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RESEARCH ARTICLE

Suppression of Monkeypox Virus by Downregulation of Fatty Acid Synthase and Upregulation of Cholesterol-25 Hydroxylase

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ABSTRACT

The World Health Organization declared the spread of monkeypox virus (MPXV) across multiple African countries a public health emergency of international concern in August 2024. This marks the second such declaration in the past 2 years in response to the MPXV transmission. The re-emergence of the MPXV 3 years after the start of the SARS-CoV-2 epidemic further emphasizes the need to develop broad spectrum antivirals that might control the spread of poorly understood pathogens. The induction of innate immune responses to a viral infection triggers rapid expression of type-I-interferon, which subsequently leads to the differential regulation of over 300 genes that collectively establish an antiviral state. While most of these "interferon-stimulated genes" (ISGs) are upregulated, some are downregulated. Notably, the expression of certain ISGs involved in lipid metabolism is suppressed, which can be exploited by viruses to facilitate their replication. Herein, we report that the expression of fatty acid synthase (FASN), an enzyme involved in de novo biosynthesis of fatty acids, was significantly reduced upon MPXV infection. Moreover, MPXV infection was impaired in FASN knockout cells, and biological inhibitors of FASN significantly inhibited MPXV. Interestingly, chosterol-25-hydroxylase was induced in MPXV-infected cells, and its enzymatic product, 25-hydroxychlosterol (25HC), blocked MPXV infection. Overall, this study suggests that 25HC and FASN inhibitors exhibit significant antiviral activity and may have therapeutic applications in combating understudied infectious diseases in early outbreak settings when targeted therapies have not yet been developed.

1 | Introduction

After 40 years without any reported endemic cases of monkeypox virus (MPXV) outside West and Central Africa, over the last 6 years there have been multiple outbreaks throughout the world, with the most recent outbreak in 2022 establishing MPXV as a global health issue [1]. The first description of MPXV in wild monkeys was reported in 1959 in Copenhagen, Denmark, while the first human MPXV case occurred in 1970 in the Democratic Republic of Congo [2]. Since its discovery, multiple outbreaks have occurred in both endemic and non-endemic countries, with the most recent outbreak occurring in 2022 [1]. As of March 2023, there were approximately 86 000 confirmed global cases, including more than 84 000 confirmed cases in countries non-endemic to MPXV [3]. Two clades of MPXV exist—the West African clade and the more severe

Congo Basin (Central African) clade [4]. The natural reservoir of MPXV remains unknown, however various animal species have been identified as susceptible, such as rope squirrels, tree squirrels, Gambian pouched rats and nonhuman primates [2]. The virus's spread to nonendemic regions may signify that human population has now itself become a significant reservoir [5]. In the Democratic Republic of Congo, the number of reported cases as of August 2024 has already surpassed last year's total, with over 15 600 cases and 537 deaths of which over 100 laboratories confirmed cases of clade 1b have been reported in countries previously unreported for MPXV. This promoted WHO to declare the rapid emergence and widespread transmission of MPXV across multiple African countries a public health emergency of international concern on August 14, 2024 [5]. This declaration represents the second time in 2 years that MPXV transmission has prompted such a response, highlighting the critical need for immediate global attention and intervention. Consequently, there is an immediate need for further research into MPXV's infectious mechanisms, host immune responses, and the development of novel antiviral therapies.

MPXV is a double stranded DNA virus with a low frequency of mutation, of the genus *Orthopoxvirus*, reminiscent of the smallpox virus with similar symptoms including rash, fever, chills, swollen lymph nodes, and exhaustion [1]. In addition, severe MPXV infection may cause damage to various immune organs and can impair the host's immune system integrity, such as by inhibiting NK-cell function [6]. It is spread through close contact by direct or indirect exposure to infectious secretions or lesions [7]. MPXV encodes approximately 190 genes. Its genomic structure consists of a core region of relatively conserved sequences flanked by variable left and right “arms” which are posited to be involved in pathogenicity in Orthpoxviruses, specifically to humans [8]. Phylogenetic analysis highlighted that strains responsible for the MPXV outbreak in 2022 originated from an MPXV strain isolated in 2018 [5]. Major mutations were identified in proteins such as MPXV DNA polymerase (OPG071), MPXV BCL-2 family protein (OPG034), MPXV poly(A) polymerase catalytic subunit (OPG063), and MPXV DNA helicase (OPG145), among others [9].

To combat MPXV outbreaks in nonendemic countries such as the US, an orthopoxvirus vaccine made from vaccinia virus has been the most effective preventive measure. This vaccine is known to be approximately 85% effective in preventing MPXV before exposure, but is much less protective when administered after exposure [6]. Beyond vaccination, the administration of repurposed antiviral drugs (tecovirimat, cidofovir, brincidofovir), has been effective at reducing viral loads in vitro and improving clinical outcomes in human studies [10].

The innate immune response is the first line of defense against invading pathogens. Detection of pathogen-associated molecular patterns (PAMPs) by select germ line encoded pattern recognition receptors (PRRs) on innate immune cells results in the induction of the type-I interferon (IFN-I) response, which establishes an antiviral state upon viral attack. The viral RNA and DNA species derived from replicative cycles function as PAMPs and are sensed by cytosolic PRRs [11–15]. RNA species are sensed by the RIG-I like family of RNA sensors and viral DNA species are recognized by cGAS, IFI16, and DDX41, resulting in the activation of the IFN-I response [16–18].

