Identifying and Targeting Immune Escape Mechanisms in Epstein-Barr Virus-Driven Lymphoproliferative Disease

DISSERTATION

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By

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Abstract

Around 12% of human cancers are caused by viral infections. Cancer-inducing viruses, called “oncoviruses”, hijack cellular machinery to avoid normal cell growth controls and detection of the infected cell by the immune system. Epstein-Barr virus (EBV), which infects over 90% of the world’s population, is the most potent of these oncoviruses and is responsible for a diverse spectrum of malignant diseases. If the immune system becomes compromised, as in patients with AIDS or undergoing organ transplant treatment, the virus can initiate its growth program and transform host B cells into malignant lymphoma. These lymphomas are typically aggressive and exhibit poor clinical outcomes in an already-vulnerable patient population. Currently there is no standard treatment for EBV-associated lymphomas, demonstrating a significant unmet need for novel preventative and therapeutic approaches. Since nearly all individuals have been infected with EBV early in life, the immune system is already able to detect these EBV-infected cells. If properly activated and released from the suppressive effects of cancer, the immune system can use this previous detection, “immune memory”, to recognize and eliminate EBV-infected tumor cells in the setting of lymphoma.

When EBV infects a cell, it uses the normal cell machinery to produce viral proteins that drive cell growth and survival signals. Dysregulation of these signals contributes to the
emergence of lymphoma. In individuals with a normal immune system, these viral proteins are recognized by a type of white blood cell called cytotoxic T lymphocytes (CTLs) that kill infected cells to prevent viral spread. Here we show that a compound called silvestrol selectively targets B cell cancers while sparing normal immune cells by blocking the production of viral cancer-promoting proteins, mainly latent membrane protein 1 (LMP1), increasing immune cell-mediated death of cancer cells. Animal models of EBV-driven lymphoma treated with silvestrol survived and exhibited no signs of disease, without discernable toxicity, while control mice either succumbed to disease or showed significant signs of lymphoma upon analysis. Interestingly, silvestrol lost its effectiveness when these mice were depleted of CTLs, demonstrating that this potent anti-tumor effect occurs through the immune system and not through a direct effect of silvestrol on the tumor.

One of the ways tumor cells can evade immune detection is by recruiting another immune cell type called macrophages, which are able to suppress anti-tumor CTL activity and promote tumor growth. We developed a model system in which normal immune cells are incubated with lymphoma cells to initiate an immune reaction. When this occurs, factors are secreted into the surroundings that promote the differentiation of cells into immunosuppressive macrophages. Silvestrol, even at extremely low concentrations, blocks both the secretion of the macrophage-inducing factors and also directly inhibits the outgrowth of macrophages even in the presence of suppressive factors. This finding indicates that protein synthesis is important to this immune evasion strategy, and provides a new approach to modulate the immune response in EBV-driven lymphoma and possibly other cancers. In summary, this work provides a strong rationale...
for continued development of this novel immunotherapeutic approach to treating this aggressive and clinically difficult type of cancer.
For Laura and Evelyn
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Fields of Study

Major Field: Comparative and Veterinary Medicine
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Chapter 1: Introduction

1.1 Overview

Epstein-Barr virus (EBV) is a ubiquitous herpesvirus, infecting over 95% of the world's adult population, and plays an important role in human diseases. EBV preferentially infects B lymphocytes and typically persists as a life-long asymptomatic infection. However, disruption of the virus-host equilibrium triggers the virus to exhibit its pathogenic potential, leading to a diverse array of acute and chronic diseases. EBV is the best known as the principle agent in the development of infectious mononucleosis (IM), or “mono”. Acute infection is typically self-limiting due to a strong, virus-specific T cell response. However, a significant number of patients exhibit prolonged symptoms and a subset will develop severe, life threatening diseases. In males with the X-linked lymphoproliferative (XLP) disease trait or with chronic active infection (CAEBV), fatal hemophagocytosis can develop. Furthermore, several autoimmune disorders, most notably multiple sclerosis (MS), are related to EBV-specific immune dysregulation. Most importantly, EBV has the capacity to malignantly transform lymphocytes and epithelial cells and is linked to a wide range of cancers. EBV is associated with Burkitt lymphoma (BL), nasopharyngeal carcinoma (NPC), Hodgkin’s lymphoma, gastric carcinoma and T/NK cell lymphoma, all of which occur with little to no prior immune
impairment\textsuperscript{11, 12}. In addition, immunocompromised individuals with human immunodeficiency virus (HIV) infection or post-transplantation are at increased risk of developing B lymphoproliferative disorders (LPD). It is clear that the immune response to EBV in different situations can either promote or disrupt pathogenesis\textsuperscript{11, 13, 14}. A better understanding of the interplay between host and viral immune response is crucial to improve immune-based strategies to combat these diseases. This chapter will review these concepts, which are central to the work laid out in this dissertation.

1.2 EBV infection and life cycle

More than 130 viruses are included in the family Herpesviridae\textsuperscript{15}. These viruses contain relatively large, double stranded DNA genomes typically encoding 100-200 genes. Humans are the primary host for eight of these viruses labeled human herpes virus (HHV) 1 through 8 (Table 1.1)\textsuperscript{3, 16}. All herpes viruses have a biphasic life cycle consisting of latency, where infected host cells contain the viral genome but are not producing virus, and lytic reactivation involving the production of new virions\textsuperscript{17}. Together, these cycles comprise the mechanism by which herpes viruses establish lifelong infections and spread to new hosts. The subfamily Gammaherpesvirinae (γ-herpes virus) was first distinguished by their cellular tropism for lymphocytes and the capacity to promote lymphoproliferation and cancers. This subfamily includes two viruses that are important to human pathology, HHV-4 (EBV) and HHV-8 (Kaposi’s sarcoma associated herpes virus)\textsuperscript{18}. In particular, EBV is notable for its high prevalence worldwide and its association with a wide range of diseases.
EBV infection is predominately restricted to humans, but has the capability to infect some non-human primates including cotton-top tamarins\(^{19,20}\). People infected with EBV shed virus into the saliva, and therefore, infection typically occurs when an EBV-negative individual is exposed to viral particles in the saliva of an EBV-infected person\(^{21}\). Viral entrance is mostly limited to cells expressing the cell surface molecule CD21, which is the receptor of complement component C3d, also known as complement receptor 2 (CR2), primarily expressed on B lymphocytes\(^{22}\). Infection is initiated by attachment of the major viral envelope glycoprotein gp350/220 to CD21 and facilitated by the binding of another glycoprotein, gp42, to the co-receptor human leukocyte antigen class II (HLA II). Other cells are known to be infected by EBV including epithelial cells, natural killer (NK) cells, T cells, macrophages and dendritic cells (DC); however, this occurs through other

<table>
<thead>
<tr>
<th>Human Herpes Virus</th>
<th>Common name</th>
<th>Subfamily</th>
</tr>
</thead>
<tbody>
<tr>
<td>HHV-1</td>
<td>Herpes simplex virus 2 (HVS1)</td>
<td>α</td>
</tr>
<tr>
<td>HHV-2</td>
<td>Herpes simplex virus 2 (HSV2)</td>
<td>α</td>
</tr>
<tr>
<td>HHV-3</td>
<td>Varicella-zoster virus</td>
<td>α</td>
</tr>
<tr>
<td>HHV-4</td>
<td>Epstein-Barr virus</td>
<td>γ</td>
</tr>
<tr>
<td>HHV-5</td>
<td>Cytomegalovirus</td>
<td>γ</td>
</tr>
<tr>
<td>HHV-6</td>
<td>Human herpes virus 6</td>
<td>β</td>
</tr>
<tr>
<td>HHV-7</td>
<td>Human herpes virus 7</td>
<td>β</td>
</tr>
<tr>
<td>HHV-8</td>
<td>Kaposi’s sarcoma-associated virus</td>
<td>γ</td>
</tr>
</tbody>
</table>

*Table 1.1: Human herpes viruses. Adapted from Fields et al.\(^2\)*
tropisms not yet fully defined\textsuperscript{23}. Recently, Ogembo et al. described an alternative, CD21-independent pathway in which CD35 mediates infection when HLA II is co-expressed\textsuperscript{23}. CD35 is expressed on B cells, but also DC, NK cells, macrophages and subsets of T cells, indicating it may be a physiologically relevant pathway of infection for non-B lymphocyte lineages\textsuperscript{24}.

The EBV nucleocapsid contains a genome consisting of a linear, double stranded DNA molecule approximately 172 kb in size. The EBV genome encodes around 100 genes important for regulating expression of viral nucleic acids, forming the structural components of the virion, and modulating the host immune response\textsuperscript{4}. Once the virus enters the host nucleus, the linear EBV genome circularizes, forming an episome, which coincides with initial gene expression\textsuperscript{25}. This initial replication of transmitted virus occurs in pharyngeal epithelium and is characterized by high rates of virus shedding into the throat\textsuperscript{26}. The infection spreads to B cells in oropharyngeal lymphoid tissue and initiates a latent, growth-transforming infection (latency III)\textsuperscript{27}. At the same time, the immune system controls viral shedding in the throat and mounts a cellular response to newly infected B cells, which down-regulate viral antigens and enter a resting state as memory B cells (latency 0)\textsuperscript{28}. This pool of long-lived infected B cells continues to circulate between the blood and lymphoid tissues with intermittent lytic activation within the oropharynx, resulting in viral shedding and potential dissemination to a new host\textsuperscript{29}. 
1.3 EBV latent infection

Following transmission to a new host, EBV begins the process of establishing a persistent infection in the B cell compartment. This is characterized by the establishment of latency, the hallmark of which is a restricted expression of viral genes that alter cellular function and proliferation\(^30\). Of the nearly 100 viral genes, latent genes represent nine, including six nuclear proteins (EBNAs 1, 2, 3A, 3B, 3C and LP), two latent membrane proteins (LMP-1 and 2) and two non-coding small RNAs (EBERs 1 and 2)\(^31\). During initial infection, B cells enter a growth phase, designated latency III, which is characterized by the expression of all latent proteins\(^32\). This pattern of gene expression pushes infected cells to become proliferating B cells and drives replication of the viral episome\(^33\). However, by limiting viral gene expression, EBV reduces the number of viral antigens available for recognition of infected cells by cytotoxic T lymphocytes (CTL)\(^34, 35\). However, this is a multi-step process that transition through transcriptional programs termed latency 2, 1 and 0, which express distinct gene expression (Table 1.2)\(^36, 37\). Immunologic pressure drives newly infected B cells to switch to latency II (default program) expressing EBNA1, LMP1 and LMP2A, which lowers immunogenicity of infected cells\(^38\). In this program LMP1 and LMP2A mimic CD40 and IgG signaling respectively, driving infected cells to transition to memory B cells\(^39\). However, these proteins are immunogenic and the host immune system eventually forces the virus into an immunologically hidden state of true latency\(^40\). This program, termed latency 0, lacks any viral protein expression, and only EBV-encoded RNAs (EBER1 and 2) are transcribed\(^38, 41\). Finally, when EBV-infected memory B cells divide, the virus switches from latency 0 to a transcriptional program only expressing EBNA1 protein (latency 1).
This is necessary for EBV persistence since EBNA1 facilitates EBV episome replication in dividing cells⁴². It is important to note that these transcriptional patterns are associated with different EBV cancers, which will be discussed in more detail below⁴³.

Lastly, EBV is sub-classified as either type 1 or type 2 based on the sequence of several latent proteins. The two subtypes are essentially identical throughout most of the viral genome, but they can be distinguished by polymorphisms primarily within EBNA2⁴⁴. Some variations also exist in genes encoding other nuclear antigens EBNA3A, 3B and 3C as well as the small non-coding RNAs EBERs 1 and 2⁴⁵, ⁴⁶. The two strains differ in geographic distribution, with type 1 EBV being prevalent across most populations and type 2 being nearly equally distributed with type 1 in equatorial Africa⁴⁷. Phenotypically, type 2 transforms B cells less efficiently. This is thought to relate to the divergence in EBNA2 sequence, which functionally replaces important cell fate-determining Notch signaling by directly binding RPB-Jκ to mediate transcriptional activation of pro-growth genes⁴⁸-⁵⁰. This last point underscores the important role of viral gene expression in the development of cancer.

<table>
<thead>
<tr>
<th>Latency program</th>
<th>Transcriptional Pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td>III (Growth program)</td>
<td>EBNA1, EBNA2, EBNA3A, EBNA3B, EBNA3C, EBNA-LP, LMP1, LMP2, and EBERs</td>
</tr>
<tr>
<td>II (Default program)</td>
<td>EBNA1, LMP1, LMP2, and EBERs</td>
</tr>
<tr>
<td>I (Episomal segregation)</td>
<td>EBNA1 and EBERs</td>
</tr>
<tr>
<td>0 (True latency)</td>
<td>EBERs</td>
</tr>
</tbody>
</table>

Table 1.2: EBV latency programs
1.4 Immune responses to EBV infection

Thus far we have alluded to the essential role of viral immune escape in the development of diseases. Understanding immune control is therefore essential, since disruption initiates these conditions. To highlight this point, it is clear that progression of primary EBV infection, and in particular, a history of IM, correlates with the development of autoimmune diseases such as systemic lupus erythematosus (SLE) and MS as well as various lymphomas. This suggests the inflammatory environment shaped by EBV infection contributes to immune dysregulation and disease later in life. However, as will be discussed in more detail later, virus-specific immune responses can be exploited to treat EBV-driven lymphomas. It is therefore important to understand the EBV immune response to fully appreciate potential targets for EBV-specific immunotherapies.

While others have described the immunology of EBV infection in great detail (see Rickinson et al. and Hislop et al.), here we briefly describe the key components of EBV immunity.

1.4.1 Innate immunity to EBV

The innate immune system, in contrast to the adaptive (antigen-specific) immune system, consists of cells capable of directly responding to pathogenic stimulus though germline encoded receptors that recognize molecular patterns. These cells are typically the first line of defense and direct the subsequent adaptive immune response.

Natural killer (NK) cells are large granular lymphocytes that have been shown to be important in the control of EBV-infected cells. This importance is demonstrated by
the increased frequency of NK cells in peripheral blood during primary infection, which is inversely correlated to EBV viral load\textsuperscript{57, 58}. Furthermore, studies in humanized mouse models of infection have shown NK cells rapidly expand in response to infection and NK depletion results in loss of viral control recapitulating IM symptoms\textsuperscript{59}. Of the NK cell subtypes, it appears less mature CD56\textsuperscript{bright}/CD16- cells, characterized by high secretion of the antiviral cytokine interferon gamma (IFNγ), are activated in the tonsils of EBV infected individuals and are capable of preventing B cell transformation \textit{in vitro}\textsuperscript{60, 61}. Furthermore, lytic programming, as observed in acute infection, appears to render the infected cells more susceptible to NK-mediated cytolysis, most likely as a result of down-regulated HLA I (resulting in “missing self”)\textsuperscript{62, 63}. As expected, the immunological pressure exerted by NK cells has resulted in the evolution of immunoevasive strategies. Recently, Griffin et al. described a mechanism in which lytic protein BILF1 targets HLA-A, B and E, but not HLA-C, the ligand for certain NK inhibitory receptors (KIR), thus mitigating both NK and T cell responses\textsuperscript{64}. This growing evidence that NK cells play a crucial role in the immune response against EBV necessitates further exploration into potential NK cell immunotherapies for treatment of EBV-driven diseases\textsuperscript{65}.

The myelomonocytic lineage is comprised of various cell populations with distinct functions that can potentiate or inhibit adaptive immune responses\textsuperscript{66}. This lineage is derived from a common myeloid progenitor and consists of immature, circulating monocytes and two groups of terminally differentiated, tissue resident cell types, macrophages and DCs\textsuperscript{67}. DCs are cells that specialize in antigen processing and initiate cell-mediated, adaptive immune responses by presenting antigen, providing co-stimulatory signals and releasing immune-mediating cytokines\textsuperscript{68}. Macrophages are
closely related to DCs and are characterized by their ability to engulf pathogens or cell debris and secrete a wide variety of immunomodulatory cytokines\textsuperscript{69}. However, macrophage populations exhibit a broad range of functions which are reflective of their tissue microenvironment and range from proinflammatory subsets (M1) to subsets that promote wound healing (M2)\textsuperscript{70}. In immune-competent hosts, elicitation of the anti-viral adaptive immune response is mediated by activation of pattern recognition receptors (PRRs), particularly toll-like receptors (TLRs) and Rig-I-like receptors (RLRs) in myeloid cells. TLR3 and TLR9 are the most well described of these and have been implicated in the sensing of EBV RNA and unmethylated DNA, respectively\textsuperscript{71}. Monocytes are capable of sensing EBV virions directly through TLR2, resulting secretion of cytokines and chemokines\textsuperscript{72}. Lastly, EBV-encoded small RNAs (EBERs) are recognized by both RLRs and TLR3, leading to an antiviral type I IFN response\textsuperscript{73, 74}. This is of interest since high concentrations of EBERs were found in the sera of patients with hemophagocytic lymphohistiocytosis (HLH), indicating a possible role of aberrant EBV-related inflammation in disease states\textsuperscript{75}. Furthermore, it is hypothesized that chronic inflammation associated with EBV infection polarizes macrophages to an immunosuppressive phenotype that may play a role the oncogenic process\textsuperscript{76}.

EBV also plays a significant role in escaping immune detection by the myeloid compartment, which in turn blunts activation of adaptive immune responses. For instance, both the latent protein LMP1 and the lytic protein BGLF5 down-regulate TLR9-mediated signaling, and consequently, the release of antiviral cytokines\textsuperscript{77, 78}. The virus also encodes a lytic protein, BARF1 (a functional homolog of CSF-1R) which binds CSF-1, thereby hampering myeloid cell differentiation\textsuperscript{79}. Considering the immune modulatory
effects of EBV outlined here as well as the role of chronic inflammation in cancer promotion and progression, it is not surprising EBV infection predisposes individuals to disease\textsuperscript{80}.

1.4.2 Adaptive immunity to EBV

Lymphocytes of the adaptive immune system have receptors that confer specificity to particular epitopes, and are characterized by their ability to differentiate into cells with immunological “memory”. This process involves learning and recall, which result in a high level of antigen specificity that takes time to develop. It is important to note that development of an adaptive immune response is dependent upon, and defined by, innate immune functions\textsuperscript{81, 82}. There are two types of adaptive immune responses: humoral immunity, mediated by antibody-producing B lymphocytes, and cellular immunity, mediated by T lymphocytes.

During acute EBV infection, B cells produce a wide range of antibodies specific to lytic cycle antigens, and in particular to structural proteins such as the viral capsid antigen (VCA)\textsuperscript{83}. Recently it was reported that serum levels of neutralizing antibodies to gp350 and gp42, which have been shown to block infection of B cells, increase very early during infection\textsuperscript{84}. Antibody levels to EBNA2 (latency III) appear soon after viral titers peak and subsequently decline, while the anti-EBNA1 (latency I, II and III) humoral response is delayed months before stabilizing in infected hosts\textsuperscript{85}. Profiling serum of healthy EBV carriers shows continued high levels of immunoglobulin G (IgG) against VCA and EBNA1, which most likely contributes to extended viral control\textsuperscript{86}. While these
studies provide insight into the kinetics of neutralizing antibody responses, deeper assessment would be valuable in the development of candidate EBV vaccines.

T lymphocytes can be subdivided into cytokine-producing helper T cells (CD4+ T cells) and cytotoxic T lymphocytes (CTLs, or CD8+ T cells) which kill infected cells to eliminate the source of pathogen54. In contrast to B cells, T cells do not directly interact with pathogens, but rather with an antigen-presenting cell (APC), such as a DC, to mount a response. Presentation by APCs is facilitated though major HLA complexes, with HLA type I interacting with the T cell receptor (TCR) of CD8+ T cells and type II interacting with the CD4+ TCR87. This interaction is complicated by the fact that HLA molecules are highly diverse and will express different antigens with different affinities, leading to diverse responses to the same antigen88. Therefore, the efficiency by which an HLA molecule binds a peptide may confer a protective advantage against viral infection. Thus, HLA type will determine the dominance of EBV proteins in eliciting an immune response and consequently, plays a role in disease protection89. While the rest of this section will focus on the general responses to EBV, it is important to note these inherent variances play a role in immunity to EBV. This particularly is salient when considering vaccine development or immunotherapies that could be exploited by the virus escaping cellular responses90, 91.

