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AFRICAN SWINE FEVER VIRUS A151R SUPPRESSES CGAS-STING-MEDIATED TYPE I INTERFERON RESPONSE VIA FERROPTOSIS-DRIVEN LIPID PEROXIDATION AND FERRITINOPHAGY

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ABSTRACT

African Swine Fever (ASF) is a truly devastating disease for pigs worldwide, causing high mortality and leaving us without effective treatments. A big part of how the African Swine Fever Virus (ASFV) wreaks havoc is by cleverly sidestepping the host's natural defenses, especially a crucial pathway called cGAS-STING, which is vital for producing antiviral signals (Type I interferons). Our study dives into a fascinating new way ASFV does this, focusing on a viral protein called A151R. We found that ASFV A151R actively shuts down the cGAS-STING pathway's ability to make IFN-β by triggering something called ferroptosis – a unique type of cell death driven by iron and fat damage.

Here's the nitty-gritty: A151R essentially kickstarts a process called ferritinophagy, which is like the cell's recycling system for iron-storing proteins. This releases a flood of iron inside the cell, leading to a lot of fat damage (lipid peroxidation). This damage then directly messes with STING, preventing it from getting activated and doing its job, ultimately silencing the antiviral response. Our lab experiments showed that if we created a version of ASFV without the A151R gene (ASFVΔA151R), the cells could partially restore their IFN-β production and cGAS-STING activation. Conversely, just adding A151R to cells was enough to suppress STING. What's more, when we used drugs to block ferroptosis or ferritinophagy, we could actually help the cells recover their IFN-β production. We even saw these protective effects in live pigs: ASFVΔA151R caused less severe disease, with higher IFN-β levels and less ferroptosis in their tissues. These findings reveal a critical, previously unknown role for ASFV A151R in outsmarting the immune system by inducing ferroptosis. This gives us fresh insights into how ASFV causes disease and points us toward exciting new targets for developing antiviral treatments.

Keywords: African Swine Fever Virus, ASFV A151R, cGAS-STING pathway, IFN-β, Ferroptosis, Lipid Peroxidation, Ferritinophagy, Innate Immunity, Viral Evasion.

INTRODUCTION

Imagine a highly contagious and often deadly disease that sweeps through pig populations, leaving behind a trail of devastation. That's African Swine Fever (ASF), caused by the formidable African Swine Fever Virus (ASFV) [2, 44]. This large, double-stranded DNA virus is a nightmare for the global swine industry, and what makes it even scarier is that we currently don't have any approved vaccines or effective treatments [2, 3, 4]. A big part of ASFV's success lies in its uncanny ability to slip past the host's innate immune system—our body's first line of defense—which is absolutely crucial for the virus to replicate and cause disease [3, 5, 62]. If we want to get a handle on this devastating illness, truly understanding how ASFV evades our immune system is the first step. The economic fallout from ASF outbreaks is simply catastrophic, leading to massive losses of livestock, crippling trade restrictions, and huge financial burdens for farmers and affected countries alike. The urgent need for a commercial vaccine or effective antiviral drugs is clear, driving us to dig deep into ASFV-host interactions and search for innovative ways to fight back.

Our innate immune system is like a vigilant guard, constantly on the lookout for invaders. It uses special sensors, called pattern recognition receptors (PRRs), to spot unique molecular signatures of pathogens (PAMPs) [6, 7]. When it comes to DNA viruses, one of the most important sensing pathways inside our cells is the cyclic GMP-AMP synthase (cGAS) and stimulator of interferon genes (STING) pathway [8, 9, 10, 13]. Think of it like this: cGAS is the initial detector. When it spots foreign double-stranded DNA (dsDNA) from a virus or even misplaced host DNA, it undergoes a shape change and produces a special messenger molecule called cGAMP. This cGAMP then latches onto and activates STING, which usually hangs out in the endoplasmic reticulum (ER) membrane [9, 10, 13]. Once activated, STING starts to clump together

and moves from the ER to the Golgi apparatus [10, 14]. In the Golgi, activated STING acts as a beacon, recruiting and activating another key player, TANK-binding kinase 1 (TBK1). TBK1 then phosphorylates (adds a phosphate group to) interferon regulatory factor 3 (IRF3), which then pairs up and moves into the nucleus. Once in the nucleus, IRF3 kicks off the production of vital antiviral signals, specifically type I interferons (IFN-I) like IFN-β, and other interferon-stimulated genes (ISGs) [10, 11, 13, 41, 47]. These IFN-I cytokines are absolutely essential for creating an antiviral state in both infected cells and their neighbors, effectively slowing down viral spread and replication. Because this pathway is so central to fighting viruses, many DNA viruses, including ASFV, have developed incredibly clever ways to counteract or shut down the cGAS-STING pathway at various points in its signaling cascade [38, 62]. ASFV, for example, produces numerous proteins that interfere with cGAS-STING components or their downstream effectors. Just to give you a few examples, MGF360-12L blocks interferon production by messing with how importin α interacts with the NF-κB pathway [1], CD2v plays a role in regulating the JAK2-STAT3 pathway and stopping cell death [34], pS273R prevents a type of inflammatory cell death called pyroptosis [35], MGF110-9L and MGF505-7R stop TBK1 degradation [36], p17 halts cell proliferation by causing ER stress and ROS [37], L83L negatively controls the cGAS-STING-mediated IFN-I pathway by recruiting tollip to promote STING breakdown [49], A137R stops type I interferon production by breaking down TBK1 through autophagy [50], pS273R blocks DNA sensing in the cGAS-STING pathway by targeting IKKε [51], C962R [52], MGF360-14L negatively regulates type I interferon signaling by targeting IRF3 [53], pI215L negatively regulates cGAS-STING signaling by recruiting RNF138 to inhibit K63-Linked ubiquitination of TBK1 [54], E301R [55], QP383R reduces type I interferon production by promoting cGAS palmitoylation [56], EP364R and C129R directly target cGAMP to inhibit the cGAS-STING pathway [57], pD345L negatively regulates NF-κB signaling by inhibiting IKK kinase activity [58], MGF505-7R interacts with interferon regulatory factor 9 to escape the type I interferon signaling pathway and boost viral replication [59], MGF360-12L inhibits host type I IFN, NF-κB, and JAK/STAT pathways [60], and MGF360-11L negatively regulates cGAS-STING-mediated Inhibition of type I interferon production [61].

More and more, we're seeing how closely connected our cell's metabolism, its internal balance of oxidants and antioxidants (redox homeostasis), and our innate immunity truly are [7, 14]. Keeping this redox balance is absolutely essential for cells to function properly and for our immune system to respond effectively. When this balance goes awry, leading to an excess of reactive oxygen species (ROS), we get what's called oxidative stress. This can damage cells and throw signaling pathways into disarray [45]. Now, enter ferroptosis, a distinct and fascinating form of regulated cell death (RCD) that's been getting a lot of attention in the world of viral infections and innate immunity [16, 18, 19, 42, 46, 74]. Unlike other forms of cell death, ferroptosis is characterized by iron-dependent accumulation of damaged fats (lipid hydroperoxides), especially those from polyunsaturated fatty acids (PUFAs) in cell membranes [42, 46]. Key players in controlling ferroptosis include glutathione peroxidase 4 (GPX4), an enzyme that repairs damaged fats, and the cystine/glutamate antiporter system Xc−, which helps bring cystine into the cell to make glutathione (GSH) [16, 20, 27, 66, 67]. GSH is a vital ingredient for GPX4 to work, so if GSH levels drop or GPX4 is inhibited, cells become much more vulnerable to ferroptosis [16, 20, 66]. On top of that, there's ferritinophagy, a specialized autophagy pathway (the cell's self-eating process) that's managed by nuclear receptor coactivator 4 (NCOA4) [22, 23, 24, 43]. NCOA4 targets ferritin, the main protein that stores iron, for breakdown in lysosomes. This releases a pool of "labile" iron that can then act as a catalyst, producing highly reactive hydroxyl radicals through the Fenton reaction, which in turn accelerates lipid peroxidation and fuels ferroptosis [22, 23, 24, 43, 46]. Recent research has shown that various viruses can either trigger or block ferroptosis to help them replicate or escape the immune system [25, 26, 28, 29, 30, 31, 32, 69, 70, 71, 72, 74, 75]. For instance, hepatitis B virus (HBV) protein X (HBx) stimulates liver cell ferroptosis by negatively regulating SLC7A11, leading to acute liver damage and boosting HBV proliferation [26]. Human papillomavirus (HPV) infection stops SLC7A11 and SLC3A2 from being expressed [27]. Japanese encephalitis virus (JEV) infection causes nerve cell ferroptosis by damaging the antioxidant system involving GSH/GPX4 [28]. Swine influenza virus (SIV) suppresses the system Xc−/GSH/GPX4 axis, reducing GSH and promoting lipid peroxidation, which then triggers ferroptosis and helps the virus replicate [29]. Bovine viral diarrhea virus (BVDV) and Newcastle disease virus (NDV) break down ferritin through NCOA4-mediated ferritinophagy, releasing iron, promoting ferroptosis, and enhancing viral replication [31, 32]. Even the V protein of human parainfluenza virus type 2 (HPIV-2) interacts with ferritin heavy chain 1 (FTH1), leading to its autophagic degradation via NCOA4-mediated ferritinophagy, which supports HPIV-2 growth [33]. These findings collectively paint a picture: viruses are master manipulators, controlling the Xc−/GPX4 axis or boosting ferritinophagy to speed up their replication and escape our immune defenses.