The IFN-I pathway is activated when IFNα and IFNβ bind to the interferon-I receptor on neighboring cells. This interaction triggers the Janus kinase-signal transducer and activator of transcription (JAK-STAT) signaling pathway, leading to the expression of over 300 genes collectively known as interferon-stimulated genes (ISGs). These ISGs work together to establish an antiviral state and inhibit viral infection. Furthermore, induction of the IFN-I pathway results in the suppression of dozens of genes which function as pro-viral genes, including those involved in fatty acid metabolism. Notably, only a subset of ISGs are involved in the inhibition of infection by a particular virus [19–23]. MPXV appears to be more sensitive to exogenous IFN treatment compared to related viruses such as variola (VARV) and vaccinia (VACV). It has been shown that pretreating cells with human IFN-β significantly inhibits MPXV replication [24]. However, MPXV has evolved several immunomodulatory proteins that counteract the type I interferon response. Notably, the F3 protein of MPXV impairs the activation of PRRs and inhibits the phosphorylation of eIF2α [25]. F3 protein is a modified homolog of the E3 protein found in VACV [26]. These studies suggest that although MPXV has evolved mechanisms to suppress IFN-I responses, there may still be some degree of IFN induction during infection.

Our lab, along with others have shown that modulating enzymes in the fatty acid synthesis pathway plays a crucial role in regulating infection by several viruses [27], including dengue virus (DENV) [28–30], hepatitis C virus (HCV) [31, 32], human cytomegalovirus (HCMV) [33], Kaposi sarcoma-associated herpesvirus (KSHV) [34]. The IFN-I-suppressed fatty acid synthase (FASN) is crucial for the replication of various viruses. Consequently, inhibitors targeting fatty acid metabolism genes have been found to impede the replication of a broad spectrum of viruses. Specifically, the antiviral effect is attributed to the reduction in protein palmitoylation and neutral lipid synthesis resulting from the inhibition of fatty acid metabolism pathways [27].

FASN is the sole enzyme in de novo fatty acid synthesis. Therefore, FASN inhibitors including C75, cerulenin, and TVB-3166 inhibit infection by multiple enveloped viruses, including herpes simplex virus-1 (HSV-1), murine-herpesvirus-68 (MHV-68), Zika virus (ZIKV), and severe acute respiratory virus-2 (SARS-CoV-2) [27]. FASN and certain genes involved in fatty acid biosynthesis play a significant role in viral infection with a wide variety of enveloped viruses, including HCMV [33], KSHV [34], DENV [28–30], chikungunya virus [9], rotavirus [35], HCV [31, 32, 36], and SARS-CoV-2 [27].

In contrast to FASN, Overexpression of cholesterol 25-hydroxylase (Ch25H) significantly inhibits infection by a broad array of viruses [20, 21, 37].

Ch25H is an enzyme involved in cholesterol and lipid metabolism which converts cholesterol to 25-hydroxycholesterol (25HC). 25HC is known to be produced by macrophages and dendritic cells in response to various toll-like receptor ligands and IFNs [13]. Mechanistically, 25HC causes depletion of

cholesterol in the plasma membrane through activation of the enzyme acyl-CoA:cholesterol acyltransferase (ACAT). Activation of ACAT leads to the esterification of cholesterol and its subsequent transport from the plasmamembrane to the endoplasmic reticulum (ER). This process effectively inhibits viral fusion with the host cell membrane [38, 39].

Herein, we report that IFN-I inversely regulates the expression of Ch25H and FASN to combat MPXV replication. Whereas FASN is suppressed, Ch25H is induced by IFN-I to block infection by MPXV. Gaining a better understanding of the infectious process by this virus emphasizes the critical need for developing broad antiviral agents to combat this understudied virus.

2 | Results

2.1 | MPXV Differentially Regulates the Expression of Genes Involved in the Innate Immune Response

NR-2500 MPXV strain US 2003 was amplified in Vero cells and the viral titer was evaluated by plaque assay (Figure S1). The expression of MPXV G2R (OPG-087) [40], a critical gene transcribed early in infection that supports viral replication by interacting with viral RNA polymerase during intermediate and late phases, was monitored at 1, 3, and 6 h-post infection (HPI) by RT-qPCR (Figure 1A). G2R serves as a key marker for early and rapid detection of MPXV infection [41–44]. Additionally, the expression levels of MPXV genes M1L (OPG-037, an early gene), G8R (OPG-093, an intermediate gene), and A5L (OPG −130, a late gene) [43] was evaluated over the infection course in both Huh 7 (Figure S2) and Hela cells (Figure S3A,B). To investigate the expression pattern of the innate immune response during MPXV infection, Huh-7 cells were infected with MPXV and analyzed at 1, 3, and 6 HPI using RT-qPCR. The results showed a time-dependent downregulation of FASN. FASN expression began to decline at 1 HPI, with a more pronounced decrease observed at 3 and 6 HPI in Huh 7 cells (Figure 1B) and 12 HPI in Hela cells (Figure S3C). Interestingly, the expression of CH25H, IFIT1, IL6, IRF1, and IFITM1 demonstrated a time-dependent upregulation upon MPXV infection (Figure 1C–G). Notably, DDX58 and IRF1 levels increased at 1 and 3 HPI but decreased by 6 HPI (Figure 1H,K).