The CTL response to EBV infection has been extensively studied92. Primary infection is associated with an early expansion of EBV-specific T cells in circulation14. Remarkably, the responses to individual lytic epitopes can exceed 50% of the total CTL population11. In this early phase lytic antigens are dominant over latent antigen specificity, which follows the temporal course of viral protein exposure to the immune system14. As acute
infection gives rise to latency, dominance of circulating T cells again follow the temporal viral expression pattern. As primary infection wanes, responses become strongest for the latent EBNA3 family proteins, which accounts for more than 60% of the coding capacity of latent viral genes. However, as mentioned previously, particular HLA alleles will induce strong response against epitopes from usually sub-dominant antigens. Lytic antigens also display a similar hierarchy of dominance across HLA types with the strongest response directed temporally against the immediate early (IE) proteins BZLF1 and BRLF1, and declining in recognition frequency to the late, structural protein antigens. In certain individuals, the latently infected B cell pool is quickly controlled, while the lytically infected cells remain high for months, suggesting that these variances in epitope immunogenicity may result in inefficient recruitment of lytic epitope-specific CTLs in particular HLA types. With the decline in circulating viral loads, the CTL compartment eventually displays an associated contraction back to its pre-infection size. This is also connected with the establishment of an EBV-specific memory pool, that even in long-term carriers can constitute up to 2% of CTL memory for certain lytic epitopes and nearly 0.5% for latent epitopes. While on the surface these numbers may seem low, when one takes into account that this amount of immunological memory is dedicated to a single pathogen out of the entire pool of pathogens one will be exposed throughout a lifetime, it is truly remarkable. It is clear that EBV-specific CTLs play an important role in actively restraining virus-infected B cells; however, it does not appear that EBV mediates an immune-evasion mechanism to escape this pressure other than indirectly by modulating the myeloid compartment (mainly DCs, as discussed earlier). In fact, it may not be in the evolutionary interest of EBV to develop such a strategy, which would result in the uninhibited growth of virus transformed B cells and result in the
death of the host\textsuperscript{97}. This is precisely what happens in patients exhibiting severe immunodeficiencies who develop B cell lymphoproliferative disorders, which are universally fatal if left untreated\textsuperscript{98}. This point strongly illustrates the critical role latent EBV antigen-specific CTLs play in control of disease.

The CD4+ helper T cell response to EBV appears to display a broader range of specificity across lytic and latent cycle proteins; including proteins that rarely, if ever elicit a CTL response such as envelope glycoproteins gp350 and gp110\textsuperscript{99}. It therefore appears that if a hierarchy of immunodominance exists within the CD4+ T cell compartment, it is not as intensely varied as in CTLs. Further contrasting with CTLs, there are minimal changes in total numbers of circulating T helper cells during acute infection, although small pools of EBV-specific CD4+ T cells can be detected\textsuperscript{100}. After resolution of primary infection, populations of EBV-specific CD4+ T cells contract, representing up to 0.1% of the total memory pool, 10 to 20-fold less than seen with CTL memory\textsuperscript{100}. CD4+ T helper cells can be most simply classified as Th1 or Th2, which are involved in immunity to intracellular and extracellular pathogens, respectively\textsuperscript{101}. It is therefore unsurprising that functional analysis of EBV-specific helper T cells shows they largely express a Th1 phenotype, producing the antiviral cytokine IFN\textgamma\textsuperscript{102}. Unlike CTLs, there is evidence that EBV employs active CD4+ T cell evasion strategies. Lytically infected cells shed a soluble form of the envelope protein gp42, which binds HLA class II, protecting the infected cell from detection by CD4+ T cells\textsuperscript{103}. Infected cells also secrete other proteins in lytic cycle, BCRF1 and BNLF2a, which are viral homologs of the anti-inflammatory cytokine IL-10\textsuperscript{104}. These proteins play a role in immune tolerance by diverting T cells to a Th2 or T regulatory cell phenotype, thus blunting the anti-viral
response. Importantly, while CTLs play a distinct protective role against EBV, the role of T helpers is less clear. In animal models of EBV-driven lymphoma, SCID mice engrafted with PBMCs depleted of CD4+ T cells fail to develop tumors, providing clear evidence for a role in of T helper cells in cancer development. It is clear the immune system plays both a protective role against EBV disease and has a hand in development of cancer. These contrasting roles are defined by complex interactions between the virus, host, environmental factors and co-factors. The next section will illustrate how these factors contribute to the development of EBV-driven cancers.

1.5 EBV-driven cancers

1.5.1 Historical perspective

In the late 1950s, Denis Burkitt, a British surgeon working in equatorial Africa, observed an increased frequency in childhood lymphoma in regions where malaria was endemic. This observation lead Dr. Burkitt to suggest that malaria or another infectious agent was responsible for the development of cancer. Several years later, Michael Epstein, Yvonne Barr and Bert Achong, working with cell cultures derived from the eponymous Burkitt's lymphoma, discovered herpes virus-like particles throughout the samples. Immunofluorescence studies demonstrated the uniqueness of the virus and it was subsequently named Epstein-Barr virus. The discovery of tumors associated with EBV created a new paradigm of understanding that human cancer could be caused by virus. This was the first of a wide range of cancers discovered to be associated with EBV.
1.5.2 Viral oncogenesis

In total, between 15 and 20% of all human cancers are caused by viruses. These viruses, termed oncoviruses, include EBV, hepatitis B and C viruses (HBV and HCV respectively), human papillomavirus (HPV), human T cell lymphotropic virus-1 (HTLV-1) and Kaposi’s sarcoma herpesvirus (KSHV), of which, EBV is the most potent\textsuperscript{111-113}. It should be noted that EBV is necessary but not sufficient for development of its associated cancers, reflecting the multistep nature of oncogenesis\textsuperscript{114}. As discussed earlier, when EBV first infects B cells it deploys a growth program (latency III) that is highly immunogenic and quickly controlled by CTLs. It is therefore unsurprising that the only setting where cancers with EBV latency III programming develop are in individuals that are immunosuppressed\textsuperscript{38}. In this scenario, T cell immunosuppression is sufficient to facilitate transformation and proliferation of B cells. However, immunocompetent hosts require additional oncogenic events such as a somatic mutation or second viral infection which can result in cancers displaying latency I or II transcriptional patterns (Table 1.3)\textsuperscript{111, 115}. However, in all these scenarios, some viral proteins are expressed that contribute to the oncogenic process to varying degrees. Here we will examine the role of EBV in the oncogenic process, including immune evasion, focusing on B cell cancers exemplifying these viral programs with particular emphasis on lymphomas in immunosuppressed individuals as modeled in later chapters.
1.5.3 Latency I cancers

As mentioned previously, endemic Burkitt's lymphoma (eBL) was first shown to be associated with EBV, which is present in all cases of this form. The disease characteristically forms tumors on the facial skeleton of children in areas of Africa and New Guinea where malaria is endemic\textsuperscript{116}. Another form of Burkitt's lymphoma is prevalent in individuals infected with human immunodeficiency virus (HIV), of which 30-40% are associated with EBV. Of note, the incidence of BL overlaps with both HIV and malaria infections\textsuperscript{117}. In this regard, these pathogens are thought to be cofactors of lymphomagenesis through chronic stimulation of the immune system\textsuperscript{118}. Furthermore, all

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Subtype</th>
<th>% EBV positive</th>
<th>EBV proteins expressed</th>
<th>Latency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymphoproliferative disease</td>
<td>Posttransplant, AIDS-related</td>
<td>100%</td>
<td>EBNA1, EBNA2, EBNA3A, EBNA3B, EBNA3C, EBNA-LP, LMP1, and LMP2</td>
<td>III</td>
</tr>
<tr>
<td>Hodgkin's lymphoma</td>
<td>Classical</td>
<td>30%</td>
<td>EBNA1, LMP1, and LMP2</td>
<td>II</td>
</tr>
<tr>
<td></td>
<td>AIDS-related</td>
<td>100%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diffuse large B cell lymphoma</td>
<td>Late posttransplant DLBCL</td>
<td>&gt;50%</td>
<td>EBNA1, LMP1, and LMP2</td>
<td>VIII</td>
</tr>
<tr>
<td></td>
<td>Elderly DLBCL</td>
<td>&gt;50%</td>
<td></td>
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<tr>
<td></td>
<td>AIDS-related</td>
<td>~50%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Burkitt's lymphoma</td>
<td>Endemic</td>
<td>100%</td>
<td>EBNA1</td>
<td>I</td>
</tr>
<tr>
<td></td>
<td>AIDS-related</td>
<td>30–40%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>T/NK cell lymphoma</td>
<td>Extralodal</td>
<td>100%</td>
<td>EBNA1 and LMP2B</td>
<td>III</td>
</tr>
<tr>
<td>Nasopharyngeal carcinoma</td>
<td>Undifferentiated</td>
<td>100%</td>
<td>EBNA1, LMP1, and LMP2</td>
<td>VIII</td>
</tr>
<tr>
<td>Gastric carcinoma</td>
<td></td>
<td>5–15%</td>
<td>EBNA1 and LMP2</td>
<td>III</td>
</tr>
<tr>
<td>Smooth muscle sarcoma</td>
<td>Of the immunosuppressed</td>
<td>100%</td>
<td>Unknown</td>
<td>Unknown</td>
</tr>
</tbody>
</table>

Table 1.3: EBV malignancies and their latency programs. Adapted from Taylor et al.\textsuperscript{10}
17

Burkitt’s lymphomas involve one of three characteristic chromosomal translocations that place the MYC proto-oncogene under the control of the B cell specific immunoglobulin heavy or light chain promoter\textsuperscript{119}. The necessity of this translocation in the pathogenesis of BL has been firmly established experimentally\textsuperscript{120, 121}. Therefore, the contribution of EBV to the oncogenic process is somewhat confounded. At presentation, most EBV+ BL show a latency I program of viral antigen expression limited to EBNA1\textsuperscript{122}. EBNA1 is necessary for replication of the viral episome; however, it is also plays a role in lymphoma persistence by directly up-regulating a protein inhibitor of apoptosis called survivin\textsuperscript{123}.

While latency I transcriptional programming is limited to a single viral protein, there is abundant expression of EBV-encoded small RNAs (EBERs). Moreover, EBERs are the most abundant viral transcripts in all latently infected lymphomas and play a role in maintenance of malignant phenotypes of BL\textsuperscript{124, 125}. This may by partly attributed to the immunomodulatory properties of EBERs such as the induction of the anti-inflammatory cytokine IL-10 and promotion of tumor progression though cancer related inflammation via chronic PRR stimulation\textsuperscript{126, 127}.

1.5.4 Latency II cancers

Hodgkin’s lymphoma (HL) is a B cell lymphoma exemplifying a latency II EBV gene program. This disease in unusual in that malignant Hodgkin/Reed-Sternberg (HRS) cells are vastly outnumbered by a non-malignant, inflammatory infiltrate\textsuperscript{128}. Unlike Burkitt’s lymphoma, HL is not associated with a particular chromosomal or genetic aberration or
viral cofactor. HL is associated with EBV in approximately 40% of cases, with mixed cellularity (MC) and the rarer lymphocyte depleted (LD) subtypes (which are defined by the reactive infiltrate) exhibiting the highest correlation. The existence of a large EBV-negative cancer population has raised questions whether EBV infection is coincidental. However, there is compelling evidence that EBV contributes not only directly to the oncogenic process, but also to the inflammatory process driving the diseases. First, epidemiological studies have demonstrated that a history of infectious mononucleosis correlates with an increased risk of developing HL later in life and elevated levels of EBV antibodies are detected preceding onset of disease. Secondly, there is an increased incidence of HL in populations that are immunosuppressed and HL in these populations are almost universally EBV-associated. Lastly, if EBV is detected within the malignant HRS cells, all of the cells exhibit monoclonal EBV DNA, suggesting the infected population arose from a single cell. These malignant cells exhibit a latency II gene pattern which is characterized by the expression of LMP1 and LMP2A in addition to the latency I genes. LMP1 is oncogenic by itself in vivo and mimics CD40, replacing the stimulus typically provided by cognate T cells and thereby constitutively activating NFκB, a key transcription factor in lymphomagenesis. Interestingly, the HRS cells of EBV- HL show a remarkably similar deregulation of NFκB, indicating this is an important feature of disease pathogenesis that can be driven by EBV. LMP2A mimics signaling from the B cell receptor, which is typically associated with antigen recognition and activates the PI3K-AKT pathway to promote survival. While LMP1 and LMP2 are considered sub-dominant they are still typically recognized by the immune system. It is important then to understand how
these cells are evading immune detection in order to develop potential immunotherapeutic strategies.

It is thought that EBV-related chronic inflammation may play a role in aberrant activation of macrophages, polarizing them into an immunosuppressive phenotype, which is a negative prognostic indicator of disease\(^{138}\). Many of these macrophages within the tumor infiltrate exhibit EBV lytic infection, which may result in further inflammation and tumor progression\(^{139}\). Lastly, several groups have reported an association with certain HLA alleles to the development of HL, which may be linked to the CTL response during acute infection\(^{140-142}\). Together, these studies demonstrate the critical role of both innate and adaptive immunity in the oncogenic process and provide reasonable insight into immune evasion strategies of malignant HRS cells.

1.5.5 Latency III cancers

Latency III cancers express the full set of oncogenic gene products EBNA1, LMP1, LMP2A, EBNA2, EBNA3A, EBNA3B, EBNA3C and EBNALP as well as the EBV microRNAs\(^{143}\). Typically cells expressing this gene pattern elicit a robust immune memory response, particularly to the EBNA3 family proteins, and are eliminated by CTLs\(^{144}\). Therefore, diseases associated with latency III are only seen in immunosuppressed individuals, represented by AIDS-associated lymphomas and post-transplantation lymphoproliferative disease (PTLD), which arises in patients as a result of immunosuppression following transplantation\(^{145}\).
Among the nine EBV latent proteins, ENBA2, EBNA3A, EBNA3C and LMP1 have been shown to be essential for transformation of B cells in vitro\textsuperscript{46}. As discussed earlier, EBNA2 is a viral protein that mimics the activated form of Notch by associating with RBP-J, which is known to drive non-viral malignancies\textsuperscript{146}. EBNA2 has also been implicated in the deregulation of MYC expression resulting in up-regulation of genes associated with increased proliferation such as D and E cyclins\textsuperscript{147}. It appears that EBNA3B is dispensable for B cell transformation, but loss of EBNA3A or EBNA3C renders the virus incapable of transformation\textsuperscript{14}. It has been experimentally shown that these isoforms associate with ubiquitin complexes that target the tumor suppressor retinoblastoma (Rb) for proteosomal degradation\textsuperscript{148,149}.

While the most important factor for the development of these latency III malignancies is T cell suppression, not everyone who is immunocompromised will develop these diseases, indicating additional events are necessary for transformation. One possibility is host genetic variability leading to differential HLA immunodominance as discussed previously. Alternatively, polymorphisms in the virus itself can determine the strength of immune detection potentially indicating differences in pathogenicity based on viral strain\textsuperscript{150}. Lastly, our group and others have shown polymorphisms in host cytokine genes correlate to development of PTLD\textsuperscript{151}.

\textbf{1.5.6 Role of lytic cycle genes in cancer}

The majority of infected B cells remain in latency, avoiding detection by the large population of lytic antigen-specific memory T cells\textsuperscript{93}. However, spontaneous reactivation
from latency to the lytic cycle frequently occurs and evidence is mounting that these proteins contribute to the oncogenicity of the virus. It was noted earlier that lytic viral proteins include viral homologs of IL-10 (BCRF1 and BNLF2a) and the anti-apoptotic gene BCL2 (BALK and BPAR, respectively). However, because of the greater numbers of lytic proteins and their typically low expression levels in cells, the contribution of most of these proteins remains unknown. Recently Arvey et al. characterized the transcriptome and epigenome of human LCL lines from over 700 publically available high-throughput sequencing data sets and found that viral lytic genes are co-expressed with many host cancer-associated pathways. The most conclusive role of a lytic involvement in development of lymphoma comes from studies with the immediate-early gene BZLF1 which acts as a switch from latent to lytic infection. Ma et al., working with BZLF1 knockout (lytically defective) or BZLF1 over expressing virus strains showed that BZLF1 increases the incidence of lymphoma in the context of a self-educated human immune system, although the exact mechanism was not revealed. Furthermore, other studies have suggested that polymorphisms in the BZLF1 promoter facilitate different clinical behavior of the virus. The increasing evidence that lytic genes play a significant role in lymphomagenesis offers another potential for EBV-specific targeted therapies.

It is clear the development of EBV+ lymphoma is driven by a complex set of interactions between the virus and the host. In the diseases described above, EBV is necessary but not sufficient for cancer development. EBV plays a role in mimicking host processes that drive cell growth and avoid immune detection; however, the presence of the virus also
offers a set of targetable proteins that are not expressed in healthy cells. Emerging therapies take advantage of this point, and will be reviewed in the next section.

1.6 Emerging treatments and unmet needs

Given the significant clinical problem of EBV-associated cancers, it is important to use what we know about the interplay between the host immune system and the virus to design novel therapies to specifically target these diseases that represent a cancer burden of 200,000 new cases a year (Table 1.4). This section will explore emerging therapies that directly target EBV. While others have significantly reviewed these topics (Merlo et al., Yong et al., and Cohen), here we will provide a brief overview of therapies that directly target these disease. PTLD, with high correlation with EBV and full expression of viral proteins, is a useful illustration for therapeutic targeting and therefore will be used as a disease model here and in chapters to follow.

<table>
<thead>
<tr>
<th>Estimated new cases of EBV-associated cancers worldwide/year</th>
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<tbody>
<tr>
<td><strong>Cancer</strong></td>
</tr>
<tr>
<td>Burkitt lymphoma</td>
</tr>
<tr>
<td>Developed Countries</td>
</tr>
<tr>
<td>Less-developed countries</td>
</tr>
<tr>
<td>Gastric carcinoma</td>
</tr>
<tr>
<td>Hodgkin lymphoma</td>
</tr>
<tr>
<td>Nasopharyngeal carcinoma</td>
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<td></td>
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</table>

Table 1.4: EBV cancer burden. Adapted from Cohen et al.
1.6.1 Non-specific therapies

PTLD is a heterogeneous group of lymphoproliferative disorders classified into 4 subtypes, all of which are associated with EBV\textsuperscript{161}. The typical treatment options for these diseases are reduction of immunosuppression, CD20 monoclonal antibody therapy and chemotherapy\textsuperscript{162}. Since EBV-PTLD is related to the immunosuppression necessary after transplantation, a reduction in these regimens can reestablish EBV-specific CTL surveillance and effectively resolve the disease. However, individuals may be at risk of rejecting the transplantation\textsuperscript{163}. In fact, studies have estimated to be upwards of 40% of patients with tapered immunosuppression will lose the graft\textsuperscript{164, 165}. Rituximab is a humanized monoclonal antibody targeted against CD20, which is expressed on the surface of mature B cells. While this treatment can be effective for CD20+ PTLD, the antigen is not just confined to malignant cells and rituximab therefore also eliminates normal B cells. This can be harmful to patients who are already immunosuppressed since it may lead to fatal viral reactivations\textsuperscript{166}. Lastly, chemotherapy regimens used for lymphoma, such as CHOP (cyclophosphamide, doxorubicin, vincristine, and prednisone) can be used in combination with rituximab therapy or as an option for patients who fail to respond to other therapies. However, these treatment regimens also have profound effects on the immune system, increasing the risk of infection and result in further loss of immune control over EBV\textsuperscript{161}.

