Hybrid Recombineering:
CRISPR/Cas9 Mutagenesis of Murine Cytomegalovirus BAC

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1. Introduction

At what point do you realize that you have contracted an illness? Is it the sign of a slight cough and low-grade fever? Maybe it is the development of a sore throat and congestion that suggest an infection. While no one enjoys being sick, we take for granted the ability of our immune system to acknowledge and respond to foreign pathogens, bringing about the aforementioned indicators of illness. Unfortunately, our immune system is incapable of effectively detecting all pathogens and can be circumvented by certain diseases. Reputably, human cytomegalovirus (HCMV) is a ubiquitous herpesviruses that can asymptomatically infect immunocompetent hosts and establish lifelong latency. HCMV is most commonly recognized as a leader in congenital disease that infects approximately 35,000 infants in the US alone, causing disabilities including mental retardation, blindness, and vision loss amongst roughly 8,000 children each year. In the National Health and Nutrition Examination Survey of 2006, the seroprevalence of CMV was measured to be ~60% in people six years of age or older, and from the age of 6 to 80+, the prevalence of CMV increased from 36% to 91% [1]. HCMV is studied to trigger severe symptomatic infections in hosts that are immunocompromised such as pre-exposed neonatal, organ transplant, and AIDS patients. HCMV is a unique pathogen as it hijacks the immune system for both infection and latency depending on its host's immunological state. While the necessity of a vaccine is an urgent matter, principal focus is positioned on the development of a comprehensive grasp and subsequent investigation of HCMV's broad dissemination processes. Current studies suggest that polymorphnuclear leukocytes play a key role in HMCV dissemination, and many current explorations specifically emphasize the role of neutrophils.

2. Effects of HCMV

2.1 Introduction and History of CMV

Cytomegalovirus (CMV) is a common betaherpesvirus that infects nearly 70% of the human population and was first discovered in 1881 by researchers who believed the CMV-infected cells were simple protozoa [2, 3]. The viral-infected cells found in 4 infants were described as large cells containing a "central nuclear body" surrounded by a clear halo; the first description of standard cytomegalic cells with intranuclear inclusions [2]. However, it was not until 1954, when human cells could be grown in culture, that HCMV could be routinely cultured *in vitro* [2]. Studies over the past 60 years have continued to elucidate how HCMV has evolved to continue to affect such a diverse population range. While the majority of those infected with the virus remain asymptomatic throughout their lives, CMV typically arises as a threat once the host has become immunocompromised [2]. Symptomatic, life-threatening HCMV is studied to be characteristically most severe in immunocompromised hosts [2]. The core host range includes neonatal individuals, organ transplant patients, and individuals diagnosed with acquired immune deficiency syndrome (AIDS).

2.2 Neonatal Population

For neonatal hosts, HCMV is the most common infectious diseases present at birth affecting 0.2-2.0% of all newborns [4]. While 90% of all infected infants are asymptomatic, the remaining 10% develop cytomegalic inclusion disease (CID) and have a mortality rate of 20-30% [4]. Of the 10% symptomatic infants infected with CMV, up to 15% of these patients eventually develop long-lasting neurological conditions including visual impairment, hearing loss, and mental retardation [4]. The confirmation of active infection and treatment of HCMV for infants and newborns is proportionally based on the phenomenon of HCMV antigenemia where antigen-positive leukocytes are detected [4]. Reports show that HCMV antigenemia of newborns *in utuero* have a low sensitivity and low positive predictive value for HCMV detection [4]. Notably, antigenemia is significantly higher at birth for symptomatic than asymptomatic newborns, but the correlation and usefulness of HCMV antigenemia as a clinical marker for intrauterine HCMV infection severity had not been studied until the work of Kaneko et al. Though this research suggests that antigenemia could be used for the evaluation of viral activation in intrauterine infection, an antigenemia assay was reported not sensitive enough for HCMV diagnosis [4]. In addition to infants, CMV actively affects people that are immunocompromised including organ transplant recipients triggering complications associated with mononucleosis, retinitis, and pneumonia, while CMV remains asymptomatic in the immunocompromised [5].

2.3 Organ Transplant Population

For individuals who undergo organ transplantation, HCMV has been identified as the most important cause of infectious disease [2]. HCMV affects 75% of all organ recipients within one year after transplantation, and Ho et al. discovered that 80% of people who were HCMV seronegative prior to their kidney transplant had become seropositive when the organ originate from a seropositive donor. Similar studies have found that when the donor is not seropositive, HCMV infections could be reactivated in recipients after kidney, cardiac, liver, and marrow transplants [2]. Rubin et al. suggest that the leading factors for HCMV pathogenesis in organ transplant recipients are due to viral latency, cell association, endothelial cell infection, and immune activation. In liver transplants specifically, one study of 143 consecutive liver transplant recipients found a 22% graft loss rate in individuals absent of HCMV, but in the presence of HCMV, graft loss was nearly double [6]. In all patients suffering from HCMV infection and simultaneous organ transplant complications, endothelial cells and leukocytes had become activated which helped to support injury in allografts both humoral and cellular in nature [6]. Additional research has shown that the presence of symptomatic HCMV disease is associated with a ten-fold increase in post-transplant lymphoproliferative disease (PTLD) and growth factors, cytokines, and chemokines associated with the immune response to HCMV infection are the cause for the relation [6].

2.4 AIDS Population

In adults, HCMV infection has been observed to be the most severe in individuals who are also AIDS patients affecting between 21 and 44% of this entire population. In specific AIDS patients, HCMV infection also causes a severe involvement with the central nervous system (CNS), which suggests that HCMV could be using the CNS as a route to evade the entire body in these individuals [2]. Alongside the degradation of the immune system that occurs in AIDS patients, the co-occurrence of HCMV infection also causes an array of organ and cell involvement, as well [2]. Retinitis caused by HCMV infection is the most disabling characteristic of HCMV infection in AIDS patients, but in regards to the nervous system, HCMV polyradiculopathy causes AIDS patients to experience weakness, loss of control the bladder and anal function, and loss of sensation or reflexes in the legs [2]. The co-contraction of HCMV for people with AIDS can also lead to HCMV encephalitis, which is difficult to distinguish from HIV dementia, but causes cognitive and motor disturbances including impaired memory, unsteadiness, forgetfulness, and confusion [2]. The scope of individuals severely affected by HCMV seems to have strong links to those that are or become immunocompromised, although the disease can also affect the immunocompetent. While HCMV is likely asymptomatically present in the myriad of the human population, the key features of this disease must lay in the virus's ability to manipulate the immune system and remain latent within its host.

3. Latency in HCMV Infection

3.1 Overview of HCMV and Latency

As a herpesvirus, HCMV persists in its host by establishing subliminal, lifelong latency. Through millions of years of coevolution alongside the human body, HCMV has essentially maintained a biological technique to evade its hosts without detection and has formulated a system that manipulates the human immune system against its elimination. The pathway for HCMV to establish such a latency within its host is largely due to the virus containing the largest gene cassette of any herpesvirus that is solely committed to altering both the innate and adaptive immune responses of the human body [7].

3.2 *Latency in the Innate Immune System*

The innate immune system's method of operation against bodily infections is the production of a range of plasma proteins that induce inflammatory mediators, phagocyte opsonization, and lysis of pathogens and infected cells [7]. Since these pathways lead to the production of chemoattractants, opsonizing factors, and the membrane attack complex as a complement for antibody-mediated lysis, herpesviruses such as HCMV genetically encode complement regulatory proteins to prevent such occurrences [7]. Another aspect of innate immune response to inhibit viral replication is the production of interferons (IFNs). During early infection, HCMV encodes a range of proteins that assuage the activation of IFN-α and IFN-β by deactivating a specific protein kinase that seeks to block protein synthesis of virus infected cells [7].

Other cells that play a significant role in eliminating virus-infected cells are natural killer (NK) cells, whose activities are also interfered with by various CMV-produced proteins [7]. HCMV also encodes for interleukin-10 (IL-10), which is a cytokine homologue that suppresses the immune system through the downregulation of inflammatory cytokine synthesis, alteration of antigen appearance, and proliferations of regulatory T-cells [7]. Through this suppression of the immune system, CMV self-creates an environment that is conducive to effective replication within the host [7]. Other cytokines homologues produced by the virus seek to avert the immune system through blocking T-cell propagation and possibly impairing lymphocyte responses [7].

In addition to the cytokines, CMV chemokine homologues seek to block host chemokines from effectively trafficking and activating leukocytes. Research has found a CMV-encoded chemokine, which is responsible for inducing chemotaxis and intracellular calcium release in human neutrophils, that is significant to CMV dissemination within a host and facilitates an important interaction between HCMV and neutrophil response to its presence [7]. As the focus of the background, the nature of involvement between HCMV and neutrophil interactions will be discussed in depth subsequently in the text. Interestingly, the same genetic locus that encodes for the previous chemokine was also found necessary for leukocyte and endothelial cell tropism, which has also explained a loss of endothelial cell tropism in strains where the specific gene for the chemokine had been mutated or deleted [7]. In addition to the previous mechanisms by which HCMV evades and suppresses the innate immune system, the virus has also evolved strategies to prevent apoptosis, which is a mechanism for programmed cell death to eliminate virally-infected cells within a host [7]. HCMV specifically prevents apoptosis damage to virus-infected cells' mitochondria by producing proteins that function to induce structural changes in the mitochondria that inhibit the release of cytochrome *c*, preventing the events of apoptosis to occur [7].

3.3 Latency in the Adaptive Immune System

The adaptive or acquire immune system takes a highly specialized response to pathogens that invade the host, and in the case of CMV infection, T-cell mediated responses are integral to the clearance of the virus [7]. More specifically, HCMV seeks to impair $CD8^+$ T-cell dependent responses through interference with antigen processing and appearance of both major histocompatibility complex (MHC) classes I and II [7]. In addition to the impairment of $CD8^+$ T cell responses, the degradation of MHC class I, predominantly derived from HCMV's encoded endoplasmic reticulum (ER) glycoproteins, causes infected cells to become less susceptible to natural killer (NK) cell toxicity, which may explain HCMV's mechanical evolution to prevent NK lysis [7]. In an investigation by Mason et al., HCMV latency was found to cause changes in the CD34+ secretome and other cellular cytokines and chemokines resulting in CD4+ T-cell chemotaxis [8]. This study suggest that HCMV latency is highly immunosuppressive and highlights a mechanism for immune response avoidance through the manipulation of specific T-cells [8].