More recent studies have hinted that ASFV is involved in regulating different types of cell death, including apoptosis [34], pyroptosis [35], and autophagy [36]. ASFV infection or its proteins can even increase markers of oxidative stress, like reactive oxygen species (ROS), 8-oxo-7,8-dihydroguanine (8-oxoG), and oxoguanine DNA glycosylase 1 (OGG1) [37]. And here's an interesting twist: if you inhibit OGG1, it actually boosts IFN-β expression and

makes antiviral agents more effective against ASFV, suggesting that ASFV might be promoting lipid peroxidation to suppress interferon production [37, 79]. But despite these clues, the exact relationship between ASFV and ferroptosis, and the molecular tricks it uses to evade immunity, largely remain a mystery.

The ASFV A151R gene codes for a nonstructural protein that has been linked to affecting TBK1 and IκB kinase ε (IKKε) function, thereby limiting IFN-β production and helping ASFV escape [38]. Plus, a modified virus without A151R (ASFV-ΔA151R) actually showed protection against the virulent parent ASFV [39]. But how A151R specifically targets ferroptosis to control interferon production is still a big question. In this study, we're putting forward a hypothesis: A151R acts as a negative regulator of the cGAS-STING pathway by promoting STING carbonylation and stopping its movement from the ER to the Golgi. We believe A151R achieves this by suppressing the system Xc-/GSH/GPX4 axis, which then leads to lipid peroxidation and ferroptosis. We also want to find out if activating GPX4 can counteract the protein carbonylation caused by A151R and boost the cGAS-STING pathway, ultimately leading to more IFN-β and ISG production. Our goal is to uncover new insights into how ASFV causes disease and lay the groundwork for understanding its fight against the innate immune response, potentially pointing us toward new therapeutic targets.

MATERIALS AND METHODS

To truly understand the intricate dance between ASFV A151R, ferroptosis, and the cGAS-STING immune response, we designed a comprehensive set of experiments. These approaches range from detailed molecular and cellular tests in the lab to studies in live animals, all to ensure our investigation is as thorough and robust as possible.

Reagents and Antibodies

First things first, we made sure to get all our essential ingredients and tools from reliable sources to keep our experiments consistent and trustworthy. For molecular cloning, we used Phanta™ Super-Fidelity DNA Polymerase and Clone Express II recombinase from Vazyme Biotech (Nanjing, China). For our Western blot and immunoprecipitation experiments, we gathered a full panel of antibodies from Proteintech (Wuhan, China). These included antibodies against Flag, HA, STING, its phosphorylated form (p-STING), TBK1, its phosphorylated form (p-TBK1), IRF3, its phosphorylated form (p-IRF3), NCOA4, GPX4, FTH (which is part of ferritin, an iron-storing protein), LC3 (a marker for autophagy), p62 (another autophagy marker), and GAPDH (a common loading control). We also got HRP goat anti-rabbit IgG (H+L) and HRP goat anti-mouse IgG (H+L) secondary antibodies from Proteintech. For visualizing things under the microscope with immunofluorescence, we used mouse IgG (H+L), green and red fluorescent secondary antibodies, and HRP-conjugated streptavidin, all from Aibotech Biotech (Wuhan, China). Our restriction enzymes came from Thermo Fisher Scientific, and our plasmid extraction kit from Cowin Biotech (Taizhou, China). Using these specific, high-quality reagents is key to making sure our results are reproducible and valid.

Cell Culture and Plasmid Transfection

For our lab-based experiments, we carefully selected and maintained specific cell lines under strict conditions. We started by taking the genetic code for ASFV A151R (its open reading frame, or ORF) from the ASFV genome. We then used Phanta™ Super-Fidelity DNA Polymerase to make copies of it and inserted these copies into a pCDNA3.1 vector. To help us detect and locate A151R later, we added Flag, HA, and EGFP tags to it. We also used pCAGGS-HA and pCAGGS-Flag plasmids, which are standard tools for expressing proteins in mammalian cells.

We grew human embryonic kidney (HEK) 293T cells, a popular choice for temporary gene expression studies because they're very good at taking up new DNA. These were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS) from Gibco, USA. For cells more relevant to pig studies, we used porcine kidney (PK-15) cells (which we referred to as 3D4/21 cells, likely a specific version) and primary porcine alveolar macrophage (PAM) cells. These were grown in RPMI 1640 medium with 10% FBS. All our cell cultures lived in a humidified incubator with 5% CO$_{2}$ at 37°C. When the cells were about 70-80% full in their plates (like 6-well plates or confocal dishes), we introduced the plasmids using Lipofectamine 3000 (Invitrogen, USA), following the manufacturer's instructions. We typically collected our samples 24 hours after this "transfection" step, giving the cells enough time to express the new proteins and for any pathway changes to begin.

Virus Infection

To get our ASFV for experiments, we grew the ASFV SY-1 strain, which is a known virulent type, in PAM cells. This helped us create high-titer viral stocks, as described in previous research [40]. We then measured the viral strength by calculating the 50% tissue culture infectious dose (TCID$_{50}$), which ensures we're using a consistent and measurable amount of virus for each challenge. For our lab infection experiments, we first washed the PAM cells with phosphate-buffered saline (PBS) to get rid of any leftover growth medium. Then, we exposed them to ASFV at a multiplicity of infection (MOI) of 1.0. This is a standard MOI that allows for efficient infection without killing too many cells too quickly. We collected the infected cells 24 hours later for various analyses. It's crucial to mention that all our work with live ASFV was done under very strict biosafety level 3 (BSL3) conditions, following rigorous safety rules to prevent any accidental spread of the virus. These procedures were all

approved by the Institutional Biosafety Committee of Huazhong Agricultural University.

For our studies in live animals, we chose seven-week-old Landrace pigs, a common breed in swine research. We gave the pigs ASFV SY-1 orally, at a dose of 2 × 10^{2} HAD$_{50}$ (hemadsorption dose 50%), or an equal amount of a control solution for the uninfected group. Giving the virus orally mimics one of the natural ways ASFV spreads, making our model more realistic. Experienced personnel (Associate Professor Jinxia Dai from Huazhong Agricultural University) handled all the infection procedures. We closely watched the pigs daily for any signs of illness, like fever, not eating, or being sluggish. We humanely euthanized the animals 23 days after infection, which is usually when severe disease signs appear with virulent ASFV. We then collected tissue samples, specifically from the lungs and spleens, as these are key organs where ASFV replicates and causes damage. The tissues were immediately preserved in 4% paraformaldehyde solution for later histological and immunohistochemical analyses. All animal work strictly followed the guidelines for laboratory animal care and use, approved by the Research Ethics Committee of Huazhong Agricultural University (license no. HZAUSW-2024-0042).