1.6.2 Antiviral treatment

Another potential approach is using existing therapies that specifically target viral proteins. This may be accomplished by targeting the EBV-specific enzymes thymidine
kinase (TK) and BGL4 with nucleoside analogs. These EBV lytic proteins phosphorylate the prodrugs ganciclovir (GCV) and acyclovir (AZT), converting them into cytotoxic products only within infected cells\textsuperscript{167}. These antiviral agents play a role in prophylaxis by blocking EBV production in donor B cells from subsequently infecting EBV negative recipients by preventing viral reactivation\textsuperscript{168, 169}. However, these are not generally effective as standalone treatments for EBV-positive tumors since most cells are expressing the latent form of EBV. Thus, it is necessary to first convert the latent form of EBV into the lytic form for this treatment to be successful\textsuperscript{170}. This can be accomplished by treating with certain chemotherapeutic agents\textsuperscript{171}

1.6.3 Adoptive cell therapy

Adoptive cell therapy to restore EBV-specific immunity is an attractive option to target the cause of these diseases. Initially, clinical trials used unselected donor lymphocytes from healthy EBV+ donors. While this strategy yielded strong results, it carries the significant risk of donor lymphocytes targeting the recipient as graft versus host disease (GvHD)\textsuperscript{172}. Because of this risk, it is now more common practice to generate and infuse donor-derived EBV-specific T cells, \textit{i.e.} donor lymphocyte infusions (DLI). While this treatment has been highly effective in patients, it takes a significant amount of time to generate patient-derived LCLs and expand EBV-specific CTLs\textsuperscript{173}. In order to generate EBV-specific CTLs more rapidly, several groups have developed protocols in which donor PBMCs are stimulated with peptides derived from EBV antigens followed by selection of peptide-specific IFNγ-secreting CTLs and expansion\textsuperscript{174, 175}. Even though these protocols have significant advantages, there are still problems that need to be
addressed. First, targeting a single peptide or small peptide repertoire can increase the likelihood of immune escape\textsuperscript{176}. Furthermore, additional studies are necessary to understand the phenotype and functional characteristics of T cells that lead to prolonged T cell persistence and expansion \textit{in vivo}\textsuperscript{177}. As with many immunotherapies, these strategies fail to address the underlying immune suppression associated with EBV infection and the tumor microenvironment which may limit their effectiveness\textsuperscript{178}. Lastly, even though this is an effective emerging therapy for EBV-PTLD, it is not generally available at most centers and requires significant upfront costs to develop\textsuperscript{179}.

\textbf{1.6.4 Emerging immunotherapies}

In order to establish long-term anti-EBV immunity, it is necessary to address the underlying mechanism of immune inhibition associated with these cancers. One method to accomplish this is though targeting immune checkpoint receptors which compromise several inhibitory pathways modulating the duration and intensity of immune responses\textsuperscript{180}. Two immune checkpoint receptors of particular interest are cytotoxic T-lymphocyte-associated protein 4 and (CTLA-4) and programmed cell-death protein 1 (PD-1)\textsuperscript{178}. While the two molecules have distinct mechanisms of action, ligation of either receptor results in termination of co-stimulation and therefore T cell activation\textsuperscript{181}. In particular, targeting the PD-1 axis is a potentially attractive immunotherapy as EBV infection induces its ligand (PD-L1) in Hodgkin lymphomas and PTLD\textsuperscript{182}. It is therefore unsurprising that PD-1 blockade has been shown to restore T cell functions in various EBV+ lymphoma models\textsuperscript{183, 184}.
As mentioned earlier, there are scenarios where the immune system actively plays a role in oncogenesis, rather than neutralizing disease\(^{185}\). Chronic EBV-related inflammation may play a role in aberrant activation of macrophages, polarizing them to an immunosuppressive phenotype. These macrophages are negative prognostic indicator in EBV-driven diseases\(^{138}\). For this reason, immunotherapies directed to myeloid cells in the tumor microenvironment have emerged as a strategy to remove the underlying immune suppression\(^{186}\). One such method is targeting CSF1, which is the major lineage regulator in most population of macrophages and acts as a major chemoattractant\(^{187}\). Elevated levels of CSF1 are detected in many tumor types and are associated with a poor prognosis. Several small molecular inhibitors of the CSF1 receptor (CSF1R) are in various stages of clinical trial\(^{178}\). A similar strategy in clinical trials is an inhibitor of the chemokine ligand CCL2 which is associated with macrophage infiltration and invasiveness of disease\(^{188}\). \(^{189}\). Lastly, treatment with agents inhibiting immunosuppressive metabolic enzymes of infiltrating cells has shown promise. One such enzyme is indoleamine 2, 3-dioxygenase (IDO) that initiates the rate-limiting step of tryptophan breakdown. Initially, IDO was thought to play an antimicrobial role by reducing the essential amino acid tryptophan\(^{190}\). However, since tryptophan is essential for T cell function, it has central role in facilitating immune escape\(^{191}\). IDO inhibitors are currently being assessed in clinical trials involving many different cancers\(^{178}\). It is likely that a combination of these immunotherapies tailored to the tumor microenvironment, in addition to adoptive cell therapy or vaccine strategies, will provide the greatest benefit to these diseases.
1.6.5 Vaccine strategies

Arguably the most effective therapy would be a prophylactic vaccine to limit viral load and protect against EBV-associated diseases\textsuperscript{192}. While vaccine against other oncoviruses (HPV and HBV) and herpes viruses (zoster) are in clinical use, the development of an effective EBV vaccine remains stubbornly elusive\textsuperscript{159}. To date, the majority of prophylactic vaccine efforts have focused on induction of neutralizing antibodies to lytic EBV glycoprotein gp350.\textsuperscript{193-195} While this is a sensible target, these vaccines have been insufficient at preventing infection. However, they have displayed minor efficacy in lowering incidence of IM.\textsuperscript{195} Similar trends were observed in a small phase I trial of a CD8$^+$ T cell-restricted peptide vaccine to EBV latency protein EBNA3.\textsuperscript{196} However, numbers of participants in both trials was insufficient to make any conclusions related to protection against viral reactivation or EBV-associated malignancies. It can be argued that developing a vaccine that completely inhibits EBV infection may be secondary to a vaccine that reduces EBV-associated diseases\textsuperscript{197}. Therefore, while not statistically significant, the trend of lowering IM in these studies is important in its own right since a history of this disease is associated with an increased risk of HL and MS\textsuperscript{11}. A vaccine including EBV antigens in combination with gp350 could be effective in preventing EBV infection, incidence of IM and future EBV-driven malignancies.\textsuperscript{198} However, while potential candidates have been proposed, no systematic studies have been performed to elucidate optimal targets. Furthermore, because humans are the only natural hosts of EBV, animal models for EBV research have proven difficult. Until recently, the majority of murine models used to study EBV have focused on mice
reconstituted with EBV-infected human immune cells to study development of lymphoma, not primary infection\textsuperscript{199}.

A different approach to an EBV vaccine involves the induction of T-cell immunity to EBV proteins\textsuperscript{196}. This approach requires a method of delivering EBV antigens for presentation on dendritic cells. DCs express endocytic receptors on their surface that internalize antigen for presentation on MHC class I and II to CTL and T helper cells, respectively\textsuperscript{200}. One such receptor is DEC-205, which is predominately restricted to DCs, but has also been shown to be expressed at similar levels on EBV-transformed B cells\textsuperscript{201}. Together, this makes DEC-205 an intriguing target for a cellular EBV vaccine. A humanized mouse model demonstrated that vaccination with an anti-DEC-205-EBNA1 fusion protein induced a protective T cell response\textsuperscript{202}. While more work is necessary, induction of EBV-specific T cell by this or other methods should be explored as a vaccination approach. Given the significant disease burden associated with EBV, it is of the upmost importance to develop new strategies to combat these diseases.

1.7 Summary

EBV is a ubiquitous γ-herpes virus that establishes a lifelong infection in B lymphocytes, typically contained by host immune controls. EBV has evolved replication strategies mimicking B cell proliferation and survival signals that have oncogenic potential. Primary infection elicits a strong immune response from both adaptive and innate immune components, forcing the virus into less immunogenic transcriptional patterns. Certain scenarios, such as immunosuppression, disrupt immune control and can lead to an array
of malignant diseases. Typically, these cancers are treated with therapies that not only target the cancer, but exhibit deleterious effects on immune components. Emerging strategies address the complex interplay between host immunity and EBV that drives disease. Therapies such as adoptive cell therapy seek to restore EBV-specific immunity to target the cause of disease. However, it is clear that the immune system plays both a protective role against EBV disease and in cancer development. Therefore, more work is needed to develop methods targeting the immunosuppressive nature of the tumor microenvironment. Ultimately, new therapies are needed that exploit cellular immune responses to target EBV-malignancies, particularly to enhance the development of a prophylactic EBV vaccine.
Chapter 2: Central goal and aims of the project

The central goal of this project is to identify therapeutic agents that target the tumor while preserving EBV-specific immunity. Using EBV lymphoproliferative disorders (LPD) as a model, the major aims of this thesis are as follows:

AIM 1: Identify mechanisms targeting EBV proteins while sparing host immunity

When EBV infects a cell, it hijacks the normal cell machinery to produce viral proteins that drive cell growth and survival signals. Dysregulation of these signals contributes to the emergence of lymphoma. Individuals with a normal immune system recognize these viral proteins via a coordinated interaction between cells that provide antigen presentation (tumor and dendritic cells) and the adaptive immune response including antigen specific helper (Th) and cytotoxic T cells (CTLs). We hypothesized that targeting these cancer-inducing viral proteins or promoting anti-viral adaptive immunity will result in the elimination of lymphoma and establishment of immunologic memory. With that considered, our group showed that a compound called silvestrol selectively targets B cell lymphomas while sparing normal immune cells by blocking the production of cancer-promoting proteins. Additional work by other groups demonstrated that a cell protein called eIF4A, which is necessary for the synthesis of many cancer-associated proteins, is the sole target of silvestrol. Early work with silvestrol in EBV-driven lymphoma cell lines showed a remarkable decrease in the main cancer-promoting oncogene of EBV
(LMP-1) indicating this drug could be useful in treating these lymphomas. The first aim will explore the effect of silvestrol on these oncogenic EBV proteins and evaluate the effect on immune function. To examine this more rigorously, we examine the anti-tumor immune response in an established mouse model of EBV lymphoma\textsuperscript{205}. This will include investigating the immune components necessary for the protective effects of silvestrol.

\textbf{AIM 2: Identify and target the mechanisms by which EBV-driven lymphomas evade immune recognition}

Cells of the immune system can have roles either in protection against cancer or in tumor promotion. As mentioned previously, CTLs can recognize virally infected and otherwise abnormal tumor cells and directly kill them. However, a hallmark of cancer is the acquisition of mechanisms that not only evade immune destruction, but actively suppress immune function\textsuperscript{115}. One of the ways tumor cells can do this is by recruiting another immune cell type called macrophages, which are able to suppress anti-tumor CTL activity and promote tumor growth. These tumor-associated macrophages (TAMs) are associated with a worse prognosis in several types of lymphoma \textsuperscript{206}. We hypothesize that targeting suppressive immune subsets will enhance the immune system’s anti-tumor response by preventing CTL suppression. We developed a model system in which normal immune cells are incubated with lymphoma cells to initiate an autologous immune reaction. Using this system we will explore the suppressive nature of the cells expanding in this model as well as investigate the potential effects of silvestrol, and therefore the role of eIF4a, in mitigating this tumor escape mechanism.
**Significance**

The lack of treatment options and poor outcome of patients diagnosed with these aggressive lymphomas creates an urgent need to find new therapeutic strategies. This project addresses several important aspects of EBV infection and biology that are highly relevant for cancer immunotherapy. These specific aims bring together novel methods to mobilize the immune system against EBV-driven lymphoma. We propose strategies to attack the lymphoma on multiple fronts with the overarching goal to deliver lasting therapeutic efficacy to individuals suffering from these diseases.
Chapter 3: Silvestrol exhibits direct anti-tumor activity while preserving innate and adaptive immunity against EBV-driven lymphoproliferative disease

3.1 Summary

Treatment options for patients with Epstein-Barr Virus-driven lymphoproliferative diseases (EBV-LPD) are limited. Chemo-immunotherapeutic approaches often lead to immune suppression, risk of lethal infection and EBV reactivation, thus it is essential to identify agents that can deliver direct anti-tumor activity while preserving innate and adaptive host immune-surveillance. Silvestrol possesses direct anti-tumor activity in multiple hematologic malignancies while causing minimal toxicity to normal mononuclear cells. However, the effects of silvestrol on immune function have not been described. We utilized in vitro and in vivo models of EBV-LPD to simultaneously examine the impact of silvestrol on both tumor and normal immune function. We show that silvestrol induces direct anti-tumor activity against EBV-transformed lymphoblastoid cell lines (LCL), with growth inhibition, decreased expression of the EBV oncogene latent membrane protein-1 and its downstream AKT, STAT1 and STAT3 signaling pathways. Silvestrol promoted potent indirect anti-tumor effects by preserving expansion of innate and EBV antigen-specific adaptive immune effector subsets capable of effective clearance of LCL tumor targets in autologous co-cultures. In an animal model of spontaneous EBV-LPD, silvestrol demonstrated significant therapeutic activity dependent on the presence of
CD8-positive T-cells. These findings establish a novel immune-sparing activity of silvestrol, justifying further exploration in patients with EBV-positive malignancies.
3.2 Introduction:

Epstein-Barr Virus (EBV) is an oncogenic B-lymphotropic virus associated with Burkitt’s lymphoma, non-Hodgkin’s and Hodgkin’s lymphomas, nasopharyngeal and gastric carcinomas, and post-transplant lymphoproliferative disease (LPD)\(^{207}\). Following primary infection, the virus establishes persistent, life-long latency in the B-cell compartment of the human host. This virus/host coexistence is controlled by a highly efficient antigen-specific adaptive immune response that protects immune-competent individuals from EBV-driven pathology. EBV-seropositive individuals who become immunocompromised are at risk for EBV reactivation and development of aggressive B-cell lymphomas. Current treatments for patients with EBV-driven lymphomas are of limited benefit and lead to further immune suppression, risk of opportunistic infections, and a loss of EBV-specific immunity due to dysregulation of immune surveillance\(^{208}\). Therefore, novel treatment approaches that target EBV-driven cancers while maintaining normal immune function are in great demand.

Silvestrol\(^{209}\) is a unique agent that possesses anti-tumor activity in multiple cancer models\(^{210-214}\). This capability is attributed to inhibition of translation initiation, which occurs when silvestrol induces aberrant dimerization of the RNA helicase eIF4A with capped mRNA. This effect interferes with normal recruitment of mRNA to the eIF4F initiation complex, thus preventing the rapid synthesis of pro-survival and pro-growth proteins leading to tumor cell death via caspase-dependent apoptosis\(^{215-218}\). Our group reported that silvestrol shows \textit{in vivo} activity in the B-cell malignancies chronic lymphocytic leukemia, acute lymphoblastic lymphoma\(^ {203}\) and mantle cell lymphoma\(^{219}\), and also that silvestrol appears to be selectively cytotoxic to malignant B-cells while
sparking normal lymphocytes\textsuperscript{203}. To date, however, the effects of silvestrol on normal immune function have not been evaluated.

Here we show that silvestrol promotes direct anti-tumor activity against EBV-LPD by blocking oncogenic pathways driven by the EBV gene product, latent membrane protein (LMP-1). Furthermore, we demonstrate that silvestrol preserves the anti-tumor function of innate immune effectors as well as antigen-specific adaptive immune effectors in both \textit{in vitro} and \textit{in vivo} models of EBV-LPD. This highly unusual activity suggests that silvestrol may provide an entirely new immune-potentiating therapeutic strategy for this histologic subset of aggressive lymphomas.
3.3 Methods and materials

3.3.1 Ethics statement

Investigations were conducted in accordance with the ethical standards and according to the Declaration of Helsinki and according to national and international guidelines, and were approved by the OSU institutional review board.

3.3.2 Reagents

Silvestrol was isolated as described\textsuperscript{209}. The active metabolite of fludarabine, 2-fluoro-ara-A, was obtained from Sigma (St. Louis, MO).

3.3.3 Cells and cultures

EBV-transformed lymphoblastoid cell lines (LCL) were derived \textit{in vivo} by engrafting severe combined immune-deficient (SCID) mice (Taconic, Hudson, NY) with human peripheral blood mononuclear cells (PBMC) from healthy EBV-positive donors\textsuperscript{220, 221} (Figure 3.1). Co-cultures (CoCx) were created in 96-well plates by mixing LCL (either non-irradiated or irradiated with 14,000 rad) with equal numbers of autologous PBMC. Cultures were grown in the presence of 10 U/ml interleukin-2 (IL-2; Prometheus, San Diego, CA), and were given a single dose of silvestrol (10 nM) before being cultured for 10-14 days.
3.3.4 Immunoblot analysis

LCL were subjected to immunoblotting as described. Antibodies were from Cell Signaling Technology (Danvers, MA) (β-actin, pSTAT1, pSTAT3, STAT1, STAT3, pAkt, or Akt) or DakoCytomation (Carpinteria, CA) (LMP-1).

3.3.5 Proliferation assays

MTS assays to measure mitochondrial function were performed using the CellTiter96 assay (Promega, Madison, WI).

3.3.6 Flow cytometric analyses

Cells were co-stained with LIVE/DEAD reagent (Invitrogen, Grand Island, NY) and hu-CD3-APC, as well as hu-CD4-PE, hu-CD8-PE, hu-CD19-PE (BD Biosciences, San Diego, CA), or hu-CD56-PE (Beckman Coulter, Brea, CA). Events were gathered by gating on cells negative for the LIVE/DEAD stain on an FC500 cytometer (Beckman-Coulter). Cell viability also was measured by flow cytometry using annexin-V-FITC and propidium iodide (PI) (BD Biosciences). Human HLA-B8 tetramers complexed with
immunodominant peptide from BZLF-1 (RAFKKQLL) and conjugated with allophycocyanin (APC) were provided by the NIAID Tetramer Facility and the NIH AIDS Research and Reference Reagent Program (Atlanta, Georgia). Where indicated, cell counting beads were used to obtain cell numbers according to manufacturer's description (BD Biosciences).

3.3.7 Cytotoxicity assays

Non-radioactive flow cytometry-based cytotoxicity assays were performed as described. CoCx (PBMC plus irradiated LCL) were incubated in the presence or absence of silvestrol (10 nM) for 14 days. Fresh autologous LCL cells were stained with carboxyfluorescein diacetate succinimidyl ester (CFSE; CellTrace, Invitrogen) prior to being mixed with effectors from the irradiated CoCx at an effector:target ratio of 20:1. Cells were stained with the viability dye 7-aminoactinomycin D (7-AAD) (BD Biosciences) and washed. Cytotoxicity was measured by gating on CFSE-positive events and measuring 7-AAD-positive cells. For antibody-dependent cell-mediated cytotoxicity (ADCC) assays, co-cultured effectors were natural killer (NK) cell-enriched and were incubated in the presence or absence of 5 µg/ml rituximab or the irrelevant control antibody herceptin (Genentech, South San Francisco, CA).

3.3.8 CFSE cellular proliferation assays

CFSE-stained PBMCs were incubated with unstained autologous LCLs, and CFSE-stained LCLs were incubated with autologous unstained PBMCs. At days 3 and 5, cells were stained with anti-CD19-APC for CFSE-LCL, or anti-CD56-APC or anti-CD8-APC for CFSE-PBMC, and analyzed by flow cytometry.
3.3.9 In vivo studies

The Hu-PBL-SCID model has been described\(^{220, \ 221, \ 225}\). PBMC were obtained from healthy EBV-seropositive donors under an Ohio State University Institutional Review Board-approved protocol. PBMC were injected intraperitoneally (IP) into SCID mice depleted of murine NK cells by pretreatment (plus weekly re-treatment) with anti-asialo (GM1) (Wako, Richmond, VA). Engraftment was confirmed by hu-IgG ELISA\(^{221}\). Treatments with vehicle (30% hydroxypropyl-β-cyclodextrin; CTD Holdings, Inc., Alachua, FL) or silvestrol (1.5 mg/kg every 48 hr IP) began 2 weeks post-engraftment.

3.3.10 Quantitative Reverse Transcription PCR

ICAM-1 (CD54) mRNA levels were determined using the Viia7 Real-Time PCR System (Applied Biosystems, Foster City, CA), using TaqMan Gene Expression Assays for ICAM-1 and TBP (control) (Applied Biosystems).

