The JAK/STAT3 signaling pathway in vaccinia virus infection

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Abstract

Poxvirus infections continue to threaten human health despite the eradication of smallpox, which was one of the most lethal infectious diseases in human history. Our objectives were to identify the host cell components/functions that are important for poxvirus infection and to gain insights into the molecular mechanism of poxvirus replication, ultimately guiding novel anti-viral development. Using vaccinia virus, the prototype poxvirus, we screened inhibitors of viral replication from over 3,000 chemical compounds, most of which have known cellular targets. This screening revealed numerous JAK/STAT3 inhibitors that could inhibit the replication of vaccinia virus. We further used multiple inhibitors of the JAK/STAT3 pathway and tested their effects on the replication of vaccinia virus in multiple primary and transformed cells through reporter assay and viral infectious particles measurement. The JAK/STAT3 inhibitors being tested were: SC144, an inhibitor of the interleukin 6(IL-6), receptor of the JAK/STAT3 signaling pathway, AZ960 (a JAK2 inhibitor), Stattic and niclosamide (inhibitors of STAT3). Overall, our data indicate the JAK/STAT3 inhibitors could repressed vaccinia virus replication in multiple cell types, suggesting that the JAK/STAT3 signaling pathway is required for the efficient replication of vaccinia virus. Moreover, we observed that STAT3 was enriched in the cell nucleus, although the phosphorylation level of STAT3 was downregulated in vaccinia virus-infected cells during the early stages of infection. This study demonstrates an important role of the JAK/STAT3 signaling pathway in the replication of vaccinia virus, providing a possible novel direction by which to intervene in poxvirus infection and related diseases.

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Chapter 1 - Literature review

Poxvirus

Poxviruses are large, enveloped, double-stranded DNA viruses that infect insects (*Entomopoxvirinae*) and chordates (*Chordopoxvirinae*). Within the *Chordopoxvirinae* subfamily, species that can infect humans are found in four genera: *Molluscipoxvirus*, *Orthopoxvirus*, *Parapoxvirus*, and *Yatapoxvirus* (Marennikova & Moyer, 2005). Several species from the *Orthopoxvirus* genus are well-known poxviruses that cause outbreaks among humans and other animals. The most notorious disease associated with poxviruses is smallpox, caused by the variola virus (*Orthopoxvirus* genus). Smallpox was the deadliest disease in human history (Marennikova & Shchelkunov, 2005), and the annihilation of smallpox through global vaccination is one of the greatest triumphs of modern medicine (Smith & McFadden, 2002). Nevertheless, poxviruses continue to pose threats to human health long after the World Health Organization certified the world free of smallpox in 1980.

Monkeypox (*Orthopoxvirus* genus) is one fatal poxvirus that also infects humans. Human monkeypox has been endemic to central and west African countries ever since it was recognized as a human disease in 1970 (Durski et al., 2018). The spread and mortality rate of monkeypox is increasing. Many countries that had not reported a single case in several decades have started to experience large-scale monkeypox outbreaks. Recently, there were 172 suspected and 61 confirmed cases of monkeypox infection reported in Nigeria in 2017 ("WHO | Monkeypox – Nigeria," n.d.). Currently, the fatality rate of monkeypox in persons without vaccination is 11% (Durski et al., 2018). Vaccination against smallpox can provide cross-protection against monkeypox ("Monkeypox | Poxvirus | CDC," n.d.); however, the majority of young adults are

susceptible to monkeypox infection due to the cessation of the smallpox vaccination program in 1980 (Smith & McFadden, 2002).

Poxviruses can also infect domestic animals and cause economic loss in many countries. For instance, goatpox and sheeppox cause skin lesions in infected animals, resulting in weight loss, reduction of milk, and loss of wool (Yune & Abdela, 2017). Moreover, poxvirus infection in domestic animals weakens the immune system, which directly or indirectly leads to the death of livestock (Babiuk, Bowden, Boyle, Wallace, & Kitching, n.d.). In addition to economic impact, poxvirus infections in animals often lead to human zoonotic poxvirus outbreaks that are potential threats to human society.

Zoonotic poxvirus outbreaks alarm the possible returning of smallpox or even more lethal poxvirus plagues. Furthermore, there have also been fears of terrorists using variola virus as a bioterrorism weapon since the eradication of smallpox (Sharma, 2002). In 2017, a team from Canada successfully generated a horsepox virus, another poxvirus currently extinct in nature, through biosynthesis and recombination (Noyce, Lederman, & Evans, 2018). Their publication suggested that it is possible to generate the variola virus or an even more deadly virus through biosynthesis and recombination technology.

Currently, there is no specific treatment for poxvirus infection, although vaccination can offer cross-protection against poxvirus infection ("Monkeypox | Poxvirus | CDC," n.d.). Several antiviral drugs have shown effective inhibition towards poxvirus infection in a laboratory setting. Tecovirimat (Berhanu et al., 2015), Cidofovir (De Clercq, 2002), and Brincidofovir (Foster, Parker, & Lanier, 2017) are recommended treatments against poxvirus infection, despite the fact that they have not yet been tested on patients ("Prevention and Treatment | Smallpox | CDC," 2017). Promising as it appears, poxvirus antiviral therapy is challenged by drug resistance.

Tecovirimat (also called ST-246), the antiviral medicine being stockpiled at the Center for Disease Control to combat potential poxvirus attacks, has been reported to interact with virion membrane protein F13L (Yang et al., 2005). Mutation within the F13L gene results in poxviruses that are resistant to ST-246 treatment (Duraffour et al., 2015). Due to the scarcity of antiviral medicines against poxvirus infection, it is necessary and essential to study the biology of poxviruses in order to assist in the discovery of antiviral drugs against current and potential poxvirus outbreaks.

Vaccinia virus

Vaccinia virus (*Orthopoxvirus* genus), an archetypal member of the poxvirus family, was used as a vaccine in the global eradication program against smallpox (Smith & McFadden, 2002). The global smallpox eradication program eliminated naturally-occurring smallpox; however, it also helped spread the vaccinia virus all over the world. Several sub-lineage strains of vaccinia virus have caused outbreaks among domestic animals and humans in various locations worldwide (Singh, Balamurugan, Bhanuprakash, Venkatesan, & Hosamani, 2012). Most recently, vaccinia virus has caused bovine vaccinia outbreaks in Brazil (Miranda et al., 2017). The cessation of the smallpox vaccine campaign has put human society in a dangerous position because the vaccinia virus has been spread all over the world. Despite vaccinia virus being one of the most well-studied viruses, the origin and natural reservoir of vaccinia virus remains a mystery (McFadden, 2005). Vaccinia virus has a large host range, infecting both wild and domestic animals, as well as humans. It is possible that more dangerous strains of vaccinia virus are being generated through evolution in nature.

Despite being a causative agent for several outbreaks worldwide, vaccinia virus is frequently used as a research tool in the study of poxviruses. Vaccinia virus, which replicates

entirely in the cytoplasm of the host cell (Moss, 2013b), possesses a transcriptional system. The vaccinia virus possesses a transgene upload capability of approximately 25 kb (Smith & Moss, 1983) that makes it a perfect vector for the expression of foreign proteins in mammalian cells (Chakrabarti, Sisler, & Moss, 1997). Recombinant vaccinia virus-based vector systems have been used to engineer vaccines to combat various infectious diseases. The vaccinia–rabies recombinant oral vaccine (Pastoret & Brochier, 1996), which has been applied to control wildlife rabies among foxes in several European countries and the United States, uses recombinant vaccinia virus as a vector. A system called "Sementis Copenhagen Vector" that is a vaccinia-based vaccine vector, allows multiple antigens to be expressed in a single vector (Prow et al., 2018). Moreover, the fact that vaccinia virus can rapidly replicate, spread to tumor tissue, and perform active lytic activity makes it a great candidate as an oncolytic agent (Mansfield et al., 2016).

The lifecycle of vaccinia virus (Figure 1-1) consists of several steps: cell entry, gene expression, DNA replication, virion assembly, and exit (Moss, 2013a). Vaccinia virus infectious particles enter the cell through direct fusion with the host cell membrane or endocytosis. Early gene expression then starts directly after the virus core enters the host cell cytoplasm (Gershon & Moss, 1990). Vaccinia virus encodes factors and enzymes for early gene expression, which are packaged in the core of infectious particles at the late stage of infection. Early genes encode factors that transactivate intermediate gene expression and DNA replication. Newly synthesized viral DNA then functions as a template for intermediate and late gene expression. The three stages of gene expression are regulated in a progressive manner (Yang, Martens, Bruno, Porcella, & Moss, 2012). Following late gene expression, viral proteins and genomes are assembled into virions in viral factories, which are granular, restricted areas in an infected cell's

cytoplasm. Viral factories are also responsible for the maturity of virions, including packaging genome and core proteins into immature virions, assembling crescent membranes with immature virions, and producing infectious particle mature virions (Condit, Moussatche, & Traktman, 2006). Mature virions can be released either by cell lysis or by transferring through the host cell endoplasmic reticulum to gain host cell plasma membranes and then exiting the cell through exocytosis. Host–viral interaction occurs throughout the lifecycle of vaccinia virus. Viral factors are released during viral entry and interact with host proteins to promote survival of the virus. A clear picture of the infection cycle of vaccinia virus in host cells provides insights for antiviral developments.

Vaccinia virus–host cell interactions

As with all other viruses, the vaccinia virus has developed numerous strategies for surviving in hosts over years of evolution. Host antiviral immune response is a critical barrier for pathogen invasion. Vaccinia virus deploys factors that directly bind to and neutralize cytokines and chemokines, or indirectly inhibit apoptosis and signaling pathways, reducing the production of factors that contribute to immune response (Smith et al., 2013).

Regarding immune evasion, vaccinia virus has been reported to interfere with the host NF-κB pathway, which is a critical signaling pathway that regulates inflammatory and immune response (Gómez et al., 2012). Multiple factors targeting different sites of the NF-κB pathway can be used to achieve total suppression. These factors include the viral factors, M2L (Gedey, Jin, Hinthong, & Shisler, 2006) that represses ERK2 phosphorylation; K1L (Shisler & Jin, 2004) that reduces IκBα degradation; and B14 (Benfield et al., 2011) that binds to IKKβ, further inhibiting NF-κB activation.

Interferon response is pivotal in innate immune response that makes it a primary target for pathogen during their invasion (Samuel, 2001). The induction of a series of effectors, named interferon-stimulated genes (ISGs), is the primary effects of interferon activation (Schoggins et al., 2011). Interferon response encompasses interferon production and interferon signaling, which further activates the Janus kinase (JAK)/signal transducer and activator of transcription 1 (STAT1) pathway, resulting in the production of ISGs (Ivashkiv & Donlin, 2014). Vaccinia virus encodes several factors that specifically interfere with interferon production and signaling pathways (Smith, Talbot-Cooper, & Lu, 2018). Vaccinia virus secretes soluble α/β interferon receptors that bind to the host membrane to prevent interferon response (Alcamí, Symons, & Smith, 2000). A dual-specificity phosphatase (H1) encoded by vaccinia virus is responsible for repressing the type I interferon response by dephosphorylating activated STAT1 during infection (Najarro, Traktman, & Lewis, 2001). Additionally, the vaccinia virus's E3 protein binds to ISG15, a ubiquitin-like modifier, to prevent its antiviral functions (Guerra, Cáceres, Knobeloch, Horak, & Esteban, 2008).

In addition to immune evasion, vaccinia virus also manipulates host machinery to benefit its replication. For example, it hijacks host translational machinery for viral protein synthesis (Moss & Salzman, 1968). Inhibition of the apoptosis pathway is another common strategy through which viruses prolong their replication within infected host cells. Vaccinia viral anti-apoptosis protein F1L, a B-cell CLL/lymphoma 2(Bcl-2) homolog, inhibits host apoptosis by blocking the release of cytochrome c (Wasilenko, Banadyga, Bond, & Barry, 2005).

The interactions among vaccinia viruses and host cells are complicated, and many unknown interplays await exploration. Understanding more about the biology of vaccinia virus

infection benefits the human species as a whole, allowing us to survive potential poxvirus outbreaks.

JAK/STAT3 signaling

Janus kinases (JAKs) are a family of non-receptor cytoplasmic tyrosine kinases that are constitutively associated with cytokine and chemokine receptors (Yu, Pardoll, & Jove, 2009). In mammals, the JAK family consists of four members: JAK1, JAK2, JAK3, and tyrosine kinase (TYK2). JAKs can phosphorylate downstream factors in the cytoplasm upon activation (Yamaoka et al., 2004). The STAT family comprises transcription factors that can be activated by JAKs and transduce signals from the cytoplasm to the nucleus (Yu, Lee, Herrmann, Buettner, & Jove, 2014). The STAT family shares several highly conserved domains (Figure 1-2), including the amino-terminal domain (N), the coil-coil domain (CC), the DNA-binding domain (DBD), the linker domain (LK), the Scr homolog domain (SH2), the tyrosine phosphorylation site (Y), and the transactivation domain (TAD). Upon ligand binding, receptors located on the cell membrane go through conformational changes and activate a pair of JAKs that are constitutively associated with their intracellular domain. Active JAKs then phosphorylate the intercellular domain of receptors and themselves to provide docking sites, and further phosphorylate specific tyrosine residues within the TAD domain of downstream STATs. Phosphorylated monomer STATs can form dimers (homo- or hetero-) through reciprocal interactions among their SH2 domain phosphorylated tyrosine residues, which are then released from receptors and translocate to the nucleus (Yu et al., 2009). After nucleus translocation, STAT dimers function as transcription factors and regulate expression of specific genes (Peng Lim & Cao, 2006). Even though members of the STAT family share similar structures and

activation pathways, specific extracellular signals preferentially stimulate certain types of STATs and exert different functions (O'Shea et al., 2015).