Hematoxylin and Eosin (H&E) Staining

To get a clear picture of the tissue damage caused by the infection, we performed Hematoxylin and Eosin (H&E) staining. Our lung and spleen samples, which had been carefully preserved in 4% paraformaldehyde for at least a day, went through a standard tissue preparation process. This involved dehydrating them through a series of alcohol solutions and then embedding them in paraffin wax. Once embedded, we sliced the tissues into very thin sections, just 4 micrometers thick, using a special instrument called a microtome. We then removed the paraffin with xylene, rehydrated the sections, and stained them. Hematoxylin stains cell nuclei a purplish-blue, while eosin stains the cytoplasm and extracellular material pink. After staining, we mounted the sections with coverslips. We then examined all the sections under a high-power light microscope (BX51, Olympus, Tokyo, Japan) to look closely at the cell shapes, how many inflammatory cells were present, any signs of bleeding, and other pathological changes that would tell us about the ASFV infection. This histological analysis gave us a broad view of the tissue damage and how the cells were responding.

Immunohistochemical Staining

Immunohistochemistry (IHC) was our go-to method for pinpointing the location and amount of specific proteins, like FTH and GPX4, within the tissue sections. Here's how we did it: First, we prepared the paraffin-embedded sections by removing the wax and rehydrating them. Then, we performed "antigen retrieval" by heating the sections in a sodium citrate buffer solution using a microwave. This step is crucial because it helps to unmask any hidden protein sites that might have been altered during the tissue preservation process. Next, we quenched any natural peroxidase activity in the tissue with 3% H${2}O{2}$ to prevent it from interfering with our detection system. To prevent our antibodies from sticking to just any random protein, we blocked non-specific binding sites by incubating the slides with 5% goat serum protein (GSA) for 30 minutes at 37°C.

Then came the primary antibodies: we applied rabbit anti-FTH (at a 1:100 dilution) from ABclonal, China, and rabbit anti-GPX4 (at a 1:100 dilution) from Biosciences, USA, to the tissue sections. These were left to incubate overnight at 4°C. After thoroughly washing away any unbound primary antibodies, we added HRP-conjugated secondary antibodies (from ABclonal, China) for 30 minutes at 37°C. The signal was then developed using DAB (3,3'-diaminobenzidine) chromogen from Proteintech, China. This chemical creates a brown precipitate exactly where our target proteins are located. To help us see the cell nuclei, we counterstained the sections with hematoxylin and then mounted them with coverslips. Finally, we examined all the sections under a light microscope (BX51, Olympus). To get a semi-quantitative measure of protein levels in the tissues, we quantified the positive brown signals (indicating protein expression) by measuring their integrated optical density (IOD) using Image-Pro Plus 6.0 software (Media Cybernetics, USA).

Luciferase Reporter Assays

To precisely measure how active the IFN-β promoter was—which tells us how much the cGAS-STING pathway is being activated—we used luciferase reporter assays. We started by transiently co-transfecting 293T cells with two main plasmids: one that had the firefly luciferase gene driven by the IFN-β promoter (IFN-β-Fluc), and another, a Renilla luciferase control plasmid (pTK-RL), which we used to normalize for any differences in how well the cells took up the plasmids. We also added plasmids encoding cGAS, STING, or A151R (or various combinations of them) to see how they affected the IFN-β promoter's activity. After 24 hours, we collected the cell samples and measured the luciferase activities using a dual-specific luciferase assay kit from Beyo-time (Shanghai, China). We then normalized the firefly luciferase activity to the Renilla luciferase activity, as per the manufacturer's instructions. If the normalized firefly luciferase activity went down, it meant that the IFN-β promoter's activation was being suppressed.

RNA Extraction and Quantitative Real-Time PCR (qPCR)

To figure out how much of our target genes were being expressed at the mRNA level, we first extracted total RNA from our cultured cells. Typically, we grew these cells in 6-well plates, either transfecting them with plasmids or infecting them with viruses for 24 hours. We then lysed the cells using TRIzol reagent (Sigma), following the manufacturer's instructions. This method is great for

getting high-quality total RNA. After extracting the RNA, we converted it into cDNA using a reverse transcription kit. Then, we performed quantitative real-time PCR (qPCR) using SYBR Green Master Mix from Servicebio (Wuhan, China) on a real-time PCR system. To calculate the relative expression of our target genes, we used the 2$^{−ΔΔCT}$ method, which is a standard way to compare gene expression. We carefully designed gene-specific primers for IFN-β, ISG15, ISG54, GPX4, B646L (which is an ASFV structural protein gene and helps us gauge viral load), A151R, cGAS, and STING. We also included GAPDH as an internal control to normalize our results, helping us account for any variations in the amount of RNA we started with or how efficiently it was converted to cDNA.

Table 1: Sequence of primers used for qPCR

Genes	Sequence (5'-3')
IFN-β	Forward: TGCAACCACCACAATTCCAGAAGG Reverse: TGACGGTTTCATTCCAGCCAGTG
ISG15	Forward: CCCTTGAGGGACTGCATGAT Reverse: GACCCTTGTCGTTCCTCACC
ISG54	Forward: GCACAGCAATCATGAGTGAGAC Reverse: GCTTGCCGTAAGCATTCCAG
GPX4	Forward: CCCTCTGTGGAAGTGGATGAAAGTC Reverse: GTAGCACGGCAGGTCCTTCTCTA
B646L	Forward: TGCACGTTCGCTGCGTATCATT Reverse: CACTTGGTCGGCCAGGAGGTAT
A151R	Forward: AGCAAAGAAGATCTTAAAGGAGCA Reverse: AAAGGATGTGCATTTAGTACACCA
cGAS	Forward: AGACGGTGCTGGAGAAGGTGAG Reverse: GCAACGCCTTTGAACTCGGACT
STING	Forward: GCTGCTGCTGTCCTGCTACTTC Reverse: CCAGTCCATGAGCCACGTTGAA
GAPDH	Forward: ACCCAGAAGACTGTGGATGG Reverse: GTAGCACGGCAGGTCCTTCTCTA

Interaction of A151R and GPX4 via Molecular Docking and Molecular Dynamics Simulations

To see if ASFV A151R and the host's GPX4 protein might directly interact, we turned to computer-based methods. First, we used AlphaFold3, a cutting-edge artificial intelligence system, to predict the detailed three-dimensional structures of both A151R and GPX4. Then, we used molecular docking software like HDOCK and Rosetta Dock. These tools are like virtual puzzle solvers, predicting the most likely ways two molecules will fit together and how strongly they'll bind. After that, Ligplot software helped us zoom in on the specific types of bonds and interactions (like hydrophobic interactions, salt bridges, and van der Waals forces) happening where the proteins meet. PyMOL software then gave us beautiful visual representations of these docked protein complexes.

To really understand how stable and dynamic this predicted interaction was, we ran molecular dynamics (MD) simulations using Gromacs 2023.3. We set up the simulation to mimic real physiological conditions, using the Amber force field and a solvent box with salt. We put the system through equilibration steps to ensure a constant temperature (37°C) and pressure. Then, the main simulation ran for a full 50 nanoseconds, taking tiny steps of 2 femtoseconds at a time. We used Python and the MDAnalysis package to dig into the data, looking at things

like the root mean square deviation (RMSD) to check structural stability and analyzing hydrogen bonds to pinpoint key interaction points over time. We also calculated the binding free energy between the two proteins, which tells us how energetically favorable the interaction is.

Coimmunoprecipitation and Western Blot

To confirm if proteins were actually interacting and to measure how much protein was present and if it was phosphorylated (a key activation step), we used Coimmunoprecipitation (Co-IP) and Western blot analyses. We started by harvesting our cells and breaking them open with ice-cold RIPA lysis buffer, making sure to add protease and phosphatase inhibitors to keep our proteins intact and their phosphorylation status preserved. We then measured the protein concentration in these lysates using a BCA assay.

For Co-IP, we took a portion of the cell lysate and incubated it with a specific antibody (for example, anti-Flag for our tagged A151R or anti-GPX4) or a control antibody (IgG) for 4 hours at 4°C, gently rotating the mixture. We also added protein G agarose beads to capture the antibody-protein complexes. After incubation, we washed the beads three times with lysis buffer containing salt to remove any proteins that had stuck non-specifically. The immunoprecipitated proteins, along with some of the original cell lysates (our "input" controls), were then separated by size using SDS-PAGE (a gel electrophoresis technique) and transferred onto special PVDF membranes.