2.2 | FASN Supports MPXV Infection

As shown in Figure 1B, the expression of FASN was suppressed during MPXV infection. To validate this finding, FASN was overexpressed in Huh-7 cells and subjected to MPXV infection. At 24 HPI, cells were harvested and the MPXV replication was quantified by RT-qPCR. The results supported the pro-viral function of FASN. Indeed, MPXV more efficiently replicated in FASN-overexpressed cells compared to the control cells (Figure 2A and Figure S5A). In addition, the small molecular inhibitor of FASN, C75, greatly inhibited MPXV infection. Huh 7 cells were infected with MPXV and treated with C75. Cells were harvested at 24HPI and subjected to RT-qPCR. The results indicated that C75 effectively inhibited MPXV replication (Figure 2B). Furthermore, the number of MPXV infectious particles after treatment with C75 was titrated by plaque forming assay (PFA) and confirmed the inhibitory effect of C75 on MPXV (Figure 2C and Figure S7I). The IC_{50}, the dose of C75 at which the rate of MPXV growth was reduced by 50% was determined to be 1.06 μM (Figure S4A).

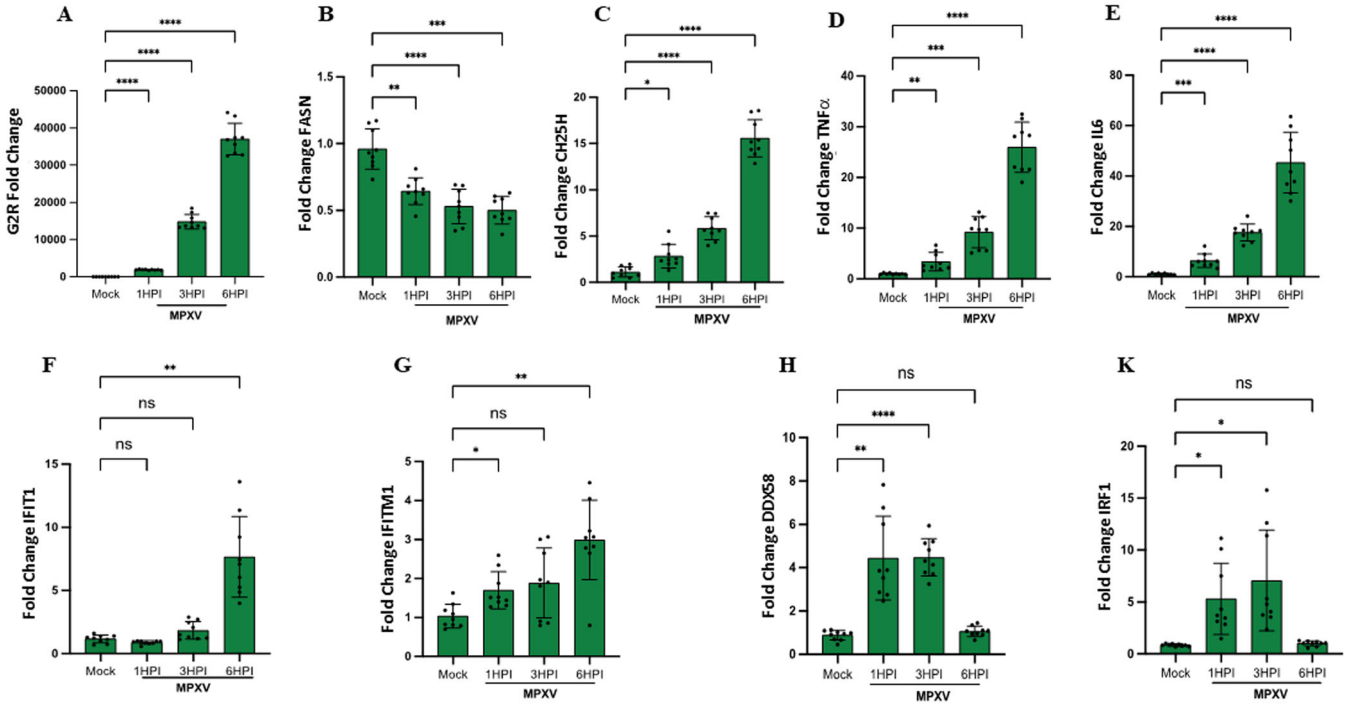

FIGURE 1 | IFN-I-regulated genes control MPXV infection. (A) Huh 7 cells were infected with MPXV and harvested at 1, 3, and 6 HPI. The expression level of (A) MPXV G2R, (B) FASN, (C) CH25H, (D) TNF-α, (E) IL6, (F) IFIT1, (G) IFITM1, (H) DDX58, and (K) IRF1 was quantified by RT-qPCR.