STAT3, a member of the STAT family, plays a central role in many physiological and pathological pathways. It interacts with various factors and has different functions based on cellular context. STAT3 regulates a broad range of physiological functions, such as development, differentiation, immunity, and metabolism, as well as exhibiting pathological over-expression in many cancer tissues (Siveen et al., 2014). STAT3 was initially described as a DNA-binding factor that can transduce signals from interferons and interleukin 6 (IL-6; Darnell, Kerr, & Stark, 1994). Many extracellular stimuli can activate STAT3, such as growth factors, cytokines, stress, and infection (Hu et al., 2018). In addition to its involvement in the classic JAK/STAT3 signaling pathway, unphosphorylated STAT3 can form dimers and regulate gene expression in the nucleus (Nkansah et al., n.d.).

STAT3 is essential for many aspects of development, especially immune development (Takeda et al., 1998). Mice with knockdown of STAT3 are more susceptible to infection due to the critical role STAT3 plays in the development of the innate and adaptive immune systems (Akira, n.d.). STAT3 is also involved in inflammation in tissues with chronic infection (Welte et al., 2003). Many cancer patients have histories of chronic viral infection and, correspondingly, highly elevated STAT3 levels. The fact the STAT3 is involved in immune response and anti-apoptosis makes this pathway both beneficial and harmful for viral infection. Vaccinia virus deploys factors to encourage anti-apoptosis in order to prolong its infection. Meanwhile, inhibitory effects on host immune response are also essential for the virus's survival in the host.

JAK/STAT3 inhibitors

The IL-6/JAK/STAT3 axis is hyperactive in many types of cancer, and inhibitors targeting this pathway have been under development for decades. Currently, there two common strategies to repress the IL-6/JAK/STAT3 pathway: blocking the phosphorylation of STAT3 by inhibiting the upstream activation pathway, or directly inhibiting the STAT3 protein by binding to its SH2 domain (Johnson, O'Keefe, & Grandis, 2018).

Several interference approaches, such as antisense oligonucleotides, small interfering RNA, peptide-based inhibitors, and small molecule inhibitors, have shown effective blocking of STAT3 activation (Furtek, Backos, Matheson, & Reigan, 2016). Among the currently available approaches, small molecule inhibitors are gaining more attention due to several features, including simple structure, permeability to the cell membrane, and stability. Below are several examples of IL-6/JAK/STAT3 small molecule inhibitors.

One small molecule, 2-(7-Fluoropyrrolo[1,2-a]quinoxalin-4-yl) 2-pyrazinecarboxylic acid hydrazide hydrochloride (SC144), binds to the IL-6 receptor GP130 and induces its phosphorylation and deglycosylation to repress IL-6/JAK/STAT3 signaling (Xu, Grande, Garofalo, & Neamati, 2013). SC144 is the first oral bioactive drug available that targets GP130, which shows selective inhibition of STAT3 activation over other STAT proteins.

Many JAK inhibitors are currently available as FDA-approved drugs to treat autoimmune diseases. Since the publication of the kinase and pseudokinase domain structures of JAK1, JAK2, and TYK2, many inhibitors have been identified that target ATP-binding sites of JAK kinase and pseudokinase domains. These include pan-JAK inhibitors (e.g., gandotinib) that non-differentially repress all JAKs, and inhibitors that selectively inhibit one type of JAK (e.g., AZ960, which selectively suppresses JAK2; Vainchenker et al., 2018). Due to the structural

similarity of STATs, activated JAKs are shared among several STATs. Inhibition of upstream JAKs has shown promising results in repressing STAT3; however, the effects are not specific. In contrast, small molecule inhibitors that directly interfere with the STAT3 protein exhibit fewer off-target effects.

Many functional domains within the STAT3 protein can be target sites for the development of inhibitors; however, currently, only inhibitors that interact with the SH2 domain of the STAT3 protein are primarily explored. Scientists have tried several strategies to drug the SH2 domain of STAT3. Some small molecules have shown efficacy with reducing STAT3 phosphorylation, dimerization, nucleus translocation, and gene expression in vivo and in vitro (Kolosenko et al., 2017). Stattic is one of the first few small molecules to directly target STAT3 and repress its activation (Schust, Sperl, Hollis, Mayer, & Berg, 2006). Stattic was thought to suppress STAT3 by interfering with the SH2 domain specifically; however, later on, it was found to alkylate the cysteine residues of many molecules in the cytoplasm (Heidelberger et al., 2013). Since the identification of Stattic, researchers have been trying to identify other small molecules that repress STAT3 through interference with the STAT3 SH2 domain with higher specificity (Yue & Turkson, 2009). With better understanding, there are currently many STAT3 inhibitors with good specificity and fewer known off-target effects (Furtek et al., 2016). For instance, niclosamide is an FDA-approved anthelmintic medicine and has shown efficient and selective inhibition effects on the STAT3 activation (Ren et al., 2010).

JAK/STAT3 signaling in viral infections

Interferons and cytokines regulate a series of physiological functions through the JAK/STAT pathway. STAT1 and STAT2 are the most well-characterized STATs that are involved in interferon response, whereas the role of STAT3 in viral infections is more

complicated (Kuchipudi, 2015). Regarding its role in immune response, STAT3 is reported to negatively regulate STAT1-mediated type I interferon response (Wang, Levy, & Lee, 2011), while also acts as a critical factor in the adaptive immune response (Takeda et al., 1998). The involvement of STAT3 in many signaling pathways, as well as its role as a central factor that regulates a broad range of physiological activities, demonstrates how complicated its role in viral infections is. Indeed, many viruses infection repressed the function of STAT3 while many other viruses infection activated it (Kuchipudi, 2015).

After the viral invasion, various factors, which are products of the host's acute phase response, can stimulate STAT3 activation. Because STAT3 signaling is related to inflammatory response and anti-apoptosis pathways, many viruses have evolved to employ STAT3 signaling to prolong their infection (Suarez, Van Renne, Baumert, & Lupberger, 2018). Human hepatitis B virus, an onco-virus, up-regulates STAT3 activation dependent and independent of the IL-6/JAK/STAT3 signaling pathway (Hösel et al., 2017). Zika virus also activates IL-6/JAK/STAT3 signaling (Zhu et al., 2017), which assists its persistence in the central nervous system and lymph nodes of monkeys (Aid et al., 2017).

Virus-mediated suppression of STAT3 is observed in many types of viral infections, most likely because STAT3 activation can upregulate expression of genes that are responsible for immune response. The mumps virus deploys a viral protein, called V, to eliminate STAT1 and STAT3 through ubiquitylation and degradation (Ulane, Rodriguez, Parisien, & Horvath, 2003). Similarly, the rabies virus antagonizes STAT3 with a viral protein, called P protein, which inhibits STAT3 nuclear translocation and GP130-dependent signaling (Lieu et al., 2013).

A couple of papers have suggested that the JAK/STAT3 pathway plays a role in antiviral defense during vaccinia virus infection. Two previously published papers have demonstrated that

repressing the JAK/STAT3 pathway, both in human keratinocytes and in a murine model, impairs host cell interferon response during vaccinia virus infection (He et al., 2014; Mahony et al., 2017). These reports are contradictory to our unbiased screening assay, which showed that many JAK/STAT3 inhibitors suppress vaccinia virus replication, as is demonstrated in the next chapter. These discrepancies will be discussed in detail, as well.

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Figures

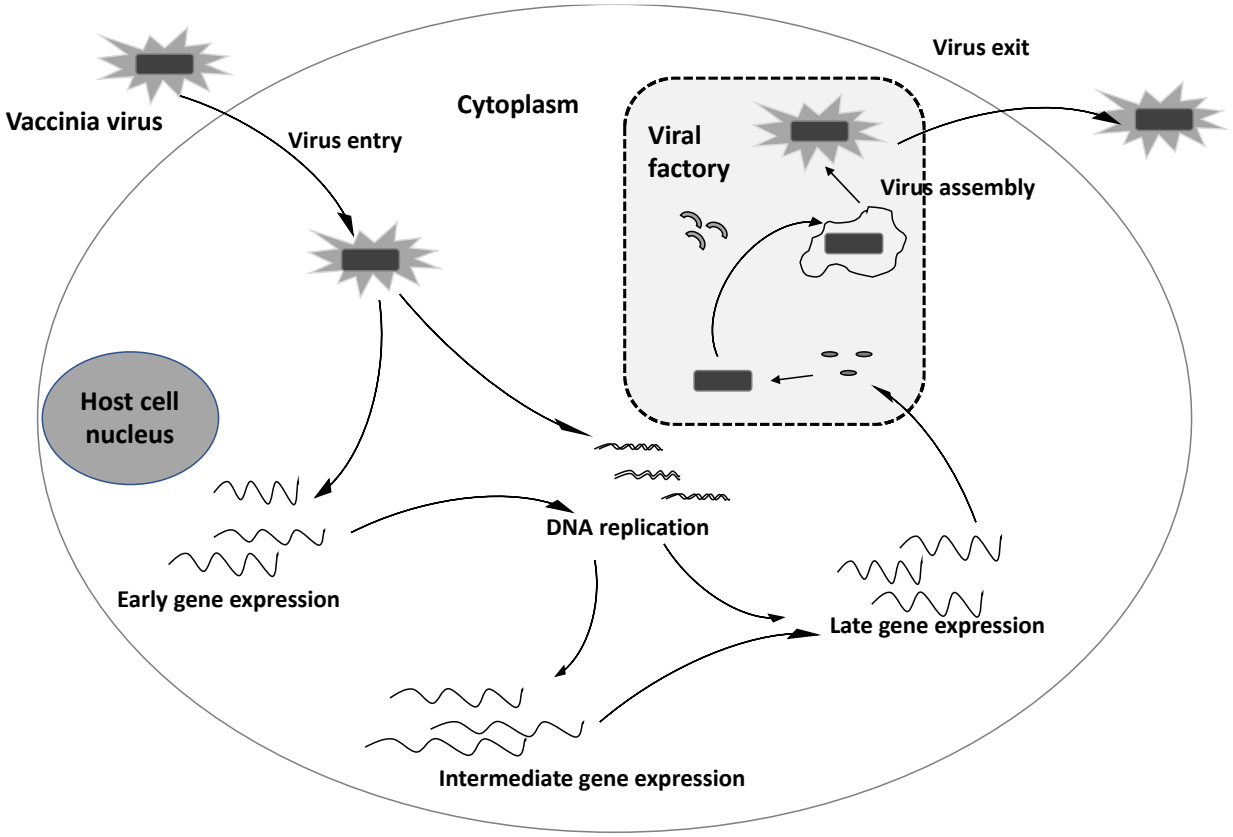

Figure 1-1 The lifecycle of the vaccinia virus

The lifecycle of the vaccinia virus includes virus entry, early gene expression, DNA replication, intermediate gene expression, late gene expression, virus assembly, and virus exit. Vaccinia virus infectious particles enter cells through membrane fusion and endocytosis. Early gene expression is initiated directly after virus entry, with the assistance of enzymes and factors packaged in the virus core. Early genes then encode factors and enzymes for DNA replication and intermediate gene expression. Newly synthesized viral DNA functions as a template for intermediate and late gene expression. Intermediate genes produce factors to regulate late gene expression. Late genes then express enzymes and factors that are required for early gene expression, and the products of late gene expression are packaged into the viral core. Vaccinia virus viral assembly takes place in a restricted area within the host cell cytoplasm, called the viral factory. In the viral factory, viral cores and crescents are assembled into mature virions and exit the cell through endocytosis or by cell lysis.

A.

B.