Next, we blocked the membranes with 5% skim milk in PBST (phosphate-buffered saline with Tween 20) for an hour to prevent our antibodies from binding to random spots. Then, we incubated the membranes with our primary antibodies (like those for p-STING, STING, p-TBK1, TBK1, p-IRF3, IRF3, protein carbonylation, GPX4, LC3, FTH, p62, NCOA4) at specific dilutions (usually 1:1000, except for GAPDH at 1:3000) for an hour at room temperature or overnight at 4°C. After washing, we added appropriate HRP-conjugated secondary antibodies (at a 1:5000 dilution) for an hour at room temperature. We then visualized the signals using enhanced chemiluminescence (ECL) on photographic film. Finally, we used Image J software (NIH, MD, USA) to quantify the protein expression levels, giving us a numerical measure of our results.

Immunofluorescence Staining

Immunofluorescence (IF) staining was our way of visually seeing where proteins were located inside the cells and observing cellular events like STING moving around. We seeded our cells into special confocal dishes, aiming for a density of 60-70% after we had transfected them with our plasmids. We then fixed the cells with 4% paraformaldehyde at 4°C for 30 minutes, made them permeable with 0.2% Triton X-100 in PBS, and blocked them with 5% BSA in PBS for an hour to reduce any unwanted antibody binding. Next, we incubated the cells with our primary antibodies (like anti-Flag for our tagged A151R and anti-HA for our tagged STING) for an hour. After washing, we added the fluorescently tagged secondary antibodies for another hour. To highlight the cell nuclei, we counterstained them with DAPI. Finally, we viewed our samples using a Zeiss LSM-800 laser scanning fluorescence microscope (Carl Zeiss AG, Oberkochen, Germany), which allowed us to capture high-resolution images and analyze if different proteins were located together.

Detection of Total Protein Carbonylation and STING Carbonylation

Protein carbonylation is a tell-tale sign of oxidative stress and protein damage, so we measured it for all cellular proteins and specifically for STING. For total protein carbonylation, we took cell lysates (which contained protease and phosphatase inhibitors to protect the proteins) and treated them with 10% biotin hydrazide (to a final concentration of 5 mM). We incubated this mixture at 37°C for 2 hours; this step essentially "labels" carbonylated proteins with biotin. Then, we prepared the samples with a special buffer, boiled them, and ran them on an SDS-PAGE gel. After transferring the proteins to PVDF membranes, we detected the total protein carbonylation by incubating the membranes with an HRP-streptavidin conjugated secondary antibody (at a 1:5000 dilution) in 5% BSA, and then visualized the signal using ECL.

For specifically detecting STING carbonylation, we first ran cell lysates on an SDS-PAGE gel and transferred them to PVDF membranes. Then, we incubated the membranes with an anti-STING antibody (at a 1:1000 dilution) in TBS with 5% BSA. After washing, we incubated the membranes with an HRP-streptavidin-conjugated secondary antibody for an hour. We made sure to wash thoroughly to remove any excess, unbound HRP-streptavidin. Finally, we used ECL on photographic film to detect the chemiluminescence. This method allowed us to specifically identify carbonylated STING protein, giving us direct evidence of this oxidative modification.

Detection of the Contents of Cellular MDA, GSH, and Iron

To quantify important indicators of fat damage (lipid peroxidation), antioxidant status, and iron levels, we used commercial assay kits. We grew our cells in 6-well plates, treated them with plasmids or viruses as needed, then rinsed them with cold PBS and broke them open. We used commercial kits from Elabscience (Wuhan, China) to measure malondialdehyde (MDA), glutathione (GSH), and iron, following their specific instructions. MDA, a key byproduct of lipid peroxidation, was measured using a spectrophotometer at 532 nm. GSH, a crucial antioxidant, was quantified at 412 nm. Intracellular iron content was determined at 593 nm. We calculated the amount of each substance based on standard curves provided by the

manufacturer. This gave us concrete numbers to understand the cell's redox state and how ferroptosis was progressing.

Detection of the Levels of Cellular ROS and Lipid ROS

To see how much general reactive oxygen species (ROS) and, more specifically, lipid ROS were building up in our cells, we used fluorescent probes. We employed the DCFH-DA (2',7'-dichlorodihydrofluorescein diacetate) fluorescent probe to detect total cellular ROS, and the C11 BODIPY 581/591 lipid peroxidation fluorescent probe for lipid-specific ROS. Both probes, sourced from Beyotime (Shanghai, China), were diluted to their working concentrations using serum-free medium, following the manufacturer's guidelines. We then incubated our cells with 1 ml of the probe solution at room temperature for 30 minutes in the dark. After incubation, we rinsed the cells three times with serum-free medium to wash away any unbound probe. Finally, we observed and photographed the fluorescent signals using a fluorescence microscope. This allowed us to visually and semi-quantitatively assess the levels of oxidative stress within the cells.

siRNA Transfection

To specifically reduce the expression of certain genes, we used small interfering RNAs (siRNAs). All the siRNAs we used, including the non-targeting negative control siRNAs, were custom-synthesized by Sangon Biotech (Shanghai, China) and are listed in Table 2. We introduced these siRNAs into our cells using Lipofectamine 3000 (Invitrogen) for 24 hours, at a final concentration of 10 nM, following the manufacturer's instructions. This technique allowed us to specifically "knock down" (reduce the expression of) genes like NCOA4 and A151R, which was crucial for understanding their exact roles in ferroptosis and immune modulation.

Table 2: Sequence of the siRNAs

siRNA	Sequence (5'-3')
siRNA-NCOA4	Sense: GGAUCUCAUCUAUCAGCUUTT Antisense: AAGCUGAUAGAUGAGAU CCTT
siRNA-Negative control	Sense: UUCUCCGAACGUGUCACGUTT Antisense: ACGUGACACGUUCGGAG AATT
siRNA-A151R	Sense: GUCGCCCAAUAUAUUCCAATT Antisense: UUGGAAUAUAUUGGGCG ACTT

Statistical Analysis

All the numerical data we collected from our independent experiments were put through a rigorous statistical analysis using GraphPad Prism 10 software. We consistently presented our results as the mean plus or minus the standard error of the mean (SEM), which gives you an idea of the data's variability and precision. To determine if there were statistically significant differences between our independent samples, we used a robust statistical test called one-way ANOVA (Analysis of Variance). If necessary, we also used post-hoc tests (like Tukey's HSD) to pinpoint specific differences between groups. We considered a result statistically significant if the P value was less than 0.05 ($P < 0.05$), with increasing levels of significance shown by more asterisks: $P < 0.05$ (), $P < 0.01$ (), $P < 0.001$ (), and $P < 0.0001$ (****). If there were no significant differences, we explicitly marked them as "ns". To ensure our findings were truly reproducible and statistically sound, we made sure to repeat all our experiments at least three times independently.

RESULTS

Our in-depth investigations into how ASFV A151R influences the host's innate immune system uncovered a really clever trick: the virus triggers ferroptosis, which then helps it shut down the cGAS-STING pathway. Let's walk through our findings step by step, starting with how A151R directly impacts IFN-β production and then diving into the molecular details.

A151R Functions as a Negative Regulatory Factor in the Production of IFN-β Triggered by the cGAS-STING Pathway

The cGAS-STING pathway is like a crucial alarm system for our cells, detecting foreign DNA and then kicking off the TBK1-IRF3 pathway. This whole process is absolutely essential for making IFN-β and getting the broader innate immune response going [41]. To understand exactly how ASFV A151R affects this vital pathway and IFN-β production, we started our experiments in 293T cells. We introduced plasmids containing HA-A151R, along with cGAS-FLAG, STING-FLAG, an IFN-β promoter-driven firefly luciferase reporter (IFN-β-Fluc), and a Renilla luciferase control (TK-Rluc). After 24 hours, we measured the IFN-β promoter activity using luciferase assays and checked the

mRNA levels of IFN-β, ISG15, and ISG54 using qPCR.

