ABSTRACT

Effects of the Induction of Pyroptosis via Topoisomerase II Inhibition in Human Papillomavirus-Associated Vaginal and Oral Cancers on Antigen-Specific Immunity

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Human papillomavirus (HPV) is the most common sexually transmitted infection worldwide, and infection with HPV may lead to several cancers. Topoisomerase II is overexpressed in HPV-positive cancers; thus, its inhibition may differentially target cancer cells. In certain cancer cell lines, topoisomerase II inhibition was found to induce pyroptosis—a Gasdermin-mediated form of cell death characterized by pore formation in cell membranes and release of cellular contents that may function as immunostimulants and neoantigens. This study sought to determine whether topoisomerase II inhibition induces pyroptosis in HPV-positive cancers and stimulates HPV-antigen-specific immunity. TC-1 and mEER cells, two established preclinical models of HPV-positive vaginal and oral cancers, respectively, were treated *in vitro* with two topoisomerase II inhibitors, etoposide and mAMSA. Western blotting was used to detect markers of pyroptosis. Splenocytes from mice given HPV-oncoprotein peptide vaccines served as sources of antigen-specific T cells and were cocultured with conditioned media from topoisomerase inhibitor treatments. Flow cytometry was used to analyze immune activity. Western blotting revealed the presence of markers of pyroptosis or immunogenic cell death in both cell lines treated with topoisomerase inhibitors. Flow cytometry analysis revealed some significant relationships in treatment with topoisomerase inhibitors and antigen-specific immune activation. The findings indicate that topoisomerase II inhibition may be a promising therapeutic option for HPV-positive cancers.

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# EFFECTS OF THE INDUCTION OF PYROPTOSIS VIA TOPOISOMERASE II INHIBITION IN HUMAN PAPILLOMAVIRUS-ASSOCIATED VAGINAL AND ORAL CANCERS ON ANTIGEN-SPECIFIC IMMUNITY

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# CHAPTER ONE

## Introduction

Human papillomavirus (HPV)-attributable cancers represent a massive global health concern. An understanding of the pathophysiology of HPV infection and progression to cancer is necessary in identifying therapeutic targets: HPV relies on host cell replication machinery, and infected cells therefore overexpress genes involved in DNA replication and metabolism, including topoisomerase II. Topoisomerase II inhibition induces pyroptosis in many cancer cell lines. Pyroptosis is form of cell death mediated by Gasdermin family proteins characterized by pore formation in the cell membrane and release of proinflammatory signals and neoantigens, leading to inflammation and immune activation, which can further aid in eliminating tumor cells. However, it is unknown whether pyroptosis can induce HPV-antigen-specific immunity.

### *Human Papillomavirus and Cancer*

Human papillomavirus (HPV) is a group of over 200 small, double-stranded DNA viruses that belong to the *Papillomaviridae* family (National Cancer Institute 2019; Kombe Kombe et al. 2021). HPV is the most common sexually transmitted infection in the world and may be transmitted through vaginal, anal, or oral sex, during passage through the birth canal, or possibly via fomites (National Cancer Institute 2019; Hellner & Münger 2011). In their lifetimes, sexually active individuals will be infected with HPV at least once (Kombe Kombe et al. 2021). Significant risk factors for HPV infection include first sexual encounter at an early age, high number of pregnancies, high number

of sexual partners, long-term use of oral contraceptives, tobacco use, and marriage at an early age (Vinodhini et al. 2011). HPVs may be classified as low risk and high risk. Low-risk HPV types generally do not cause disease, although some types cause warts at the site of infection. There are 14 types of high-risk HPVs which can cause many types of cancer (National Cancer Institute 2019; Hellner & Münger 2011). Long-term infection with high-risk HPVs can cause cancer at the site of infection, including the cervix, oropharynx (including the oral, tonsil, and throat areas), vagina, vulva, penis, and anus. HPV infects the squamous cells lining the surfaces of these organs; thus, many cancers associated with HPV are classified as squamous cell carcinomas. However, HPV may also infect glandular cells of the cervix, leading to adenocarcinoma (National Cancer Institute 2019). Due to the high prevalence of HPV infection, preventing and treating HPV and its associated cancers is vital for the global health community.

*Epidemiology*

The distribution of HPV infection varies significantly around the world. Several geographic, socioeconomic, and cultural factors as well as intrinsic individual factors including age, gender, site of infection, and health status affect the morbidity and mortality associated with HPV infection. While there are several prophylactic HPV vaccines currently available and approved for reducing infection and HPV-related disease incidence, the burden of HPV-related disease, including cancer, remains high (Vinodhini et al. 2011). Between 2000 and 2011, overall prevalence of HPV in 576,281 women with normal and abnormal cervical cytology worldwide was estimated to be 32.1% (Vinodhini et al. 2011). The prevalence of HPV infection in men globally is similar to that in

women. In women, HPV prevalence is generally highest among adolescents and women under the age of 25, whereas in men, prevalence varies little with age (Kombe Kombe et al. 2021). Prevalence of HPV infection is higher in low- and middle-income countries than in more developed regions of the world (Hellner & Münger 2011). The prevalence of each type of HPV, including low-risk and high-risk types, varies around the world by age, gender, race, vaccination status, and several other factors, which carries important implications for the prevalence of disease-associated HPV, including HPV-associated cancers.

In 2008, of the approximately 12.7 million cancers occurring worldwide, 610,000 cases were attributable to HPV infection. Of the HPV-attributable cancers, cervical cancer accounted for 530,000 cases, and anogenital cancers including vaginal, vulvar, penile, and anal cancers and head and neck cancers accounted for the remaining 80,000 cases (Forman et al. 2012). HPV is the cause of virtually all cervical cancers (National Cancer Institute 2019). Cervical cancer accounted for 83% of HPV-attributable cancer in 2012, with two-thirds of cases occurring in low- and middle-income countries. Other HPV-attributable cancers include anogenital cancers and head and neck cancers. In 2012, 8,500 vulvar cancers, 12,000 vaginal cancers, 35,000 anal cancers, and 13,000 penile cancers were found to be attributable to HPV infection, and in the head and neck, 38,000 cancers could be attributed to HPV infection. Of these head and neck cancers, 21,000 were oropharyngeal cancers occurring in high-income countries (de Martel et al. 2017). In the United States, most oropharyngeal cancers (70%), anal cancers (over 90% penile cancers (over 60%), vaginal cancers (75%), and vulvar cancers (70%) are caused by HPV (National Cancer Institute 2019). The incidence of HPV-attributable oropharyngeal

cancer incidence in the United States has grown steadily over the past five decades and has surpassed cervical cancer as the most frequently diagnosed HPV-attributable cancer (LeConte et al. 2018). Though progression from HPV infection to cancer is uncommon, HPV-attributable cancers have remained prevalent throughout the world.

*Pathophysiology of HPV Infection and Cancer Progression*

HPV infects cutaneous and mucosal epithelia (Graham 2017); HPV has evolved so that its life cycle may contend with the differentiation of cells within stratified epithelia (Moody 2017). HPVs generally infect basal epithelial cells (the layer of undifferentiated cells adjacent to the basement membrane). There, they establish their genomes in the nuclei by producing numerous copies of their episomes using the host cells' own replication machinery, which is abundant in proliferating basal cells (Moody 2017; Longworth & Laimins 2004). As the basal cells divide, infected daughter cells may begin the differentiating and advancing through the suprabasal layer toward the epithelial surface, triggering changes in viral gene expression resulting in a productive infection. HPV infection, even by high-risk types, is not sufficient to cancer formation. Though most individuals will experience infection by a high-risk type of HPV during their lifetimes, they are generally detected and cleared by the immune system over several months. Therefore, such transient infections will not progress to clinically significant disease or cancer. However, HPV infection may become persistent due to mutations in the viral genome or the genome of the host cell. Progression to cancer occurs when the infection is not recognized and cleared by the immune system, and the expression of viral

oncoproteins is increased leading to cell cycle activation, inhibition of apoptosis, and accumulation of DNA damage in host cells (Graham 2017).

All types of HPV possess and episomal double-stranded DNA genome which is approximately eight kilobytes in size. The genome contains three functional sections: the early (E) region, the late (L) region, and the long control region (LCR), also known as the upstream regulatory region (URR). The early region encodes at least seven viral proteins—E1, E2, E4, E5, E6, E6, and E8—which have several regulatory functions in infected cells. The late region encodes the structural proteins of the virus—L1 and L2. The viral capsid is comprised of 360 copies of the L1 protein with one copy of the L2 protein at the center. The long control region contains regulatory sequences which control viral DNA replication, transcription, and post-transcriptional control (Graham 2017).

HPV can bind receptors on the epithelial cell surface or enter the epithelium through microabrasions and bind receptors on the basement membrane or on the surface of basal layer cells. Virions may bind heparin sulfate proteoglycans (HSPGs) through interactions with the L1 capsid protein. Upon binding, the viral capsid undergoes a conformational change leading to the exposure and cleavage of the L2 protein, which can then bind to a secondary receptor. The virus then enters the cell via endocytosis (Raff et al. 2013). After entering the cell, the virus is trafficked to the nucleus, where it may enter via nuclear pores or during mitosis after the breakdown of the nuclear membrane. Once it has reached the nucleus, the L1 capsid is disassembled and the L2 protein and viral genome associate with nuclear bodies (Graham 2017). Following HPV infection, the viral replication and transcription factors E1 and E2 are the first viral transcripts to be detected. The E1 and E2 proteins are necessary in replicating viral DNA and modulating

transcription (Ozbun 2002). Early expression of the viral transcription factor E2 also allows correct regulation of the viral promoter necessary in directing expression of the E6 and E7 regulatory proteins, which ensure continued survival of infected cells (Graham 2017).

Upon entering the nucleus, the viral E1 and E2 proteins along with host cell replication machinery carry out replication of the viral genome. This transient round of replication, also known as "establishment replication", results in between 50 and 100 copies of the viral genome in each cell. These viral episomes are thereafter maintained in number within the undifferentiated basal cells by replicating along with the cellular chromosomes (Moody 2017; Longworth & Laimins 2004). The expression of viral proteins remains low in infected basal cells due to repression of transcription by the viral E2 protein, which inhibits access of transcription factors to the viral p97 promoter and alters the conformation of the viral genome (Graham 2017). When an infected basal cell replicates, one daughter cell migrates toward the suprabasal layer, carrying the viral genomes, and begins differentiation (Longworth & Laimins 2004). Upon differentiation, the productive phase of the HPV life cycle is activated, leading to the amplification of viral genome replication to thousands of copies per cell and activation of expression of the late genes necessary for the generation and release of progeny virions (Graham 2017; Moody 2017). The low levels of viral genome replication and gene expression allow the virus to remain undetected within the basal layer, and amplification of viral gene expression and virion production are limited to the uppermost epithelial layers, which are not heavily surveilled by the immune system. This regulation of the life cycle of HPV

allows the virus to evade immune detection, permitting infection to last several decades (Moody 2017; Longworth & Laimins 2004).

Within undifferentiated basal cells, HPV relies on the readily available host DNA replication machinery to produce episomal copies because its genome does not encode the factors required to replicate its own DNA. Differentiation of epithelial cells normally leads to their exit from the cell cycle, which limits the availability of replication machinery. Yet, epithelial differentiation is necessary for entry to the productive phase of the viral life cycle. Therefore, HPV must push differentiating cells back into the cell cycle in order to amplify viral genome replication, gene expression, and virion assembly and generation (Moody 2017). This is accomplished through a number of mechanisms. The viral E7 protein binds and inactivates proteins of the retinoblastoma family (pRb) and other proteins (p107 and p130) which regulate entry into the S-phase of the cell cycle (and therefore entry of differentiated epithelial cells into the cell cycle). pRb, p107, and p130 form an inhibitory complex containing the E2F transcription factor, which activates the transcription of several genes related to the cell cycle (Graham 2017; Moody & Laimins 2010). Once E7 binds pRb, p107, and p130, the E2F transcription factor may constitutively activate transcription of genes encoding the factors necessary for the transition from the G1-phase to the S-phase of the cell cycle (Graham 2017). E7 activates the cell cycle in epithelial cells that otherwise would have undergone terminal differentiation and thereby maintains the host cells' capacity to replicate viral DNA. The viral E5 protein also contributes to productive viral replication by binding B cell receptor-associated protein 31 in the endoplasmic reticulum, allowing for control of protein trafficking,  by binding ATPases within endosome to control the turnover of

epidermal growth factor receptors and thereby permitting constitutive activation of receptor tyrosine kinase signal transduction networks, including growth factor signaling pathways, and stimulating protein kinase activity involved in these pathways (Graham 2017; Moody & Laimins 2010). The viral E4 protein also activates growth factor signaling by stimulating protein kinase activity and facilitates in sustaining a G2-arrested environment in host cells upon differentiation, which further increases the cellular capacity for viral replication (Graham 2017).