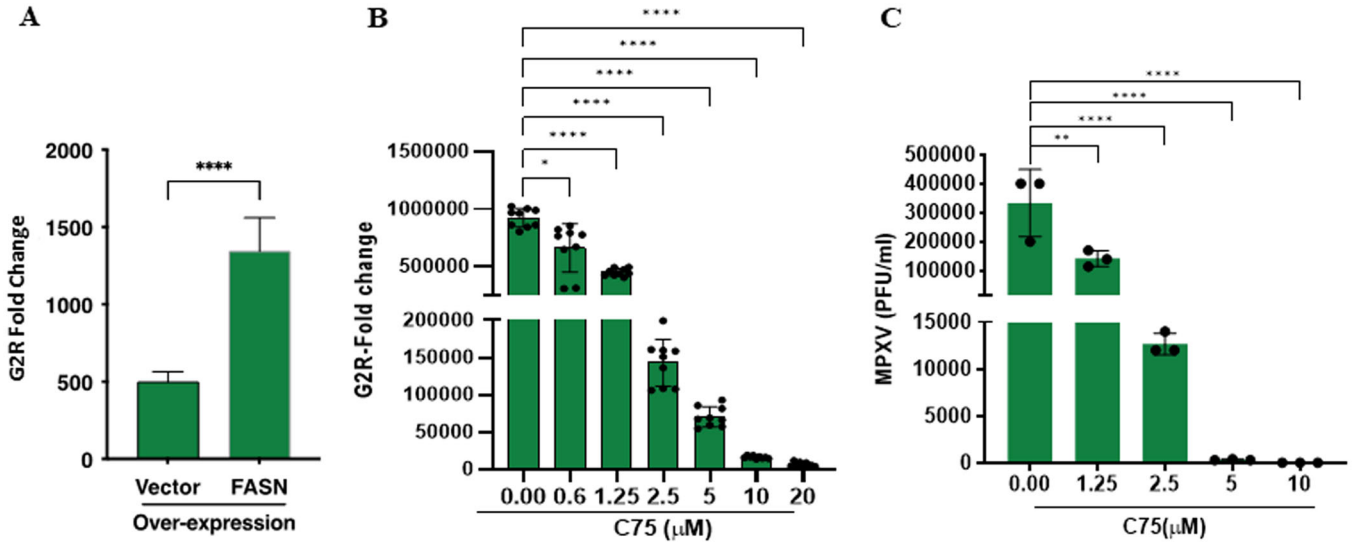

FIGURE 2 | The impact of FASN on MPXV infection. (A) Huh 7 cells were transfected with control plasmid (vector only) or expression plasmid, FASN for 24 h before MPXV infection (MOI = 0.5) and cells were harvested to assess the rate of viral replication at 24HPI by RT-qPCR. (B) The rate of MPXV infection in the presence of C75 measured by RT-qPCR. (C) MPXV titration in cells treated with different concentration of C75 by plaque assay.

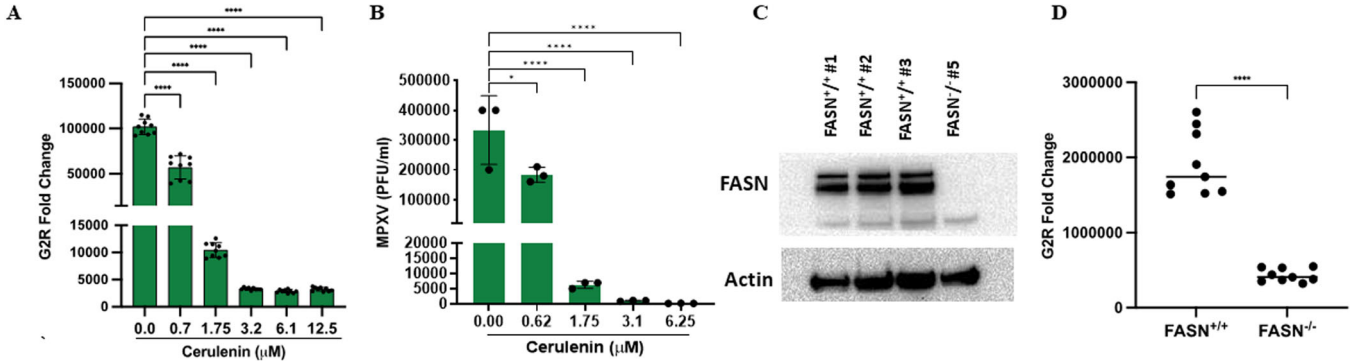

FIGURE 3 | Blocking the MPXV replication by cerulenin. Huh 7 cells were treated with indicated dose of cerulenin 1-h post-MPXV infection. The cell lysate was subjected to (A) RNA extraction to evaluate the level of viral RNA transcripts by RT-qPCR (B) MPXV titration in cells treated with different concentration of cerulenin by plaque assay. (C) FASN knockout cells were generated by using CRISR/CAS9 technology and the efficiency of the procedure was investigated by Western blot analysis as indicated. Actin was used as loading control. (D) Evaluation of MPXV G2R in $FASN^{+/+}$ and FASN Knockout ($FASN^{-/-}$) #5 at 24HPI by RT-qPCR.