Largely, HCMV's coevolution alongside the human host has allowed for the manipulation, modulation, and overall alteration to the immune system, and its mechanisms have allowed for the virus to establish lifelong latency. Holistically, the virus and the immune system have effectively established a relationship that ensures a "mutually assured survival" rather than mutually assured destruction [7]. Vast research has revealed that a large range of viral proteins and host cells play a complex role in the immune system's overall alteration, manipulation, and suppression which allows for HCMV to be effectively disseminated throughout the human body.

4. Neutrophil Function, Importance, and Role in CMV

4.1 Introduction

While a number of host cells are suspected of having an intimate relationship with HCMV dissemination, some studies have suggested that neutrophils play a substantial role in the movement of the virus throughout the body. Characteristically, neutrophils are quite complex cells. They are significant to the host immune system, ironically involved in the pathology of various diseases, and are heterogeneous, meaning that subpopulations of neutrophils exist in diverse stages ranging from fully active to dormant [9]. Neutrophil activity is regulated by its local microenvironment and in conjunction to a multitude of mediators such as cytokines, neuroendocrine hormones, and bioactive lipids, and a vast range of research has and will continue to be completed to identify these essential mediators and their interactions upon neutrophils [9].

Neutrophils belong to the large group of white blood cells of the immune system known as polymorphnuclear leukocytes (PMNLs), which includes neutrophils, eosinophils, and basophils. Representing 50 to 60% of circulating leukocytes, neutrophils travel throughout the bloodstream and are readily accessible by the immune system as the "first line of defense" against bacteria, fungi, protozoa, viruses, virally infected cells, and tumor cells [9]. In their immune processes, neutrophils form combinations of reactive oxygen species (ROS) and various enzymes that can be influenced both positively and negatively by the array of mediators previously mentioned [9]. While they are primarily thought to be effector cells that act in response to other immune functions, neutrophils have also been found to secrete their own mediators, such as cytokines, to play a role in host immunity [9].

As members of the nonspecific immune system, the major role of neutrophils is to phagocytose and obliterate infectious agents that are foreign to the host, but they also restrict the growth of some microbes, which buys time for specific immunological responses to develop [9]. While extensive research has elucidated the importance of neutrophils in fighting bacterial and fungal infections, there has been restricted focus placed on their involvement in viral infections. In the case of influenza, neutrophils seem to be the principal cells responsible for blocking the initial stage of infection in mice, and they also appear to play an important role in diminishing the infection of herpes by opsonizing the virus and virally-infected cells via antibodies [9]. This is interesting to note because it supports the idea that neutrophils contribute to the defense

against the herpesvirus, while other extensive research supports the idea that neutrophils aid in the virus's latency and spreading [10-13].

4.2 Recruitment of Neutrophils

While monocytes appear to be the major cell-type harboring HCMV in immunocompetent seropositive persons, various research reports that neutrophils are a major reservoir of infectious HCMV in the immunocompromised as well as in the immunocompetent [10]. In peripheral blood of viremic patients, infectious virus has more often been associated with neutrophils than monocytes, and the overall magnitude of CMV DNA present in polymorphnuclear leukocytes is more significant than in mononuclear cells [10]. While the origin of the virus detected in neutrophils is unknown, CMV-infected endothelial cells are prime candidates as the source of CMV infection of neutrophils in vivo [10]. The speculation of the research by Grundy et al. is that since they are the first immune cells recruited to sites of CMV-infection in the endothelium, neutrophils must procure infectious virus from the interaction with endothelial cells. Findings that show that neutrophils were able to harbor the virus in its infectious state lead to the conclusion neutrophils must play an important role in HCMV dissemination during acute infection [10].

It had previously been assumed that neutrophils acquire CMV through phagocytosis of virions or debris form infected cells, but the previously cited study proposes that neutrophils obtain the virus through direct contact with CMV infected endothelial cells [10]. The release of CXC chemokines and other neutrophil chemoattractants occur simultaneously with the release of viral particles from infected endothelial cells, initiating the infection process of neutrophils [10]. Once neutrophils make direct contact with CMV infected endothelial cells, the virus is transmitted to neutrophils, which can then disseminate throughout the body via the bloodstream [10].

4.3 HCMV Chemokine for Neutrophil Signaling

HCMV contains 82 open reading frames (ORFs) in its genome that are unnecessary for viral replication *in vitro* but may play a role in its immune evasion in vivo [11]. The ORFs *UL146* and *UL147* are found to have a very restricted homology to host CXC chemokines, yet, the *UL146* protein of the Toledo HCMV strain (vCXCL-1_{Toledo}) acts as a functional CXC chemokine that binds to chemokine receptors (CXCR1 and CXCR2), prompts calcium mobilization, and induces neutrophil chemotaxis [11]. However, the UL146 gene has been found to be one of the HCMV genome's most variable [11]. The hypothesis for this was that hypervariable vCXC-1s manufactured from HCMV-infected endothelial cells recruit neutrophils with alterations in their bindings, activations, and functions that aid to HCMV dissemination and possibly pathogenesis [11].

Of the nine HCMV strains uses by Heo et al., the Towne strain produced a vCXCL-1 with a low affinity for CXCR2, a lower calcium flux, minimal ability for chemotaxis, and no signaling compared to the virulent Toledo strain vCXCL-1 [11]. While the evidence is

circumstantial, vCXCL-1 differences in peripheral blood neutrophils (PBNs) could be a major factor to HCMV virulence [11]. The specific role of vCXCL-1 in pathogenesis is difficult to clarify without knowing the concentrations of these chemokines in an active in vivo infection [11]. The study proposes two nonexclusive models for how HCMV vCXCL-1s could function in vivo through either the "neutrophil shuttle model" and/or the "neutrophil amplifier model" [11].

In the "neutrophil shuttle model," neutrophils are suggested to function as vehicles for HCMV dissemination [11]. Similar to the process suggested in [9], this model proposes that PBNs acquire HCMV during neutrophil endothelial transmigration and transmit infectious HCMV to fibroblasts [11]. In the "neutrophil amplifier model," focus is placed on vCXCL-1's induction of neutrophilic granules and the secretion of specific chemokines and cytokines [11]. The presence of these inflammatory mediators could cause inflammatory responses that consequently recruit other infiltrating immune cells as better vehicles for HCMV dissemination [11].

4.4 Replication of HCMV in PMNLs

While it is known the PMNLs are not the site of HMCV persistence in the immunocompetent population, results suggest that infectious virus and late viral products were detected in PMNLs 1-3 hours after cocultivation with clinical HCMV strain-infected human umbilical vein endothelial cells (HUVEC) [12]. However, the study found that HCMV virions and viral material detected in PMNLs are not the result of a complete viral replication process occurring within PMNLs, but the experiment upholds that the presence of HCMV in PMNLs is due to direct cell-to-cell contact with infected endothelial cells [12]. Their conclusion is supported by finding that HCMV DNA is distributed in comparable amounts between the nucleus and cytoplasm of cocultured PMNLs, which suggests that some virions that enter the PMNLs are uncoated when entering the nucleus, initiating immediate-early (IE) transcription of HCMV [12]. The results indicate that all HCMV material detected in PMNLs were gained via transfer directly from infected cells, and that infectious virus nor late viral genes are markers of active virus replication in PMNLs [12].

In the same study, cell-to-cell transmission of HCMV was responsible for 100% of the structural virion phosphoprotein pp65 antigen and infectious virus and more than 90% of the nucleic acids of HCMV in PMNLs cocultured with clinical-strain HCMV-infected HUVEC [12]. This phenomenon was completely absent with the use of laboratory-adapted HCMV strains, which were found to have a slower replication rate in stromal cells compared to the clinical HCMV strains [12]. Because of this, the study argues that active transfer of HCMV must be mediated by precipitous microfusion events [12]. However after a 24-hour coculture period, the quantity of PMNLs positive for CMV DNA approximated 100%, specifically measuring high for pp65 and p72 (the CMV IE antigen) and IE-mRNA but low for infectious CMV [12].

In all, the study showed that HCMV and viral materials identified in PMNLs were permissively transferred via infected endothelial or human fibroblast cells from in immunocompetent individuals, but only abortive (partial) replication was detected within the

PMNLs [12]. This phenomenon is mediated via rapid microfusion events of PNNLs and infected cells, only occurring from the wild-type HCMV strain rather than laboratory-adapted strains of HCMV [12]. With majority of PMNLs being positive for HCMV DNA over a 24 hour coculture period, it was concluded that virtually all PMNLs come into contact with HCMV infected cells and receive a minute amount of HCMV-infected material during an observed lag time [12].

4.5 Neutrophils as HMCV "Trojan Horse"

Prior to 2001, the relative contribution of different leukocytes to the overall viral load of HMCV remained a contested debated amongst researchers. For those who support PMNLs as the main population involved, their studies have been implicated by research that suggest that HCMV's presence in this cell type only due to their phagocytic abilities and not productive infection [14]. However, the detection of immediate early and late transcripts in PMNLs, monocytes and lymphocytes in vivo has lead to conflicting results [15-16].

In support of active infection of HCMV in PMNLS in addition to other white blood cells, the work of Hassan-Walker et al. [2001] supports that active HMVC DNA can be detected in PMNLs, monocytes, B-cells, and T-cells [17]. In their groundbreaking study, Hassan-Walker et al. extended the work of previous studies by assessing the relative contribution of the aforementioned leukocyte fractions to the overall viral load in the blood and found that PMNLs contribute more to the overall viral load in the blood with median viral load of $10^{5.37}$ genomes/ml of blood compared to $10^{4.40}$ genomes/ml of blood for monocytes [17]. These results are consistent with the findings that PMNLs (mostly neutrophils) constitute 55-60% of the total peripheral leukocyte population [18]. The overall HMCV DNA load for each leukocyte population can be illustrated via Figure 1 [17].

In addition, Hassan-Walker et al. found that while PMNLs carried most of the HCMV load in the blood, monocytes were found to carry a higher viral load of DNA/cell equivalent to 0.65 genomes/monocyte compared to 0.11 genomes/PMNL, which details the importance of monocytes during HMCV viraemic episodes [17]. The high load of viral DNA/cell for monocytes is consistent with previous studies that suggest that monocytes are sites for HCMV reactivation as the cells differentiate into macrophages [19-21]. Hassan-Walker agrees that the greater viral load/cell supports a productive infection in monocytes greater than all other cell types and is consistent with previous observations by Yurochko and Huang (1999) [22].

