes), 14 of which are co-expressed as a cluster. A striking feature of KSHV is the number (at least 14) of ORFs that encode cellular orthologues. Identified ORFs and encoded proteins are indicated in the figure. Putative latent transcripts are indicated in green, and cellular orthologues in yellow. Kb = kilobase [figure modified from 77].

Figure I.1

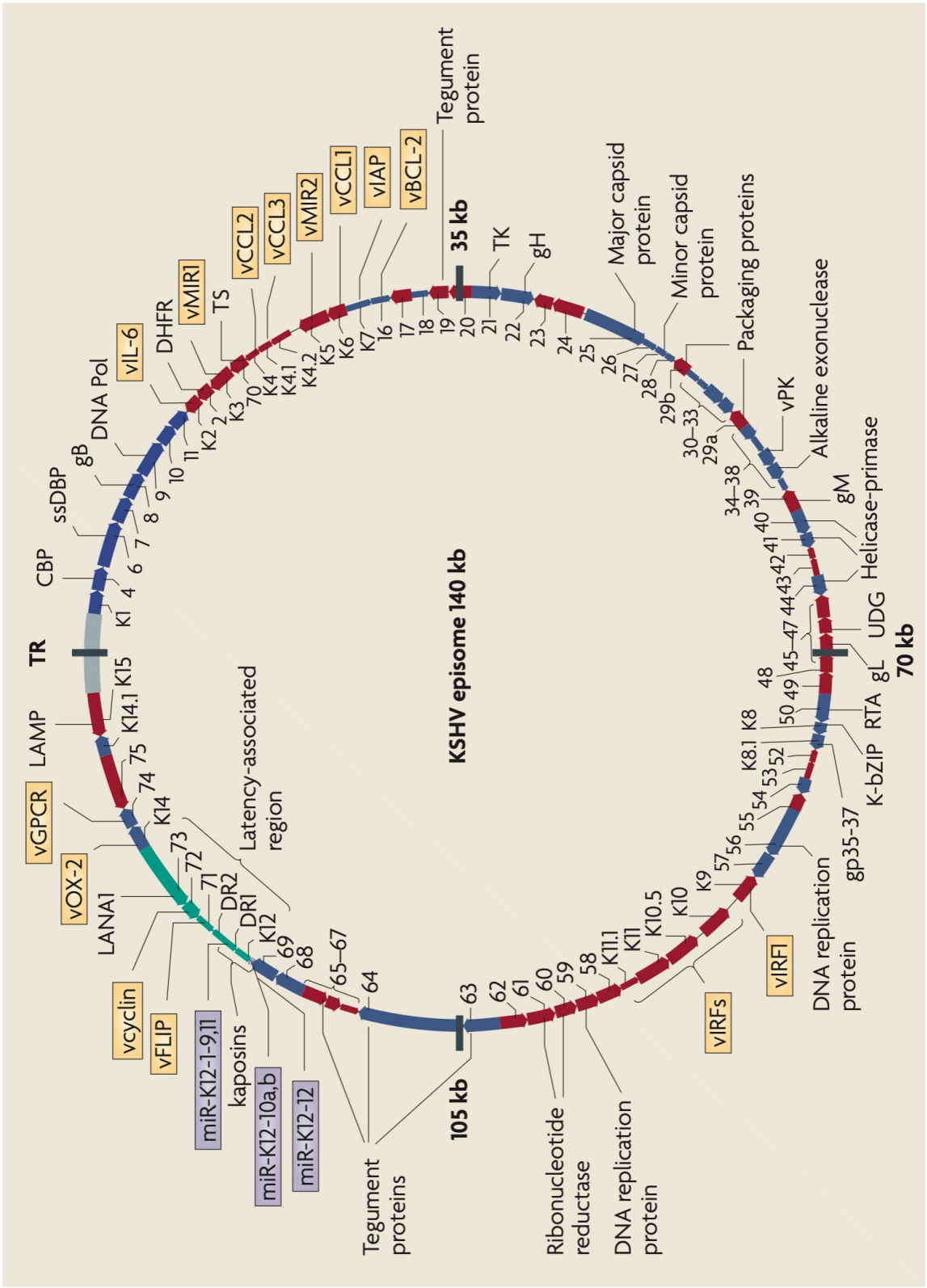

KSHV episome 140 kb
35 kb
70 kb
105 kb
TR
Tegument protein
TK
gH
Major capsid protein
Minor capsid protein
Packaging proteins
vPK
Alkaline exonuclease
gM
Helicase-primase
UDG
gL
RTA
K-bZIP
gp35-37
DNA replication protein
vIRF1
vIRFs
DNA replication protein
Ribonucleotide reductase
Tegument proteins
miR-K12-12
miR-K12-10a,b
kaposins
miR-K12-1-9,11
vFLIP
vcyclin
LANA1
vOX-2
vGPCR
LAMP
K14.1 K15
CBP
ssDBP
gB
DNA Pol
vIL-6
DHFR
vMIR1
TS
vCCL2
vCCL3
vMIR2
vCCL1
vIAP
vBCL-2
Latency-associated region
K1 4 6 7 8 9 10 11 K2 2 K3 70 K4 K4.1 K4.2 K5 K6 K7 16 17 18 19 20 21 22 23 24 25 26 27 28 29b 30–33 29a 34–38 39 40 41 42 43 44 45–47 48 49 50 K8 K8.1 52 53 54 55 56 57 K9 K10 K10.5 K11 K11.1 58 59 60 61 62 63 64 65–67 68 69 K12 DR1 DR2 71 72 73 K14 74 75

Figure I.2 *Schematic of Epstein Barr virus genome*

Letters on the inner edge of the circular genome denote BamHI digestion fragments. Cis-acting elements within the genome, including the origin of plasmid replication (oriP), the two origins of lytic replication (oriLyt), and the terminal repeats formed when the linear genome is circularized, are denoted in blue squares. Lytic genes are shown in orange boxes. Coding exons for the latency genes are shown in green boxes. EBV latent mRNAs can be initiated from different promoters depending on latency type and time after infection: the W promoter (Wp), the C promoter (Cp), the Q promoter (Qp, only in latency I), and the LMP promoters are labeled. The unspliced pre-mRNAs driven from these promoters are shown as a dotted line. EBV-encoded noncoding RNAs, such as the miR-BHRF1 cluster, the miR-BART cluster, and the EBERs, are shown as red triangles. Kb = kilobase [figure modified from 78].

Figure I.2

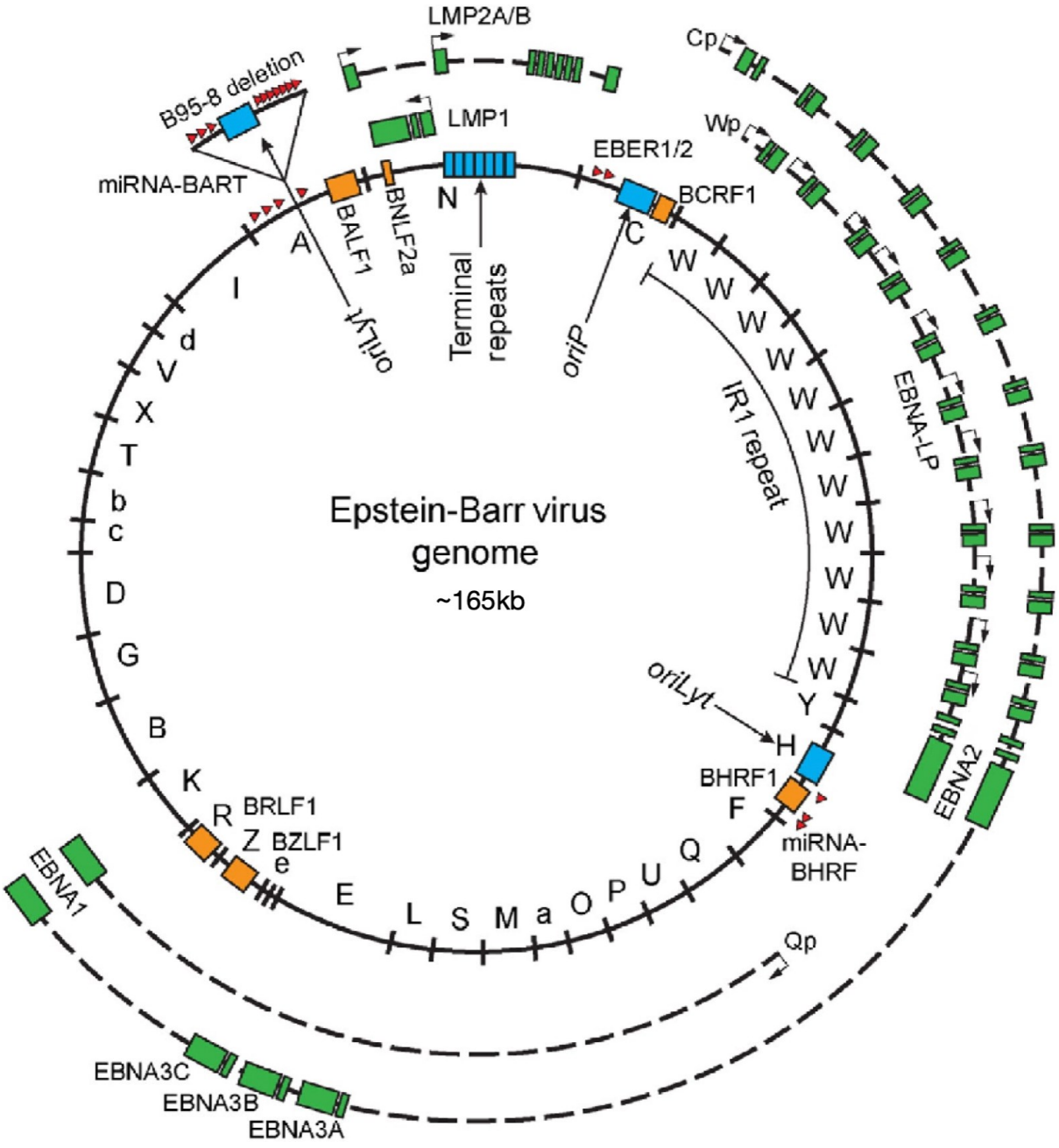

LMP2A/B
Cp
B95-8 deletion
LMP1
Wp
EBER1/2
miRNA-BART
BNLF2a
BALF1
N
BCRF1
C
A
oriLyt
Terminal repeats
oriP
W
W
W
W
W
W
W
W
W
W
W
W
W
W
W
IR1 repeat
EBNA-LP
I
d
V
X
T
b
c
D
G
B
K
R
BRLF1
Z
BZLF1
e
E
L
S
M
a
O
P
U
Q
F
BHRF1
H
Y
oriLyt
miRNA-BHRF
EBNA2
Epstein-Barr virus genome
~165kb
EBNA1
Qp
EBNA3C
EBNA3B
EBNA3A

Figure I.3 *EBV Latency types and gene expression*

In EBV-associated tumors, the virus establishes a latent infection marked by the restricted expression of specific viral latent genes. The distinct expression patterns of these latent genes give rise to three unique latency programs, each associated with a particular tumor type. EBV-encoded small RNAs (EBERs) and BAMHI-A rightforward transcripts (BARTs), six nuclear antigens (EBNA1, EBNA2, EBNA3A, EBNA3B, EBNA3C, and EBNA-LP) and three latent membrane proteins (LMP1, LMP2A, and LMP2B). Lymphoblastoid cell lines (LCLs), Post-transplant lymphoproliferative disease (PTLD), Hodgkin lymphoma (HL), Nasopharingeal Carcinoma (NPC), Burkitt lymphoma (BL) and Gastric Cancer (GC) [78].

Figure I.3

Latency type	Viral gene expressed	Associated disease
Latancy 0	EBERs, miR-BARTs	Burkitt's lymphoma
Latancy I	EBERs, miR-BARTs, EBNA1	Burkitt's lymphoma
Latancy II	EBERs, miR-BARTs, EBNA1, LMP1, LMP2A/B	Hodgkin’s lymphoma, NPC, Gastric cancer
Latancy III	EBERs, miR-BHRF1s, miR-BARTS, EBNA1, EBNA-LP, EBNA2, EBNA3s, LMP1, LMP2A/B	AIDS-associated DLBCL, PTLD, LCL
Wp-restricted latency	EBERs, miR-BARTS, EBNA1, truncated-EBNA-LP, EBNA3s, BHRF1	Burkitt's lymphoma

Figure I.4 Morphology and phenotype of PEL

Morphologic examination of the pleural effusion showed abundant pleomorphic to anaplastic discohesive lymphoma cells (**A**), Diff-Quick stain. (**B**). H&E stained cell block. The lymphoma cells were positive for CD45 (**C**), MUM1 (**D**), HHV8 (**E**), Ki-67 (**F**), EBER (**G**), and monotypic Kappa by in situ hybridization (**H,I**). Original magnification ×500 [figure modified from 79].