To further elucidate the role of FASN in MPXV growth, we evaluated the effects of two additional FASN inhibitors, cerulenin and TVB-3166, on MPXV replication. Huh-7 cells were infected with MPXV and treated with cerulenin at concentrations ranging from 0.7 to 12.5 μM. RT-qPCR results revealed a significant reduction in MPXV replication with cerulenin (Figure 3A, and Figure S6A,B), yielding an IC_{50} value of 1.3 μM (Figure S4B). This finding was corroborated by plaque assays (Figure 3B and Figure S7II). Additionally, TVB-3166, another FASN inhibitor, demonstrated a significant efficacy in inhibiting MPXV replication (Figure S6C). To further validate the antiviral effects of FASN inhibition, we generated FASN knockout Huh cells (Figure 3C and Figure S5B). We evaluated the rate of MPXV G2R expression in both $FASN^{-/-}$ clone #5 (Figure 3C) and clone#9 but only the MPXV growth rate in clone #5 was shown in Figure 3D. This indicates that MPXV replication was markedly reduced in FASN knockout ($FASN^{-/-}$) cells compared to wild-type ($FASN^{+/+}$) cells (Figure 3D)

2.3 | CH25H Blocks MPXV Infection

As shown in Figure 1C, the level of Ch25H was significantly elevated throughout the course of MPXV infection. To further investigate the impact of CH25H on MPXV infection, Huh 7 cells were transfected with Ch25H or a vector control for 24 h and subsequently infected with MPXV. At 24HPI, the infected cells were harvested and the MPXV replication was evaluated by RT-qPCR. The results indicated that CH25H inhibited MPXV replication and significantly blocked viral growth compared to the control cells transfected with the vector. (Figure 4A). Ch25H catalyzes the conversion of cholesterol to 25HC [13]. To further investigate the antiviral function of CH25H, Huh 7 cells were infected with MPXV and subsequently treated with varying concentrations of 25HC. RT-qPCR analysis revealed a significant reduction in MPXV infection upon 25HC treatment (Figure 4B), with an IC_{50} value of 0.56 μM (Figure S4C). These

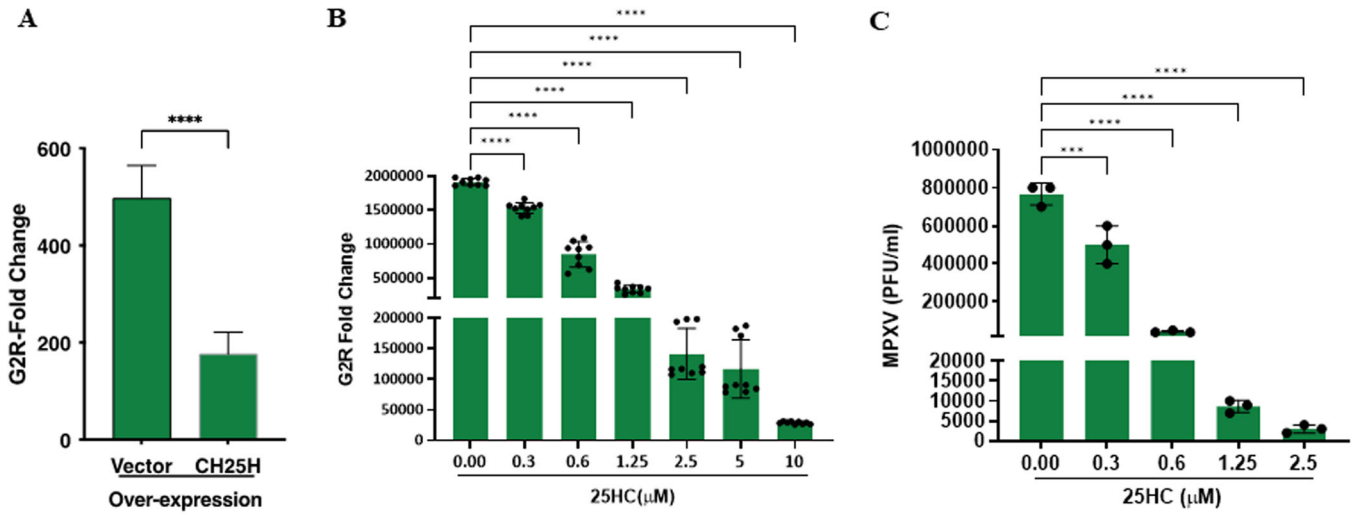

FIGURE 4 | Overexpression of CH25H, or treatment with 25HC, can inhibit MPXV infection. (A) Huh 7 cells were transfected with expression plasmids, vector only or the vector expressing CH25H for 24 h before MPXV infection (MOI = 0.5) and cells were harvested to assess the rate of viral replication at 24HPI. (B) The Huh 7 cells were infected with MPXV and treated with the indicated dose of 25HC 1 h-post infection. The cell lysate was subjected to RNA extraction to evaluate the level of viral RNA transcripts (C) MPXV titration in cells treated with different concentration of 25HC by plaque assay.

findings were corroborated by plaque assays (Figure 4C and Figure S7III)

2.4 | Identification of CC_{50}Values for FASN Inhibitors

To identify the potential cytotoxic effect of the compounds used to block infection with MPXV, the 50% cytotoxic concentrations (CC_{50}) of C75, cerulenin, and 25HC were evaluated by treating Huh 7 cells with increasing doses of C75 (Figure S8A), cerulenin (Figure S8B), or 25HC (Figure S8C) for 24 h.

We did not detect any apparent cytotoxicity at the concentrations of the compounds used to inhibit MPXV infection in the present research. Indeed, the effective doses for viral inhibition for the antiviral compounds used in this study were significantly lower than that of the CC_{50}.