In their conclusion Hassan-Walker contends that although monocytes harbor the most productive viral replication amongst the white blood cells, PMNLs must contribute significantly to viral dissemination in multiple tissues due to the detection of high viral loads and HMCV glycoprotein-B transcripts that are indicative of active HMVC viraemia [17]. This conclusion is consistent with a study by von Laer et al. (1995) where HCMV immediate early and late mRNA were found in PMNLs in addition to monocytes to provide strong evidence for active HCMV infection in these cells types [23]. *In vitro* studies like the aforementioned and by Grundy et al. (1998) have shown that HCMV can infected PMNLs and induce the secretion of chemokines that are neutrophil attractants, which has led Grundy et al. to proposed that neutrophils do not retrieve

HCMV via phagocytosis but through transmigration through HCMV-infected endothelial cells [10]. More recent than the Grundy et al. (1998) study, Gerna et al. (2000) utilized an *in vitro* model to propose that the presence of HCMV in PMNLs was due to either the major mechanism consisting of transmigration across infected endothelial cells or via the acquisition of HCMV RNA and DNA via endocytosis [12].

4.6 Plan to Investigate Neutrophils

Although we suspect that neutrophils play a significant role in the dissemination of HCMV, we agree with the claim by Hassan-Walker et al. (2001) that the current *in vitro* systems do no tell the complete story of HCMV dissemination or reflect the environment of PMNLs in vivo [17]. Knowing that chemokine vCXCL-1 encoded by *UL146* in the HCMV genome is primarily associated with neutrophils and functions as a neutrophil-attracting agent that induces chemotaxis, we plan to utilize this gene to further investigate CMV dissemination. However to begin this work, we must first develop a system to assess the role of neutrophils in the dissemination of CMV in a laboratory *in vivo* model within mice. Instead of *UL146,* murine CMV (MCMV) encodes for a murine chemokine (MCK-2) that primarily attracts macrophages on secretion [24]. The current MCMV model does not encode for neutrophil-specific chemokines to allow assessment of the role of neutrophils in the dissemination of CMV *in vivo*. We plan to mutate the current MCMV model by replacing the MCK-2 genes with the genes corresponding to the HCMV neutrophil chemokine utilizing the CRISPR-Cas9 system.

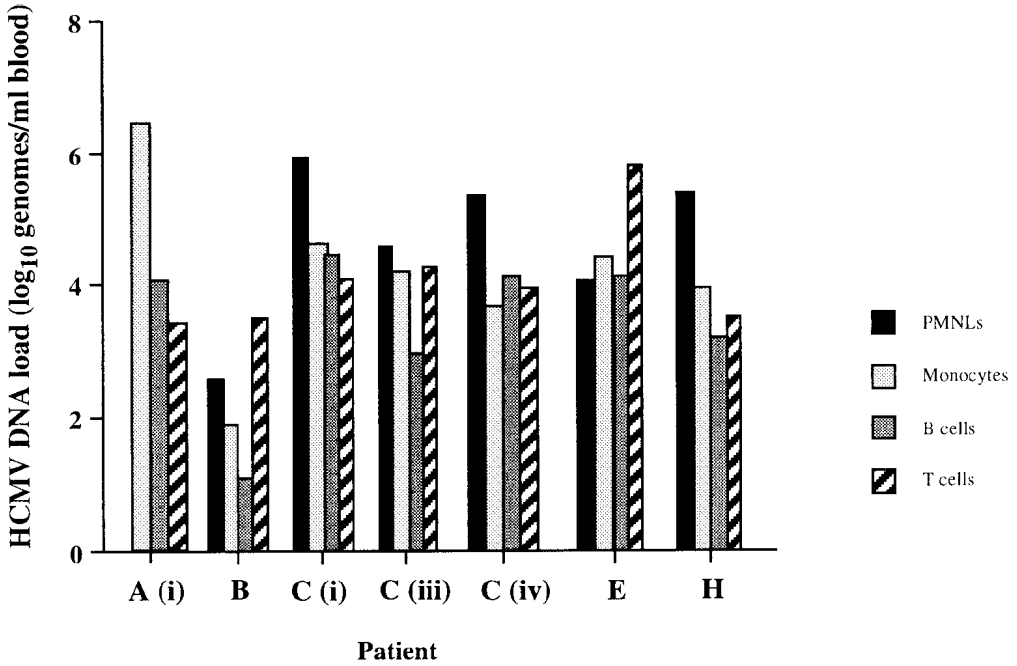

Figure 1. Bar graph of HCMV DNA load in leukocyte subpopulations. Samples are from patients A, B, C (i, iii, and iv), E, and H, and all data shown is expressed as genomes/ml of whole blood [17].

5. CRISPR-Cas9 Mutagenesis System

5.1 Introduction and Function

Although clustered regularly interspaced short palindromic repeats (CRISPR) was discovered in 1987 in *Escherichia coli,* the interest in this genetic element boomed in the 2000s along with many CRISPR-associated (Cas) proteins [25-27]. The most important aspect in its discovery was the ability of short spacers, originating from extra-chromosomal DNA within the repeats of CRISPR, to provide resistance for bacteria from bacteriophage and plasmid transformations [28-30]. Utilizing previous *in silico* analyses that suggested that spacers were being derived from *S. thermophilus* plasmids and that the CRISPR/Cas system interferes with plasmid transfer in this species, Garneau et al. hypothesized that the CRISPR/Cas system was responsible for the shortage of plasmids in wild-type strains of *S. thermophilus* and investigated the *in vivo* activity of CRISPR/Cas system against bacteriophage and plasmid DNA [30-34]. In the previously mentioned study, Garneau et al. found that the *S. thermophilus* CRISPR/Cas system cleaves both bacteriophage and plasmid DNA *in vivo* through endonuclease activity, which is responsible for the observed resistance [34]. The pathway for the CRISPR/Cas system functions in three phases including acquisition/adaptation of CRISPRs, expression/biogenesis of CRISPR RNAs (crRNAs), and interference/silencing of invader nucleic acids [35-36].

During adaptation, a fragment or copy of nucleic acid foreign to the host will be acquired and integrated into the CRISPR locus (Figure 2) [35]. These integrated fragments are termed protospacers and integrated into the CRISPR sequence immediately following the leader sequence and provides a chronological order of previous infections or exposure to invaders (Figure 2) [35]. Following acquisition of foreign nucleic acids, the CRISPR loci undergoes transcription to express numerous individual crRNAs and subsequent translation to produce Cas proteins that are constitutively expressed to create an 'immune system' within prokaryotes that operates in 'surveillance mode' to detect the nucleic acid of previous invaders [35]. Once transcribed, crRNAs are incorporated into effector complexes that guide the system to invading nucleic acids to silence invading RNA or DNA, which requires the targeting of a protospacer adjacent motif (PAM) in the DNA target (Figure 2) [35]. To ensure that the CRISPR system does not target its own spacer identical to the protospacer in the invader, the PAM recognized by the CRISPR/Cas system of the invader is not present in the repeat spacer's that are incorporated into the CRISPR locus [35].

Within the CRISPR Locus, there are groups of conserved protein-encoding genes (*cas* genes) that have identifiable helicases, nucleases, polymerases, and RNA-binding proteins that suggest that they may collectively be part of a novel DNA repair system [37]. Since the order, orientation, and grouping of *cas* genes are extremely variable and continue to grow in complexity as the number of associated genomes increases, attempts to classify Cas proteins have been made but have proven to be difficult [38-40]. The major Cas proteins have been identified as Cas1 – Cas10, and based on their respective but integrated phylogeny, sequence, locus organization, and content, three types of CRISPR/Cas systems have been identified [39].

Of these, the most pertinent to our mutagenesis system is the Cas9 protein under CRISPR/Cas Type II system.

The Type II CRISPR system is represented by the Cas9 signature protein, which is a large multifunctional protein that has the ability to biosynthesize crRNA and target bacteriophage and plasmid DNA for degradation [34]. Type II is described as the simplest of the CRISPR/Cas systems because it only has 4 genes that compose its operon including *cas9, cas1, cas2,* and either *cas4 or csn2* [36]. The best-studied model of Type II CRISPR/Cas is in that of *S. thermophilus* against bacteriophage and plasmid DNA [29, 34]. In 2011, it was discovered that a *trans*-encoded small CRISPR RNA (tracrRNA) is utilized to process pre-crRNA into crRNA within the Type II system through the creation of a duplex with the CRISPR repeat sequence [41]. It was later found that Cas9 with mature crRNA is enabled to interfere with matching foreign dsDNA by homology-driven cleavage within the protospacer sequence in the vicinity of the PAM, which allows phages and plasmids to circumvent the CRISPR 'immunity' due to mismatches at the 3' end of the protospacer and/or the PAM [34, 42].

5.2 Uses on Large Viral Genomes

In recent years, the CRISPR/Cas9 system has been applied on a variety of large viral genomes to induce genomic editing on a range of targets. In 2014, Bi et al. utilized CRISPR/Cas9 on recombinant adenovirus and type I herpes simplex virus to show that this mutagenesis system is valuable for editing large DNA viruses [43]. In this study, they confirmed the effectiveness of CRISPR/Cas9 by initiating site-specific insertions or deletions (indels) and inserting a foreign gene into an adenovirus vector (ADV) and type I herpes simplex virus (HSV-1) in a single step [43]. They found that the CRISPR/Cas9 system interfered with viral replication and efficiency of genome mutation and recombination increased significantly, which demonstrated the system's versatility [43]. The aforementioned experiments suggest that the CRISPR/Cas9 system can control the site of mutation efficiently through a high cleavage efficiency that inhibits viral replication and induces non-homologous end joining (NHEJ) and homology-directed repair (HDR) [43]. Knowing that repair efficiency can be a major concern for viral genomes, Bi et al. demonstrated that large quantities of the viral genome inside of a nucleus could not be repaired after cleavage due to the presence of hundreds of viral genomes inside a single cell, however they also show that some of the cleaved genomes could be repaired during the early stages of infection [43].

Also in 2014 following studies that showed that CRISPR/Cas9 could be used to mutate large viral genomes, Suenga et al. advanced these mutations to develop an efficient method for editing large viral DNA genomes [44]. In their experiments, the CRISPR/Cas9 system was used to edit the large 152 kb genomes of HSV-1 to generate specific mutations at a high frequency in less than two weeks without unexpected mutations versus traditional methods where mutant virus are created by spontaneous homologous recombination that typically takes several weeks [44]. Although previous studies were able to edit HSV-1 and adenovirus, Suenga et al. was able to generate a gene-ablated virus in addition to a revertant virus from the gene-ablated virus

(knock-in virus), which is essential for studying viral specific genes [43-44]. To push viral genome editing even further, Yuan et al. utilized the versatility of CRISPR/Cas9 to modify the vaccinia virus (VACV) by utilizing three guide RNAs (gRNAs) to target the viral genome and generate specific double-stranded breaks (DSBs) in the DNA of VACV to increase the efficiency of editing and creating new vectors of the virus [45]. They found that the gRNA-guided Cas9 could induce homologous recombination across multiple sites on the VACV genome simultaneously and rapidly without evident off-target effects [45].