3.3.11 Statistics

To account for correlations among observations from the same donor, linear mixed effects models were used to estimate the effect of silvestrol on apoptosis and cell proliferation. Similarly, differences in rituximab-mediated NK cell ADCC activity in effectors expanded in the presence versus absence of silvestrol were examined using mixed effects models. Differences in cell numbers of each of the four subsets (CD3-/CD19+, CD3+/CD8+, CD3-/CD56+, and CD3+/CD4+) were compared between cells incubated with versus without silvestrol using paired t-tests. Differences in surface expression of immunological synapse proteins in activated effectors incubated with versus without silvestrol were also examined using paired t-tests, with an adjusted
significance level of 0.01 to control overall Type I error. The proportions of mice alive at
d end of study were compared between the vehicle control and silvestrol-treated groups
using Fisher’s exact test. Finally, spleen size differences between silvestrol-treated and
control mice were assessed using a two-sample t-test. All analyses were performed
3.4 Results

3.4.1 Silvestrol promotes direct anti-tumor activity against LCL

We first evaluated silvestrol’s direct anti-tumor activity in LCL derived from malignant EBV-LPD tumors that spontaneously developed in SCID mice engrafted with PBMC from EBV-seropositive donors (Figure 3.1)\textsuperscript{220, 221, 225}. Six different LCL were plated in the presence or absence of silvestrol, and cell viability (annexin/PI negativity; Figure 3.2A) and growth inhibition (MTS assay; Figure 3.2B) were evaluated at 24, 72, and 120 hr. Moderate but significant anti-tumor activity was noted both in growth inhibition and viability assays (p<0.001 and p=0.006, respectively, in silvestrol treated vs. vehicle control), with a 50% growth inhibitory concentration (IC\textsubscript{50}) of approximately 40 nM at 72 hr. Recent pharmacokinetic work in mice indicates that a 10 nM plasma concentration of silvestrol is attainable \textit{in vivo}\textsuperscript{226}. Therefore, 10 nM and lower doses were used in subsequent studies.
Figure 3.2 Silvestrol promotes direct anti-tumor activity in lymphoblastoid cell lines (LCL). *In vivo*-derived LCL (N=6) were incubated with indicated concentrations of silvestrol or vehicle for 24, 72, or 120 hr. (A) Viability was examined by annexin/propidium iodide (PI) flow cytometry. Data are shown as percent annexin-negative and PI-negative cells relative to the time-matched vehicle control group. Bars show mean ± standard deviation (SD). Differences with silvestrol treatments were significant (p<0.001). (B) Proliferation was measured by the MTS assay. Data are shown relative to the vehicle control at each time point. Bars show mean ± SD. Differences with silvestrol treatments were significant (p=0.006).
3.4.2 Silvestrol induces LMP-1 depletion in LCL

The virally-encoded transmembrane oncoprotein LMP-1 acts as a constitutively active receptor of the TNF-R family\(^{227}\), promotes multiple growth and survival pathways, suppresses immune-activating cytokines, and is essential for B-cell transformation\(^{228,229}\). These properties make it a potentially valuable therapeutic target for LMP-1-expressing Type II or III EBV-driven malignancies\(^{230-234}\). Therefore, we evaluated expression of LMP-1 protein, as well as its trans-activator EBNA-2, in eight LCL lines (including the six lines used in the viability and proliferation assays above) by immunoblot 72 hr after treating with silvestrol (Figure 3.3A). We observed a notable drop in LMP-1 expression across all LCL tested, and a corresponding decrease in EBNA-2 in six of the eight. As shown in a representative LCL (DC9; Figure 3.3B), LMP-1 levels fall incrementally as a function of time after a single 10 nM dose of silvestrol, even though the effect on EBNA-2 in this LCL was minor. Silvestrol had varying effects on the latent EBV gene products EBNA-3A and -3C, however, and did not induce the expression of the lytic transcription factor BZLF-1 (Figure 3.3B). Lysates from Akata cells (Type I latency) incubated with anti-IgG to induce lytic cycle and BL41-B95.8 cells (Type III latency) were included as controls\(^{235-237}\).
Figure 3.3: Silvestrol modulates EBV LMP-1 and LMP-1-driven signaling pathways in LCL. 
(A) LCL (N=8) were incubated with 0 or 10 nM silvestrol for 72 hr, and whole cell lysates were immunoblotted for LMP-1 and EBNA-2. β-actin was included as a loading control. (B) Lysates from LCL incubated 24, 72, and 120 hr with 0 or 10 nM silvestrol were evaluated by immunoblot. Akata cells (latency I) were treated with 7.5µg/ml anti-IgG for 24 hr to induce lytic cycle. BL41-B95.8 were incubated for 24 hr untreated. Results shown are representative of 3 different LCL. (C) LCL incubated as in (B) were immunoblotted for phosphorylated and total STAT1 and STAT3. Results were representative of 3 LCL. (D) LCL incubated as in (B) were immunoblotted for phosphorylated and total AKT. Results were representative of 3 LCL.
LMP-1 is known to constitutively activate multiple pro-survival signaling pathways including NF-κB, PI3K/AKT, STAT1 and STAT3 through its cytoplasmic C-terminal-activating regions (CTAR1 and 2), biologically mimicking the TNF family receptor CD40. Thus, LMP-1 promotes tumor cell survival and growth through diverse mechanisms. To investigate the effects of silvestrol on these LMP-1-induced pathways, LCL were incubated for 24, 72 and 120 hr with vehicle or 10 nM silvestrol and cell extracts were analyzed by immunoblot. While total STAT1 and STAT3 levels remained unchanged, the levels of their phosphorylated (activated) forms decreased (Figure 3.3C). Decreases of both total AKT and its activated, phosphorylated form (Figure 3.3D) were also observed. Unexpectedly, NF-κB p65 phosphorylation increased with silvestrol treatment, suggesting activation, although total p65 levels remained relatively unchanged (Figure 3.4A). Total levels of NF-κB components p50, p105 and IκBα were unchanged, as were NF-κB targets Bcl-2 and Bax, and despite the silvestrol-induced phosphorylation of p65, none of this phosphorylated p65 was evident in the nuclear fraction (Figure 3.4A, 3.4B, 3.4C). Together, these data suggest that changes in STAT and AKT, rather than NF-κB, may underlie the direct anti-tumor effects of silvestrol.
Figure 3.4: Silvestrol modulates LMP-1 and downstream targets. LCL were incubated 24, 72, and 120 hr in the presence of 10 nM silvestrol or vehicle. Whole cell lysates were immunoblotted for: (A) LMP-1, phosphorylated and total p65, p105/p50, and IKBα; (B) Bax and Bcl-2. β-actin was included as a loading control. Results are representative of four individual LCL. (C) Cytoplasmic and nuclear extracts were prepared from LCL incubated 24, 72, and 120 hr in the presence of 10 nM silvestrol or vehicle and immunoblotted for phosphorylated and total p65. α-tubulin and Brg1 were included as cytoplasmic and nuclear controls, respectively. Results are representative of four individual LCL. (D) Lysates from four LCL incubated 24 hr with vehicle or 10 nM silvestrol were immunoblotted for cyclin D3, CDK4 and β-actin. (D) A representative LCL was incubated 24 and 72 hr with vehicle or 10 nM silvestrol, and lysates were immunoblotted for phosphorylated and total Rb, cyclin D3 and CDK4. β-actin was included as a loading control.
We next examined the effects of silvestrol on known short half-life proteins, as we and others have reported in other cell types\textsuperscript{218, 219}. Cyclin D3 and CDK4 were each notably reduced by 24 hr in three out of four LCL tested (Figure 3.4D). It is unclear why this effect differed in one the four LCL (JD22), although this relative lack of protein effect also corresponded with reduced growth inhibition by silvestrol in this LCL (MTS assay; not shown). As an expected consequence of the silvestrol-mediated depletion of cyclin D3 and CDK4, phosphorylation of Rb was notably diminished (Figure 3.4E), consistent with our earlier findings\textsuperscript{219}.

### 3.4.3 Immune effector function is preserved in the presence of silvestrol in irradiated co-cultures

To explore the immune modulatory activity of silvestrol in EBV-LPD, co-cultures (CoCx) were created by lethally irradiating LCL and plating in the presence of autologous PBMC (1:1 ratio). Under these conditions, memory adaptive components of PBMC become activated and expand in response to the antigenic stimuli from the LCL\textsuperscript{220, 221, 225}. CoCx created using three LCL and their respective autologous PBMC were treated once with 0 (vehicle control), 2, 5, or 10 nM silvestrol and incubated for 14 days. Although the total cell numbers expanded in CoCx in the presence of 5 or 10 nM silvestrol appeared to be lower than in the vehicle-treated CoCx, these differences did not reach significance, and all CoCx conditions exhibited total cell numbers equal to or greater than unstimulated PBMCs alone (Figure 3.5A). As no irradiated LCL remain by day 14 (not shown), this result demonstrates that addition of silvestrol still allows for expansion of normal effector
populations following exposure to LCL. Immunophenotyping of the resulting populations showed no statistically significant differences in both CD8\(^+\) cytotoxic T-cells (CTL) and CD4\(^+\) helper T-cells with silvestrol treatment (Figure 3.5B, 3.5C; results shown relative to untreated CoCx). Similarly, no significant differences were seen with silvestrol treatment in the LCL-induced expansion of CD56\(^+\) NK cells, the majority of the expanded population (Figure 3.5D). These results indicate that silvestrol exposure under these conditions is permissive for viability and growth of innate and adaptive immune effector cells.
Figure 3.5: T cell and NK cell populations are maintained in the presence of silvestrol in irradiated LCL co-cultures. CoCxs were created by mixing irradiated LCL with equal numbers of autologous PBMC. CoCxs (or PBMC alone) were incubated in the presence of 10 U/ml IL-2, and given a single dose of 0 (vehicle only), 2, 5, or 10 nM silvestrol. Flow cytometric analysis was conducted on day 14. Live cells were gathered by gating on cells negative for the LIVE/DEAD stain. Data are expressed as percentage of viable population, relative to the vehicle CoCx condition, for: (A) total cells; (B) CD3+/CD8+ cells; (C) CD3+/CD4+ cells; (D) CD3-/CD56+ cells. Results shown are the averages from three individual CoCxs; bars show ± SD.
3.4.4 Silvestrol leads to depletion of LCL in non-irradiated co-cultures

We next examined how silvestrol impacts viable, non-irradiated LCL in the presence of immune effectors. LCL were incubated 1:1 with their respective autologous PBMC for 10 days after adding a single dose of silvestrol (0, 2, 5 or 10 nM). Figure 3.6A shows representative flow cytometry data for LCL+PBMC CoCx generated from one donor (data from additional donors are presented in Figure 3.7). LCL cells (CD3⁻/CD19⁺) appear in the bottom right quadrant of each plot; PBMC-derived effector populations (CD19⁻) appear in the left quadrants. In untreated (vehicle control) conditions, transformed LCL cells proliferated and matched the expansion of effector cell subsets, approximately maintaining the 1:1 ratio (Figure 3.6A upper right panel; all events gated on the viable population). However, with a one-time addition of silvestrol, a dose-dependent ablation of viable LCL was observed (Figure 3.6A, lower three panels and Figure 3.7). Results from three separate experiments are quantified in Figure 3.6B and demonstrate a significant loss of LCL in the presence of silvestrol (p=0.025 for 0 versus 10 nM). Percentages of CD4⁺ T-cells appeared to be reduced with silvestrol treatment as well (Figure 3.6C), although the differences were not statistically significant (p=0.537 for 0 versus 10 nM). In contrast, CD8⁺ T-cells (Figure 3.6D) and CD56⁺ NK cells (Figure 3.6E) expanded significantly in the presence of silvestrol (p=0.019 and p=0.032, respectively, for 0 versus 10 nM silvestrol). Importantly, this significant effect was not observed when LCL were incubated without PBMC in 5 or 10 nM silvestrol under otherwise identical conditions (Figure 3.6F). Although a trend toward lower cell numbers (enumerated using counting beads) was observed in the presence of silvestrol, this
effect did not reach significance (p=0.072 for 0 versus 10 nM), consistent with the modest growth inhibitory effects previously mentioned (Figure 3.2).
Figure 3.6 Silvestrol leads to depletion of non-irradiated LCL in co-cultures while permitting expansion of T and NK cells. CoCx (N=3) were created by mixing non-irradiated LCL with equal numbers of autologous peripheral blood mononuclear cells (PBMC). CoCx or PBMC alone were incubated in the presence of 10 U/ml IL-2 and given a single dose of 0 (vehicle only), 2, 5, or 10 nM silvestrol. Flow cytometric analysis was conducted on day 10. For all results, live events were gathered by gating on cells negative for the LIVE/DEAD stain. (A) Representative flow cytometry dot plots of mononuclear cells from CoCx. Cells were stained for CD3 (y-axis) and CD19 (x-axis) and gated on live events. LCL (CD3⁻/CD19⁺) are shown in the bottom right quadrant of each panel. (B-E) Data are expressed as percentage of total viable population expressing: (B) CD3⁻/CD19⁺ (LCL); (C) CD3⁺/CD4⁺ (helper T-cells); (D) CD3⁺/CD8⁺ (cytotoxic T cells); (E) CD3⁺/CD56⁺ (NK cells). All results are averages of three individual CoCx; ns = not significant. (F) Four different LCL were cultured using the same conditions as in the above co-cultures, but without the addition of PBMCs, and incubated with a single dose of silvestrol. Viable LCL were enumerated by flow cytometry, using cell counting beads and gating on cells negative for the LIVE/DEAD stain, and are shown relative to the untreated (vehicle) control. Differences with silvestrol treatment (0 versus 10 nM) were not significant (ns).
Similar cultures were set up and treated with a single dose of fludarabine (active metabolite 2-fluoro-ara-A), as this agent has been used clinically to treat LPD. However, unlike silvestrol, 2-fluoro-ara-A treatment resulted in outgrowth of LCL (Figure 3.8A, 3.8B). It also exhibited indiscriminate cytotoxicity toward the effector populations, leading to dramatically fewer effectors in the 2-fluoro-ara-A treated versus untreated conditions (Figure 3.8B). This is in stark contrast to the nearly 100% viability of effectors in the silvestrol treated CoCx.
Figure 3.8: Fludarabine does not control outgrowth of LCL. CoCx (N=3) were created by mixing non-irradiated LCL from with equal numbers of autologous PBMC. CoCx or PBMC alone were incubated in the presence of 10 U/ml IL-2 and given a single dose of 0, 2, 5 or 10 nM silvestrol, or 5 or 10 µM 2-fluoro-ara-A (active metabolite of fludarabine). Flow cytometric analysis was conducted on day 10 to assess viable cells (i.e. cells negative for the LIVE/DEAD stain). (A) Data are expressed as percentage of CD3⁺/CD19⁻ (LCL) in the total viable population. Bars show mean ± SD. (B) Dot plots showing viable LCL (CD19-positive cells negative for LIVE/DEAD stain (FL5)).
We next worked to identify which effector subsets mediate the anti-LCL effect in the presence of silvestrol. Cells expressing CD8 (CTL), CD14 (monocytes) or CD56 (NK cells) were depleted from PBMC using immunomagnetic beads; mock depletion with biotin-only beads was included as a control. Effector cell numbers equivalent to the total number in the mock-depleted condition were added 1:1 with autologous LCL and incubated with 0 or 10 nM silvestrol for 10 days. Depleted cultures in the absence of silvestrol showed varying outgrowth of LCL; however, silvestrol-treated CoCx all produced a similar loss of LCL. These results indicate that each of these immune cell subsets participates in the anti-tumor activity of the autologous effector population (Figure 3.9).
Figure 3.9: Depleted effector subsets still allow for ablation of LCL in silvestrol-treated CoCx. PBMC were subjected to immunomagnetic bead depletion (negative selection) for: CD8 (cytotoxic T cells), CD14 (monocytes) or CD56 (NK cells). Biotin-only conjugated beads were used for the control (mock-depleted). Efficiency of depletion was verified to be greater than 90% by flow cytometry. CoCx were created by mixing non-irradiated LCL from three individual EBV-positive donors with equal volumes of autologous PBMC from each depletion condition. CoCx were incubated in the presence of 10 U/ml of IL-2 and given a single dose of 0 (vehicle only) or 10 nM silvestrol. Flow cytometric analysis was conducted on day 10. Live LCL were assessed by gating on CD19+ cells negative for the LIVE/DEAD stain.
To further explore the differential effect of silvestrol on the proliferative capacity of tumor targets and effector populations, LCL or PBMC were stained with the membrane dye CFSE and equal numbers were plated with their unstained autologous counterparts (LCL-CFSE with PBMC, and LCL with PBMC-CFSE). Cultures were treated with 0 or 10 nM silvestrol for 3 or 5 days (Figure 3.10). LCL-CFSE cultures were then stained for CD19, and PBMC-CFSE cultures were stained for either CD8 or CD56. Gates were set on the subset of interest and analyzed for CFSE intensity (proliferating cells lose CFSE intensity with each cell division). At 3 and 5 days, LCL showed nearly a 50% decrease in proliferation with silvestrol treatment under these CoCx conditions, consistent with growth arrest (Figure 3.10A, 3.10B). Conversely, the proliferation rate of both adaptive (CD8+) and innate (CD56+) immune effectors remained unchanged in silvestrol-treated versus untreated CoCx (Figure 3.10C-3.10F), as indicated by little or no change in CFSE intensity. These results indicate that silvestrol differentially affects cell proliferation in tumor cells versus immune effector subsets.
Figure 3.10: Differential anti-proliferative effect of silvestrol on LCL versus immune effector subsets. PBMC or LCL were stained with CFSE. Equal numbers were plated with unstained autologous counterparts (LCL-CFSE with PBMC and LCL with PBMC-CFSE) and then treated with 0 or 10 nM silvestrol for 3 or 5 days. At each time point, CoCxs were stained with LIVE/DEAD cell stain and antibodies to CD19 (A, B) for LCL-CFSE CoCxs, or CD8 (C, D) or CD56 (E, F) for PBMC-CFSE CoCxs. Loss of CFSE mean fluorescence intensity (MFI) indicates an increase in proliferation. Results shown are representative of 3 individual experiments.
3.4.5 Silvestrol preserves cytotoxic function of adaptive and innate immune effectors

We next analyzed immune cell function as part of the indirect anti-tumor activity of silvestrol. Viable LCL cells were stained with CFSE, and then incubated at an effector-to-target ratio of 20:1 with autologous PBMC that had been expanded in the presence of irradiated LCL, with or without silvestrol, for 14 days. After a 4 hr incubation, cells were stained with the viability dye 7-AAD and washed. Direct cytotoxic activity of effectors against the CFSE-labeled LCL targets was measured by gating on CFSE-positive events and measuring 7-AAD-positive cells. As shown in Figure 3.11A, effector cells expanded in the presence of silvestrol largely maintained their cytotoxic activity against autologous LCL compared to effector cells expanded in the absence of silvestrol (N=3; differences between 0 and 10 nM silvestrol were not significant).

Given that LCL cells were depleted and effector cell cytotoxic function was maintained in non-irradiated CoCx, we hypothesized that silvestrol lowers the apoptotic threshold of LCL. To address this, PBMC were co-cultured with irradiated LCL for 14 days. These expanded immune effector cells (comprised of both CD8+ T-cells and CD56+ NK cells) and fresh, viable LCL were then incubated separately with silvestrol or vehicle control for 18 hr and washed. Cells were then combined, and after a 4 hr incubation, cytotoxicity assays were performed as described above. Silvestrol-treated effectors showed no decrease in their ability to kill LCL targets compared to untreated effectors (p=0.287; Figure 3.11B). However, LCL target cells pre-treated with silvestrol were more efficiently killed compared to untreated targets (p=0.002). These data indicate that silvestrol, even
at concentrations that show minimal direct cytotoxicity, significantly increases the sensitivity of tumor cells to effector cell-mediated killing.

