

Cytokine signaling and innate host defense in modulation of viral infections and the viral evasion

Edited by

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Published in

Frontiers in Cellular and Infection Microbiology
Frontiers in Pharmacology



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ISSN 1664-8714
ISBN 978-2-8325-6915-3
DOI 10.3389/978-2-8325-6915-3

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Cytokine signaling and innate host defense in modulation of viral infections and the viral evasion

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Citation

Rui, Y., Li, Z., Delpino, M. V., Quarleri, J., eds. (2025). *Cytokine signaling and innate host defense in modulation of viral infections and the viral evasion*.

Lausanne: Frontiers Media SA. doi: 10.3389/978-2-8325-6915-3

Table of contents

- 04 **Editorial: Cytokine signaling and innate host defense in modulation of viral infections and the viral evasion**
M. Victoria Delpino and Jorge Quarleri
- 07 **Infectious bursal disease virus affecting interferon regulatory factor 7 signaling through VP3 protein to facilitate viral replication**
Zhiyuan Wang, Yang Chen, Yanyan Chen, Rui Chen, Weiwei Wang, Shichen Hu, Yihai Li, Hongjun Chen, Ping Wei and Xiumiao He
- 20 **Self-assembled epitope-based nanoparticles targeting the SARS-CoV-2 spike protein enhanced the immune response and induced potential broad neutralizing activity**
Yue Liu, Chenxi Li, Zirui Wu, Yu Zhao, Tieyan Yin, Xiaopan Liu, Jiaru Hui, Qingyu Wang, Yi Pan, Yaming Shan and Xinglong Qu
- 34 **Associations between type III interferons, obesity and clinical severity of COVID-19**
Dana Alalwan, Alejandro Abner Garcia Leon, Gurvin Saini, Colette Gaillard, Riya Negi, Camille Heckmann, Grace Kenny, Eoin Feeney, Aoife G. Cotter, Christine Kelly, Michael Carr, Eoghan de Barra, Obada Yousif, Mary Horgan, Corinna Sadlier, Alan Landay, Gabriel Gonzalez and Patrick W. G. Mallon, on behalf of the All-Ireland Infectious Diseases Cohort Study
- 44 **The emerging roles of ubiquitin-like modifications in regulating HIV replication and host defense**
Jinsong Yuan, Huihan Wang, Xiaodong Sun and Chen Huan
- 61 **The GPR4 antagonist NE-52-QQ57 increases survival, mitigates the hyperinflammatory response and reduces viral load in SARS-CoV-2-infected K18-hACE2 transgenic mice**
Xin-Jun Wu, Karen A. Oppelt, Ming Fan, Mona A. Marie, Madison M. Swyers, Ashley J. Williams, Isabelle M. Lemasson, Rachel L. Roper, Paul Bolin and Li V. Yang
- 75 **HIV infection drives proinflammatory adipocyte differentiation in an *in vitro* model and reveals a new inflammatory pathway**
Rosa Nicole Freiburger, Cynthia Alicia Marcela López, Franco Agustin Sviercz, Patricio Jarmoluk, María Belén Palma, Marcela Nilda García, Jorge Quarleri and M. Victoria Delpino
- 93 **Tryptophan metabolic reprogramming modulates cytokine networks in nucleos(t)ide analogue-treated chronic hepatitis B patients**
Xuedan Gao, Xiaojuan Wu, Yi Li, Xinhua Dai and Bei Cai
- 106 **Detecting early kidney injury due to host–virus interaction response in treatment-naïve CHB—a pilot study**
Puneet Gandhi, Kavita Peter, Subodh Varshney, Mahendra Kumar Atlani and Kewal Krishan Maudar
- 117 **Ferroptosis and mitochondrial ROS are central to SARS-CoV-2-induced hepatocyte death**
Cintia Cevallos, Patricio Jarmoluk, Franco Sviercz, Rosa Nicole Freiburger, Cynthia Alicia Marcela López, M. Victoria Delpino and Jorge Quarleri



OPEN ACCESS

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RECEIVED 28 August 2025
ACCEPTED 08 September 2025
PUBLISHED 15 September 2025

CITATION

Delpino MV and Quarleri J (2025)
Editorial: Cytokine signaling and innate
host defense in modulation of viral
infections and the viral evasion.
Front. Cell. Infect. Microbiol. 15:1694793.
doi: 10.3389/fcimb.2025.1694793

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Editorial: Cytokine signaling and innate host defense in modulation of viral infections and the viral evasion

M. Victoria Delpino* and Jorge Quarleri*

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KEYWORDS

cytokine signaling, innate immunity, viral evasion, immunometabolism, host-pathogen interaction

Editorial on the Research Topic

Cytokine signaling and innate host defense in modulation of viral infections and the viral evasion

Viral infections do more than passively replicate within host cells. They reprogram cellular metabolism, alter immune signaling pathways, and reshape tissue homeostasis. These changes influence both the acute manifestations of disease and long-term health outcomes (Heaton and Randall, 2011; Sanchez and Lagunoff, 2015; Thaker et al., 2019). The articles in this Research Topic explore how viruses and the body's defenses interact, how viruses get around the body's defenses that are triggered by cytokines produced after sensing the infection, and how we might use this information to come up with better ways to diagnose and treat infections.

A common thread is how infections change with the cell's metabolism and what happens to the cell due to the changes. Cevallos et al. show that SARS-CoV-2 infection of hepatocytes disrupts mitochondrial homeostasis and lipid metabolism, promoting ferroptosis as a big factor in how the virus causes disease. It also suggests that controlling mitochondrial ROS and iron might help reduce liver problems tied to infection. Gao et al. add to this by reporting that chronic patients on antiviral therapy have changed metabolism. Their work connects changes in metabolites with changes in cytokine networks, which suggests that immunometabolism can serve as a biomarker and regulator of antiviral immunity.

The importance of metabolism in viral persistence is supported by Freiburger et al., who show that HIV infection can push mesenchymal stromal cells toward becoming proinflammatory adipocyte-like. This state involves changes in the way of how the body handles lipids and releases inflammatory mediators, which sheds light on why people living with the problem experience metabolic and adipose tissue issues.

Taken together, these papers emphasize the strong interconnection between metabolic pathways and innate immunity and showed that metabolic pathways decide how cytokine networks are turned on, reshaped, or thrown off balance during short-term and long-term infections.

Innate immunity doesn't act the same way everywhere in the body, and how organs respond to cytokines is another topic covered here. [Wu et al.](#) highlights GPR4 antagonism as a promising therapeutic avenue in severe COVID-19. Using a SARS-CoV-2-infected K18-hACE2 mouse model, the authors show that pharmacological inhibition of GPR4 improves survival, attenuates cytokine-driven inflammation, and reduces viral burden in both lung and brain tissues. These findings position GPR4 blockade as a potential dual-action strategy—modulating detrimental inflammation while limiting viral replication—that warrants further investigation for COVID-19 and related viral diseases.

These results show that tweaking the body's inflammatory sensors could lead to treatments that help with acute infection. At the same time, [Gandhi et al.](#) underscore the value of biomarker-based screening to detect subclinical renal injury in patients with chronic hepatitis B (CHB). By analyzing serum and urinary proteins in treatment-naïve individuals, the authors identify several informative indicators, with a combined biomarker panel achieving high sensitivity and specificity for early kidney injury. Importantly, this approach allows detection of renal impairment before conventional markers such as serum creatinine become abnormal, offering a noninvasive tool to improve risk stratification and prognosis in CHB. This highlights how immune activation and interactions between the virus and the body can quietly hurt organs before symptoms show up.

These articles together stress that cytokines are a double-edged sword: they're needed to keep viruses in check, but they can be harmful if they're overactivated or misdirected.

At the molecular level, some articles in this section highlight the interaction back-and-forth between the body's virus sensors and how viruses get around them is also discussed. [Yuan et al.](#) review the emerging importance of ubiquitin-like (UBL) modifications in shaping HIV–host interactions. Beyond acting as simple antiviral defenses or viral tools, UBLs play dual roles by regulating both host restriction factors and viral proteins, thereby influencing HIV replication, immune evasion, and intracellular signaling. By synthesizing recent advances, the authors position UBLs as central players in the ongoing virus–host arms race and as potential targets for innovative therapeutic strategies against HIV by pointing host pathways that are less likely to be escaped by the virus as a treatment approach.

Other papers back up how viruses interfere with innate immune pathways. [Wang et al.](#) uncovers a novel mechanism by which infectious bursal disease virus (IBDV) undermines host antiviral defenses in chickens. The authors demonstrate that the viral VP3 protein interacts with and promotes proteasome-mediated degradation of IRF7, thereby suppressing type I interferon signaling and facilitating viral replication. Notably, this effect is pronounced with very virulent IBDV strains but absent in attenuated forms. These findings highlight IRF7 as a pivotal target of viral immune evasion and provide new insights into IBDV pathogenesis, with potential implications for vaccine design and antiviral strategies in poultry health.

Similarly, [Liu et al.](#) show an epitope-based nanoparticles (NPs) as a promising next-generation vaccine strategy against SARS-CoV-2. By incorporating conserved spike protein epitopes into ferritin-based NPs, the authors achieved durable antibody responses, neutralizing activity, balanced Th1/Th2 cellular immunity, and antibody-dependent cellular cytotoxicity in animal models. These findings highlight the potential of epitope-focused nanovaccines to overcome challenges posed by spike protein variability, offering a path toward broader and more durable protection against COVID-19.

The interaction between innate immunity and other comorbidity is shown at the clinical study level. [Alalwan et al.](#) identifies type III interferons as key modulators of COVID-19 severity. While IFN λ 4 expression showed no association with disease outcome, elevated IFN λ 2 levels correlated strongly with severe disease, independent of major demographic and clinical factors. Notably, this association was evident only in non-obese individuals, suggesting that obesity may obscure IFN λ 2's predictive value. These findings highlight IFN λ 2 as a potential biomarker of COVID-19 severity and underscore the need to consider host metabolic status when evaluating immune correlates of disease progression. The study clearly highlights the importance of the translational studies, and reminds that body physiology—whether metabolic, inflammatory, or structural—changes the outcome of infections as much as viral make up does.

When put together, this section shows a connected view of viral immunopathogenesis. Pathogens alter metabolic pathways, hijack post-translational changes and modify stromal or adipose cell identities. These aren't separate problems. They spread through immune networks and organ function, creating both risks for pathology and chances for intervention. Some works in this Research Topic show how metabolic or cytokine biomarkers can be used for diagnosis, while others pinpoint therapeutic spots where changing the body's response could lessen viral disease.

A unifying idea from this section is that cytokine signaling and innate host defense are inherently double-edged. They offer the necessary means by which pathogens are recognized and controlled. At the same time, their dysregulation contributes to tissue injury, systemic inflammation, and long-term issues. Viral evasion strategies—from degradation of IRF7 to subversion of UBL signaling—remind us that pathogens get around this balance for their own gain, keeping up persistence or increasing spread.

All these studies bring up key questions. Can metabolic pathways such as tryptophan catabolism or lipid remodeling be tweaked to adjust cytokine responses without causing pathology? How might organ-specific changes of innate sensors, such as GPR4 inhibition, be used to reduce damage in acute infections? What vaccine strategies will do the best job of predicting viral change while knowing about immune evasion? Maybe most importantly, how do the health issues—such as obesity to chronic viral carriage—reshape innate immunity in ways that change how likely we are to catch new viral threats?

The works in this Research Topic give not only mechanical depth but also translational hope. By showing how viruses interact with cytokine signaling and innate defense, they map out paths toward accurate immunomodulation—approaches that try to boost protective immunity while lowering damage to tissues.

Author contributions

MD: Writing – original draft, Writing – review & editing. JQ: Writing – original draft, Writing – review & editing.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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- Thaker, S. K., Ch'ng, J., and Christofk, H. R. (2019). Viral hijacking of cellular metabolism. *BMC Biol.* 17, 59.



OPEN ACCESS

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RECEIVED 16 November 2024

ACCEPTED 23 December 2024

PUBLISHED 13 January 2025

CITATION

Wang Z, Chen Y, Chen Y, Chen R, Wang W,
Hu S, Li Y, Chen H, Wei P and He X (2025)
Infectious bursal disease virus
affecting interferon regulatory factor 7
signaling through VP3 protein to
facilitate viral replication.
Front. Cell. Infect. Microbiol. 14:1529159.
doi: 10.3389/fcimb.2024.1529159

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Infectious bursal disease virus affecting interferon regulatory factor 7 signaling through VP3 protein to facilitate viral replication

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Interferon regulatory factor 7 (IRF7)-mediated type I interferon antiviral response is crucial for regulating the host following viral infection in chickens. Infectious bursal disease virus (IBDV) is a double-stranded RNA virus that induces immune suppression and high mortality rates in chickens aged 3–6 weeks. Previous studies have shown that IBDV infection antagonizes the type I interferon production to facilitate viral replication in the cell, and IRF7 signaling might play an important role. However, the underlying mechanisms that enable IBDV to block the IRF7 pathway remain unclear. In this study, we found that IRF7 and IFN- β expression were suppressed in DF-1 cells during infection with very virulent IBDV (vvIBDV), but not with attenuated IBDV, while the virus continued to replicate. Overexpression of IRF7 inhibits IBDV replication while knocking down IRF7 promotes IBDV replication. Overexpression of IRF7 couldn't compensate the IRF7 protein level in vvIBDV-infected cells, which suggested that IRF7 protein was degraded by IBDV infection. By using inhibitors, the degradation of IRF7 was found to be related to the proteasome pathway. Further study revealed that IRF7 was observed to interact and colocalize with the IBDV VP3 protein. Consistent with IBDV infection results, IBDV VP3 protein was observed to inhibit the IRF7-IFN- β expression, affect the degradation of IRF7 protein via proteasome pathway. All these results suggest that the IBDV exploits IRF7 by affecting its expression and proteasome degradation via the viral VP3 protein to facilitate viral replication in the cells. These findings revealed a novel mechanism that IBDV uses to evade host antiviral defense.

KEYWORDS

infectious bursal disease virus (IBDV), interferon regulatory factor 7 (IRF7), antiviral response, IBDV VP3 protein, viral replication

1 Introduction

Infectious bursal disease (IBD) is a highly contagious and immunosuppressive viral disease caused by infectious bursal disease virus (IBDV), posing a serious threat to the global poultry industry. IBDV is a double-stranded RNA virus belonging to the Birnaviridae family and the Avibirnavirus genus, primarily infecting chicks aged 3–6 weeks. IBDV is classified into two serotypes, serotype 1 and serotype 2. Serotype 1, which is pathogenic to chickens can be further divided into four phenotypes, including classical IBDV (cIBDV) (Cosgrove, 1962), very virulent IBDV (vvIBDV) (Chettle et al., 1989), attenuated IBDV (attIBDV) (Winterfield et al., 1981), antigenic variant IBDV (avIBDV) (Jackwood and Saif, 1987), and recently identified Chinese novel variant IBDV (nvIBDV) (Fan et al., 2019; Wang et al., 2022b). The key pathological manifestation of IBDV infection in chicks is the destruction of the bursa of Fabricius, leading to immune suppression in the flock, increased susceptibility to other pathogens, and ultimately, death (Fan et al., 2022; Wang et al., 2022b). In recent years, as IBDV strains continue to mutate, they have caused significant economic losses to the poultry farming industry (Wang et al., 2022a). However, to date, there is no completed effective method for preventing IBDV infection. Therefore, understanding the innate antiviral immune mechanisms from the perspective of virus-host interactions and enhancing the animals' antiviral capabilities will be an effective approach to control IBDV infection. However, research on the interactions between the host and IBDV remains insufficient.

Viral infection involves a continuous struggling between the virus and the host (Hage and Rajsbaum, 2019; Qin and Zheng, 2017). During this process, the virus utilizes its proteins to interact with host factors, thereby promoting its replication. Type I Interferons (IFN- α/β) are known to be the critical factors in fighting viral infections, constituting the first line of defense in both animals and humans (Chen et al., 2019). Interferon regulatory factor (IRF) 3 and 7 are two key transcription factors that modulate type I IFN expression upon viral infection and have been extensively researched in mammals (Krishnan et al., 2019; Liu et al., 2024; Ma et al., 2023; Matthews et al., 2014; Šestan et al., 2024; Xu et al., 2015; Zhan et al., 2017). Following virus infection, viral genome is sensed by several pattern recognition receptors (PRRs), including toll like receptors (TLRs), and RIG-Like receptors (RLRs) and cytosolic DNA sensors. These receptors signal through mitochondrial antiviral-signaling protein (MAVS) or stimulator of IFN genes (STING) to activate IKK kinase epsilon (IKKi) and Tank binding kinase 1 (TBK1) (Cui et al., 2014). IKKi and TBK1 phosphorylate IRF3/7, leading to its dimerization, nuclear import, and binding to the promoters to induce expression of IFN- β . IRF3/7 was eventually degraded through the proteasome pathway, thus ending their role in antiviral responses. However, the function of IRF3/7 upon virus infection in chickens is still limited. Studies revealed that IRF3 is genetically deficient in chickens and other avian species (Cheng et al., 2019). Conversely, IRF7 has been confirmed to reconstitute corresponding IFN signaling to respond to both DNA and RNA viral infections in chicken (Cheng et al., 2019; Kim and Zhou, 2015).