Although the aforementioned studies [43-45] have showed that CRISPR/Cas9 can be utilized to edit and mutate large viral genomes, Bierle et al. were the first to apply this mutagenesis system on CMV [46]. Compared to previous studies that implemented CRISPR/Cas9 on large viral genomes, CMVs are described to be much more difficult to manipulate due to their size since HCMV is ~235-kbp with hundreds of proteins and noncoding RNAs [47-48]. Bierle et al. utilized a transfection/infection-based method to use CRISPR/Cas9 to introduce mutations into the genome of guinea pig CMV (GPCMV) by incorporating a simple viral-specific gRNA to induce indels at a predicted Cas9 cleavage site [46]. In addition, this study also cotransfected multiple gRNAs to generate markerless gene knockout, nonsense, and knock-in mutations by HDR to create specific changes to the GPCMV genomes [46]. The largest limitation of the study was the possibility of off-site targets of CRISPR/Cas9 on CMV genomes, but this study utilized predictive algorithms to exclude potential CRISPR sites with possible homology to other areas of the genomes [46]. Although a previous study found that the CRISPR/Cas9 mutagenesis affected the speed of viral replication and contributed to the limitations of its efficacy [43], Bierle et al. found only a modest decrease in the recovery of progeny virus transfected with specific gRNAs compared to non-targeting gRNAs [46]. It has been suggested that CRISPR/Cas9 may be more suited for mutagenesis on CMV compared to other large viral genomes due to its slow replicative cycle, which is possibly due to the size of CMV genomes [49-50]. In all and most importantly, CRISPR/Cas9 has been shown to be able to mutate CMV genomes and is described as a technically simple alternative to BAC recombineering, which is the current and most frequently utilized strategy to mutate CMV although it is extremely time consuming and challenging due to CMV's genome size [46].

5.3 Adaptation for MCMV Mutagenesis

Ran et al. proposed an in depth protocol that describes how the CRISPR/Cas9 can be used to facilitate efficient genome editing and engineering via NHEJ or HDR in mammalian cells [51]. Although we will utilize CRISPR/Cas9 to mutate a bacterial artificial chromosome (BAC) containing MCMV, the protocol by Ran et al. provides a guide for our adapted study. Figure 3 shows two ways that the mutagenesis system can be utilized for genome editing [51]. In an alternative project, we utilize the typical CRISPR/Cas9 system to induce NHEJ within the MCMV genome at the N-Deacetylase/N-Sulfotransferase 2 gene (NDST-2), which is a gene described to be important to the attachment/fusion of herpesviruses' viral envelop to host cell membranes during infection (Figure 3A) [51, 52]. By inducing the typical NHEJ by CRISPR/Cas9 on this gene, we can create an NDST-2 knockout cell line of MCMV for a variety of *in vivo* studies. For the focus of this experiment, we will use CRISPR/Cas9 to edit our MCMV BAC by an alternative DNA repair pathway described by Ran et al. that utilizes HDR [51]. In this pathway, new genes can be inserted in place of genes within the original DNA (in our case the MCMV BAC) via homologous regions flanking the insertion sequence that will trade places

following DSBs induced by the Cas9 system (Figure 3B) [51].

To elaborate on how we plan to utilize the system described by Ran et al. in Figure 3B, our first step will be to PCR produce a selectable gene that allows the bacteria to utilize galactose to reproduce (*galK*), which is created to have homologous flanking regions synonymous to *m128 and m129* on our MCMV BAC (Figure 4). Once this PCR product is created, BAC Recombineering is utilized to induce the uptake of *galK* into the BAC via homologous recombination via transformation (Figure 4, Step 1). Prior to step 2, a PCR product has to be created for the 2A-Tol/*UL146* gene that has homologous flanking regions synonymous to *m128 and m129* on our now mutated MCMV BAC with *galK* (Figure 4). Step 2 is where we will incorporate CRISPR/Cas9 mediated HDR that will create DSBs and allow for the recombination that will insert our HCMV genes into our MCMV BAC (Figure 4).

One limitation of our proposed utilization of CRISPR/Cas9 is that the system's extended presence can induce off-target effects along with Cas9 cytotoxicity, which can inhibit subsequent genetic mutations [53]. In this study, Wang et al. designed a 'suicide' CRISPR/Cas9 that is suitable for gene complementation via cis-arrangement of gDNA and Cas9 cassettes to on side of the homologous arms of the deletion construct with the rationale being that this would be a counter elimination process where the flanking DNAs are degraded by double crossover in homologous recombination, which was completed after showing that the CRISPR/Cas9 system induced mutations on their fungal DNA [53]. Wang et al. later tested this 'suicide' CRISPR/Cas9 system with a larger fragment to be eliminated that was over 5.0 kb in length and found that the gDNA and Cas9 could still be readily eliminated despite their size while maintain their editing functions [53]. This suggested suicidal concept increases the applicability of the CRISPR/Cas9 system because it has the ability to induce a complementation mutation to validate the function-phenotype relationship of the targeted genes, which would allow our experiment to avoid the need to sequence the entire genome following mutations [53]. We plan to later adapt this 'suicidal' CRISPR/Cas9 system in order to avoid off-target effects following our initial successes with the system's foundational mutagenesis.

In all, we will utilized *galK* Recombineering and the CRISPR/Cas9 mutagenesis system to mutate our MCMV BAC to insert the HCMV neutrophil attracting chemokine gene *UL146* to assess the role of neutrophils in CMV dissemination via the outline diagramed in Figure 4. This mutagenesis will be accomplished through the use of CRISPR/Cas9-mediated mutagenesis in addition to the common and universally utilized BAC recombineering system protocol that utilizes a *galK* insertion.

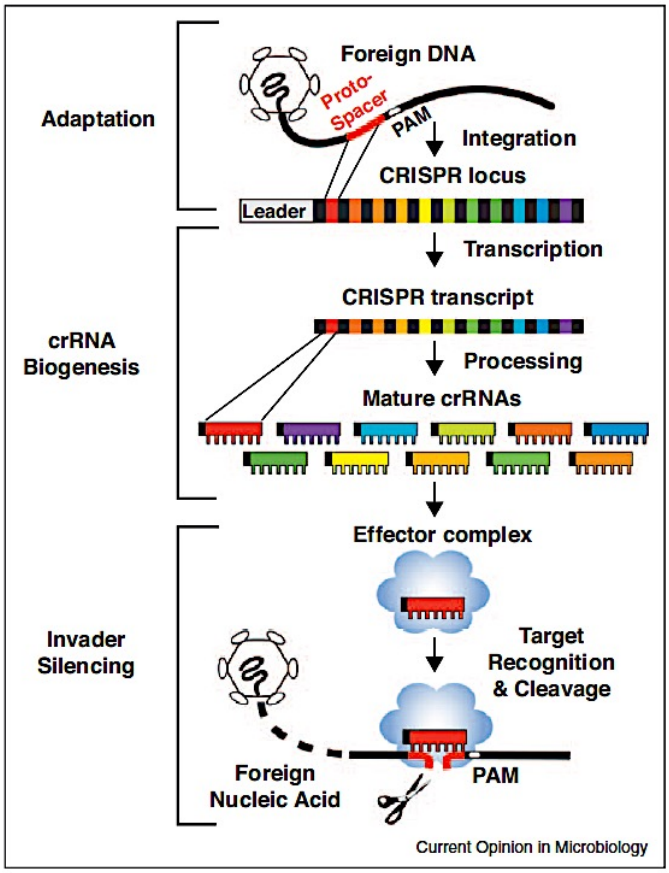

Figure 2. Overview of CRISPR/Cas defense pathway and function [35]. In adaptation, fragments of foreign DNA (protospacer) is acquired and integrated into host CRISPR locus. In crRNA biogenesis, CRISPR transcripts are processed to generate RNA products that target different genetic sequences. In silencing, crRNA-Cas proteins form complexes that recognize foreign nucleic acids and cleave targeted sites using PAM sequences as signals.

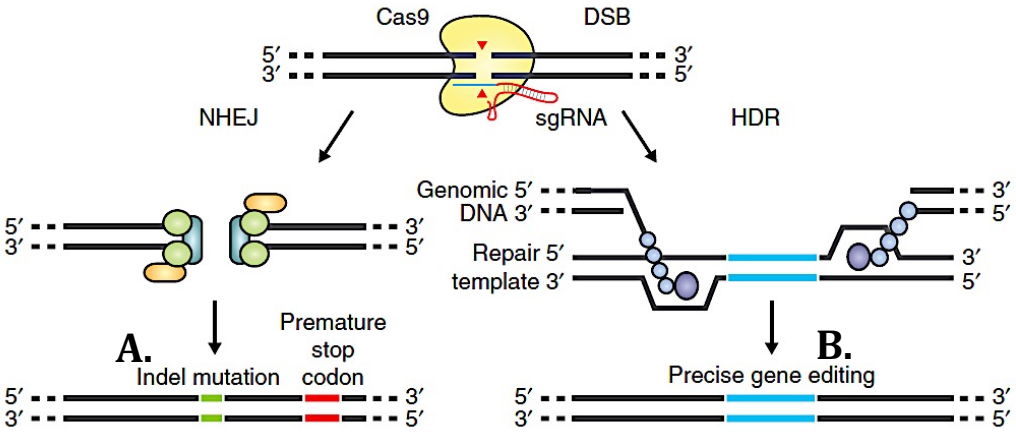

Figure 3. Two major pathways for DNA damage repair upon cleavage by Cas9 [51]. (a) NHEJ that leaves scars and can be used to generate gene knockout mutations. (b) HDR that can be utilized to create precise mutations at a target location.