The actions of the viral E4, E5, and E7 proteins are necessary in keeping differentiated epithelial cells active in the cell cycle and thus allowing viral replication, gene expression, and virion generation and release. In addition, along with the viral E6 protein, they may contribute to cancer progression. By contributing to unscheduled S-phase reentry, E4, E5, and E7 lead to an increase in p53 expression, which, under normal circumstances, leads to cell cycle arrest in the G1-phase or apoptosis. However, E6 stimulates ubiquitinylation and proteasome-dependent degradation of the tumor suppressor protein p53, allowing the cell to avoid cell cycle arrest or programmed cell death as well as blocking the negative effects of p53 on productive viral replication (Moody 2017; Moody & Laimins 2010). Moreover, E6 activates telomerase expression, which is necessary in immortalizing the host cell. Together, E6 and E7 generate chromosomal instability through several mechanisms, such as inducing abnormal centrosome replication and interfering with histone deacetylases, resulting in mutations and damage to the host cell's genome, and affect cytokine expression, allowing for control of cell proliferation and contributing to evasion of immune detection (Moody & Laimins 2010). The vital change needed to progress from HPV to cancer is increased

expression of the viral E6 and E7 oncoproteins in dividing epithelial cells. The activity of the E6 and E7 oncoproteins stimulates cell growth, inhibits apoptosis, and induces genomic instability, which together result in tumorigenesis (Graham 2017). Furthermore, these oncoproteins have been indicted in promoting angiogenesis and inducing characteristics of cancer involved in the process of epithelial to mesenchymal transition, which leads to malignancy and metastasis (Hellner & Münger 2011).

*Current Treatments and Potential Therapeutic Targets for HPV-Positive Cancers*

For cervical and oropharyngeal cancers, current standard treatments involve surgery, radiation, chemotherapy, or a combination of these therapies, and immunotherapies are newer options for advanced, recurrent, or metastatic cancers. However, the decline of the mortality rate for cervical cancer following the introduction of screening measures has slowed to less than 1% over the past decade, while the mortality rate for oropharyngeal cancers has increased due to rising numbers of deaths from cancers driven by HPV (American Cancer Society 2021). Still, patients with oral and oropharyngeal cancers generally receive the same treatment regimens regardless of HPV status (Kim et al. 2018). In light of this and that many current treatments for HPV-positive cancers cause systemic toxicities, leading to significant detriments to quality of life, more effective, targeted treatments must be pursued.

The fundamental aim of cancer treatment is to target cancer cells while leaving healthy cells undamaged. Given the foreign nature of HPV, there are several potential therapeutic options that would allow for sensitive and specific targeting of cancer cells, including interference with E6 and E7 oncoprotein expression using antisense and RNA interference approaches; identification of small molecules that interfere with the

association of E6 or E7 proteins with their cellular targets, such as the p53 or pRb tumor suppressors; targeting cellular enzymes that are redirected by E6 and E7 expression, including protein kinases, histone deacetylases, and components of the ubiquitin-proteasome system—the latter is particularly appealing because inhibitors are already in use as cancer therapies; and inhibition of cellular pathways and signal transduction network that are disrupted by viral oncoprotein expression, such as protein trafficking and receptor tyrosine kinase and growth factor signaling (Hellner & Münger 2010). In addition, the E6 and E7 oncoproteins are ideal foreign antigen targets for therapeutic vaccination. Their sustained expression drives HPV-positive cancers, as they are necessary and sufficient for malignant transformation, and they are only expressed in infected cells but are present both in premalignant lesions and cancers (Hellner & Münger 2010, Tampa et al. 2020). Moreover, while immune checkpoint inhibitor therapies alone have resulted in tumor regression in only a minority of patients, they have shown promising results in combination with other therapies, including peptide vaccines targeting the HPV E6 and E7 oncoproteins (Massarelli et al. 2019).

Additional therapeutic targets for HPV-positive cancers may be identified based on differences among HPV-positive and negative cancers. For instance, a study conducted in 2018 determined through differential gene expression analysis that in contrast to HPV-negative head and neck cancers, many genes were overexpressed in HPV-positive head and neck cancers, specifically genes involved in DNA replication and DNA metabolic processes. This is unsurprising given that HPV depends on its host's replication machinery and manipulates differentiated epithelial cells to maintain their capacity for DNA replication. Among the overexpressed genes were thymidylate

synthase, which is involved in the metabolism of the nucleotide thymidine, and topoisomerase II, which relieves or induces supercoiling and is essential in processes such as DNA replication, transcription, chromosomal segregation, and chromatin remodeling. Additionally, both thymidylate synthase and topoisomerase II are well-known targets with clinically established inhibitors. The findings of the study were verified by transfecting HPV-negative oral cancer cells with E6 and E7 oncogenes; increased expression of thymidylate synthase and topoisomerase II was found in all cell lines following transfection. In addition, two thymidylate synthase inhibitors and one topoisomerase II inhibitor, etoposide, were shown to have greater cytotoxicity in HPV-positive than HPV-negative cancer cell lines (Kim et al. 2018).

### *Topoisomerase Function and Inhibition*

DNA topoisomerases solve topological problems associated with DNA replication, transcription, recombination, and chromatin remodeling by catalyzing the formation of transient breaks in the DNA to relieve or induce supercoiling. There are several types of human topoisomerases, which are classified by their substrate—including DNA, RNA, and the mitochondrial genome—and mechanism (Pommier et al. 2016). Type I topoisomerases catalyze the creation and ligation of single-stranded DNA breaks independent of ATP, whereas type II topoisomerases create double-stranded DNA breaks in an ATP-dependent manner (McKie et al. 2021). There are two isoforms of topoisomerase II, TOP2α and TOP2β. TOP2α is necessary in DNA replication and chromosomal segregation during mitosis, and its expression is regulated by the cell cycle. TOP2β is necessary for normal development and important in transcriptional regulation but is not essential in certain types of cells. Because of their roles in DNA replication,

transcription, chromosome segregation, and maintaining genomic stability, topoisomerase II is heavily involved in both cancer prevention and survival of cancer cells (Nitiss 2009).

Topoisomerase II (TOP2) emerged as a therapeutic target when it was discovered that active anti-cancer drugs, including etoposide and doxorubicin, inhibited TOP2 (Liu 1989). Drugs that target TOP2 may be classified either as TOP2 poisons and TOP2 catalytic inhibitors. Many TOP2 poisons are well-established clinically active chemotherapeutic drugs. TOP2 poisons are known as such because they bind DNA or TOP2 while they are covalently bound, generating lesions including the broken DNA strands and the enzyme, which induces DNA damage and interferes with replication and transcription, effectively converting TOP2 into an agent of cellular damage. TOP2 poisons may be further subdivided into intercalating and non-intercalating poisons. Intercalating TOP2 poisons include a chemically diverse group of drugs including doxorubicin and other anthracyclines, mitoxantrone, m-amsacrine (mAMSA), and several other compounds not currently in clinical use. While these agents employ a variety of mechanisms, they each inhibit TOP2 activity by intercalating DNA and trapping TOP2. Conversely, non-intercalating TOP2 poisons do not interact strongly with DNA; rather, they have been suggested to bind covalent complexes of TOP2 and DNA. The epipodophyllotoxins etoposide and teniposide and fluroquinolones, which primarily target prokaryotic TOP2, are non-intercalating TOP2 poisons. Both subgroups of TOP2 poisons increase the levels of TOP2-DNA covalent complexes and DNA cleavage, whereas TOP2 catalytic inhibitors repress TOP2 activity but do not lead to increased DNA cleavage. Many TOP2 catalytic inhibitors target the ATPase activity of TOP2; inhibiting ATP hydrolysis can trap TOP2 in a closed clamp conformation while bound to

DNA, inhibiting its activity while leaving DNA undamaged. However, most TOP2 catalytic inhibitors are not specific for TOP2 and do not produce a DNA damage response with short term exposure; therefore, they are not often used in clinical practice (Nitiss 2009).

Two TOP2 inhibitors that induced greater cytotoxicity in HPV-positive than HPV-negative cancer cell lines were identified: etoposide and mAMSA. Etoposide treatment leads to cell death by binding and stabilizing a complex of the enzyme covalently bound to cleaved DNA, referred to as the cleavage complex, which is an intermediate of the catalytic mechanism of TOP2. As cleavage complexes accumulate in etoposide-treated cells, permanent DNA strand breaks are generated, triggering recombination and repair pathways, mutations, and chromosomal translocations. If this accumulation of breaks overwhelms the cell, it can initiate cell death pathways. Etoposide thereby converts TOP2 from a vital enzyme to a potent toxin which destroys the genome (Baldwin & Osheroff 2005). mAMSA similarly acts as a TOP2 poison, increasing the levels of covalently bound enzyme and cleaved DNA complexes. However, mAMSA intercalates DNA, which increases its affinity for the cleavage complex (Ketron et al. 2012). Both drugs generate high levels of cleavage complexes, blocking transcription and replication, and cell death pathways are subsequently initiated. There are several mechanisms of cell death, and the type that a cell undergoes is contingent upon a variety of intrinsic and extrinsic factors. Topoisomerase II inhibition with etoposide has been shown to induce apoptosis—a mechanism of regulated, anti-inflammatory cell death—in many cancer cell lines (Nitiss 2009; Bertheloot et al. 2021). In addition, etoposide treatment has been found to induce pyroptosis in melanoma cell lines; pyroptosis is a

gasdermin protein family-mediated pathway of cell death which has pro-inflammatory and immunogenic effects, including stimulation of anti-tumor immune activity (Erkes et al. 2020). This presents an interesting therapeutic opportunity for HPV-positive cancers, as topoisomerase II inhibition may selectively target and kill cancer cells overexpressing TOP2, and in doing so, attract immune cells which may further attack cancer cells.

### *Cell Death Pathways*

Forms of cell death may be classified as regulated, or programmed, and unregulated, and the effects of cell death on their surroundings have generally been viewed as a dichotomy: necrosis is a passive form of cell death with strong pro-inflammatory effects, while apoptosis is concisely organized and immunologically inert. However, other mechanisms of cell death, including pyroptosis, which are both highly coordinated and immunostimulatory, have come to light (Bertheloot et al. 2021). Therefore, for cancer cells, the type of cell death pathway has significant implications for immune activation, including anti-tumor immune activity.

Apoptosis is a genetically regulated form of programmed cell death mediated by the activation of several caspases, including caspases 3, 6, and 7. Apoptotic cells are typically scavenged by macrophages in a highly organized manner, rendering them immunologically inert, but if they are not scavenged, these cells may progress to secondary necrosis, a process involving lysis of the dying cell, inflammation, and immune infiltration (Rogers et al. 2017). The caspases involved in apoptosis may activate pyroptotic pathways under certain cellular conditions.

Pyroptosis may be induced in several ways. The canonical inflammasome-dependent pathway occurs in macrophages upon microbial infection or endogenous

damage, which may be sensed by the presence of double-stranded DNA crystals or toxins in the cytosol. When bacterial infection, pattern-associated molecular patterns (PAMPs), or endogenous damage-associated molecular patterns (DAMPs) are sensed, a multi-protein complex known as an inflammasome is assembled. Pro-caspase-1 is then sensed by inflammasome domains, leading to self-cleavage and caspase-1 activation. Caspase-1 may then cleave Gasdermin D (GSDMD), activating it and allow it to form pores in the cell membrane and cleave pro-inflammatory cytokines, leading to their maturation. In neutrophils, GSDMD may also be activated by caspase-4, caspase-5, and caspase-11 via the non-canonical inflammasome dependent pathway. Cytosolic lipopolysaccharides may bind these caspases directly, leading to their activation and cleavage of GSDMD (Tan et al. 2021; Wang et al. 2017).