Increasing evidence demonstrates that many avian viral and viral proteins evade the host's IFN response by interfering the IRF7-IFN- α/β signaling pathway. Kim (Kim et al., 2020) observed a significant increase in viral titers when DF-1 cells lacking IRF7 were infected with avian influenza virus, indicated a crucial role of IRF7 in regulating the replication process of avian influenza virus. Gao (Gao et al., 2023) discovered that the σA protein of avian reovirus suppresses the activation of IRF7, thereby inhibiting the production of IFN-I and facilitating the immune evasion process of the virus. In our previous studies, we found that following IBDV infection in specific-pathogen-free (SPF) chickens, the activation of TLR3 continuously increased at 8- and 12-hours post-infection (hpi). However, compared to 8 hpi, the expression of IRF7 in the TLR pathway was downregulated at 12 hpi (Chen et al., 2022). Ouyang's group (Ouyang et al., 2018) found that MicroRNAs (miRNAs) gga-miR-142-5p reduced the expression of the chMDA5 protein, promoting IBDV replication via an IRF7-dependent pathway in DT40 cells. Therefore, IRF7 is speculated to play an important role in the pathogenic mechanism of IBDV. The aim of this study was to investigate the specific regulation effects of IBDV infection on the IRF7 signaling pathway and to elucidate the underlying mechanism. The findings will provide a theoretical foundation for understanding the role of IRF7 in the antiviral immune response following IBDV infection.

2 Materials and methods

2.1 Viruses, cells and antibodies

The vvIBDV strain used in this study was NN1172 starin which was isolated and identified by our group (He et al., 2014). The attenuated virulent IBDV strain was a commonly used commercial attenuated live vaccine strain B87 which was purchased from HLJ Animal-use Biological Products Co., Ltd., Beijing, China. Dulbecco's modified Eagle's medium (12100061; Gibco, USA) supplemented with 10% fetal bovine serum (12483020; Gibco, USA) was used to culture chicken fibroblast cell line DF-1 cells. Cells were grown at 37°C and 5% CO₂. Anti-IBDV VP2 mouse monoclonal antibody (mAb) was prepared in our laboratory. Other antibodies used in our study were anti-IRF7 rabbit polyclonal antibody (pAb) (bs-2994R, BIOSS, China), anti-FLAG mouse mAb (CW0287, CWBIO, China), anti- β -actin mouse mAb (CW0096M, CWBIO, China), anti-His mouse mAb (M20001M, Abmart, China), AbBox Fluor 594-labeled goat anti-rabbit IgG (BD9279, Biodragon, China), FITC-labeled goat anti-mouse IgG (BF05001, Biodragon, China), goat anti-rabbit IgG H&L antibody (CW0103S, CWBIO, China), goat anti-mouse IgG H&L antibody (CW0102S, CWBIO, China). Other reagents were the proteasome inhibitor MG132 (A2585, APEXBIO, China), ubiquitin inhibitor PYR41 (B1492, APEXBIO, China), autophagosome formation inhibitor Wortmannin (A8544, APEXBIO, China), protease inhibitors (CW2200S, CWBIO, China), SYBR Green qPCR Mix (11201ES, YEASEN, China), DAPI Staining Solution (C1005, Beyotime, China).

2.2 Plasmid construction

To construct the VP3 expression plasmid, the VP3 gene was amplified using vvIBDV strain NN1172 cDNA and cloned into the p3×FLAG-CMV-14 vector with the Flag fused to its 3' end to yield VP3-Flag. Plasmid harboring chicken IRF7 (GenBank accession no. KP096419) was constructed by cloning the synthesized sequence into pcDNA3.1 with the His tag fused to the 3' ends to yield IRF7-His.

2.3 IBDV infection

DF-1 cells were seeded onto plates/dishes at a density of 1.2×10^5 cells. Upon reaching approximately 90% confluent, the growth medium was removed. The cell monolayers of each well were then inoculated with 100 μ L of serially diluted virus in DMEM without foetal bovine serum (FBS). Following a 1-hour adsorption period at 37°C, the cells were washed twice with PBS and maintained with DMEM containing 1% FBS at 37°C.

2.4 Quantitative real-time PCR

Total RNA from the indicated cells was extracted using Trizol (CW0580, CWBIO, China) reagent, and reverse transcribed into cDNA using reverse transcriptase (CW2020M, CWBIO, China). To detect the relative RNA quantities changed fold of target genes (IBDV, IRF7, and IFN- β), the quantitative Real-time PCR was performed using the SYBR qPCR Mix with an Applied Biosystem (Thermo, USA) according to the manufacturer's instructions. RT-qPCR was performed using the following cycling conditions 93 °C for 3 min, 95°C for 15 s, 60°C for 15 s, and 72°C for 20 s, followed by 40 cycles. The results were analyzed using the $2^{-\Delta\Delta C_t}$ method. The RT-PCR primers were listed in [Table 1](#).

2.5 Enzyme-linked immunosorbent assay

The levels of IFN- β in cells cultures were analyzed using an ELISA kit for chicken IFN- β (HEA222Ga, USCN Life Science, China) according to the manufacturer's instructions.

2.6 Western blot

Cell samples were harvested and the expression of denatured proteins was analyzed using SDS-PAGE. Cells were lyse with RIPA buffer (P0013B, Beyotime, China) containing Protease Inhibitor Cocktail (CW2200S, CWBIO, China). The lysates were mixed with $5 \times$ SDS loading buffer (CW0027S, CWBIO, China), boiled for 10 minutes, and separated on 10% SDS-PAGE gels. Proteins were subsequently transferred onto a nitrocellulose membrane (ISEQ00010, Solarbio, China). The membrane was then blocked in 5% (w/v) skim milk for 2 h, followed by incubation with monoclonal or polyclonal antibodies for 2 h. After being washed three times (10 min each) with TBST, the membrane was incubated with either goat anti-rabbit IgG (H+L) or goat anti-mouse IgG (H+L) antibody for 1 h., the protein blots were finally visualized using the WD-9423BC automatic chemiluminescence imaging system (LIUYI, China) for further analysis.

2.7 Transfection

DF-1 cells were seeded in dishes and grown to over 90% confluence, then the cells were transfected with different plasmids or small interfering RNA(siRNA), using the Lipo8000TM transfection reagent (C0533, Beyotime, China) according to the manufacturer's instructions.

2.8 Knockdown of IRF7 by RNA interference

A specific siRNA targeting chIRF7 mRNA was designed by GenePharma (Shanghai,China). The siRNA sequences used in the experiment were as follows: siIRF7 (Sense: GCA CAG AGC UCC GGG ACU UUU; Antisense: AAG UCC CGG AGC UCU GUG CUU), and Non-specific control (Sense: UGU UAA CCA CCG CAU CCU U; Antisense: GGA UGC GUG GUU AAG CAU U). DF-1 cells were transfected with the siRNA. At 6 h after transfection, cells were infected with 1 MOI vvIBDV. Cells were then collected at different time points after infection to evaluate the knockdown efficiency of IRF7 by Western blot, replication level of

TABLE 1 List of primers used in RT-qPCR.

Genes	Direction	Sequence	Product (bp)	Accession no. in GenBank
IBDV	Forward	ACCGGCACCGACAACCTTA	117	FJ615511.1
	Reverse	CCCTGCCTGACCACCACTT		
IRF7	Forward	ACCACATGCAGACAGACTGACACT	146	AF268079
	Reverse	GGAGTGGATGCAATGCTGCTCTT		
IFN- β	Forward	TTCTCCTGCAACCATCTTC	82	NM001024836.1
	Reverse	GAGGTGGAGCCGTATTCT		
β -actin	Forward	CAACACAGTGCTGTCTGGTGGTA	205	NM_205518.2
	Reverse	ATCGTACTCCTGCTTGCTGATCC		

IBDV based viral load detection by RT-qPCR and VP2 protein level by Western blot.

2.9 Inhibitor treatment

DF-1 cells were transfected with p3×FLAG-CMV14-VP3 plasmid or infected with vvIBDV (1 MOI). At 6 h after transfection or 2 h after infection, the DF-1 cells were treated with PYR-41 (a ubiquitin inhibitor), Wortmannin (an autophagy inhibitor), or MG132 (a proteasome inhibitor) for an additional 24 h, and cell lysates were subjected to determination of the protein levels of IRF7 and VP3 or VP2 by Western Blot. In the VP3 transfection experiment, PolyI:C should be added as an immune activator when the inhibitors were added.

2.10 Co-immunoprecipitation and immunofluorescent staining

Co-Immunoprecipitation (Co-IP) was conducted to determine whether IBDV-VP3 interacts with IRF7. DF-1 cells were co-transfected with pcDNA3.1-His-IRF7 and p3×FLAG-CMV14-VP3 plasmids. After 48 hours post transfection, Co-IP was performed as follows: cells were washed three times with ice-cold PBS and lysed in 500 μ L of RIPA lysis buffers (P0013, Beyotime, China) for 30 min. After 12,000×g centrifugation, the supernatants of cell lysates were incubated with 3 μ L anti-Flag mouse mAb or control mouse IgG overnight. Subsequently, 30 μ L protein A/G agarose (80104G, Invivogen, FR) was added to the lysate mixture for 6–8 h. The beads were collected by centrifugation at 3,000 ×g for 5 min at 4°C and washed five times with ice-cold PBS.

Immunofluorescent (IF) staining was conducted to determine whether IBDV-VP3 co-localized with IRF7 in the cell. DF-1 cells were co-transfected with p3×FLAG-CMV-14-VP3 and pcDNA3.1-His-IRF7 plasmids. At 36 h after transfection, the cells were fixed with 4% paraformaldehyde for 20 min at room temperature and permeabilized for 15 min with 0.25% Triton X-100. After being blocked with 5% skim milk, cells were incubated with anti-His mouse mAb and anti-FLAG rabbit mAb overnight at 4°C. After being washed three times with PBS, cells were further incubated with FITC-labeled goat anti-mouse IgG and AbBox Fluor 594-labeled goat anti-rabbit IgG secondary antibody at room temperature for 1 h. Cellular nuclei were stained with DAPI for 10 min and viewed with an LAS X laser scanning confocal microscope (Leica, Cologne, Germany).

2.11 Statistical analysis

Statistical significance was calculated using the Student's t-test for individual paired comparisons or one-way ANOVA whenever multiple groups were compared. For individual comparisons of multiple groups, the Student–Newman–Keuls *post-hoc* test was used to calculate p-values. All values are reported as means $\pm$

standard errors (SEM). All statistical calculations were performed using Primer of Biostatistics.

3 Results

3.1 vvIBDV infection suppress IRF7 expression in DF-1 cells

To understand the impact of IBDV infection on IRF7, we infected DF-1 cells with 1 MOI of vvIBDV or attenuated IBDV, respectively. The expression of IRF7 was detected by RT-qPCR, and the results are shown in Figure 1. In the vvIBDV-infected group (Figure 1A), viral replication levels significantly increased at 24 hpi. The expression levels of IRF7 and IFN- β were significantly upregulated earlier at 16 hpi, but the expression of IRF7 significantly decreased between 24–32 hpi, while the expression level of IFN- β peaked at 24hpi and then down regulated. In the attenuated IBDV-infected group (Figure 1B), the viral replication level peaked at 24 hpi, consistent with that in vvIBDV group. However, the mRNA expression of IRF7 showed no significant changes at the detected time points in the attenuated IBDV group. The expression of IFN- β significantly increased only at 18 hpi. The results suggest that vvIBDV suppresses the IRF7 mRNA expression.

In order to further explore the impact of IBDV on IRF7 and IFN- β expression, we infected DF-1 cells with different IBDV titers (0.5, 1 and 1.5 MOI), respectively. RT-qPCR were used to detect IRF7, IFN- β and IBDV VP2 mRNA expression levels. The results, shown in Figure 1C, indicate that viral replication peaked at 24 hpi under all the indicated viral titers infection, with highest viral load in 1 and 1.5 MOI group. The mRNA levels of IRF7 were significantly lower in the cells infected by 1 and 1.5 MOI vvIBDV between 12–32 hpi, compared to the levels observed in 0.5 MOI infection group. The mRNA levels of IRF7 significantly lower in 1.5 MOI group than that in 1 MOI group at 12 and 32 hpi. While the expression of IFN- β were the lowest level in the 1.5MOI group at all the detection time point. IRF7 protein and its downstream IFN- β protein levels in the cells were further detected correspondingly by Western Blot and ELISA, respectively. The IRF7 protein was found to be degraded beginning at 18 hpi following all the titers IBDV infection (Figures 1D–F), which suggest that the IRF7 protein degradation response to vvIBDV. Consistently, the IFN- β protein peaks at 18 hpi and subsequently declines, with no differences between groups with different dose of IBDV infection (Figure 1G). These results suggested that IBDV infection down regulates IRF7 expression and its downstream factor IFN- β while virus replication.

3.2 Overexpression of IRF7 inhibits IBDV replication

To further confirm if IRF7 affect IBDV replication, we transfected DF-1 cells with pcDNA3.1-His-IRF7 plasmid and

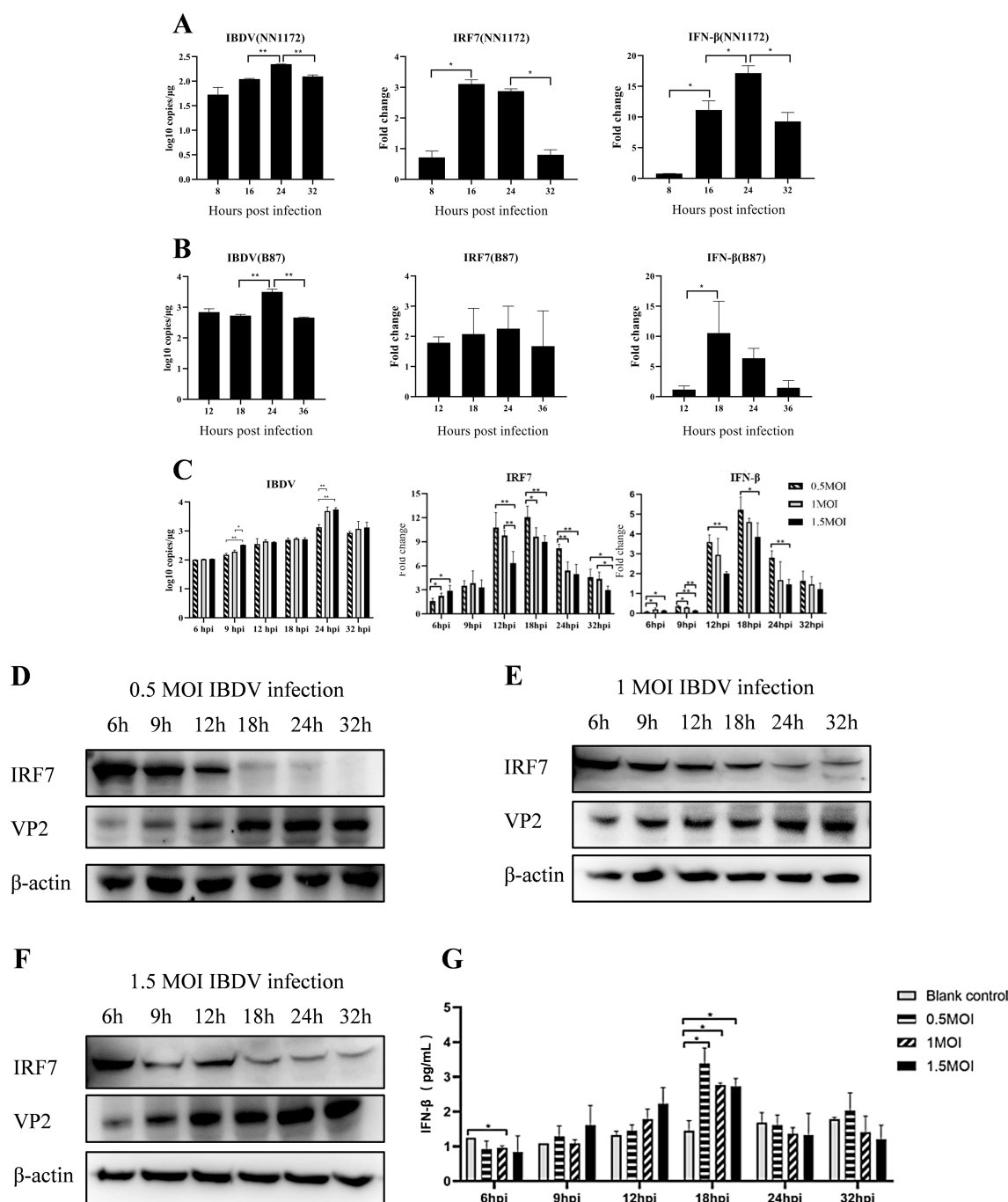


FIGURE 1

IBDV infection can suppress the expression of IRF7. IBDV viral load, relative expression levels of the IRF7 and IFN-β gene (detected by RT-qPCR) in DF-1 cells infected with 1 MOI vIBDV NN1172 (A) or attenuated IBDV B87 (B) at different time points. (C) IBDV viral load, relative expression levels of the IRF7 and IFN-β gene in DF-1 cells infected with 0.5, 1, or 1.5 MOI vIBDV at different time points. (D-F) The IRF7 and VP2 protein levels in DF-1 cells (detected by western blot) infected with 0.5, 1, 1.5 MOI vIBDV at different time point. (G) The protein levels of IFN-β at different time points after varying titers of IBDV infection. Three independent experiments were conducted, and the data are presented as the mean ± standard deviation of three replicates from a representative experiment. * $p < 0.05$; ** $p < 0.01$.

pcDNA3.1-His plasmid, respectively. Following transfection, cells were infected with vIBDV, and samples were collected at different time points for western blot analysis. As shown in Figure 2, in the group transfected with the IRF7 plasmid, the expression of IRF7 protein was significantly higher compared to the empty vector control group as expected. Interestingly, the expression of IBDV

VP2 protein was lower in this group than control group (Figure 2A). RT-qPCR analysis revealed that cells transfected with pcDNA3.1-His-IRF7 plasmid and then infected with IBDV showed a lower viral load compared to cells transfected with the pcDNA3.1-His plasmid (Figure 2B). These results indicated that over expressing of IRF7 is capable of inhibiting IBDV replication.