3 | Discussion

MPXV causes a rare viral disease that is similar to smallpox but generally less severe. It is primarily a zoonotic infection which often transmitted through direct contact with infected animals or animal products. The recurrent outbreaks of mpox since 2022, after a long period without endemic cases, particularly during the global battle against SARS-CoV-2 raise significant concerns [5]. Managing multiple infectious disease outbreaks simultaneously can strain healthcare systems and resources.

MPXV is a double stranded DNA virus, therefore the mutation rate is much lower than RNA viruses, which helps in developing vaccines that may have long term effectiveness in controlling the viral threat [6].

Here, we have shown that MPXV infection promoted the expression of multiple pro-inflammatory genes including TNF-α and IL6. In addition, a few genes including Ch25H and IFIT1 were transcriptionally upregulated at early time point-post MPXV infection. It has been well established that viral-mediated-activation of IFN-I pathway triggers the JAK-STAT signaling which leads to the expression of over 300 interferon-stimulated genes (ISGs) that establish an antiviral state and inhibit viral infection [21, 27]. The cGAS-STING pathway plays a crucial role in sensing cytosolic DNA from poxviruses, facilitating the production of cGAMP and triggering type I interferon responses [45, 46] In addition, mice lacking cGAS or STING showed reduced type-I IFN levels, higher viral loads, and increased susceptibility to mousepox [47]. However, virulent poxviruses, including vaccinia virus, can inhibit STING dimerization and phosphorylation, effectively blocking downstream signaling. Additionally, other poxvirus proteins, such as K7, C11, B18, and B19, may also contribute to the inhibition of the cGAS-STING pathway [46]. MPXV demonstrates greater sensitivity to exogenous interferon (IFN) treatment than related viruses like VARV and vaccinia (VACV) and pre-treating cells with human IFN-β significantly inhibits MPXV replication [24]. However, MPXV has evolved several immunomodulatory proteins that effectively counteract IFN-I responses. Among these, the F3 protein, a homolog of the E3 protein found in VACV [26]. inhibits the activation of PRRs and the phosphorylation of eIF2α [25]. These studies may suggest that while MPXV has developed mechanisms to suppress IFN-I responses, there may still be limited IFN induction during infection. The balance between viral evasion strategies and host IFN responses is likely crucial in determining disease outcomes of MPXV infection. For instance, Sendai virus (SeV) blocks IFN-α signaling but can simultaneously trigger the phosphorylation of TBK1 and IRF3, leading to the induction of certain ISGs [48]. Similarly, HCV attenuates the IFN response by activating NS3/NS4A protease, which disrupts RIG-I and toll-like receptor signaling [49]. Thus, the interplay between viral antagonism and host responses results in the activation of a subset of ISGs that help limit viral growth.

Additionally, the IFN-I pathway suppresses pro-viral genes, including those related to fatty acid metabolism [21, 27]. In this study, we demonstrated that while Ch25H is induced, FASN is

suppressed upon MPXV infection in Huh 7 cells, and this effect is time dependent. We used a low MOI to better mimic the natural cell-to-cell spread of the virus. It has been shown that MPXV spread is attenuated in vitro in the presence of IFN-β under low MOI conditions [24]. It is possible that virus MOI may influence replication kinetics and the induction of interferon [50].

We examined the effects of various compounds on MPXV infection using Hela and Huh7 cells. While HeLa cells are widely used in poxvirus research [24, 51]. Our results indicated that Huh7 cells were more susceptible to DNA transfection and better suited for studying virus-host interactions.

We have shown that 25HC and biological inhibitors of FASN significantly suppress MPXV infection. Our lab, along with others, has demonstrated that to establish an infection, viruses manipulate the metabolic network of the host cell, hijacking the lipid and glucose metabolic pathways and the nucleic acid biosynthetic pathways [21, 27, 52]. To complete their life cycle, some viruses even alter the cellular metabolism of host cells. For instance, CH25H is a major gene in the regulation of cholesterol through its hydroxylation to 25HC. Many studies have shown that CH25H is upregulated during viral infection and functions as an ISG to inhibit viral replication. In addition, 25HC which is a natural oxysterol, inhibits infection by a broad range of viruses including both RNA and DNA viruses [53, 54]. Fatty acids are also an integral component of virtually all cells. They are involved in structuring the cell membrane, forming certain membranous structures for facilitating viral replication, signaling, and energy storage. FASN specifically catalyzes the production of palmitate, a 16-carbon fatty acid involved in the modulation of the phospholipid bilayer. FASN inhibition results in a drastic shift in the structure of the cell membrane [36]. While the expression of FASN is under the control of sterol regulatory element-1 (SREBP-1), the expression of CH25H is under the control of SRBP-2 [55]. Our laboratory and others have shown the role of Ch25H as a critical ISG which is upregulated during infection by a broad range of viruses, including HCV, HSV, MHV, West Nile Virus, VSV, HIV, Ebola, Rift Valley Fever Virus, Yellow Fever Virus, Russian spring summer encephalitis virus, and SARS-CoV-2 [21, 27, 56].