Our results were very clear: A151R significantly put a damper on IFN-β promoter activation (Fig. 1A, as shown in the original PDF's figure). The more A151R we added, the more pronounced this suppression became. At the same time, A151R consistently reduced the mRNA expression of IFN-β (Fig. 1B), ISG15 (Fig. 1C), and ISG54 (Fig. 1D). These genes would normally be strongly activated when cGAS and STING are co-expressed in 293T cells. This gave us strong initial evidence that A151R is a powerful negative regulator of the cGAS-STING-mediated IFN-I response.

To make sure these findings were relevant to pigs, we repeated the experiments in 3D4/21 cells (a porcine kidney cell line). We co-transfected these cells with cGAS and STING plasmids, along with different amounts of HA-A151R plasmids (0, 0.5, 1.0, and 1.5 μg). Western blot confirmed that cGAS, STING, and A151R proteins were all expressed as expected, with A151R showing a clear dose-dependent increase (Fig. 1E). Our qPCR analysis then showed that A151R effectively reversed the mRNA levels of IFN-β (Fig. 1F), ISG15 (Fig. 1G), and ISG54 (Fig. 1H) that were normally triggered by cGAS-STING, again in a clear dose-dependent fashion.

Most importantly, our Western blot analysis of key signaling molecules confirmed that A151R was indeed putting the brakes on the cGAS-STING pathway. When A151R was present, we saw a significant drop in the phosphorylation of STING (Fig. 1I, J), TBK1 (Fig. 1I, K), and IRF3 (Fig. 1I, L). Phosphorylation is like the "on" switch for these proteins, so a reduction means they're not getting activated properly. This directly tells us that A151R interferes with the core signaling events of the pathway. Putting all this together, our findings unequivocally show that A151R is a powerful negative regulator that effectively suppresses cGAS-STING signaling, blocking the downstream TBK1-IRF3 pathway and thereby shutting down IFN-β expression. This antagonistic role clearly positions A151R as a key viral weapon in ASFV's immune evasion strategy.

A151R Promotes Lipid Peroxidation, Facilitates STING Carbonylation, and Restricts the Translocation of STING from the ER to the Golgi

Viral infections often bring with them oxidative stress and damage to fats (lipid peroxidation) within our cells. This can be a sneaky way for viruses to suppress our antiviral responses. A major consequence of this oxidative stress is something called protein carbonylation—an irreversible damage to proteins that usually renders them inactive. In the world of the cGAS-STING pathway, STING carbonylation has been shown to stop STING from moving from the endoplasmic reticulum (ER) to the Golgi apparatus, a crucial step it needs to take to get activated [14, 17]. To see if A151R's ability to block IFN-β production was connected to lipid peroxidation and STING carbonylation, we carefully measured various markers of oxidative stress and STING modifications.

Our results clearly showed that A151R significantly increased the buildup of total reactive oxygen species (ROS) in cells, which we detected with the DCFH-DA probe (Fig. 2A, B). Even more specifically, A151R dramatically boosted the levels of lipid ROS, which we identified using the C11 BODIPY 581/591 probe (Fig. 2A, C). These findings tell us that A151R is actively creating a state of oxidative stress, particularly targeting the fats within the cell.

Digging deeper, we found that these high ROS levels led to significant protein damage. The levels of total protein carbonylation were substantially elevated in cells that had been co-transfected with A151R, cGAS, and STING plasmids (Fig. 2A, D). And, more importantly, when we specifically looked for STING carbonylation, we saw a clear increase in damaged STING protein when A151R was present (Fig. 2A, E). At the same time, and consistent with A151R's inhibitory role, the phosphorylation levels of STING were noticeably reduced (Fig. 2A, F). This strongly suggests a direct link between A151R-induced oxidative stress, STING damage, and impaired STING activation.

A critical step for cGAS-STING to get going is for STING to move from the ER to the Golgi. Our immunofluorescence assays gave us compelling visual proof that A151R actively blocked this vital movement. In cells expressing A151R, STING (which we stained red) showed much less overlap with Golgi markers (stained green), meaning it wasn't moving to the Golgi as it should (Fig. 2G). This blocked movement is a direct result of STING being inactivated, most likely due to its carbonylation. In contrast, simply adding cGAS or STING plasmids alone didn't significantly affect cellular ROS, lipid ROS, or total protein carbonylation, confirming that A151R is the main culprit behind these oxidative changes.

Putting all these findings together, we can see a crucial mechanism at play: ASFV A151R promotes the carbonylation of the STING protein by boosting lipid peroxidation in the cell. This oxidative damage, in turn, stops STING from making its essential journey from the ER to the Golgi, which then leads to the cGAS-STING pathway being shut down and, ultimately, the antiviral IFN-β response being suppressed. This is a sophisticated viral strategy to disarm a central part of the host's innate immunity through oxidative damage.

A151R Inhibits the System Xc−/GSH/GPX4 Axis, Promotes Lipid Peroxidation, and Induces Ferroptosis

The system Xc−/GSH/GPX4 axis is a truly vital antioxidant pathway that actively stops fat damage (lipid peroxidation) and is the core mechanism cells use to resist ferroptosis. This pathway is absolutely essential for keeping the cell's internal balance (redox homeostasis) in check, and by extension, for STING to function properly [14, 66]. To really dig into how A151R influences ferroptosis, we carefully examined its effects on this crucial antioxidant system.

Our initial experiments showed that A151R significantly reduced the expression of SLC7A11 (Fig. 3A, B), a key part of the system Xc− antiporter that brings cystine into the cell. It also suppressed the expression of GPX4 (Fig. 3A, C), the enzyme that's critical for detoxifying damaged fats. We saw this reduction happen in a dose-dependent way, meaning the more A151R we added, the more pronounced the effect. As you'd expect with these protective components being inhibited, the amount of glutathione (GSH)—a major antioxidant—inside the cells dropped noticeably (Fig. 3E). At the same time, the level of malondialdehyde (MDA), a reliable marker of lipid peroxidation, significantly increased in a dose-dependent manner (Fig. 3F). These findings collectively pointed to one thing: A151R was shutting down the key ferroptosis resistance pathway, the system Xc−/GSH/GPX4 axis, thereby triggering fat damage and creating an environment ripe for ferroptosis.

To further explore how A151R interacts with the cGAS-STING pathway in the context of lipid peroxidation, we co-transfected cells with cGAS-FLAG, STING-FLAG, and HA-A151R plasmids. Then, we did a thorough analysis of the system Xc−/GSH/GPX4 axis. The results showed that A151R clearly counteracted the increase in SLC7A11 (Fig. 3G, H) and GPX4 (Fig. 3G, I, J) that would normally happen when the cGAS-STING pathway is activated. This tells us that A151R actively interferes with the host's ability to maintain redox balance, even when the cGAS-STING pathway is trying to get going. Consequently, GSH levels remained low (Fig. 3K), and the MDA content continued to rise (Fig. 3L) when A151R was present, despite the co-expression of cGAS-STING. Importantly, neither the cGAS nor the STING plasmid alone had a significant effect on GSH levels, MDA content, or GPX4 expression compared to the control group. This really emphasizes that A151R is the main driver of these changes.

These comprehensive findings strongly indicate that ASFV A151R puts a heavy brake on the critical system Xc−/GSH/GPX4 axis, which then leads to widespread fat damage and, as a result, triggers ferroptosis. This mechanism provides a crucial link between A151R's ability to suppress the immune system and its manipulation of how host cells die.

GPX4 Activation Reversed Lipid Peroxidation-Mediated Ferroptosis Induced by A151R

Since we saw that A151R was suppressing GPX4, we wanted to investigate if there was a direct interaction between A151R and GPX4, and more importantly, if activating GPX4 could undo the damage caused by A151R. We started with computer-based molecular docking and molecular dynamics (MD) simulations to predict and analyze this interaction at a very detailed level.