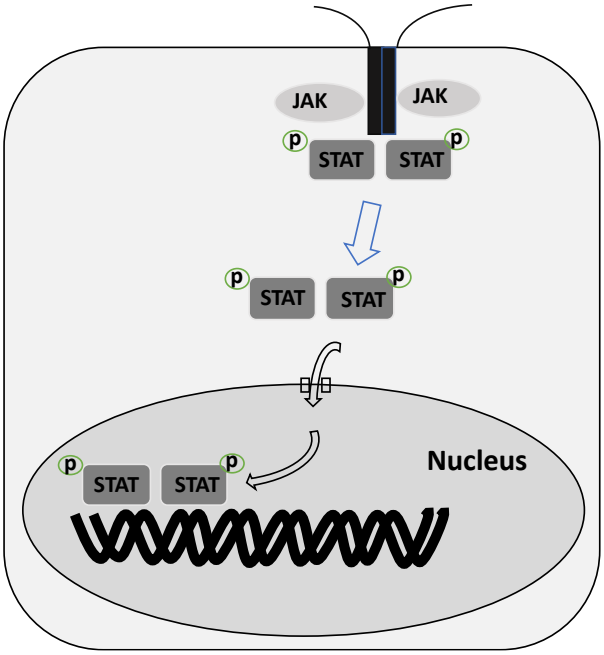

Figure 1-2 The JAK/STAT pathway

A: STAT family members share several highly conserved structures: the amino-terminal domain (N), the coil-coil domain (CC), the DNA-binding domain (DBD), the linker domain (LK), the Scr homolog domain (SH2), the tyrosine phosphorylation site (Y), and the transactivation domain (TAD).

B: Extracellular stimuli can activate receptors on the cell membrane, resulting in the receptors undergoing conformational change. This conformational change brings JAKs together and allows self-phosphorylation. Phospho-JAKs recruit and phosphorylate downstream STATs by interacting with the SH2 domain. Phosphorylated STATs dimerize and translocate to the cell nucleus. Nuclear STAT dimers bind to DNA regulatory elements and function as transcriptional factors, modulating gene expression.

Chapter 2 - JAK/STAT3 inhibitors repress vaccinia virus infection

Abstract

Poxviruses continue to threaten human health even after the eradication of the historical smallpox plague through a global vaccination program. There is no specific treatment for poxvirus infection. A better understanding of critical host cell functions in poxvirus replication is vital for drug development against poxvirus infection. Our goal is to identify host functions/factors that are essential for vaccinia virus infection. To achieve this objective, we developed an assay suitable for high throughput screening of chemical inhibitors based on the vLGluc recombinant vaccinia virus expressing the secreted gaussia luciferase gene. We screened vaccinia virus inhibitors from two libraries, bioactive compounds (Selleck Chemicals) and FDA-approved drugs, totaling over 3000 chemical compounds, most of which have known cellular targets. The screening identified and confirmed numerous inhibitors of vaccinia virus, many of which target the JAK/STAT3 signaling pathway. We further investigated the effects of multiple JAK/STAT3 inhibitors on vaccinia virus infection. Our results demonstrated that these JAK/STAT3 inhibitors significantly inhibited vaccinia virus replication, suggesting that the JAK/STAT3 pathway is essential for efficient replication of the vaccinia virus.

Introduction

Poxviruses continue to pose threats to human health after the eradication of smallpox, one of the most lethal infectious diseases in human history (Smith & McFadden, 2002). There are concerns about using variola virus, a causative agent of smallpox, as it could be transformed into a bioterrorism weapon. Indeed, the successful laboratory synthesis of a horsepox virus indicates the possibility of biosynthesizing smallpox (Noyce et al., 2018). In addition, zoonotic poxviruses undermine the safety of human society, such as the monkeypox virus (Durski et al., 2018), which causes infection in both humans and domestic animals. Currently, there is no specific treatment for poxvirus infection. Understanding more about the interaction between host cells and poxviruses is vital for antiviral therapy development against potential poxvirus attacks.

Vaccinia virus, the archetypal member of the poxvirus family, is an enveloped, double-stranded DNA virus that contains an approximately 200-kb genome (Moss, 2013b). It replicates entirely in the host cell cytoplasm and has its own transcriptional system (Smith et al., 2013). Vaccinia virus was used as a vaccine to eradicate smallpox; now, it is used as a vector in vaccine development for other infectious diseases and as a research tool in biological studies investigating the features of poxviruses (Moss, 2013a).

Over thousands of years of evolution, viruses have evolved numerous tactics for manipulating host cell machinery to support viral replication. Our objective was to identify host cellular components that are essential for poxvirus infection and replication. The Yang laboratory established an assay suitable for high-throughput screening based on a reporter recombinant vaccinia virus expressing the secreted gaussia luciferase reporter gene. After screening over 3000 bioactive compounds and FDA-approved drugs with known cellular targets, numerous inhibitors of vaccinia virus replication were identified, including chemicals that target

cellular functions known to be essential for vaccinia virus replication (Dr. Peng Chen, personal communication; Figure 2-1). Many of these inhibitors target the Janus kinase (JAK)/signal transducer and activator of transcription 3 (STAT3) pathway, suggesting a supporting role of this cell signaling pathway in the replication of vaccinia virus.

STAT3 is a crucial regulator of many physiological processes, including development, differentiation, immunity, and metabolism, as well as being over-expressed in many types of cancer tissues (Siveen et al., 2014). STAT3 can be activated through the JAK/STAT3 pathway to enact different cellular functions, such as anti-apoptosis, anti-inflammation, proliferation, and immune response, based on cell type and extracellular signals (Darnell et al., 1994). The JAK/STAT3 pathway is known to be activated during pathogen invasion as a part of the acute phase response (Cray, Zaias, & Altman, 2009). Because the JAK/STAT3 pathway plays a pivotal role in innate and adaptive immune response, many types of viruses are known to antagonize it during infection through various methods; however, this pathway is also reported to be hyperactive during various kinds of viral infection due to its effect on cell proliferation and anti-apoptosis (Gong, Waris, Tanveer, & Siddiqui, 2001; Hösel et al., 2017).

Our screening results suggest that JAK/STAT3 is required for vaccinia virus replication. Here, we employed multiple inhibitors that target different sites of the pathway and tested their effects on viral replication using normal human dermal fibroblasts (NHDFs), HeLa cells, and keratinocytes. Our results indicate that inhibitors that target multiple components of the JAK/STAT3 pathway indeed repress the replication of vaccinia virus.

Materials and methods

Cell cultures

All cells were cultured in a 37°C incubator with a supplement of 5% CO_2. HeLa cells (ATCC CCL-2) and normal human dermal fibroblasts (NHDFs; ATCC PCS 201-010) were both cultivated in Dulbecco's Modified Eagle Medium (DMEM) with the addition of 10% fetal bovine serum (FBS), 2 mM glutamine, and 1% penicillin-streptomycin. BS-C-1 cells were cultured in Eagle's Minimum Essential Medium (EMEM) with the addition of 10% FBS, 2mM glutamine, and 1% penicillin-streptomycin. Primary human keratinocytes (HFKs; kindly provided by Dr. Nicholas Wallace) were cultured in defined Keratinocyte-SFM medium.

Chemical inhibitors

The chemical inhibitors Stattic, AZ960, and SC144 were purchased from Selleck Chemicals. Niclosamide was purchased from Sigma.

MTT assay

A 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) assay was performed to measure the cytotoxicity of chemical inhibitors on cells. NHDFs, HFKs, or HeLa cells were placed in a 96-well plate at a density of 3.2×10^4 per well. NHDFs and HeLa cells were refreshed with pre-warmed DMEM medium with supplement of 2.5% FBS, 2 mM glutamine, and 1% penicillin-streptomycin in the presence of dimethyl sulfoxide (DMSO) or the desired concentration of chemical inhibitors, and then incubated for 8 or 24 hours. HFKs were refreshed with pre-warmed Gibco medium in the presence of DMSO or chemical inhibitors, and then incubated for 24 hours. After incubation, 10 ul MTT reagent was added to each well and cells were incubated for another three hours. Next, 100 ul crystal resolving solution was added to each well. Absorbance at 595 nm was measured for each well after 18 hours of incubation at

37°C. Three biological replicates were conducted for each test and representative data were selected to present.

Viruses, viral infection, and plaque assays

The Western Reserve strain (ATCC VR-1354) of vaccinia virus was amplified in HeLa cells and purified according to a previously-published protocol (Cotter, Earl, Wyatt, & Moss, 2015). Three recombinant vaccinia viruses, vEGluc, vIGluc, and vLGluc (kindly given by Dr. Bernard Moss), were used, which expressed the secreted gaussia luciferase reporter gene under well-defined early, intermediate, and late promoters, respectively.

For the amplification and purification of vaccinia virus, a monolayer of HeLa cells was infected with vaccinia virus at a multiplicity of infection (MOI) of 0.01 for 48 hours. The infected cells were then collected, and the cell lysate was obtained through three freeze–thaw cycles. The cell lysate was then sonicated, and vaccinia virus stock was purified from it via sucrose gradient centrifugation.

For viral infection, NHDFs or HeLa cells were placed in a 12-well plate at a density of 0.4×10^6/well one day before infection. Next, cells were incubated with virus-containing medium (containing 2.5% FBS, 2 mM glutamine, and 1% penicillin-streptomycin) for the desired amount of time before samples were collected for titration. For HFKs, viruses were diluted in Gibco medium and incubated for the desired time.

Viral titer was determined via plaque assay. For this assay, BS-C-1 cells were also prepared in the same way as described above, then infected with serially-diluted viral samples and incubated in EMEM medium (containing 2.5% FBS, 2 mM glutamine, 1% penicillin-streptomycin, and 0.5% methyl cellulose) for 48 hours before staining with 0.1% crystal violet to count the number of plaques.

Gaussia luciferase assay

NHDFs or HeLa cells were placed in a 12-well plate at a density of 0.4 x 10^5/well. The next day, cells were refreshed with medium containing DMSO or different chemical inhibitors, and then infected with vEGluc, vIGluc, or vLGluc at a MOI of 1. A 100-uL aliquot of medium was taken from each well after the desired incubation time and gaussia luciferase activity was measured with the Pierce Gaussia Luciferase Flash Assay Kit (Thermo Scientific). Next, 30 uL of media from each well were placed in a white plate and equal amounts of fresh luciferase substrates were added to each well. The reaction between luciferase and its substrates produced luminescence, which was measured with the GloMax Navigator System (Promega). Three biologal replicates were conducted for each experiment. The inhibition rate of gene expression was calculated by comparing gaussia luciferase light unit values for each sample to a control.

Plaque assay

Plaque assays were performed to measure the inhibition effects of chemical inhibitors on the replication of vaccinia virus. First, NHDFs or HeLa cells were placed in a 12-well plate at a density of 0.4 x 10^5/well. Attached cells were then infected with vaccinia virus (MOI of 1) and inoculated with DMSO or a serial concentration of chemical inhibitor. After a 24-hour incubation, cell lysates were obtained to measure viral titer via plaque assay. Three biological replicates were conducted and the average viral titer for each sample was calculated.

Statistical analysis

All experiments had three biological replicates and representative data were used for statistical analysis. T-tests were performed to assess differences between two groups, and significance was defined by a p-value ≤ 0.05.

Results

Identification of JAK/STAT3 inhibitors that suppress replication of vaccinia virus

To identify host components that are essential for the replication of vaccinia virus, we first generated an assay suitable for high-throughput screening based on a recombinant reporter vaccinia virus (vLGluc) that expresses secreted gaussia luciferase under the well-defined viral late promoter F17R. Over 3000 chemical compounds with known cellular targets from bioactive compound and FDA-approved drug libraries were tested for vaccinia virus inhibitors (Figure 2-1; Dr. Peng Chen, personal communication). Next, we tested the cytotoxicity of the primary hits on HeLa cells and observed their effects on the gaussia luciferase enzyme alone. In the meantime, we tested the antiviral effects of these inhibitors on the replication of vaccinia virus using vLGluc at MOIs of 0.01 and 2. The four parallel experiments confirmed that the change in gaussia luciferase luminescence in each well represented the effects of the different chemicals on the replication of vaccinia virus. We identified and confirmed numerous inhibitors of vaccinia virus, many of which target the JAK/STAT3 pathway. Based on our chemical screen results, we propose that the JAK/STAT3 pathway is essential for the replication of vaccinia virus.

Chemical inhibitors of the JAK/STAT3 pathway repress gene expression in vaccinia virus

To test the effect of inhibiting the JAK/STAT3 pathway on the replication of vaccinia virus, we chose small molecule inhibitor SC144 (IL-6 receptor inhibitor), AZ960 (JAK2 inhibitor), and stattic and niclosamide (STAT3 inhibitors). The JAK/STAT3 pathway is closely related to cell proliferation and apoptosis, and the inhibitors of this pathway have inhibitory effects on cell viability at certain concentrations (Yu et al., 2009). All inhibitors used here are reported to inhibit cell viability for several types of cell lines (Gozgit et al., 2008; Ren et al., 2010; Schust et al., 2006; Xu et al., 2013). Because an elevated apoptosis rate in the host cell

suppresses the replication of vaccinia virus (Kvansakul et al., 2008), we first optimized the working concentrations of chemical inhibitors at which there was no significant toxic effect on cell viability. MTT assays were performed to test the effects of JAK/STAT3 inhibitors on cell viability. NHDFs were seeded in a 96-well plate and incubated with media containing chemical inhibitors for 8 hours. MTT assays were then performed according to the manufacturer's protocol and absorbance from each well was measured using a microplate reader. SC144, AZ960, and Stattic exhibited no significant toxicity effects on NHDFs at their indicated concentrations (1 uM, 3 uM, and 3 uM, respectively), whereas niclosamide treatment significantly (>30%) improved NHDF cell viability at a concentration of 1 uM (Figure 2-3 D).