Similarly, the role of FASN in supporting the replication of a wide range of viruses including HCMV [33], KSHV [57], HIV [31], HCV [31], and SARS-CoV-2 [37, 38], has been reported. Moreover, the primary determinant of the HCV assembly is based on the core protein's droplet-binding domain [58]. It has also been shown that both KSHV and West Nile virus induce lipogenesis [57]. Herein, we have shown that both Ch25H and FASN play a pivotal role in MPXV infection. The altered expression of either gene may lead to immense changes in lipid metabolic pathways. While Ch25H blocks the MPXV infection rate, FASN promotes it.

Currently, the majority of antiviral drugs target specific viral properties or proteins, such as the HIV reverse transcriptase or influenza neuraminidase, and are the result of years of research and development. For instance, the anti-SARS-CoV-2 drug nirmatrelvir [PF-07321332] approved by the FDA for emergency use against COVID-19 [59, 60], unsurprisingly has no inhibitory effect on MPXV (Figure S9). However, in the past two decades the world has faced a number of dangerous pathogenic viruses such as ZIKV, MERS, SARS, Ebola, SARS-CoV-2 and MPXV, and we are likely encounter more in the years ahead. We may not be able to afford to wait years to produce tailored antiviral agents against these threats, therefore broadly acting antiviral agents should also be considered for further development. Although viruses have evolved to hijack a cell's metabolic pathways, our bodies have also evolved mechanisms to counteract them by upregulating and downregulating different ISGs [20, 33, 34] In this manuscript we have provided evidence that 25HC, already used as part of the natural innate immune cell defense, and chemical inhibitors of FASN, can be used as broad-spectrum antiviral agents against numerous types of viruses, including SARS-CoV-2 and MPXV. Beyond inhibiting viral pathogens, downregulation of FASN may be a key step to providing therapeutic relief to a number of distinct diseases. In the case of diabetes, previous studies have shown that knocking out FASN in mice resulted in a drastic reduction in diet-induced insulin resistance and chronic inflammation [61]. Ultimately, increasing our understanding of the relationship between the innate immune system and other host metabolism pathways may be a promising area of research with applications in developing broad spectrum antiviral agents and in combating many metabolic diseases. In August 2024, the WHO declared the spread of MPXV across multiple African countries a public health emergency of international concern for the second time in 2 years [5]. This decision highlights the urgent need for the development of broad-spectrum antiviral agents to combat emerging viruses.

3.1 | Materials and Methods

3.1.1 | Viruses

NR-2500 MPXV strain US 2003 - isolated during the 2003 US outbreak and is not a currently circulating strain- was obtained from BEI resources via a UCLA MTA.

3.1.2 | Reagents

Lipofectamine™ 2000 (Invitrogen) was used for DNA transfection at a 1:2 DNA to the reagent ratio. C75 and cerulenin were from Cayman and 25HC was from Sigma. All the experiments were repeated at least three times with biological triplicates within each experiment.

3.1.3 | Cell Lines

Hela, Huh 7, and Vero cells were purchased from ATCC and were grown in the standard DMEM (ThermoFisher) with 10% FBS (HyClone), and 1% Penicillin/Streptomycin (GIBCO) at 37°C in a 5% CO_2 humidified atmosphere.

Propagation and titration of MPXV: NR-2500 MPXV strain US 2003 was obtained from BEI resources, propagated in Vero or Hela cells. Briefly, A T75 flask of Vero or Hela cells at 80%

TABLE 1 | Primers used in this study.

M1L-F	MPXV	5′–AAC GGA CCA CAT CCT TCT TC–3′	PMID: 27467578
M1L-R	MPXV	5′–ATC CAA ACG CGT GTG ATA AA–3′	
G8R-F	MPXV	5′–GCG GAT CTG TAA ACA TTT GG–3′	PMID: 27467578
G8R-R	MPXV	5′–CCT TGG ACA CTG GAA GGT TAA A–3′	
A5L-F	MPXV	5′–CTT CCA TCC GAT TGT TGT GT–3′	PMID: 27467578
A5L-R	MPXV	5′–AGT ACA CTC CTT CCA GCG TT–3′	
G2R-F	MPXV	5′-GGAAAGTGTAAAGACAACGAATACAG-3′	
G2R-R	MPXV	5′-GCTATCACATAATCTGAAAGCGTA-3′	
IL6-F	Human	5′-GGG GCT GCT CCT GGT GTT G-3′	
IL6-R	Human	5′-CTG AGA TGC CGT CGA GGA TGT A-3′	
TNFa-F	Human	5′-CAC TAA GAA TTC AAA CTG GGG C-3′	
TNFa-F	Human	5′-GAG GAA GGC CTA AGG TCC AC -3′	
CXCL10-F	Human	5′-GTG GCA TTC AAG GAG TAC CTC-3′	
CXCL10-R	Human	5′-TGA TGG CCT TCG ATT CTG GAT T3′-	
IFITM 1-F	Human	5′-AGC ATT CGC CTA CTC CGT GAA G-3′	
IFITM 1-R	Human	5′-CAC AGA GCC GAA TAC CAG TAA CAG-3′	
ISG15-F	Human	5′-CAC AGC CCA CAG CCC ACA G-3′	
ISG15-R	Human	5′-GCT CAG GGA CAC CTG GAA TTC G-3′	
IRF1-F	Human	5′-ACC CTG GCT AGA GAT GCA GA-3′	
IRF1-R	Human	5′-TGC TTT GTA TCG GCC TGT GT-3′	
DDX58-F	Human	5′-CTG GAC CCT ACC TAC ATC CTG-3′	
DDX58-R	Human	5′-GGC ATC CAA AAA GCC ACG G-3′	
IRF1-F	Human	5′-CCT CCC TGG AAA ATC TAG GCT CT-3′	
IRF1-R	Human	5′-GTA AAG TGA CAT CTC AAT TGC TCC AGA-3′	
CH25H-F	Human	5’-CGA CGG AAC GTG TCG GAG CG-3’	
CH25H-R	Human	5’-GTG TGA CGT TCA TCA TGT CG-3’	
FASN-F	Human	5’-ACT CCA CAG GTG GGA ACA AG-3’	
FASN-R	Human	5’-CCC TTG ATG AAG AGG GAT CA-3’	
L32-Forward	Human	5′-CGT CCC TTC TCT CTT CCT CGG-3′	
L32-Reverse	Human	5′-ACA TAT CGG TCT GAC TGG TGC-3′	