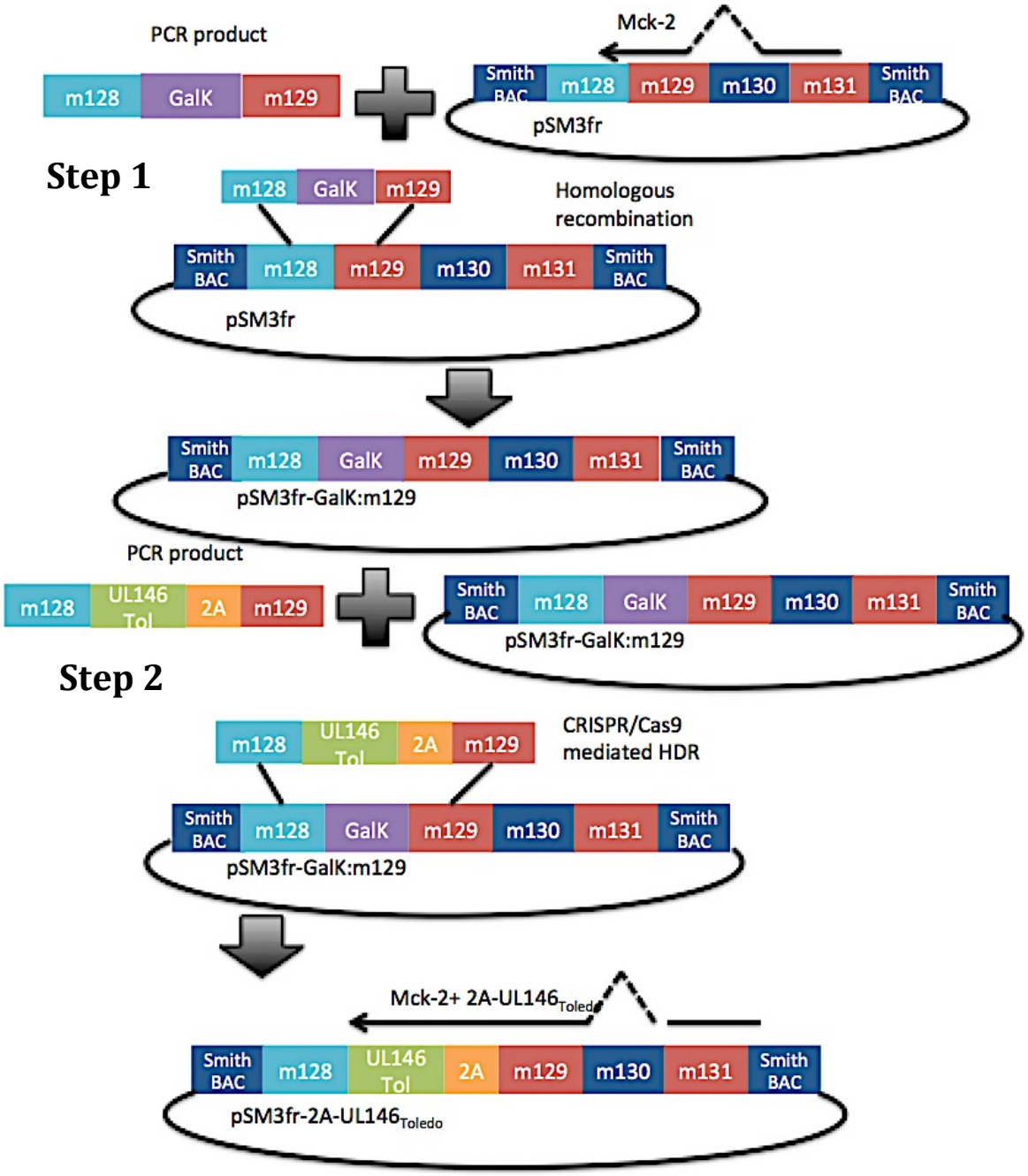

Figure 4. Schematic representation of mutation approach. This schematic shows the steps involved in the mutagenesis to produce mutant MCMV that expressing vCSCL-1 from the HCMV gene *UL146.* Step one involves PCR producing out *galK* insert product and transforming it into the Smith BAC labeled 'pSM3fr.' Step two involves utilizing CRISPR/Cas9 mediated HDR to insert our *2A-Tol/UL146* PCR product into the pSM3fr-GalK construct.

Materials & Methods

BAC

The Smith BAC containing the MCMV genome encodes for the MCK-2 chemokine homologue with a fixed point mutation named 'pSM3fr'. This point mutation allows for the expression of MCK-2 with low expression levels. This BAC was present in all experiments and was prepared by following the "BAC Miniprep" protocol outlined by Pranay Dogra.

The Smith BAC had to be prepared for transformation using the "BAC Miniprep" protocol. Overnight (O/N) cultures of the DH10B containing the BAC were grown at 37°C and centrifuged for 10 minutes at 4000rpm. After decanting, the pellet was resuspended in 200µl of P1, washed using 300µl of P2, and incubated at room temperature for 4 minutes to lyse the cells. 300µl of cold P3 was added to the mixture to precipitate out proteins and unneeded cell components, incubated for 5 minutes, and centrifuged at 4°C for 7 minutes at 13000 rpm. Clear supernatant was decanted, the pellet was washed with 700µl of ethanol, and recentrifuged for 7 minutes at 13000 rpm at 4°C. Supernatant was decanted and aspirated off. BAC preparations were allowed to air dry for 30 minutes and resuspended in 75µl TE plus RNAse. Whenever, BAC preparations are needed, this protocol is followed.

Cells

For efficient chemical transformations during the CRISPR experiments, DH10B MG1655 competent *E. coli* cells are used. For the *galK* recombineering experiment, SW105 *E. coli* cells are used for the transformations by electroporation. The SW105 cells are derived from DH10B-DY380-EL250 cells that possess a temperature-sensitive red recombinase and dysfunctional gal operon (*galK*-) for selection.

Plasmids

For the CRISPR experiments, the Cas9 plasmid containing kanamycin (Kan) resistance was purchased online from addgene© to mediate the experimental mutagenesis. The Fozo Lab supplied the ampicillin (AMP) resistant TOPO MCherry (MC) gRNA that was utilized by the CRISPR/Cas9 system throughout the length of the experiment.

PCR Products

For the *galK* recombineering experiments, *galK*-restoring sequences had to be PCR amplified from previously stored stocks. The MCK-2 *galK* m129 2A linker was designed by Joseph Jackson and then subsequently amplified via PCR. An alternate MCK-2 *galK* m129 link, labeled at T4, was designed by Tom Masi and subsequently amplified via PCR. For the CRISPR experiments, Joseph Jackson supplied the MC gene sequence that was later PCR replicated for ligation in conjunction with the Smith BAC for later mutagenesis.

CRISPR/Cas9 Mutagenesis Protocol

To recognize a MCherry sequence for mutation, the gRNA for MCherry had to be ligated into the Cas9 plasmid to enable it for MCherry recognition. The Cas9 plasmid was digested using the restriction enzyme BSA-I. The restriction digest included 1µl Cas9 plasmid, 1µl BSA-I (NEB), 5µl 10X NEB buffer, 0.5µl 100x BSA, and 42.5µl of ddH_20. The CRISPR/Cas9 digest was gel purified and then utilized in ligation to the MCherry oligos. The MCherry oligos, created by Joseph Jackson, were diluted to 1:5 and 1:10. The ligation mixture, consisting of 1µl of BSA-I digested Cas9, 1µl of 1:1, 1:5, or 1:10 MCherry oligo, 2µl 10x T4 Ligase buffer (NEB), 1µl T4 ligase, and 15µl ddH_20, were incubated overnight at room temperature.

Using the DH10B chemically competent cells, the ligation mixture was transformed using standard transformation protocol via heat shock. DH10B competent cells were thawed on ice while LB agar plates with CM/Kan antibiotics are warmed to 37°C. 3µl of the ligation

mixture is incubated with 20µl chemically competent cells on ice for 30 minutes, heat shocked at 42°C for 45 seconds, placed back on ice for 5 minutes, rescued with 800µl of SOC media, and placed in a shaking incubator for 1 hour at 37°C. After the rescue period, cells are pelleted and plated on the agar plates aforementioned. Colonies are observed after O/N incubation at 37°C and selected colonies are prepped for DNA sequencing. A similar CRISPR/Cas9 mutagenesis protocol was followed to incorporate the 2A-Tol gene product with Smith BAC homology into the Smith BAC to create a mutated virus product.

galK Recombineering Protocol

Joseph Jackson began this protocol by preparing/designing/ordering the needed *galK* primers to amplify the *galK* cassette and prepared all the needed agar plates (MacConckey) for galactose metabolism and minimum media agar plates (2-deoxy-galactose – DOG).

The aforementioned Smith BAC was transformed into SW105 cells and subsequently prepped and digested using the HIND-III restriction enzyme. All transformations in the *galK* recombineering protocol followed the "Transformation by Electroporation" protocol by Aixia Zhang, adapted by Liz Fozo, and modified by Joseph Jackson. At 30°C, an O/N culture of SW105 electrocompetent cells were grown. The next day, the O/N culture was diluted into 25mls of LB broth and incubated at 30°C until optical density of the culture reached 0.4 – 0.6 OD. Cells were pelleted at 4°C and 3600 rpm for 10 minutes, washed with equal amounts of ice-cold tissue culture water and repelleted twice, and then resuspended in 800µl of ice-cold tissue culture water. The cells were then pelleted at 13000 rpm at 4°C for 3 minutes and subsequently, the supernatant is aspirated off to resuspend the pellet in 90µl of ice-cold water.

The transformation mixture of ~45µl of competent cells and 1.5µl of Smith BAC is added to new ice-cold eppendorff tubes to be adequately mixed before being added to freezer-chilled Gene Pulse Cuvettes. Once added, cuvettes are removed of any residual water, and the transformation mixture is electroporated, rescued using 700µl of LB broth only, and incubated at 30°C for 1 hour. After the incubation period, the cells are pelleted and resuspended in 100µl of LB, diluted to 1:10, 1:100, and 1:1000, plated on CM/Kan LB agar plates, and incubated overnight at 32°C.

The next step in this protocol involves transforming the *galK* expression cassette in place of the MCK-2 encoding region using the PCR amplified products 2A linker and T4. The same transformation by electroporation protocol was following with any modifications mentioned below. After making two separate O/N and reaching the required OD, one of the cultures must be induced by placing the flask at 45°C for exactly 15 minutes while the other (uninduced) remains at 30°C. The remainder of the protocol is followed, but the transformation mixtures will contain 1.5µl of T4, 2A linker, or 4A linker (labeled later as L4, but is identical to L2). The transformations are then rescued for 4.5 hours instead of 1 hour, and then plated on DOG plates at dilutions of 1:1, 1:10, 1:100, and 1:1000.

Results

CRISPR/Cas9 Mutagenesis

To ensure that the Cas9 plasmid has been successfully digested for later ligations, the digested DNA was analyzed via electrophoresis. Lanes 1-5 show undigested Cas9 plasmids, while lanes 7 and 8 show the BSA-I digested Cas9 plasmid with two distinct bands instead of one (Figure 5). Following the transformation of the TOPO MCherry gRNA to the Cas9 plasmid, the DNA was sent off for sequencing. In Figure 6, the red highlighted portion of the sequence shows the sequence of the MCherry gRNA leading to the confirmation of a successful ligation and transformation.