Figure I.4

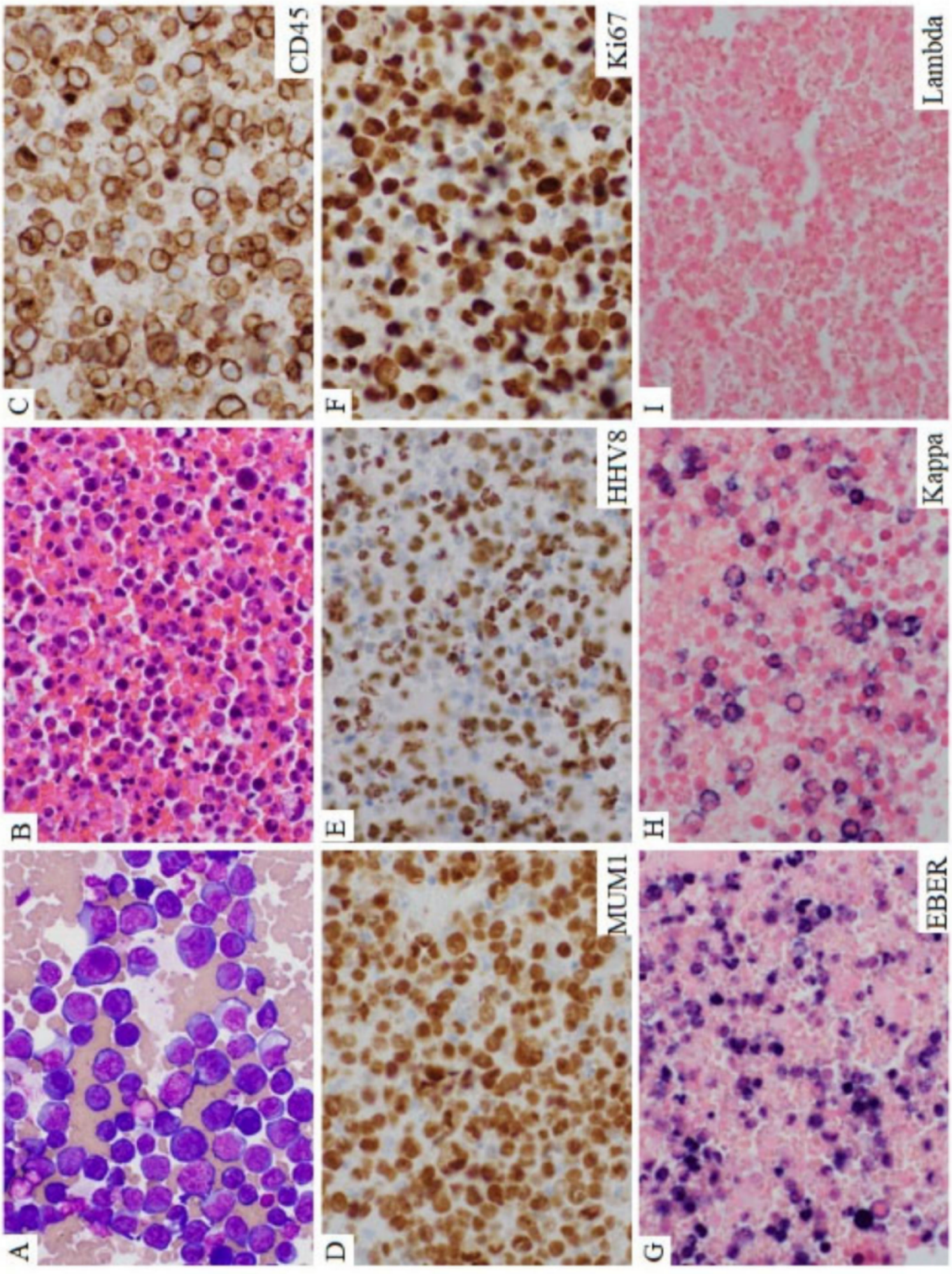
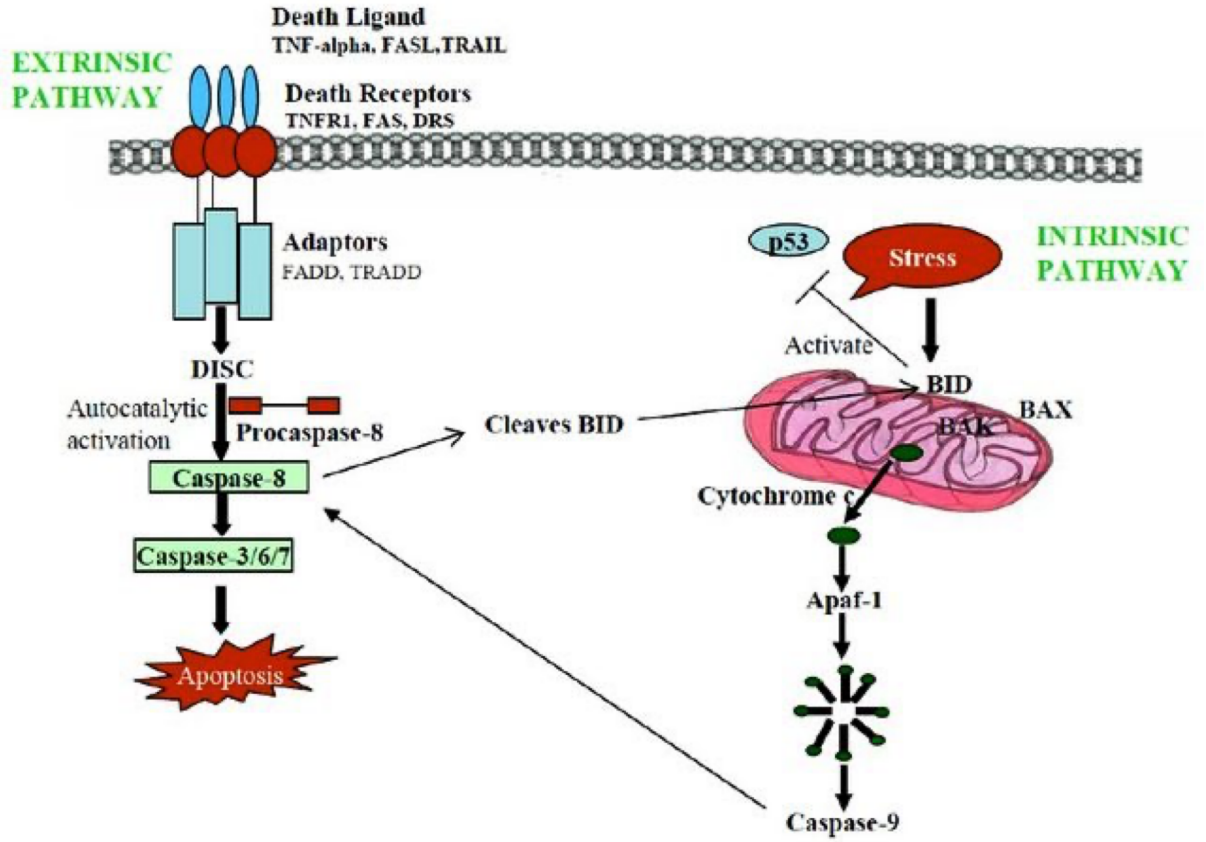


Figure I.5 *Extrinsic and Intrinsic pathway of apoptosis*

In the extrinsic pathway, death ligands bind to death receptors, clustering them on the cell surface and recruiting adaptor proteins to form the death-inducing signaling complex (DISC). DISC assembly recruits procaspases, facilitating their autocatalytic activation. Once activated, they initiate the caspase cascade into the cytoplasm. Active caspase-8 also mediates the cleavage of proapoptotic protein BH3 interacting-domain death agonist (BID), which subsequently releases mitochondrial proapoptotic factors linking the two pathways. In the case of the intrinsic pathway, stress signal causes the binding of cytoplasmic proteins, bcl-2 associated X protein (BAX) and BID to the outer membrane of mitochondria. Another mitochondrial protein Bcl-2 homologous antagonist/killer (BAK) interacts with BAX and BID causing the release of cytochrome c into the cytosol. This binds to apoptotic protease activating factor 1 (Apaf-1) which then forms the apoptosome that triggers the activation of procaspase-9. Activated caspase-9 further initiates the caspase cascade, ultimately leading to apoptosis. Tumor suppressor p53 protein serves as a cellular stress sensor and plays a crucial role in initiating apoptosis by transcriptionally activating proapoptotic proteins such as BID and BAX [47, figure modified from 80].

Figure I.5


Death Ligand
TNF-alpha, FASL,TRAIL
EXTRINSIC PATHWAY
Death Receptors
TNFR1, FAS, DRS
Adaptors
FADD, TRADD
DISC
Autocatalytic activation
Procaspase-8
Caspase-8
Caspase-3/6/7
Apoptosis
p53
Stress
INTRINSIC PATHWAY
Activate
BID
BAX
BAK
Cleaves BID
Cytochrome c
Apaf-1
Caspase-9

CHAPTER II.

Materials and methods

Cells and culture conditions

The PEL-derived B cell lines used in this study include one cell line positive for KSHV (BCBL-1) and two cell lines dually positive for KSHV and EBV (JSC-1 and BC-1). Other cell lines used in this study are BJAB (Burkitt's lymphoma-derived cell line, negative for EBV), 721 (lymphoblastoid cell line, positive for EBV) and Jurkat (immortal human T lymphocytes), PTLD-1 (derived from post-transplant lymphoproliferative disorder) and Oku-1 [1], Sav-1 [2], and AG876 (derived from Burkitt lymphomas) [3]. Adherent cells used in this study include 293T**,** expressing SV40 T-antigen to enhance plasmid replication with an SV40 origin. These cell lines were cultured in either Dulbecco's modified Eagle's medium (DMEM) (Gibco) (adherent cells) or in Roswell Park Memorial Institute 1640 medium (RPMI) (Gibco) (suspension cells), supplemented with 10% fetal bovine serum (FBS) (Cytiva), 200 units/ml of penicillin and 200 µg/ml of streptomycin at 37°C in a humidified 5% carbon dioxide (CO_2) atmosphere.

Growth curve

Cells were plated at a starting concentration of 5 x 10^4 cells/mL in culture medium in a 12 multiwell plate and viable cells were counted every day for 14 days using a hemacytometer. To discriminate viable cells from non-viable ones, cells were stained with eosin yellow (with a 1:1 dilution of 0.1% eosin yellow dissolved in 1x PBS). When cells were confluent, they were diluted 1:20 and plated for further time points.

Small molecule inhibitor, cell viability assay and apoptosis

About 1 × 10^4 cells (in 200 µl) were seeded in triplicate in 96-well flat-bottom microtiter plates. Cells were treated with various concentrations of Narciclasine (#SML2805, Sigma-

aldrich), Cytarabine (#S1648, Selleck), Abemaciclib (#LY2835219, Selleck), Palbociclib (#PD-0332991, Selleck), GSK682753A (#502260393, Fisher), NIBRI89 (#S0183, Selleck), saJM589 (#SML2198, Sigma), AZD-5991 (#28926, Cayman Chemical), ABT-263 (#A3007, APExBIO) and Camptothecin (#159732, MP Biomedicals). All drugs were resuspended in dimethyl sulfoxide solution (DMSO) and tested at a final concentration of 0.1% DMSO. Cell survival was determined with Cell Titer Glo (#G9242, Promega). The Cell Titer Glo determines the number of viable cells in a culture based on quantitation of the adenosine triphosphate (ATP) present, which signals the presence of metabolically active cells, using light as a surrogate marker. Relative lights units (RLUs) were measured using the Victor X5 luminometer (PerkinElmer). The results are reported as the mean normalized RLUs of the DMSO-treated control cells. Apoptosis was measured with the Caspase 3/7 reagent (#G8090, Promega). The Caspase 3/7 assay measures activity by caspase cleavage of the substrate and generation of a “glow-type” luminescent signal, produced by luciferase. Relative lights units (RLUs) were measured using the Victor X5 luminometer (PerkinElmer). The results are reported as the mean normalized RLUs of the DMSO-treated control cells.

Transfection

293T cells were plated in 10 cm dishes and grown to about 70% confluency. For each dish, 10µg of a vector was diluted in 500 µl of Opti–Modified Eagle’s Medium (MEM) (Gibco) and then combined with prediluted lipofectamine 2000 (#11668027, Invitrogen) Opti-MEM mixture (30µl of lipofectamine was diluted in 500 µl of Opti-MEM and incubated for 5 minutes at room temperature). DNA – lipofectamine complexes were incubated for 25 minutes at room temperature. Cells were washed once with DMEM and plated with 4 mL of DMEM and

1 mL of transfection mixture and incubated for 4 hours in air containing 5 % CO_2 at 37 °C. Following the incubation, the medium was replaced with 8 mL of fresh D10F. Cells were collected after 48 hours. The efficiency of transfection was determined by western blot or by percentage of fluorescent positive cells.

Western blot

Approximately 5 x 10^6 cells were harvested, washed twice with PBS and resuspended in Radioimmunoprecipitation assay (RIPA) buffer (10mM Tris (pH 8.0), 1 mM EDTA, 0.5 mM EGTA, 1 % Triton X-100, 0.1% Sodium deoxycholate (SDO), 0.1% SDS, 140mM NaCl, 1 X protease inhibitor cocktail). The cell suspension was sonicated using a Qsonica Q700 sonicator at 100 mA for at least 1 min and centrifuged at 20,000 rcf for 30 min, 4°C, and the supernatant was collected for immunoblotting. The collected supernatant was mixed at a 1:1 ratio with 2 X SDS sample buffer (0.12M Tris pH 6.8, 138.7mM (4%) SDS, 20% Glycerol, 0.3mM Bromophenol blue, 5% beta-mercaptoethanol). Approximately 5 x 10^5 cell equivalents per sample per well were loaded onto an SDS-PAGE gel (#4569033, Bio-Rad), the gel was run, and the proteins were transferred to a 0.22µm nitrocellulose membrane (#10600006 Amersham, Cytiva). The membranes were blocked in Tris-buffered saline (TBS) (Gibco) with 0.1% Tween 20 (Fisher Scientific) (TBST) containing 5% (w/v) powdered milk and incubated with the primary antibody overnight at 4°C (Table II.1). Following treatment with a primary antibody, the membranes were washed with 1 X Tris-buffered saline with 0.1% Tween 20 (TBST) and incubated with the secondary antibody (Table II.1) for 1h at room temperature. All antibody dilutions were prepared in a 5% milk solution in TBST. Membranes were washed in TBST, developed using Pierce enhanced chemiluminescence (ECL) western blotting substrate

(#W1001, Promega) according to the manufacturer's protocol, and visualized using the ChemiDoc imaging system (Bio-Rad).