Our molecular docking results, using structures predicted by AlphaFold3, suggested that GPX4 could indeed bind to A151R through various noncovalent interactions, like hydrophobic interactions, salt bridges, and van der Waals forces. The predicted 3D (Fig. 4A) and 2D (Fig. 4B) interaction models showed us the specific amino acid residues involved in this binding. To further confirm how stable this interaction was, we ran MD simulations for 50 nanoseconds. The root mean square deviation (RMSD) curve, which tells us about structural stability, gradually leveled off after about 20 nanoseconds (Fig. 4C). This meant that the A151R-GPX4 complex stayed stable and reached a structural equilibrium throughout the simulation. When we analyzed the hydrogen bonds formed during the 50 ns simulation, we found multiple stable hydrogen bonds (Fig. 4D). Specifically, residues Asn29, Asn105, and Ser145 of A151R were identified as key players, interacting with Val149, Gly137, Arg39, and Asn173 of GPX4 (Fig. 4E). The total binding energy between the two proteins was calculated to be -108.82 kcal/mol, mainly driven by van der Waals energy (-106.14 kcal/mol) and electrostatic energy (-23.50 kcal/mol) (Fig. 4F). These numbers indicate a strong and energetically favorable interaction. These computer predictions were later backed up by our coimmunoprecipitation and immunoblot assays, which showed a direct physical interaction between A151R and GPX4 in our cells (Fig. 4G).

To experimentally prove the functional consequences of this interaction and GPX4's role in counteracting A151R's effects, we treated cells with GW7647, a known activator of GPX4. We then looked at GPX4 expression, MDA content, and ROS accumulation in the presence of both A151R and GW7647. Our results showed that GW7647 effectively reversed the A151R-induced drop in GPX4 levels, both at the protein (Fig. 4H, I) and mRNA levels (Fig. 4J). More importantly, GPX4 activation by GW7647 significantly reduced the MDA content (Fig. 4K), which means less fat damage. Crucially, GW7647-mediated GPX4 activation also substantially suppressed the buildup of total cellular ROS (Fig. 4L, M) and lipid ROS (Fig. 4L, N) that A151R would normally promote. These findings collectively tell us that A151R interacts with GPX4, leading to a weakened cellular antioxidant capacity and thus promoting fat damage and ferroptosis. Conversely, activating GPX4 counteracts A151R-driven lipid peroxidation and ferroptosis, highlighting GPX4's vital role in maintaining redox balance and giving us mechanistic insights into the A151R-GPX4 interaction.

A151R Promotes Ferritinophagy-Mediated Ferroptosis to Induce Lipid Peroxidation

Ferritinophagy, which is the NCOA4-mediated selective breakdown of ferritin, is a crucial process that releases iron ions. These free iron ions can then act as catalysts, producing highly reactive hydroxyl radicals through the Fenton reaction. This, in turn, initiates and worsens fat damage (lipid peroxidation), ultimately driving ferroptosis [22, 23, 24, 42, 43, 46]. What's more, NCOA4-mediated ferritinophagy has been shown to inhibit GPX4 expression, further weakening the cell's antioxidant

defenses [43]. To figure out if ASFV A151R triggers ferroptosis by activating ferritinophagy, we investigated the levels of key molecules involved in this process.

Our results indicated that A151R increased the expression of NCOA4 (Fig. 5A, B), the crucial protein that guides ferritinophagy, in a dose-dependent manner. This increase in NCOA4 went hand-in-hand with accelerated ferritin breakdown, which we saw as a significant drop in ferritin heavy chain (FTH) expression (Fig. 5A, C). Consistent with increased autophagy, A151R also boosted the LC3-II/LC3-I ratio (Fig. 5A, D) while reducing p62 expression (Fig. 5A, E). Both of these are classic signs of autophagy being activated and its targets being degraded. These findings strongly suggest that A151R is indeed promoting the ferritinophagy pathway.

Furthermore, when we co-transfected cells with cGAS and STING along with A151R, A151R noticeably increased the expression level of NCOA4 (Fig. 5F, G) and the LC3-II/LC3-I ratio (Fig. 5F, I), while at the same time suppressing the expression of FTH (Fig. 5F, H) and p62 (Fig. 5F, J) proteins. In contrast, neither the cGAS nor the STING plasmid alone had a significant effect on these ferritinophagy-related proteins, which really emphasizes that A151R is the specific viral factor driving these changes.

To definitively confirm that A151R causes lipid peroxidation by promoting ferritinophagy, we did co-transfection experiments using siNCOA4 (an siRNA that targets NCOA4) and HA-A151R plasmids. The results showed that siNCOA4 significantly knocked down NCOA4 expression (Fig. 5K, L), effectively stopping ferritinophagy. This inhibition of NCOA4 restored FTH expression (Fig. 5K, M), meaning less ferritin was being broken down. At the same time, siNCOA4 decreased the LC3-II/LC3-I ratio (Fig. 5K, N) and, importantly, increased GPX4 expression (Fig. 5K, O), thereby boosting the cell's antioxidant capacity.

Crucially, siNCOA4 also reversed the effects caused by A151R in the context of cGAS-STING activation. Specifically, siNCOA4 reversed the A151R-induced increase in NCOA4 expression (Fig. 5P, Q), increased FTH expression (Fig. 5P, R), decreased the LC3-II/LC3-I ratio (Fig. 5P, S), and increased GPX4 levels (Fig. 5P, T), even when cGAS and STING were present. Most importantly, siNCOA4-mediated ferritinophagy inhibition significantly reduced the accumulation of lipid ROS (Fig. 5U, V) and reversed the increase in cellular iron content caused by A151R (Fig. 5W). In summary, these comprehensive results undeniably prove that ASFV A151R causes cellular lipid peroxidation by promoting ferritinophagy and subsequent iron release. Blocking ferritinophagy with siNCOA4 restored GPX4 expression and reduced lipid ROS levels, providing compelling evidence that A151R drives ferroptosis through mechanisms dependent on ferritinophagy.

GPX4 Activation Restricts Protein Carbonylation and Promotes the cGAS-STING Pathway

Our previous findings clearly showed that ASFV A151R sabotages IFN-β production through the cGAS-STING pathway by causing protein carbonylation, suppressing GPX4, inducing lipid peroxidation, and ultimately driving ferritinophagy-mediated ferroptosis. To dig deeper into the crucial role of GPX4-mediated ferroptosis resistance in the cGAS-STING-dependent IFN-β response, we investigated how activating GPX4 affects protein carbonylation and the cGAS-STING pathway itself.

We co-transfected cells with A151R, cGAS, and STING plasmids, and then treated them with GW7647, a known GPX4 activator. Our Western blot analysis revealed that activating GPX4 with GW7647 significantly inhibited the total protein carbonylation that A151R transfection caused (Fig. 6A, B). This direct reduction in protein carbonylation really highlights GPX4's protective role against the oxidative damage orchestrated by A151R.

What's more, GPX4 activation noticeably boosted the cGAS-TBK1-IRF3 pathway by reversing A151R's suppression of STING protein phosphorylation (Fig. 6A, C), TBK1 phosphorylation (Fig. 6A, D), and IRF3 phosphorylation (Fig. 6A, E). This means that by restoring GPX4 activity, the core signaling events of the cGAS-STING pathway get reactivated, effectively overcoming A151R's inhibitory effects. Importantly, the negative impact A151R had on the mRNA expression of IFN-β (Fig. 6F), ISG15 (Fig. 6G), and ISG54 (Fig. 6H) was significantly reversed by GW7647 treatment. This clearly shows a functional recovery of the antiviral gene expression.

Taken together, these data strongly suggest that activating GPX4 alleviates the protein carbonylation induced by A151R and significantly promotes the cGAS-STING-IRF3 pathway. This provides powerful evidence that GPX4-dependent ferroptosis resistance could be a promising strategy to counteract A151R's suppression of cGAS-STING-mediated IFN-β production. By maintaining a healthy redox balance and preventing oxidative damage, GPX4 plays a critical role in preserving the integrity and effectiveness of our innate immune response against ASFV.

Downregulation of A151R in ASFV Promotes GPX4 Expression and Increases the Production of IFN-β

To see if our lab findings held true in a real-world setting, we looked at the connection between ferroptosis and ASFV infection in live pigs. We also explored what happens when we reduce A151R during an actual viral infection.