The lifecycle of the vaccinia virus consists of cell entry, gene expression, DNA replication, virion assembly, and exit (Moss, 2013a). Vaccinia virus gene expression has three stages and is regulated in a cascading manner (Yang, Maruri-Avidal, Sisler, Stuart, & Moss, 2013). Each stage of vaccinia virus gene expression produces different factors and enacts different functions to interact with host cell components (Van Vliet et al., 2009). To test which stages of vaccinia virus gene expression were affected by inhibiting the JAK/STAT3 pathway, we used three recombinant reporter vaccinia viruses (vEGluc, vIGluc, and vLGluc) expressing gaussia luciferase under well-defined early, intermediate, and late vaccinia virus promoters, respectively. NHDFs were infected with vEGluc, vIGluc, or vLGluc at MOIs of 1 in the presence of four chemical inhibitors. Culture media from each well were collected at 4, 8, and 12 hours after infection and measured for gaussia luciferase activity. Early, intermediate, and late gene expression was measured 4, 8 and 12 hours post infection, respectively.

All four JAK/STAT3 inhibitors used here were able to significantly reduce all three stages of vaccinia virus gene expression at concentrations that did not decrease cell viability

(Figure 2-3 A – C). SC144, an IL-6 receptor (GP130) inhibitor, can repress intermediate and late gene expression by approximately 80-fold and 30-fold, respectively, and inhibit early gene expression up to 3-fold. AZ960, a selective JAK2 inhibitor, displayed a similar trend in regulating all three stages of gene expression, with an inhibition effect of approximately 8-fold. The two STAT3 inhibitors, Stattic and niclosamide, showed different effects on early gene expression, but similar inhibitory effects on intermediate and late gene expression (approximately 20-fold and 8-fold, respectively). Stattic repressed early gene expression by approximately 8-fold, whereas niclosamide repressed early gene expression by approximately 15-fold. Even with different extensions, all four inhibitors showed significant inhibitory effects towards all three stages of vaccinia virus gene expression. Because vaccinia virus gene expression is regulated in a cascading manner, these results indicate that the JAK/STAT3 pathway was required for vaccinia virus replication starting from the early gene expression stage.

JAK/STAT3 inhibitors repress gene expression of vaccinia virus in keratinocytes

In a previously-published paper, the inhibition of the JAK/STAT3 pathway resulted in increased replication of vaccinia virus in keratinocytes (He et al., 2014), which contradicts our findings. To clear this discrepancy, we tested the effects of two STAT3 inhibitors on vaccinia virus late gene expression, as well as their effects on replication in HFKs. As described previously, HFKs were seeded in a 24-well plate and infected with vLGluc at a MOI of 1 for 12 hours. Culture media were collected and used for the gaussia luciferase assay. Both Stattic and niclosamide effectively reduced vaccinia virus late gene expression by approximately 5-fold (Figure 2-4), indicating a suppressing effect in a different cell type.

The cytotoxicity of niclosamide on NHDFs and the inhibitory effects of niclosamide on replication of vaccinia virus

Niclosamide, a small chemical inhibitor that targets STAT3, is also an FDA-approved drug against parasitic infection. In previous experiments, we noticed that niclosamide showed potent inhibitory effects on vaccinia virus gene expression at 1 uM concentration. Niclosamide therefore has the potential to be repurposed as an antiviral drug for poxvirus infection. To further characterize this chemical, we measured the cytotoxicity of niclosamide on NHDFs with an MTT assay and its inhibitory effects on vaccinia virus replication with a plaque assay.

NHDFs were treated with a series of concentrations of niclosamide. An MTT assay was performed to measure cell viability in each well. In the 0 – 50 uM concentration range, niclosamide had no significant toxicity effects on NHDFs. To determine the inhibitory effects of niclosamide on vaccinia virus replication, NHDFs were plated in a 12-well plate and infected with wild-type vaccinia virus (Western Reserve strain) at a MOI of 1 in the presence of niclosamide. A series of concentrations of niclosamide were used to determine the inhibitory effects of niclosamide on vaccinia virus replication and a plaque assay was performed. Niclosamide started to repress vaccinia virus at a concentration of 1 uM and reached its maximum effect at a concentration of 1.5 uM, which caused approximately a 10-fold reduction in viral titer (Figure 2-5).

The cytotoxicity of SC144 on NHDFs and the inhibitory effects of SC144 on replication of vaccinia virus in NHDFs

In addition to niclosamide, another small molecule inhibitor, SC144, also had strong effects on vaccinia virus gene expression in our previous experiments. SC144 is a bioactive compound that specifically targets GP130, which is a subunit of the IL-6 receptor, and further

blocks activation of the JAK/STAT3 pathway (Xu et al., 2013). SC144 can repress vaccinia virus gene expression at a concentration of 1 uM. Our objective was to further characterize SC144 and measure the inhibitory effects of SC144 on vaccinia virus and the cytotoxicity effects of SC144 on NHDFs. In NHDFs, SC144 started to decrease cell viability at a concentration of 5 uM and reached its maximum effect at a concentration of 30 uM. SC144 repressed replication of vaccinia virus at 1 uM and reached its maximum effect at a concentration of 2.5 uM (Figure 2-6).

The cytotoxicity of SC144 on HeLa cells and the inhibitory effects of SC144 on vaccinia virus in HeLa cells

To test if the inhibitory effect of SC144 on the replication of vaccinia virus is cell type-dependent, we measured the cytotoxicity effects of SC144 on HeLa cells and the inhibitory effects on vaccinia virus replication in HeLa cells. MTT assays and plaque assays were performed with HeLa cells to measure the effect of SC144 on HeLa cell viability and vaccinia virus replication in HeLa cells. Compared to NHDFs, SC144 showed lower toxicity on HeLa cells, starting to show cytotoxicity to HeLa cells at a concentration of 25 uM. SC144 repressed vaccinia virus replication at a concentration of 2.5 uM (Figure 2-7).

Discussion

The objective of this study was to identify host cell components that were required for efficient replication of the vaccinia virus. Our chemical screening of vaccinia virus inhibitors revealed that the JAK/STAT3 pathway is required for vaccinia virus replication. This phenomenon was consistent when we tested the effects of four inhibitors of the JAK/STAT3 pathway on vaccinia virus replication in NHDFs and HFKs. The JAK/STAT3 signaling pathway is involved in many physiological processes, including immune response and cell proliferation (Takeda et al., 1998); however, the role of the JAK/STAT3 pathway regarding viral infections is

complex (Kuchipudi, 2015). Our unbiased chemical screening data suggest that this pathway is required for efficient vaccinia virus replication. In addition, we tested which steps of vaccinia virus replication required the JAK/STAT3 signaling pathway using a reporter recombinant vaccinia virus. Our data suggest that inhibition of the JAK/STAT3 signaling pathway suppresses all three stages of gene expression for the vaccinia virus. Since the gene expression of vaccinia virus is regulated in a cascading manner (Yang et al., 2013), we believe that vaccinia virus requires the JAK/STAT3 pathway from the early gene expression stage, but may also require it during later gene expression.

The role of the JAK/STAT3 signaling pathway in vaccinia virus replication has previously been shown by He et al. (2014), who observed that mice developed larger vaccinia lesions and higher viral titers when topically treated with Stattic, a STAT3 inhibitor. Cultured human and murine keratinocytes produced more infectious particles when treated with Stattic, as well. He et al. argued that the inhibition of STAT3 repressed the type I interferon response in keratinocytes, and therefore the JAK/STAT3 signaling pathway suppressed vaccinia virus replication. Our unbiased chemical screening results and data regarding multiple JAK/STAT3 inhibitors are not consistent with their finding. We also tested two STAT3 inhibitors, Stattic and niclosamide, on vaccinia virus gene expression in human keratinocytes. We found that both Stattic and niclosamide treatment repressed vaccinia virus replication in human keratinocytes after 24 hours of incubation. The discrepancy in effects of JAK/STAT3 inhibitors on vaccinia virus replication could be explained by the fact that the replication of vaccinia virus requires STAT3 signaling at the cellular level, whereas inhibition of STAT3 activity damages adaptive immune response in animals, leading to more virulent symptoms. Although we cannot explain the discrepancy between our findings and the aforementioned study using a cell culture system,

we reproducibly observed the same results using multiple JAK/STAT3 inhibitors and multiple cell types. Thus, we have concluded that the JAK/STAT3 pathway is required for efficient replication of the vaccinia virus in culture cells.

Highly elevated STAT3 activation is a marker of poor clinical outcomes for several types of cancer patients. Vaccinia virus is an oncolytic agent that has been studied to treat cancer. STAT3 activation supporting vaccinia virus replication explains why vaccinia virus preferentially replicates in cancer cells (Zeh et al., 2015). The link between STAT3 and vaccinia virus is supported in this study, providing some insights concerning how vaccinia virus manipulates the JAK/STAT3 pathway. Vaccinia virus is reported to inhibit host immune response and apoptosis, and the JAK/STAT3 pathway is involved in both of these physiological processes. Activation of the JAK/STAT3 pathway is both advantageous and disadvantageous for the replication of vaccinia virus. We have demonstrated that vaccinia virus somehow utilizes the JAK/STAT3 pathway to promote virus replication.

In addition, we observed that two inhibitors of the JAK/STAT3 pathway, niclosamide (STAT3 inhibitor) and SC144 (GP130 inhibitor), can repress vaccinia virus replication at a very low working concentration (1 uM). Further characterization showed that these two chemicals have great potential to treat poxvirus infections. Regarding niclosamide, this FDA-approved drug has already shown anti-viral effects on multiple types of viral infections (Jurgeit et al., 2012). In addition, niclosamide can effectively inhibit vaccinia virus replication in NHDFs and HFKs, which makes it a promising candidate for antiviral drug development. Similarly, SC144, a bioactive that targets GP130, repressed vaccinia virus replication in NHDF, HFKs, and HeLa cells. SC144 has inhibitory effects on several types of cancer cells (Xu et al., 2013), and our data suggest that SC144 has the potential to treat poxvirus infection as well.

In summary, we have demonstrated that the JAK/STAT3 pathway is required for efficient replication of the vaccinia virus and furthermore, two JAK/STAT3 inhibitors, niclosamide and SC144, have the potential to be antiviral drugs to treat poxvirus infection.

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Figures

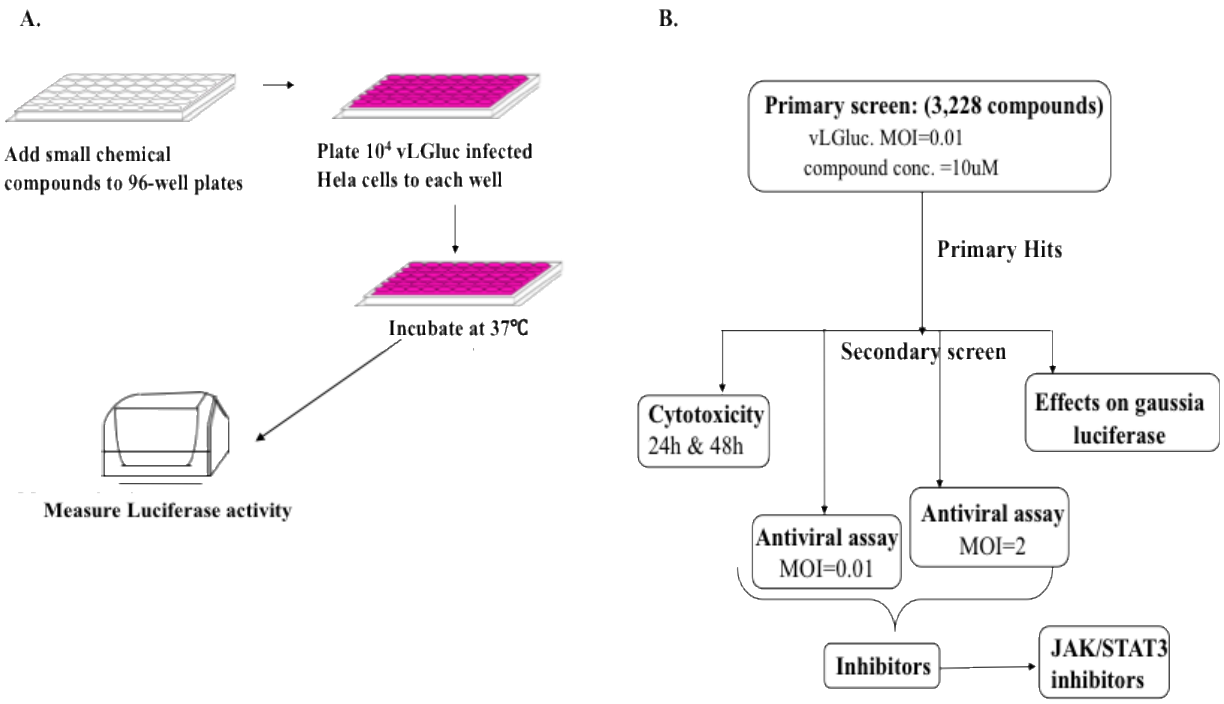

Figure 2-1 Schematic of chemical screen for inhibitors of vaccinia virus

A: Trypsinized HeLa cells were infected with vLGluc at a MOI of 0.01 and placed in a 96-well plate. vLGluc-infected HeLa cells were then treated with different chemicals for 24 hours and gaussia luciferase activity was measured in the culture medium.