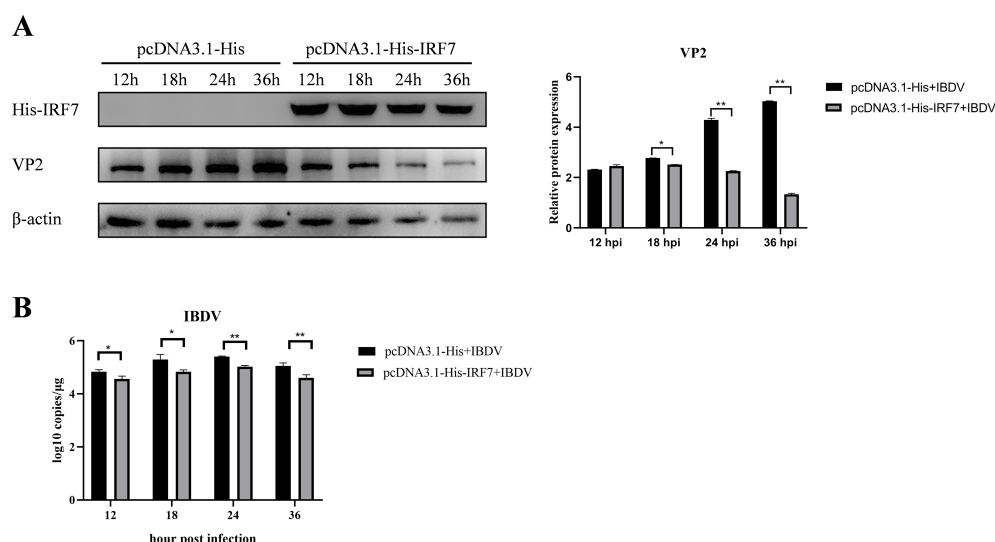


FIGURE 2

Overexpression of IRF7 inhibits IBDV replication. (A) After transfecting DF-1 cells with the pcDNA3.1-His-IRF7 plasmid for 6 hours, cells were infected with 1 MOI vvIBDV. Samples were collected at various time points, and western blot was used to measure the expression levels of IRF7 and IBDV VP2 proteins. Alternatively, relative intensities of VP2 were normalized to β-actin. (B) RT-qPCR was employed to measure the IBDV viral load at various time points. Three independent experiments were conducted, and the data are presented as the mean ± standard deviation of three replicates from a representative experiment. * $p < 0.05$; ** $p < 0.01$.

3.3 Knocking down IRF7 promotes IBDV replication

To further confirm the affecting of IRF7 on IBDV replication, siRNA targeting IRF7 were used to transfect DF-1 cells, a non-specific (NS) siRNA used as control. Following siRNA transfecting, the cells were infected with vvIBDV, and samples were collected at different time points for western blot analysis. As shown in Figure 3, as expected, the expression of IRF7 protein was significantly lower in the cells transfected with IRF7 siRNA compared to the NS control. Consistently, the expression of IBDV VP2 protein was higher in the IRF7 siRNA-transfected group than the NS control group (Figure 3A). RT-qPCR analysis further confirmed that cells transfected with IRF7 siRNA had a significantly higher IBDV viral load than the NS control group (Figure 3B). These results indicated that knocking down IRF7 promotes IBDV replication.

3.4 Overexpression of IRF7 couldn't inhibit IRF7 degradation in vvIBDV-infected cells

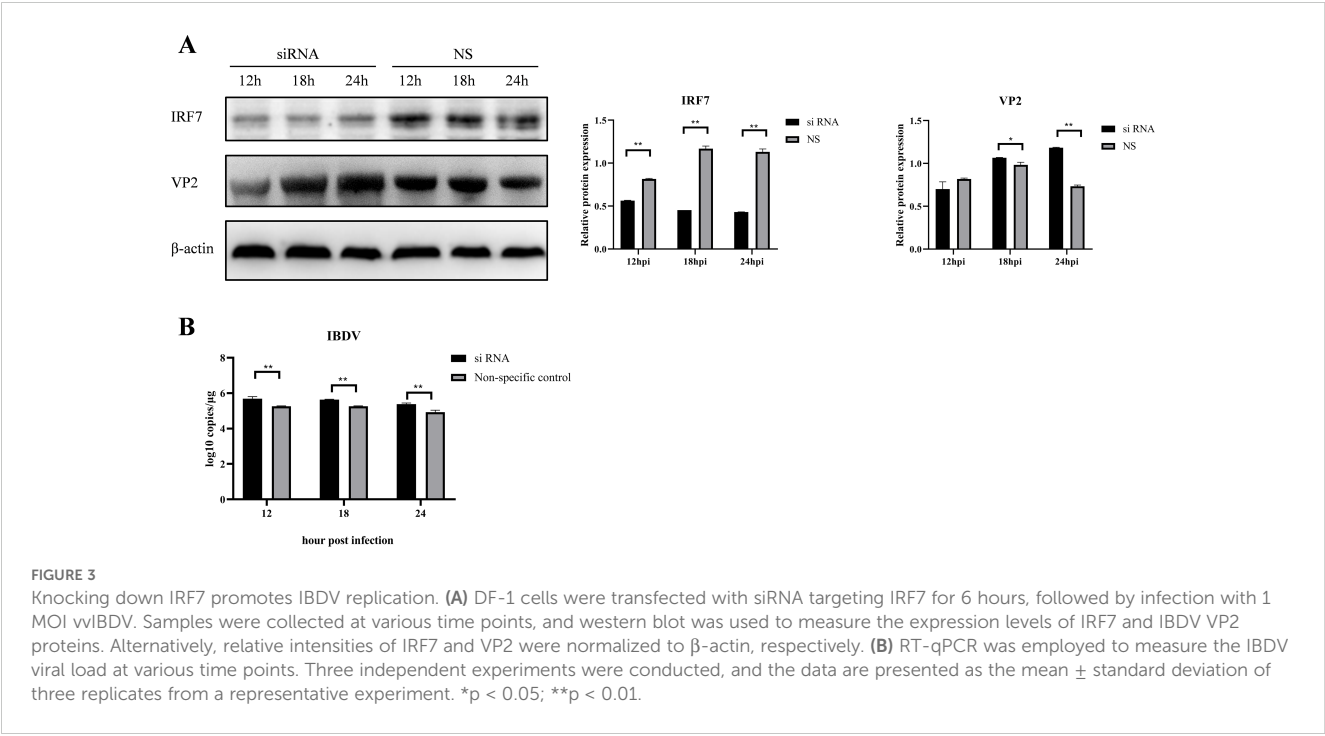
To further confirm if overexpression of IRF7 could compensate the IRF7 protein level when infected by vvIBDV, DF-1 cells were transfected with the pcDNA3.1-His-IRF7 plasmid and then infected with 1 MOI of vvIBDV. The IRF7 protein was detected by western blot. The results showed that the IRF7 protein gradually increased at the early time points, and peaked at 12hpi; however, the expression of IRF7 protein significantly decreased at 18 hpi and afterward, compared to the early time points and uninfected control. As expected, the IRF7 protein gradually increased in the uninfected group until the end of the experiment at 24 hpi (Figure 4A), which

suggested that the IRF7 protein in the IBDV infected group was degraded.

We also further detected if there were changes in IRF7 protein level in B87-infected group when overexpress the IRF7. DF-1 cells were transfected with pcDNA3.1-His-IRF7 plasmid followed by infection with 1 MOI attenuated IBDV, B87. The western blot analysis results indicated that as the infection time extended, the expression of IRF7 protein gradually increased. However, when comparing the infected group to the uninfected group, the differences in the IRF7 protein production at various time points were not statistically significant (Figure 4B) which is different from that in vvIBDV group.

3.5 vvIBDV promotes IRF7 degradation through the proteasomal pathway

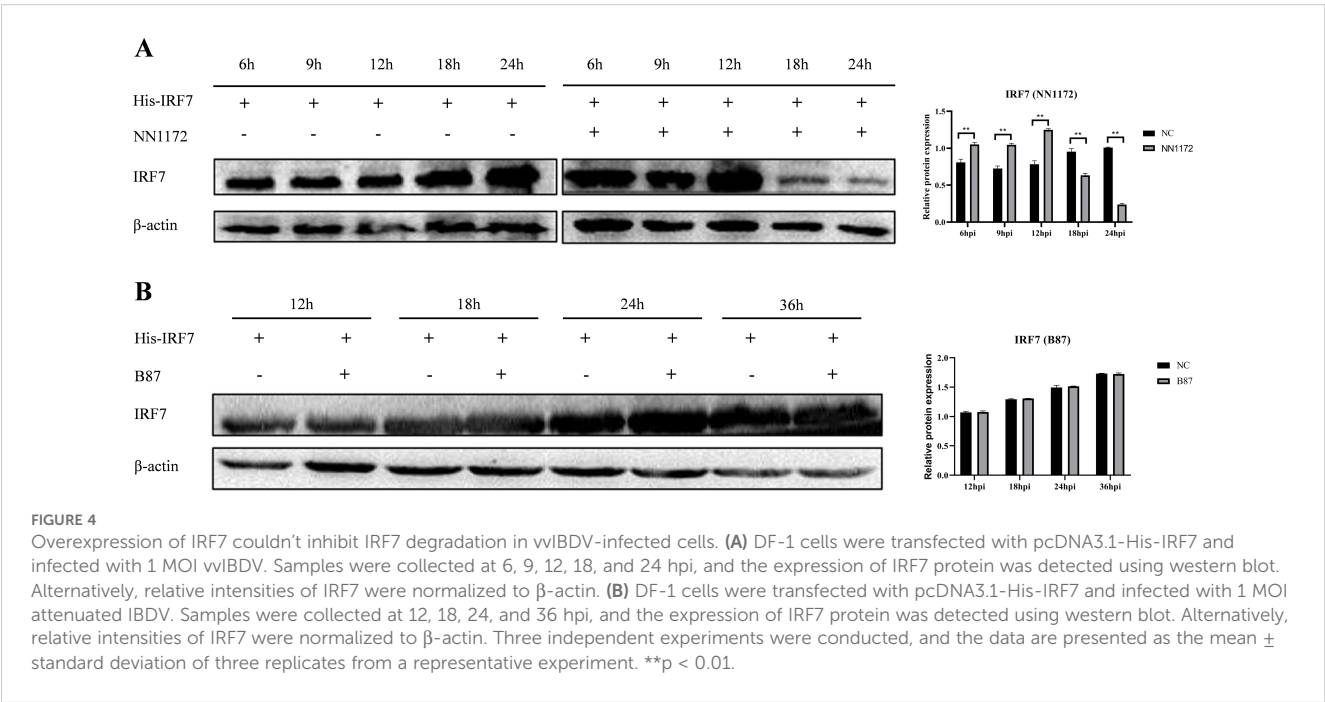
To explore which pathway IBDV employs to affect IRF7 degradation, the cells were treated with PYR-41, Wortmannin, or MG132 after infected with 1 MOI of vvIBDV, respectively. IRF7 expression and viral VP2 were detect by western blot. The results are shown in Figure 5, the protein level of IRF7 in cells treated with MG132 was significantly higher compared to other inhibitor treated groups, and showed no different with untreated uninfected control. Additionally, the protein level of IBDV VP2 was lower in this group compared to other experimental groups, which indicated lower level of viral replication in the cells treated with proteasome inhibitor MG132. These results suggest that the degradation of IRF7 protein level induced by IBDV infection is mediated through the proteasome pathway.

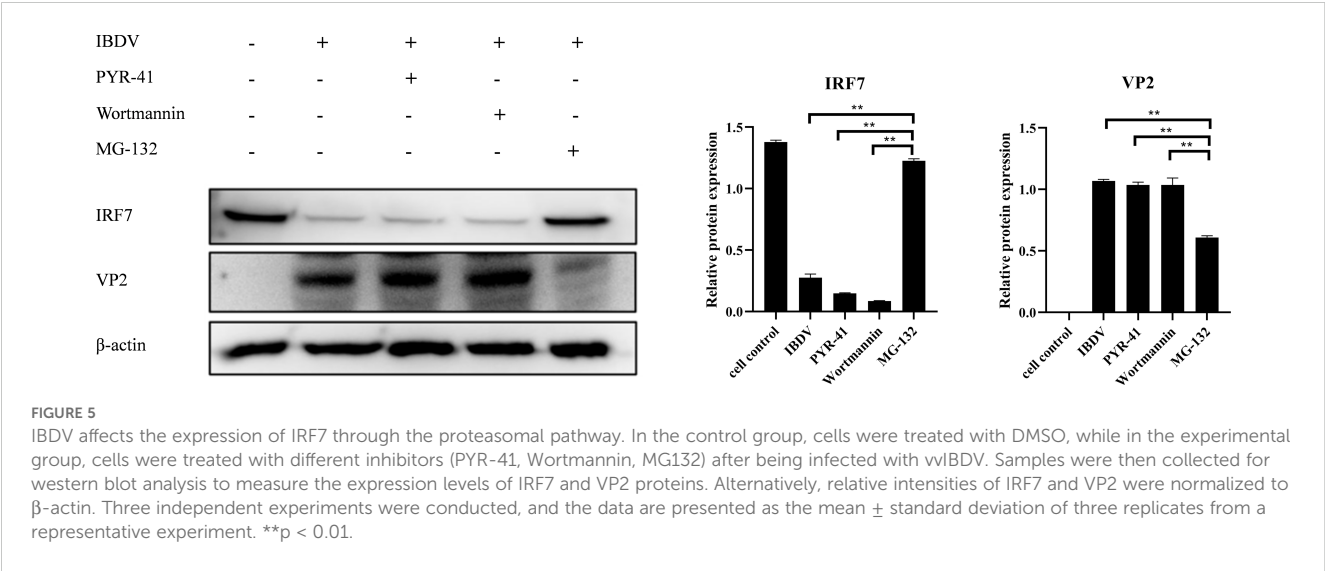


3.6 IBDV VP3 interacts with IRF7

It has been reported by [Ye et al. \(2014\)](#) that IBDV VP3 can suppress the expression of IFN-β. However, whether IBDV VP3 can interact with chicken IRF7 remains unknown. To investigate this interaction, we co-transfected DF-1 cells with pcDNA3.1-His-IRF7 and p3×FLAG-CMV14-VP3. Following transfection, we performed

CO-IP using Flag-tag and His-tag antibodies. The results are shown in [Figure 6A](#), the Flag-tag antibodies precipitated Flag-VP3 and concurrently, His-IRF7, demonstrating an interaction between IBDV VP3 protein and IRF7 protein. To further validate the relationship between IRF7 and VP3, an immunofluorescence staining assay was conducted. We observed that exogenous viral protein VP3 colocalized with exogenous IRF7 in the cytoplasm of DF-1 cells ([Figure 6B](#)).

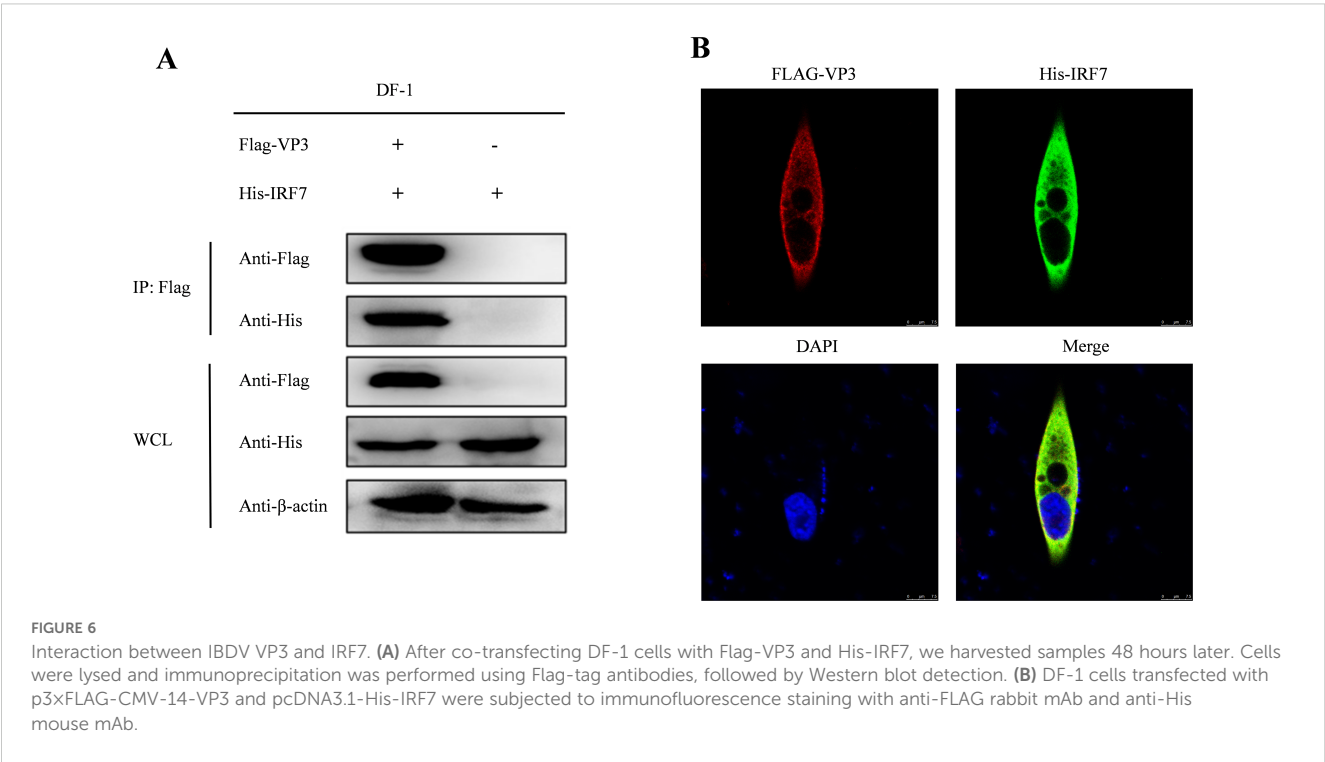




3.7 Overexpression of IBDV VP3 inhibits the expression of IRF7

Since IBDV VP3 interacts with IRF7, we sought to investigate the affecting of IBDV VP3 on IRF7 mRNA and protein levels. DF-1 cells were transfected with either the p3×FLAG-CMV14-VP3 plasmid or the p3×FLAG-CMV14 plasmid, following transfection, the cells were stimulated with polyI:C, an immune activator. Cell samples were collected at various time points post-stimulation, IRF7 and IFN- β expressions were analyzed using RT-qPCR. As

shown in [Figure 7A](#), compared to the empty vector control group, the mRNA levels of IRF7 and IFN- β in cells transfected with the IBDV VP3 plasmid were significantly reduced after 24 hours of stimulation. Western blot analysis was further used to detect the changes in IRF7 protein levels at 24h post transfection. As shown in [Figure 7B](#), the IRF7 protein levels were significantly lower in the IBDV VP3-transfected cells compared to the empty vector control group, consistent with the mRNA level results. These findings indicate that in cells with activated IRF7 signaling, the IBDV VP3 protein reduces both the gene and protein levels of IRF7.