Pyroptosis may also be induced in epithelial cells through a Gasdermin E (GSDME) mediated pathway in response to tumor necrosis factors binding cellular receptors or to chemotherapeutic drugs. Tumor necrosis factors or chemotherapies can activate caspase-3-mediated apoptosis. Caspase-3 then cleaves GSDME, diverging from the apoptotic pathway. Activated GSDME may then perforate the cell membrane and activate inflammasomes, which in turn activates caspase-1 and GSDMD and subsequently leads to maturation of inflammatory cytokines (Tan et al. 2021; Wang et al. 2017). These cytokines, including interleukin-1 alpha (IL-1$\alpha$), interleukin-1 beta (IL-1$\beta$), and interleukin-18 (IL-18), are released through the membrane pores generated by GSDME (Tan et al. 2021; Tsuchiya et al. 2021). In addition, DAMPs such as high mobility group box protein 1 (HMGB1)—a nuclear chromatin-binding factor and secreted protein—are released from the cell through pores where they may bind immune

cells in the tumor microenvironment—which is composed of cellular components including tumor cells, stromal cells, endothelial cells blood and lymphatic vessels, neurons, and infiltrating immune cells—and promote inflammation (Erkes et al. 2020; Scaffidi et al. 2002). Here, caspase-3-mediated pyroptosis differs from apoptosis progression to secondary necrosis, because the latter does not involve the release of HMGB1 (Scaffidi et al. 2002). In addition, caspase-3 may also cleave poly-(ADP-ribose) polymerase (PARP), which regulates the release of HMGB1 by upregulating the acetylation of HMGB1 (Morris et al. 2017; Yang et al. 2014). Released HMGB1 binds toll-like receptors on dendritic cells, which are antigen-presenting cells necessary for adaptive immunity, thereby promoting inflammation and stimulating the immune response (Erkes et al. 2020). The pyroptotic cells also release neoantigens into the tumor microenvironment, providing stimuli for dendritic cells, which may then become activated and present antigens to cytotoxic T lymphocytes (CD8+ T cells) via antigen cross-priming (Tan et al. 2021). Because of these immunostimulatory effects, pyroptosis is considered a form of immunogenic cell death.

### *Pyroptosis and Anti-Tumor Immunity*

Induction of pyroptosis with chemotherapeutic drugs is a promising method for generating anti-tumor immunity. A study conducted in 2020 demonstrated that inducing pyroptosis with active Gasdermin conjugated to a cancer-imaging probe using nanoparticle-mediated delivery in less than 15% of tumor cells was sufficient for complete regression of murine mammary carcinoma (4T1) tumor grafts. In immune-deficient or T-cell depleted mice, tumor clearance was absent, validating the importance of pyroptosis in regression. Furthermore, partial induction of pyroptosis with an

ineffective dose sensitized 4T1 tumors to immune checkpoint inhibition (ICI) (Wang et al. 2020). A synergistic effect was also found when pyroptosis induction was combined with ICI in ICI-resistant tumors, demonstrating the capacity of pyroptosis to transform the tumor immune microenvironment (TME) from an immunosuppressive or inactive environment which hinders treatment to a stimulated, anti-tumorigenic environment (Tang et al. 2020).

The TME plays an essential role not only in determining the efficacy of immunotherapies but in regulating tumor growth and invasion and in allowing evasion of immune surveillance. Immunosuppressive cells, including regulatory T cells and myeloid derived suppressor cells, within the tumor microenvironment generate a pro-tumorigenic effect, whereas CD4+ helper T lymphocytes, cytotoxic CD8+ T cells, and natural killer cells promote an anti-tumorigenic immune environment. The balance of these two types of cells within the TME is important in determining whether the immune system can effectively attack cancer. Induction of pyroptosis can alter the TME by activating anti-tumor immunity, in addition to directly inducing cancer cell death. Immune cells, including natural killer cells and cytotoxic CD8+ T lymphocytes, may further induce pyroptosis of tumor cells via granzyme protease-mediated cleavage of certain gasdermin protein family members. This leads to pore formation in the tumor cell membrane and magnification of inflammation signals in the TME, further amplifying anti-tumor immunity (Tan et al. 2021). Because cytotoxic CD8+ T cells are primed by dendritic cells, they can specifically attack cancer cells sharing antigens with those that have released neoantigens during pyroptosis. This presents a unique opportunity for targeting HPV-positive cancers because these tumor cells possess foreign antigens, such as the E6

and E7 oncoproteins. Therefore, topoisomerase inhibition may differentially induce pyroptosis in HPV-positive tumor cells and promote HPV-antigen-specific immunity simultaneously.

### *Purpose and Overview*

The purpose of this study was to determine whether pyroptosis is induced through topoisomerase II inhibition in HPV-positive cancers and leads to enhancement of antigen-specific immune activity. Two topoisomerase II inhibitors, etoposide and mAMSA, which induced greater cytotoxicity in HPV-positive cell lines than HPV-negative cells were identified using cytotoxicity assays. Established preclinical models of vaginal and oral HPV-positive cancers include the TC-1 and mEER cell lines, respectively, which express the HPV-16 E6 and E7 oncogenes. These tumor cells were cultured and treated *in vitro* with physiologically attainable doses of etoposide and mAMSA. Western blotting was then used to detect protein markers of pyroptosis within the cells and secreted into the cell supernatant. Splenocytes from mice immunized with a therapeutic HPV peptide vaccine were then cocultured with the cell supernatants, or conditioned media, from treatment with topoisomerase II inhibitors. Flow cytometry was used to measure maturation, proliferation, and activity of cytotoxic T lymphocytes and HPV antigen-specific T lymphocytes.

*Hypotheses*

1. Treatment with etoposide and mAMSA, relative to vehicle control groups, would induce pyroptosis in TC-1 and mEER cells, demonstrated by increased Gasdermin E cleavage and greater amounts of HMGB1 and IL-1α in the cell supernatant in TC-1 and mEER cells.
2. Splenocytes cocultured with conditioned media from TC-1 or mEER cells subjected to etoposide or mAMSA treatment, relative to vehicle control groups, would induce greater activation, proliferation, and cytotoxic activity of HPV antigen-specific cytotoxic T lymphocytes.

# CHAPTER TWO

## Methods

Two cell lines—both established preclinical models of HPV-positive cancers—were cultured, then treated with topoisomerase II inhibitors. Following treatment, cell lysates and supernatants were probed using Western blotting for markers of pyroptosis. Splenocytes from E7-immunized mice were cocultured with conditioned media from topoisomerase treatment, and maturation, proliferation, and activity of cytotoxic T lymphocytes and HPV-antigen-specific cytotoxic T lymphocytes were measured using flow cytometry.

### *Cell Cultures*

Two types of HPV-positive murine cancer cell lines, TC-1 and mEER, were cultured for this experiment. TC-1 cells were derived from murine primary lung epithelial cells and cotransformed with the HPV-16 E6 and E7 oncogenes and the c-Ha-Ras oncogene, which is involved in signal transduction promoting mitosis and cellular differentiation (Lin et al. 1996). The TC-1 cell line is an established preclinical model for HPV-positive vaginal cancers. TC-1 cells were cultured in RPMI supplemented with heat-inactivated fetal bovine serum, penicillin, streptomycin, and gentamycin and incubated at 37° C in 5% CO2. The mEER cell line is an established preclinical model of HPV-positive oropharyngeal squamous cell carcinoma and was derived from murine oropharyngeal epithelial cells and similarly transformed with HPV-16 E6 and E7 and H-Ras oncogenes (Vermeer et al. 2016). mEER cells were cultured in DMEM supplemented

with Ham's F12 nutrient mixture, heat-inactivated fetal bovine serum, hydrocortisone, transferrin, insulin, triiodothyronine, epidermal growth factor, Cholera toxin, penicillin, and streptomycin and incubated at 37° C in 5% $CO_2$.

Both cell types were cultured in 10 cm plates with 10 mL of their respective media. Confluency was checked daily, and cells were split every two to three days. To split cells, media was decanted, and 2-3 mL of trypsin was added to each plate. Cells were incubated until they detached from the surfaces of the plates. Then, plates were washed with media to remove trypsin by centrifugation at 1,500 rotations per minutes (rpm) and 20° C for five minutes. The supernatant was decanted, and cells were resuspended in their respective media. Cells were then plated at a ratio of 1:2 to 1:4 depending upon the degree of confluency in 10 mL of their respective media.

### *Etoposide and mAMSA Treatment*

Prior to treatment with Topoisomerase II inhibitors, TC-1 and mEER cells were removed from media containing fetal bovine serum using trypsin, washed with phosphate-buffered saline, and cultured in their respective media without fetal bovine serum (in order to reduce nonspecific binding in Western blotting). In three 6-well plates (35 mm in diameter), $0.5 \times 10^6$ cells were plated in each well. In each plate, three wells contained TC-1 cells, and three wells contained mEER cells. In each plate, all cells were treated either with etoposide (10 μM), mAMSA (3 μM), or dimethyl sulfoxide (DMSO), with the latter serving as a vehicle control. These concentrations were selected based on previous cytotoxicity assays using human preclinical models of HPV-positive cancers. The treated cells were then incubated for 72 hours at 37° C in 5% $CO_2$. After incubation, the conditioned media was collected using serological pipettes and micropipettes. Cells

were harvested using phosphate-buffered saline (PBS) and cell scrapers in order to maximize sample recovery, then centrifuged at 1,500 rpm and 20° C. The supernatant was removed using a micropipette and added to the conditioned media. Cells were resuspended stored in phosphate-buffered saline, and the conditioned media was stored separately.

### *SDS-PAGE and Western Blotting*

To prepare the samples for Western blotting, cells were lysed using lysis buffer, protease inhibitor, and phosphatase inhibitor in order to prevent protein and phosphoprotein degradation. Conditioned media were centrifuged in protein concentrators at 1,500 rotations per minute in order to remove small polypeptides and debris. Then, a bicinchoninic acid assay was conducted to determine the protein concentration for each sample. After the concentration of protein was calculated, the appropriate amounts of each sample, Laemmli buffer, and β-mercaptoethanol were combined, then boiled for five minutes at 95° C to ensure that proteins denatured to their primary structures. After cooling, samples and a molecular weight size marker mix were each loaded into individual wells of the SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) gels in tris-glycine buffer. The electrophoresis chamber was set to 130 V for 60-70 minutes. The gels were subsequently removed and placed in transfer buffer.

Following electrophoresis, the nitrocellulose membrane and two transfer sponges were soaked in transfer buffer. One transfer sponge was placed into the cassette of the blotting apparatus, then the nitrocellulose membrane, SDS-PAGE gel, and remaining transfer sponge were placed on top, respectively. Bubbles were removed after each step

using a roller. The cassette was then inserted into the transfer apparatus to begin the blotting process.

After the transfer was complete, the membrane was rinsed with tris-buffered saline (TBS). Then, the membrane was incubated in blocking buffer (5% nonfat dry milk in TBS) for one hour on a rocker to prevent antibodies from nonspecifically binding to the membrane. The membrane was then rinsed with TBS, and the primary antibodies were added to the membrane in blocking buffer. For the cell lysates, the primary antibodies used were specific to GSDME, cleaved caspase 3, and PARP. Antibodies specific to β-actin, a housekeeping protein, was used as a loading control. For the conditioned media, or cell supernatant, anti-HMGB1 and anti-IL-1α were used as primary antibodies. The membranes were incubated with their respective primary antibodies for 24 hours at 4° C on a gentle rocker. Following incubation, the membranes were washed three times for ten minutes with TBS with gentle rocking to remove excess antibodies, then incubated at room temperature with gentle rocking with the appropriate secondary antibody (anti-rabbit or anti-mouse) in blocking buffer. After one hour, the membranes were washed with TBS three times for ten minutes each to remove unbound secondary antibodies. Throughout this process, the membrane was not allowed to dry in order to reduce background and improve signal clarity.