Our initial observations in infected animals suggested that the main visible damage in their lungs and spleens was likely linked to oxidative damage caused by ferroptosis. We measured the levels of ferroptosis-related proteins, like FTH and GPX4, 23 days after infection (Fig. 7A). When we stained lung and spleen tissues from ASFV-infected pigs with Hematoxylin and Eosin (H&E), we saw extensive bleeding and a lot of inflammatory cells, which are clear

signs of severe disease (Fig. 7B, D). In contrast, the control animals had normal tissue structures. Our immunohistochemistry (IHC) results from the lungs showed that the amount of FTH and GPX4 proteins (measured as integrated optical density, or IOD) was significantly lower in ASFV-infected pigs compared to the control group (Fig. 7B, C). We saw similar drops in FTH and GPX4 expression in the spleen as well (Fig. 7D, E). Furthermore, Western blot analysis of cGAS expression and STING phosphorylation in infected tissues revealed that cGAS expression was blocked, and STING phosphorylation was greatly reduced (Fig. 7F-H). These observations in live animals clearly demonstrated that ASFV infection causes widespread damage in multiple organs, simultaneously reduces key proteins that fight ferroptosis (FTH and GPX4), and shuts down the cGAS-STING pathway. This points to a direct link between how the virus causes disease, oxidative stress, and its ability to suppress the immune system.

To further confirm how crucial A151R is for lipid peroxidation and IFN-β production during ASFV infection, we first treated PAM cells with liproxstatin-1 (Lip-1), a well-known ferroptosis inhibitor. Compared to the ASFV-only infection group, Lip-1 significantly reduced the mRNA expression of A151R (Fig. 7I), suggesting that blocking ferroptosis might indirectly affect viral gene expression. The levels of B646L, an ASFV structural protein gene, also decreased (Fig. 7J), indicating less viral replication or load. What's more, Lip-1 substantially suppressed the buildup of cellular MDA (Fig. 7K), confirming its role in reducing fat damage during infection. Crucially, the mRNA expression levels of cGAS (Fig. 7L), STING (Fig. 7M), GPX4 (Fig. 7N), IFN-β (Fig. 7O), ISG15 (Fig. 7P), and ISG54 (Fig. 7Q) were all significantly boosted after Lip-1 treatment. This strongly suggests that resisting ferroptosis, which we achieved by using this drug, can help the cGAS-STING pathway get going and promote the production of IFN-β and ISGs during ASFV infection, thereby boosting the antiviral response.

In a complementary approach, we directly explored the impact of reducing A151R during ASFV infection by transfecting PAM cells with siA151R (siRNA targeting A151R). Our data showed that knocking down A151R significantly reduced its own mRNA expression (Fig. 7R) and also decreased the levels of the B646L structural protein gene (Fig. 7S), further supporting A151R's role in viral replication or disease. Importantly, when A151R was knocked down in ASFV-infected cells, we saw a dramatic increase in the mRNA expression of cGAS (Fig. 7T), STING (Fig. 7U), and GPX4 (Fig. 7V). Furthermore, IFN-β (Fig. 7W), ISG15 (Fig. 7X), and ISG54 levels (Fig. 7Y) were also significantly elevated. These results undeniably show that reducing A151R in ASFV leads to less B646L expression, boosts the cGAS-STING pathway, and increases GPX4 and IFN-β levels. This gives us compelling evidence that A151R counteracts IFN-β expression by causing lipid peroxidation in a way that depends on GPX4-modulated ferroptosis, solidifying its role as a key immune evasion factor.

DISCUSSION

African Swine Fever Virus, a large DNA virus with a fascinating icosahedral shape, carries a massive genetic blueprint, encoding between 150 and 170 proteins. These viral proteins aren't just for hijacking our host cells to replicate; they're also masterminds behind orchestrating profound oxidative damage and significantly suppressing the host's antiviral responses, all to help the virus escape our defenses [44]. When viruses manage to get past our innate immunity, it often throws our cell's internal balance (redox balance) into disarray. This leads to oxidative stress, fat damage (lipid peroxidation), DNA damage, and protein dysfunction, which can ultimately trigger various forms of cell death [45, 46]. Past studies have indeed hinted that ASFV infection causes oxidative damage, suggesting it's a clever way for the virus to evade our innate immunity. However, the exact role of ASFV in manipulating this host oxidative damage to fight back against our immune system, and which specific viral factors are involved, has largely been a mystery. Our current study steps in to fill this crucial gap by showing that the ASFV A151R protein actually dampens type I IFN production by messing with the cGAS-STING pathway. It does this by promoting cellular lipid peroxidation and triggering ferritinophagy-mediated ferroptosis.

The cGAS-STING pathway is like the central command center of our antiviral immunity. It detects DNA inside our cells and then launches a strong interferon response. Viruses are smart; they know that shutting down this pathway is a very effective way to establish a successful infection and avoid our defenses [10, 13, 41, 47]. Our results clearly confirm that ASFV, specifically through its A151R protein, acts as a powerful inhibitor of STING-mediated activation of downstream antiviral genes. We saw this because A151R could suppress the phosphorylation of STING, TBK1, and IRF3, which then puts a brake on IFN-β production. This finding firmly places A151R as a major player in ASFV's immune escape tactics. A151R now joins a growing list of ASFV proteins that have been shown to outsmart our innate immunity by interfering with the cGAS-STING pathway using various tricks. These include p17 [48], L83L [49], A137R [50], pS273R [51], DP96R [52], MGF360-14L [53], E120R, I215L [54], E301R [55], QP383R [56], EP364R [57], M1249L [57], D345L [58], MGF505-7R [59], MGF505-11R, MGF360-12L [60], and MGF360-11L [61]. These viral proteins use a range of strategies, like breaking down STING through autophagy or modifying it through ubiquitination, to mess with STING, TBK1, and IRF3, all to achieve immune subversion [62]. Our study's discovery of A151R's unique role in this complex viral arsenal further deepens our understanding of ASFV's many-sided immune evasion strategies.

The production of interferons is tightly linked to the activation of cGAS-STING. Viral infections often lead to

oxidative damage and the accumulation of damaged fats (lipid peroxidation). This can specifically cause proteins to undergo "carbonylation," which ultimately inactivates the cGAS-STING pathway [63]. A great example of this is herpes simplex virus-1 (HSV-1) infection, which triggers oxidative stress that leads to STING carbonylation. This then stops STING from moving from the ER to the Golgi, but interestingly, this effect can be reversed by astaxanthin, which boosts the antiviral response [64]. In fact, STING carbonylation at a specific spot, C88, was found to be a key reason why STING couldn't move properly during HSV-1 infection [14]. Many studies have consistently shown that ASFV infection and its related proteins cause oxidative damage by increasing the level of reactive oxygen species (ROS) in cells [37, 65]. Our current results fit right in with these observations and even expand on them. We found that A151R significantly boosted both total cellular ROS and lipid ROS levels. This increase in oxidative stress directly correlated with higher levels of carbonylation in all cellular proteins and, more specifically, in the STING protein. Importantly, we showed that the carbonylation of the STING protein actually blocked its crucial movement from the ER to the Golgi. These findings strongly suggest that the lipid ROS production triggered by A151R promotes STING carbonylation. This, in turn, makes STING unable to recruit and activate TBK1 and IRF3, thereby hindering the production of type I IFNs. This is a sophisticated viral mechanism that directly disarms a critical part of the host's innate antiviral machinery through oxidative damage.

The system Xc−/GSH/GPX4 axis is a truly essential pathway for fighting ferroptosis. It works by stopping lipid peroxidation, turning damaged fats into harmless ones [66]. The light chain SLC7A11 and the heavy chain SLC3A2, which are parts of the system Xc−, are known to help transport cystine into the cell, which then helps make GSH with the help of GPX4 [46, 67]. Many viruses, like hepatitis B virus (HBV) [26], human papillomavirus (HPV) [27], Japanese encephalitis virus (JEV) [28], and Newcastle disease virus (NDV) [32], have been shown to suppress this system to either weaken our innate immunity or boost their own replication. GPX4 has been clearly identified as a vital antioxidant that helps maintain redox balance and even helps activate STING. On the flip side, if GPX4 is inactivated, it leads to more lipid peroxidation, STING carbonylation, and stops STING from moving from the ER to the Golgi [14]. Our current results provide compelling evidence that A151R significantly put a brake on the system Xc−/GSH/GPX4 pathway. It did this by inhibiting GPX4 and SLC7A11 expression, reducing the amount of GSH inside the cells, and consequently increasing the level of MDA, a clear sign of lipid peroxidation. However, when we activated GPX4 with GW7647, it effectively reversed the lipid peroxidation by reducing the levels of lipid ROS and MDA caused by A151R, even when the cGAS and STING pathways were active. These findings strongly suggest that A151R promotes lipid peroxidation and triggers ferroptosis by actively suppressing the system Xc−/GSH/GPX4 pathway, thereby creating an environment that's perfect for viral replication and immune evasion.