Synthesis of MMLV retroviral/lentiviral particles

293T cells were transfected with DNA-expressing retroviral proteins for synthesizing, packaging and releasing retroviral particles. Cells were split the day before transfection for a confluency of 80 – 90% (~ 5 x 10^6 cells per 10 cm plate). For transfection, cells were washed with serum-free DMEM (Gibco) and replenished with 4 ml of serum-free DMEM (Gibco) and stored at 5% CO_2, 37°C until transfection complexes were generated (~ 30 – 60 minutes). To generate retroviral-lipophilic complexes for transfecting a 10 cm plate of 293T cells, a mixture was prepared containing 10 µg of retroviral vector, 3 µg of Gag/polymerase (p2843), 1 µg of NFkB (p1238), 1 µg of vesicular stomatitis virus (VSV) glycoprotein (p2842) and 0.5 ml of Opti-MEM (Gibco). In the case of lentiviral vectors, the combination consisted of 10 µg of retroviral vector, 12 µg of psPAX.2, 4 µg of VSV glycoprotein (p2842) and 0.5 ml of Opti-Mem (Gibco). A second mixture containing 30 µl of Lipofectamine 2000 (Invitrogen) and 0.5 ml of Opti-Mem (Gibco) was prepared. Five minutes after each reaction mix was prepared, they were combined and incubated for at least 25 minutes at room temperature. The combined mixture was then carefully added dropwise on top of the previously prepared 293T cells containing 4 ml of DMEM. Four hours following transfection, the media was removed and replaced with 4 ml of DMEM 10% FBS (D10F) / 50mM Hepes. The medium was collected 24 and 48 hours post-transfection and stored at 4°C no longer than 1 day after the final collection. The medium was cleared of cellular debris by centrifugation (300rcf, 10 minutes) and filtered with a 50 ml syringe fitted with a 0.8-micron filter. Retroviral particles were concentrated by adding one

volume of supernatant and one volume of concentrator Polyethylene Glycol – 8000 (PEG) (#BP233-100, Fisher scientific) and sodium chloride (NaCl) solution. The concentrations of PEG-8000 and NaCl in the stock solutions are 20% (w/v) and 0.6 M, respectively. The mix was incubated with constant rocking at ~ 60 RPM overnight at 4°C. The samples were spun down at 1600rcf for 60 minutes, the supernatant removed and the retroviral particles resuspended in 1 ml (concentrated 1:20) of RPMI and stored at -80°C.

Retroviral transduction

B cells were resuspended and plated 5 x 10^5 in 800 µL of R10F in 6 well clusters and 200µl concentrated retrovirus was added to each well. Cells and viruses were centrifugated at 950g for 90 minutes at room temperature (spinoculation). Additional medium (1ml) was added to each well after 24 hours, and cells and virus were incubated for an additional 24 hours at 37 °C.

Cell sorting

Single cells were sorted on BD FACSAria “Jack” with standard settings of 20 psi pressure and a 130µm nozzle tip size. BCBL-1 and JSC-1 cells transduced with retroviral vectors were sorted for their levels of BCL2A1-mRFP by first selecting all mRFP-positive cells, then isolating single cells expressing the lowest 20% and the highest 20% levels of mRFP, were collected via FACS.

RNA isolation and cDNA synthesis

RNA was isolated using 500µl of TRIzol (#15596018, Ambion) according to the manufacturer's instructions (#R2062, Direct-zol RNA Microprep, Zymo research). In brief, an equal volume of ethanol (96 – 100%) was added to a sample lysed in TRIZOL and the solution was transferred into a Zymo-spin IC column and centrifuged at 16.000 g for 1 minute. After a wash with 400µl of RNA Wash Buffer, the column was treated with DNase I to remove contaminating DNA for 15 minutes at room temperature. The RNA was collected by adding 15µl of DNAse/RNase free water after two washes with 400µl of Direct-zol RNA Pre-Wash and one wash with 700µl of RNA wash buffer.

Around 500 ng of total RNA was reverse transcribed using a First Strand cDNA synthesis kit (#E6560, New England biolabs) following the manufacturer's instructions. In brief, template RNA was added to a mix containing random primers (final concentration of 3µM), ProtoScript II reaction Mix (1X), ProtoScript II enzyme mix (1X) and water to a final volume of 20µl. A no reverse transcriptase negative control reaction was prepared for each sample by not adding the ProtoScript II enzyme mix. The reverse transcription (RT) of 2x-myc-BCL2A1 were performed with gene-specific primers at a final concentration of 3µM for experiments that required its detection (Table II.2).

RT-qPCR

Reverse-transcribed cDNA was amplified and detected by real time PCR under the following conditions: 1X AmpliTaq Gold PCR Master Mix (#4398881, Applied Biosystems), 0.5µM for each primer, 0.2µM probe, 1X ROX reference dye (#12223-012, Invitrogen) and water to 20µl. PCR cycling conditions were 50 °C for 2 minutes, 95 °C for 10 minutes, 40 cycles

of 95 °C for 15 seconds, 60 °C for 60 seconds and 72°C for 2 minutes, then 72 °C for 7 minutes. Probes were labeled with fluorescein amidite (FAM) and either carboxytetramethylrhodamine (TAM) or Iowa Black FQ quencher (IABkFQ) at their 5' and 3' ends, respectively, and were ordered from IDT DNA (Coralville, IA). Sequences for all primers and probes used are listed in Table II.2. Measurements were made with the ABI Prism 7900 thermocycler and analyzed with SDS 2.4.2 software (Applied Biosystems). The expression of the mRNAs was determined in three steps. First, the cycle at which the amplification curve passed a threshold of detection (Ct) was calculated. Second, the Ct-value of the amplified cDNA for each sample was compared to the Ct-value of a control sample by the delta (Δ)ΔCt method [4]. Finally, the expression of all reverse transcribed and amplified mRNAs was normalized to the amount of input RNA. The results are reported as the mean expression of the mRNA relative to a control sample unless otherwise noted.

dCas9-KRAB system (CRISPRi)

Inactive Cas9 (dCas9), fused either with KRAB-MeCP2 or KRAB-ZIM3 repressor domains in a vector expressing antibiotic resistance to blasticidin, was transduced into A++9D and JSC-1. The cell lines transduced with these dCas9 derivatives underwent selection with 3µg/µl and 15µg/µl blasticidin, respectively, for over a month. Subsequently, they were transduced with a short-guide RNA targeting the promoter of BCL2A1, MCL1, or Rhodopsin (Table II.3), which was cloned into the BsmbI-digested LentiCRISPRv2-mScarlett vector. Transduced cells were selected three days later through fluorescence-activated cell sorting (FACS) for mScarlett-positive cells. These cells were then resuspended either in R10F for cell viability assays or lysed in TRIZOL for RT-qPCR.

Statistics

It is noteworthy that the majority of my assays were conducted with technical triplicates. For those instances where time permitted the execution of biological replicates, I ensured a thorough statistical analysis.

Table II.1 Antibodies used for proteins detection via western blot

Primary	Host	Monoclonal/Polyclonal	Company	Dilution
Alpha-Tubulin 50KDa	Mouse	M	Sigma #T61199	1:3000
EBNA3A 145 KDa	Sheep	P	Exalpha #F115P (Provided by Makoto Ohashi)	1:500
LMP1 50KDa	Mouse	M	Abcam #AB78113 (Provided by Makoto Ohashi)	1:500
MCL-1 40KDa	Rabbit	P	Santa Cruz (S-19) sc-819	1:400
BCL2A1 20KDa	Rabbit	P	Abcam #AB75887	1:250
BCL2A1 20KDa	Rabbit	M	Abcam #AB287160	1:200
BCL2A1 20KDa	Rabbit	M	Cell signalling #14093S	1:300
Secondary				
Anti-Rabbit HRP	Goat	P	Promega #W401B	1:2500
Anti-Mouse HRP	Goat	P	Promega #W402B	1:2500
Anti-Sheep HRP	/	/	Provided by Makoto Ohashi	1:5000

Table II.2 Primers and probes used for detection of mRNAs via RT-qPCR

mRNA	Primer	Sequence (5' to 3')
BCL2A1	RT	Random primers
	Forward PCR	CAG CAC ATT GCC TCA ACA GCT TCA
	Reverse PCR	TTG CTT GGA CCT GAT CCA GGT TGT
	TaqMan probe	FAM - AAG ACT TTG CTC TCC ACC AGG CAG AA - TAM
BCL2A1, set 2 (used to amplify 2x-myc-BCL2A1)	RT	TCA ACA GTA TTG CTT CAG GAG AG
	Forward PCR	ACA CAG GAG AAT GGA TAA GGC
	Reverse PCR	TCA ACA GTA TTG CTT CAG GAG AG
	TaqMan probe	FAM - AAC GGA GGC TGG GAA AAT GGC T – TAM
MCL1	RT	Random Primers
	Primer 1 PCR	CAG CGC AAC CAC GAG AC
	Primer 2 PCR	TCC TAC TCC AGC AAC ACC T
	TaqMan probe	FAM - AAA GCC AGC / ZEN / AGC ACA TTC CTG ATG / 3IABkFQ

Table II.3 Oligos sequences for CRISPRi

mRNA	Primer	Sequence (5’ to 3’)
MCL1 sg1	Oligo 1	CACCGGGAAGACCCCGACTCCTTAC
	Oligo 2	AAACGTAAGGAGTCGGGGTCTTCCC
MCL1 sg2	Oligo 1	CACCGGCCGCCTGGCTGAGAAAACT
	Oligo 2	AAACAGTTTTCTCAGCCAGGCGGCC
BCL2A1 sg1	Oligo 1	CACCGGCATTGCCTCAACAGCTTCA
	Oligo 2	AAACTGAAGCTGTTGAGGCAATGCC
BCL2A1 sg2	Oligo 1	CACCGGTCTGTCATCTTCTGCCTGG
	Oligo 2	AAACCCAGGCAGAAGATGACAGACC
Rhodopsin sg1	Oligo 1	CACCGCCCACCCAAGAATGCTGCGA
	Oligo 2	AAACTCGCAGCATTCTTGGGTGGGC

CHAPTER III.

Characterizing anti-apoptotic pathways in PELs and PEL-LCs

Acknowledgment Chapter III

In expressing my gratitude, I extend thanks to the fine scientist, Mitchell Hayes, whose generosity in sharing Figure III.6A has significantly enriched this work. His invaluable contribution has added depth and clarity to the content, and I genuinely appreciate his collaboration.

Introduction

I investigated whether Primary Effusion Lymphoma cells (PELs) and the PEL-Like cells (PEL-LCs) exhibit similar apoptotic pathways. PEL cell lines, including JSC-1, BC-1 and BCBL-1, were derived from primary tumors [1]. JSC-1 and BC-1 are co-infected with EBV and KSHV, while BCBL-1 is solely infected with KSHV. On the other side, PEL-LCs, including A++9D, A++11F and 2C2, represent stably transformed *in vitro* models of peripheral cells dually infected with EBV and KSHV [2]. Notably, A++9D and A++11F originate from the same blood donor.

To begin, I conducted a survey on these cell lines using small molecule inhibitors that target various molecules involved in apoptosis. Apoptosis is a regulated cell death process that can be initiated either through the extrinsic pathway, involving the activation of death receptors or via the cell-intrinsic pathway triggered by various forms of cellular stress. In the cell-intrinsic pathway, apoptotic signals converge on mitochondria, leading to the release of cytochrome C (cyto C) into the cytosol. This event, in turn, induces caspase activation, ultimately resulting in cell death [3]. The intrinsic apoptotic pathway is tightly regulated by the BCL-2 family proteins. Members of this family contain BCL-2 homology (BH) regions and can be broadly separated into two groups: pro-survival proteins (such as BCL-2, MCL-1, BCL-XL, BCL2A1/BFL-1 and BCL-W) and pro-apoptotic proteins. The pro-apoptotic proteins further divide into BH3-only proteins (e.g. BAD, BID, BIK, BIM, BMF, HRK, NOXA and PUMA) and BAX/BAK-like proteins [4].

The literature, documents well that PELs rely on the antiapoptotic MCL-1 protein for their survival. This finding is supported by the work of Manzano and colleagues, who performed a CRISPR-Cas9 screening and identified MCL-1 as one of the essential genes in PELs [5]. Moreover, Dunham and colleagues conducted knockout experiments targeting different

antiapoptotic proteins that are endogenously expressed (MCL1, BCL2, or BCL-xL) in the BC-3 PEL cell line, which is infected only with KSHV. These experiments revealed that, despite the expression of other BCL2 family proteins, PELs predominantly rely on MCL1 for their survival. In addition, Dunham and colleagues generated clonal BAX and BAK double knockout (DKO) cell lines from BC-1 (infected with KSHV and EBV) and BC-3 (KSHV-only) cells to inactivate the intrinsic apoptotic pathway. The formation of pores by BAX or BAK is considered a necessary step to irreversibly initiate intrinsic apoptosis. When BC-3 and BC-1 DKO cells were treated with an MCL1 inhibitor (S63845), they were resistant even at the maximum dose used (20 mM) compared to WT BC-1 and BC-3, highlighting the specific role of MCL-1 in inhibiting intrinsic apoptosis in these cells [6].

I included other cell lines infected with EBV in my screens, such as PTLD-1 (derived from post-transplant lymphoproliferative disorder) and Oku-1 [7], Sav-1 [8], and AG876 (derived from Burkitt lymphomas) [9]. Among the inhibitors tested, the only one that exhibited a difference in response between PELs and PEL-LCs was AZD-5991, an inhibitor targeting the antiapoptotic protein MCL-1 [10]. Notably, I observed that only PEL-LCs and PTLD-1 were resistant to high concentrations of the MCL-1 inhibitor, whereas all the other cell lines tested were sensitive to lower concentrations of it.