B: Schematic of chemical screening process. For primary screen, vLGluc-infected HeLa cells (at MOI of 0.01) were placed in a 96-well plate in the presence of DMSO or different chemical compounds (at a concentration of 10 uM) from Selleck Chemicals and FDA-approved drug libraries. To confirm that the primary hits affect vaccinia virus replication, we performed a second screen and assessed the toxicity effects of the primary hits on the gaussia luciferase enzyme and HeLa cell viability. In addition, we used high (MOI of 2) and low (MOI of 0.01) amounts of vLGluc to infect HeLa cells and to test the effects of the primary hits on vaccinia virus replication. For cytotoxicity effects, HeLa cells were incubated with DMSO or chemical compounds for 24 or 48 hours and an MTT assay was performed to measure cell viability after treatment. Gaussia luciferase enzymes were incubated with DMSO or chemical compounds, and a gaussia luciferase assay was performed to measure gaussia luciferase activity after treatment. Secondary screening revealed numerous inhibitors of vaccinia virus replication, many of which targeted the JAK/STAT3 pathway.

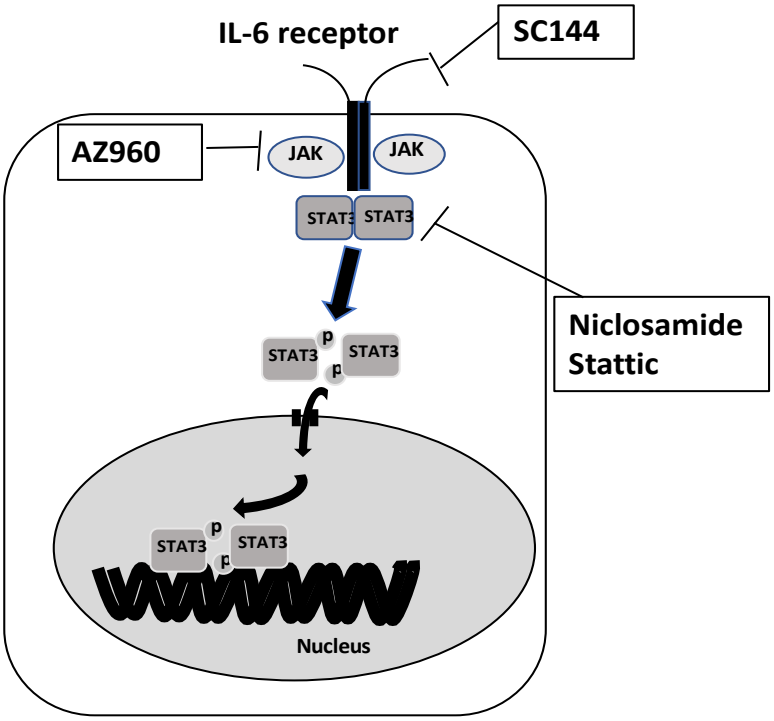

Figure 2-2 Small chemical inhibitors target the JAK/STAT3 pathway

SC144 inhibits GP130, a subunit of the IL-6 receptor, and further represses activation of the JAK/STAT3 pathway. AZ960, another small chemical inhibitor, competitively binds to ATP-binding sites of JAK2 and blocks the JAK/STAT3 signaling pathway. In contrast, niclosamide and Stattic directly target the STAT3 protein by binding to its SH2 domain.

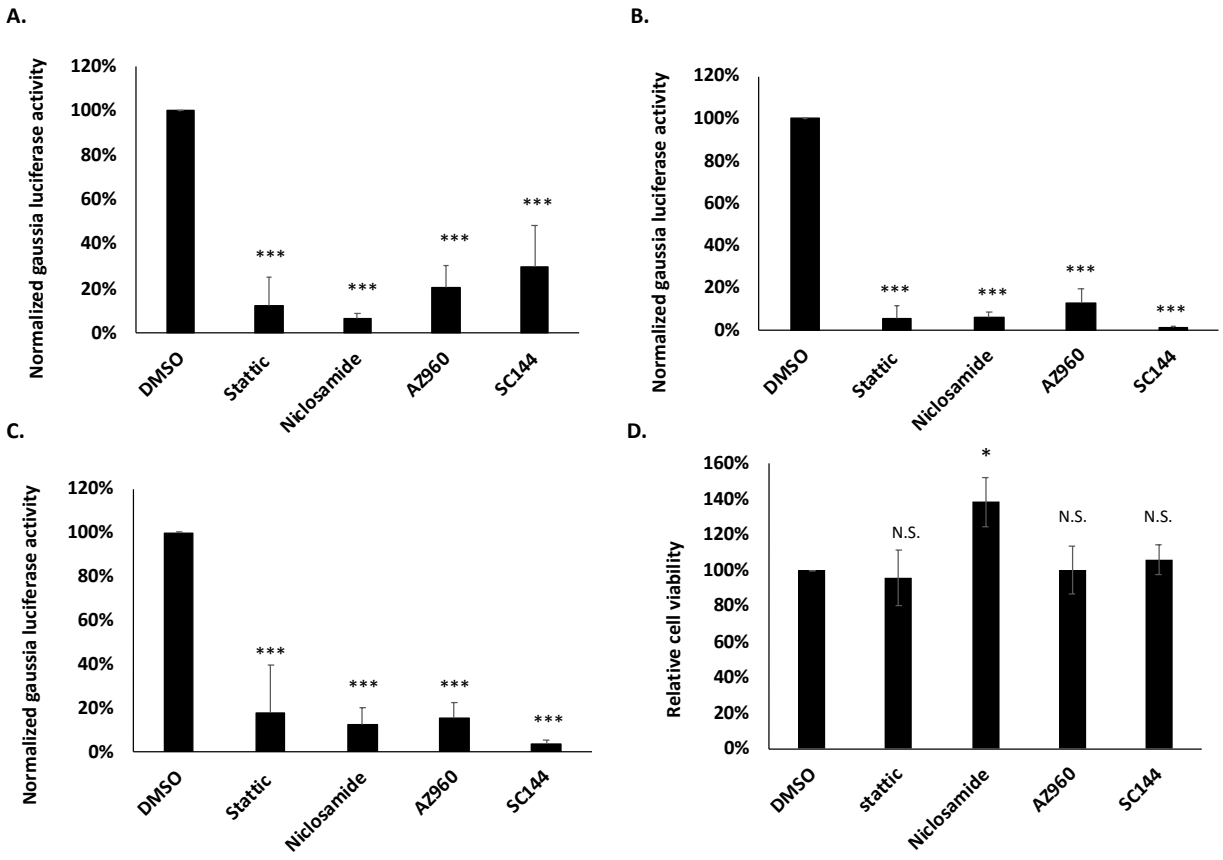

Figure 2-3 Effects of inhibitors of the JAK/STAT3 pathway on gene expression in vaccinia virus

A – C: Effects of inhibitors of the JAK/STAT3 pathway on early, intermediate, and late gene expression by vaccinia virus. Normal human dermal fibroblasts (NHDFs) were plated in a 12-well plate and infected with vEGluc (early), vIGluc (intermediate), or vLGluc (late) vaccinia virus at a MOI of 1 in the presence of DMSO, Stattic (3 uM), niclosamide (1 uM), AZ960 (3 uM), or SC144 (1 uM). Culture media from each well were used to measure gaussia luciferase activity 4, 8, and 12 hours after infection. (A) Early gene expression (vEGluc) was measured based on gaussia luciferase activity. (B) Intermediate gene expression (vIGluc) was measured 8 hours post infection. (C) Late gene expression (vLGluc) was measured 12 hours post infection. **D:** Toxicity effects of inhibitors of the JAK/STAT3 pathway on NHDFs. NHDFs were placed in a 96-well plate and incubated with DMSO, Stattic (3 uM), niclosamide (1 uM), AZ960 (3 uM), and SC144 (1 uM) for 8 hours. An MTT assay was performed to measure cell viability. Three biological replicates were conducted, and representative data are presented here.

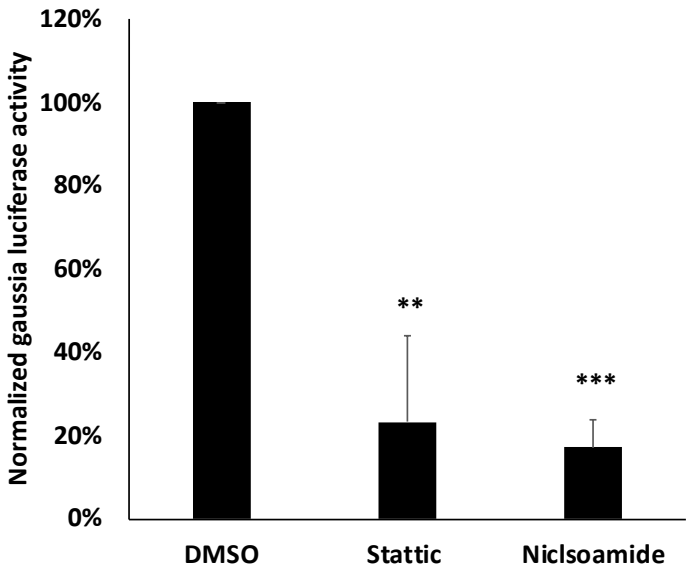

Figure 2-4 Effects of STAT3 inhibitors on late gene expression of vaccinia virus in primary human keratinocytes (HFKs)

HFKs were placed in a 24-well plate and infected with the recombinant vaccinia virus vLGluc at a MOI of 1 in the presence of DMSO, Stattic (5 uM), or niclosamide (1 uM). At 12 hours post infection, a gaussia luciferase assay was performed with culture media from each well to measure vaccinia virus late gene expression.

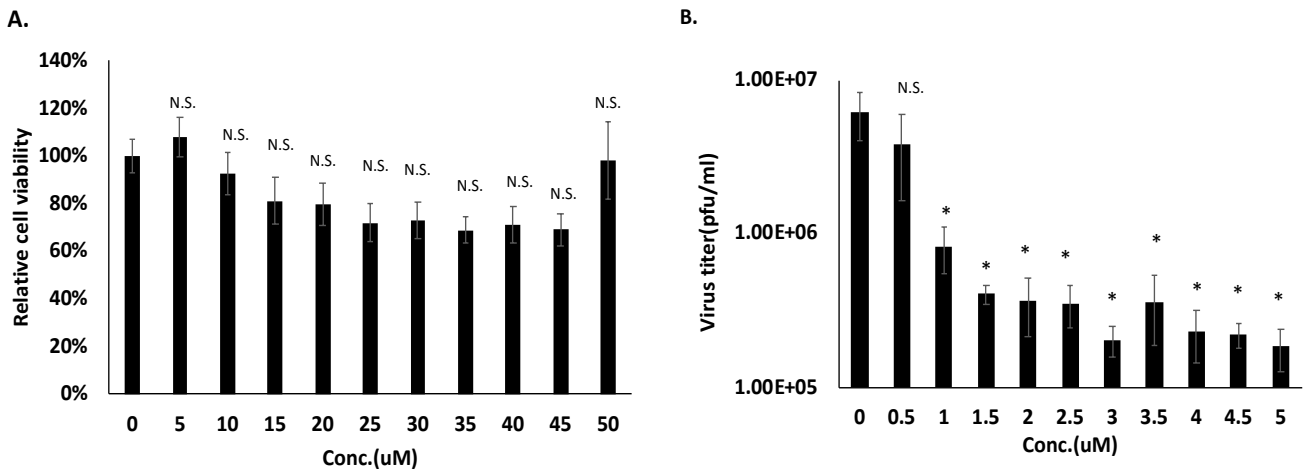

Figure 2-5 The cytotoxicity and the inhibitory effects of niclosamide on normal human dermal fibroblasts (NHDFs) and replication of vaccinia virus

A: NHDFs were placed in a 96-well plate and incubated with a series of concentrations of niclosamide for 24 hours. An MTT assay was then performed to measure cell viability of NHDFs.

B: NHDFs were placed in a 12-well plate and infected with vaccinia virus (Western Strain) at a MOI of 1 in the presence of DMSO or the indicated concentration of niclosamide. A plaque assay was then performed to measure viral titer.