The enhanced chemiluminescence substrate solution was prepared, and the membrane was incubated with the solution for five minutes. Subsequently, the membrane was imaged with a chemiluminescence imaging system. Following imaging, the membranes were washed with and stored in TBS and the procedure was repeated for the remaining primary antibodies.

*Splenocyte Coculture*

Immunocompetent (C57B1/6) mice were vaccinated with a mixture of HPV-oncoprotein peptides and adjuvants. Blood samples collected after two weeks were processed for flow cytometry by staining with a mixture of antibodies against CD3, CD4, CD8, and a tetramer reagent specific to a peptide corresponding to an immunodominant epitope in the HPV E7 protein. Following the confirmation of the presence of CD8+ E7+ T lymphocytes via flow cytometry, mice were euthanized, and their spleens were harvested. Splenocytes were filtered to remove fat and debris, then centrifuged in RPMI supplemented with fetal bovine serum, penicillin, streptomycin, and gentamycin at 1,500 rpm and 20° C. The supernatant was decanted, splenocytes were resuspended in 2 mL ACK (Ammonium-Chloride-Potassium) buffer to lyse red blood cells and left to rest for three minutes, then topped with 10 mL media and centrifuged again. Splenocytes were resuspended in media and enumerated for viable cells using the Trypan blue dye exclusion methodology. In a 96-well plate, $1.0 \times 10^6$ spleen cells were added per well in 160 μL of media and incubated with culture supernatants from TC-1 and mEER tumor cells treated with topoisomerase II inhibitors (as described earlier). Treatments were added in 40 μL media, giving a total volume of 200 μL per well. There were twelve treatment groups, with five replicates for each group, and the cultures were incubated for either 24 or 48 hours. The different cultures included media only (which served as a negative control), 20% DMSO-treated TC-1 supernatant, 20% etoposide-treated TC-1 supernatant, 20% mAMSA-treated TC-1 supernatant, 20% DMSO-treated mEER supernatant, 20% etoposide-treated mEER supernatant, 20% mAMSA-treated supernatant, Concanavalin A (which induces T cell proliferation and served as a positive

control), Dynabeads Mouse T-Activator CD3/CD28 (which stimulates activation and proliferation of murine T cells and served as an additional positive control), DMSO, etoposide (10 μM), and mAMSA (3 μM). After 24 and 48 hours, cells were removed from their respective treatments and stained with antibodies for flow cytometry.

### *Flow Cytometry*

To remove cells from their respective treatments, the 96-well plates were centrifuged for five minutes at 1,500 rpm and 20° C. The supernatant was decanted, and cells were resuspended in PBS and pulsed with a vortex to mix. The plates were centrifuged again for five minutes, after which the supernatant was decanted and cells were resuspended in 50 μL of the antibody cocktail and mixed, containing antibodies at their respective amounts (listed below) in FACS (fluorescence-activated cell sorting) buffer, which is PBS containing 2% fetal bovine serum and 2mM EDTA.

| Laser | Filter | Fluorophore | Antibody | Amount |
|---|---|---|---|---|
| UV Laser 355 nm | Filter 379/28 | BUV395 | CD8 | 1:100 |
| UV Laser 355 nm | Filter 740/35 | BUV737 | CD3 | 1:200 |
| Violet Laser 405 nm | Filter 525/50 | BV510 | Live/Dead | 1:300 |
| Violet Laser 405 nm | Filter 610/20 | BV605 | CD19 | 1:300 |
| Violet Laser 405 nm | Filter 670/30 | BV650 | GR-1 | 1:300 |
| Violet Laser 405 nm | Filter 780/60 | BV786 | CD11b | 1:300 |
| Blue Laser 488 nm | Filter 710/50 | PerCP-Cy5.5 | NK1.1 | 1:300 |
| Green Laser 561 nm | Filter 586/15 | PE | MHC (I-A/I-E) | 1:300 |
| Green Laser 561 nm | Filter 610/20 | PE-Dazzle | CD11c | 1:300 |
| Green Laser 561 nm | Filter 780/60 | PE-Cy7 | CD4 | 1:300 |
| Red Laser 640 nm | Filter 670/30 | APC | E7 Tetramer | 1:200 |
| Red Laser 640 nm | Filter 780/60 | APC-Cy7 | CD62-L | 1:100 |
| | | | Mouse Fc Block | 1:250 |

Table 1. Lasers exciting each fluorophore, filters directing certain wavelengths of light emitted by excited fluorophores to their respective detector, fluorophore names and their conjugated antibodies, and the ratio of the volume of the antibody to the total volume of the solution are listed. When excited by the laser, the fluorophore bound to a cell via the antibody emits photons with a specific wavelength. All of the filters listed here are bandpass filters; photons with wavelengths within a given range indicated by the second number (nm) above or below the first number (nm) may pass through a given filter and are subsequently directed to the detector.

The cells in the antibody cocktail were incubated for 30-45 minutes at room temperature, protected from light. Then, they were topped with 200 μL of FACS buffer and centrifuged for five minutes. The supernatant was decanted, then cells were resuspended in 50 μL of fixation buffer containing formaldehyde and mixed. Cells were

then incubated for 15-30 minutes at room temperature, protected from light, after which 200 μL of permeabilization buffer was added to each well. The plates were centrifuged for five minutes, and the supernatant was discarded. The cells were resuspended in 50 μL of antibody cocktail, containing intracellular cytokine (ICC) flow cytometry antibodies at their respective amounts (listed below) prepared in permeabilization buffer. Cells are fixed and permeabilized prior to ICC staining to allow antibodies to bind intracellular components.

| Laser | Filter | Color | Antibody | Amount |
|---|---|---|---|---|
| Violet Laser 405 nm | Filter 450/50 | BV450 | GnzB | 1:300 |
| Violet Laser 405 nm | Filter 710/50 | BV711 | IFNγ | 1:300 |
| Blue Laser 488 nm | Filter 530/30 | FITC | Foxp3 | 1:200 |
| Red Laser 640 nm | Filter 730/45 | APC-R700 | Ki-67 | 1:200 |

Table 2. Lasers exciting each fluorophore, filters directing certain wavelengths of light emitted by fluorophores to the appropriate detector, fluorophore names and their conjugated antibodies, and the ratio by volume of the antibodies in solution are listed. These ICC antibodies were used to detect expression of target protein antigens within the cells.

The cells were incubated with the ICC antibodies for 30 minutes, protected from light, after which 200 μL were added to each well. The plates were then centrifuged for five minutes, and the supernatant was discarded. Cells were resuspended in 100 μL of FACS buffer, then stored at 20° C until the samples were analyzed with flow cytometry.

A Fortessa X-20 instrument was used for flow cytometry sample acquisition, and compensation was adjusted and distinct cell populations were gated using FlowJo software. Microsoft Excel and GraphPad software were used to plot and analyze the data.

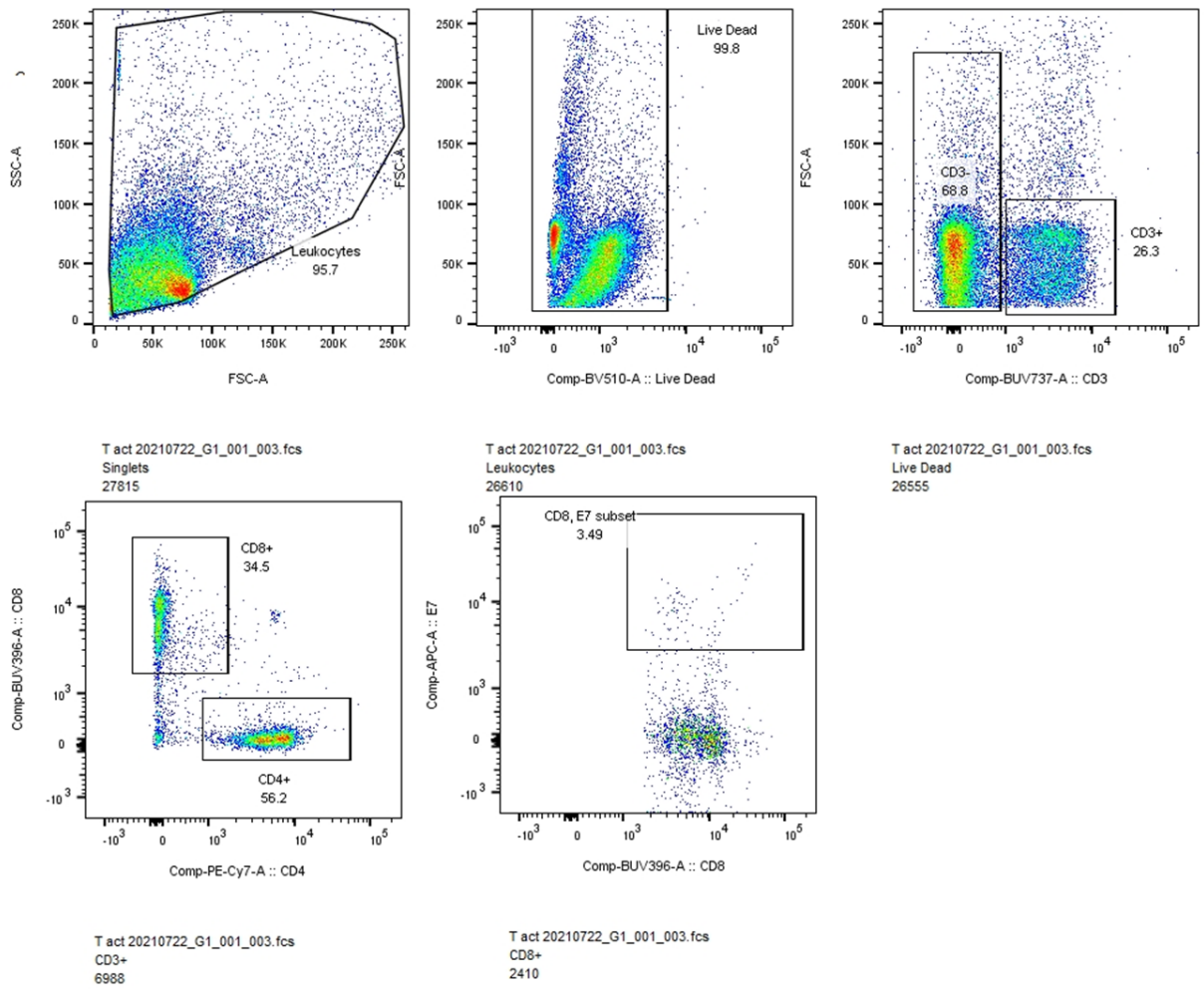


Figure 1. An example of the gating strategy used for data analysis. Forward and side scatters were used to gate for single cells (leukocytes), which was confirmed using forward scatter and the live/dead marker. The CD3 marker was used to gate for lymphocytes (CD3+). From the lymphocyte population, the CD8 marker was used to gate for cytotoxic T lymphocytes (CD8+, CD4-). Within the cytotoxic T cell population, the E7 marker was used to gate for HPV E7-specific cytotoxic T lymphocytes (E7+).

# CHAPTER THREE

## Results

### *Western Blot*

TC-1 and mEER cell lysates were probed for markers of pyroptosis, including GSDME, cleaved Caspase 3, and PARP, and β-actin, which was selected as a housekeeping protein to ensure equal loading during SDS-PAGE and equal transfer of proteins from the gel to the membrane.