In this Chapter, I identified BCL2A1 as the antiapoptotic protein that is upregulated in PEL-LCs compared to PELs. Furthermore, I identified two EBV genes expressed in latency III as potential candidates that provide a survival advantage to dually infected PEL-LCs, perhaps, by regulating BCL2A1.

Results

Screening small-molecule inhibitors targeting diverse apoptotic molecules

In my initial exploration, I surveyed PELs (BC-1, JSC-1 and BCBL-1) and PEL-LCs (A++9D and A++11F) cell lines with a variety of small-molecule inhibitors [Table III.1] to determine if any cellular pathways distinguished PEL-LCs from PELs, at three different concentrations: 0.3 µM, 1µM and 3µM . Additionally, I included other cell lines infected with EBV, such as PTLD-1 derived from post-transplant lymphoproliferative disorder and Oku-1, Sav-1, and AG876 derived from Burkitt lymphoma. Notably, I found that it appeared that the mechanism by which these cells regulated apoptosis differed between them, so I subsequently focused my research on this mechanism. Among the inhibitors tested, AZD-5991 (#28926, Cayman Chemical) stood out as the sole agent showcasing a discernible difference between PELs and PEL-LCs. This particular inhibitor is designed to target the antiapoptotic protein MCL-1 [Figure III.1].

Apoptosis hinges on a delicate balance between proapoptotic and antiapoptotic regulators to ensure the proper survival of cells and tumors which often exhibit dysregulation in antiapoptotic proteins. Intriguingly, my findings revealed that only PEL-LCs and PTLD-1 demonstrated resistance to high concentrations of AZD-5991 (~ 3µM). In contrast, all other tested cell lines displayed sensitivity to lower concentrations (~ 0.3µM).

MCL-1 inhibitor induces apoptosis in PELs

MCL-1, a prominent member of the BCL2 family, plays a crucial role in promoting the survival of malignant cells when overexpressed [11,12]. In this study, I investigated the inhibitor responses in both PELs and PEL-LCs. Notably, PEL-LCs exhibited approximately a 200-fold greater resistance to the MCL-1 inhibitor (IC_{50} ~ 6.5 µM) compared to PELs (IC_{50} ~ 0.03

μM) [Figure III.2A]. To confirm the induction of apoptosis in PELs upon MCL-1 inhibition, I exposed them to a lethal dose of the MCL-1 inhibitor (1μM) for 24 hours. Subsequently, I measured apoptotic markers using cell titer glo and caspase 3/7 luciferase assays. As a positive control, I included a lethal dose of camptothecin (10μM), a specific inhibitor of DNA topoisomerase I, known to induce activation of caspase-3, -8, and -9 [13] for 6 hours. The results revealed a reduction in live cells upon exposure to the MCL-1 inhibitor or camptothecin in PEL cells, compared to the dimethyl sulfoxide (DMSO) control and A++9D (PEL-LC resistant to the inhibitor) [Figure III.2B]. This decrease correlated with a proportional increase in cells undergoing apoptosis, as evidenced by the rise in caspase 3/7 activities [Figure III.2C]. Thus, these data show that pharmacological inhibition of MCL-1 induces apoptotic cell death in PELs.

Expression of MCL1 in PEL and PEL-LC cell lines

The existing literature extensively demonstrates that PELs depend on the antiapoptotic MCL-1 protein for their survival, as indicated by various studies [5, 6, 10]. Indeed, of all the BCL2 family members, only MCL1 showed a strong and consistent requirement in all PEL cell lines. Subsequently, I investigated variations in MCL-1 expression in PEL-LCs, which serve as a stable *in vitro* model representing peripheral B cells dually infected with EBV and KSHV [2], in comparison to traditional PELs. The RT-qPCR analysis revealed an approximately 5-fold difference in mRNA expression [Figure III.3A]. However, this disparity was insufficient to account for the significant contrast observed in MCL-1 resistance as reported earlier. Intriguingly, the western blot analysis unveiled a substantial ~25-fold difference at the MCL-1 protein level [Figure III.3B]. These findings suggest, at least in part,

that PEL-LCs are resistant to MCL-1 pharmacological inhibition because they they do not rely on MCL-1 for their survival.

Characterizing anti-apoptotic pathway in PEL-LCs

PEL-LCs transcribe MCL-1 mRNA but do not translate it detectably into proteins. This raises the question of which antiapoptotic protein is responsible for the survival of PEL-LCs. To address this, I investigated the sensitivity of both PELs and PEL-LCs to ABT263, a potent small molecule inhibitor targeting BCL-2, BCL-xL, and BCL-w [10, 14]. As a positive control, the Jurkat cell line was included [15]. Consistent with the dependence of PELs on MCL-1, treatment with ABT-263 resulted in an IC_{50} of ~ 4.5 μM in proliferating cells. Interestingly, when ABT-263 was added to PEL-LCs, they exhibited resistance resulting in an IC_{50} of approximately 4 μM [Figure III.4]. Therefore, this suggests that PEL-LCs may rely on a distinct mechanism for their survival.

My preliminary screening revealed that PTLD-1 and PEL-LCs were the only cell lines resistant to MCL-1 inhibition (AZD-5991) (Figure III.1). Those are infected with EBV in Latency III, one of the distinct latent infection statuses in which the full range of EBV latent genes is transcribed. Given that various EBV genes expressed in PEL-LCs are associated with anti-apoptotic functions, I speculate that one or more EBV proteins expressed in latency III play a vital role in their survival. I hypothesized that EBV provides resistance of PEL-LCs on BCL-2, BCL-xL, BCL-w and MCL-1 inhibition by upregulating another BCL-2 family member. From the literature on EBV proteins involved in survival mechanisms, LMP-1 (16) and EBNA3A (17) are candidates for being responsible for the resistance to AZD-5991 and ABT-263 in PEL-LCs by upregulating the antiapoptotic protein BCL2A1 (A1, BFL-1).

Expression of BCL2A1 in PEL and PEL-LC cell lines

To assess whether the resistance of PEL-LCs to AZD-5591 and ABT-263 is mediated by BCL2A1, I initially examined variations in BCL2A1 expression between PEL-LCs and PELs. Surprisingly, the RT-qPCR analysis revealed a ~250-fold higher expression of BCL2A1 in PEL-LCs compared to PELs [Figure III.5A]. However, BCL2A1 protein was only detectable through western blot analysis when I transfected an expression vector for BCL2A1, which was tagged with myc, into the 293T cell line. Notably, none of the three antibodies I tested recognized BCL2A1 in any sample [Figure III.5B]. This lack of detection can be attributed to an unexpected low affinity of the commercial antibodies. The 293T cell line expresses SV40 T-antigen to augment plasmid replication with an SV40 origin. Consequently, only in the transfected 293T cells, which express about 1000 times more protein, was I able to detect the BCL2A1 protein.

Following that, I investigated the expression of viral genes in PEL-LCs and PELs through a combination of RNAseq data analysis and western blot analysis. The results indicated that PEL-LCs exhibited expression of both viral proteins, LMP1 and EBNA3A, in contrast to PELs [Figure III.6A-B]. These data support a hypothesis that the resistance of PEL-LCs to AZD-5591 and ABT-263 might be mediated by BCL2A1 and identify EBNA3A and LMP1 as potential candidates that provide a survival advantage to dually infected PEL-LCs, perhaps, by regulating BCL2A1.

Table III.1 Preliminary screening to distinguish PEL-LCs from PELs.

I surveyed PELs (BC-1, JSC-1 and BCBL-1) and PEL-LCs (A++9D and A++11F) cell lines with a variety of small-molecule inhibitors to determine if any cellular pathways distinguished PEL-LCs from PELs, at three different concentrations: 0.3 µM, 1µM and 3µM . Additionally, I included other cell lines infected with EBV, such as PTLD-1 derived from post-transplant lymphoproliferative disorder and Oku-1, Sav-1, and AG876 derived from Burkitt lymphoma. Among the inhibitors tested, AZD-5991 stood out as the sole agent showcasing a discernible difference between PELs and PEL-LCs.

Table III.1

Name	Mechanism of action
Narciclasine	Blocking protein biosynthesis
AZD-5991	Anti-MCL1
Cytarabine	Competes with cytidine incorporation
Abemaciclib	CDK4/6 inhibitor
Palbociclib	CDK4/6 inhibitor
GSK682753A	EBI-2 Inhibitor
NIBRI89	EBI-2 Inhibitor
saJM589	Disrupting MYC-MAX heterodimerization

Figure III.1 *Effects of AZD-5991 (MCL-1 inhibitor) on the growth of lymphoma cell lines*

Among the inhibitors tested, the MCL-1 inhibitor was the only one showing a discernible difference between PELs and PEL-LCs. I included other cell lines infected with EBV, such as PTLD-1 derived from post-transplant lymphoproliferative disorder and Oku-1, Sav-1, and AG876 derived from Burkitt lymphoma. The green color signifies inhibitor sensitivity of the cell line (~ 0.3 µM), while yellow indicates resistance (~ 3 µM). Burkitt lymphoma (BL), Primary effusion lymphoma (PEL), Primary effusion lymphoma like cell (PEL-LC).

Cell Line	Disease	Viral state	EBV Latency	MCL-1 Inhibitor AZD-5991
PTLD1	EBV-related post-transplant lymphoproliferative disorder	EBV	Latency III	Resistant
A++11F	PEL-LC	KSHV-EBV	Latency III	Resistant
A++9D	PEL-LC	KSHV-EBV	Latency III	Resistant
BCBL-1	PEL bona fide	KSHV		Sensitive
BC-1	PEL bona fide	KSHV-EBV	Latency I	Sensitive
JSC-1	PEL bona fide	KSHV-EBV	Latency I	Sensitive
Sav-1	BL	EBV	Latency I	Sensitive
Oku-1	BL	EBV	Wp- restricted Latency	Sensitive
AG876	BL	EBV	Latency II	Sensitive

Figure III.1

Figure III.2 *AZD-5991 (MCL1 inhibitor) effects on the growth and induction of apoptosis in PELs* (**A**) Different MCL-1 inhibitor response between PELs and PEL-LCs. The green color signifies inhibitor sensitivity of the cell line, while yellow indicates resistance. In this context, IC_{50} is defined as the concentration required to inhibit half of the maximum biological response of the drug. (**B**) Percentages of live cells after 24 hours treatment of 1 μM MCL-1 inhibitor (AZD-5991) or 10 μM camptothecin (6 hours) as determined by cell titer glo assay. (**C**) In parallel, caspase 3/7 activities were measured using a luciferase assay from cells in B. Error bars, standard error of mean.

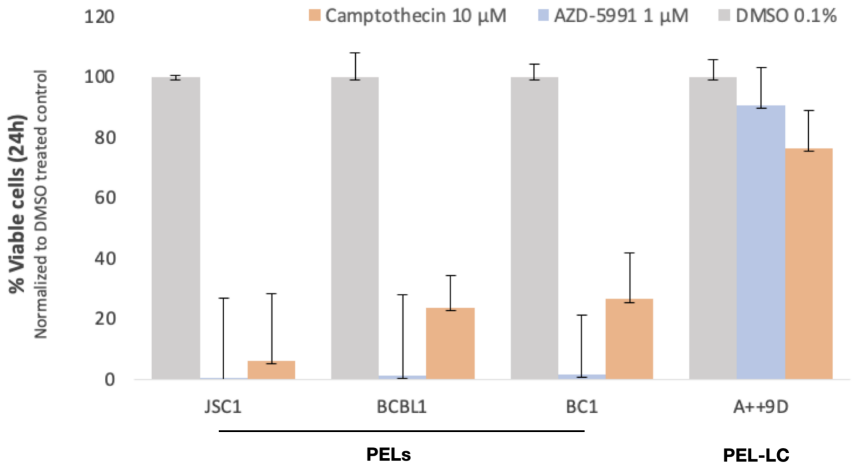
Figure III.2

A

Model	Cell line	MCL-1 Inhibitor IC50 AZD-5991
PEL-LCs	A++11F	7 μM
	A++9D	7 μM
	2C2	6.5 μM
PELs	BCBL-1	0.03 μM
	BC-1	0.03 μM
	JSC-1	0.03 μM

Sensitive
Resistant

B



C

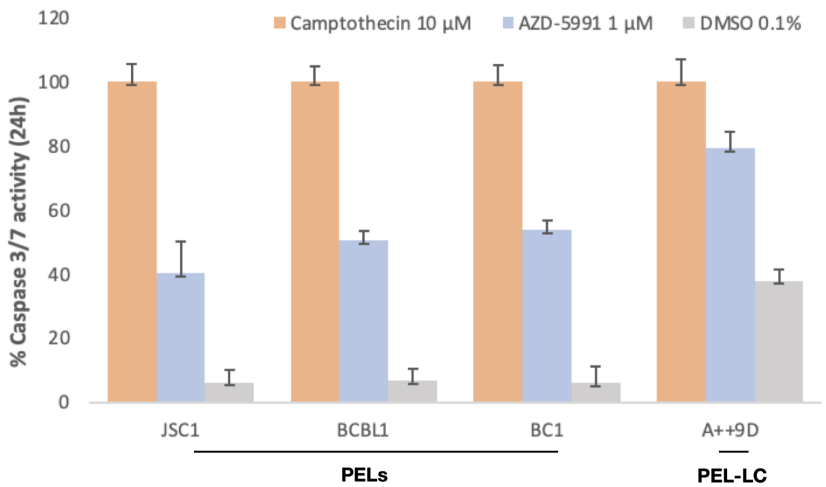


Figure III.3 *Expression levels of MCL-1 in PELs and PEL-LCs*

(**A**) Results of RT-qPCR showing expression of MCL-1. The values have been normalized to the mRNA levels. Error bars, standard error of mean. (**B**) Western blot analysis showing the protein expression of MCL1. The values have been normalized to the expression of tubulin. The levels of significance were determined using an unpaired two-tailed t-test, by GraphPad Prism software 8.0.1. ** = $0.001 < P < 0.01$. A representative example of three independent experiments is shown.