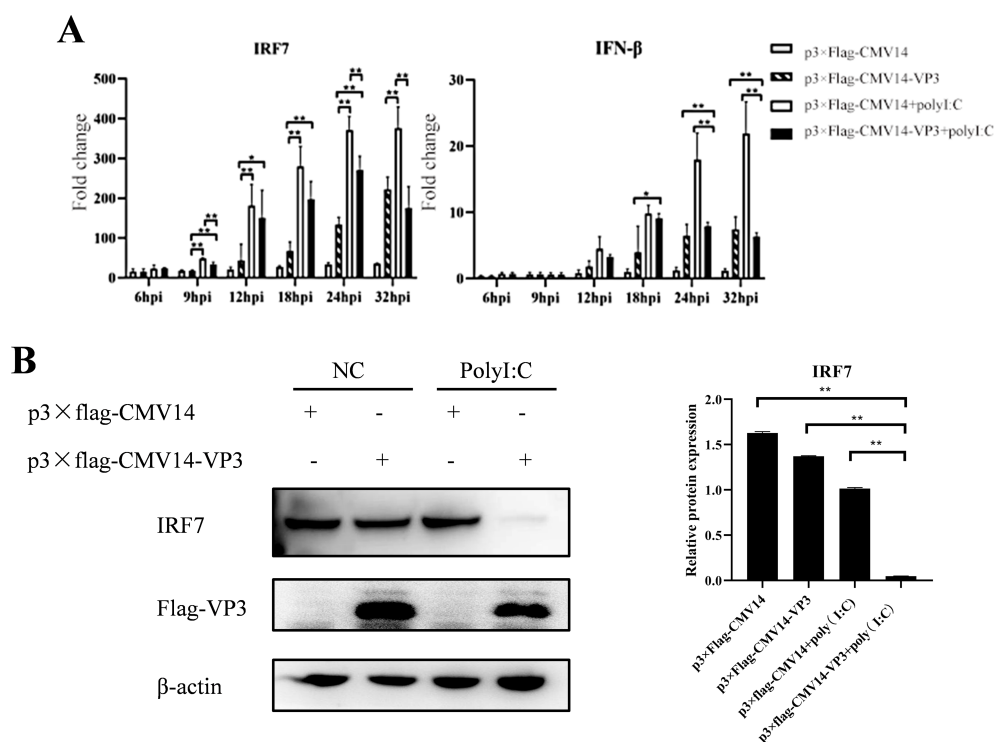


FIGURE 7

Overexpression of IBDV VP3 inhibits IRF7 expression. (A) DF-1 cells were transfected with the p3×FLAG-CMV14-VP3 plasmid for 6 hours and then stimulated with polyI:C. RT-qPCR was used to detect the gene expression levels of IRF7 and IFN-β at various time points post-infection. (B) DF-1 cells were transfected with the p3×FLAG-CMV14-VP3 plasmid for 6 hours and then stimulated with polyI:C for 24 h. Western blot analysis was performed to detect the protein expression levels of IRF7 and Flag-VP3. Alternatively, relative intensities of IRF7 were normalized to β-actin. Three independent experiments were performed, and the data are presented as the mean ± standard deviation of three replicates from a representative experiment. *p<0.05; **p<0.01.

3.8 IBDV VP3 promote IRF7 degradation through the proteasome pathway

To further explore how IBDV VP3 affects the IRF7 protein level, we transfected DF-1 cells with the p3×FLAG-CMV14-VP3 plasmid. Post-transfection, the cells were treated with MG132, Wortmannin, and PYR-41, respectively. Following this, the cells were stimulated with polyI:C to activate the antiviral immune signaling pathway. After 18 hours, cells were collected for detecting IRF7 protein using Western blot. As shown in Figure 8, cells transfected with the p3×FLAG-CMV14-VP3 plasmid showed an increase in IRF7 protein level when treated with the proteasome inhibitor MG132. In contrast, the IRF7 protein was degraded in the other inhibitor treated groups. This indicates that IBDV VP3 protein induces the degradation of IRF7 protein through the proteasome pathway.

4 Discussion

In recent years, as the field IBDV strains continue to mutate, an increasing number of gene reassortant strains, gene recombination strains, and Chinese novel variant strains have been confirmed (Fan et al., 2019; Wang et al., 2022a, b), IBDV still poses a threat to the poultry industry worldwide, with traditional vaccines failing to provide full protection (Fan et al., 2020), presenting significant

challenges to the control of IBD. IBDV infection represents a complex interplay between the virus and the host, hence understanding the interaction between IBDV and the host can aid in the development of novel vaccines. Since the vital role of IRF7 inducing IFN-α/β antiviral responses both in mammals and chickens (Gao et al., 2019, 2023), it is imperative to investigate the interaction between IBDV and IRF7 to further comprehend the pathogenic mechanism of IBDV.

IRF7 is a crucial transcription factor that regulates the production of IFN-β and located downstream of the TLRs and MDA5 receptors (He et al., 2017; Yu et al., 2022). It has been established that the antiviral activity of the host is usually associated with the elevation of IRF7 and IFN-β levels in the cells after virus infection (Lan et al., 2017). Our study reveals that IBDV triggers an early IRF7-IFN-β response during infection of DF-1 cells. Consistent with this, IBDV, being an RNA virus, has been reported to trigger the TLR3 (He et al., 2017; Quan et al., 2017), TLR7 (Yu et al., 2019) and MDA5 (Chen et al., 2024; Hui and Leung, 2015; Lee et al., 2014; Yu et al., 2022) innate antiviral pathway *in vitro* and/or *in vivo*, which is accompanied by upregulation of IFN-β. Furthermore, IRF7 was also found to be upregulated in chicken thymus at early IBDV infection stages, as demonstrated by both transcriptomic and proteomic analysis (Chen et al., 2024). Taken together, our results confirm that IRF7-IFN-β innate antiviral pathway was activated early after IBDV infection,

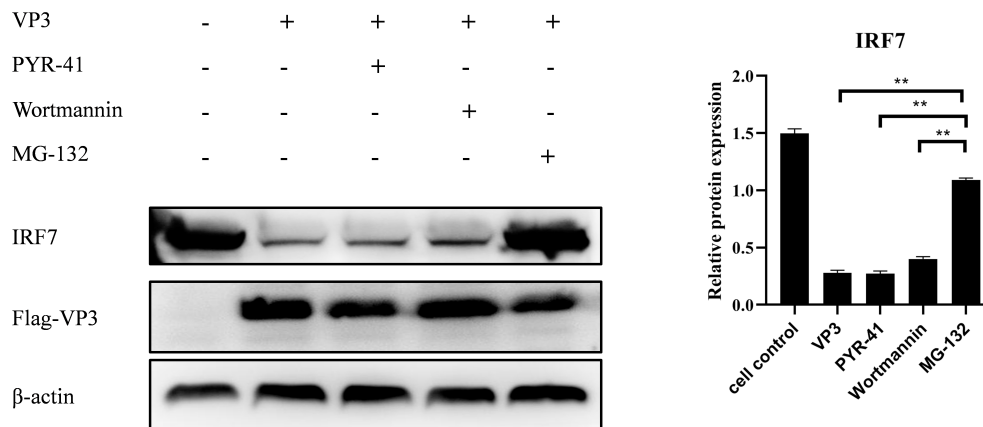


FIGURE 8

IBDV VP3 promote IRF7 degradation through the proteasome pathway. After transfecting DF-1 cells with the p3xFLAG-CMV14-VP3 plasmid for 6 hours, cells were treated with inhibitor: PYR-41, Wortmannin, or MG132, respectively. Samples were collected 24 h after polyI:C treatment and the IRF7 and VP2 protein levels were analyzed using Western blot. Alternatively, relative intensities of IRF7 were normalized to β -actin. Three independent experiments were conducted, and the data are presented as the mean $\pm$ standard deviation of three replicates from a representative experiment. ** $p < 0.01$.

suggesting that the host cell, including DF-1 cells, attempting to combat IBDV infection by activating the interferon signaling pathway.

Interestingly, we observed that the mRNA levels of IRF7 decreased in DF-1 cells following vvIBDV infection at later time points (≥ 24 hpi), compared to earlier time points and uninfected controls (Figure 1A). Further analysis revealed that the reduction in IRF7 mRNA was dependent on the IBDV titer, with higher titers inducing a more pronounced decrease (Figure 1C). However, this trend was not reflected at the protein level. As we observed that the protein levels of IRF7 were significantly reduced during the later stages of infection (≥ 18 hpi), regardless of the IBDV titer (Figures 1D–F). Consistently, the protein level of IFN- β , a downstream factor of IRF7, mirrored the trend of IRF7 (Figure 1G). Studies based on integrating proteomics and transcriptomics have shown that transcriptional and protein levels often do not directly correlate (Abdulghani et al., 2019; Chen et al., 2024; Song and Wang, 2019). Current evidence suggests that such discrepancies may result from regulatory mechanisms, including methylation modifications (Cieřla et al., 2023), alternative splicing (Chembazhi et al., 2023), and post-translational modifications (Xu et al., 2021). Therefore, whether the expression of IRF7 in IBDV-infected cells is regulated by these mechanisms requires further investigation. Notably, the trend of IRF7 expression found in the DF-1 cells is consistent with our previous findings from intestinal lamina propria (ILP) cells of chicken infected with vvIBDV which were detected on mRNA level (Chen et al., 2022). All these consistent results suggest that IRF7 transcription is inhibited by IBDV infection. Many viruses have been evolved to block IRF3/7 signaling to inhibit the production of IFN- β , thereby facilitating virus replication within the cells. Examples include Hepatitis C virus (HCV) (Raychoudhuri et al., 2010), Marek's disease virus (Gao et al., 2019), and avian reovirus (Gao et al., 2023). In Ouyang's research (Ouyang et al., 2018), attenuation of IRF7 signaling promoted the replication of

IBDV in DT40 cells. We also observed that IRF7 knockdown in DF-1 cells enhanced IBDV replication (Figure 3), while IRF7 overexpressed inhibited IBDV replication in DF-1 cells (Figure 4). Given that type I IFN has been reported to be antagonized after IBDV infection in many studies (He et al., 2017; Lee et al., 2015; Rauf et al., 2011; Smith et al., 2015), it is suggested that IRF7 signaling is suppressed by IBDV, which inhibits IRF7 transcription and protein levels, potentially serving as a strategy for IBDV to evade the host immune system and promote viral replication and dissemination. Taken together, IRF7 is one of important factors that exploited by IBDV to block cell mediated innate antiviral response to facilitate viral replication.

It has been confirmed that upon receiving signals of viral infection, IRFs undergoes phosphorylation, leading to their dimerization and translocation into the cell nucleus, where they binds to the IFN- β promoter and enhances the expression of the IFN- β (Arnold et al., 2013). Many RNA viruses have been shown to regulate the IRF pathway either directly or indirectly, with different viruses exhibited different regulatory mechanisms. For instance, Kaposi's sarcoma associated herpesvirus (KSHV) blocks the phosphorylation and accumulation of IRF7 during viral infection (Liang et al., 2012), Epstein-Barr virus inhibits the IRF7 dimerization (Wang et al., 2020), SARS coronavirus (SARS-CoV) blocks IRF3 signaling at a step after phosphorylation, but does not inhibit IRF3 dimerization, nuclear localization or DNA binding, the blocking were further confirmed to be related to IRF3 ubiquitination (Matthews et al., 2014). In this study, we observe that IRF7 protein levels, decreased by IBDV infection, were blocked when infected cells were treated with the proteasome inhibitor MG132, suggesting that IRF7 degradation is promoted by IBDV through the proteasome pathway. In 2024, Niu and his peers revealed in a study that IBDV has the power to inhibit the nuclear entry activity of IRF7, thus resulting in the suppression of interferon expression. Taken together, IBDV appears to inhibit the IRF7 signaling by multiple steps, including reducing IRF7

expressing, promoting proteasomal degradation as observed by us and nuclear entry activity reported by Niu et al., 2024. Since IRFs function involves several important critical steps, including phosphorylation, dimerization, nuclear localization or DNA binding, further investigation is required to determine the impact of IBDV infection on other aspects of IRF7 activation.

RNAi technique has emerged as the most desired method for researchers who wish to silence a specific gene of interest and has been extensively used in the scientific researches. RNAi is also an effective antiviral mechanism which include important regulation molecules such as Dicer, Drosha, Exportin5 and Ago2 (Poirier et al., 2021; Shah et al., 2021; van Rij et al., 2006). Mammals express Dicer1 and Ago1 for the biogenesis of both miRNAs and siRNAs but retain the cleavage activity essential for RNAi (Bartel, 2018; Shah et al., 2021). In our study, we only detected IFN- β response to reveal the IRF7 interference on the replication of IBDV. As Shah et al. (2021) concerned that Whether IFN and RNAi contribute in these ways to antiviral immunity in avian remains controversial, since Shah et al. (2021) also found that the expressions of dicer and ago2 were basically blocked in the IBDV infection, further detection of more key genes in the RNAi pathway will be needed to gain a more comprehensive understanding of the interaction mechanism between IBDV and host cells.

IBDV VP3 is the viral protein that has been reported to regulate viral replication through various mechanisms. In 2020, research by Zhang's group indicated that the IBDV VP3 protein inhibits autophagy during the early stages of IBDV replication (Zhang et al., 2020). Other studies have shown that IBDV VP3 inhibits the host antiviral response, thereby facilitating viral replication and pathogenesis (Busnadiego et al., 2012; Deng et al., 2022). In 2014, Ye and his fellows found that IBDV VP3 can compete with MDA5 to bind intracellular viral genomic dsRNA, suppressing the production of IFN- β . In another study, it was demonstrated that VP3 works synergistically with the chicken RNA binding protein Staufen1 (STAU1) to suppress IFN- β production (Ye et al., 2019). Deng's group revealed that IBDV VP3 hinders the formation of the TRAF3-TBK1 complex by reducing K33-linked polyubiquitination of lysine 155 on TRAF3, thereby inhibiting MDA5-dependent, IRF3-mediated signaling and ultimately facilitating viral replication (Deng et al., 2021). In this study, we found that IRF7 interacts and colocalizes with IBDV VP3 protein in the cells. Further, we observed that IBDV VP3 protein down-regulates the IRF7 protein production, blocking the proteasome pathway halts IRF7 degradation. These findings are consistent with those observed in DF-1 cells infected with IBDV. Since many viruses have been confirmed to regulate the IRFs signaling by viral proteins (Gao et al., 2023; Liang et al., 2012; Wang et al., 2020), and given our consistent results from both IBDV infection and IBDV-VP3 transfection studies, it can be concluded that IBDV inhibits IRF7 signaling through its VP3 protein to facilitate viral replication. However, the specific mechanism underlying the interaction between VP3 and IRF7 remains unclear. Future research should focus on identifying the key interaction sites of VP3 to elucidate its mechanism of action. Additionally, it is worth investigating whether VP3 affects the stability and function of IRF7 through other pathways, such as

phosphorylation, dimerization, or ubiquitination modifications. Animal studies are also needed to assess the impact of VP3 protein on IBDV pathogenicity, immune response and disease outcome. These investigations will help evaluate the feasibility and effectiveness of targeting the VP3 protein for antiviral therapy. Collectively, these studies could provide deeper insights into the molecular mechanisms involved and potentially facilitate the development of antiviral drugs aimed at the VP3 protein.

It is worth noted that infections with different virulent strains of IBDV differing in their impact on IRF7 expression was observed in our study. In DF-1 cells infected with vvIBDV, IRF7 was initially upregulated and then degraded. Conversely, in cells infected with the attenuated vaccine strain B87, IRF7 gene expression levels did not significantly change at various time points, nor did IFN- β levels. We only observed a transient upregulation of IFN- β at 18 hpi in B87-infected DF-1 cells which is consistent with our previous research, that study showed transient activation of IFN- β antiviral response at 4 hpi in the Gut-associated lymphoid tissues (GALT) cells isolated from B87-infected chickens (Chen et al., 2022). The differential effect of different virulent IBDV on antiviral pathways have been noted by many researchers. Our group demonstrated that infection with the intermediate virulent strain NN040124 and vvIBDV strain NN1172 showed a down-regulating effect on IFN- β expression in chicken peripheral blood mononuclear cells and three-week-old chickens, respectively (He et al., 2017). In contrast, B87 infection showed initially downregulated and later upregulated IFN- β levels in chickens. However, in 2006, Eldaghayes research group found no significant change in IFN- β mRNA expression after infection of SPF chicken with classical strain F52/70 (Eldaghayes et al., 2006). In contrast, UK661 infection initially down-regulated but later recovered to baseline. These results indicate that B87 infection does not significantly affect the innate antiviral response, including the IRF7 signaling step, which might be related to the easier replication and dissemination of B87, and consistent with the biology of this vaccine strain as we concerned (Chen et al., 2022). This result further confirms the crucial role of the IRF7 signaling in the pathogenicity of IBDV.