Next, we utilized ADCC assays to measure the innate immune response of NK cells to LCL tumor targets in the presence of silvestrol. Effector cells were expanded in the presence of irradiated LCL, with or without 10 nM silvestrol, for 14 days prior to enriching for NK cells via negative selection. Fresh CFSE-labeled LCL targets were incubated with rituximab (anti-CD20, expressed on LCL targets) or the negative control antibody herceptin (anti-HER2) in the presence of NK cells. After 4 hr, ADCC was evaluated by gating on CFSE events (stained targets) and analyzing the percentage of 7-AAD positive cells. Rituximab-mediated ADCC of NK cells was similar between effectors expanded with versus without silvestrol (p=0.838; Figure 3.11C). To determine the acute effect of silvestrol on ADCC, fresh (non-autologous) NK cells were obtained from healthy donors, incubated 18 hr with or without 10 nM silvestrol, then washed and mixed with CFSE-stained LCL. As shown in Figure 3.11D, silvestrol pre-incubation had no significant effect on the ADCC activity of freshly isolated NK cells (p=0.854).
Figure 3.11: Effector cell cytotoxic activity is maintained in the presence of silvestrol. (A) Cytotoxicity assays were performed using autologous effectors expanded 14 days in the presence of irradiated LCL and 10 nM silvestrol. Targets were fresh CFSE-stained LCL at an effector:target (E:T) ratio of 20:1. After a 4 hr incubation, cells were stained with 7-AAD and washed, and cells positive for both CFSE and 7-AAD were enumerated by flow cytometry. Data are shown relative to the vehicle-only control and are the averages of three independent experiments. Bars show mean ± SD. Differences were not significant (ns). (B) Effectors were expanded in the absence of silvestrol. LCL and effectors (effctr) were then incubated separately for 18 hr without or with 10 nM silvestrol (+S). Cytotoxicity assays were then performed as in (A). Data are shown relative to the vehicle-only control and are the averages of three independent experiments. Bars show mean ± SD. (C) Antibody-dependent cell-mediated cytotoxicity (ADCC) assay using NK cells (effctr) from cocultures grown in the absence or presence of 10 nM silvestrol (+S) mixed with CFSE-stained autologous LCL targets at an E:T ratio of 20:1. Cells were incubated without (No Rx) or with 5 µg/ml rituximab (ritux) or herceptin (irrel). Cytotoxicity was measured by CFSE and 7-AAD dual-positive events. Data are shown relative to the positive control (effectors + targets + ritux, without silvestrol) and are the averages of three individual experiments. Bars show mean ± SD. (D) ADCC assays were performed as in (C), but using fresh non-autologous NK cells incubated 18 hr without or with 10 nM silvestrol (+S). Data are shown relative to the positive control (effectors + targets + ritux) and are the averages of three individual experiments. Bars show mean ± SD.
3.4.6 Antigen-specific immune responses are maintained in the presence of silvestrol

We next examined the effect of silvestrol on the development of EBV antigen-specific CTL. Effector populations were expanded for 14 days in the presence of irradiated LCL with or without silvestrol. A flow cytometric tetramer assay was used to analyze the ability of antigen-specific CTL to detect the immunodominant HLA-B8-restricted RAK epitope of the EBV lytic protein BZLF-1, as previously described\(^{107}\). BZLF-1 has been shown to play an important role in the development of B cell lymphomas, although the precise mechanism remains unclear\(^{156}\). The CTL present in baseline PBMC not exposed to irradiated LCL exhibit very low-level detection of EBV BZLF-1 antigen; however, effectors expanded in the presence of irradiated LCL show significant increases in BZLF-1-specific CTL (p<0.001; **Figure 3.12A**). Regardless of silvestrol treatment, all PBMC cultures exposed to irradiated LCL showed significant expansion of BZLF-1-specific CD8+ T-cells (p<0.001 for each condition relative to baseline PBMC), although the expansion with 10 nM silvestrol was less than in the untreated (vehicle control) condition (p = 0.019). These results demonstrate that at least at these low concentrations, silvestrol does not block the development of antigen-specific CTL (**Figure 3.12A**).

Since effectors expanded in the presence of silvestrol maintain the ability to recognize HLA-presented viral peptide, we next evaluated the expression of immunological synapse proteins on effectors and targets, as modulation of these proteins has been shown as a mechanism for tumor immune escape\(^{242,243}\). First, LCL from 3 donors were incubated for 48 hr with 0 or 10 nM silvestrol. After incubation, LCL were analyzed for
MHC class I (HLA-ABC), co-stimulatory molecules (CD70, CD80 and CD86), and integrin-binding protein CD54 (ICAM-1). Incubation with silvestrol did not significantly alter the intensity of expression of class I MHC, nor did it have a significant impact on the expression of co-stimulatory molecules or ICAM-1 on tumor cells (Figure 3.12B). Next, we analyzed the cognate immunological synapse molecules on the surface of activated immune effector cells. PBMC from 3 donors were incubated with 0 or 10 nM silvestrol for 24 hr followed by overnight stimulation with anti-human CD3. Cells were incubated with labeled antibodies to the molecules of interest and evaluated by flow cytometry, gating on live, CD8+ events. Silvestrol did not significantly affect surface levels of CD8 (T-cell co-receptor and MHC class I binding protein), CD27 (co-stimulatory molecule that binds CD70), CD28 (CD80/86 activating binding partner), or LFA-1 (integrin that is bound by ICAM-1 to stabilize the synapse). Although silvestrol treatment decreased expression of the inhibitory molecule CTLA-4 on some CTL, these effects were more variable between samples and thus overall did not reach significance (Figure 3.12C). As LMP-1 has been shown to induce the transcription of ICAM-1, we analyzed ICAM-1 transcript levels by real-time RT-PCR. Unexpectedly, despite the silvestrol-mediated depletion of LMP-1, ICAM-1 transcript was moderately but significantly increased with silvestrol treatment (p=0.047; Figure 3.12D). Together, these data demonstrate that silvestrol does not significantly impact activating components of the immunological synapse or co-stimulatory molecules on tumor targets. Importantly, CTL expanded in the presence of silvestrol retain the ability to recognize and proliferate in response to EBV recall antigens presented on LCL tumor cells.
Figure 3.12: Antigen-specific immune responses are maintained in the presence of silvestrol. (A) Effectors were expanded 14 days in the presence of irradiated LCL, with or without silvestrol as indicated, using LCL and autologous PBMC from one HLA-B8 donor. A flow cytometric HLA-tetramer assay was used to analyze the ability of antigen-specific CTL to detect EBV BZLF-1 protein bound to MHC class I. The increase in BZLF-1-specific CTL following exposure to irradiated LCL was significant under all conditions (p<0.001 for each 0, 2, 5 and 10 nM silvestrol treatment compared to non-exposed, baseline PBMC), although the expansion with 10 nM silvestrol was less than in the 0 nM (vehicle control) condition (p=0.019). (B) LCL were incubated in 0 or 10 nM silvestrol for 48 hr and analyzed for surface expression of immunological synapse and co-activation proteins. Change in MFI when treated with silvestrol is shown. Bars show mean ± SD from 3 individual donors; differences with silvestrol treatment relative to vehicle were not significant. (C) PBMC from the same 3 donors as in (B) were incubated with 0 or 10 nM silvestrol for 24 hr followed by stimulation with 1 µg/ml anti-hu-CD3 for 18 hr. PBMCs were gated on live, CD8+ T-cells, and cognate molecules to those shown in (B) were analyzed for change in MFI with silvestrol treatment. Bars show mean ± SD; differences with silvestrol treatment were not significant. (D) Real-Time RT-PCR analysis of ICAM-1 expression relative to TBP (control gene) in LCL (N=3) incubated 48 hr with 0 or 10 nM silvestrol. Fold change was calculated using the ΔΔCt method \(^1\). The silvestrol-induced increase in ICAM-1 at the mRNA level was borderline significant (p=0.047).
3.4.7 *In vivo* efficacy of silvestrol in the hu-PBL-SCID model of EBV-LPD

The hu-PBL SCID model has been used to identify experimental therapeutic strategies to prevent or treat spontaneous EBV-LPD\(^{220, 221, 245-248}\). PBMC from a healthy EBV-positive donor were injected into a group of SCID mice that had been pretreated with anti-asialo (GM1) to deplete murine NK cells. Mice were randomized (N=14/group), and IP treatments with vehicle or silvestrol (1.5 mg/kg) every 48 hr began 2 weeks after engraftment. Two weeks after treatment began (4 weeks post-engraftment), an ELISA was performed to evaluate human IgG plasma levels. All 28 mice were shown to produce human IgG (*Figure 3.13A*), with no significant difference between the silvestrol-treated versus vehicle-treated groups. This finding not only confirmed successful engraftment, but also showed that silvestrol did not adversely affect production of human IgG by xenografted B-lymphocytes, suggesting that silvestrol also may allow the preservation of adaptive humoral immune responsiveness. At 4 and 8 weeks post-engraftment, flow cytometry analysis (hu-CD45\(^+\)/CD19\(^+\)/CD3\(^-\)) was performed on spleen cells from 2 mice in each group. Mice in the vehicle control group showed substantial tumor infiltration of the spleen without obvious nodal involvement, whereas no tumor cells could be detected in the spleens of mice from the silvestrol-treated group (not shown). By 8 weeks post-engraftment, spleens from mice in the control group showed profound splenomegaly characterized by human B-cell infiltration, while spleens from silvestrol-treated animals exhibited normal spleen mass (*Figure 3.13B*). Spleens from both treatment groups were also obtained from each animal as euthanasia criteria were met, or for the silvestrol-treated mice, at the end of study (140 days). Mice in the vehicle control group showed significantly enlarged spleens relative to silvestrol-treated mice.
Finally, silvestrol significantly prolonged survival compared to vehicle (7/7 silvestrol-treated mice vs. 2/9 control mice alive at day 140, \( p<0.004 \); Figure 3.13D). At the end of the study, all animals were examined for presence of EBV-LPD by necropsy as well as flow cytometric evaluation of spleen cells. While the remaining vehicle-treated mice exhibited substantial tumor infiltration of the spleen, no such lymphocyte infiltration could be detected in the spleens of any of the silvestrol-treated mice, nor did these animals exhibit any other obvious signs of lymphoma upon necropsy (not shown). We also evaluated normal blood cell populations following silvestrol treatment (1.5 mg/kg IP every other day for 28 days) in healthy, immune-competent C57BL/6 mice. No significant changes were found in total leukocyte, lymphocyte, and erythrocyte subsets, although a moderate (approximately 1.4 fold) increase in platelets was observed (Figure 3.14A). Total spleen cells from these healthy mice were also investigated for effects on normal lymphocyte subsets; no differences were found with silvestrol treatment on murine CD4+ or CD8+ (T-cell), CD19+ (B-cell) or NK1.1+ (NK cell) subsets (Figure 3.14B).
Figure 3.13: Evaluation of in vivo therapeutic activity of silvestrol in hu-PBL SCID model. SCID mice depleted of murine NK cells were injected intraperitoneally with $5 \times 10^7$ PBMC from a healthy EBV-seropositive human donor. Treatment with vehicle or 1.5 mg/kg silvestrol (N=14 per group) every other day by the IP route began two weeks post-engraftment. (A) Human Ig ELISA on peripheral blood from all mice (N=28) 4 weeks after engraftment; averages for both groups are shown. Bars show mean ± SD. Differences were not significant (ns). (B) Spleens are shown from two mice from each group 8 weeks post-engraftment, with weights in mg. (C) Spleen weights were recorded from all mice upon euthanasia or end of study (day 140 post-engraftment) (N=9 for vehicle control and N=7 for silvestrol group). Bars show mean ± SD; differences were significant (p=0.008). (D) Kaplan-Meier analysis of overall survival (vehicle control N=9; silvestrol-treated N=7; p<0.01). (E) Specific lymphocyte subsets were depleted from PBMC directly prior to engraftment using immunomagnetic bead depletion for CD8 (cytotoxic T cells), CD14 (monocytes), or CD56 (NK cells). Biotin-only conjugated beads were used for the control (mock-depleted) condition. Efficiency of depletion was verified by flow cytometric analyses and show to be greater than 90%. Equivalent numbers of depleted PBMC preparations ($5 \times 10^7$ cells in the mock-depleted condition) were engrafted by intraperitoneal injection into murine NK cell-depleted SCID mice (N=5 per group). After 4 weeks, human Ig levels were assessed by ELISA, and only mice showing engraftment by this parameter were included in the study. Kaplan-Meier analysis of overall survival was then performed. The differences between the non-depleted vehicle control (N=3) vs. non-depleted silvestrol-treated (N=4) groups, and between the CD8-depleted silvestrol-treated vs. non-depleted silvestrol-treated groups (N=4 each), were significant (p=0.029 each). The difference between CD56-depleted silvestrol-treated (N=5) vs. non-depleted silvestrol-treated (N=4) was not significant.
3.4.8 CD8+ T lymphocytes are necessary for in vivo efficacy of silvestrol

To further investigate the importance of immune effector cells in silvestrol's dramatic protective effect in EBV-LPD, the previous in vivo experiment was repeated with prior depletion of specific human lymphocyte subsets. Before engraftment, PBMC were...
subjected to immunomagnetic bead depletion for cells carrying CD8 (CTL), CD14 (monocytes), or CD56 (NK cells). Mock depletion was performed using biotin-conjugated beads as a negative control. Efficiency of depletion was verified by flow cytometric analyses to be greater than 90% (Figure 3.15). A total of 5x10^7 mock-depleted PBMC, or equivalent percentages from each of the subset depletions as described previously\textsuperscript{107}, were engrafted into SCID mice depleted of murine NK cells by anti-asialo injections (N=5/group). As with the prior experiment, IP treatment with 1.5 mg/kg silvestrol or vehicle every 48 hr began 2 weeks after engraftment. Prior experience with this model shows PBMC depleted of CD3/CD8+, CD56+, or CD14+ cells do not affect human cell engraftment and production of human IgG levels\textsuperscript{221}. Two weeks after treatment began (4 weeks post-engraftment); an ELISA was performed to evaluate human IgG. Only mice shown to be engrafted by production of human IgG were used in the study. The mock-depleted mice showed results similar to the previous experiment, with 0/3 surviving in the vehicle group and 4/4 surviving in the silvestrol-treated group (p=0.029) (Figure 3.13E). Notably, when CD8\textsuperscript{+} T-cells were depleted, silvestrol no longer provided effective anti-tumor protection (0/4 surviving in silvestrol-treated CD8-depleted group versus 4/4 in the silvestrol-treated mock-depleted group; p=0.029), nor did it reduce tumor burden as measured by spleen mass (not shown). Mice receiving CD14\textsuperscript{+} (monocyte) or CD56\textsuperscript{+} (NK cell) depleted engraftments exhibited an intermediate response to silvestrol treatment (2/4 and 4/5 surviving, respectively) that did not reach statistical significance with this number of animals. These data indicate that silvestrol is indirectly mediating protection through immune surveillance mechanisms, and that at least CD8\textsuperscript{+} T-lymphocytes are essential in the silvestrol-mediated clearance of tumor.
Prior to engraftment in SCID mice, PBMC were subjected to immunomagnetic bead depletion (negative selection) for CD8 (cytotoxic T-cells), CD14 (monocytes) or CD56 (NK cells). Biotin-only conjugated beads were used for the mock-depleted condition. Efficiency of depletions was verified by flow cytometric analysis gating on viable cells (i.e. negative for LIVE/DEAD stain) and evaluating each of the markers. The y-axis shows forward scatter (FS).
3.5 Discussion

EBV is associated with a broad spectrum of benign and malignant diseases that, collectively, represent a growing number of cases in immunocompromised individuals and in our aging population\textsuperscript{192, 249, 250}. Current therapies for EBV-LPD often lead to profound immune suppression and subsequent development of life-threatening opportunistic infections and EBV reactivation. Thus, an ideal agent to treat patients with EBV-LPD would possess direct anti-tumor activity while preserving the host anti-tumor immune function. Previous studies showed the direct anti-tumor activity of the translation inhibitor silvestrol in leukemia and lymphoma\textsuperscript{203, 209, 212, 217-219}, and selective cytotoxicity to malignant B-cells compared to normal lymphocytes\textsuperscript{203}. This activity appears to be due to the loss of short half-life protective proteins resulting from direct translation inhibition. Here we show that, in addition to this activity, silvestrol is also an immune-preserving agent. We provide a characterization of the unique anti-tumor and immune-potentiating properties of this drug using \textit{in vitro} and \textit{in vivo} models of EBV-LPD.

Our principle findings show that direct anti-proliferative activity of silvestrol toward LCL at physiologically achievable concentrations is moderate. However, these low concentrations of silvestrol, when added to co-cultures of LCL and autologous PBMC, produce complete ablation of the malignant LCL cells. Importantly, the \textit{in vivo} EBV-LPD model revealed a remarkable survival advantage with silvestrol treatment, far greater than we and others have previously shown with silvestrol using xenografts in immune deficient mice\textsuperscript{203, 219}. Together, these results indicate that the indirect effects of silvestrol on tumor cells via innate and adaptive immune components may be at least as important as its direct effects. As most \textit{in vivo} studies with silvestrol to date have used tumor
xenografts in immune deficient mice, this crucial aspect was previously unknown.

The direct growth inhibitory activity of silvestrol as well its ability to sensitize LCL to immune-mediated killing appears to be associated with a loss of the LMP-1 oncoprotein. Drugs and cytotoxic T-cell preparations directly targeting LMP-1 have been shown to prevent metastasis, promote apoptosis and enhance radiosensitivity, identifying LMP-1 as a potential therapeutic target for EBV-driven malignancies. The ablation of LMP-1 protein has been shown to interfere with several downstream signaling pathways including AKT as well as STAT1 and STAT3, and our results support these findings. Inhibition of these pathways may lower tumor apoptotic threshold and allow sensitization to effector immune cell anti-tumor activity. Certainly, we observed that low doses of silvestrol enhanced sensitivity of LCL tumor targets to innate and adaptive immune effector-mediated killing while producing little to no effect on those effector cells’ functional capabilities. We are presently investigating the mechanism accounting for this reduced apoptotic threshold in LCL. It was previously reported that the classical chemotherapeutic agents paclitaxel, cisplatin, and doxorubicin sensitize several types of solid tumor cells to cytotoxic T-lymphocytes by increasing tumor cell permeability to granzyme B.

It has been previously reported that silvestrol sensitizes tumor cells to classical chemotherapeutic agents including doxorubicin. However, as standard chemotherapies used in the treatment of lymphoma impair adaptive and innate cell-mediated immunity, such combinations may potentially circumvent the immune-preserving advantage of silvestrol. Here we describe an entirely new characteristic of silvestrol, which is the maintenance of adaptive immune effectors (EBV-specific CTL)
and innate immune effectors (NK cells) using concentrations that have direct, albeit moderate, anti-tumor activity. These studies employed an established in vitro model in which EBV-driven lymphoma cells (LCL) are incubated with autologous PBMC. Left untreated, the LCL ultimately outpace the expanding effector populations, and overtake the culture. With the addition of as little as 10 nM silvestrol, we observed complete ablation of viable LCL in these autologous co-cultures while effector cell populations (i.e. CD8+ CTL and CD56+ NK cells) were maintained; importantly, this effect of silvestrol on LCL is greatly reduced in the absence of effector cells. Furthermore, proliferation assays in silvestrol-treated co-cultures show a substantial decrease in proliferation of LCL targets without a corresponding change in proliferation of effector cells. We are currently investigating the differential activity of silvestrol on cell cycle checkpoints in tumor versus immune effector populations.

Besides affecting absolute cell numbers, classical chemotherapy is known to have a deleterious effect on cancer-relevant immune cell function including NK cell-mediated ADCC\(^ {253, 254} \). Our experiments show that silvestrol does not significantly impact NK cell ADCC activity, either when NK cells were expanded in the presence of silvestrol or when silvestrol was added immediately prior to the ADCC assay. Additionally, EBV proteins also mediate multiple effects to evade adaptive immune detection of virally-infected cells, and appropriate adaptive immune recognition of EBV proteins by CTL is essential to avoid development of EBV-related malignancies\(^ {255} \). CTL exposed to silvestrol retained the ability to recognize presented viral peptide in the context of class I MHC, and showed little or no change in cytotoxic function or in expression of immune synapse components. As silvestrol treatment does not impede the expansion of EBV-specific CTL
or alter the expression of immune synapse or co-stimulatory molecules, this may explain how silvestrol treatment can preserve immunological responsiveness against EBV-LPD.

Our *in vivo* studies using the hu-PBL SCID model provide significant insight into the efficacy of silvestrol in treating EBV-LPD. Importantly, our *in vivo* experiment using grafts depleted of effector cell subsets establishes that CD8+ CTL are necessary for full silvestrol efficacy in this model. The moderate loss of protection when other lymphocyte subsets are depleted suggests that silvestrol is sensitizing tumor cells for clearance through other immune effectors as well, although a larger study is required to address this. Furthermore, *in vivo* experiments using animals with intact immune systems did not show significant alteration in total lymphocytes or modulation in lymphocyte populations with silvestrol treatment, consistent with previously reported data\(^2\). Together, these studies provide further evidence of silvestrol's limited toxicity to immune effectors.