confluency was infected with MPXV (MOI = 0.1) and harvested at 60 HPI. After three freeze-thaw cycles, the viral samples were centrifuged to remove cell debris. The stock was titrated on Vero cells. Briefly, the media was removed from the confluent monolayer of Vero cells followed by infecting cells with the serially diluted virus and incubated for 1 h at 37°C in a 5% CO_2 humidified atmosphere. Cells were then washed with 1X PBS and covered with agarose-overlay for 60 h. Subsequently, the agarose-overlay was removed, cells were washed with 1X PBS, fixed with 4% paraformaldehyde (PFA) for 1 h. Viral plaques were visualized by staining with a solution of 0.5% crystal violet (m/v) in 4% PFA (v/v) for 30 min. The yield of MPXV was much higher in Vero cells compared to that of the Hela cells. To investigate the effect of the compounds on the MPXV growth rate by plaque assay, the infected cells were washed with 1× PBS and incubated with TE buffer to facilitate detachment. Subsequently, the cells were gently scraped using a cell scraper. The detached cells were then collected in an Eppendorf tube and subjected to two cycles of freeze-thawing to prepare them for the plaque assay.

3.2 | All the MPXV Based Experiments Were Performed at the UCLA BSL3 Facility

3.2.1 | Generation of *FASN*$^{-/-}$ Huh 7 Cells Using CRISPR/Cas9 Technology

The *FASN* knockout Huh 7 cell line was created using the Invitrogen TrueCut Cas9 Protein v2. TrueGuide.

3.2.2 | Western Blotting Analysis

Cells were harvested in ice-cold lysis buffer (50 mM Tris-Cl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40) supplemented

with complete protease inhibitors (Roche). Total protein was immunoblotted with the indicated FASN, Actin, or GAPDH antibodies (cell signaling) to measure the level of the target proteins.

3.2.3 | RT-qPCR

Cells were collected in TRIzol reagent (Invitrogen, Cat# 1559608), and RNA was isolated using standard isopropanol precipitation according to the Invitrogen protocol. RNA was quantified and 1 μg of RNA was reverse-transcribed using iScript (BioRad) according to the manufacturer's instructions with random hexamer as primers to generate complementary DNA (cDNA). qPCR analysis was conducted on cDNA using the iCycler thermocycler (Bio-Rad) in a final volume of 20 μL using gene-specific primers. Amplification conditions were 95°C (3 min), 40 cycles of 95°C (20 s), 55°C (30 s), 72°C (20 s). Expression values were normalized to L32 (RPL32), a suitable housekeeping gene for gene expression analysis and fold induction was subsequently normalized to the untreated control samples.

3.2.4 | Viral RNA Detection

For detection of viral RNA, cells were infected with MPXV, collected in Trizol and RNA was isolated and reverse-transcribed using MPXV-specific primers listed in Table 1.

3.2.5 | Identification of CC_{50} and IC_{50} Values

A day before performing the cytotoxicity assay, 8×10^3 Huh 7 cells or Hela cells were seeded in a 96 Well White/Clear Bottom Plate, TC Surface (Thermo Fisher). Cells were treated with twofold serial dilutions (100–1.5 μM) of C75, (160–2.5 μM), of cerulenin, and (50–0.05 μM) of 25HC for 20 h. The cell viability was determined using the Cell Titer-Glo Luminescent cell viability assay (Promega). IC_{50} and CC_{50} values were calculated by nonlinear regression analysis using GraphPad 10.

3.3 | Statistical analysis

The data were analyzed by unpaired student *t*-test using Prism software (GraphPad). All the data are shown as mean ± SEM from three independent experiments. $^*p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$.

Author Contributions

Saba R. Aliyari was involved in the design and execution of the project in the BSL3 and in writing the manuscript, Jean Shanaa and Daniel Yu were involved in performing the experiments and writing the first draft of the manuscript. Lulan Wang, Kaitlyn N. Bateman, and Mari Yamamoto assisted with performing the experiments. Genhong Cheng and Michael E. Jung are involved in overall project design and manuscript preparation.

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Data Availability Statement

The data that support the findings of this study are available in Pubmed at https://pubmed.ncbi.nlm.nih.gov. These data were derived from the following resources available in the public domain: - Pubmed, https://pubmed.ncbi.nlm.nih.gov

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.