Following the ligation, the transformation of the BAC + Cas9 plasmid + MCherry gRNA was digested using HIND-III and analyzed via electrophoresis. Compared to the BAC only HIND-III digestion, the BAC + Cas9 + MCherry gRNA digestion showed that the insert was complete with a multitude of DNA fragments that were much shorter in length (Figure 7). The BAC only digestion has two strong bands near 10K bp, with one around 8k bp, a cluster of three bands around 6k bp, and two other bands near 2700 bp and 2200 bp (Figure 7). Comparatively, the mutated plasmid digestion has all of the previous bands, of much less visual significance, minus the two smallest bands at 2700 and 1200 bp but with the addition of 1 band around 3400 bp, 1 band near 2500 bp, and 1 band near 1400 bp (Figure 7).

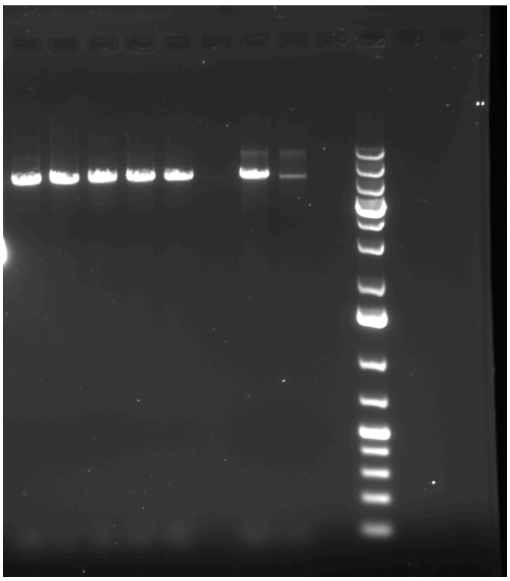

Figure 5. BSA-I Linearized Cas9. The Cas9 plasmid was linearized using the restriction enzyme BSA-I.

1:5 #6 Mcherry:

CGCARATGAAGAtATTTCTTATAaCTAAaAATATGGTATAATACTCTTAATAAATGCAGTAATACAGGGGC
TTTTcAAGACTGAAGTCTAGCTGAGACAAATAGTGCGATTACGAAATTTTTTAGACAAAAATAGTCTACG
AGGTTTTAGAGCTATGCTGTTTTGAATGGTCCCAAAACGCGCCTACAACGTCAACATCGTTTTAGAGCTA
TGCTGTTTTGAATGGTCCCAAAACTTCAGCACACTGAGACTTGTTGAGTTCCATGTTTTAGAGCTATGCTG
TTTTGAATGGACTCCATTCAACATTGCCGATGATAACTTGAGAAAGAGGGTTAATACCAGCAGTCGGAT
ACCTTCCTATTCTTTCTGTTAAAGCGTTTTCATGTTATAATAGGCAAAAGAAGAGTAGTGTGATCGTCCAT
TCCGACAGCATCGCCAGTCACTATGGCGTGCTGCTAGCGCTATGTGCGTTGATGCAATTTCTATGCGCAC
CCGTTCTCGGAGCACTGTCCGACCGCTTTGGCCGCCGCCCAGTCCTGCTCGCTTCGCTACTTGGAGCCAC
TATCGACTACGCGATCATGGCGACCACACCCGTCCTGTGGATCCTCTACGCCGGACGCATCGTGGCCGG
CATCACCGGCGCCACAGGTGCGGTTGCTGGCGCCTATATCGCCGACATCACCGATGGGGAAGATCGGG
CTCGCCACTTCGGGCTCATGAGCGCTTGTTTCGGCGTGGGTATGGTGGCAGGCCCCGTGGCCGGGGGA
CTGTTGGGCGCCATCTCCTTGCATGCACCATTCCTTGCGGCGGCGGTGCTCAACGGCCTCAACCTACTAC
TGGGCTGCTTCCTAATGCAGGAGTCGCATAAGGGAGAGCGTCGACCGATGCCCTTGAGAGCCTTCAACC
CAGTCAGCTCCYTYCCGGTGGGCGCGGGGCATGACTATCGTCGCCGCACTTATGACTGTCTTCTTTATCA
TGCAACTCGTAGGACAGGKGCCGGCAGCGCTCTGGGTMATTTYCGCGAGGACCGCTTYSCTGGAGCGC
GACGATGATCSGCCYTGYSCTTKSCGTATTCGGATCTTTGCACGYCCTYSCTYCAAGSCCTTCGKTMMCTG
GTTCCCGCACCAAACKGTTTCGGSGAGAAGCAGGCMATTWATCGCCGGTCATGYSGGCCGACGCSCCT
GGGGCTAMCGTCTCTGGCTKGGGCGTTS

Figure 6. Sequence Conformation of MCherry. Sequence coding for MCherry fluorescent protein highlighted in red.

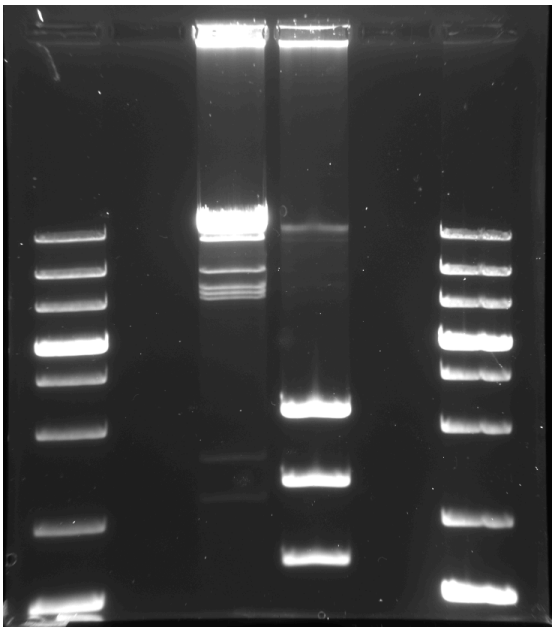

Figure 7. HIND-III Digest. Digest of 2D BAC with MCherry is shown in the middle left lane. Digest of 2D BAC with Cas9 plasmid with Kan and MCherry guide RNA is shown in the middle right lane.

To further analyze the mutated Cas9 plasmid, the DNA was plated on LB agar plates in the presence of chloramphenicol and kanamycin. The CRISPR/Cas9 system carries kanamycin resistance while the MCMV BAC carries the chloramphenicol resistance. The original TOPO MCherry was streaked out on LB agar plates with ampicillin (plate 1), and the colonies that grew have the ability to express the MCherry gene causing the colonies to turn light red in color (Figure 8). Plates 2-4 are LB agar plates with Kan + CM, and the experimental colonies plated are originally clear colonies from a previous transformation of the mutated Cas9 plasmid (original plate not shown). From this initial plate, many of the colonies had a red-tint, but plates 2-4 are selected clear/white colonies that grew amongst the red colonies on the initial

transformation plate (Figure 8). Plates 2 and 4 grew white colonies only, and plate 3 grew colonies that had a mild red tint that was significantly attenuated compared to plate 1 (Figure 8). Following the growth from Figure 8, one colony from each of plate 2-4 were cultured and prepped for DNA sequencing to evaluate the functioning of the Cas9 + MCherry gRNA. Of the three samples sent for sequencing, one sequence came back negative for the MCherry gene with a deletion of a cytosine base at the 9th residue causing a frameshift mutation in the MCherry gene, which resulted in the observed lack of red coloring in the colony growth (Figure 8 and 9).

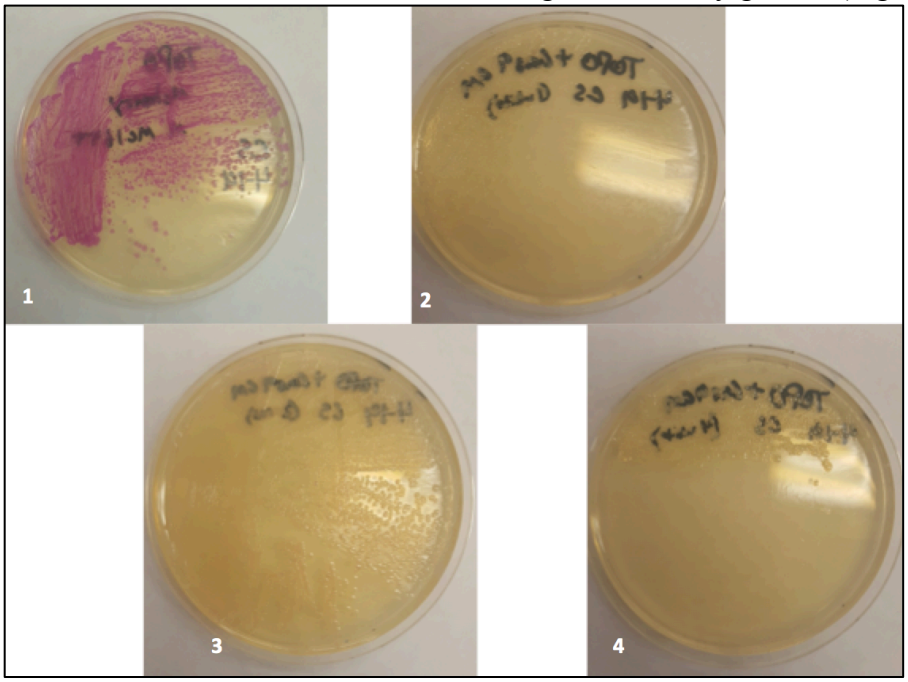

Figure 8. Streaked out colonies of constructed plasmids. Plate 1: BAC with MCherry ligation. Plates 2 and 4 show clear colony growth of the MCherry gRNA insert on the Cas9 plasmid. Plate 3 shows red-tinted/clear colony growth of the MCherry gRNA insert on the Cas9 plasmid.