In summary, we present new information regarding the efficacy of the translation inhibitor silvestrol in EBV-LPD and demonstrate that this efficacy is largely due to the maintenance of number and function of both innate and adaptive anti-tumor immune components. Additionally, silvestrol promotes modest direct anti-tumor activity against EBV-transformed lymphoma cells, likely by depleting the oncogenic viral protein LMP-1 and interfering with downstream survival pathways. Other chemotherapeutic regimens currently in use for the treatment of EBV-LPD display a non-specific cytotoxicity both to the tumor target as well as immune effectors, leading to elimination of potent memory anti-tumor responses. This effect leaves patients vulnerable to disease relapse and at increased risk of serious and potentially lethal opportunistic infections. The ability of silvestrol to simultaneously exert direct anti-proliferative activity against EBV-transformed
tumor cells while sparing adaptive and innate immune effector function is highly unusual if not unique, and shows promise for future clinical translation.
Chapter 4: The role of suppressive macrophages in EBV-driven disease

4.1 Summary

Epstein-Barr virus (EBV) is an oncogenic herpes virus associated with the development of a range of B-cell lymphomas with poor outcomes. Current treatment options are typically immune suppressive, increasing the risk of EBV reactivation and lethal opportunistic infections. These complications make it essential to identify novel therapeutic approaches targeting mechanisms of immune evasion while promoting tumor immune surveillance. Immune avoidance by tumors is supported by aberrant ligand and cytokine expression within the microenvironment, and tumor-associated macrophages (TAM) play an important role in inhibiting anti-tumor immune responses. TAMs are known to suppress T cell activation, and increased TAM density and ratio to cytotoxic T lymphocytes (CTL) are negative prognostic indicators. However, little is known about the mechanisms of macrophage polarization in the context of EBV-lymphoma. Here we show that when EBV+ tumor cells are cultured with autologous peripheral blood mononuclear cells (PBMC), soluble factors are released that drive development of macrophages with a M2-like phenotype with potent cytotoxic capabilities against activated T cells. This polarization can be abrogated by treatment with translation inhibitor silvestrol. Together, our findings suggest a novel tumor immune escape model driven by immune recognition of EBV-driven lymphoma that results in the polarization of monocytes to a suppressive macrophage phenotype.
4.2 Introduction

Cells of the immune system can have roles either in protection against cancer or in tumor promotion. As mentioned previously, CTLs can recognize virally infected and otherwise abnormal tumor cells and directly kill them. However, a hallmark of cancer is the acquisition of mechanisms to not only evade immune detection, but to actively suppress immune function\textsuperscript{115}. While activation of tumor-specific adaptive immune cells result in tumor eradication, chronic activation of innate immune cells has the ability to stimulate tumor progression\textsuperscript{256}. The chronic nature of cancer-induced inflammation results in a modified cellular milieu surrounding the tumor. This tumor microenvironment consists of complex interactions between malignant and non-malignant cells, which include a significant leukocyte population, predominately comprised of macrophages\textsuperscript{257-259}. The interaction of tumor and innate immune cells results in further recruitment and differentiation of monocytes into TAMs\textsuperscript{260}. Once activated by cancer cells, TAMs release a variety of soluble factors that promote tumor growth and metastasis, in part by suppressing T cell activation and anti-tumor response\textsuperscript{258, 261-263}. As in vivo evidence of these effects, increased TAM density and ratio to cytotoxic T lymphocytes (CTL) in the tumor microenvironment are negative prognostic indicators in several types of EBV-driven lymphomas\textsuperscript{264, 265}.

Conventional treatments as well as immunotherapies are remarkably affected by the presence of TAMs within the microenvironment\textsuperscript{261}. Emerging immunotherapies for EBV-driven lymphomas such as EBV-specific adoptive cell therapy, chimeric antigen receptor and dendritic cell vaccines fail to address the underlying local immunosuppression within the tumor microenvironment that blocks the anti-tumor response\textsuperscript{266, 267}. It is therefore
essential to have a better understanding of TAM activation to develop novel therapeutic strategies to address the immunosuppressive nature of the tumor microenvironment. These strategies, combined with conventional and immune-based therapies, would provide an innovative approach to improve cancer treatment outcomes\textsuperscript{186, 268}.

We previously reported that the eIF4A-specific translation initiation inhibitor silvestrol elicits marked anti-tumor activity against EBV-driven lymphoma. To do this, we employed an \textit{in vitro} co-culture (CoCx) system in which EBV-transformed lymphoblastoid cell lines (LCL) are cultured with autologous peripheral blood mononuclear cells (PBMC). In this variation of a mixed-lymphocyte reaction, interactions between tumor and autologous immune cells can be examined in detail. We found that silvestrol's anti-tumor effects occur via its facilitation of the activation, proliferation and cytotoxic activity of anti-tumor immune subsets including EBV-specific CTL. This earlier work focused on T-cells, however, and did not address the role of the myeloid compartment. Here, we exploit this CoCx system to investigate how monocytes and macrophages promote or impede the development of an anti-tumor immune response. We found that the interaction of LCL with autologous PBMC stimulates the outgrowth of a differentiated macrophage population with an M2-skewed phenotype. This effect is reproduced using just the media from LCL+PBMC CoCx, demonstrating it is mediated by soluble factor(s) in the conditioned media. These differentiated macrophages express multiple immunosuppressive markers including PD-L1 and IDO, and are cytotoxic toward activated but not non-activated T cells. Given that silvestrol restores anti-tumor activity in this system, we use this agent as a biological probe to investigate the mechanism by which LCL+PBMC CoCx drives the proliferation and differentiation of M2-like
macrophages. We found that the addition of 5 nM silvestrol into the initial CoCx blocked the release of the soluble factors causing monocyte/macrophage differentiation, and also that adding silvestrol to the CoCx conditioned medium, after the soluble factors are already present, still completely prevented M2 cell development. These findings indicate that selective targeting of translation initiation may block TAM formation in the setting of EBV-driven lymphoma at multiple stages, providing a new potential therapeutic strategy.
4.3 Methods and Materials

4.3.1 Cells and cultures

Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Paque Plus gradient centrifugation from healthy, EBV+ adult volunteers. Informed consent was obtained from the volunteers prior to participation in accordance with IRB guidelines. EBV-transformed lymphoblastoid cell lines (LCL) were derived in vivo by engrafting these cells into severe combined immune-deficient (SCID) mice (Taconic, Hudson, NY) donors. When mice exhibited signs of disease, they were sacrificed and spleocytes were cultured resulting in outgrowth of EBV transformed LCLs. Cells were maintained in RPMI 1640, with 10% fetal bovine serum (FBS) and 1% GlutaMAX (Thermo Fisher, Waltham, MA) under a humidified 5% CO₂ atmosphere at 37°C.

4.3.2 Generation of conditioned media (CM)

Conditioned media (CM) were created by incubating CellGro serum free media (CellGenix) alone or with PBMC, LCL, or autologous PBMC+LCL (co-cultures; CoCx) (5x10⁵ cells/mL for each), in 200 μL/well of 96 well U-bottom plates. Supernatants were collected after 48 hours, centrifuged at 800xg for 10 minutes to remove cells, and frozen in aliquots at -80°C. Aliquots were used once for experiments and not re-frozen (Figure 4.1).
4.3.3 Cytokine Arrays

Membrane-based antibody arrays were used for screening expression levels of cytokines and chemokines released into CM. CM was collected from co-cultures treated 48 hrs with 0 or 10 nM silvestrol, and arrays were performed according to the manufacturer’s instructions using Proteome Profiler Human Cytokine Array detecting relative expression of 36 human cytokines (R&D systems, Minneapolis, MN) or Human Cytokine Array C2000 detecting expression of 174 cytokines (RayBiotech, Norcross, GA). Briefly, membranes were incubated at overnight at 4°C and chemiluminescent signal was visualized by x-ray film (Human Proteome Profiler) or digitally (Human Cytokine Array C2000). Densitometry data was collected and analyzed using the RayBiotech analysis tool.
4.3.4 Proliferation assays

PBMC were re-suspended in EasySep buffer containing PBS, pH 7.4, 2% FBS and 1 mM EDTA. Monocytes were isolated by magnetic immunoselection using EasySep Human Monocyte Enrichment Kits (StemCell Technologies, Vancouver BC). Enrichments were tested for purity by flow cytometric analysis of CD14+ events. Monocytes were re-suspended in CM to a density of 7.5x10^5 cells/mL, and 150 µL/well was plated in 96 well flat-bottom plates. On day 3, wells were supplemented 75 µL fresh CM. On day 6 MTS assays were performed to measure mitochondrial function as a surrogate of cell proliferation (CellTiter96, Promega, Madison WI).

4.3.5 Transwell Assays

Transwell co-cultures were created by incubating PBMC and LCL (5x10^5 cells/mL) together or on either side of a 0.4 µm pore polycarbonate transwell membrane. Supernatants were collected after 48 hours, centrifuged at 800xg for 10 minutes to remove cells, and frozen in aliquots at -80°C.

4.3.6 Flow cytometry

5x10^5 were transferred to a 5 mL polystyrene round-bottom tube and washed with cell staining buffer (PBS, pH 7.4, with 5% mouse serum and 0.09% sodium azide) followed by centrifugation at 300xg for 10 minutes. Pellets were re-suspended with 50 µL mouse serum and incubated 10 minutes at room temperature to block Fc receptors. Cells were then washed and re-suspended in 100 µL cold staining buffer containing fluorescence-labeled antibodies: CD14 (TUK4), CD62L (145/15), HLA-DR (AC122) (Miltenyi), CD3 (UCHT1), CD4 (RPA-T4), CD8 (RPA-T8), CD11c (B-ly6), CD16 (3G8), CD40 (5C3),
CD80/B7.1 (BB1), CD86/B7.2 (FUN-1) (BD Biosciences), CD11b (M1/70), CD163 (GHI/61), CD206 (19.2), and CD274/PD-L1 (29E.2A3) (BioLegend) with near IR LIVE/DEAD cell viability dye (ThermoFisher). Cells were incubated 30 minutes at 4°C followed by washing with 1 mL staining buffer. Cells were re-suspended in 350 µL staining buffer and analyzed on a Beckman Coulter Gallios flow cytometer. Recorded data was exported and analyzed with Beckman Coulter Kaluza Analysis 1.3 software.

4.3.7 Cell depletion and enrichments

Depletions were performed using positive immunomagnetic selection. Cells were re-suspended in ice-cold MACS buffer (PBS, pH 7.4, 0.5% FBS, 2 mM EDTA) per 1x10⁷ cells, and MicroBeads conjugated to CD4, CD8, CD14, CD19, CD56 or biotin (mock) (Miltenyi) were added. Suspensions were incubated for 15 minutes at 4°C, washed with 1 mL MACs buffer per 1x10⁷ cells, and centrifuged at 300xg for 10 minutes. Cells were re-suspended in 500 µL buffer and magnetically separated using LD Depletion Columns (Miltenyi). Enrichments were performed using negative immunomagnetic selection with EasySep Human Monocyte, T cell, CD4+ T Cell or CD8+ T Cell Enrichment Kits (StemCell Technologies). PBMC suspensions were prepared in a 5 mL polystyrene round bottom tube at a concentration of 5x10⁷ cells/mL in EasySep Buffer (PBS, pH 7.4, 2% FBS and 1 mM EDTA). Enrichment cocktail (or EasySep buffer for mock selected conditions) was added according to the manufacturer’s instructions. Cells were incubated 10 minutes at 4°C followed by addition of magnetic particles and an additional incubation at 4°C for 5 minutes. Cell suspensions were brought up to 2.5 mL with EasySep buffer and placed into the separation magnet for 2.5 minutes followed by inversion of the magnet and tube to decant the desired cell fraction. All populations were
confirmed by flow cytometry for CD3+/CD4+ (T helper cells), CD3+/CD8+ (CTL), CD3-/CD56+ (NK cells), CD3-/CD14+ (monocytes), CD3-/CD19+ (B cells)

4.3.8 Quantitative T cell flow cytometry

Isolated T cells were re-suspended to 2x10^6 cells/mL in the presence of 40 U/mL IL-2, and half were stimulated with Dynabeads Human T-Activator CD3/CD28 (25 µL/10^6 T cells). Solutions (0.5 mL per well) were plated in a 48 well plate. Macrophages that had been expanded in CoCx CM for 6 days were added to the T cells at ratios of 1:5, 1:10 and 1:20 macrophages:T cells, with media alone as a negative control. At 24 and 48 hours, cells were transferred to a 5 mL polystyrene round-bottom tube and washed with cell staining buffer (PBS, pH 7.4, with 5% mouse serum and 0.09% sodium azide) followed by centrifugation at 300xg. Pellets were re-suspended with 100 µL cold staining buffer containing fluorescence-labeled antibodies: CD14-VioGreen, CD3-PerCP-Vio700 (Miltenyi), CD4-FITC, and CD8-PE (BD Bioscience) with LIVE/DEAD near IR cell viability dye. Absolute numbers of cell populations were assessed by re-suspension in 350 µL staining buffer containing 25 µL calibrated suspensions of fluorescent CountBright Absolute Counting Beads (ThermoFisher). CD14-/CD3+/CD4+ (T helper) and CD14-/CD3+/CD8+ (CTL) were calculated from the number of cellular events compared with the known concentration of fluorescent microspheres. Gating strategy is shown in Figure 4.2.
4.3.9 Immunoblot analysis

LCL were subjected to immunoblotting as described previously. Antibodies to β-actin (13E5), Notch1(D1E11), IDO-1 (D5J4E), eIF4a (C32B4), eIF4e (C46H6), phospho-eIF4e (Ser209), RIG-I (D14G6), MDA5 (D74E4), IRF-3 (D614C), phospho-IRF-3 (Ser396), IRF-7, (D2A1J) phospho-IRF-7 (Ser477), IKKε (D20G4), phospho- IKKε (Ser172), TBK1 (D1B4), phospho-TBK1 (Ser172) were purchased from Cell Signaling Technology.
4.3.10 AmpliSeq

Isolated monocytes were incubated CoCx conditioned medium, and RNA was isolated by Trizol extraction at 0, 1, 3, or 6 days. cDNA libraries were constructed by reverse transcription and amplification using the Ion AmpliSeq Transcriptome Human Gene Expression Kit (ThermoFisher). Prepared libraries were loaded onto an AmpliSeq Whole Transcriptome chip and run on an ion sequencer. Data was analyzed using the Torrent Suite analysis software (ThermoFisher).
4.4 Results

4.4.1 EBV co-cultures release myeloid-associated factors

Our group previously characterized the unique anti-tumor and immune-potentiating properties of the translation inhibitor silvestrol. Our findings showed that silvestrol has only moderate direct anti-tumor activity against EBV-driven LCL lines. However, when added at low concentrations to co-cultures of LCL and autologous PBMC, a complete ablation of the malignant LCLs was observed, indicating silvestrol increases immune cell-mediated death of cancer cells. To examine this *in vivo*, we employed an established mouse model that recapitulates human EBV-driven lymphoproliferative disorder (EBV-LPD). Mice with EBV-driven lymphoma that were treated with silvestrol survived and exhibited no signs of disease, while control mice succumbed to disease. However, silvestrol lost its effectiveness when these mice were depleted of CTLs, demonstrating that this potent anti-tumor effect occurs via the immune system and not through a direct effect of silvestrol on the tumor.

To investigate how silvestrol facilitates this anti-tumor immune effect, we began by examining the how silvestrol altered the interaction between tumor cells (LCL) and immune effectors from autologous PBMC. Based on our earlier work, we hypothesized that silvestrol treatment was leading to activation of anti-tumor immune subsets though modulation of soluble factors responsible for anti-tumor T and NK cell activation. We therefore used sandwich-based membrane antibody arrays to profile expression levels of proteins released into the supernatant of co-cultures. CoCx CM was generated by incubating equal numbers of LCL with autologous PBMC in serum-free medium 48 hrs in
the presence of 0 or 10 nM silvestrol. Immune assay membranes were incubated overnight in the CoCx CM, and chemiluminescent signals were visualized by x-ray film (R&D Systems Human Proteome Profiler, Figure 4.3A) or digitally (RayBiotech Human Cytokine Array C2000, Figure 4.3B). These protein screens indicated that CM from cultures treated with silvestrol exhibited a loss of growth factors and chemokines responsible for myeloid activation and recruitment (CXCL1, CXCL8, CCL2, CC7, CCL8 and CCL9). The release of many of these factors has been associated with the recruitment and differentiation of circulating monocytes and differentiation of suppressive myeloid subsets, including tumor-associated macrophages (TAMs) and myeloid-derived suppressor cells (MDSC)\textsuperscript{270, 271}. This led us to consider a unique tumor immune escape mechanism involving cells of the myelomonocytic lineage.
Figure 4.3: Co-culture cytokine analysis. Co-culture conditioned media (CoCx CM) was created by incubating autologous PBMC and LCL for 48 hrs in the presence or absence of 10 nM silvestrol. Cells were removed by centrifugation and analyzed by two different cytokine arrays: A) R&D systems Human Proteome Profiler; B) RayBiotech C2000 Human Cytokine Array.
4.4.2 Conditioned media from co-cultures promotes monocyte proliferation

To further investigate the role of soluble factors released by the interaction of tumor cells and immune effectors, we generated CM by incubating serum-free media alone, PBMC alone, LCL alone, or CoCx containing equal numbers of LCL and PBMC for 48 hrs. Supernatants were centrifuged to remove cells, and used to culture monocytes previously isolated by negative selection from three healthy donors. Cells were incubated without the addition of exogenous cytokines for six days (Figure 4.1). Proliferation was assessed by MTS assay and results are shown relative to the media-only condition (Figure 4.4A). Microscopy indicated that monocytes cultured with CoCx CM formed non-adherent, proliferating colonies, whereas monocytes incubated in media alone, PBMC CM, or LCL CM remained adherent (Figure 4.4B). We examined the role of contact dependence of the release of growth-promoting factors by employing transwell assays. We compared the proliferation of monocytes cultured in CoCx CM (LCL+PBMC in contact) to monocytes cultured in CM generated by LCL and PBMC separated by a 0.4 µm pore polycarbonate transwell membrane (Figure 4.4C). Monocytes incubated in CM from the transwell plates exhibited markedly lower proliferation compared to the CM from LCL+PBMC together, demonstrating that the release of monocyte-differentiating soluble factors into the media is contact-dependent.
Figure 4.4: Proliferation of monocytes in CoCx CM: CM from PBMC, LCL, or PBMC/LCL (CoCx) incubated 48 hrs were used to culture purified autologous monocytes for 6 days. A) MTS assays showing monocyte proliferation in CoCx CM relative to PBMC CM or LCL CM (n=3). B) Monocytes in CoCx CM formed non-adherent, proliferating colonies. C) Proliferation of monocytes in CoCx CM created by LCL+PBMC together or separated by a permeable membrane was assessed by MTS assay.
4.4.3 Depletion of T lymphocytes decreases monocyte proliferation

With evidence that a cell contact-dependent mechanism was involved, we next examined the subsets necessary for the release of the factors driving monocyte proliferation. PBMC were immunomagnetically depleted of CD8+ (CTLs), CD4+ (T helper cells), CD14+ (monocytes), CD19+ (B cells), or CD56+ (NK cells) prior to incubation with autologous LCLs. Mock depletion (biotin beads) was used as a control. CM from these cultures were collected and centrifuged as before. Purified, autologous monocytes were incubated for 6 days in these CM and proliferation was assessed by MTS assay. Depletion of CD8+ or CD4+ T lymphocytes from the PBMC led to a decrease in proliferation of monocytes, indicating a significant role of T cells in this phenomenon (Figure 4.5). Depletion of CD14+, CD19+ or CD56+ subsets from co-cultures did not have a significant effect on the proliferation of monocytes.
To further elucidate the interactions between T cells and LCs in co-culture, we depleted and selected for T cell subsets from PBMC prior to incubation with LCL. PBMC were immunomagnetically depleted of CD4+ (T helper cells), CD8+ (CTLs), or both CD4+ and CD8 T cells prior to incubation with autologous LCLs. As above, mock depletion using biotin beads was included as a control. Purified, autologous monocytes were incubated for 6 days in these CM and proliferation measured by MTS assay. Compared with the mock-depleted condition, both CD4 and CD8 depletion resulted in loss of monocyte proliferation.