In summary, we demonstrated for the first time that IBDV antagonizes host IFN- β by regulating the IRF7 signaling via IBDV VP3 protein. This regulation involves down regulating IRF7 expression, promoting IRF7 degradation through the proteasome pathway, thereby facilitating viral replication within the cells. Different virulent strains of IBDV exhibit varying regulatory effects on the cellular IRF7 signaling pathway, with vvIBDV has a significant effect and attenuated strain has no effect. These findings may expand our current knowledge about the mechanisms that IBDV exploits to evade host antiviral innate immunity and promote the development of more effective strategies for prevention and control of IBD in clinical practice.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

Ethical approval was not required for the studies on animals in accordance with the local legislation and institutional requirements because only commercially available established cell lines were used.

Author contributions

ZW: Conceptualization, Data curation, Methodology, Writing – original draft. YC: Conceptualization, Data curation, Methodology, Writing – original draft. YYC: Formal analysis, Investigation, Writing – original draft. RC: Formal analysis, Investigation, Writing – original draft. WW: Formal analysis, Investigation, Writing – original draft. SH: Formal analysis, Investigation, Writing – original draft. YL: Formal analysis, Investigation, Writing – original draft. HC: Formal analysis, Investigation, Writing – original draft. PW: Writing – review & editing. XH: Conceptualization, Funding acquisition, Methodology, Validation, Writing – review & editing.

Funding

The author(s) declare financial support was received for the research, authorship, and/or publication of this article. This research was funded by the National Natural Science Foundation of China (32460875, 32160824 and 31560706).

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Acknowledgments

We acknowledge Professor Jianqiang Ye from Yangzhou University, China, for technical assistant.

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OPEN ACCESS

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RECEIVED 14 January 2025

ACCEPTED 20 March 2025

PUBLISHED 09 April 2025

CITATION

Liu Y, Li C, Wu Z, Zhao Y, Yin T, Liu X, Hui J,
Wang Q, Pan Y, Shan Y and Qu X (2025) Self-
assembled epitope-based nanoparticles
targeting the SARS-CoV-2 spike protein
enhanced the immune response and induced
potential broad neutralizing activity.
Front. Cell. Infect. Microbiol. 15:1560330.
doi: 10.3389/fcimb.2025.1560330

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Self-assembled epitope-based nanoparticles targeting the SARS-CoV-2 spike protein enhanced the immune response and induced potential broad neutralizing activity

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Introduction: The ongoing COVID-19 has caused a global pandemic, resulting in millions of infections and deaths. While current vaccines target the SARS-CoV-2 spike (S) protein, its high mutation rate significantly compromises vaccine efficacy. We aimed to evaluate the potential of epitope-based nanoparticles (NPs) to induce broad cross-protection and durable immune responses against SARS-CoV-2.

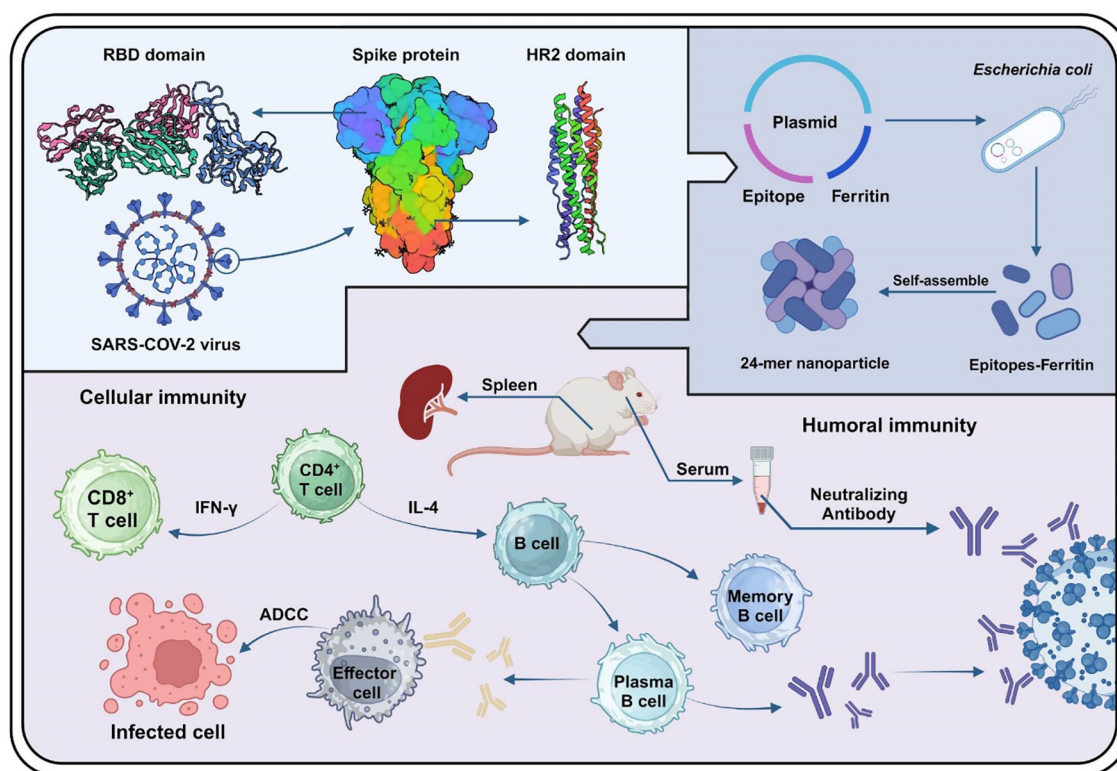
Methods: Four conserved epitopes derived from the receptor-binding domain (RBD) and S2 subunit of the spike protein were integrated into *Helicobacter pylori* ferritin to create epitope-based NPs named S18-F, RBM-F, UH-F, and HR2-F. The immunogenicity of the epitope-based NPs was evaluated through animal experiments to measure epitope-specific antibody titers and assess neutralizing activity against SARS-CoV-2 pseudovirus. To characterize cellular immune responses, splenic lymphocyte proliferation following epitope stimulation was measured, and cytokine secretion profiles including IFN- γ , IL-2, IL-4, and IL-10 were analyzed to determine Th1/Th2 immune polarization. Antibody-dependent cellular cytotoxicity (ADCC) assays were performed to evaluate NP-enhanced recognition and elimination of infected target cells.

Results: These NPs induced high titers of epitope-specific antibodies lasting three months post-immunization. Sera from the RBM-F, UH-F, and HR2-F groups exhibited neutralizing activity against the SARS-CoV-2 pseudovirus WH-1 *in vitro*. Splenic lymphocytes from the S18-F, RBM-F, and UH-F groups showed significantly increased proliferation. Lymphocytes from the RBM-F group demonstrated increased secretion of IFN- γ , IL-2, IL-4, and IL-10 cytokines, indicating a balanced Th1 and Th2 immune response. Immune sera from the S18-F and mixed-immunized groups exhibited antibody-dependent cellular cytotoxicity.

Discussion: The results indicate that these NPs induce robust humoral and cellular immune responses, potentially offering a promising strategy for effective vaccine development against SARS-CoV-2.

KEYWORDS

SARS-CoV-2, ferritin, self-assembled nanoparticles, key epitopes, immunogenicity study



GRAPHICAL ABSTRACT

1 Introduction

At the end of 2019, COVID-19 emerged as a global pandemic (Rothan and Byrareddy, 2020; Zhu et al., 2020). The International Committee on Taxonomy of Viruses identified the causative pathogen as SARS-CoV-2 (The species Severe acute respiratory syndrome-related coronavirus: classifying 2019-nCoV and naming it SARS-CoV-2, 2020). Phylogenetic analysis revealed that SARS-CoV-2 shares 79% sequence similarity with SARS-CoV and over 95% homology with the bat coronavirus RaTG13 (Lu et al., 2020). According to statistics of World Health Organization, SARS-CoV-2 has resulted in approximately 700 million infections and over six million deaths.

Existing vaccines have effectively prevented SARS-CoV-2 infection, playing a crucial role in controlling viral transmission

and alleviating symptoms (Tregoning et al., 2021). These vaccines target the spike (S) protein, eliciting protective immune responses (Li et al., 2022). However, the high mutation rate of the S protein has led to reduced alignment with emerging variants, which exposed the limitations of traditional drug discovery methods (Zhang et al., 2022). Researchers increasingly rely on bioinformatics analyses for the development of vaccines and antibodies. Bioinformatics has proven invaluable in predicting numerous conserved epitopes (Khan et al., 2023). Recent findings emphasize the critical roles of linear epitopes in virus vulnerability and conservation, as well as their importance as components of conformational epitopes necessary for inducing virus neutralization. For example, a linear epitope of Human Immunodeficiency Virus (HIV) demonstrated broad-spectrum

protective effects and could be pivotal in developing universal vaccines (Kong et al., 2019). Grifoni et al. identified potential B and T cell epitopes for SARS-CoV-2, which hold promise as targets for immune recognition against the virus (Grifoni et al., 2020).

The S protein is integral to viral host cell infection and comprises two subunits, S1 and S2. The receptor-binding domain (RBD) of S1 contains a core structure and a receptor-binding motif (RBM) that interacts with Angiotensin-Converting Enzyme 2 (ACE2). Subunit S2 consists of the heptad repeat (HR1), heptad repeat 2 (HR2), and the central helix (CH), which mediate virus-cell membrane fusion (Shang et al., 2020). Consequently, RBD and S2 are primary targets for designing vaccine candidates based on the S protein (Huang et al., 2020). Epitope-based vaccine strategies have demonstrated stronger immune responses and improved safety compared with those based on the full-length S protein (Soria-Guerra et al., 2015). Most neutralizing antibodies against SARS-CoV-2 primarily target the S1 subunit, particularly the RBD, to block virus attachment to host cells (Barnes et al., 2020). The RBD remains a key focus for COVID-19 vaccines aimed at eliciting neutralizing antibodies (Dickey et al., 2022). In contrast, the S2 subunit exhibits greater sequence conservation across various SARS-CoV-2 variants of concern compared to S1 (Olukitibi et al., 2023). Given that the structural transition of the S protein from prefusion to post-fusion states is critical for virus-host membrane fusion, antibodies directed against the S2 stem helix region are expected to impede SARS-CoV-2 infection (Wang et al., 2021). Recent studies have highlighted that antibodies targeting specific epitopes within the S2 subunit of the SARS-CoV-2 S protein frequently demonstrate broad neutralizing capabilities (Pinto et al., 2021; Hurlburt et al., 2022; Zhou et al., 2022).

The low immunogenicity of short peptides presents a significant challenge for epitope-based vaccine development. Nanoparticles (NPs) offer a promising solution by serving as effective delivery vehicles and immune enhancers (Walls et al., 2020; Wang et al., 2020). Among these, ferritin NPs stand out due to their excellent safety and biocompatibility (Bou-Abdallah, 2010; Truffi et al., 2016; Arosio et al., 2017; Li et al., 2023), which enhance immunogenicity by improving antigen presentation efficiency and stability. Utilizing ferritin to display trimeric conformations of the HIV gp120 protein notably enhances the immunogenicity and protective efficacy of HIV vaccines (He et al., 2016). Researchers are increasingly leveraging ferritin as a carrier based on experimental findings that consistently demonstrate enhanced immune responses. Ferritin has been successfully employed to carry various antigens, including the

A helix and CD helix from the influenza virus hemagglutinin stem (Qiao et al., 2022), influenza virus hemagglutinin trimer (Kanekiyo et al., 2013), as well as full-length S protein and RBD sequences of SARS-CoV-2 (Powell et al., 2021). Studies indicate that NPs such as ferritin (24-mer), mi3 (60-mer), and I53–50 (120-mer) induce more robust immune responses compared to RBD monomers (Kang et al., 2021). Specifically, ferritin-displayed antigens from influenza A virus have demonstrated robust and efficient immune responses (Kanekiyo et al., 2013).

In this study, we developed and characterized NPs based on conserved linear epitopes from the SARS-CoV-2 S protein RBD and S2 domain. Using IEDB and Bepipred Linear Epitope Prediction 2.0, we identified four conserved epitopes (S18, RBM, UH, and HR2). These epitopes were integrated with *Helicobacter pylori* ferritin to create epitope-based NPs named S18-F, RBM-F, UH-F, and HR2-F. The immunogenicity of the epitope-based NPs was assessed through animal experiments to measure epitope-specific antibody titers and evaluate neutralizing antibody levels against the SARS-CoV-2 pseudovirus. Additionally, splenic lymphocyte proliferation in response to epitope stimulation was analyzed to assess the cellular immune response. The secretion levels of Th1/Th2-associated cytokines were assessed to determine whether the epitopes could elicit a balanced immune response. Furthermore, antibody-dependent cell-mediated cytotoxicity (ADCC) activities was measured to evaluate the NPs' ability to stimulate splenic cells in recognizing and eliminating infected cells. We hope that epitope-based NPs induce robust humoral and cellular immune responses, potentially providing enduring protection. Utilizing epitope-based self-assembled ferritin could thus present an effective strategy for vaccine development.

2 Materials and methods

2.1 Animals and cells

Specific pathogen-free 6–8-week-old female BALB/c mice were procured from Liaoning Changsheng Biotechnology Co., Ltd. (Liaoning, China).

Human embryonic kidney 293T cells were obtained from the American Type Culture Collection (Manassas, VA, USA), and Huh7 cells were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan). Briefly, the cells were propagated in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY,

TABLE 1 Conservation sequences from RBD and S2 subunit.

Name	Amino Acid Sequence
RBM	SNNLDSKVGNNYNYLRLFRKSNLKPFRDISTEIYQAGSTPCNGVEGFNCYFPLQSYGFQPTNGVGYQPY
HR2	PDVDLGDISGINASVVNIQKEIDRLNEVAKNLNESLIDLQEL
UH	VNNTVYDPLQPELDSFKEELDKYFKNHTSPDVDLGDISGI
S18	GKIADYNYKLPPDDFTGCVIAWNSNNL

USA) supplemented with 10% heat-inactivated fetal bovine serum (Gibco, Grand Island, NY, USA) and 1% penicillin-streptomycin (Sinopharm Chemical Reagent, Beijing, China).

2.2 Conservation analysis of the RBD and S2 protein

Full-length amino acid sequences of different SARS-CoV-2 surface glycoprotein (n=37) from 2019 to 2024 were downloaded from the National Center for Biotechnology Information (NCBI) in the FASTA format. The sequences were aligned using MEGA 11. The conservation of RBD and S2 subunit was analyzed using two approaches. The aligned sequences were loaded into the MEME Suite (<https://meme-suite.org/meme/>) to assess amino acid frequency at each site. Alternatively, the aligned sequences were imported into Weblogo (<https://weblogo.threeplusone.com/>) and a logo created to indicate amino acid conservation based on the height of each individual amino acid. Four linear epitopes listed in Table 1 were identified based on these analyses, named S18, RBM, UH, and HR2. The peptides, S18, RBM, UH and HR2 were synthesized (GL Biochem, Shanghai, China) with > 90% purity.

2.3 Immunogen design and gene synthesis

The genes encoding the RBM, HR2, UH, and S18 epitope sequences were cloned into pET-20b by GenScript Biotech (Nanjing, China). *Helicobacter pylori* ferritin (GenBank: NP_223316) was inserted into the plasmids at the C-terminus of the epitopes, followed by a 6 × His tag at the C-terminus of ferritin for affinity purification through the NdeI and XhoI restriction sites.

2.4 Protein expression and purification

The recombinant plasmids were transformed into *E. coli* BL21 (DE3) pLysS competent cells. When the *E. coli* culture entered the logarithmic growth phase, 0.1 mM isopropyl β-D-1-thiogalactopyranoside was added to induce protein expression, and the culture was maintained for another 12 hours at 16°C. The harvested cells were re-suspended in Tris-buffered saline buffer (50 mM Tris, 150 mM NaCl, pH 8.0) and disrupted by sonication at 4°C. The expressed proteins were then purified from the culture supernatant using affinity chromatography on a His-trap column (GE Healthcare, USA). The purified proteins were detected using the bicinchoninic acid method.

2.5 NP characterization

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed to identify the protein monomers. The samples were mixed with a four-fold loading buffer, heated for 10 min at 98°C, and separated on 12% polyacrylamide gel. A total of

5 μg of each protein was loaded onto the gel. The proteins were stained with Coomassie Brilliant Blue (Sigma, St. Louis, Missouri, USA) and decolorized in double-distilled water with 45% methanol and 10% glacial acetic acid until a clear background appeared. They were visualized and recorded using a Tanon-5200 Chemiluminescent Imaging System (Tanon Science and Technology, Shanghai, China).

The morphology of NPs was evaluated using transmission electron microscopy (TEM; H-7650, Hitachi, Japan) at an accelerating voltage of 100 kV. The diameter distribution of the NPs was determined by dynamic light scattering (DLS) at 25°C using a Zetasizer NANO ZS90 instrument (Malvern, Worcestershire, UK).

2.6 Animal immunization

The BALB/c mouse immunity experiment was approved from the Experimental Animal Ethics Committee of Jilin University (approval number: YN2021094). To assess the immunogenicity of the NPs, BALB/c mice were randomly assigned to 7 groups (n = 6 per group). The NPs were combined with an aluminum adjuvant (InvivoGen, San Diego, CA, USA) at a ratio of 3:1 for immunization. Mice received subcutaneous immunizations with 20 μg of ferritin, S18-F, RBM-F, UH-F, HR2-F, or a mixture of NPs. The mixed-immunized group received 5 μg of each NP to ensure consistent immunization doses across all groups. Phosphate-buffered saline (PBS) was used as a negative control. Following the initial immunization, blood was collected every 14 days, and the serum was stored at −20°C.