Figure III.3

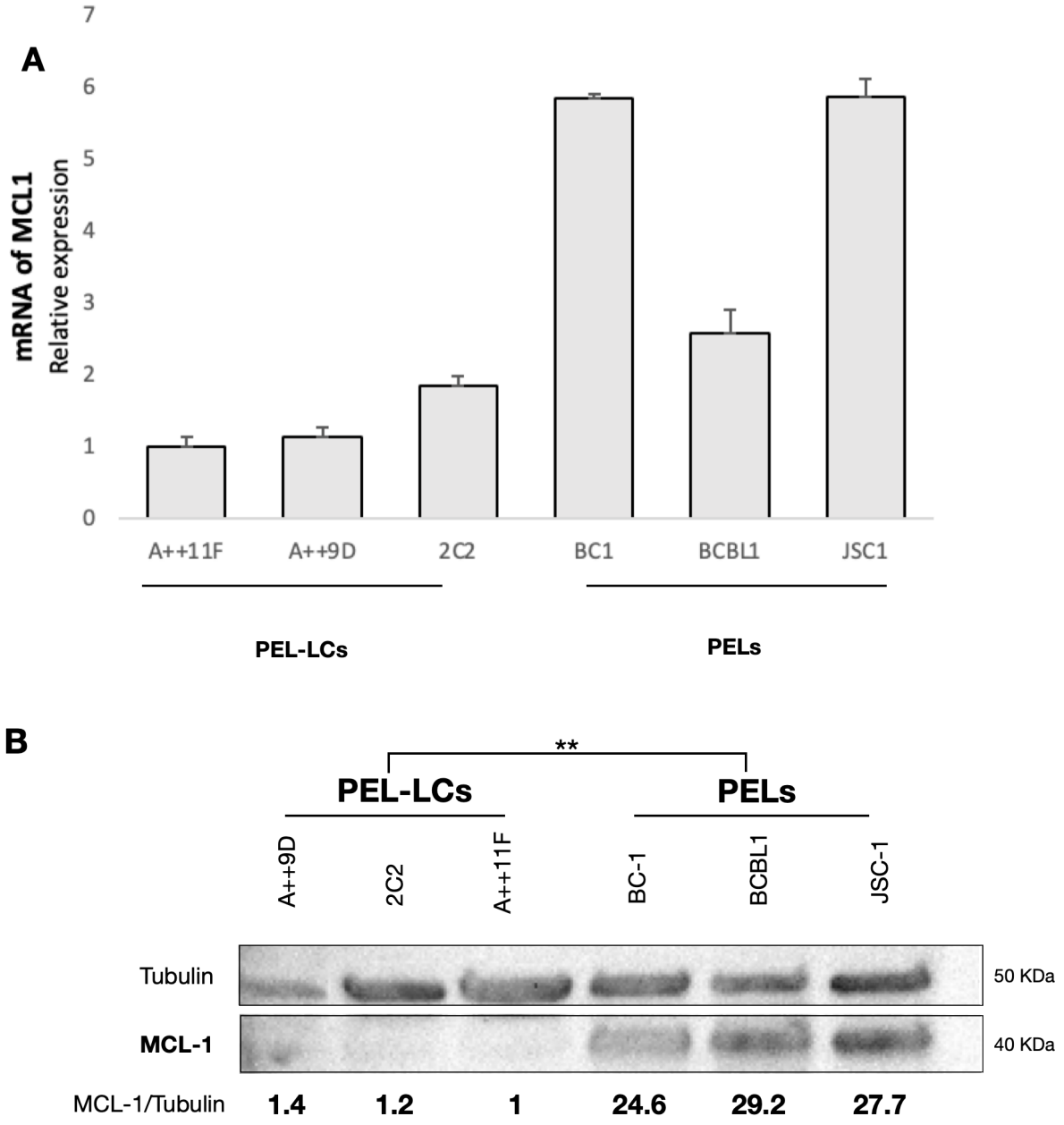

A
mRNA of MCL1
Relative expression
7
6
5
4
3
2
1
0
A++11F
A++9D
2C2
BC1
BCBL1
JSC1
PEL-LCs
PELs
B
**
PEL-LCs
PELs
A++9D
2C2
A++11F
BC-1
BCBL1
JSC-1
Tubulin
MCL-1
50 KDa
40 KDa
MCL-1/Tubulin
1.4
1.2
1
24.6
29.2
27.7

Figure III.4 *Effects of ABT-263 (BCL-2, BCL-xL, and BCL-w) on the growth of PELs and PEL-LCs*
Identical BCL-2, BCL-xL, and BCL-w inhibitor resistance between PELs and PEL-LCs. The green color signifies inhibitor sensitivity of the cell line, while yellow indicates resistance. In this context, IC_{50} is defined as the concentration required to inhibit half of the maximum biological response of the drug.

Figure III.4

Model	Cell line	BCL2, BCL-XL and BCLw Inhibitor IC50 ABT-263
PEL-LCs	A++11F	4 μM
	A++9D	4.5 μM
PELs	JSC-1	5.5 μM
	BC-1	4.5 μM
	BCBL-1	4.5 μM
Immortal human T lymphocytes	Jurkat	0.04 μM

Sensitive
Resistant

Figure III.5 *Expression levels of BCL2A1 in PELs and PEL-LCs*

(**A**) Results of RT-qPCR showing expression of BCL2A1. The values have been normalized to the mRNA levels. Error bars, standard error of mean. (**B**) Western blot analysis showing the protein expression of BCL2A1 and (**C**) myc-tag. 293T cell line expresses SV40 T-antigen to augment plasmid replication with an SV40 origin. Consequently, only in the transfected 293T cells, which express up to 1000 times more protein, could the BCL2A1 protein be detected, and then only with an antibody to the *myc* tag.

Figure III.5

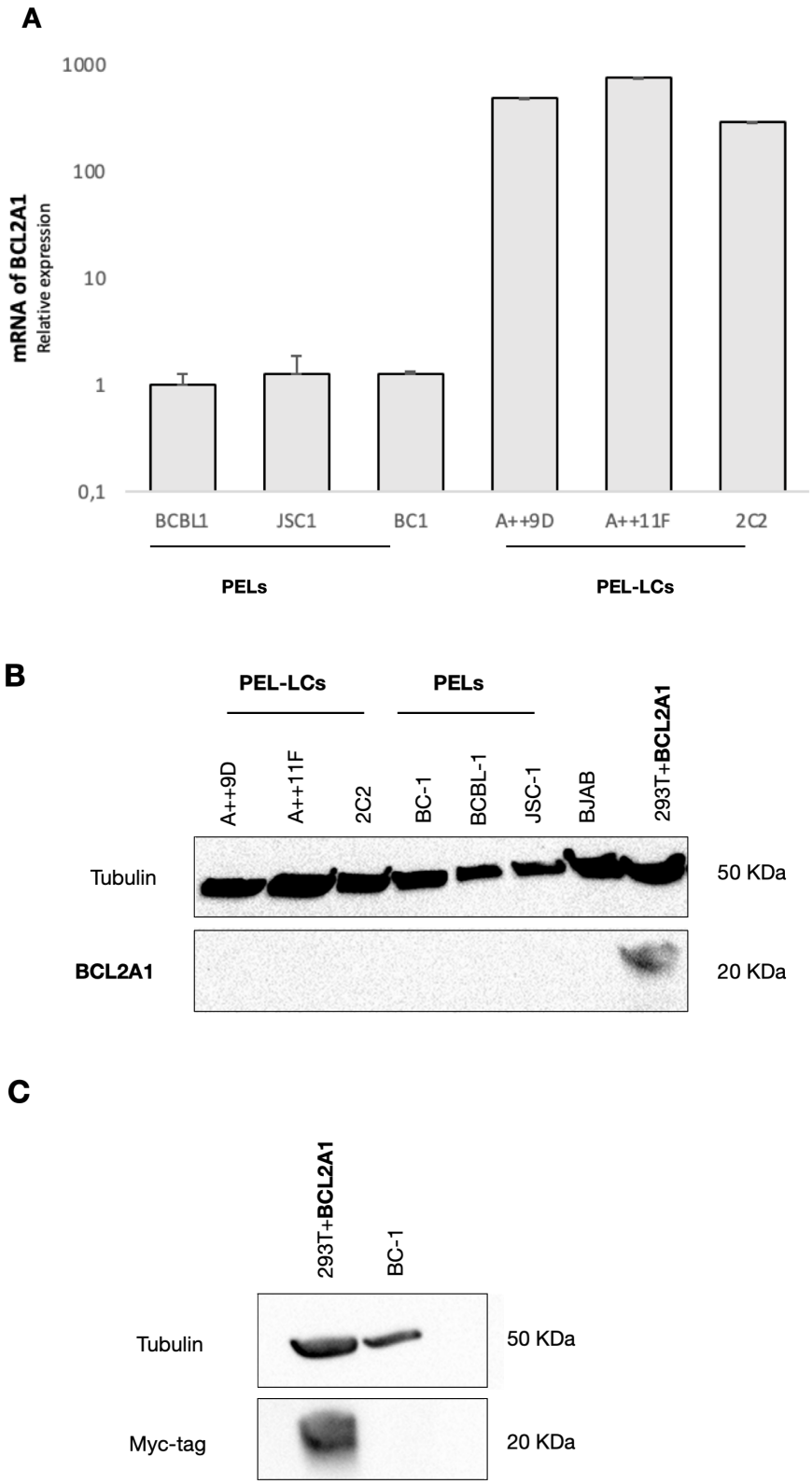
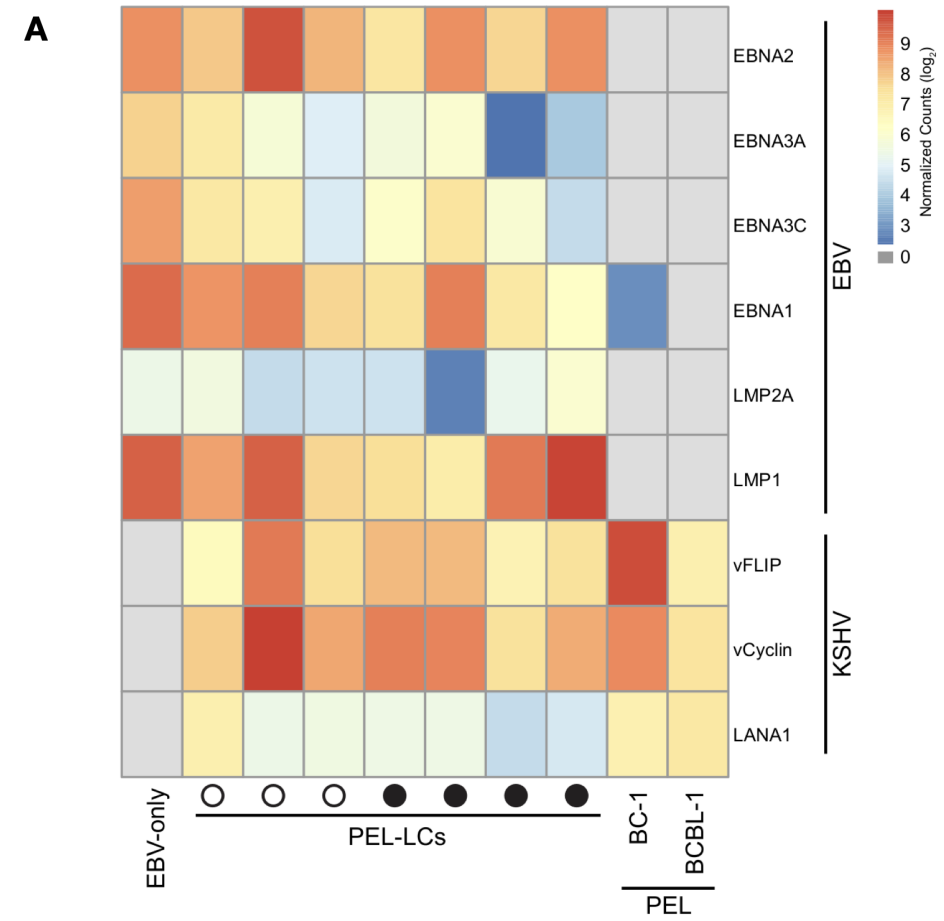
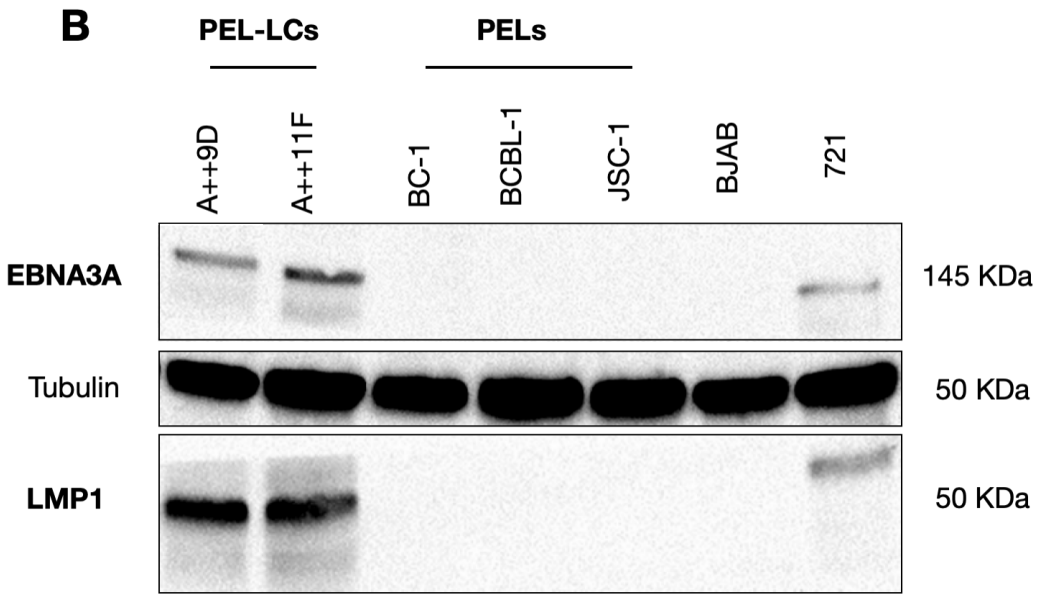

A
1000
100
10
1
0,1
mRNA of BCL2A1
Relative expression
BCBL1
JSC1
BC1
A++9D
A++11F
2C2
PELs
PEL-LCs
B
PEL-LCs
PELs
A++9D
A++11F
2C2
BC-1
BCBL-1
JSC-1
BJAB
293T+BCL2A1
Tubulin
50 KDa
BCL2A1
20 KDa
C
293T+BCL2A1
BC-1
Tubulin
50 KDa
Myc-tag
20 KDa

Figure III.6 *Expression levels of viral genes in PELs and PEL-LCs*

(**A**) Heatmap showing the viral gene expression *in vitro* measured by RNA-Seq in cells infected only with EBV, in populations (white circle) and clones (black circle) of PEL-LCs and PEL cell lines. From blue to red there is a 60-fold difference. (**B**) Western blot analysis showing the protein expression of LMP1 and EBNA3A in PEL-LCs.