4.4.4 T lymphocytes in co-cultures are necessary and sufficient for monocyte proliferation

Figure 4.5 Depletion of T cells from co-cultures decreases monocyte proliferation: PBMC from three donors were immunomagnetically depleted of CD8+, CD4+, CD19+, CD56+ or CD14+ subsets prior to incubation with autologous LCL. PBMC were also mock-depleted using biotin-only beads. CM from these CoCx, collected after 48 hrs, was used to culture purified autologous monocytes for 6 days. Monocyte proliferation in CM from CoCx depleted of various cell subsets is shown by MTS assay (n=3).
proliferation, with an even more pronounced decrease when both T cell subsets were depleted (Figure 4.6A). Flow cytometry was used to confirm depletion status of PBMC prior to incubation with LCL (Figure 4.6B) (make this the first figure in this section). As additional confirmation, T cells were selected using negative immunomagnetic selection to obtain CD4+ T cells, CD8+ T cells or total T cells. PBMC were also mock selected in the presence of immunomagnetic beads without the addition of antibody cocktails. Equivalent numbers of cells from each depleted PBMC preparation were added to the LCL. CM was collected from these cultures after 48 hrs and added to purified autologous monocytes. MTS assays after 6 days showed no significant differences between the non-depleted PBMC and T-cell depleted conditions, indicating that T cells in co-cultures are sufficient to induce proliferation of monocytes (Figure 4.6C). Selected PBMC subsets were analyzed by flow cytometry to confirm enrichment of T cell subsets prior to generation of conditioned media (Figure 4.6D)
Figure 4.6: T cells in co-cultures are necessary and sufficient for monocyte proliferation. A) PBMC were immunomagnetically depleted of CD8+, CD4+ or both CD4+ and CD8+ T cells prior to incubation with autologous LCL. PBMC were also mock-depleted using biotin-only beads. CM from these co-cultures was collected after 48 hrs and used to culture purified autologous monocytes for 6 days. Monocyte proliferation is shown by MTS assay. B) Depleted PBMC were analyzed by flow cytometry to confirm removal of T cell subsets. C) PBMC were selected for T helper cells, cytotoxic T cells (CTL) or total T cells by immunomagnetic positive selection. Cells were also mock selected in the presence of immunomagnetic beads without antibody. Equivalent cell numbers of depleted PBMC were added to autologous LCL. CM was collected after 48 hrs and used to incubate purified autologous monocytes for 6 days. Monocyte proliferation was assessed by MTS assay. D) Selected PBMC subsets were analyzed by flow cytometry to confirm enrichment of T cell subsets. Data is representative of two experiments.
4.4.5 Conditioned media from co-cultures polarizes monocytes to an M2-like phenotype

Cells of the myeloid lineage have a remarkable plasticity that permits a tailored immune response to environmental signals\textsuperscript{272}. Two distinct functionally polarized activation states of macrophages are typically used that mirror T\textsubscript{H}1 and T\textsubscript{H}2 T helper cell polarization: classically activated pro-inflammatory (M1) macrophages and alternatively activated immunosuppressive (M2) macrophages\textsuperscript{273}. While these two functional phenotypes are useful to describe polarization, macrophages are quite heterogeneous and will typically fall somewhere between the typical M1 and M2 extremes\textsuperscript{274}. Tumor-associated macrophages (TAMs) typically exhibit a phenotype skewed towards an M2-like polarization, however, they may also exhibit a low level of M1-associated protein expression, consistent with the chronic nature of cancer inflammation\textsuperscript{263, 275, 276}. The outcome of this smoldering inflammation is aberrant functional reprogramming that results in an immunosuppressive and tumor promoting microenvironment\textsuperscript{277, 278}. We therefore examined the immunophenotype of this monocyte-derived population after six days in culture by flow cytometry. Cells from these cultures maintained surface expression of CD14 as well as the monocyte/macrophage integrin, CD11b. Canonical M1 markers (CD80 and CD86) were expressed at low levels, while M2 (CD206 and CD163) antigens were highly elevated\textsuperscript{279}. High expression levels of CD16 and HLA-DR indicate differentiation to a macrophage rather than MDSC\textsuperscript{280}. Furthermore, immune checkpoint molecules PD-L1 and IDO-1 were predominantly expressed (Figures 4.7A). Compared with isolated monocytes prior to incubation in CM, the differentiated macrophages are much larger and more granular as seen on forward scatter/side scatter.
plots (Figure 4.7C). Also, compared with day 0, cultured cells acquire significant expression of surface antigens CD16, HLA-DR, PD-L1, CD11b, and CD206. Together, these findings indicate that monocytes cultured in CoCx CM are polarized to an immunosuppressive M2-like phenotype.
Figure 4.7 Conditioned media from co-cultures polarizes monocytes to an immunosuppressive M2-like phenotype. Freshly isolated monocytes (Day 0) were compared with isolated monocytes incubated in CoCx CM (Day 6). A) Flow cytometric analysis of surface markers on CM-derived cells. B) Forward scatter (FS) and side scatter (SS) analysis of CM-derived cells. C) Comparison of median fluorescent intensity (MFI) in freshly isolated monocytes and monocytes incubated in the presence of CoCx CM and analyzed by flow cytometry (n=3).
4.4.6 Expanded macrophages exhibit cytotoxic activity against activated T cells

TAMs generally have an M2-like phenotype that is capable of suppressing the anti-tumor immune response\textsuperscript{281}. We therefore examined the functional phenotype of the macrophages acquired during the process of differentiation CoCx CM. We assessed the activity of these macrophages on T cells by incubating purified T cells alone or with increasing ratios (1:20, 1:10 or 1:5) of autologous, CoCx CM-derived macrophages in the presence or absence of anti-CD3/CD28 conjugated beads to recapitulate the activation of T cells within the tumor microenvironment. At 24 and 48 hrs, T cell numbers were assessed by quantitative flow cytometry (Figure 4.2). At both timepoints, a small decrease in total numbers of CD4+ and CD8+ T cells was seen in unstimulated conditions compared to T cells cultured alone. However, if T cells were first activated, we saw a loss of total numbers of viable T cells, with increasing concentration of macrophages, when compared with activated T cells alone. By 48 hrs the condition with the highest ratio of macrophages to T cells (1:5) lost 70% of CD4+ T cells (Figure 4.8A) and 90% of CD8+ T cells (Figure 4.8B). These data show that macrophages expanded from CoCx CM are not passively suppressing proliferation, but exhibit potent cytotoxic activity against activated T cells.
Figure 4.8: Expanded macrophages show cytotoxic activity against activated T cells. Purified T cells were incubated with increasing ratios of autologous, CoCx CM-derived macrophages in the presence or absence of T cell activating anti-CD3/CD28 beads (n=3). T cell numbers were assessed by quantitative flow cytometry at 24 and 48 hours by gating on live A) CD3+/CD4+ T helper cells or B) live CD3+/CD8+ CTLs. The y-axes show cell numbers relative to the T cell only conditions (* p<0.05, **p<0.01).
4.4.7 Hypothetical immune escape mechanism

Together these data indicate an immune escape mechanism where EBV-specific T cells interact with EBV transformed B cells, resulting in the release of cytokines that drive the differentiation of TAMs in the tumor microenvironment. These growth factors and inflammatory signals are launched by aberrant ligand interactions resulting in the polarization of monocytes to an immune suppressive M2-like macrophage. This macrophage population expresses immune checkpoint proteins PD-L1 and IDO-1 and is potently cytotoxic to the activated T cells that initiated the cascade. Transcriptome data indicates these macrophages express cytokines that recruit myeloid cells (CCL2) and T regulator cells (CCL22), which are associated with increased tumor growth\textsuperscript{282, 283} (Figure 4.9).

\textbf{Figure 4.9: Hypothetical immune escape mechanism}. EBV-specific T cells interact with an EBV-transformed B cell resulting in the release of soluble factors that recruit monocytes to the tumor microenvironment and subsequently polarize to an M2-like phenotype. These macrophages express immunosuppressive proteins (PD-L1 and IDO-1) as well as exhibit cytotoxic activity to activated (EBV-specific) T cells. These macrophages also express cytokines that are capable of recruiting more myeloid cells as well as suppressive T cell subsets.
4.4.8 Silvestrol blocks monocyte proliferation

Translation dysregulation contributes to malignant transformation and is therefore an emerging target for cancer therapies. This process is tightly controlled in part by the eukaryotic initiation complex eIF4F, a complex composed primarily of three proteins: 5’ cap-binding protein eIF4E, DEAD box RNA helicase eIF4A and scaffolding protein eIF4G. Silvestrol is a plant-derived natural product identified and characterized by Kinghorn’s group. This agent was shown to block the initiation step of translation by causing an abnormal chemical dimerization of eIF4A with mRNA that results in its sequestration away from the initiation complex. Our group and others reported that silvestrol has direct anti-cancer effects in several diseases including chronic lymphocytic leukemia and mantle cell lymphoma by inhibiting the translation of select cancer genes, particularly those with short half-lives (c-Myc, Mcl-1, cyclin D1). However, in models of EBV-driven lymphoma, our results showed the anti-tumor effects of silvestrol were mediated through the immune system, not through a direct effect of silvestrol on the tumor. As stated above, PBMC+LCL co-cultures treated with silvestrol exhibited a loss of factors involved myelomonocytic activation and recruitment (Figures 4.3A and 4.3B). We therefore examined the effect of silvestrol on the polarization and proliferation of monocytes in CoCx CM. Purified monocytes were incubated in media alone or CoCx CM, treated with a single dose of 0, 1.25, 2.5, 5, or 10 nM silvestrol, and incubated 6 days. Silvestrol directly inhibited the outgrowth of macrophages in a dose-dependent manner (Figure 4.10A). We next examined the temporal expression of proteins within the eIF4F complex during the polarization process, in the absence of silvestrol. Immunoblot analysis of purified monocytes incubated in CoCx conditioned medium for 0,
1, 3, and 6 days showed little modulation of total levels eIF4A and eIF4E during the polarization process. Phosphorylation of eIF4E on Ser209, which has been reported to enhance the translation of cap-dependent transcripts\textsuperscript{285}, went down as a function of time (Figure 4.10B). This observation suggests that efficient translation is of greater importance earlier in the polarization process, consistent with our finding that adding silvestrol after 3 days has little effect on the outgrowth of M2-like macrophages.

**Figure 4.10: Silvestrol blocks monocyte proliferation.** Conditioned media were created by culturing LCL with autologous PBMC. **A** CM was used to culture purified autologous monocytes for 6 days in the presence of a single dose of 0, 1.25, 2.5, 5 or 10 nM silvestrol and proliferation was assessed by MTS (n=3). **B** Purified monocytes were incubated in CoCx CM. Lysates were collected at 0, 1, 3 and 6 days and immunoblotted for the eIF4F translation complex proteins eIF4a (target of silvestrol) as well as phosphorylated and total eIF4E.
4.4.9 IDO-1 inhibition does not block macrophage proliferation

The enzyme indoleamine 2, 3-dioxygenase 1 (IDO-1) is an immune-inhibitory molecule expressed by tumor cells as well as infiltrating myeloid cells. It catalyzes the rate-limiting step of tryptophan degradation, leading to a local reduction in tryptophan concentration. Previous studies suggest that IDO-1 expression by cells in the microenvironment is a mechanism of immune escape, causing cell cycle arrest, anergy and apoptosis of responding T cells. Furthermore, evidence suggests that there is a role of EBV in mediating IDO-1 expression in tumor stroma of EBV+ nasopharyngeal carcinoma. IDO-1 may also play a role in macrophage polarization by increasing the expression of M2 markers in THP-1 cells, while knockdown of IDO expression restores an M1 phenotype. To investigate the expression level of IDO-1 during macrophage polarization, purified monocytes were incubated with autologous CoCx CM and whole cell protein lysates were collected at 0, 1, 3, and 6 days. Freshly isolated monocytes (day 0) exhibited no detectable IDO-1 protein expression. However, by one day in culture significant levels of IDO-1 protein were observed and maintained through 6 days in culture. PMA-differentiated, IFNγ-induced THP-1 cells were used as positive control. To elucidate the potential role of IDO-1 in M2-polarization in this system, monocytes incubated in CoCx CM were treated with 1, 0.5 or 0.25 µM of the IDO-1 inhibitor, 1-methyl-D-tryptophan (1-DMT) at day 0 and day 3. Inhibition of IDO-1 did not affect macrophage proliferation as measured by MTS assay.
Notch blockade does not result in loss of macrophage proliferation.

The Notch signaling pathway regulates the development and function of immune cells and has been implicated in terminal differentiation of both inflammatory and suppressive myeloid subtypes. Ligation of Notch receptors by their ligands leads to proteolytic cleavage by γ-secretase to release the Notch intracellular domain (NICD) which...
translocates to the nuclease where it interacts with the DNA-binding protein, RBP-J, leading to upregulation of target genes\textsuperscript{297, 298}. Recently, it was reported that Notch is an eIF4A-dependent and thus silvestrol-sensitive transcript\textsuperscript{288}. We therefore explored the role of Notch in M2 macrophage polarization in our co-culture system. Monocytes were incubated in media alone or CoCx CM, and lysates were collected at 1, 3, 5 and 7 days. Immunoblot analysis showed that Notch-1 protein levels were increased by incubation in CoCx CM, but not in the media alone condition (Figure 4.12A). Additionally, incubation in PBMC or LCL CM did not lead to as robust Notch-1 protein expression compared to CoCx CM 24 hrs. (Figure 4.12B). As expected, monocytes incubated with CoCx CM in the presence of silvestrol lost Notch-1 protein in a dose-dependent manner (Figure 4.12C). Given the role of Notch-1 during macrophage polarization, we next evaluated Notch signaling in our experimental system. While silvestrol inhibited macrophage proliferation, blocking either Notch-1 or Notch 2/3 receptors with antibodies did not affect proliferation. We next tested the γ-secretase inhibitor $N\{N\{3,5$-difluorophenacetyl\}$L$-alanyl\}$-S$-phenylglycine t$-butyl ester (DAPT), which blocks Notch signaling by preventing its cleavage and is currently the only form of Notch inhibitor in clinical trials\textsuperscript{299}. Addition of silvestrol prevented macrophage proliferation as before; however, inhibition of the Notch signaling by DAPT did not. Analysis of transcripts for Notch-1 target genes HES1 and HEY1 showed no difference between CM from PBMC, LCL, and PBMC+LCL CoCx. Together, these experiments indicate Notch-1 does not play a role in M2 macrophage polarization in this system.
Figure 4.12: Notch-1 blockade does not result in loss of macrophage proliferation: The expression of Notch-1 was assessed by immunoblot in lysates from: A) Purified monocytes incubated in media alone (-) or CoCx CM (+) for 1, 3, 5 or 7 days; B) Purified monocytes incubated 24 hrs in media alone or CM from PBMC, LCL or PBMC+LCL CoCx; C) Purified monocytes incubated in CoCx CM for 24 hrs in the presence of 0, 1, 2.5 or 5 nM silvestrol. D) Proliferation of isolated monocytes incubated 6 days in media alone or CoCx CM with/without silvestrol (sil) or Notch-1 neutralizing antibodies was assessed by MTS assay. E) Proliferation of isolated monocytes incubated 6 days in media alone or CoCx CM with/without silvestrol (sil) or γ-secretase inhibitor DAPT was assessed by MTS assay (representative of two experiments).
4.4.11 Inhibiting IKKε/TBK1 blocks proliferation of macrophages in conditioned media.

To better grasp the molecular changes occurring during the process of macrophage differentiation and polarization in CoCx CM, we conducted a transcriptome analysis using AmpliSeq. After a 24 hr incubation in CoCx CM, monocytes displayed nearly 400 transcripts elevated greater than 10-fold compared with freshly isolated monocytes. Several elevated transcripts had already been confirmed though our immunophenotype analysis such as CD274 (PD-L1) and IDO1 (elevated over 200 and 500 fold, respectively). Several highly elevated transcripts such as CCL22, CCL24 and CCL2 (all elevated over 500-fold by 24 hrs) encode proteins that have been associated with M2 and TAMs.

Interestingly, pathway analysis of our AmpliSeq data pointed to a potential role of cytosolic pattern recognition receptors (PPRs) such as DDX58 (RIG-I) and IFIH1 (MDA5). Inflammation is initiated by PRRs, which sense pathogen-associated molecular patterns (PAMPs) or danger-associated molecular patterns (DAMPs) leading to an immune response to foreign infection or intrinsic stresses, respectively. In cases of continued exposure to PAMPs/DAMPs, infiltrating myeloid cells fail to withdraw and differentiate into M2 macrophages or TAMs. RIG-I-like receptors (RLRs) RIG-I and MDA-5 are cytosolic PRRs that detect viral RNA and trigger type-1 interferon response through though activation kinases TBK1 and IKKε. Furthermore, these kinases phosphorylate and activate interferon-regulator factors (IRFs), which determine gene transcription programs of macrophage polarization.
Transcriptome analysis showed an increase in transcripts of RIG-I, MDA5 as well as their positive regulator DDX60 at day one and continued expression throughout polarization compared with day 0 monocytes. Transcripts of downstream kinases TBK1 and IKKε were slightly elevated, but not to the same extent as the nucleic acid-sensing molecules. TLR3, which can also signal through the same pathway as the RLRs, showed low expression levels on day one compared to fresh monocytes, but increased during the polarization process (Figure 4.13A). Interferon-induced protein with tetratricopeptide repeats (IFIT) transcripts, which are triggered by viral infection, were highly expressed. However, transcripts for type I interferons (IFNB) were not observed (Figure 4.13B). To determine if these PRRs play a role in macrophage polarization, we incubated monocytes in CoCx CM and treated them with silvestrol, the TBK1/IKKε inhibitor BX795, or the endosomal TLR (including TLR3) inhibitor chloroquine. At higher doses, inhibition of TBK1/IKKε with BX795 blocked proliferation of macrophages. However, chloroquine did not impact proliferation compared to cells in media alone. These results together indicate a potential role of RLRs, but not TLRs, in M2 polarization (Figure 4.13C). We therefore investigated the pathway involved in RLR signaling. Cells were incubated in media alone, CoCx CM, or CoCx CM treated with 5 nM silvestrol or 1 µM BX795 for 24 hrs. As expected from the transcriptome analysis, cells incubated in media alone did not express RLRs (RIG-I and MDA5), but these molecules were highly expressed in the CoCx CM conditions (Figure 4.13D). Phosphorylation of downstream kinase IKKε was increased by incubation in CoCx CM and abrogated by BX795, but not silvestrol. Transcription factors IRF3 and IRF7 were increased by incubation with CoCx CM, but not changed by treatment with silvestrol or BX795. Phosphorylation of IRF3 and IRF7, which is mediated by TBK1 and IKKε, was difficult to detect by immunoblot, preventing
conclusions regarding these factors. Together, these experiments indicated a potential role of RLRs in driving TAM differentiation; however, further experiments are necessary to evaluate the role of silvestrol in this pathway.
Figure 4.13: Inhibition of IKKε/TBK1 blocks proliferation of macrophages: Ion Torrent AmpliSeq transcriptome analysis was performed on RNA from purified monocytes incubated with CoCx CM for 0, 1, 3 or 6 days. Shown is a selection of transcripts involved in: A) sensing of viral nucleic acids by RIG-I/MDA5, or B) Interferon-stimulated genes. C) CM was used to culture purified autologous monocytes for 6 days in the presence of media alone, CoCx CM, or CoCx CM with 5 nM silvestrol, 10, 100, or 1000 nM BX-795, or 1, 10 or 100 µM chloroquine. D) Purified monocytes were incubated in CoCx CM. Lysates were collected at 24 hrs and immunoblotted for RIG-I/MDA5 pathway proteins: phospho (ser172) and total IKKε; phospho (ser172) and total TBK-1; phospho (ser396) and total IRF3; phospho (ser477) and total IRF7; MDA5; RIG-I.
4.5 Discussion

EBV is an oncogenic, human γ-herpesvirus that has the capacity to transform epithelial cells and lymphocytes and is associated with the development of nasopharyngeal carcinoma and several lymphoproliferative disorders (LPD) including Hodgkin’s (HL) and non-Hodgkin lymphomas (NHL). EBV-LPD often manifests as opportunistic malignancies that arise in patients with profound immune deficiency, and are associated with poor prognosis. Collectively, EBV-associated cancers are estimated to afflict nearly 200,000 individuals worldwide\textsuperscript{192}. Existing treatment options for EBV-associated cancers are immunosuppressive and increase the risk of potentially lethal opportunistic infections. Currently there is no standard treatment for EBV-associated cancers, demonstrating a significant unmet need for novel preventive and therapeutic approaches. To have lasting efficacy, such approaches must target the tumor’s immune evasion mechanism while promoting anti-tumor immune surveillance. The tumor microenvironment of EBV-associated lymphomas has been characterized as containing a cell infiltrate comprised mainly of tumor-associated macrophages (TAMs). It has been reported that TAMs suppress T cell activation, and increased TAM density and TAM to cytotoxic T lymphocytes (CTL) ratios in the tumor microenvironment have been shown to be negative prognostic indicators in diseases like HL and diffuse large B-cell lymphoma.\textsuperscript{267, 304, 305} To this end, developed an \textit{in vitro} co-culture (CoCx) system in which EBV-transformed lymphoblastoid cell lines (LCL) are cultured with autologous peripheral blood mononuclear cells (PBMC) to recapitulate the immune interactions of the tumor microenvironment. Here we demonstrate cell-free conditioned media from
these cultures has the capacity to polarize monocytes to a suppressive M2-like macrophage, which we can target using the translation inhibitor silvestrol.