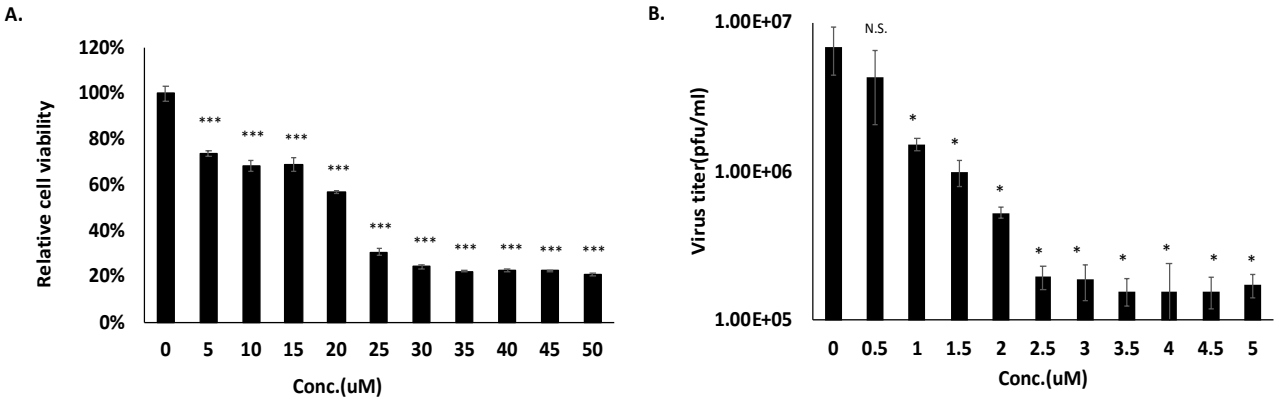

Figure 2-6 The cytotoxicity and the inhibitory effects of SC144 on NHDFs and replication of vaccinia virus

A: NHDFs were placed in a 96-well plate and incubated with a series of concentrations of SC144 for 24 hours. Cell viability was measured by MTT assay.

B: NHDFs were placed in a 12-well plate and infected with vaccinia virus at a MOI of 1 in the presence of DMSO or the indicated concentration of SC144. Viral titer was measured by plaque assay.

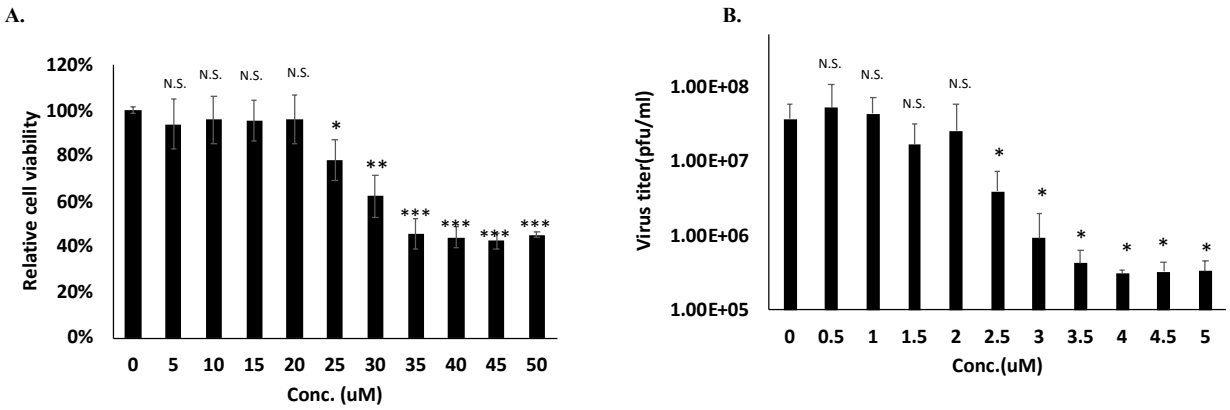

Figure 2-7 The cytotoxicity and the inhibitory effects of SC144 on HeLa cells and replication of vaccinia virus in HeLa cells

A: HeLa cells were placed in a 96-well plate and incubated with the indicated concentration of SC144 for 24 hours. Cell viability was measured by MTT assay.

B: HeLa cells were placed in a 12-well plate and infected with vaccinia virus at a MOI of 1 in the presence of DMSO or the indicated concentration of SC144. Viral titer was measured by plaque assay.

Chapter 3 - Construction of recombinant vaccinia virus with an IPTG-inducible phosphatase, H1

Abstract

We observed that the phosphorylation of STAT3 was downregulated in the early stages of vaccinia virus infection. Vaccinia virus encodes a dual-specific viral phosphatase, H1, and we proposed that H1 is responsible for the dephosphorylation of STAT3. To test this hypothesis, we generated a recombinant vaccinia virus, viH1L, based on a hybrid vaccinia virus system (Vac/T7). In the absence of the inducer isopropyl β-D-1-thiogalactopyranoside (IPTG), the expression of the viral phosphatase H1 was suppressed (viH1), whereas in the presence of IPTG, the expression of viral phosphatase could be restored (viH1+IPTG). We found that recombinant viH1L produced more infectious particles in the presence of IPTG in comparison to viH1L infection alone without IPTG treatment.

Introduction

The phosphorylation and dephosphorylation of proteins play an important role in modulating physiological activities in eukaryotic cells. Viruses are known to manipulate host cell machinery by regulating host cellular phosphorylation events. Vaccinia virus encodes two kinases, B1 and F10, and one phosphatase, H1, which indicates that regulation of phosphorylation events is also important for vaccinia virus infection (Greseth, Carter, Terhune, & Traktman, 2017). The dual-specificity phosphatase H1, an essential factor for vaccinia virus infection, regulates the dephosphorylation of viral and host proteins. Repression of H1 results in vaccinia virus with low infectivity, whereas loss of H1 leads to non-viable virus (Liu, Lemon, & Traktman, 1995). H1 is released from the viral core during viral core activation, which is characterized by the disulfide-bonded viral core protein being reduced and releasing factors and enzymes encapsulated in the viral core.

H1 can also dephosphorylate host the signal transducer and activator of transcription factor 1 (STAT1) protein to repress host interferon response (Najarro, Traktman, & Lewis, 2001). Type I interferon response, which is the first immune defense system against viral evasion, is mediated by the Janus kinase (JAK)/STAT1 pathway. Activating the JAK/STAT1 pathway induces the expression of interferon stimulated genes (ISGs), which further activates innate and adaptive immune responses, and leads the host to an antiviral state (Ivashkiv & Donlin, 2014). Upon phosphorylation, reciprocal interaction between a phosphorylated tyrosine (701Y) residue and the SH2 domain leads to the formation of a STAT1 dimer. Following dimerization, STAT1 translocates to the nucleus and binds to DNA to regulate gene expression. H1 is reported to dephosphorylate the STAT1 protein and block its nucleus translocation in a dose-dependent manner (Koksal, Nardozzi, & Cingolani, 2009). Structural analysis has revealed

that dimeric H1 is capable of recognizing STAT1 after its dimerization, dephosphorylating its tyrosine (701Y) residue, and blocking its nuclear translocation.

Signal transducer and activator of transcription 3 (STAT3), which shares many common features with the STAT1 protein, is also involved in vaccinia virus infection. To determine the dynamic of phosphorylation level change of STAT3 during vaccinia virus infection, we used immunoblotting analysis and blotted vaccinia virus-infected cell lysate against tyrosine-phosphorylated STAT3 antibodies. Immunoblotting results revealed that STAT3 phosphorylation is downregulated in vaccinia virus-infected cells in the early stage of infection, although the level is recovered later. We therefore proposed that viral phosphatase H1 is responsible for the dephosphorylation of STAT3 in the early stage of vaccinia virus infection. To test this hypothesis, we generated a recombinant viH1L vaccinia virus in which the expression of H1 was experimentally controlled. We expected that repression of H1 in vaccinia virus would interfere with the dephosphorylation events of STAT3 in vaccinia virus-infected cells.

Materials and methods

Cell cultures

All cells were cultured in a 37°C incubator with a supplement of 5% CO_2. HeLa cells (ATCC CCL-2) and normal human dermal fibroblasts (NHDFs; ATCC PCS 201-010) were both cultivated in Dulbecco's Modified Eagle Medium (DMEM) with the addition of 10% fetal bovine serum (FBS), 2 mM glutamine, and 1% penicillin-streptomycin. BS-C-1 cells were cultured in Eagle's Minimum Essential Medium (EMEM) with the addition of 10% FBS, 2 mM glutamine, and 1% penicillin-streptomycin.

Viral infection and titration

The Western Reserve (WR) strain (ATCC VR-1354) of vaccinia virus was amplified and purified, as described previously (Cotter et al., 2015). For viral infection, the desired amount of viral stock was diluted in culture medium (contain 2.5% FBS), and cells were incubated with infectious medium for an hour before the medium was refreshed. A plaque assay was performed with BS-C1 cells to determine the viral titer.

Construction of an isopropyl β-D-1-thiogalactopyranoside (IPTG)-inducible recombinant vaccinia virus, viH1L

Due to the insertion of edited green fluorescence protein (eGFP) into the viH1L construct, which was a foreign gene, we split the whole construct into three pieces. The upstream fragment contained 500 nucleotides of H1L gene, the middle fragment contained 20 nucleotides overlapped with the H1L gene and eGFP gene, and the downstream fragment contained 20 nucleotides overlapped with the middle fragment and 500 nucleotides upstream of H1L (vH2R gene; Figure 3-4). PCR products of the three fragments were run on a DNA gel, and then the three PCR products were annealed and used as a template to amplify the whole construct via recombinant PCR technology. The viH1L construct possessed approximately 1800 nucleotides (Figure 3-4). HeLa cells were transfected with the viH1L construct for an hour and then infected with Vac/ T7 vaccinia virus (provided by Dr. Bernard Moss). Samples were collected for further purification and isolation.

Preparation and isolation of the recombinant viH1L vaccinia virus

BS-C-1 cells were infected with a serially-diluted sample of recombinant viH1L for 48 hours. A single plaque that expressed green fluorescence was then picked and used to reinfect BS-C-1 cells. The steps described above were repeated for three rounds until all plaques in a

single well were green. The purified viH1L was then amplified in HeLa cells, as described previously (Cotter et al., 2015).

Immunoblotting analysis

NHDFs were seeded in a 12-well plate at a density of 0.4 x 10^6/well. Cells were infected with viH1L or WR vaccinia virus at a MOI of 1, either in the presence or absence of IPTG for 24 hours. Cells were lysed with 80 ul RIPA buffer and agitated for 30 min. Twenty ul of 5x western blot loading buffer was added and samples were incubated at 100°C for 5 minutes, followed by a cool down on ice before loading. Next, 30 ul of sample was loaded onto each 10-well 10% SDS-PAGE gel and run for one and a half hours before transferring to a SDS-PAGE membrane for another hour. After the membrane was immunoblotted with anti-phos-STAT3 (Y705), anti-STAT3, and GAPDH, the membrane was developed with chemiluminescent substrate (National Diagnostics, Atlanta, GA, United States).

Results and discussion

Phosphorylation of STAT3 decreases in vaccinia virus-infected cells, whereas total STAT3 is enriched in the cell nucleus

The JAK/STAT3 pathway can transduce extracellular signals to the cell nucleus via a series of intracellular phosphorylation events. The first step of STAT3 activation by JAKs is tyrosine phosphorylation at a single site near the C-terminal (Y705) of STAT3 in response to extracellular stimuli. Following tyrosine phosphorylation, a pair of STAT3 proteins dimerizes and translocates to the cell nucleus to regulate gene expression by binding to DNA regulatory elements. After establishing the fact that inhibitors of the JAK/STAT3 pathway repress vaccinia virus infection and replication, we were also interested in its activation level in NHDFs during vaccinia virus infection. After infecting NHDFs with WR vaccinia virus at a MOI of 1, we

determined the STAT3 tyrosine (Y705) phosphorylation level in NHDFs after vaccinia virus infection using immunoblotting analysis (Figure 3-1B). Another lab member, Dr. Shuai Cao, was conducting research on the localization of STAT3, however, and observed that the total STAT3 was enriched in the cell nucleus during vaccinia virus infection. The confocal image showed that STAT3 translocated to the cell nucleus 4 hours after vaccinia virus infection (Figure 3-1B; Dr. Shuai Cao, personal communication). It has previously been reported that unphosphorylated STAT3 can also function as a signal transducer and be activated by extracellular signals (Yang et al., 2007), and our data indicated that unphosphorylated STAT3 might play an important role in vaccinia virus infection. The dephosphorylation of STAT3 in the early stage of vaccinia virus infection shared many similarities with the dephosphorylation of STAT1 in the early stage of vaccinia virus infection (Najarro et al., 2001), and it has been reported that viral H1 is responsible for the dephosphorylation of STAT1. The objective of this project was to generate a recombinant vaccinia virus, viH1L, in which the expression of H1 could be experimentally controlled.

Construction of the IPTG-inducible recombinant viH1L vaccinia virus expressing green fluorescent protein (GFP) reporter gene

It has been previously reported that vaccinia virus encodes the phosphatase H1 to dephosphorylate STAT1 during infection. Because STAT1 and STAT3 share similar structures and activation pathways, we propose that viral phosphatase H1 is also responsible for the reduction of STAT3 phosphorylation during vaccinia virus infection. To investigate whether repression of H1 expression results in a reduction of STAT3 phosphorylation, we first generated an IPTG-inducible viH1L recombinant vaccinia virus in which H1 expression was controlled by the presence of the inducer in culture medium.