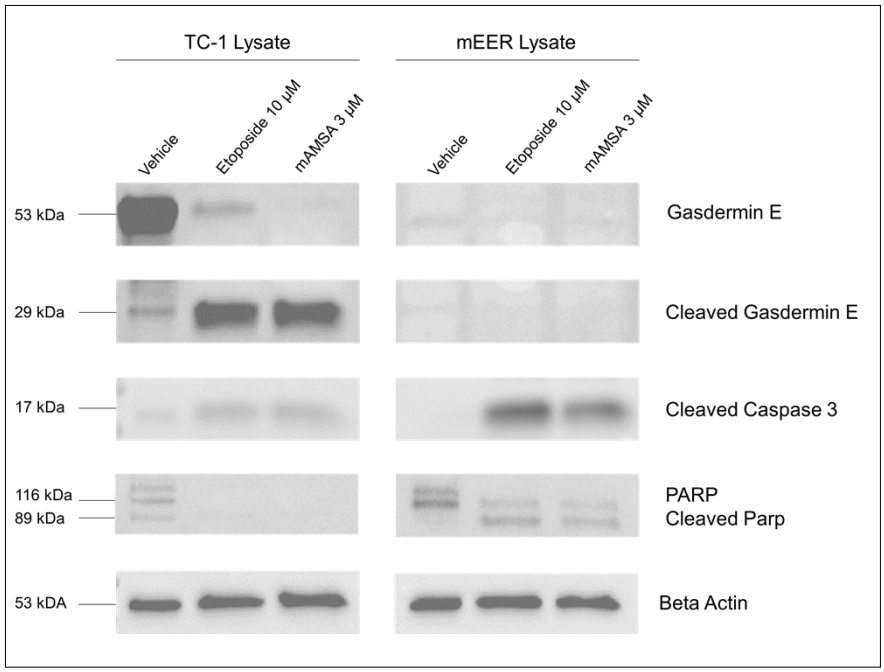


Figure 2. Western blot results for TC-1 and mEER cell lysates. Topoisomerase II inhibition may induce GSDME-mediated pyroptosis in TC-1 cells, as indicated by decreased amounts of full length GSDME and increased amounts of cleaved GSDME and cleaved Caspase 3 in groups treated with etoposide and mAMSA compared to the vehicle control group. Amounts of both full length and cleaved PARP were increased in the group treated with DMSO compared to groups treated with topoisomerase inhibitors. Amounts of β-actin were consistent across all groups, indicating approximately equal loading and transfer. Topoisomerase inhibition in mEER cells is associated with greater

cleavage of Caspase 3 and PARP than treatment with DMSO. However, amounts of both full length and cleaved GSDME are too low to effectively compare. Again, levels of β-actin are similar for all groups, indicating near-equal loading and transfer.

The conditioned media, or cell supernatant, from culture and treatment of TC-1 and mEER cells was probed for secreted proteins that have been identified as markers of pyroptosis, including HMGB1 and interleukin-1α. The cell supernatants were not probed for any housekeeping proteins, because secreted proteins are not ubiquitously expressed.

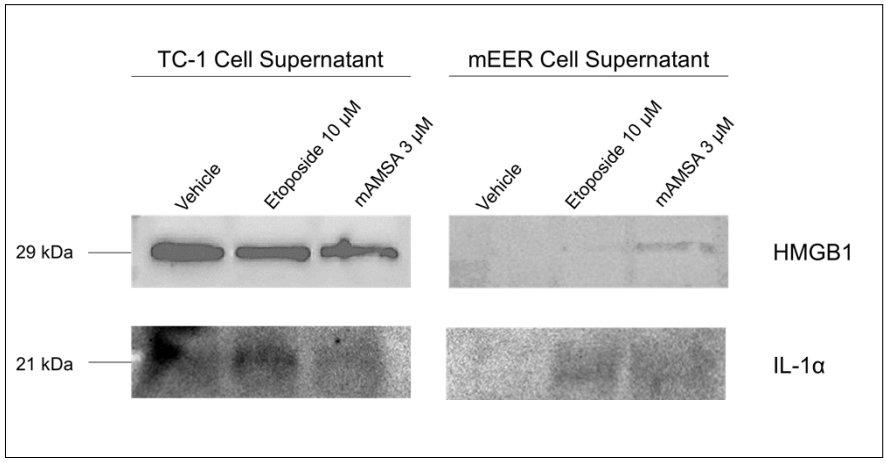


Figure 3. Western blot results for TC-1 and mEER cell supernatants. HMGB1 and IL-1α were both present in the TC-1 cell supernatant in similar amounts in the vehicle control group and etoposide- and mAMSA-treated groups. HMGB1 and IL-1α were also found in the mEER cell supernatant at in etoposide- and mAMSA-treated groups.

In TC-1 cells, increased levels of GSDME cleavage and cleaved Caspase 3 indicate that topoisomerase inhibition with etoposide and mAMSA may have induced pyroptosis mediated by GSDME cleavage. Both full length and cleaved PARP were not present in sufficient amounts in treated groups to effectively compare, however, PARP cleavage is not necessary for pyroptosis to occur. HMGB1 and IL-1α were present in the

cell supernatants of all groups, but it is impossible to compare the quantities because no loading controls were used.

In mEER cells, both full length and cleaved GSDME were not detected in sufficient amounts to compare, therefore it cannot be concluded that cells of any group underwent GSDME-mediated pyroptosis. This may be due to low expression of GSDME in mEER cells. Greater amounts of cleaved Caspase 3 and cleaved PARP in the treated groups than in the vehicle control group do demonstrate that topoisomerase II inhibition induced some mechanism of cell death. Moreover, mEER cells likely underwent a form of immunogenic cell death, as indicated by the presence of HMGB1 and IL-1α in the cell supernatants.

### *Flow Cytometry*

Flow cytometry was used to analyze activation and proliferation of cytotoxic T lymphocytes (CD8+) and HPV-E7 antigen specific cytotoxic T lymphocytes (CD8+ E7+) in splenocytes from HPV E6/E7 immunized mice cocultured with conditioned media or control treatments.

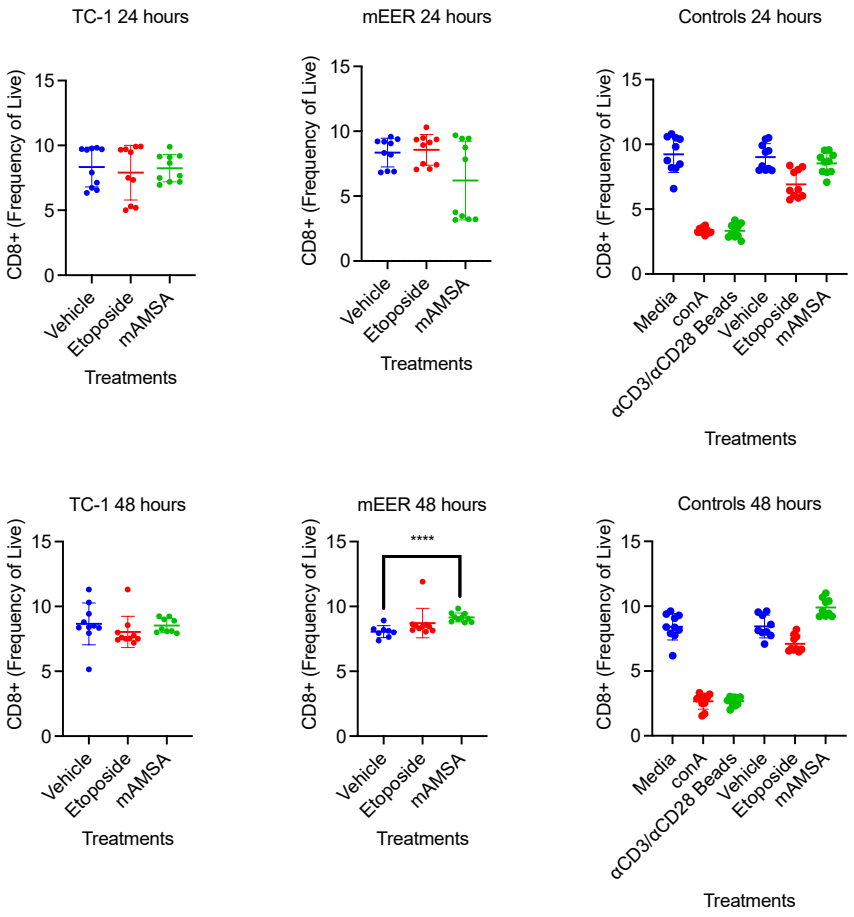


Figure 4. Frequency of live CD8+ lymphocytes. The frequency of CD8+ T lymphocytes was significantly higher ($p \leq 0.0001$) in the splenocytes cocultured with mAMSA-treated mEER cell supernatant for 48 hours than with vehicle-treated cell supernatant.

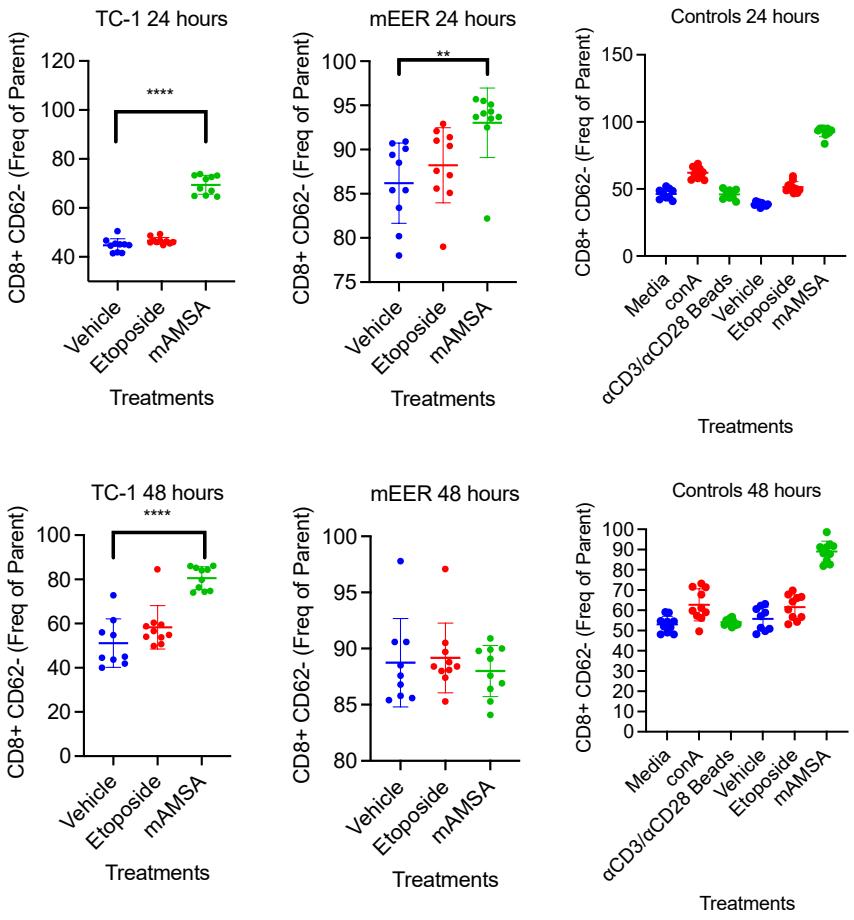


Figure 5. Frequency of CD8+ CD62L- T lymphocytes. Shedding of the CD62L marker indicates T cell maturation and activation. Therefore, the frequency of activated CD8+ T cells is significantly higher ($p \leq 0.0001$) in splenocytes cocultured with mAMSA-treated TC-1 cell supernatant for 24 and 48 hours than with vehicle control-treated cell supernatant. In addition, the frequency of activated CD8+ T cells is significantly higher ($p \leq 0.01$) in splenocytes cocultured with mAMSA-treated mEER cell supernatant for 24 hours than with vehicle control-treated cell supernatant, but there is no significant difference after 48 hours of coculture.

These findings indicate that cellular contents of TC-1 and mEER cells released in response to mAMSA treatment may enhance activation of cytotoxic CD8+ T lymphocytes.