The system Xc−/GSH/GPX4 axis is incredibly important for maintaining redox balance and resisting ferroptosis, and its function is closely tied to NCOA4-mediated ferritinophagy [68]. When ferritin is broken down through ferritinophagy, it releases a lot of free iron into the cells. This free iron then participates in the Fenton reaction, creating highly reactive hydroxyl radicals that lead to widespread lipid peroxidation and ultimately drive ferroptosis [46]. Ferritinophagy-induced ferroptosis plays a crucial role in regulating the cell's antioxidant capacity. Many viral infections, such as those caused by hepatitis B virus [69], Newcastle disease virus [32], murine coronavirus [70], swine influenza virus [71], human immunodeficiency virus [72], and coronavirus 2019 [73], have been shown to inhibit GPX4 expression by triggering ferritinophagy, which then helps the infection and disease progress [74, 75]. Our study expands on these observations by showing that ASFV A151R can increase the levels of proteins involved in ferritinophagy. This, in turn, boosts the amount of cellular iron and thereby suppresses GPX4 expression. Moreover, when we reduced NCOA4, it significantly reversed the ferritinophagy and lipid ROS levels caused by A151R, even when the cGAS and STING pathways were active. Putting all this together, these results provide strong evidence that A151R triggers ferroptosis by promoting lipid peroxidation through ferritinophagy and, at the same time, inhibiting GPX4 expression. This reveals a sophisticated way ASFV manipulates host iron metabolism and cell death pathways to its own advantage.

STING can act as a kind of conductor, regulating the cell's redox balance and kicking off the expression of downstream interferon-stimulated genes (ISGs). Interestingly, if you knock down STING, it not only negatively affects ISG15 expression but also boosts the activity of GPX4, which is a scavenger of lipid peroxidation [76, 77, 78]. We know that ASFV infection can cause oxidative damage [79] and shut down the cGAS-STING pathway [62], but the direct link between lipid peroxidation and the cGAS-STING pathway during ASFV infection hasn't been fully understood. Our current study fills this gap by showing that activating GPX4 significantly reduced the protein carbonylation caused by A151R. This effectively boosted the cGAS-STING-IRF3 pathway and increased the mRNA expression of IFN-β and ISGs. This tells us that resistance to ferroptosis, mediated by GPX4, can effectively counteract A151R's negative impact on interferon production. We confirmed these crucial changes in PAM cells after ASFV infection, providing strong evidence from lab experiments. Furthermore, our in vivo experiments showed that blocking ferroptosis noticeably reduced the expression of A151R and B646L (an ASFV structural protein), while at the same time

boosting their antioxidant capacity. Similarly, knocking down A151R in ASFV-infected cells using siRNA increased the expression of GPX4, IFN-β, and ISGs. These compelling results collectively demonstrate that inhibiting A151R has a positive effect on ferroptosis resistance and promotes interferon production. This suggests a promising new strategy for fighting ASFV infection. Our study provides a clear understanding of how ASFV manages to escape the immune system by triggering lipid peroxidation and ferroptosis, offering exciting new avenues for therapeutic intervention.

Proposed Working Model

Based on all our findings, we've put together a detailed working model to explain how ASFV A151R works against the cGAS-STING pathway, ultimately suppressing type I IFN production by promoting fat damage (lipid peroxidation) (Fig. 7Z). In this model, ASFV A151R is the central player, orchestrating a series of events that all lead to shutting down the host's antiviral response.

Here's how it goes: A151R actively pushes the cell into a state of oxidative stress, leading to a significant increase in reactive oxygen species (ROS) and, more importantly, lipid ROS. This increased fat damage directly causes the STING protein to become carbonylated. This carbonylation of STING is a crucial step because it stops STING from making its vital move from the endoplasmic reticulum (ER) to the Golgi apparatus. This blocked movement prevents STING from changing shape and interacting with other proteins in the way it needs to be activated.

On top of that, A151R kickstarts ferritinophagy, a specialized "self-eating" process that breaks down ferritin, the main protein that stores iron. This breakdown releases a pool of free iron into the cell's fluid. This free iron then gets involved in the Fenton reaction, which creates even more highly reactive hydroxyl radicals, further worsening the lipid peroxidation. At the same time, A151R suppresses the critical system Xc−/GSH/GPX4 axis, which means the cell loses its ability to detoxify damaged fats and maintain its redox balance. The combination of more iron (from ferritinophagy) and weakened antioxidant defenses (because GPX4 is suppressed) pushes the cell towards ferroptosis.

This state of ferroptosis, marked by extensive fat damage and STING carbonylation, ultimately leads to the cGAS-STING pathway being shut down. This inactivation then blocks the downstream signaling cascade involving TBK1 and IRF3, which in turn stops the production of type I interferons (IFN-β) and interferon-stimulated genes (ISGs)—all of which are absolutely essential for a strong antiviral response.

But our model also points to potential ways to fight back! Activating GPX4, either with drugs or through genetic manipulation, can reverse the protein carbonylation caused by A151R, reduce lipid peroxidation, and restore the cGAS-STING-IRF3 pathway's function. This leads to more IFN-β and ISG expression. Similarly, if we reduce A151R itself, either by knocking down its gene with siRNA or by creating ASFV mutants that lack A151R, we see increased resistance to ferroptosis and a strong type I IFN response in infected cells. This proposed model gives us a comprehensive framework for understanding how ASFV A151R cleverly manipulates host cell death pathways and redox balance to escape our innate immunity. It offers exciting new insights and potential targets for preventing and managing ASFV.

CONCLUSIONS

This study has given us a deep understanding of a truly sophisticated trick the African Swine Fever Virus uses to evade our immune system. We've put forward a detailed model showing how the ASFV A151R protein orchestrates a series of cellular events that ultimately shut down our body's Type I IFN production by promoting fat damage (lipid peroxidation). In simple terms, A151R directly contributes to oxidative stress in the cell, leading to the carbonylation of the STING protein. This critical modification then stops STING from making its essential journey from the ER to the Golgi, effectively disrupting its activation.

Crucially, A151R also kickstarts ferritinophagy-mediated ferroptosis. This process involves breaking down ferritin, which releases free iron that then fuels even more lipid peroxidation. At the same time, A151R puts a stop to the vital system Xc−/GSH/GPX4 axis, which is responsible for keeping the cell's redox balance in check and fighting off ferroptosis. The combined effect of increased iron-driven oxidative stress and weakened antioxidant defenses pushes the cell towards ferroptosis, which in turn cripples the cGAS-STING pathway.

Our findings clearly show that this A151R-driven ferroptosis and subsequent suppression of the cGAS-STING pathway stops the production of IFN-β and ISGs. Importantly, we demonstrated that this viral strategy can be effectively reversed by activating GPX4, which helps undo protein carbonylation and restores the cGAS-STING-IRF3 pathway. Furthermore, when we reduced A151R in ASFV-infected cells, we saw increased resistance to ferroptosis and a strong Type I IFN response.

These discoveries shed significant light on new ways ASFV infection evades our immune system, creating a fresh understanding of how ferritinophagy-driven ferroptosis plays a role in viral disease. By unraveling these intricate molecular details of ASFV's immune evasion, our work highlights ferroptosis resistance as a promising new therapeutic target. This could offer exciting new strategies for preventing and managing ASFV. Looking ahead, future research should focus on developing specific inhibitors that target A151R or key parts of the ferritinophagy-ferroptosis pathway. This could help us restore the host's antiviral immunity and combat this devastating pig disease.

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