Figure III.6


A
EBNA2
EBNA3A
EBNA3C
EBNA1
LMP2A
LMP1
vFLIP
vCyclin
LANA1
EBV
KSHV
Normalized Counts (log2)
9
8
7
6
5
4
3
0
EBV-only
PEL-LCs
BC-1
BCBL-1
PEL


B
PEL-LCs
PELs
A++9D
A++11F
BC-1
BCBL-1
JSC-1
BJAB
721
EBNA3A
145 KDa
Tubulin
50 KDa
LMP1
50 KDa

Discussion

Primary infection with KSHV remains poorly characterized, partly due to its lower infection rate. While endothelial and fibroblast cells can be efficiently infected with KSHV *in vitro*, attempts to infect lymphoblastic cell lines have proven unsuccessful [18-21]. Even when lymphoblastic cells can be infected, KSHV does not induce transformation. Notably, in these studies, neither EBV-negative nor EBV-positive cell lines could be infected. In contrast, EBV can infect and transform peripheral B cells *in vitro*, promoting their long-term proliferation [22, 23]. Although it is widely accepted that the development and sustained viability of PELs are primarily driven by the latent genes of KSHV, an intriguing observation arises from Mack and colleagues. Their attempt to evict EBV from dually infected PELs, resulted in the failure of colony formation under limiting dilution, supporting the idea that EBV is essential for sustaining proliferation [24]. Several latent KSHV genes inhibit apoptosis in PEL cells [25-33], as well as numerous EBV genes, including those expressed in PEL cells [34-37]. Understanding the significance of dual infection and the distinct roles each virus plays in the tumorigenesis of this lymphoma poses a complex challenge.

Recently, Faure and colleagues demonstrated that EBV facilitates KSHV infection in peripheral B cells, with optimal efficiency achieved within 24 hours of EBV infection. The dually infected proliferating cells characterized in their study can maintain both KSHV and EBV for years in culture [2]. This in vitro transformation of peripheral B cells by both viruses offers an opportunity for detailed mechanistic analysis of the viral and cellular genes orchestrating the initial stages of the progression towards PEL. My study aims to elucidate the specific contributions of each virus to the characteristics of PEL-LCs compared to PELs.

Apoptosis depends on a delicate balance between proapoptotic and antiapoptotic regulators. Tumors often exhibit dysregulation in antiapoptotic proteins. My findings, along

with existing literature, document well that PELs rely on the antiapoptotic MCL-1 protein for survival [5, 6, 10]. The fact that PELs, whether KSHV-only or dually infected, depend on MCL-1 suggest that a latent gene expressed by KSHV might upregulate MCL-1, although a mutation in a cellular gene cannot be entirely excluded. Researchers are actively identifying which KSHV gene may be responsible for MCL-1 dependency in PELs [5,6,33].

In my studies, PEL-LCs exhibited higher resistance to MCL-1 pharmacological inhibition compared to PELs, despite both being dually infected with EBV and KSHV. My results indicate a substantial ~25 fold difference at the MCL-1 protein level, suggesting that PEL-LCs resist MCL-1 inhibition because they do not rely on MCL-1 for their survival. PEL-LCs express the same KSHV genes as PELs (data not shown), but their EBV latent patterns differ. PTLD-1 and PEL-LCs were the only cell lines resistant to MCL-1 inhibition in my preliminary screening, both infected with EBV in latency III, a status where the full range of EBV latent genes is transcribed, compared to PEL infected with EBV in latency I. Considering the association of various EBV genes in PEL-LCs with anti-apoptotic functions, I speculate that one or more EBV proteins expressed in latency III play a crucial role in their survival. From the literature on EBV proteins involved in survival mechanisms, LMP-1 (16) and EBNA3A (17) emerge as candidates responsible for the resistance to MCL-1 (AZD-5991), BCL-2, BCL-XL and BCL-W (ABT-263) in PEL-LCs by upregulating the antiapoptotic protein BCL2A1 (A1, BFL-1). Surprisingly, my RT-qPCR analysis revealed a ~250-fold higher expression of BCL2A1 in PEL-LCs compared to PELs.

These data suggest that the resistance of PEL-LCs to AZD-5591 and ABT-263 might be mediated by BCL2A1 and identify EBNA3A and LMP1 as potential candidates that provide a survival advantage to dually infected PEL-LCs, possibly by regulating BCL2A1.

CHAPTER IV.

Assessing the roles of antiapoptotic proteins detected in Chapter III in PELs and PEL-LCs

Introduction

As shown in Chapter III, the survival of PELs relies on the antiapoptotic protein MCL-1. I have identified BCL2-related protein A1 (BCL2A1) as the upregulated antiapoptotic protein in PEL-LCs compared to PELs. BCL2A1 was firstly identified from fetal liver and thus named Bcl-2 related gene expressed in fetal liver (Bfl-1), highlighting its involvement in early hematopoiesis [1]. Following this discovery, in 1996, it was found that BCL2A1 can suppress p53-induced apoptosis like other Bcl-2 family members, thereby elucidating its role as a pro-survival protein involved in the regulation of apoptosis [2]. BCL2A1, an antiapoptotic homolog of BCL-2, contributes to the survival of lymphocytes and lymphomas. The expression of BCL2A1 correlates directly with the resistance of B-cell Chronic Lymphocytic Leukemias (B-CLLs) to chemotherapeutics. Conversely, the repression of BCL2A1 expression sensitizes B-CLLs to apoptosis induced by chemotherapeutics [3-6 and reviewed in 7]. Furthermore, BCL2A1's protein exhibits mitochondria localization and immunoprecipitates with BAK but not BAX [8]. Its interaction with BAK prevents BAX from adopting its active conformation, influencing the activities of both pro-apoptotic proteins [8, 9]. Additionally, BCL2A1 selectively binds the active, truncated form of BH3 interacting domain death agonist (BID), likely inhibiting its pro-apoptotic activities [9, 10].

In this Chapter, I set out to identify whether BCL2A1 is responsible for survival in PEL-LCs using two complementary approaches. The first involves the ectopic introduction of BCL2A1 fused to mRFP into PELs, followed by sorting for mRFP-positive cells. Since infected cells exhibit heterogeneity in protein expression levels, a second sorting step was performed to select the highest 20% and the lowest 20% mRFP-positive cells. This enabled the detection of differences in BCL2A1 levels. This approach shows that ectopically introduced BCL2A1 provides resistance to MCL-1 inhibitor in PELs according to mRNA levels. The second approach

consists of silencing BCL2A1 gene expression in PEL-LCs and MCL-1 in PELs by Clustered Regularly Interspaced Short Palindromic Repeat interference (CRISPRi). CRISPRi utilizes an inactive Cas9 (dCas9), fused to the Krüppel-associated box (KRAB) repressor domain, and a customizable single guide RNA (sgRNA) [11, 12]. The KRAB domain induces silencing by interacting with TRIM28/KAP1, a scaffold protein that assembles a protein complex with chromatin regulators such as CBX5/HP1α and SETB1 [13]. The dCas9-sgRNA complex binds to DNA elements complementary to the sgRNA and the interaction with a KRAB domain recruits KAP1/TRIM28 and assembles a KAP1-associated heterochromatin complex in specific regions of chromatin, including many well-known cofactors involved in heterochromatin formation: histone H3 lysine 9 (H3K9) methyltransferase SETDB1, heterochromatin protein 1, histone deacetylase containing nucleosome remodeling complex and DNA methyltransferases, resulting in the repression of the target gene [13, 14].

In this chapter, I observed enhanced survival rates in PELs based on the expression levels of BCL2A1, identifying BCL2A1 as the potential candidate protein that fosters survival in PEL-LCs.

Results

The constitutive, exogenous expression of BCL2A1 inhibited apoptosis in PELs

As shown earlier, I identified BCL2-related protein A1 (BCL2A1) as the upregulated antiapoptotic protein in PEL-LCs compared to PELs. BCL2A1 was a promising candidate for inhibiting apoptosis due to its ability to bind and inhibit both truncated BID (tBID) and BAK, which are proapoptotic proteins [8-10]. Consequently, BCL2A1's ability to inhibit the activities of tBID and BAK could potentially block the apoptosis triggered by the inhibition of MCL1 (AZD-5991) in PELs. To validate this hypothesis, I conducted tests to assess BCL2A1's efficacy in inhibiting apoptosis induced by AZD-5991 treatment.

PEL cells (BCBL-1 and JSC-1) were transduced with a vector expressing 2x-myc-BCL2A1 fused to mRFP. Subsequent sorting facilitated the selection of the top 20% and bottom 20% of mRFP-positive cells, allowing for the discernment of variations in BCL2A1 levels. These cells were then subjected to MCL-1 inhibitor response testing (AZD-5991). The results demonstrated an increase in viable cells corresponding to the increased expression of BCL2A1 in BCBL-1 (Figure IV.1A) and JSC-1 (Figure IV.1C).

Furthermore, RT-qPCR analysis revealed a 4-fold- increase in the mRNA encoding the 2x-myc-BCL2A1 protein in BCBL-1 high RFP compared to low RFP (Figure IV.1B) and at 8-fold-increase in JSC-1 high RFP compared to low RFP (Figure IV.1D). These findings suggest that BCL2A1 effectively inhibits apoptosis induced by AZD-5991.

CRISPRi approach for targeting BCL2A1 in PEL-LCs and MCL1 in PELs

The exogenous expression of BCL2A1 inhibited apoptosis induced by MCL-1 inhibition in PELs. I hypothesized that BCL2A1 upregulation in PEL-LCs protects against mitochondrial-dependent apoptosis. PEL-LCs, unlike PELs like JSC-1, have wild-type p53 alleles (data not

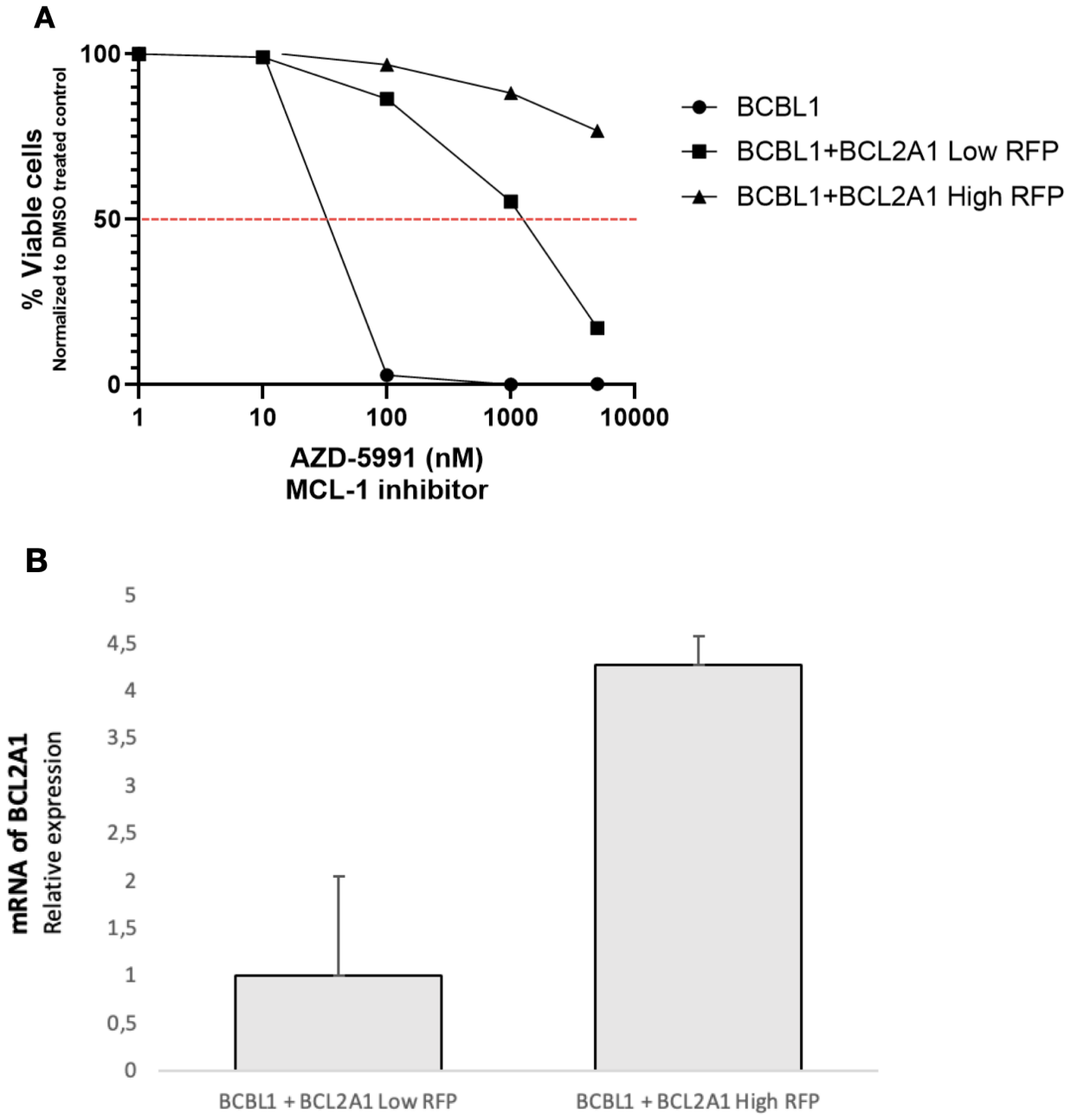
shown). From our previous experiments (data not shown), we know that introducing double-strand breaks into the genome, as in CRISPR/Cas9 editing, is toxic to cells with wild-type p53 [Figure IV.2]. Therefore, knocking out genes was not feasible. This led me to explore an alternative to reduce BCL2A1 expression in PEL-LCs. Initially, I considered using the shRNA tool, but it is well-established that this approach is less effective and prone to off-target effects [15-17] compared to CRISPR interference (CRISPRi) [18]. CRISPRi would recruit many well-known cofactors involved in heterochromatin formation, resulting in the repression of the target gene [13,14] [Figure IV.2].