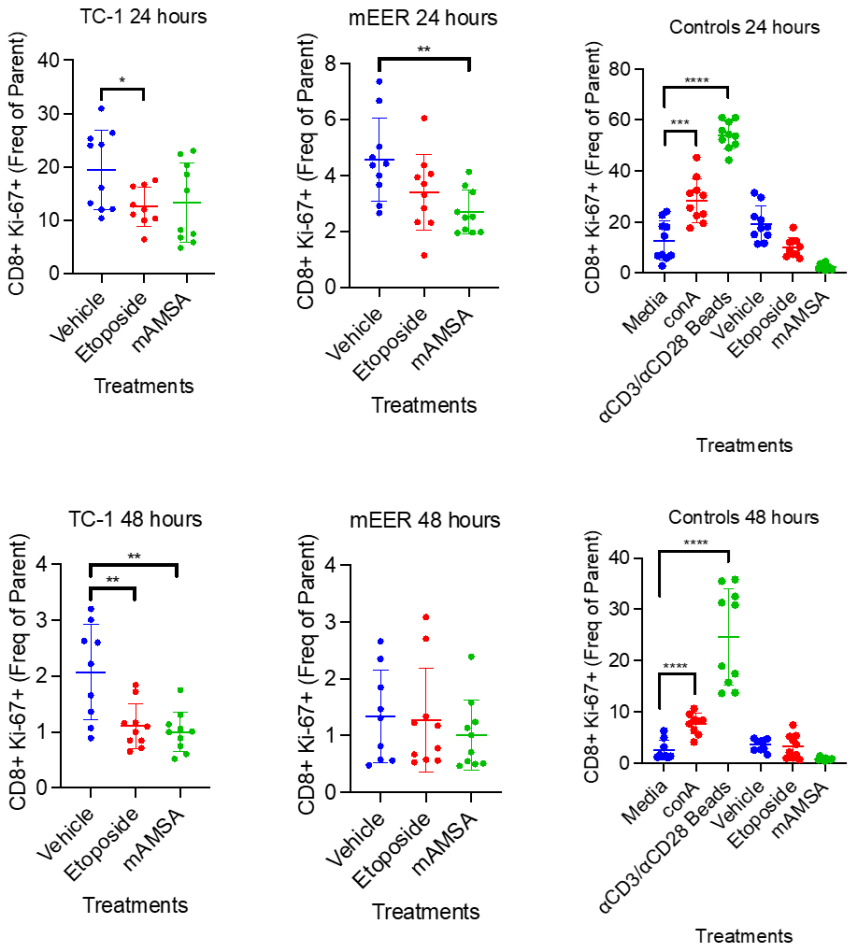


Figure 6. Frequency of CD8+ Ki-67+ T lymphocytes. Ki-67 is a marker of T cell proliferation. Thus, the frequency of proliferating CD8+ T cells is significantly lower ($p \leq 0.05$) in splenocytes cocultured with etoposide-treated TC-1 cell supernatant four 24 hours than with the vehicle control-treated cell supernatant. The frequency is also significantly lower ($p \leq 0.01$) in splenocytes cocultured with etoposide- and mAMSA-treated TC-1 cell supernatant for 48 hours than with vehicle-treated cell supernatant. The frequency of proliferating CD8+ T cells is significantly lower ($p \leq 0.01$) in splenocytes cocultured with mAMSA-treated mEER cell supernatant for 24 hours than with DMSO-treated cell supernatant. The frequency of proliferating CD8+ T cells is significantly higher in splenocytes cultured with conA and anti-CD3/anti-CD28 beads—which served as positive controls—for 24 and 48 hours than splenocytes cultured in media alone. This indicates that the *in vitro* model used in this study induces immune activity which could be detected with flow cytometry.

These findings indicate that cellular contents of TC-1 and mEER cells release in response to treatment with topoisomerase II inhibitors may hinder the proliferation of cytotoxic CD8+ T cells.

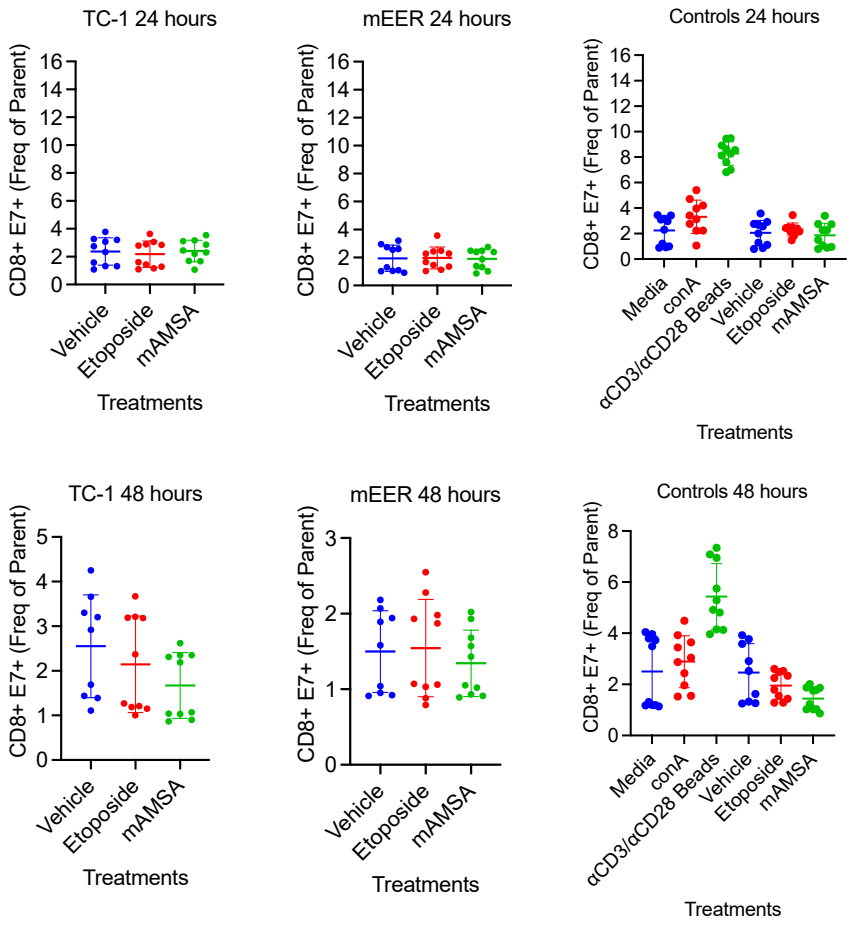


Figure 7. Frequency of CD8+ E7+ T lymphocytes. There were no significant differences in the frequency of CD8+ E7 antigen-specific T cells between splenocytes cocultured with etoposide, mAMSA, or vehicle control-treated TC-1 or mEER cell supernatant.

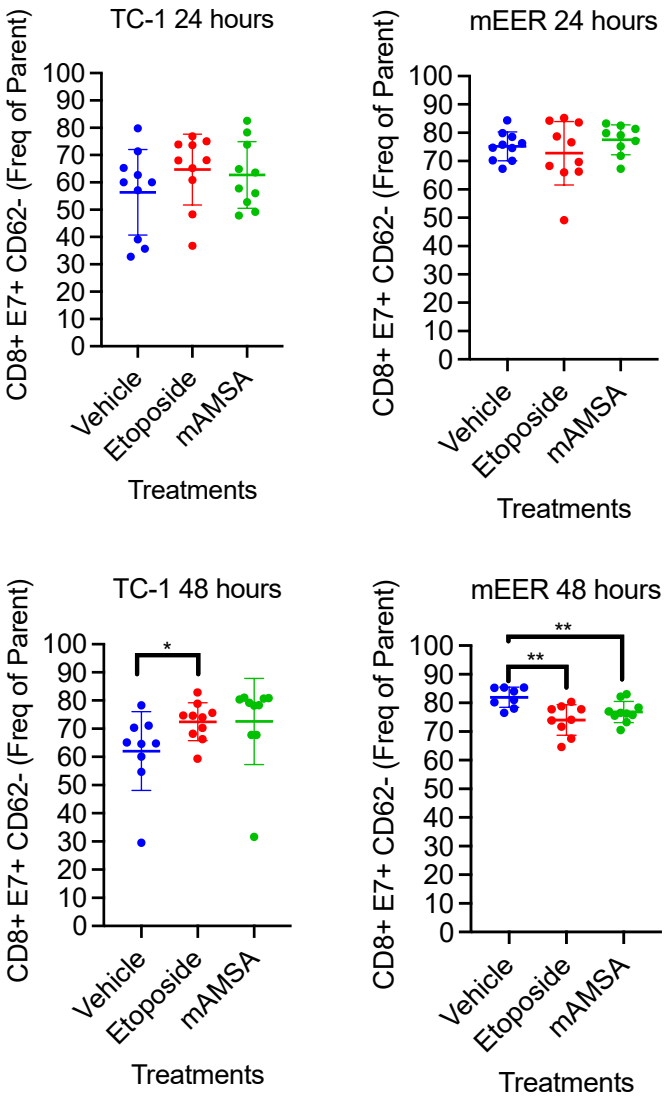


Figure 8. Frequency of CD8+ E7+ CD62L- T lymphocytes. The frequency of activated CD8+ E7 antigen specific T cells is significantly higher ($p \leq 0.05$) in splenocytes cocultured with etoposide-treated TC-1 cell supernatant for 48 hours than with vehicle control-treated cell supernatant. The frequency of activated CD8+ E7 antigen-specific T cells in splenocytes cocultured with etoposide- and mAMSA-treated mEER cell supernatant for 48 hours was significantly lower ($p \leq 0.01$) than in the vehicle control group.

These findings suggest that cellular contents of TC-1 cells released in response to etoposide treatment may induce activation of HPV E7-specific cytotoxic T lymphocytes.

However, cellular factors released by mEER cells in response to treatment with topoisomerase II inhibitors may hinder activation.

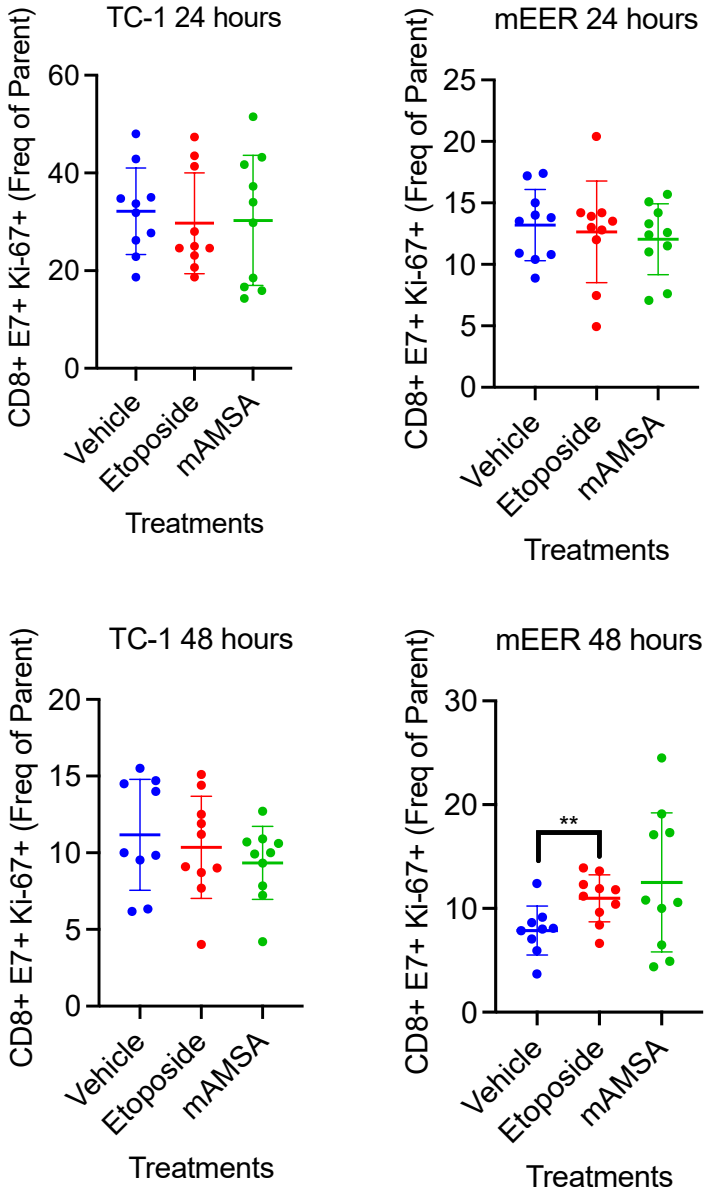


Figure 9. Frequency of CD8+ E7+ Ki-67+ T lymphocytes. The frequency of proliferating CD8+ E7 antigen-specific T cells is significantly higher ($p \leq 0.01$) in splenocytes cocultured with etoposide-treated mEER cell supernatant for 48 hours than with vehicle control-treated cell supernatant.