2.7 Enzyme-linked immunosorbent assay

S18, RBM, UH, and HR2 peptides (100 ng/well) were used to detect specific antibody titers. After blocking with 200 μL 5% bovine serum albumin (BSA; GENVIEW, Beijing, China) at 37°C for 2 h, serially diluted serum samples were added in duplicate and incubated at 37°C for 1 h. The plates were then incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (Beijing Dingguo, China) for 1 h. Subsequently, 3,3',5,5'-tetramethylbenzidine (Tiangen Biotech, Beijing, China) was added to develop the color and the reaction was stopped using 2 M H₂SO₄. The absorbance at 450 nm was measured using an iMark™ microplate reader (BioRad, Hercules, California, USA).

For the antibody isotyping assay, the ELISA plates were coated with 100 ng of S18, RBM, UH, or HR2 peptides per well. The plates were blocked with PBS containing 3% BSA, followed by incubation with serum samples (1:1000 dilution). After incubation with anti-mouse IgG1, IgG2a, IgG2b, IgG3 or IgM antibodies (1:5000 dilution; Sigma, St. Louis, Missouri, USA), alkaline phosphatase-conjugated anti-sheep IgG (Beijing Dingguo, China) was added. Subsequently, para-nitrophenyl phosphate was added as a chromogenic substrate and the reaction was terminated with 3 M NaOH. The absorbance at 405 nm was recorded immediately.

2.8 Splenocyte isolation and T cell activation assay

Two weeks after the final immunization, spleens were collected from the immunized mice, and splenic lymphocytes were isolated using a Mouse Spleen Lymphocyte Isolation Kit (Solarbio, Beijing, China).

The CD3⁺CD4⁺ and CD3⁺CD8⁺ T cells in the spleen were counted using flow cytometry. Briefly, splenic lymphocytes were blocked using the TruStain FcXTM PLUS (anti-mouse CD16/32) antibody (BioLegend, San Diego, California, USA). Splenic lymphocytes were incubated with fluorescein isothiocyanate-conjugated anti-mouse CD3 antibody, PE-conjugated anti-mouse CD4 antibody, or allophycocyanin-conjugated anti-mouse CD8 antibody (BioLegend, San Diego, California, USA). After washing with cell staining buffer, 5 μ L of 7-aminoactinomycin D (7-AAD; BioLegend, San Diego, California, USA) was added and the number of CD3⁺CD4⁺ and CD3⁺CD8⁺ lymphocytes was determined by CytoFLEX cytometry (Beckman Coulter, Brea, California, USA).

2.9 Splenic lymphocyte proliferation assay

Splenocytes isolated from immunized mice were added to a 96-well culture plate (3×10^5 cells/well). Then cells were stimulated with S18, RBM, UH, or HR2 peptides (10 μ g/mL) for 72 h at 37°C. Cell proliferation was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma, St. Louis, Missouri, USA) assay. Briefly, after treatment, 20 μ L of MTT (5 mg/mL) was added and incubated for another 4 h in the dark. Then, 100 μ L of dimethyl sulfoxide was added after removing the supernatant carefully, and the absorbance was measured at 490 nm. The stimulation index (SI) was defined as the ratio of the optical density of stimulated wells at 490 nm to that of unstimulated wells.

2.10 Antibody-dependent cellular cytotoxicity assay

The S protein in pCDNA3.1(+) (GENEWIZ, Beijing, China) was transfected into HKE 293T cells using polyethylenimine (PEI; Sigma, St. Louis, Missouri, USA) at a ratio of 1:4 (mg DNA/mg PEI). The transfected cells were used as target cells and incubated with 1 μ M carboxyfluorescein diacetate succinimidyl ester (CFSE; Invitrogen, USA). CFSE-labeled target cells were added to 96-well plates and incubated with a 1:1000 dilution of serum samples. After incubation, splenic lymphocytes from naïve mice were used as effector cells and added to the appropriate wells. Finally, cells were stained with 7-AAD. Flow cytometry was performed using a CytoFLEX cytometer. Percent ADCC activity was calculated as follows: ADCC activity % = [(experimental lysis % – spontaneous lysis %)/(positive control lysis % – spontaneous lysis %)] $\times$ 100.

2.11 Amplification determined by enzyme-linked immunosorbent spot assay

The number of IFN- γ -secreting lymphocytes was quantified using Enzyme-Linked Immunospot Assay (ELISPOT), which assessed cytokine secretion by splenic lymphocytes isolated from the immunized mice. Splenic lymphocytes from immunized mice were added to pre-coated ImmunoSpot plates, along with S18, RBM, UH, HR2 peptides, or their mixture. Negative controls received serum-free medium. After incubation, cells were removed, and biotin-conjugated anti-IL-4 or anti-IFN- γ antibodies were added. Plates were further incubated with diluted streptavidin-horseradish peroxidase, followed by a substrate solution of 3-amino-9-ethylcarbazole to develop spots. Spot counting was performed using an ImmunoSpot analyzer (Cellular Technology Ltd., Cleveland, OH, USA).

2.12 Serum cytokine detection

The concentration of cytokines INF- γ , IL-2, IL-4, and IL-10 in serum was measured using mouse Th1/Th2 Uncoated ELISA Kits (Invitrogen, USA). Splenic lymphocyte suspension (500 μ L) was added to 24-well plates, and the cells were stimulated with peptide S18, RBM, UH, HR2 peptides, or their mixture (10 μ g/mL) at 37°C for 72 h. The culture medium was centrifuged and precipitated, the corresponding antibody was added to the supernatant, and the OD450nm value was determined using an enzyme-labeled apparatus (ELX800, Bio-TEK, Windsor, Vermont, USA).

2.13 Quantification of neutralizing antibodies against SARS-CoV-2 pseudotyped virus

At week 8, neutralizing activities of sera from all animals were assessed using a pseudotyped virus-based neutralization assay, as this time point demonstrated the highest titers of epitope-specific antibodies. The assay protocol was previously described in Nature Protocols (Nie et al., 2020) and conducted by Feifan Biotechnology (Jiangsu) Co., Ltd. Serum samples were serially diluted two-fold and mixed with the pseudotyped virus, followed by incubation at 37°C for 1 hour. The serum-virus mixture was then added to Huh7 cells in 96-well culture plates and incubated at 37°C for an additional 28 hours. After the incubation period, 150 μ L of supernatant was aspirated from each well, and 100 μ L of luciferase detection reagent was added. The reaction was allowed to proceed for 2 minutes at room temperature. Following the reaction, protein levels were detected using a multimode microplate reader (PE Ensign; Bio-TEK, Windsor, Vermont, USA). The cell control (CC) with only cells and the virus control (VC) with virus and cells are set up in each plate.

Neutralization inhibition rate = [1 - (mean luminescence intensity of sample group - mean luminescence intensity of blank control CC group)/(mean luminescence intensity of negative VC group - mean luminescence intensity of blank control CC group)] $\times$ 100%.

2.14 Safety evaluation of NPs *in vivo*

The immunized mice were euthanized two weeks after immunization. The main mouse tissues (heart, liver, spleen, lungs, and kidneys) were fixed in 4% paraformaldehyde. Fixed samples were embedded in paraffin, sliced, and stained with H&E.

2.15 Statistical analysis

Data are presented as mean $\pm$ standard deviation (SD). Groups were compared using a one-way analysis of variance (ANOVA) with Tukey's *post hoc* test. The statistical significance compared to the ferritin group was denoted as follows: (ns (not significant), * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$).

3 Results

3.1 Conservation analysis and plasmid design

Epitope prediction prioritizes linear B-cell epitopes that are immunogenic, accessible, and preferably located within conserved

regions of the protein. The conservation of RBD (PDB: 7C8J_2) and S2 (PDB: 7E9T_1) were evaluated separately from SARS-CoV-2 using IEDB and Bepipred Linear Epitope Prediction to identify potential B-cell epitopes. The B-cell epitopes in the RBD and S2 regions are depicted in Figure 1. Considering the surface accessibility of RBD₇₀₋₉₅, RBD₈₄₋₁₀₉ was identified as a target epitope due to its higher surface accessibility, suggesting it is more likely to act as an exposed epitope within the RBD structure. The RBM, located between RBD₁₀₆₋₁₇₆, contains the epitopes RBD₁₀₈₋₁₅₃ and RBD₁₆₁₋₁₇₄. Given that longer segments often induce stronger immune responses, the entire length of RBM was selected as the target epitope. The allosteric folding of HR2 from S2₄₇₁₋₅₁₂ is crucial during virus-target cell membrane fusion. As HR2 contains epitopes S2₄₇₈₋₄₈₅ and S2₄₈₉₋₅₀₂, HR2 was also chosen as a target epitope. Following comprehensive screening of various factors, four potential epitopes—RBD₈₄₋₁₀₉, RBM, S2₄₃₁₋₄₇₀, and HR2—were identified through immunoinformatics and labeled as S18, RBM, UH, and HR2. These epitopes are detailed in Table 1 and Supplementary Table 1. The conservation analysis of SARS-CoV-2 variants of concern (VOCs) and variants of interest (VOIs) provided by the WHO from 2020 to 2024 was conducted using MEGA 11. The results are presented in Supplementary Figures 1–5.

RBM (71 aa), UH (40 aa), HR2 (42 aa), or a triple-copy of S18 (78 aa) were individually fused to the N-terminus of ferritin in an

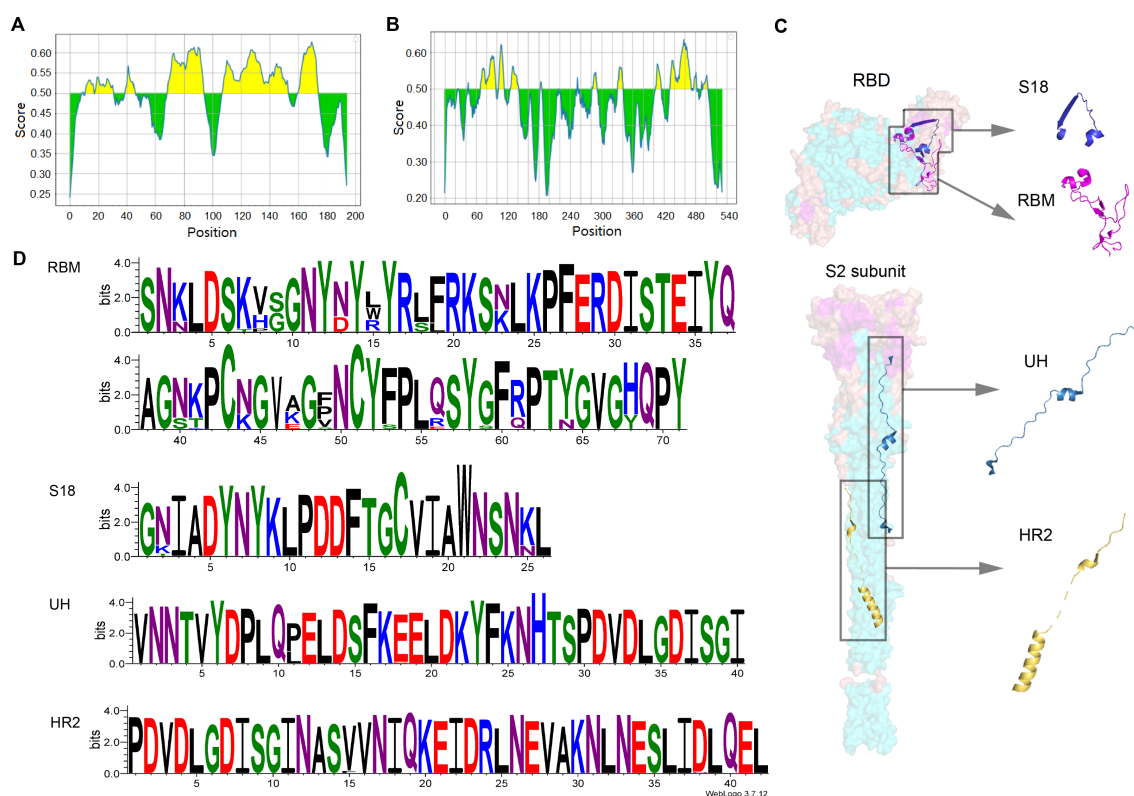


FIGURE 1

Analysis of sequence conservation and prediction of linear B-cell epitopes. (A) RBD and (B) S2 subunit linear B-cell epitope prediction using Bepipred Linear Epitope Prediction 2.0 method. (C) Structure RBD (ACCESSION: 7C8J_B) and S2 (ACCESSION: 7E9T_C) subunit. (D) Sequence logos of the multiple sequence alignments of RBM, HR2, UH and S18.

antigen-ferritin tandem format using a GSG linker. Subsequently, a 6× His-tag was attached via a flexible (GGGGS)₃ linker at the C-terminus of ferritin (Figure 2A).

3.2 Self-assembled epitope-based NPs detected *in vitro*

Protein molecular weight and purity were identified by SDS-PAGE and Coomassieblue staining. The theoretical molecular weights of these NP monomers are as follows: S18-F (27.44 kDa), RBM-F (26.77 kDa), UH-F (23.41 kDa), and HR2-F (23.37 kDa). The molecular weights of each NP monomer were approximately

consistent with the theoretical values. The purity of the NPs exceeded 90% (Figure 2B).

Particle size detection results (Figure 2C) indicated that the diameters of NPs were approximately 17.87 nm, 16.6 nm, 15.19 nm, and 18.25 nm, respectively. The size and morphology of the NPs were further characterized using TEM.

The NPs exhibited a hollow, spherical structure resembling ferritin protein (Figure 2D). The particle diameter ranged approximately from 10 to 20 nm, consistent with the particle size detection results. These findings indicate successful expression of ferritin-supported NPs in the *E. coli* system, where they self-assembled into NPs. Importantly, the addition of epitope did not impair ferritin's self-assembly capability.

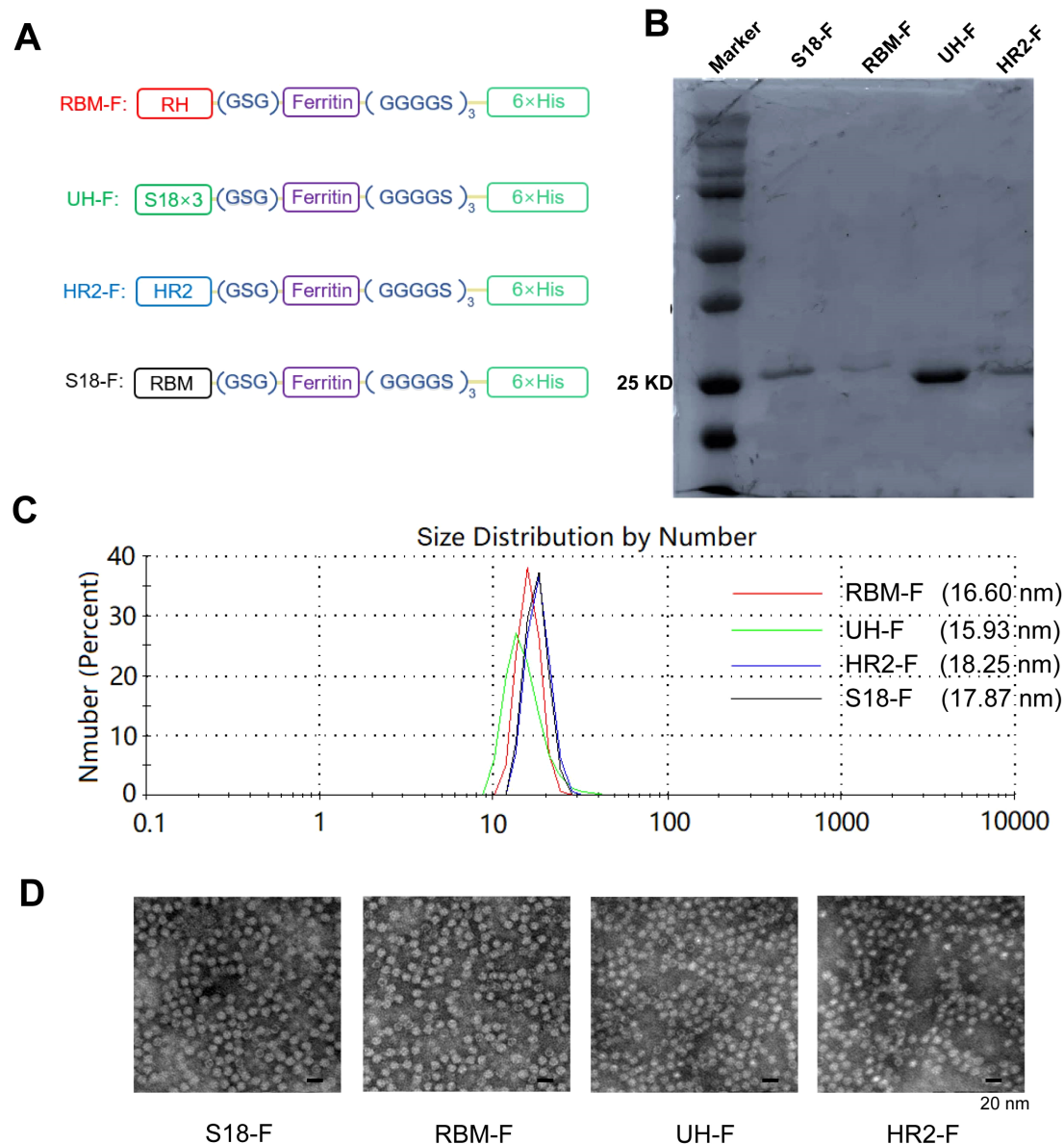


FIGURE 2

Characterization of self-assembled epitope-based NPs. (A) Schematic of conserved epitope-based NPs. (B) SDS-PAGE analysis of NPs. (C) DLS size distribution of NPs. (D) TEM images of NPs.