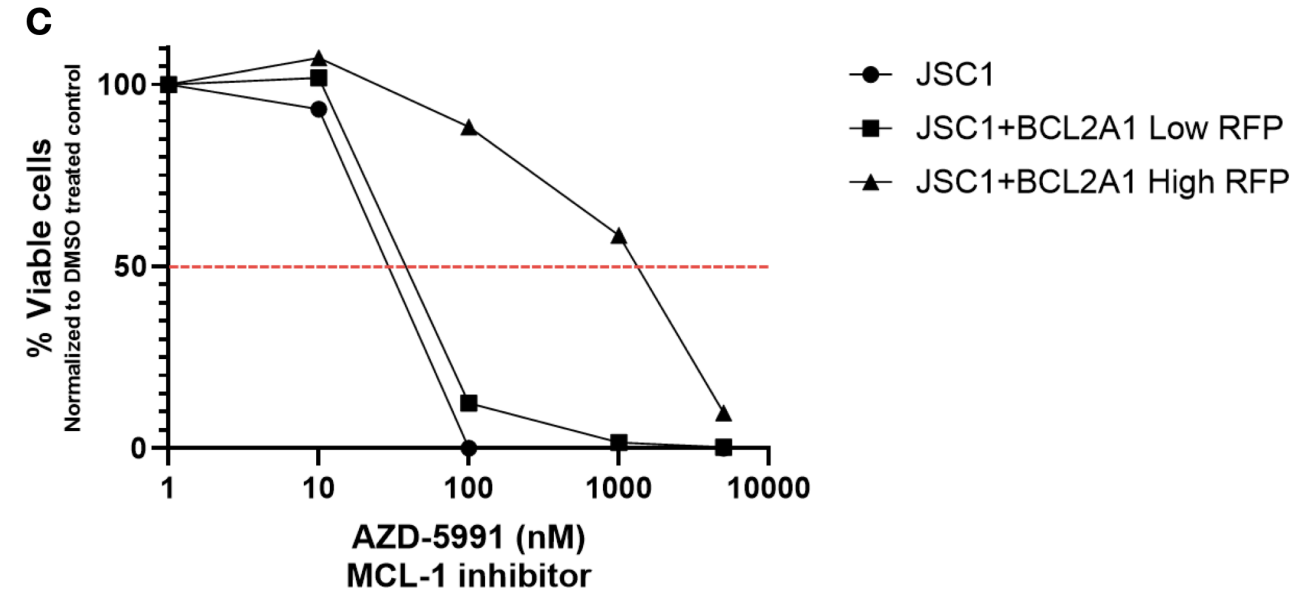
I used a two-step process involving dCas9 derivatives and short-guide RNA targeting specific genes. I generated A++9D (PEL-LC) and JSC-1 (PEL) stably expressing dCas9-KRAB constructs (either MeCP2 or ZIM3) that targeted TSS (transcription start site) of BCL2A1 in A++9D and of MCL-1 in JSC-1. Unfortunately, RT-qPCR analysis showed that none of the guides designed for either BCL2A1 or MCL1 significantly ablated the expression levels compared to the rhodopsin-targeting control [Figure IV.3].

Those cell lines were first transduced with one of the two dCas9 derivatives and underwent selection of blasticidin over a month. Subsequently, they were transduced with a short-guide RNA targeting the promoter of BCL2A1, MCL1, or Rhodopsin, cloned into the LentiCRISPRv2-mScarlett vector. Three days later, transduced cells were selected through fluorescence-activated cell sorting (FACS) for mScarlett-positive cells. Unfortunately, the guides themselves did not produce the desired effects. A more recent study by Dai et al. demonstrated that in a similar system, dCas9-KRAB significantly reduced BCL2A1 (BFL-1) mRNA levels in a lymphoblastoid cell line (LCL) infected with EBV compared to KRAB-only cells [19].

Figure IV.1 *The constitutive, exogenous expression of BCL2A1 inhibited apoptosis in PEL cells.* Ectopic introduction of BCL2A1 fused to mRFP into PELs, followed by sorting by flow cytometry for the 20% of cells expressing the lowest (Low 20%) or highest (High 20%) levels of BCL2A1-mRFP. Dose-response curves to assess AZD-5991 (MCL-1 inhibitor) sensitivity in proliferating (**A**) BCBL-1 and (**C**) JSC-1 cells. The expression of the BCL2A1-mRFP for (**B**) BCBL-1 and (**D**) JSC-1 was normalized to mRNA levels and is represented as the fold change relative to the expression in cells expressing the lowest levels of BCL2A1-mRFP. Error bars, standard error of mean.

Figure IV.1


A
% Viable cells
Normalized to DMSO treated control
100
50
0
1
10
100
1000
10000
AZD-5991 (nM)
MCL-1 inhibitor
BCBL1
BCBL1+BCL2A1 Low RFP
BCBL1+BCL2A1 High RFP
B
mRNA of BCL2A1
Relative expression
5
4,5
4
3,5
3
2,5
2
1,5
1
0,5
0
BCBL1 + BCL2A1 Low RFP
BCBL1 + BCL2A1 High RFP


C
% Viable cells
Normalized to DMSO treated control
100
50
0
1
10
100
1000
10000
AZD-5991 (nM)
MCL-1 inhibitor
JSC1
JSC1+BCL2A1 Low RFP
JSC1+BCL2A1 High RFP

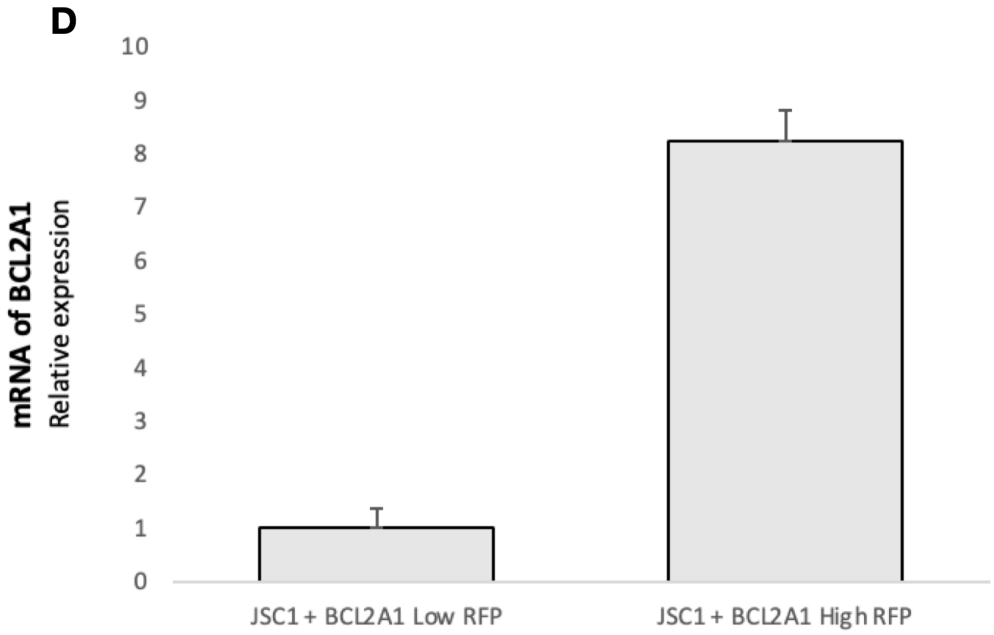
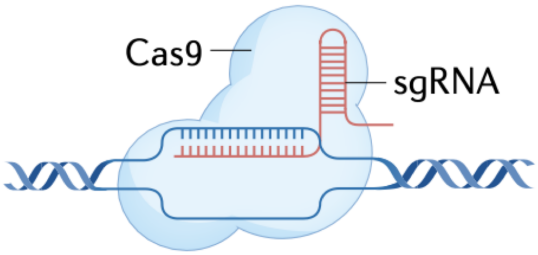

D
mRNA of BCL2A1
Relative expression
10
9
8
7
6
5
4
3
2
1
0
JSC1 + BCL2A1 Low RFP
JSC1 + BCL2A1 High RFP

Figure IV.2 *Difference between CRISPR/Cas9 and CRISPRi*

Introducing double-strand breaks into the genome, as in CRISPR/Cas9 editing, is toxic to cells with wild-type p53. CRISPRi utilizes an inactive Cas9 (dCas9), fused to the Krüppel-associated box (KRAB) repressor domain, and a customizable single guide RNA (sgRNA). CRISPRi would recruit many well-known cofactors involved in heterochromatin formation, resulting in the repression of the target gene without introducing any breaks into the genome [Figure modified from 20].

CRISPR/Cas9

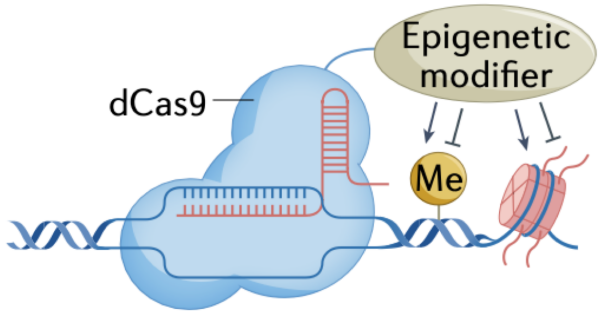


CRISPR–Cas9 editing

CRISPR–Cas9 induces double strand breaks at targeted proto-spacer with NGG PAM recognition sequence in mammalian cells that result in indel formation

Toxic when p53 is WT

CRISPR interference



Epigenetic regulation

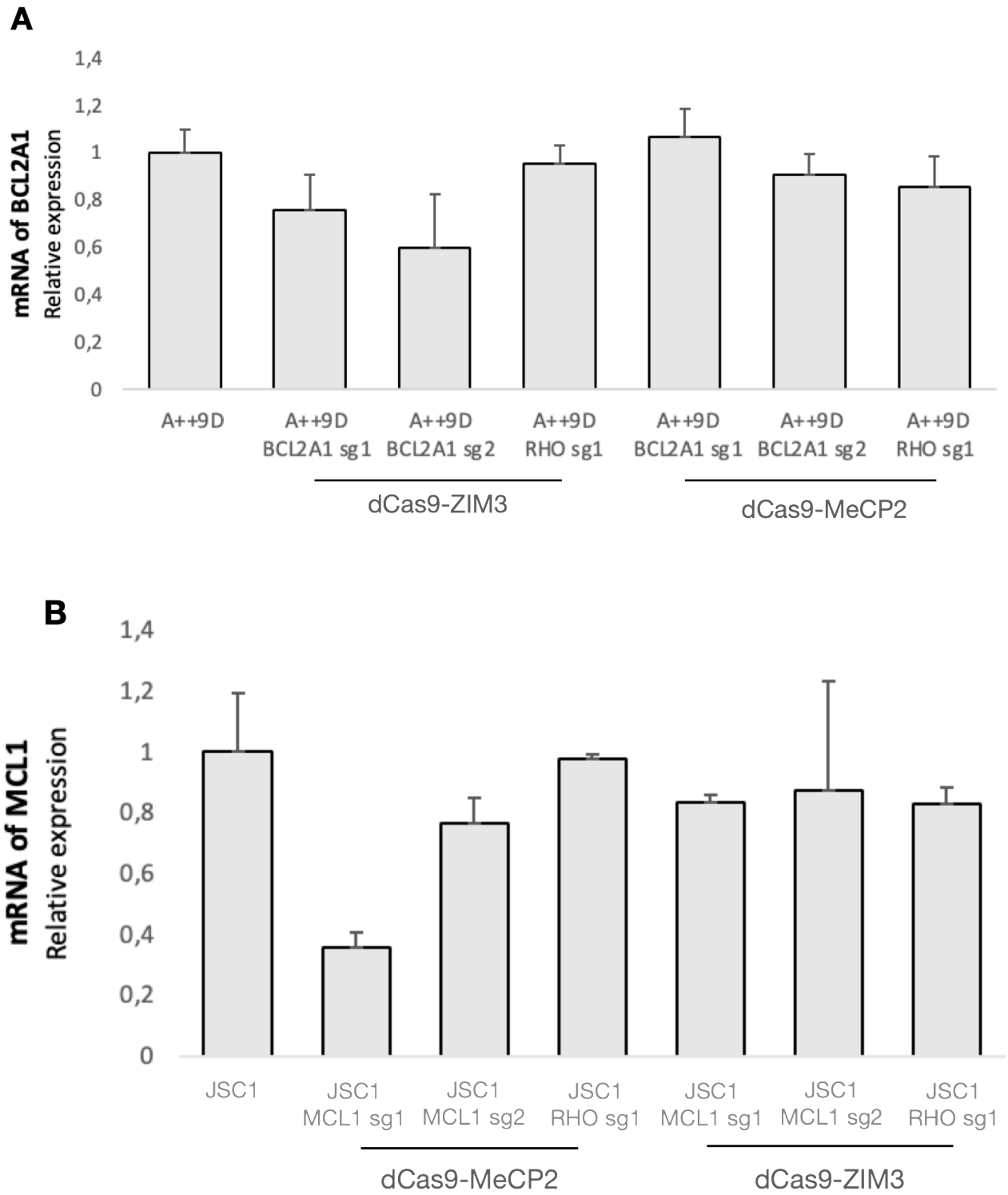
dCas9 combined with an epigenetic modifier can regulate gene expression by tethering methyltransferases and acetyltransferases

Figure IV.2

Figure IV.3 *Downregulation BCL2A1 in PEL-LC and MCL-1 in PEL*

RT-qPCR of (**A**) BCL2A1 levels in A++9D (PEL-LC) and (**B**) of MCL-1 levels in JSC1 (PEL) stably expressing either dCas9-MeCP2 or dCas9-ZIM3. Rhodopsin (RHO) is a not essential gene used as negative control. The expression of BCL2A1, MCL1 and RHO was normalized to mRNA levels. Error bars, standard error of mean. Sg=single guide.