The recombinant viH1L vaccinia virus is based on a hybrid vaccinia virus system (Vac/ T7) described previously (Alexander, Moss, & Fuerst, 1992). The Vac/ T7 vaccinia virus system consists of a constitutively expressed *Escherichia coli lac* repressor and a *lac* operon, which is inserted before the T7 polymerase gene. In the presence of the inducer (IPTG), *lac* repressors are inactive and lose their repression effect on the *lac* operon and T7 polymerase. The expression of T7 polymerase can thus induce T7 promoter-controlled gene expression (Figure 3-2). Homologous recombination frequently occurs in DNA viruses and plays an important role in viral evolution. When DNA fragments share similar sequences with the vaccinia virus genome during viral infection, these sequences can exchange with each other. Recombinant viH1L was generated based on homologous recombination (Figure 3-4A). After several rounds of purification and isolation, recombinant viH1L that expressed reporter eGFP was successfully generated (Figure 3-4B). Then we confirmed the recombinant viH1L with PCR technology and Sanger sequencing (Figure 3-5&6).

Repression of H1 phosphatase in vaccinia virus decreases the production of infectious particles

To characterize this viH1L recombinant virus, we first amplified viH1L viruses either in the presence or absence of the inducer IPTG in culture media, and then determined viral titer using a plaque assay. In the presence of IPTG, viH1L produced 7.5-fold more infectious particles than in the absence of IPTG (Figure 3-7). In conclusion, inhibition of H1 gene expression in vaccinia virus reduced the production of infectious particles (Figure 3-7). This is consistent with a previous study (Liu et al., 1995), which reported that viral H1 phosphatase was essential for vaccinia virus replication, as deletion of H1 resulted in non-viable virus. Our data confirmed that repression of H1 in vaccinia virus leads to decreased production of infectious particles. In

conclusion, we successfully generated a recombinant viH1L vaccinia virus in which the expression of H1 could be controlled.

Future perspectives

Viral phosphatase has inhibitory effect on the STAT1 phosphorylation, and we proposed that this viral protein could dephosphorylate STAT3 during vaccinia virus infection as well. With successful generation of the IPTG inducible viH1L recombinant vaccinia virus, we can test the effects of H1 repression on the phosphorylation of STAT3 during infection. Next step for this project would involve confirm that the expression of H1 can be induced by additional IPTG using Western blotting analysis. Since the STAT1 was dephosphorylated by H1, the repression of vH1 should increase the phosphorylation level of STAT1 in vaccinia virus-infected cells (Koksal, Nardozzi, & Cingolani, 2009). We proposed to test the phosphorylation level of STAT3 in the presence of absence of IPTG by Western blotting analysis in viH1L-infected NHDFs and used the phosphorylated STAT1 level change as a control. If the H1 phosphatase was responsible for the dephosphorylation of STAT3, repression of H1 would reduce the dephosphorylation of STAT3 in vaccinia virus-infected NHDFs.

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Figures

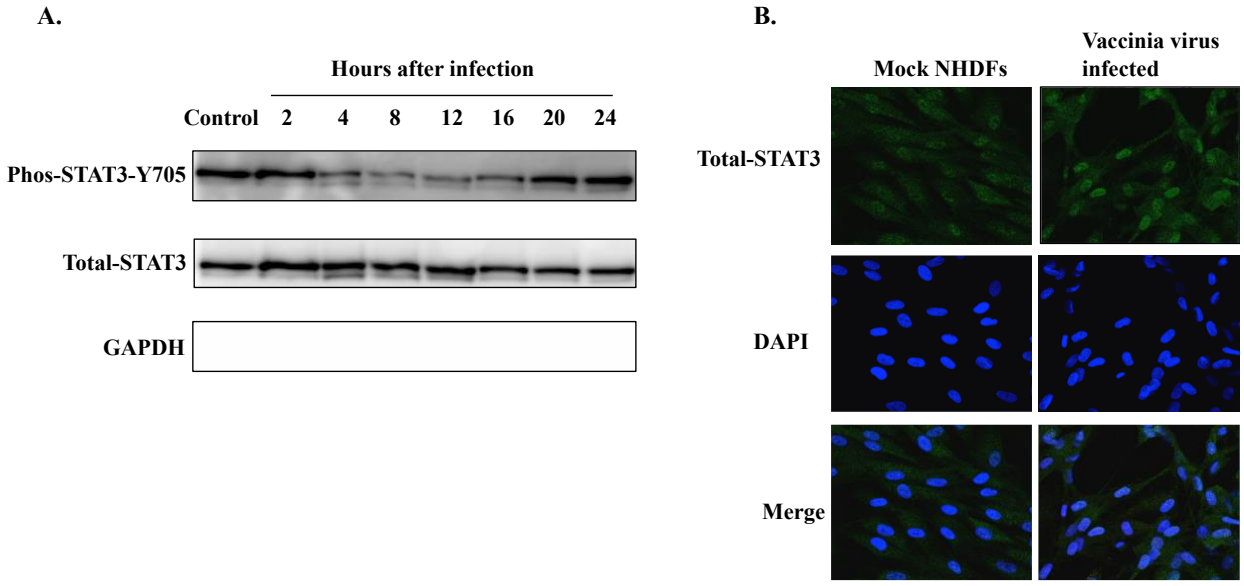


Figure 3-1 Phosphorylation of STAT3 in vaccinia virus-infected cells and total STAT3 in the cell nucleus

A: Immunoblotting image of the phosphorylated tyrosine (Y705) showing changes in STAT3 levels during vaccinia virus infection. Normal human dermal fibroblasts (NHDFs) were infected with vaccinia virus at a MOI of 1 and cell lysate was collected at the indicated time points after infection for immunoblotting analysis.

B: Confocal image of mock and vaccinia virus-infected NHDFs. Mock and vaccinia virus-infected NHDFs were stained with total STAT3 antibodies 4 hours post infection and the cell nucleus was stained with DAPI. (This experiment in panel B was conducted by Dr. Shuai Cao and the image was obtained through personal communication).

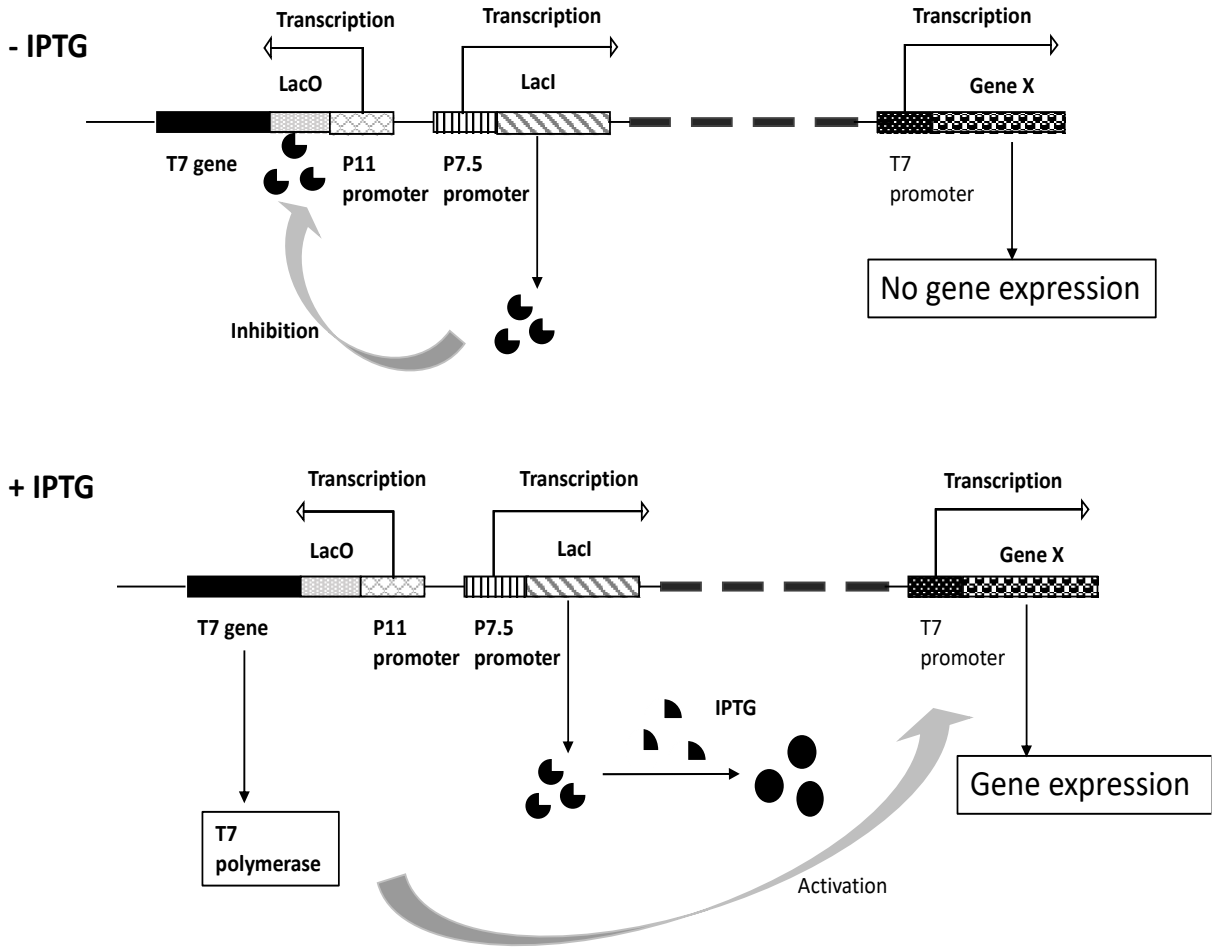


Figure 3-2 IPTG-inducible Vac/T7 recombinant vaccinia virus system

In the absence of IPTG, the Vac/T7 recombinant vaccinia virus constitutively expresses *lac* repressor (lacI) that bind to *lac* operon (lacO) and inhibit the expression of T7 polymerase. In the presence of IPTG, *lac* repressors were released from the *lac* operon which lead to the expression of T7 polymerase, resulting in the expression of genes that was placed under T7 promoter.

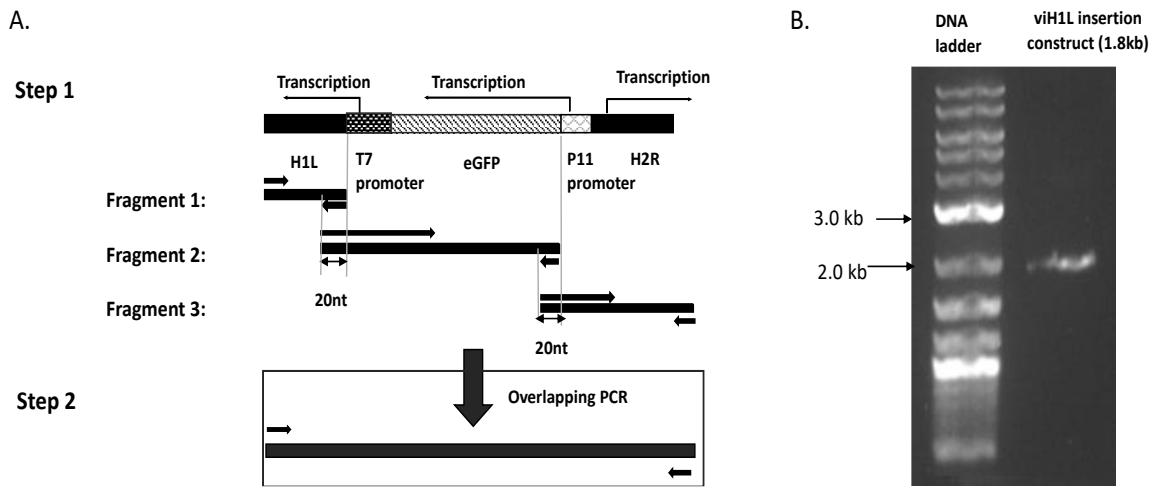
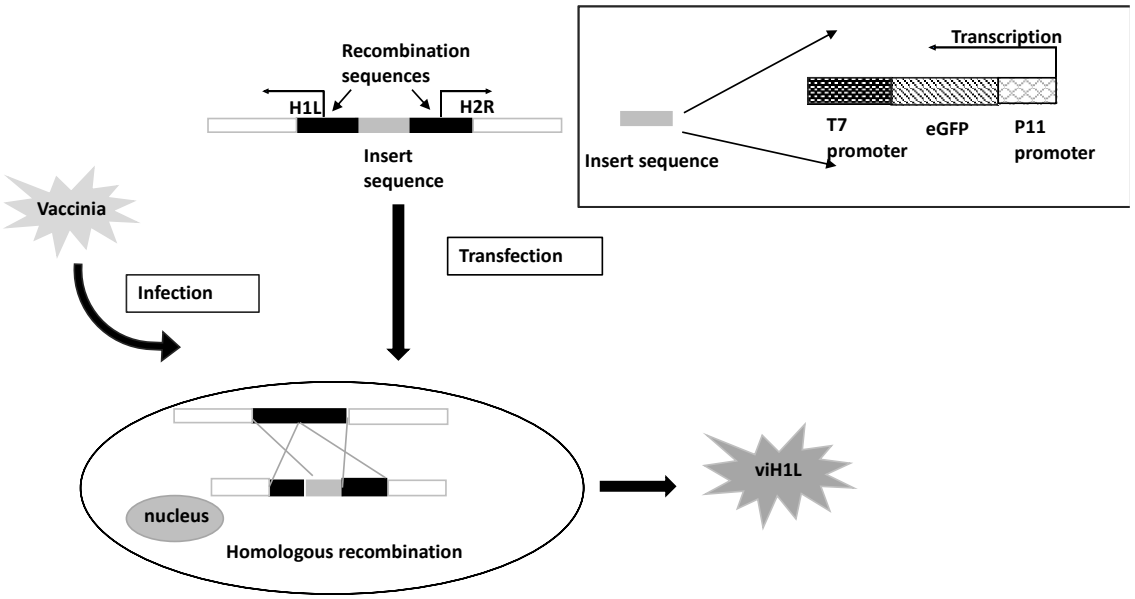


Figure 3-3 Generation of the recombinant vaccinia virus viH1L

A: Schematic showing generation of the viH1L construct via PCR technology. Fragments 1 and 3 were generated through PCR using vaccinia virus as a template, whereas fragment 2 used edited green fluorescent protein (eGFP) as a template. Overlapping PCR was then used to generate the full viH1L insertion construct.