MC Knockout

CCGGKWRAAGCWGTCATCTT**GATGTTGA**GTTGTAKGCGCCGGGCARCTGCA
CGGGCWTCWTGGCCTWGTAGGTGGTCTTGACCTCAGCGKCGWARTGGCCGC
CGTCCTTCASCTTCAGCCTCTGCTTGATCTCRCCCTWCARGGCGCCKTCCTCG
GGGTACATCCSCTCGGAWGAGGCCTCCCAKCCCAWGGTCTTCTWCTGCATTA
CKGGGCCKTCGGAGGGGAAGTTGGTGCCGCRCAKCTTCAMCTTGTAAATGAA
CTCRCSKTMCTGCAKGGAGGAGTCCTGGGTCACGGTCACCACGCCKCCATCC
TCAAARTTCATCACGCGCTCCCACTTGAARCCCTCGGGGAAGGACAGCTTCA
ASTAKTCRGGGATGTMGGCGGGGTGCTTCACGTAGGCCTTGGAGCCGTACAT
GAACTGAGGGGACAGGATGTCCCAGGCGAARGGCAKGGGGCCACCMTTGGT
CACCTTCASCTTGGCGRTCWGGGTGMCCTCATAGGGGCGGCCMTCACCCTCG
CCCWCSATCTCKAACTCGTGGCCGTTCACGGAGCCCTCCATGTGCMMCCTTG
AAGCGCATGAACTCCTTGATGATGGAGGTTTWAAMCWTG

Figure 9 MCherry Knockout Sequence. Bolded portion of the sequence shows the beginning of the MCherry sequence while the underline portion shows the rest of the sequence with a missing 9th based compared to the confirmed MCherry sequence: **GATGTTGA**CGTTGTAGGCGC. Highlight code shows the mutated base.

galK Recombineering

As an alternative form of mutagenesis, BAC recombineering can be utilized via the introduction and manipulation of the galK cassette in specific cell types. For this experiment, SW105 *E. coli* cells are used as the host of the Smith MCMV BAC and subsequent mutations.

The first step in this process is to transform the Smith BAC into the SW105 cell strain via electroporation. After transformation, the Smith BAC + SW105 DNA was digested using the HIND-III restriction enzyme. Similar to the data from in the left lane of Figure 11, the BAC HIND-III restriction gives us a tight bundle of fragments around 25k bp with a distinct band around 10k bp and three fragments between 7k and 8k bp, which compares significantly to the *in silico* HIND-III digestion simulated by DNASTAR, a software for Molecular Biology sequence analysis (Figure 10).

Following the transformation of the Smith BAC into SW105, the BAC needed to undergo experimental mutation to replace the MCK-2 gene function (m129, m130, m131) with genes (*galK*+) necessary to metabolize galactose. Before the additional transformations, the *galK*+ genes had to be PCR amplified to increase chances of a successful transformation. Using their respective forward and reverse primers, Tom's MCK-2 *galK* m129 link construct (T4) and the MCK-2 *galK* m129 2A linker (L2, identical to L4) were PCR amplified and purified via electrophoresis to increase quality and analyze sequence lengths (Figure 11). The MCK-2 PCR fragment alone is the smallest of the PCR products at ~1k bp (Figure 11). T4 and L2/L4 are all larger DNA fragments because they utilized the MCK-2 fragment to insert the *galK* function. T4 is analyzed to be 1300 bp and L2/L4 are both ~2200 bp in length (Figure 11).

The PCR amplified T4 and L2 (2A) are then transformed into the BAC containing SW105 cells using electroporation earlier described. This transformation involves inducing a portion of the SW105s to activate the red recombinase function *in vivo* to replace the MCK-2 genes with the PCR products. To analyze the PCR amplified transformations, the DNA extractions of SW105 + 2A and SW105 + T4 were electrophoresed. Compared to Figure 10, the 2A linker has similar fragment placements besides the bottom two bands between 10K bp (Figure 12). The 2A *galK* PCR insert has only a total of 3 bands below 10k bp with the addition of the ~2200 bp insert that has caused the combination of the two of the four bands found in the SW105 + BAC HIND-III only digest (Figure 12, left panel). Compared to the SW105 + Smith BAC HIND-III digest alone, the T4 + SW105 transformation replaced two of the three DNA fragments below10K bp with a single brighter band at ~13000 bp, similar to the T4 PCR product (Figure 12, right panel).

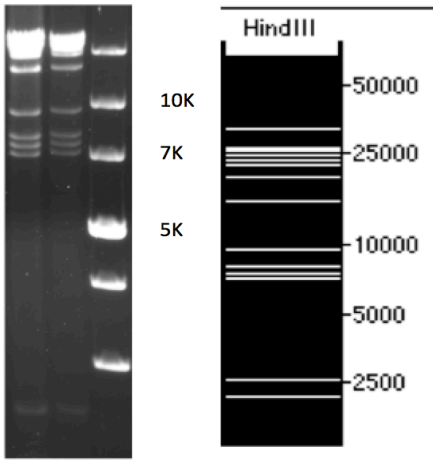

Figure 10. HIND-III digest of SW105 with Smith BAC. Digest shows cluster of fragments near 25k base pairs with 4 distinct fragments between 7K and slightly below 10k base pairs.

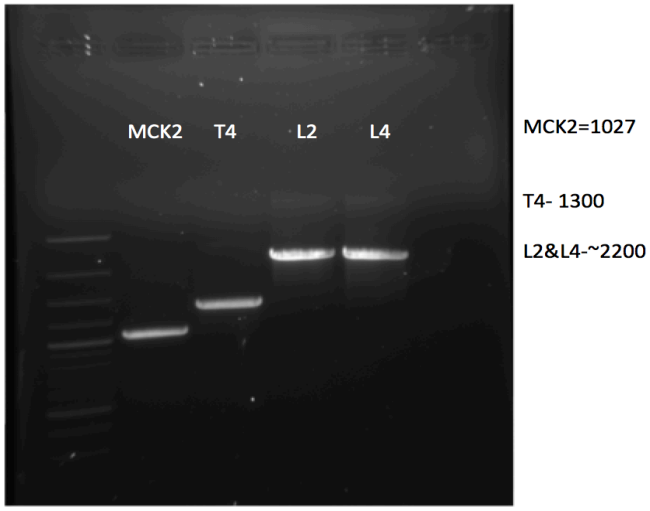

Figure 11. PCR Fragments of insert components. The natural MCK-2 product is 1027 base pairs. The T4 insert product is 1300 base pairs. Both the L2 and L4 linker insert are roughly 2200 base pairs.

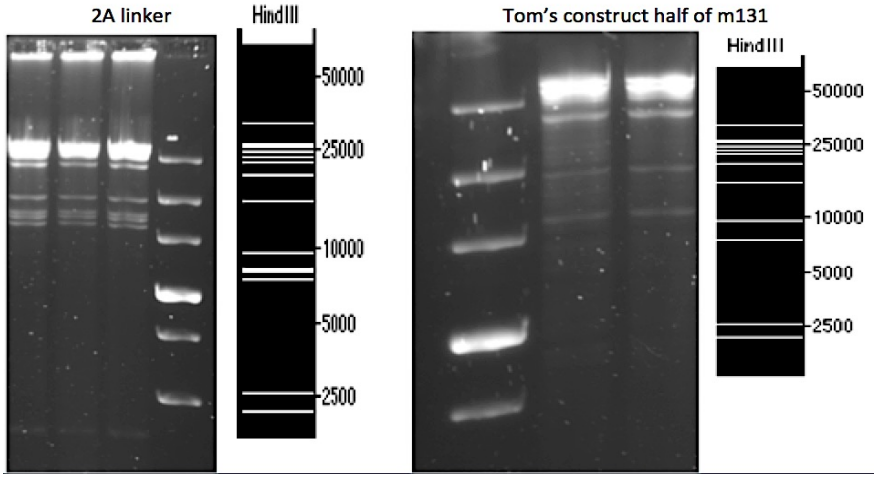

Figure 12. HIND-III Digest of Smith SW105 with 2A linker and T4 mutations. Left panel shows the digest and *in silico* digest of the MCK-2 *galK* m129 2A linker insert. The right panel shows the digest and *in silico* digest of Tom's/T4 MCK-2 *galK* m131 construct insert.

Combining CRISPR/Cas9 Mutagenesis and galK Recombineering

Following the initial experiments utilizing the CRISPR/Cas9 mutagenesis and *galK* recombineering separately to assess their respective functionalities in relation to our model, we combined the two mutagenesis systems as described in Figure 4. In order to begin the BAC recombineering phase for Step 1 (Figure 4), we created two PCR products with homology to *m128* and *m129* of our initial Smith BAC, one being the insert with *galK* and the other being UL146/2A-Tol (Figure 4). These PCR products were ran on gel electrophoresis to analysis their respective sizes compared to the gene site of interest on the wild type (wt) Smith BAC (pSM3fr) (Figure 13). From this gel, we can see that our largest to smallest PCR product was the *galK,* UL146/2A-Tol, and then the wt BAC (Figure 13). The lane labeled MCK2 Rescue shows a PCR product following the removal of *galK* from the BAC, which is why it displays a band size identical to the wt BAC (Figure 3).

Figure 14 shows the DNA digestions of our mutated BACs with respect to our 2A-Tol insert using CRISPR/Cas9, the natural wt BAC with similarities to the MCK2 Rescue, and the *galK* inserting using traditional recombineering. The top three bands of each lane are similar in size likely due to the Smith BAC backbone, but the wt Smith BAC and MCK2 Rescue both have 3 additional bands in their lane while the other two BAC digestions with mutations (2A-Tol and *galK*) only have two distinct lower bands with contrasting expressions (Figure 14). These two mutations look similar in their digestions likely due to the replacing or disrupting of one of the restriction sites in the wt BAC sequences after one of the mutation steps, but we know that these two mutations are independent of one another because they were completed sequentially and also display contrasting bands lengths from their PCR products (Figure 13).

To ensure that our mutations could be reversed without off-target effects and to ensure the integrity of our mutagenesis system, we also induced a *galK* repair using homologous recombination to repair the mutations made in step one of Figure 4 and recreate the original wt BAC. Figure 15 reflects the PCR of the *galK* Repair, which has similar band size to the MCK2 Rescue and wt BAC band on Figure 13.

To determine the efficacy of our CRISPR/Cas9 mutated Smith BAC with 2A-Tol/UL146 inserted via HDR, this mutant was extracted and replicated to assess its growth versus the original Smith BAC MCMV virus. To assess the mutant's growth, each virus titer was measured via plaque assay over the course of 7 days post-infection, and the subsequent growth curve was produce (Figure 16). Figure 16 shows the growth of the original MCMV virus (red) in comparison to the 2A-Tol/UL146 CRISPR/Cas9 mutated virus (blue). The difference between the two growth curves was not statistically differently, showing that the two viruses grew at similar rates over time.