Figure IV.3


A
mRNA of BCL2A1
Relative expression
1,4
1,2
1
0,8
0,6
0,4
0,2
0
A++9D
A++9D BCL2A1 sg1
A++9D BCL2A1 sg2
A++9D RHO sg1
A++9D BCL2A1 sg1
A++9D BCL2A1 sg2
A++9D RHO sg1
dCas9-ZIM3
dCas9-MeCP2
B
mRNA of MCL1
Relative expression
1,4
1,2
1
0,8
0,6
0,4
0,2
0
JSC1
JSC1 MCL1 sg1
JSC1 MCL1 sg2
JSC1 RHO sg1
JSC1 MCL1 sg1
JSC1 MCL1 sg2
JSC1 RHO sg1
dCas9-MeCP2
dCas9-ZIM3

Discussion

As shown in chapter III, my findings along with existing literature, document well that PELs rely on the antiapoptotic MCL-1 protein for survival. My data show that PEL-LCs exhibited higher resistance to MCL-1 pharmacological inhibition compared to PELs and suggest that this resistance might be mediated by BCL2A1 and identify EBNA3A and LMP1 as potential candidates that provide a survival advantage to dually infected PEL-LCs, possibly by regulating BCL2A1. BCL2A1 is an antiapoptotic protein similar to BCL-2, playing a crucial role in the survival of lymphocytes and lymphomas. In B-cell Chronic Lymphocytic Leukemias (B-CLLs), high expression of BCL2A1 is associated with resistance to chemotherapeutics. Suppressing BCL2A1 expression enhances sensitivity of B-CLLs to chemotherapy-induced apoptosis.

Additionally, I observed enhanced survival rates in PELs based on the expression levels of BCL2A1, identifying it as the potential protein that fosters survival in PEL-LCs. Notably, I found that the mRNA expression of Bcl2a1, as opposed to Mcl1, is greater in PEL-LCs than in PELs. My findings revealed that the introduction of exogenous BCL2A1 inhibited apoptosis in PEL cells treated with an MCL-1 inhibitor, underscoring the potential significance of BCL2A1 in the context of PEL pathogenesis. Expanding on this, existing literature supports the notion that EBV viral genes, LMP1 and EBNA3A, are implicated in the upregulation of BCL2A1, reinforcing the intricate relationship between viral infection and the antiapoptotic machinery in PEL.

Recognizing BCL2A1 as a key cofactor in PEL-LCs, the loss of BCL2A1 was expected to impact cellular survival. To directly compare the requirements for BCL2A1 and MCL1 in PEL-LCs and PELs, respectively, I aimed to suppress each antiapoptotic protein using CRISPR interference [21, 22]. In CRISPRi, endonuclease-deactivated Cas9 (dCas9) is fused with the transcriptional repressor domain Krüppel-associated box (KRAB) and directed close to the

transcription start site (TSS) of target genes through sgRNAs, inducing epigenetic transcriptional silencing (knockdown). I opted for CRISPRi over CRISPR/Cas9 due to observed gene function-independent toxicity when targeting with the Cas9 endonuclease with p53 wild-type. I also favored it over shRNA due to its greater effectiveness and reduced risk of off-target effects. Furthermore, I utilized two distinct dCas9-KRAB constructs, MeCP2 or ZIM3. ZIM3 is known for its highly potent KRAB domain, demonstrating increased effectiveness in target gene silencing and reduced sensitivity to gRNA selection compared to existing systems [11]. Besides its potency, ZIM3 KRAB is smaller than the KRAB–MeCP2 construct, offering an advantage for approaches using viral delivery methods limited by insert length. Despite this efforts, CRISPRi-mediated knockdown of BCL2A1 or MCL-1 did not lead to a significant downregulation. The sgRNAs were designed from the CRISPRi Dolcetto library (23). Moreover, it is not possible to determinate from my data which of the two dCas9-KRAB constructs is more efficient. A more recent study by Dai et al. demonstrated that in a similar system, dCas9-KRAB significantly reduced BCL2A1 (BFL-1) mRNA levels in a lymphoblastoid cell line (LCL) infected with EBV compared to KRAB-only cells [19]. In their study, researchers report that BCL2A1 expression is not crucial for LCL survival in the absence of extrinsic apoptotic signals [19, 24]. They hypothesized that BCL2A1 upregulation in LCLs protects against mitochondrion-dependent extrinsic apoptosis. To test it, they generated BCL2A1 knockdown LCL proliferating cells that were significantly more sensitive to increasing doses of FasL and TRAIL, activating the extrinsic apoptosis, compared to negative LCLs that expressed not targeting dCas9-KRAB. Their findings suggest two potential courses of action: utilizing the same sgRNA they employed and considering that the sole loss of BCL2A1 might not affect cellular survival.

In fact, adding another layer of complexity, the lack of essentiality identified for BCL2A1 in existing CRISPR screens introduces challenges in establishing a control scenario

where BCL2A1 downregulation unequivocally results in cell death, but this discovery opens up the possibility that this gene may act as a synthetically lethally gene. In such scenario, the combination of two genetic events would lead to cell death.

In this *in vitro* model, EBV is seen as an active participant rather than a mere passenger in PEL, potentially orchestrating the upregulation of BCL2A1 to provide an antiapoptotic signal. This intricate interplay may be further influenced or compensated by the deregulation of MCL-1, adding layers of complexity to the apoptotic regulatory network in PEL.

CHAPTER V.

Discussion and future directions

Primary effusion lymphoma (PEL), an aggressive non-Hodgkin's lymphoma, primarily affects immunocompromised individuals [1-3]. While KSHV and EBV co-infection is common in PEL, the role of each virus in tumor maintenance is complex. The primary infection of KSHV remains enigmatic, hindered by its low infection rate. Additionally, while KSHV efficiently infects endothelial and fibroblast cells *in vitro*, attempts to infect lymphoblastic cell lines have proven unsuccessful [4-7]. In contrast, EBV displays efficacy in infecting and transforming peripheral B cells, emphasizing its role in long-term proliferation[8, 9]. Despite observations which indicate that the development and viability of PELs are primarily driven by KSHV latent genes, attempts to eliminate EBV from dually infected PELs resulted in the failure of colony formation, highlighting the essential role of EBV in sustaining proliferation [10]. Faure et al. demonstrated EBV's facilitation of KSHV infection in peripheral B cells, offering a unique *in vitro* model for investigating the progression towards PEL [11]. My research aims to uncover the specific contributions of each virus to the characteristics of PEL-LCs compared to PELs.

The delicate balance between proapoptotic and antiapoptotic regulators is crucial for cellular survival [12]. PELs, whether KSHV-only or dually infected, heavily rely on the antiapoptotic MCL-1 protein [13-15]. This dependence on MCL-1, regardless of EBV infection status, support the hypothesis that a latent KSHV gene may play a role in the upregulation of MCL-1. Examining PEL-LCs, my research discovered their higher resistance to MCL-1 inhibition compared to PELs, indicating an alternative survival mechanism. Notably, this resistance is linked to elevated BCL2A1 expression in PEL-LCs, infected with EBV in latency III, compared to PELs, infected with EBV in latency I (when present), and suggests a role for specific EBV proteins in their survival. LMP-1 [16] and EBNA3A [17] emerge as candidates influencing resistance to MCL-1 inhibition in PEL-LCs, potentially by upregulating BCL2A1.

My study investigates the potential significance of BCL2A1 in PEL-LCs, identifying it as

a key factor for cellular survival. My findings revealed that the introduction of exogenous BCL2A1 inhibited apoptosis in PEL cells treated with an MCL-1 inhibitor, underscoring the potential significance of BCL2A1 in the context of PEL pathogenesis. Recognizing BCL2A1 as a key cofactor in PEL-LCs, the loss of BCL2A1 was expected to impact cellular survival. Attempts to suppress BCL2A1 and MCL-1 using CRISPR interference reveal complexities and challenges. Despite my efforts, CRISPRi-mediated knockdown of BCL2A1 or MCL-1 did not lead to their significant downregulation in PEL-LCs and PELs, respectively. Moreover, the lack of essentiality identified for BCL2A1 by others and in available CRISPR screens introduces challenges in establishing a control scenario where BCL2A1 downregulation unequivocally results in cell death and introduces the possibility of its “synthetic lethality”, in which the combination of two genetic events would lead to cell death. These findings suggest two potential courses of action: employing the same guide RNA used by Dai et al. for CRISPRi to downregulate BCL2A1 [18] and acknowledging that the exclusive loss of BCL2A1 may not impact cellular survival. In this context, it would be intriguing to investigate whether PEL-LCs continue to survive after the downregulation of BCL2A1 and, consequently, assess any alterations in their resistance to inhibition of MCL-1, BCL-2, BCL-XL, and BCL-W.

Therefore, I speculated a potential role for latent EBV proteins, LMP1 or EBNA3A, in conferring antiapoptotic advantages to PEL-LCs, mirroring EBV's role during initial infection and transformation in PEL. In experiments led by Pratt and colleagues, the introduction of LMP1 into cell lines, both EBV-positive and EBV-negative, exhibited a clear association with heightened levels of the antiapoptotic protein BCL2A1 and LMP1. Conversely, transfecting two EBV-negative epithelial cell lines with escalating doses of an LMP1-encoding vector did not yield detectable BCL2A1 expression via RT-qPCR [16]. Price and colleagues conducted a study using both wild-type (WT) EBV and an EBV strain lacking EBNA3A (ΔEBNA3A) to infect

and transform B cells (LCLs). Notably, cells infected with ΔEBNA3A exhibited sensitivity to ABT-737, an inhibitor targeting antiapoptotic proteins BCL-2, BCL-xL, and BCL-w, in contrast to WT EBV-infected cells. This distinctive phenotype was rescued upon the introduction of ectopic expression of EBNA3A. Furthermore, employing chromatin conformation capture assays, they unveiled that two genomic regions upstream of the BCL2A1 gene were strongly associated with the transcription start site (TSS) in WT EBNA3A, whereas this association was less pronounced in ΔEBNA3A LCLs. In support of this, ChIP-Seq data revealed the binding of EBNA3A within a known EBV Super Enhancer located 39 kb upstream of the BFL-1 TSS [17].

To assess their roles in PEL-LCs compared to PEL, I propose employing two complementary approaches. The first approach involves the ectopic introduction of LMP1 or EBNA3A fused to a fluorescent protein into PELs, and the selection of the highest 20% and the lowest 20% fluorescent-positive cells. This selection allows for the examination of the association between with levels of the antiapoptotic protein BCL2A1 and LMP1 or EBNA3A. This approach underscores the potential significance of EBV in the context of PEL pathogenesis. The second approach involves their knockdown or downregulation. This approach presents some complexities. As mentioned earlier, CRISPR/Cas9 is toxic in cells presenting wild-type p53 as in PEL-LCs. On the other hand, CRISPRi is not feasible due to shared promoters controlling most EBV-encoded transcripts. To address these challenges, I propose testing and employing CRISPR/CasRx, which utilizes a deactivated dCas9 targeting RNA sequences [19]. However, it’s important to note that the latter experiment can’t exclude the possibility that these proteins contribute to survival through mechanisms other than apoptosis. Roles beyond inhibiting apoptosis have emerged for LMP1 and EBNA3A, including inhibition of gene expression, induction of autophagy and cell proliferation [20, 21 and reviewed in 22]. To dissect the specific role of these viral proteins, a double downregulation

of BAX and BAK should be initially performed. This process inactivates the intrinsic apoptosis pathway since pore formation by BAX or BAK1 is considered the necessary step to irreversibly initiate intrinsic apoptosis. By inhibiting intrinsic apoptosis, it becomes possible to test whether the downregulation of LMP1 or EBNA3A leads to apoptosis, and if so, determine whether this is attributable to other mechanisms.

My research poses the bases to dissect the intricate interplay between KSHV, EBV, and the apoptotic regulatory network in PEL. BCL2A1 emerges as a potential key player, influenced by EBV proteins, adding layers of complexity to our understanding of PEL pathogenesis.

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Chapter I. Background and introduction

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Chapter II. Material and methods

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Chapter III. Characterizing anti-apoptotic pathways in PELs and PEL-LCs

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Chapter IV. Assessing the roles of antiapoptotic proteins detected in Chapter III in PELs and PEL-LCs

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Chapter V. Discussion and future directions

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