3.3 Strong humoral immune responses-induced by NP antigen

To assess the immunogenicity of NPs, all mice underwent four vaccinations at 2-week intervals in a prime-boost regimen (Figure 3A). Serum samples were collected every two weeks, and titers of epitope-specific IgG were evaluated using ELISA. The NPs elicited robust titers of epitope-specific antibodies, especially in the RBM-F and UH-F groups. By 8 weeks, specific IgG titers exceeded 10^5 , significantly higher than those in the PBS- and ferritin-immunized groups ($P < 0.0001$) (Figure 3B). Strong epitope-specific immune responses were also observed in the mixed-immunized group. However, due to the lower immunization dose of each NP ($5 \mu\text{g}$ per NP), the titers of specific antibodies for each epitope were relatively diminished compared to the group immunized with NPs alone. This underscores the critical need for future research to refine multi-epitope NP formulations with the aim of enhancing overall immunogenicity.

To assess the long-term changes in antibody levels, serum samples were collected at 12 and 20 weeks post-primary immunization to track the dynamics of antigen-specific antibodies. As shown in Figure 3C, the epitope-specific antibody levels in the S18-F and HR2-F groups peaked at 12 weeks and then slightly decreased but remained high. In contrast, the epitope-specific antibody levels in the RBM-F and UH-F groups peaked two weeks after immunization, with the RBM-F group showing a continued decrease by week 12. For the mixed-immunized group, antibody levels against all epitopes peaked two weeks after the four-week immunization period and then began to decline. In summary, all NP immunogens induced specific antibodies that maintained high binding titers for three months following the last immunization.

The antibody titers of epitope-specific IgG1 and IgG2a were evaluated using an antibody isotyping assay to assess the Th1/Th2 bias in the immune response. The IgG1/IgG2a ratio was calculated to indicate the relative balance between Th1 and Th2 immune

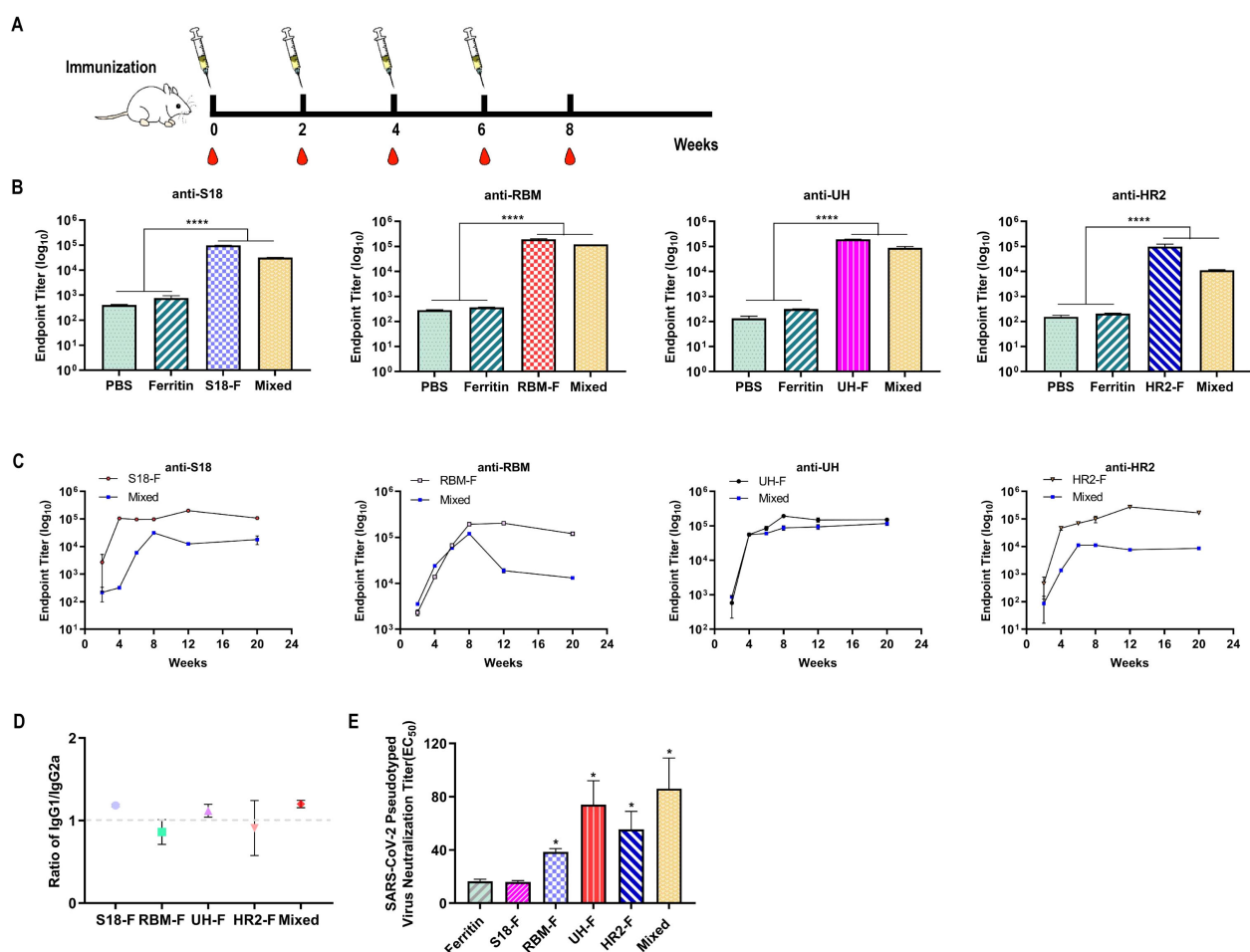


FIGURE 3

Epitope-based NPs induced robust humoral immune responses in mice. (A) Schematic diagram of immunization, and blood collection in BALB/c mice. (B) Epitope-specific IgG titers in sera collected two weeks after the final immunization ($n = 6$). (C) Epitope-specific IgG titers in sera measured from week 0 to week 20 following the initial immunization ($n = 6$). (D) The ratio of epitope-specific antibodies in sera. (E) Serum neutralizing antibody titers against SARS-CoV-2 pseudovirus WH-1 ($n = 6$). (ns, not significant; * $P < 0.05$, **** $P < 0.0001$, compared to the ferritin group).

responses (Lee et al., 2018; Kim et al., 2020). As shown in Figure 3D, the IgG1/IgG2a values in the immunized mouse sera in the S18-F, RBM-F, UH-F, HR2-F, and mixed-immunized groups were close to 0.99, indicating that NPs immunization induced relatively balanced Th1 and Th2 immune responses.

Neutralizing antibodies bind to the surface antigen of a virus, preventing it from adhering to target cell receptors and thus blocking infection. These antibodies are crucial indicators of vaccine-induced humoral immunity. In this study, we employed a pseudovirus neutralization test to determine the neutralization titer of immunized sera. The results indicated that compared to the ferritin-immunized group, serum from the S18-F group did not exhibit a neutralizing effect against the SARS-CoV-2 pseudovirus WH-1 (Figure 3E). However, the neutralization titers in the RBM-F, UH-F, HR2-F, and mixed-immunized groups were significantly higher than those in the ferritin-immunized group ($P < 0.05$), with the mixed-immunized group demonstrating the highest neutralization capacity.

3.4 Potent cellular immune responses induced by NPs in mice

CD3⁺, CD4⁺, and CD8⁺ T cells in the splenic lymphocytes of mice were stained two weeks after the fourth immunization, and the number of these T cells in the splenic lymphocytes was detected by flow cytometry. As shown in Figures 4A, B, CD3⁺ and CD4⁺ T cells were significantly increased in all NP-immunized groups compared to the PBS-immunized group. A significant increase in the number of CD8⁺ cells was observed in the HR2-F group. These results indicate that specific epitopes stimulate T cell activation and proliferation.

Lymphocytes are important cellular components of the immune response in the body. A series of changes in cell proliferation occurs when stimulated by peptides *in vitro*. The lymphocyte proliferation rate can determine the reactivity and functional status of lymphocytes to relevant stimuli, and is one of the indicators used to evaluate cellular immunity. Two weeks after the last immunization, splenic lymphocyte proliferation in immunized mice was stimulated by polypeptides, and proliferation was detected using the MTT assay. As shown in Figure 4C, the splenic lymphocytes of mice in the S18-F, RBM-F, and UH-F groups were significantly increased only under the stimulation of specific epitope peptides compared with those in the PBS-immunized group, whereas no clear proliferation was observed under the stimulation by other peptides. Splenic lymphocytes in the HR2-F group also proliferated slightly upon stimulation by the HR2 peptide. These results indicated that specific epitopes can activate lymphocytes in mice and induce cellular immune responses.

Cytokine secretion by splenic lymphocytes after epitope stimulation was detected using a Th1/Th2 cytokine detection kit. As shown in Figure 4D, IFN- γ secretion increased in S18-F and UH-F groups, which may induce TH1-biased immune response. IFN- γ , IL-2, IL-4 and IL-10 cytokines secreted by splenic lymphocytes of mice in RBM-F and mixed-immunized groups were significantly

increased, indicating that RBM-F and mixed protein immunization may induce balanced Th1 and Th2 immune responses, which was consistent with the results of antibody typing analysis.

The number of splenic lymphocytes secreting IFN- γ was detected using the ELISPOT method. As shown in Figures 4E, F, only splenic lymphocytes from RBM-F immunized mice exhibited a significant increase in IFN- γ secretion upon stimulation with the RBM epitope compared to the control group ($P < 0.001$). The number of lymphocytes secreting IFN- γ in S18-F, UH-F and mixed-immunized groups was slightly higher than that in PBS-immunized group, but the difference was not statistically significant.

ADCC is an important immune defense mechanism of the immune system against viral infections. By specifically binding to the surface antigens of target cells, antibodies recruit effector cells such as natural killer (NK) cells to kill target cells. In this study, the immunized serum-mediated effects of ADCC were examined in mice. The results indicate that the ADCC effect in the UH-F, HR2-F, and mixed-immunized groups was slightly higher compared to the PBS-immunized group (Figure 4G), but this difference did not reach statistical significance. Among all the immunized sera, the S18-F-immunized group showed the highest ADCC activity.

3.5 NP safety in mice

Histopathological sections of major organs from mice, including the heart, liver, spleen, lungs, and kidneys, were examined using H&E staining to assess the safety profile of the NPs developed in the experiment. As shown in Figure 5, no significant pathological changes were observed in the major organ tissues of mice injected with mixed-immunized NPs, indicating the safety of NPs both when used alone and in combination.

4 Discussion

SARS-CoV-2 variants have emerged (Greaney et al., 2021), posing challenges for vaccines targeting S or RBD proteins. Epitope-based vaccines offer advantages, such as rapid production in *E. coli* and the ability to adjust specific epitopes (Li et al., 2014) to counter new strains. They can induce targeted immune responses while minimizing adverse reactions. However, these vaccines face challenges due to epitope instability and lower immunogenicity, necessitating carefully designed adjuvants and delivery systems.

In this study, immunoinformatics tools were used to screen potential epitope sequences (S18, RBM, UH, and HR2). A self-assembling NP vaccine was designed using ferritin as a carrier. Due to variations in immunogenicity among these epitopes, the time required for specific antibodies to reach peak levels varied across groups that might be crucial for optimizing vaccine design and efficacy.

To evaluate the immunogenicity of these epitope NPs, mice were immunized four times at two-week intervals. Mice immunized with NPs produced high-titer specific antibodies against the targeted epitopes, with antibody titers exceeding 10^4 . In contrast,

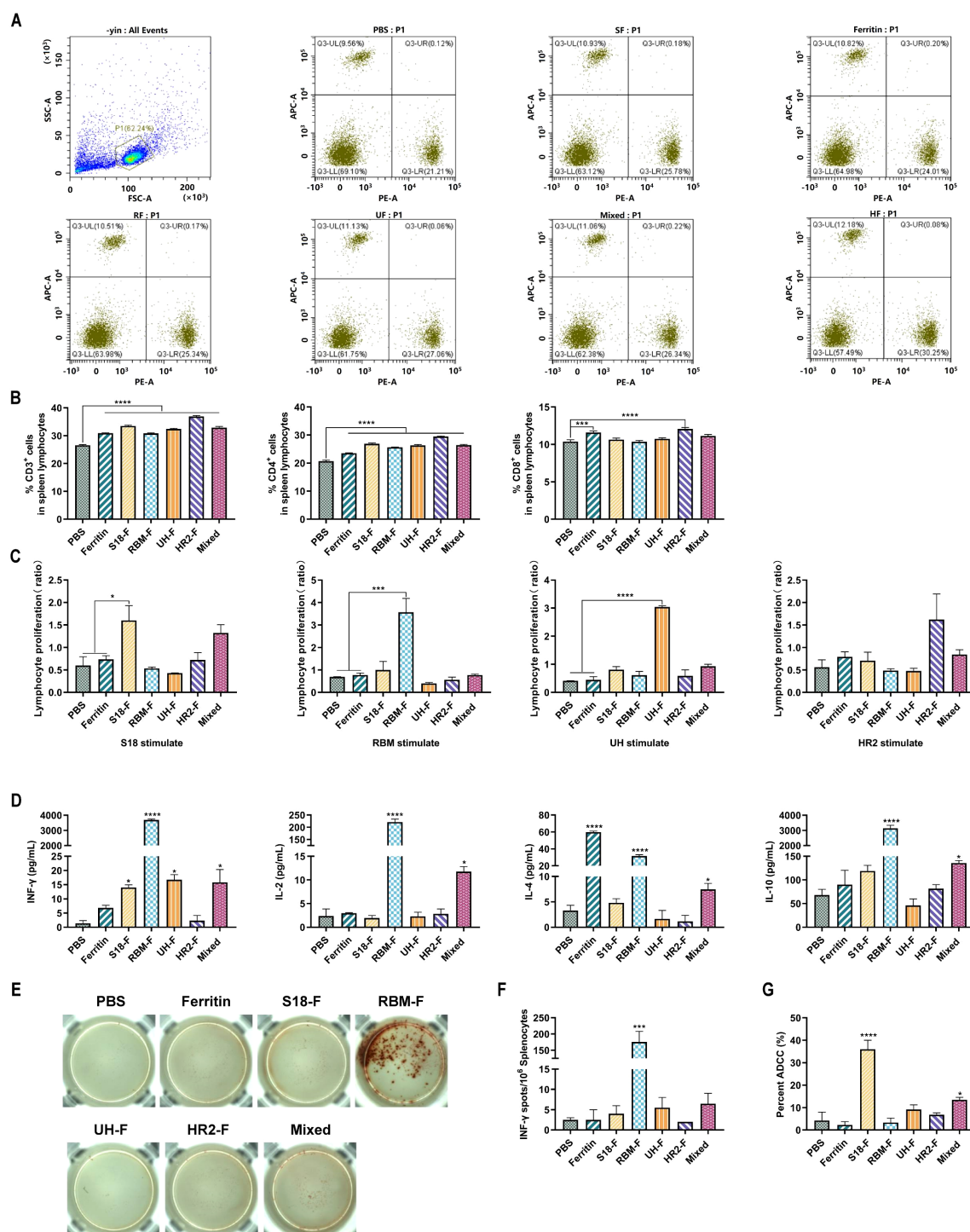


FIGURE 4

Epitope-based NPs induced robust cellular immune responses in mice. (A) Representative flow cytometric analysis of CD3⁺CD4⁺ and CD3⁺CD8⁺ T cells in the splenic lymphocytes. (B) Quantification of CD3⁺, CD4⁺, and CD8⁺ T cell proportion in the splenic lymphocytes (n = 6). (C) Peptide-stimulated splenic lymphocyte proliferation levels. (D) The concentrations of IL-2, IL-4, IL-10 and IFN-γ in serum (n = 6). (E) The spot wells of IFN-γ cytokine-secreting lymphocytes. (F) IFN-γ-secreting lymphocytes isolated from spleens were determined by ELISpot (n = 6). (G) ADCC effect of serum (n = 6). (ns, not significant; *P < 0.05, ***P < 0.001, ****P < 0.0001).

mice in the control group immunized with ferritin or PBS did not produce specific antibodies against these epitopes. Qi S et al. observed significant differences in binding antibody titers in the plasma of New Zealand white rabbits immunized with different

epitopes, with some epitopes failing to induce binding antibody production (Sun et al., 2022).