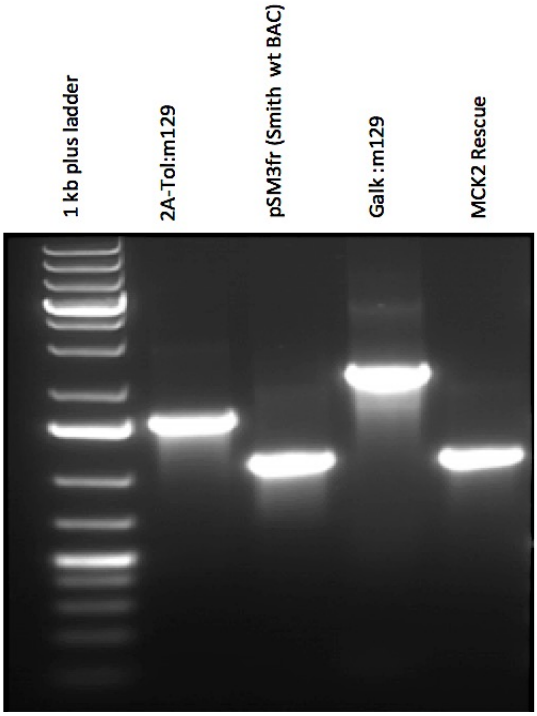

Figure 13. PCR products for target mutations. Starting from the left, there is the 1kb ladder for reference, the *2A-Tol* insert, the natural Smith BAC mutation sequence, the *galK* insert, and in the last column is the rescue of MCK-2 going from GalK:m129 back to the original Smith BAC sequence.

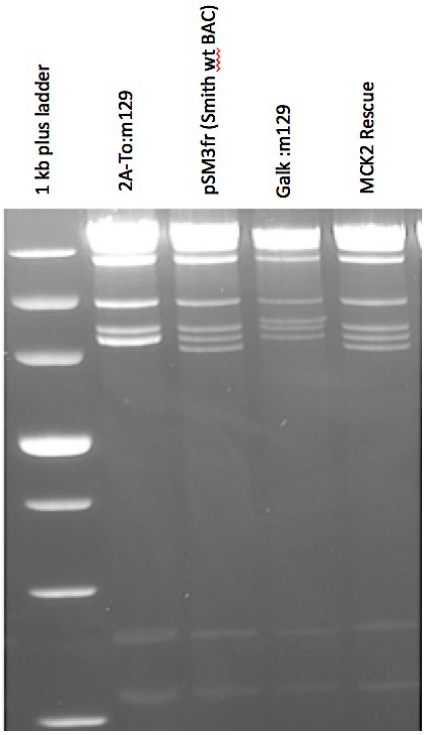

Figure 14. Post-Transformation DNA Digestions for Assessment. Starting from the left, there is the 1kb ladder for reference, the 2A-Tol insert into the wt BAC, the natural Smith BAC, the Smith BAC reflecting *galK* insert, and in the last column is the rescue of MCK-2 going from GalK:m129 back to the original Smith BAC.

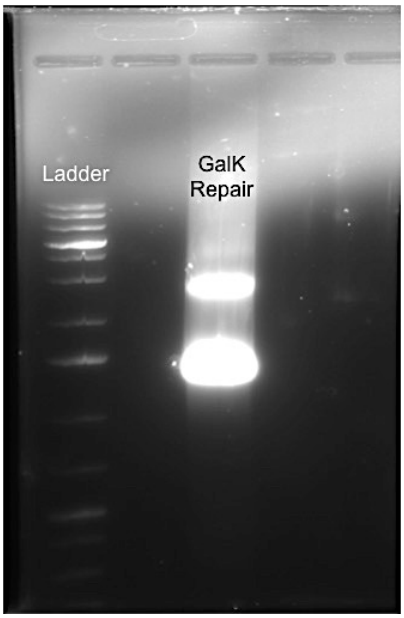

Figure 15. PCR product of the *galK* repair template. This gel reflects the total repair of the Smith BAC to its original sequence post *galK* recombineering.

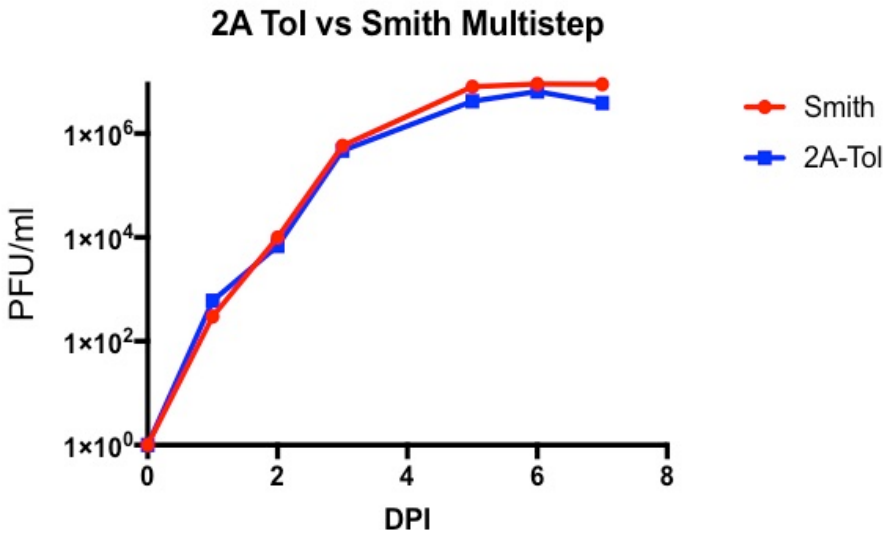

Figure 16. Growth curve analysis of Smith BAC vs. BAC with 2A-Tol insert. Curves reflect the growth of the respective viruses in vitro over the course of 7 days post infection.

Discussion

While HCMV infections amongst the immunocompromised and its congenital effects remain significant health concerns around the globe, the specifics to how this virus manipulates and disseminates throughout humans continues to be unsolved. Although many aspects of the immune system have been elucidated to play roles in the dissemination of CMV, the key to HCMV distribution has not been completely solved. Because of the significance of neutrophils to the human immune response, these particular leukocytes are the focus on the Sparer lab as the main culprits for HCMV dissemination. Because of this focus, we are determined to discover how to effectively investigate the role of neutrophils in HCMV dissemination using our MCMV model.

To evaluate the role of neutrophils in our mouse model, we must construct a mutant MCMV BAC that replaces the current MCMV MCK-2 sequence with the HCMV *UL146* gene, which encodes for the neutrophil-specific chemokine vCXCL-1. Once this is complete, the MCMV infections will express the neutrophil chemokine under physiological conditions, allowing a significant analysis of the role of neutrophils in CMV dissemination. This model will allow us to extrapolate how the virus interacts with neutrophils in human hosts, which could possibly move HCMV research closer to creating an effective vaccine to suppress viral dissemination. In order to create the neutrophil attracting MCMV mutant virus, our MCMV BAC must be mutated to allow for the replacement of the MCK-2 genes.

One of the novel modes of modern genetic mutations involves the use of the CRISPR/Cas9 mediated mutation system. The Cas9 nuclease in the CRISPR-Cas system can combine the Cas9 protein with a select guide RNAs to create a desired double-stranded break. Since the 1980s through the early 2000s, this system has been heavily researched for its versatile capabilities in genome editing with high specificity [54]. Through this project, CRISPR-Cas9 mutagenesis is used in addition to *galK* BAC Recombineering to induce mutations in our Smith BAC MCMV laboratory model.

In the preliminary phase of the project, the intended result was to observe the Cas9 nuclease utilize the MCherry gRNA to completely remove or inactivate the MCherry sequence from the BAC (Figure 8). However on the initial transformation incubation, little to no colonies grew completely white/clear. After waiting several days up to a week, few of the colonies amongst the red MCherry-containing growths lost the red color and grew to be completely clear/white colonies (Figure 8). After analysis of one of the white colonies, a mutated MCherry sequence was observed leading to the cell's lost ability to express the MCherry gene. Although initially considered futile because of the perceived inability to recombine the mutated sequence with the use of a $RecA^-$ *E. coli* strain, the CRISPR-Cas9 mediated mutagenesis of the MCMV BAC has shown promising results that we later incorporated to mutate our BAC. Since the use of the CRISPR-Cas9 system has not yet been used to mutate a BAC, this experiment as a whole is fundamental to BAC mutations and fundamental to the future assessment of neutrophils in CMV dissemination.

To achieve our desired outcome, we decided to combine the traditional BAC recombineering techniques, a more established method of mutagenesis through BAC recombineering with *galK* selection, with the CRISPR/Cas9 mutagenesis system. Compared to the CRISPR/Cas9 system, BAC recombineering is much more time consuming and can produce classical errors. Using the Recombineering protocol by J. Upton and modified by Joseph Jackson, the introduction of a *galK* cassette to the Smith MCMV BAC was successfully transformed into the SW105 strain. However due to a subsequent experiment not mentioned,

both the induced and uninduced transformations had variable, unexpected growths with large colonies as well as background plate-covering growth. Because of these unpromising results, the direct recombineering-only of the MCMV BAC had to be manipulated.

Following semi-successful mutagenesis results with each system independently, the CRISPR/Cas9 and traditional BAC recombineering using *galK* selection system were combined as described in Figure 4 by Joseph Jackson. Using a two-part system, traditional BAC recombineering was utilized to induce homologous recombination in our bacteria via transformation with a *galK* PCR product with Smith BAC homology at *m128 and m129* (Figure 4). Following this selection, the only available cells were those that adequately utilized galactose as their carbon source to grow. Following successful BAC recombineering, the CRISPR/Cas9 system was utilized to create a DSB in the Smith BAC *in vitro* to allow the insertion of our gene of interest (UL146/2A-Tol) via HDR. Figure 16 is a foundational component to this project since it shows that our CRISPR/Cas9 mutated BAC can still infect cells and produce virus at the same rate as the wt virus strain.

The future of this project will require the sequencing of each of the mutations shown in Figure 4 to verify that we have completed our mutations without any off-target effects or additional mutations that seem fairly likely to arise through each of our utilized mutagenesis systems. Knowing that off-target effects may be a serious issue and limitation in our *in vivo* model utilizing CRISPR/Cas9 [45, 53], we may eventually have to construct an additional system where the CRISPR/Cas9 functionality eventually is excised from the DNA of the cell completely in order to restrict later, unintended mutations [53]. The next phase of the project will include verifying and testing the efficiency and validity of the 2A/UL146 + Smith BAC design before evaluating how this mutated virus functions *in vivo*. The final phase will focus on how the virus functions within our laboratory mice in order to analyze the level of mutant viral interaction with murine neutrophils and hopeful MCMV dissemination.

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