These findings indicate that cellular contents release by mEER cells treated with etoposide may promote proliferation of HPV E7-specific cytotoxic T lymphocytes.

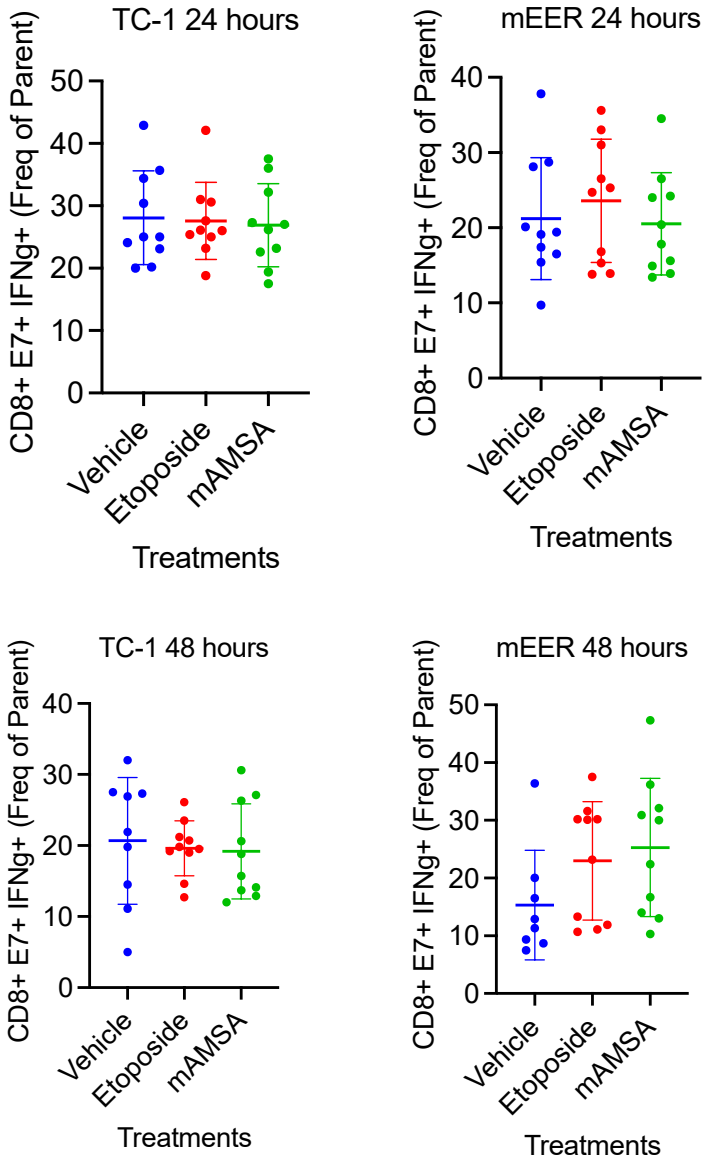


Figure 10. Frequency of CD8+ E7+ IFNγ+ T lymphocytes. There were no significant differences in interferon gamma (IFNγ) production between splenocytes cocultured with etoposide-, mAMSA-, or vehicle control-treated TC-1 or mEER cells.

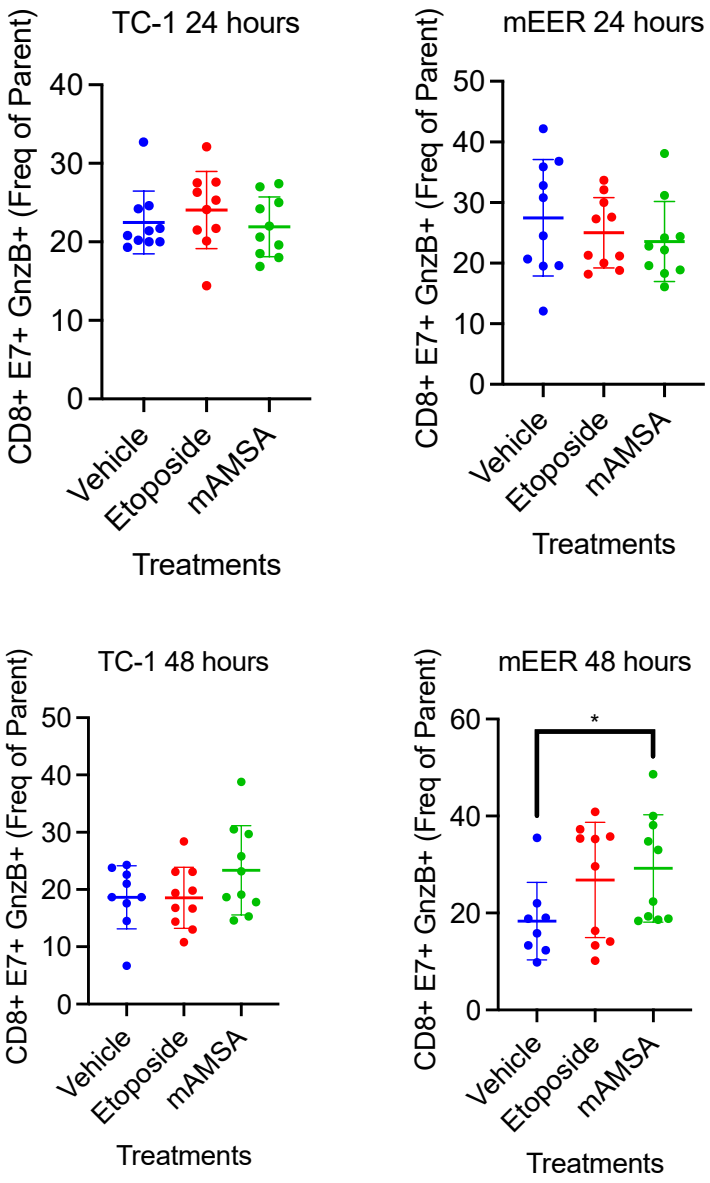


Figure 11. Frequency of CD8+ E7+ GnzB+ T lymphocytes. The frequency of granzyme B (GnzB)- producing CD8+ E7 antigen-specific T cells was significantly higher ($p \leq 0.05$) in splenocytes cocultured with mAMSA-treated mEER cell supernatant than in the vehicle control group.

These findings indicate that cellular contents of mEER cells released in response to treatment with mAMSA may enhance cytotoxic activity of HPV E7-specific cytotoxic T cells.

Flow cytometry analysis revealed some associations of activation and proliferation of cytotoxic CD8+ T lymphocytes and HPV E7 antigen specific CD8+ T lymphocytes with certain treatment groups. Notably, the frequency of mature and active CD8+ CD62L- T lymphocytes was significantly higher in splenocytes cocultured with conditioned media from mAMSA-treated TC-1 cells for 24 and 48 hours and from mAMSA-treated mEER cells for 24 hours than with the vehicle control group at each timepoint. In addition, the frequency of mature and active CD8+ E7 antigen-specific CD62L- T lymphocytes was significantly increased in splenocytes cocultured for 48 hours with conditioned media from etoposide-treated TC-1 cells than DMSO-treated cells. Proliferation of E7 antigen-specific CD8+ T lymphocytes was significantly increased in splenocytes cocultured for 48 hours with conditioned media from etoposide-treated mEER cells. The frequency of E7 antigen specific CD8+ T lymphocytes producing granzyme B was significantly increased in splenocytes cocultured for 48 hours with conditioned media from mEER cells treated with mAMSA, indicating increased anti-tumor cytotoxic activity. These results demonstrate that topoisomerase II inhibition with etoposide and mAMSA may induce some antigen-specific immune activity, warranting further study.

# CHAPTER FOUR

## Conclusions

The aim of this study was to determine whether treatment with topoisomerase II inhibitors induces pyroptosis in HPV-positive cancers and enhances HPV-antigen-specific immunity. Induction of pyroptosis with topoisomerase II inhibitors would lead to the release of cellular factor that may function as immunostimulants or neoantigens, which could attract and activate immune cells and prime cytotoxic T lymphocytes to target and attack cancer cells.

The Western blot results suggest that topoisomerase II inhibition with etoposide and mAMSA may induce pyroptosis in TC-1 cells, as indicated by cleavage of GSDME and Caspase 3 and the presence of HMGB1 and IL-1α in the cell supernatant. It cannot be concluded that topoisomerase II inhibition with etoposide and mAMSA induced pyroptosis in mEER cells due to the very low levels of both full length and cleaved GSDME, possibly due to cells undergoing an alternative mechanism of cell death, low expression of GSDME by mEER cells, or methodological issues. However, Western blot results did reveal a correlation with treatment with topoisomerase II inhibitors and the increased presence of markers of immunogenic cell death in mEER cells as indicated by Caspase 3 and PARP cleavage and the presence of HMGB1 and IL-1α in the cell supernatant. These results indicate that topoisomerase II inhibition with etoposide and mAMSA may induce immunogenic cell death in two established preclinical models of HPV-positive vaginal and oral cancers.

The results of flow cytometry analysis indicate that treatment of TC-1 cells with etoposide and coculture with splenocytes significantly increased maturation and activation of HPV-E7 antigen-specific cytotoxic CD8+ T lymphocytes, as demonstrated by the increased frequency of CD8+ E7+ CD62L- lymphocytes. Splenocytes cocultured with conditioned media from mAMSA-treated TC-1 cells showed significantly increased frequency of CD8+ CD62L- T lymphocytes, indicating that mAMSA treatment of TC-1 cells leads to maturation and activation of cytotoxic T lymphocytes. In mEER cells, treatment with etoposide and coculture significantly increased proliferation of E7 antigen-specific cytotoxic T lymphocytes, demonstrated by the greater frequency of CD8+ E7+ Ki-67+ lymphocytes. Coculture with conditioned media from mEER cells treated with mAMSA significantly increased both maturation and activation of cytotoxic T lymphocytes and cytotoxic activity of E7 antigen-specific T lymphocytes, as indicated by increased frequency of CD8+ CD62L- lymphocytes and CD8+ E7+ GnzB+ lymphocytes, respectively. In order to conclude that topoisomerase II inhibition enhances antigen-specific immunity in HPV-positive cancers, it would be necessary to demonstrate increased frequency of CD8+ E7+ T cells, CD8+ E7+ CD62L- T cells, CD8+ E7+ Ki-67+ T cells, CD8+ E7+ IFNg+ T cells, and CD8+ E7+ GnzB+ T cells relative to vehicle control groups; this would demonstrate that HPV-antigen-specific cytotoxic T lymphocytes are being activated, proliferating, and functioning to attack target cells in response to cellular factors released during treatment of HPV-positive cancer cells with topoisomerase II inhibitors. While these results do not conclusively demonstrate that treatment of TC-1 or mEER cells with etoposide or mAMSA induce greater HPV

antigen-specific immunity, they do show some promise in promoting anti-tumor immune activity.

### *Limitations*

In order to more accurately evaluate the effect of topoisomerase II inhibition on immune activity, the limitations of this study should be addressed, and the methodology should be optimized. The number of cells cultured and treated should be optimized to produce adequate amounts of protein for detection and comparison in Western blotting and for splenocyte coculture. Further, multiple concentrations of etoposide and mAMSA should be used in order to determine the optimal dosage (within physiologically attainable concentrations) for inducing pyroptosis and stimulating anti-tumor immunity. During the Western blotting process, Coomassie or Ponceau staining should be used to assess the loading or transfer of proteins, especially for secreted proteins; this will allow for normalization and comparison of the quantities of proteins released into the cell supernatant. Finally, the amount of conditioned medium used in coculture should be optimized to assess the effectiveness of immunostimulants at varying concentrations and to represent physiological conditions.