B: DNA gel image of viH1L insertion construct (1.8 kb).

A.



B.

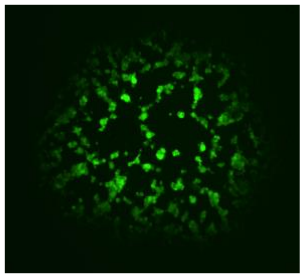


Figure 3-4 Generation of the recombinant vaccinia virus viH1L through PCR and recombinant technology

A: Schematic showing the generation of the recombinant vaccinia virus viH1L through PCR and recombinant technology.

B: HeLa cell were transfected with viH1L insertion constructs and infected with Vac/T7 recombinant vaccinia virus for 24 hours. Plaque assay was performed to isolate the recombinant viH1L. Under microscope, recombinant viH1L infected BS-C1 cells exhibit bright green fluorescence when exposed to blue light. A single green plaque was picked for further amplification.

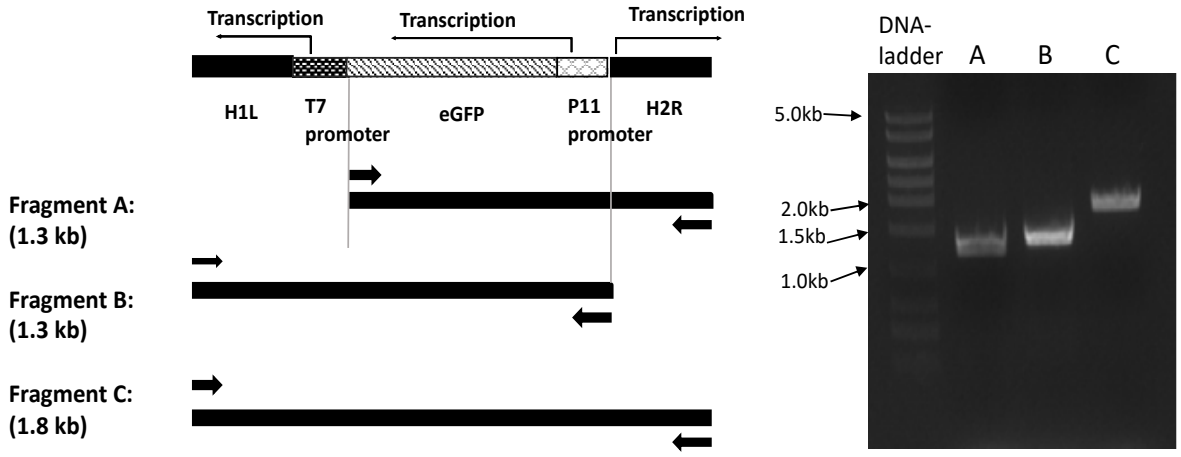


Figure 3-5 Confirmation of successful generation of the three fragments of recombinant vaccinia virus viH1L by PCR

HeLa cells were infected with recombinant viH1L for 24 hours and cell lysates were used to extract the recombinant viH1L DNA. We used the DNA extracts as template and primer pairs as shown above to amplify fragment A, B, and C, respectively. Next, the amplified fragments were run in a DNA gel.

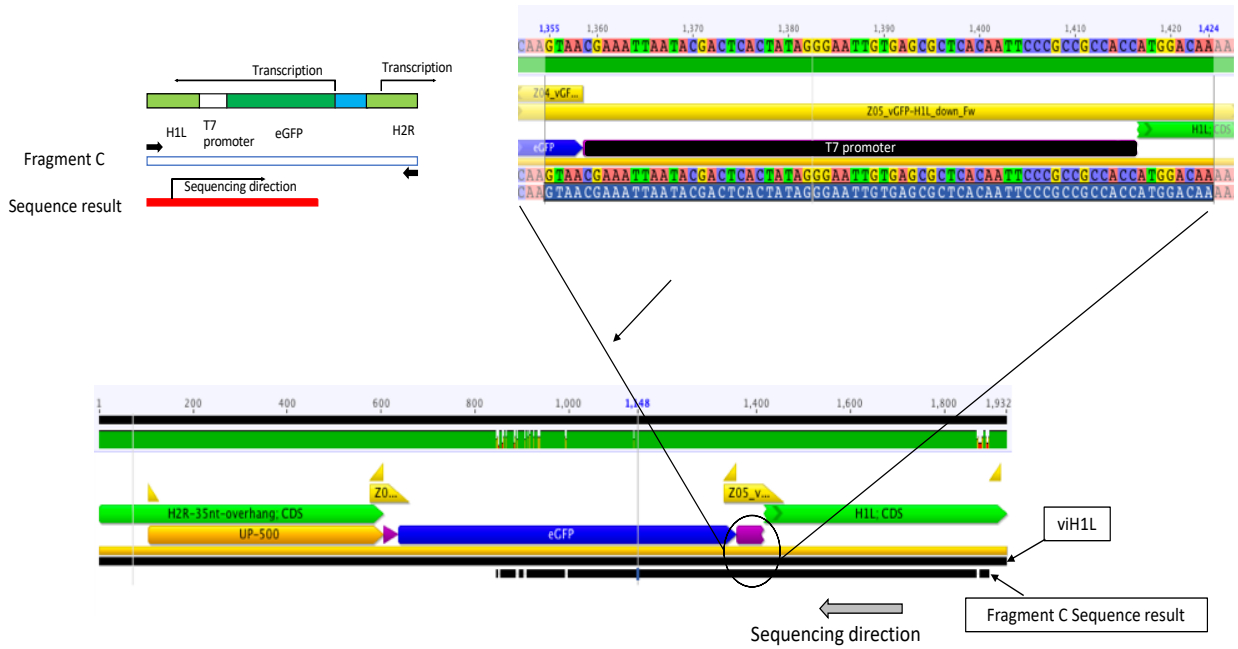


Figure 3-6 Confirmation of successful generation of the recombinant vaccinia virus viH1L by Sanger sequencing

Fragment C from Figure 3-5 was used as template for sanger sequencing and sequenced from the right end (H1L gene end). The sanger sequencing result was analyzed with Genesis software. The T7 promotor region in both sequencing result and template was enlarged and shown above.

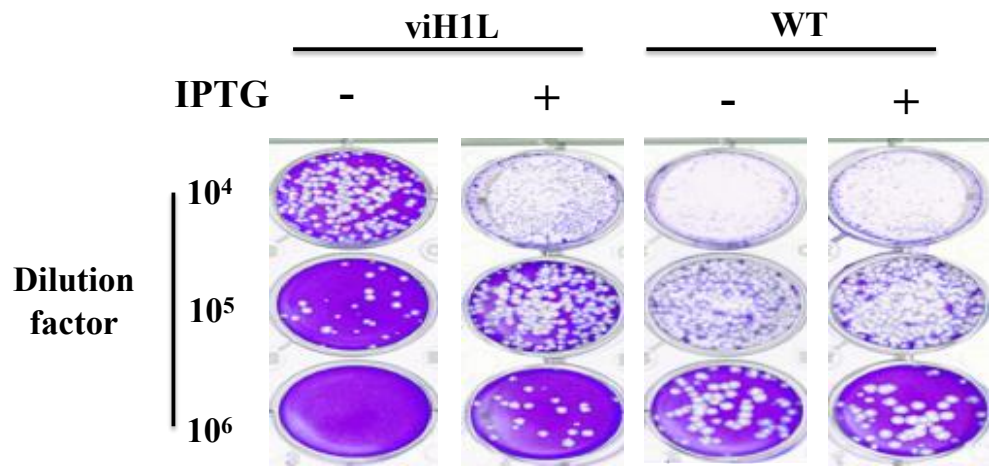


Figure 3-7 Effects of the presence of IPTG on the production of infectious particles by the recombinant vaccinia virus viH1L and the Western Reserve (WR) strain of vaccinia virus

HeLa cells were infected either with WR vaccinia virus or recombinant viH1L in the presence or absence of 100 uM IPTG for 24 hours. Virus samples were collected and diluted with cell culture medium. Ten-fold dilutions of virus samples were used to infect BS-C1 cells and plaque assay was performed to determine the virus titer of each sample.

Chapter 4 - Summary and future perspectives

The objective of this study was to identify the host cell components that were required for vaccinia virus to successful infect a cell. We identified and verified that the JAK/STAT3 pathway is essential for efficient replication of the vaccinia virus. Moreover, we discovered that STAT3 was enriched in the cell nucleus while phosphorylated (Y705) STAT3 was downregulated in vaccinia virus-infected cells.

The JAK/STAT3 pathway regulates both the host immune response and cell proliferation (Johnson et al., 2018). Vaccinia virus has been reported to repress both the host immune response and the cell apoptosis pathways during infection (Seet et al., 2003). Activation of the JAK/STAT3 signaling pathway is both advantageous and disadvantageous for viral replication (Suarez et al., 2018). Our data suggest that the vaccinia virus requires the JAK/STAT3 pathway during infection. Contrary to common knowledge regarding the JAK/STAT3 signaling pathway, our confocal image showed that STAT3 was enriched in vaccinia virus-infected NHDFs, whereas our immunoblotting image revealed that phosphorylated (Y705) STAT3 was downregulated during infection. The activation of STAT3 commonly involves it being phosphorylated on its tyrosine (Y705) residue, followed by its dimerization and translocation to the cell nucleus to enact its functions. The enrichment of STAT3 in the cell nucleus in vaccinia virus-infected cells while its total phosphorylation level was downregulated suggests that unphosphorylated STAT3 plays an important part in vaccinia virus replication. Indeed, unphosphorylated STAT3 has been reported to regulate gene expression (Yang et al., 2007), although the physiological role of unphosphorylated STAT3 is not fully explored. Our data suggest that unphosphorylated STAT3 is involved in vaccinia virus infection. Questions remain, however. How does vaccinia virus manipulate the level of phosphorylated STAT3 protein during

infection? What is the role of the JAK/STAT3 signaling pathway in vaccinia virus replication? The unphosphorylated form of the STAT3 protein has been reported to interact with the NF-κB pathway (Yang et al., 2007), which is known to be repressed during vaccinia virus replication. Unphosphorylated STAT3 may therefore regulate the replication of vaccinia virus by interacting with the NF-κB pathway. Another hypothesis is that unphosphorylated STAT3 may directly regulate the host cell's gene expression in a way that benefits vaccinia virus replication. We plan to test this particular theory in the future.

In addition, we have shown that niclosamide and SC144 have the potential to be used as antiviral drugs to treat poxvirus infection. Niclosamide is an FDA-approved drug that is reported to inhibit multiple types of viral infections (Jurgeit et al., 2012). Here, we have reported that niclosamide can repress titers of vaccinia virus up to 10-fold at a concentration of 1.5 uM. The SI of niclosamide in NHDFs is over 100, which indicates that the chemical is a suitable candidate for drug development to treat poxvirus infection. SC144 is a novel small molecule inhibitor of the IL-6 receptor that is reported to inhibit certain types of cancer (Xu et al., 2013), but little is known regarding its effect on viral infection. Here, we have reported a novel function for this bioactive chemical and have shown that its effect on viral replication is cell type-independent. In the future, we intend to test these two chemicals on vaccinia virus replication in murine models. Both niclosamide and SC144 have the potential to be developed as anti-viral medicines, and further experiments could include investigating their effects on different cell types, testing them in a murine model, and even clinical trials.

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