Our findings show that interactions between EBV-transformed LCL and autologous PBMC result in the release of growth factors and cytokines associated with recruitment and differentiation of myeloid cells. In particular, increased CCL2 levels in the tumor microenvironment is associated with increased TAM density and neutralization has been shown to inhibit tumor progression and metastasis\textsuperscript{306, 307}. Incubation of isolated monocytes in CoCx conditioned medium, but not conditioned media from PMBC alone or LCL alone, results in cells forming non-adherent proliferating colonies. Significantly, this proliferation occurs in serum-free media, without the addition of exogenous cytokines, and separation of PBMCs and LCL abrogates macrophage proliferation. We show that T cells in co-cultures are both necessary and sufficient for this macrophage proliferation and polarization. Immunophenotyping revealed a homogenous population of large, granular cells expressing M2 macrophage markers. Together, these results indicate the interaction of T cells with LCL results in release soluble factors capable of activating and polarizing monocytes to a suppressive M2-like macrophage.

TAMs generally have an M2-like phenotype that is capable of suppressing the anti-tumor immune response. We therefore examined functional activity by incubating T cells alone or with autologous M2 macrophages derived from CoCx conditioned media. Remarkably, quantitative flow cytometric analysis showed the macrophages displayed potent cytotoxic activity against T cells activated by CD3/CD28 bead stimulation and EBV-specific T cells, but not non-activated T cells. These results suggest a novel immune escape mechanism in which LCLs, when recognized by T cells, secrete factors
that recruit circulating monocytes that are then polarized to an M2-like macrophage with potent cytotoxic potential against activated T cells. Thus, we hypothesize this may account for the poor prognosis associated with an increased ratio of TAM to CTL in tumor infiltrates of EBV-associated lymphomas.

Silvestrol is a translation inhibitor with powerful anti-cancer activity \textit{in vitro} and \textit{in vivo}. Silvestrol inhibits eIF4A RNA helicase activity, preferentially disrupting the translation of mRNAs containing highly structured 5’ UTR motifs. While silvestrol inhibits tumor cell growth directly, we recently showed that its most striking anti-tumor effects are mediated through its impact on adaptive immunity. Here, silvestrol-treated CoCx conditioned medium was unable to drive proliferation or expansion of M2 macrophages. This observation suggests that selective targeting of the eIF4F translation initiation complex can block TAM formation in the setting of EBV-driven lymphoma. Interrogation of the temporal expression of eIF4F proteins indicated stable levels of eIF4A and eIF4E. However, phosphorylation of eIF4E, which is associated with efficient translation initiation, went down as a function of time, demonstrating the importance of translation early in polarization. However, it should be noted that while phosphorylation of eIF4E has been implicated in tumorigenesis, its role in normal translation is under debate\textsuperscript{308}. Furthermore, our data do not show that translation inhibition is necessarily the mechanism by which silvestrol blocks the outgrowth of immune suppressive macrophages. Multiple studies have failed to identify a cellular target of silvestrol other than eIF4A\textsuperscript{309}; however, the related agent rocaglamide was reported to inhibit MAPK signaling via its effect on prohibitins\textsuperscript{310}. Thus we cannot rule out a translation inhibition-
independent function of silvestrol in this context, and further mechanistic studies are needed to answer this question.

To better understand the mechanism of polarization we began by looking the role of IDO-1 in our system. IDO-1 has been shown to suppress T cell function in EBV-driven cancers and been implicated in M2 macrophage polarization. Incubation of monocytes in CoCx conditioned media showed marked increase in IDO-1 expression by one day, further exemplifying the suppressive nature of these macrophages. However, inhibition of IDO-1 by tryptophan analog 1-DMT did not block the proliferation of macrophages in culture, indicating IDO-1 is not the mechanism of polarization. Similarly, Notch-1 signaling has been implicated in tumor-associated macrophage polarization, and is a eIF4A-dependent, and therefore silvestrol-sensitive target. Notch-1 protein levels become elevated early and remain elevated throughout the polarization process and were lost with silvestrol treatment. Surprisingly, neutralization of Notch receptors or blockade of notch signaling by γ-secretase inhibitors did not show a loss of proliferation. Furthermore, Notch-1 target gene transcripts were not elevated in CoCx conditioned media compared with normal monocytes. We therefore concluded that Notch-1 signaling is not the driver of M2 polarization in this system. Finally, transcriptome analysis showed an increase in RIG-I-like receptors (RLRs). Several studies have shown EBV-encoded small RNA (EBER) trigger inflammation via RLRs, making them a reasonable target for further analysis. RLR-induced gene transcripts (IFITs) were increased and inhibition of downstream kinases IKKɛ and TBK1 blocked proliferation of monocytes incubated in CoCx conditioned medium. Endosomal TLR may also signal through IKKɛ and TBK1. However, inhibition by choloquine did not inhibit proliferation, further
implicating RLR signaling. Protein analysis showed an increase in RLRs and IRFs when exposed to CoCx conditioned media; however silvestrol did not modulate these proteins. It is therefore difficult to conclude if silvestrol has a direct effect on the RLR pathway or if it is acting through an independent mechanism. Further studies are necessary to assess the role of RLR signaling in M2 polarization.

In summary, we have identified an immune evasion mechanism by which direct contact of T cells with EBV+ lymphoma cells results in release of soluble factors that drive expansion of a TAM-like macrophage population with potent cytotoxic activity against activated T cells. Low-dose silvestrol treatment abrogates the outgrowth of this TAM-like population and allows expansion of EBV-specific CTL. This finding provides a new approach to modulate the immune response in this challenging group of EBV-related diseases.
Chapter 5: Discussion and directions

Epstein-Barr virus (EBV) infects more than 90% of individuals worldwide and is responsible for a diverse spectrum of diseases. These diseases are normally held in check by host immunity. However, certain events cause the loss of immune control, allowing the virus to hijack cellular machinery, bypassing normal growth controls. These diseases are typically aggressive and exhibit poor clinical outcomes in an already-vulnerable patient population. Currently there is no standard treatment for EBV-associated lymphomas, demonstrating a significant unmet need for novel preventative and therapeutic approaches. Traditional therapies for lymphoma including chemotherapy and immunotherapy kill cancer cells, but are also toxic to normal host immunity. When one considers that immunosuppression is a driver of these lymphomas, it is unsurprising these therapies can result in reactivation of the virus and lead to often fatal opportunistic infections. An alternative strategy is cancer immunotherapy, which seeks to harness the immune system to eradicate tumors, and has already displayed impressive results in cancers numerous including PTLD. The central hypothesis of this dissertation is that EBV-driven lymphomas are likely to be vulnerable to immunotherapy, as they express viral proteins that are absent in healthy tissue and thus are recognized as "non-self" by the immune system. To exploit this vulnerability, this project was designed with the overarching goal of enhancing immune-based therapies against EBV cancers.
Following primary infection, B cells enter a viral growth program (latency III) that is characterized by expression of proteins mimicking B cell proliferation and survival signals that have oncogenic potential \(^{27}\). Typically cells expressing these proteins are quickly recognized by CTLs, thus eliminating the infected cells\(^{144}\). The critical role of CTLs in containing the virus is exemplified in PTLD patients who develop disease as a result of the T cell-suppressive drugs given to prevent graft rejection. A reduction in these immunosuppressive regimens can reestablish EBV-specific CTL surveillance and effectively resolve the disease, however, up to 40% of these patients will lose the graft\(^{163}\). Therefore, the results of aim one, identifying treatments either targeting the cancer-inducting viral proteins or promoting anti-viral CTL activity, would have a profound effect on eliminating the disease.

With this in mind, our group previously showed the translational inhibitor, silvestrol, selectively targets B cell cancers while sparing normal immune cells by blocking the production of certain cancer-promoting proteins\(^{203}\). Silvestrol inhibits eIF4A RNA helicase activity, preferentially disrupting translation of mRNAs containing eIF4A-dependent motifs\(^{204}\). Together, this provided rationale to examine the effects of silvestrol on EBV-transformed lymphoblastoid cell lines (LCLs). When used at physiologically achievable concentrations, we observed a remarkable decrease in the main cancer-promoting protein LMP1. While these concentrations were not directly cytotoxic to LCL, when added to co-cultures of LCL and autologous PBMC, we observed a complete ablation of the malignant cell lines. The loss of LCLs was concomitant with expansion of CTLs and NK cells in culture. This low dose of silvestrol did not affect antibody-dependent cellular cytotoxicity of NK cells or the direct cytotoxic function of CTLs. CTLs
retained the ability to recognize viral peptides and, while not statistically significant, trended towards a decrease in the T cell inhibitory molecule CTLA-4. To examine this phenomenon more rigorously, we employed an established mouse model of EBV PTLD\textsuperscript{205}. Strikingly, mice with EBV-driven lymphoma that were treated with silvestrol survived and exhibited no signs of disease, without discernable toxicity, while control-treated mice either succumbed to disease or showed significant signs of lymphoma upon analysis. Survival is extremely rare in this model using standard treatments, underscoring the potential importance of this finding. Interestingly, silvestrol lost its effectiveness when these mice were depleted of CTLs, demonstrating that this potent anti-tumor effect occurs through the immune system and not through a direct effect of silvestrol on the tumor.

We went on to investigate the mechanism by which silvestrol facilitates immune-mediated protection. From our previous results, we hypothesized that co-cultures treated with silvestrol would alter the release of soluble factors responsible for anti-tumor T and NK cell activation, however, our results showed a profound modulation of growth factors and chemokines responsible for myeloid activation and recruitment.

It is becoming increasingly clear that the interplay between immunity and disease is far more complex than initially thought. There are situations where EBV-induced immunity actively promotes cancer development rather than contain it. This is particularly well described by the association of IM to Hodgkin’s disease, but certainly plays a role in other diseases\textsuperscript{14}. This chronic inflammatory signals associated with long-term infection, can result in the recruitment of macrophages, which generally play a role a pro-tumoral
role within the microenvironment by suppressing anti-tumor CTL\textsuperscript{264}. These tumor-associated macrophages (TAMs) are associated with a worse prognosis in several types of lymphoma\textsuperscript{206}. These data therefore provided the basis of the second aim of this dissertation, to identify and target inflammatory based mechanisms by which EBV-driven cancer evade immune recognition.

The use of our \textit{in vitro} co-culture system provides conditions that more accurately mimic the tumor microenvironment \textit{in vivo}. The direct interaction between tumor and host cells results the release of factors into the millieu that promote the differentiation of normal monocytes into an M2-like macrophage expressing immunosuppressive molecules such as PD-L1 and IDO-1, consistent with a TAM phenotype. Functional examination revealed these macrophages have potent cytotoxic activity against activated, but not resting T cells. Together these data indicate a novel immune escape mechanism where virus-specific T cells interact with EBV-transformed B cells resulting in the release of cytokines that recruit circulating monocytes to the site of tumor. Within the tumor microenvironment, growth factors and inflammatory signals, launched by aberrant ligand interactions, result in a polarization of recruited monocytes to an immune suppressive M2-like macrophage.

Though the mechanisms are not yet fully understood, the addition of 5 nM silvestrol blocks the proliferation and differentiation of monocytes into immune suppressive macrophages. Given the role of EBV specific proteins in aiding in the immune evasion process, evaluating the virus’s contribution in driving this TAM phenotype is of particular interest. Many TAMs in EBV-related tumors are infected with the virus. Therefore, it is
possible EBV-related inflammation plays a role in polarization though aberrant activation of PRRs\textsuperscript{139}. This may be the explanation for the loss of macrophage proliferation when inhibiting the RIG-I-like receptor pathway. While additional work is needed to fully elucidate the mechanism of differentiation, these finding indicate that selective targeting of the eIF4F translation initiation complex, which includes eIF4A, may block TAM formation in the setting of EBV-driven lymphoma.

The work outlined here shows the ability of silvestrol to target two facets of immune escape by re-establishing CTL control and abrogating suppressive immune subsets. Taken together, these two attributes justify further exploration of silvestrol in combination immunotherapy. One example is combination with adoptive T cell therapy using EBV-specific donor lymphocytes\textsuperscript{172, 312}. Even with the re-establishment of CTL surveillance, this treatment fails to address the underlying local immunosuppression that blocks the anti-tumor response\textsuperscript{266, 267}. Trials relating TAM infiltration to efficacy of adoptive cell therapy are lacking, however, ancillary data suggests they play a role. First, TAM density correlates with worse prognosis in EBV lymphoma\textsuperscript{313}. Secondly, several studies have shown inhibition of TAM recruitment (by anti-CFS-1R) or blockade of the PD-1/PD-L1 axis significantly improves antitumor efficacy of adoptive cell transfer\textsuperscript{314, 315}. Co-treatment with silvestrol could effectively promote anti-tumor CTL activity while removing the immunosuppressive brakes. Silvestrol may be preferable over other combination immunotherapies as it affects cells expressing several immunotherapeutic targets including checkpoints (CTLA-4, PD-L1 and IDO) and myeloid secreted factors (CFS-1 and CCL2). Furthermore, as noted earlier, NK cells play a significant role in controlling acute EBV infection. We observed a marked expansion of functionally active NK in co-
cultures treated with silvestrol. Therefore, future studies should focus on NK-based immunotherapies combined with silvestrol such as adoptive NK transfer or CAR-NK therapies.316

Finally, our data suggest silvestrol may be a powerful adjuvant in the development of a prophylactic EBV vaccine. As described in detail in section 1.6.5, to date most EBV vaccine efforts have focused on mechanisms to prevent viral infection, all of which have been unsuccessful. Smaller trials have focused on harnessing vaccination to enhance CTL-mediated immunity to EBV to eradicate malignant cells.192 These trials suggest that a therapeutic vaccination could be effective, but they have focused on a very limited set of EBV proteins. However, our group and others have shown that lytic proteins only expressed in cells with actively replicating viruses elicit a strong immune response and are good candidates for vaccine development.317 We have access to a novel delivery platform to target EBV proteins directly to the endocytic receptor DEC205. We hypothesize that delivering EBV proteins to DCs will promote the expansion of CTLs that have the capacity to recognize and kill EBV lymphoma. This strategy is similar to EBV-specific adoptive T-cell therapy, however, our strategy has enormous advantages as currently only a handful hospitals are approved for this treatment and it requires the extremely expensive and laborious preparation of EBV-specific T cells. Our preliminary data shows that vaccinating DC with a lytic EBV protein (BZLF1) targeting DEC205 elicits a much stronger tumor-specific response, as measured by the expansion of tumor-specific CTLs, than adding the protein alone (rBZLF1) to DCs. We propose to test other EBV proteins using this vaccine platform to elicit a robust and tumor-specific CTL response. Furthermore, our preliminary experiments also indicate that low dose silvestrol
promotes an EBV-specific CTL expansion. We hypothesize that using this vaccine technology to target EBV antigens to DCs in the presence of silvestrol will promote a tumor-specific response while simultaneously driving immune-mediated kill of the tumor and enhancing CTL function by abrogation of the immune suppressive TAMs.

No discussion would be complete without a critical appraisal of the research. Despite the overall strength of this work, some areas could have been bolstered by further experimental validation. In particular, while others have demonstrated silvestrol disrupts eIF4A RNA helicase-dependent translation, we do not directly demonstrate this is the mechanism underlying silvestrol’s activity in our model systems\textsuperscript{266, 318}. Ideally this would have included ribosome footprinting analysis. In brief, silvestrol or vehicle control samples are subjected to nuclease digestion followed by RNA sequencing (RNA-seq). Ribosome-protected RNA fragments (actively translated transcripts) are then compared to total transcript levels revealing changes in translation as a result of treatment\textsuperscript{319}. This technique would be useful to identify gene products whose translation is most effected by silvestrol treatment in both tumor cell lines and co-culture conditioned media-derived macrophages. These experiments would have the potential to elucidate not only the mechanisms of silvestrol activity, but also the potential to identify new therapeutic targets. On a more limited scope, polysome isolation followed by generation of a cDNA library and quantitative PCR could be useful to confirm the effects on translation of transcripts such as LMP1. However, indirect evidence indicated that translation inhibition was most likely the reason we saw a decrease in LMP1 protein following silvestrol treatment. Quantitative PCR analysis of LMP1 transcripts showed a moderate decrease in mRNA levels with treatment, but, a complete ablation of protein levels by 72 hrs.
The hu-PBL-scid mouse model is a powerful tool to identify therapeutic strategies to treat spontaneous EBV-LPD. Treatment of these mice with silvestrol significantly prolonged survival. In fact, all of the mice treated with silvestrol survived and did not exhibit signs of lymphoma at the end of study. While this in and of itself is an impressive result, these experiments could have been strengthened by the addition of multiple donor engraftments. The experiments as described here were carried out by engrafting PBMC from one healthy EBV-positive donor into a group of SCID mice. Ideally, instead of a single donor, PBMC from ten pre-identified donors would be used to engraft two mice per donor. One animal from each donor engraftment would be randomly assigned to each treatment arm, which would increase the genetic diversity and relevance of the treatment outcome.

Though the experiments outlined above would undoubtedly reinforce the findings of this work, it nonetheless makes a substantive contribution towards developing new therapeutic strategies to treat EBV-driven lymphomas. Our group had previously shown silvestrol selectively targets malignant B cells in CLL and AML. However, these studies focused on resting lymphocytes; here we explored the effects on activated cells in a model of EBV-driven lymphoma. This distinction is important since the stimulation of resting T cells results in a marked increase in the rate of protein synthesis. However, we showed that silvestrol has minimal effects activated, antigen-specific lymphocytes while sensitizing tumors to immune-mediated death. Our animal model bolstered these findings, showing CTLs are necessary for the activity of silvestrol. Together these finding indicate silvestrol, or other translation initiation inhibitors, may have a positive impact
when used with cellular immunotherapies such as adoptive T or NK cell therapy or CAR therapy.

Secondly, this dissertation describes the development of a novel autologous co-culture system mimicking the cellular interactions of the tumor microenvironment to generate conditioned medium. These conditioned media have allowed us to better understand the microenvironmental effects on myeloid populations, specifically, the differentiation and polarization of monocytes to suppressive M2-like macrophages. While many pre-clinical studies use exogenous cytokines to polarize macrophages, our system uses only the soluble factors produced through the tumor-immune interaction, and therefore more relevant to the disease. This model could potentially be useful in the development of strategies specifically targeting the outgrowth of TAMs in other cancer types. Lastly, while we do not yet fully understand the mechanism by which silvestrol inhibits the outgrowth of suppressive macrophages, the completion of this work will provide insight into therapeutic targets to prevent TAM polarization. Cancer immune evasion is a major obstacle to effective immunotherapeutic strategies and therefore, targeting these mechanisms will result in more effective treatment of these diseases.

In conclusion, this dissertation addresses several important aspects of EBV infection and biology that are highly relevant for cancer immunotherapy, including targeting oncogenic viral proteins, reestablishing EBV surveillance and ablation of suppressive immune subsets. We have not only characterized key mechanisms for immune-mediated killing of EBV driven cancers, but also improved our understanding of the complex interactions between host and virus that drive oncogenesis. Together, this work provides transnationally relevant information that will lead to novel immunotherapeutic
approaches providing positive and widespread impact on individuals suffering from EBV lymphomas.


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