### *Future Studies*

Though optimization is needed, this study suggests the potential of topoisomerase II inhibition in inducing pyroptosis and immunogenic cell death in HPV-positive cancers *in vitro* and enhancing HPV antigen-specific anti-tumor immunity. The significant

relationships found between coculture with cell supernatants from topoisomerase II inhibitor treatment and activation, proliferation, and cytotoxic activity relative to vehicle control groups warrant further study. Treatment of HPV-negative and HPV-positive cancers with topoisomerase II inhibitors may demonstrate whether they differentially induce cell death and anti-tumor immune activity based on HPV status. For instance, treatment of HPV-negative murine oral cancer (MOC) cell lines and MOC cells transfected with HPV E6 and E7 oncogenes with topoisomerase II inhibitors and subsequent coculture may reveal differential cytotoxicity of etoposide and mAMSA and further elucidate the effect of HPV status on the tumor immune microenvironment. Treating mice with induced HPV-positive tumors (such as TC-1 or mEER tumors) with etoposide or mAMSA and analyzing survival, tumor volume, and tumor-infiltrating T lymphocytes may demonstrate whether topoisomerase II inhibition induces tumor regression and provide a more robust understanding of the role of T lymphocytes in the anti-tumor immune response. Further, treatment of immunocompetent and immune-deficient or T cell-depleted mice implanted with HPV-positive tumors with etoposide or mAMSA could elucidate the role of cytotoxic T lymphocytes in tumor regression. Finally, *in vivo* models should be used to assess the efficacy of topoisomerase II inhibition in combination with immunotherapies including immune checkpoint inhibitors such as anti-PD1/PDL1 and anti-CTLA-4, agonistic anti-4-1BB/4-1BBL monoclonal antibodies, and therapeutic HPV peptide vaccines. Because induction of pyroptosis in small proportions of tumor cells has been shown to be effective in inducing tumor regression, the effects of subtherapeutic doses of topoisomerase II inhibitors may be evaluated alone or in addition to immunotherapies (Wang et al. 2020). Lower doses of

topoisomerase II inhibitors may have the added benefit of reducing systemic toxicities from chemotherapy. Overall, further investigation of topoisomerase II inhibition as a therapy for HPV-positive cancers may lead to a better understanding of cell death mechanisms and their relationships with the tumor immune microenvironment and even to effective treatments for patients with cancers caused by HPV.

# BIBLIOGRAPHY

American Cancer Society. 2021. Cancer Facts and Figures.

Baldwin EL, Osheroff N. 2005. Etoposide, Topoisomerase II and Cancer. Current Medicinal Chemistry. 5(4):364-372. doi:10.2174/1568011054222362.

Bertheloot D, Latz E, Franklin BS. 2021. Necroptosis, Pyroptosis and Apoptosis: An Intricate Game of Cell Death. Cellular and Molecular Immunology. 18(5):1106-1121. doi:10.1038/s41423-020-00630-3.

de Martel C, Plummer M, Vignat J, Franceschi S. 2017. Worldwide Burden of Cancer Attributable to HPV by Site, Country, and HPV Type. International Journal of Cancer. 141(6): 664-670. doi:10.1002/ijc.30716.

Erkes DA, Cai W, Sanchez IM, Purwin TJ, Rogers C, Field CO, Berger AC, Hartsough EJ, Rodeck U, Alnemri ES, et al. 2020. Mutant BRAF and MEK Inhibitors Regulate the Tumor Immune Microenvironment via Pyroptosis. Cancer Discovery. 10(2):254-269. doi:10.1158/2159-8290.cd-19-0672.

Forman D, de Martel C, Lacey CJ, Soerjomataram I, Lortet-Tieulent J, Bruni L, Vignat J, Ferlay J, Bray F, Plummer M, et al. 2012. Global Burden of Human Papillomaviruses and Related Diseases. Vaccine. 30:F12-F23. doi:10.1016/j.vaccine.2012.07.055.

Graham SV. 2017. The Human Papillomavirus Replication Cycle, and its Links to Cancer Progression: A Comprehensive Review. Clinical Science. 131(17):2201-2221. doi:10.1042/cs20160786.

Hellner K, Münger K. 2011. Human Papillomaviruses as Therapeutic Targets in Human Cancer. Journal of Clinical Oncology. 29(13): 1785-1794. doi:10.1200/jco.2012.28.2186.

Ketron A, Denny W, Graves DE. 2012. Amsacrine as a Topoisomerase II Poison: Importance of Drug-DNA Interactions. Biochemistry. 51(8):1730-1739. doi:10.1021/bi201159b.

Kim YR, Lee B, Byun MR, Lee JK, Choi JW. 2018. Evaluation of Pemetrexed and Etoposide as Therapeutic Regimens for Human Papillomavirus-Positive Oral and Oropharyngeal Cancer. PLoS One. 13(7):e0200509. doi:10.1371/journal.pone.0200509.

Kombe Kombe AJ, Li B, Zahid A, Mengist HM, Bounda G-A, Zhou Y, Jin T. 2021. Epidemiology and Burden of Human Papillomavirus and Related Diseases, Molecular Pathogenesis, and Vaccine Evaluation. Frontiers in Public Health. 8. doi:10.3389/fpubh.2020.552028.

LeConte BA, Szaniszlo P, Fennewald SM, Lou DI, Qiu S, Chen N-W, Lee JH, Resto VA. 2018. Differences in the Viral Genome Between HPV-Positive Cervical and Oropharyngeal Cancer. PLoS One. 13(8):e0203403. doi:10.1371/journal.pone.0203403.

Lin KY, Guarnieri FG, Staveley-O'Carroll KF, Levitsky HI, August JT, Pardoll DM, Wu TC. 1996. Treatment of Established Tumors with a Novel Vaccine that

Enhances Major Histocompatibility Class II Presentation of Tumor Antigen. Cancer Research. 56(1):21-26.

Liu LF. 1989. DNA Topoisomerase Poisons as Antitumor Drugs. Annual Review of Biochemistry. 58:351-375. doi:10.1146/annurev.bi.58.070189.002031.

Longworth MS, Laimins LA. 2004. Pathogenesis of Human Papillomaviruses in Differentiating Epithelia. Microbiology and Molecular Biology Reviews. 68(2): 362-372. doi:10.1128/mmbr.68.2.362-372.2004.

Massarelli E, William W, Johnson F. 2019. Combining Immune Checkpoint Blockade and Tumor-Specific Vaccine for Patients with Incurable Human Papillomavirus 16-Related Cancer: A Phase 2 Clinical Trial. Journal of the American Medical Association Oncology. 5(1):67-73. doi:10.1001/jamaoncol.2018.4051.

McKie SJ, Neuman KC, Maxwell A. 2021. DNA Topoisomerases: Advances in Understanding of Cellular Roles and Multi-Protein Complexes via Structure-Function Analysis. BioEssays. 43(4). doi:10.1002/bies.202000286.

Moody CA. 2017. Mechanisms by which HPV Induces a Replication Competent Environment in Differentiating Keratinocytes. Viruses. 9(9):261. doi:10.3390/v9090261.

Moody CA, Laimins LA. 2010. Human Papillomavirus Oncoproteins: Pathways to Transformation. Nature Reviews Cancer. 10(8):550-560. doi:10.1038/nrc2886.

Morris G, Walker AJ, Berk M, Maes M, Puri BK. 2017. Cell Death Pathways: A Novel Therapeutic Approach for Neuroscientists. Molecular Neurobiology. 55:5767-5786. doi:10.1007/s12035-017-0793-y.

National Cancer Institute. 2019. HPV and Cancer. National Cancer Institute. https://www.cancer.gov/about-cancer/causes-prevention/risk/infectious-agents/hpv-and-cancer.

Nitiss JL. 2009. DNA Topoisomerase II and its Growing Repertoire of Biological Functions. Nature Reviews Cancer. 9(5):327-337. doi:10.1038/2608.

Nitiss JL. 2009. Targeting DNA Topoisomerase II in Cancer Chemotherapy. Nature Reviews Cancer. 9(5):338-350. doi:10.1038/nrc2607.

Ozbun MA. 2002. Human Papillomavirus Type 31b Infection of Human Keratinocytes and the Onset of Early Transcription. Journal of Virology. 76(22)11291-11300. doi:10.1128/jbi.76.22.11291-11300.2002.

Pommier Y, Sun Y, Huang SN, Nitiss JL. 2016. Roles of Eukaryotic Topoisomerases in Transcription, Replication, and Genomic Stability. Nature Reviews Molecular Cell Biology. 17(11):703-721. doi:10.1038/nrm.2016.111

Raff AB, Woodham AW, Raff LM, Skeate JG, Yan L, Da Silva DM, Schelhaas M, Kast WM. 2013. The Evolving Field of Human Papillomavirus Receptor Research: A Review of Binding and Entry. Journal of Virology. 87(11):6062-6072. doi:10.1128/jvi.00330-13

Rogers C, Fernandes-Alnemri T, Mayes L, Alnemri D, Cingolani G, Alnemri AS. 2017. Cleavage of DNAG5 by Caspase-3 during Apoptosis Mediates Progression to Secondary Necrotic/Pyroptotic Cell Death. Nature Communications. 8(1). doi:10.1038/ncomms14128.

Scaffidi P, Misteli T, Bianchi ME. 2002. Release of Chromatin Protein HMGB1 by Necrotic Cells Triggers Inflammation. Nature. 418(6894):191-195. doi:10.1038/nature00858.

Tampa M, Mitran CI, Mitran MI, Nicolae I, Dumitru A, Matei C, Manolescu L, Popa GL, Caruntu C, Georgescu SR. 2020. The Role of Beta HPV Types and HPV-Associated Inflammatory Processes in Cutaneous Squamous Cell Carcinoma. Journal of Immunology Research. 1-10. doi:10.1155/2020/5701639.

Tan Y, Chen Q, Li X, Zeng Z, Xiong W, Li G, Li X, Yang J, Xiang B, Yi M. 2021. Pyroptosis: A New Paradigm of Cell Death for Fighting Against Cancer. Journal of Experimental and Clinical Cancer Research. 40(153). doi:10.1186/s13406-021-01959-x.

Tang R, Xu J, Zhang B, Liu J, Liang C, Hua J, Meng Q, Yu X, Shi S. 2020. Ferroptosis, Necroptosis, and Pyroptosis in Anticancer Immunity. Journal of Hematology and Oncology. 13(1):110. doi:10.1186/s13045-020-00946-7.

Tsuchiya K, Hosojima S, Hara H, Kushiyama H, Mahib MR. Kinoshita, Suda T. 2021. Gasdermin D Mediates the Maturation of IL-1α Downstream of Inflammasomes. Cell Reports. 34(12): 108887. doi:10.1016/j.celrep.2021.108887.

Vermeer DW, Coppock JD, Zeng E, Lee KM, Spanos WC, Onken MD, Uppaluri R, Lee JH, Vermeer PD. 2016. Metastatic Model of HPV+ Oropharyngeal Squamous Cell Carcinoma Demonstrates Heterogeneity in Tumor Metastasis. Oncotarget. 7(17):24194-24207. doi:10.18632/oncotarget.8254.

Vinodhini K, Shanmughapriya S, Das BC, Natarajaseenivasan K. 2012. Prevalence and Risk Factors of HPV Infection Among Women from Various Provinces of the

World. Archives of Gynecology and Obstetrics. 285(3):771-777. doi:10.1007/s00404-011-1255-8.

Wang Y, Gao W, Shi X, Ding J, Liu W, He H, Wang K, Shao F. 2017. Chemotherapy Drugs induce Pyroptosis through Caspase-3 Cleavage of a Gasdermin. Nature. 547(7661):99-103. doi:10.1038/nature22393.

Wang Q, Wang Y, Ding J, Wang C, Zhou X, Gao W, Huang H, Shao F, Liu Z. 2020. A Bioorthogonal System Reveals Antitumor Immune Function of Pyroptosis. Nature. 579(7799):421-426. doi:10.1038/s41586-020-2079-1.

Yang Z, Li L, Chen L, Yuan W, Dong L, Zhang Y, Wu H, Wang C. 2014. PARP- 1 Mediates LPS-Induced HMGB1 Release by Macrophages through Regulation of HMGB1 Acetylation. Journal of Immunology. 193(12):6114-6123. doi:10.4049/jimmuol.1400359.