Long-term monitoring of specific antibody levels is influenced by various factors, including the induction of targeted antigens,

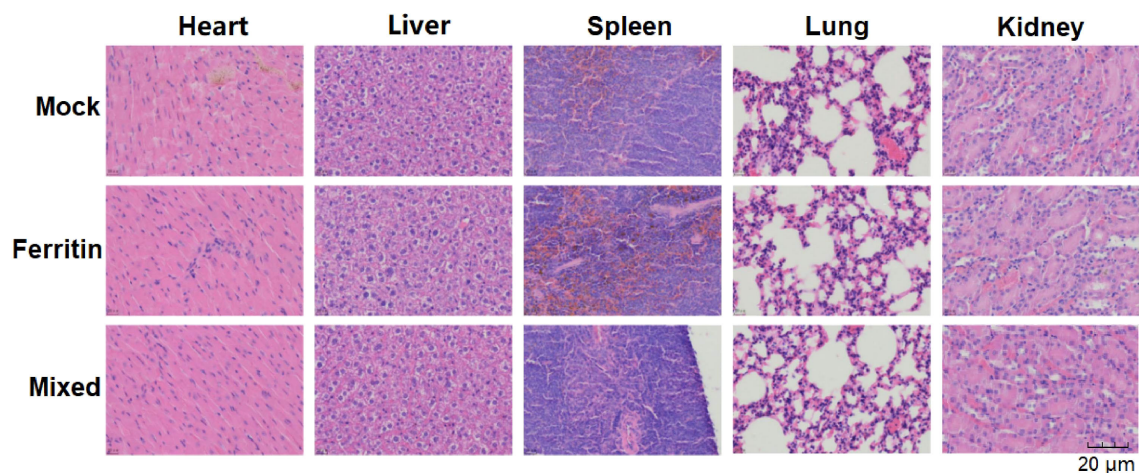


FIGURE 5
H&E staining of mouse tissue histopathological sections.

engagement of immune response mechanisms—especially $CD4^+$ and $CD8^+$ T cells—and the generation of memory cells (Tillmann et al., 2022; Perez-Saez et al., 2023). Specific antibody titers were sustained for three months in the serum of immunized mice. Although a fourth immunization might sustain antibody production, our findings suggest that the long-term effect is not solely dependent on the fourth immunization. These results indicate that the four epitope-based NPs can induce potent and long-lasting antigen-specific humoral immune responses.

Antibody typing was conducted to determine the types and subtypes of antibodies produced, with the ratio of IgG1 to IgG2a reflecting the immune response bias. Analysis of antibody types in the serum of mice immunized with NPs revealed a close to 1 ratio of IgG1 to IgG2a, indicating a balanced Th1 and Th2 immune response induced by NPs. However, it is important to note that relying solely on IgG1 and IgG2a ratios may not suffice; comprehensive analysis requires integration with other indicators to fully understand the immune response.

Neutralizing antibodies serve as critical indicators of viral vaccine efficacy, capable of binding to SARS-CoV-2 and preventing viral cell infection. This study evaluated the serum's ability from immunized mice to neutralize SARS-CoV-2 using a pseudovirus neutralization assay. Results showed that mice immunized with PBS, ferritin, and S18-F did not produce neutralizing antibodies against the wild-type pseudovirus WH-1. In contrast, sera from the RBM-F, UH-F, HR2-F, and mixed-immunized groups effectively neutralized pseudovirus WH-1. Despite RBM being considered the primary region for inducing neutralizing antibodies against SARS-CoV-2 (Hastie et al., 2021), the RBM epitope used in this study did not yield high neutralizing antibody titers. Previous studies (Long et al., 2022) have shown that sera from mice immunized with RBM did not exhibit RBD-ACE2 blocking activity, in contrast to sera from monkeys immunized with RBM, which demonstrated neutralizing antibody titers. Comparative studies between different animal models, including

primates and rodents, could continue to elucidate species-specific immune responses and further refine vaccine development strategies against emerging viral variants.

Predicting potential binding peptides of mouse major histocompatibility complex (MHC) II molecules for the entire RBD revealed that mouse MHC II molecules had significantly weaker recognition capabilities for the RBM epitope compared to human MHC II molecules. This suggests potential species differences in RBM epitope antigenicity and immunogenicity.

Cellular immunity is crucial for evaluating the efficacy of antiviral vaccines. In this study, splenic lymphocytes from immunized mice were stimulated with specific antigens to assess immune responses. $CD3^+$, $CD4^+$, and $CD8^+$ T cells, fundamental subgroups in immune responses, were analyzed. Post-immunization, all groups showed significant increases in $CD3^+$ and $CD4^+$ T cells, with the HR2-F group notably increasing $CD8^+$ T cells, enhancing viral resistance.

Stimulation with RBM epitopes in the RBM-F group led to increased secretion of $IFN-\gamma$, IL-2, IL-4, and IL-10 cytokines by splenic lymphocytes. S18-F and UH-F immunizations enhanced antiviral capabilities primarily through increased $IFN-\gamma$ levels. Furthermore, ADCC assays demonstrated that sera from S18-F and mixed-immunized groups exhibited ADCC activity, indicating their ability to bind to infected cell surface antigens and activate NK cells or leukocyte Fc- γ receptors to kill infected cells.

Serum from the UH group demonstrated potent neutralization of the SARS-CoV-2 pseudovirus, highlighting its substantial neutralizing capability. Located within the S2 subunit, the UH epitope elicited the highest titers of neutralizing antibodies and exhibits greater conservation across SARS-CoV-2 variants. Targeting this epitope may confer broad cross-neutralizing activity against diverse variants of SARS-CoV-2, though further experimental validation is needed.

The mixed-immunized group demonstrated robust and comprehensive immune responses, characterized by the highest

neutralizing antibody titers among all groups, indicating enhanced cross-neutralizing potential. Despite lower specific antibody titers for individual epitopes due to reduced doses (5 µg per epitope), the group exhibited balanced Th1/Th2 immune responses, significant T cell activation (increased CD3⁺ and CD4⁺ T cells), and durable humoral immunity lasting up to three months post-immunization. Additionally, the mixed-immunized group showed potent ADCC activity and broad-spectrum neutralizing capabilities, suggesting that multi-epitope nanoparticles might synergistically induce a more comprehensive and effective immune response against SARS-CoV-2.

Single-epitope immunogens induced limited neutralizing antibody titers, suggesting that enhanced efficacy could be achieved through optimized combinations of multiple epitopes. Xiancai Ma et al. used ferritin to deliver full-length RBD and HR domains of SARS-CoV-2, inducing neutralizing antibodies in pseudovirus assays (Ma et al., 2020). This highlights the potential of RBD and S2 domains to elicit broad-spectrum neutralizing antibodies and cross-reactive immune responses. However, no significant difference in neutralizing antibody titers was found between mixed- and single-protein immunized groups. This emphasizes the potential of conserved linear epitopes derived from the S protein in the development of multi-epitope SARS-CoV-2 vaccines. In our study, the mixed-immunized group, containing multiple effective epitopes, induced higher neutralizing antibody titers against the SARS-CoV-2 pseudovirus (WH-1) compared to single-epitope immunized groups. This suggests that multi-epitope nanoparticles (NPs) may synergistically elicit a more comprehensive and effective immune response against SARS-CoV-2. Future research will optimize these epitopes for a multi-epitope nanoparticle (NP) vaccine and evaluate its cross-protective efficacy through challenge experiments using variants of concern (VOCs) and bat coronaviruses (bat-CoVs). Additionally, evidence suggests that ferritin carriers might enhance mucosal immune responses, potentially serving as self-adjuvants in mucosal immunity (Shah et al., 2021). Verification of these epitope NPs' effectiveness in eliciting immune responses awaits further study.

5 Conclusion

Potential epitopes of the S protein were predicted using immunoinformatics. Recombinant epitopes from the S protein were fused with ferritin NPs and produced using an *E. coli* expression system. Subcutaneous immunization of BALB/c mice with these NPs successfully induced robust epitope-specific humoral immunity. The RBM-F, UH-F, and HR2-F also elicited potent neutralizing antibodies, which persisted for more than 3 months' post-immunization. The RBM-F, UH-F, and HR2-F demonstrated robust neutralizing antibody responses against the WH-1 pseudovirus. Moreover, stimulation with corresponding epitopes significantly enhanced splenic lymphocyte proliferation in the S18-F, RBM-F, and UH-F groups, underscoring the

promotion of vigorous cellular immune responses by these epitopes. The RBM epitope induced increased secretion of IFN-γ, IL-2, IL-4, and IL-10 cytokines in lymphocytes, suggesting its capacity to elicit a balanced Th1/Th2 immune response. Furthermore, immune sera from the S18-F and mixed-immunized groups exhibited substantial ADCC activity, indicating enhanced splenic cell-mediated recognition and elimination of infected cells. As This study demonstrates that ferritin NPs, incorporating selected epitopes of the SARS-CoV-2 S protein, induce strong and durable immune responses in mice. These findings underscore the potential of this approach for developing effective vaccines against COVID-19.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was approved by Institutional Animal Care and Use Committee of Jilin University. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

YL: Conceptualization, Methodology, Writing – review & editing. CL: Conceptualization, Methodology, Writing – review & editing. ZW: Formal Analysis, Writing – review & editing. YZ: Writing – review & editing. TY: Writing – review & editing. XL: Writing – review & editing. JH: Writing – review & editing. YP: Formal Analysis, Writing – review & editing. QW: Writing – review & editing. YS: Supervision, Visualization, Writing – review & editing. XQ: Conceptualization, Methodology, Supervision, Writing – original draft.

Funding

The author(s) declare that financial support was received for the research and/or publication of this article. This work was supported by Science and Technology Department of Jilin Province (Grant Nos. 20210101270JC and YDZJ202201ZYTS115).

Conflict of interest

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fcimb.2025.1560330/full#supplementary-material>

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OPEN ACCESS

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RECEIVED 24 October 2024

ACCEPTED 01 April 2025

PUBLISHED 22 April 2025

CITATION

Alalwan D, Leon AAG, Saini G, Gaillard C,
Negi R, Heckmann C, Kenny G, Feeney E,
Cotter AG, Kelly C, Carr M, de Barra E,
Yousif O, Horgan M, Sadlier C, Landay A,
Gonzalez G and Mallon PWG (2025)
Associations between type III interferons,
obesity and clinical severity of COVID-19.
Front. Immunol. 16:1516756.
doi: 10.3389/fimmu.2025.1516756

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Associations between type III interferons, obesity and clinical severity of COVID-19

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Introduction: Severe COVID-19 is characterized by hyperimmune host responses contributing to airway damage and acute respiratory distress syndrome. Type III interferons (IFN), including IFN lambda 4 (IFNλ4), expressed in individuals harboring the rs368234815-ΔG allele, are implicated in host immune responses to viral infections, including SARS-CoV-2.

Methods: We investigated associations between IFNλ4 expression through genotyping and COVID-19 disease severity in 853 laboratory-confirmed SARS-CoV-2 cases enrolled in the All-Ireland Infectious Diseases Cohort. Additionally, we measured plasma levels of Type I, II and III IFN using quantitative immunoassays along with IFNλ4 expression and COVID-19 disease severity in a sub-group [n=321 (37.6%)] with samples available within 10 days of symptom onset. IFNλ4 was expressed in 382 (44.8%) but expression was not significantly associated with COVID-19 disease severity.

Results: Within the sub-group, we found no consistent associations between IFNλ4 expression and circulating IFNs. However, we observed significantly increased expression of IFNλ1 and IFNλ2 in severe COVID-19 (P<0.01), with IFNλ2 remaining significantly associated after adjustment for age, sex, ethnicity, and comorbidities, including obesity (BMI≥30 kg/m²) (P<0.001). Interestingly, although IFNλ2 levels were significantly higher in subjects with obesity, the association between higher IFNλ2 and COVID-19 disease severity was only observed in individuals without obesity (P<0.01).

Conclusion: These data reveal an important role for IFN λ 2 as an immune correlate that predicts COVID-19 disease severity, which may be masked in those with obesity.

KEYWORDS

SARS-CoV-2, COVID-19, interferons, IFN λ 2, IFN λ 4, obesity

1 Introduction

At the onset of the COVID-19 pandemic, despite limited genetic variation in the SARS-CoV-2 genome, some individuals developed respiratory failure, while others remained asymptomatic or experienced only mild symptoms (1), even before treatments or prophylactic vaccines became widely available. Older age and presence of clinical comorbidities such as obesity are associated with an increased risk of severe COVID-19 (2), particularly in the pre-vaccine era, possibly mediated through an impaired cellular environment resulting in distorted expression of interferons (IFNs) and an impaired antiviral immunity (3).

IFNs, a principal group of antiviral cytokines, are broadly classified into three main types based on their specific receptors and structural homologies, including IFN types I, II, and III (4). Type I IFNs include IFN α , β , ϵ , κ , ω , and δ , type II comprises IFN γ (5), while type III IFNs comprise the interferon lambda (IFN λ) family and were initially termed IL-29, IL-28A, and IL-28B, but later renamed IFN λ 1, 2, and 3, respectively (6).

Genome-wide association studies (GWAS) have identified single nucleotide polymorphisms (SNPs) that are associated with expression of a fourth type III IFN; IFN λ 4. Together, the IFN λ family have been shown to impact on antiviral immune responses and correlate to infectious disease outcomes in HCV, HIV, CMV, and influenza viral infection (7, 8).

IFN λ 4 expression is determined by two major SNPs: rs368234815 and rs117648444. The former harbors a frameshift mutation that changes the genotype from Δ G-expressing to TT-non-expressing. The Δ G genotype expression allows for the production of a functional IFN λ 4 open reading frame for a functional IFN λ 4 protein, while the TT allele results in a truncated non-functional protein which abolishes the expression of IFN λ 4 (9). Importantly, IFN λ 4 expression is found in only 50% of the world population with striking ethnic differences; 50% of Europeans, 90% of Africans, and 10% of Asian individuals (10).

The genotype of a neighboring SNP, rs117648444 (G/A: IFN λ 4 P70/S70), can also modify the biological activity of rs368234815- Δ G (10). The rs117648444 activity variant SNP results in a missense mutation that gives rise to a non-synonymous mutation P70S (11). This hypomorphic rs117648444-A allele only occurs when the Δ G is present in rs368234815. The two SNPs can thus differentiate

individuals into three genotypic groups: IFN λ 4-null (rs368234815-TT/TT, rs117648444-G/G), IFN λ 4-strong (rs368234815-TT/ Δ G and Δ G/ Δ G, rs117648444-G/A), and IFN λ 4-weak (rs368234815-TT/ Δ G and Δ G/ Δ G, rs117648444-A/A) (10).

Obesity and a high body mass index (BMI) were classified as significant predictors of COVID-19 outcomes and severity (12). The relationship between inflammation and metabolism has recently become evident, as changes in metabolism, such as those seen in obesity, can lead to inflammation. Conversely, inflammation resulting from viral infections can also induce metabolic changes (13). Teran-Cabanillas et al. have demonstrated impaired interferon responses in individuals with obesity, specifically IFN α , IFN β and IFN λ , affecting their immune function (14). This indicates the role that obesity plays in modulating immune responses to viral infections.

Dysregulated IFN responses are deemed a key factor in COVID-19 pathogenesis, but conflicting results are observed in different studies examining COVID-19 disease severity and IFN responses. Early induction of type I and III IFNs may produce a favorable outcome of COVID-19 disease progression (15). While type I IFNs act rapidly to induce an immune response via the expression of chemokines and cytokines (16), type III are considered to be tissue protective and usually lack the accompanying pro-inflammatory response induced by type I IFNs (17). However, a number of studies have also associated type I IFN responses with an increased expression of interferon stimulated genes (ISGs), proinflammatory genes, and cytokines in individuals with severe COVID-19 (18). Kwon et al. have shown inflated type I/II IFNs (IFN α and IFN γ) in severe COVID-19 within 5-10 days of symptom onset, notably IFN α tended to correlate with the viral load in their cohort (19). Conversely, other studies have shown a greatly weakened type I and III IFN response in early COVID-19 infection (20). The timing of IFN induction whether type I or III, may also be an integral part in regulating the outcome of COVID-19 disease.

Given this lack of clarity on the role of IFNs in COVID-19 infection, we sought to investigate what role, if any, expression of IFN λ 4 plays in modifying disease severity in COVID-19 and additionally to explore relationships between early infection levels of other circulating type I, II, and III IFNs and COVID-19 disease severity, considering important clinical predictors such as age and presence of comorbidities such as obesity.

2 Materials and methods

2.1 Study design and participants

This analysis was conducted within the All-Ireland Infectious Diseases Cohort (AIID Cohort), a prospective, multicenter, observational cohort that recruits individuals attending hospitals in Ireland for issues relating to infectious diseases, including COVID-19. The study was approved by the National Research Ethics Committee as part of The AIID Cohort (20-NREC-COV-056), and all subjects provided written informed consent for the collection of samples and clinical data for further research. This analysis was restricted to participants with confirmed PCR-positive SARS-CoV-2 with samples collected between March 2020 and June 2021. The participants were grouped according to the WHO COVID-19 severity scale into mild, moderate, and severe/critical (21). Within the analytical group, a subgroup of biobanked samples collected within 10 days of symptom onset was selected for the analysis of early IFN responses. This timeframe was chosen to capture the critical window of early immune activation, which may occur before the onset of severe symptoms, typically observed on or after day 10.

2.2 Nucleic acid extraction

Genomic DNA was extracted from Biobanked buffy coats obtained from 3 mL sodium citrate blood, or cellular free DNA was extracted from plasma samples obtained from 10 mL ethylenediaminetetraacetic acid (EDTA) blood were utilized for DNA extraction using the MagNA Pure 96 instrument and large volume kit (Roche Diagnostics, Rotkreuz, Switzerland), as per manufacturer's instructions.

2.3 Genotyping assay

IFN λ 4 SNPs rs368234815 and rs117648444 genotyping was performed employing the TaqMan SNP genotyping assay using custom made detection mixes (Applied Biosystems, Waltham, Massachusetts, USA), as described previously (22). Briefly, the SNP genotyping assays are dependent on differently labelled fluorescent probes that discriminate the target nucleotide sequence. The VIC dye detects Allele 1 (Allele X) wild-type sequence, and the FAM dye detects Allele 2 (Allele Y) mutant sequence. Heterozygous samples contain an equal signal contribution of both FAM and VIC dyes.

2.4 Immunoassay biomarkers measurement

Type I IFN (IFN α 2a and IFN β), Type II IFN (IFN γ) and Type III IFN (IFN λ 1) were measured using a multiplex electrochemiluminescence assay (Meso Scale Discovery, Rockland, MD, USA, Cat no. K15094K-2). IFN λ 2 and IFN λ 3 were measured

using the Luminex MAGPIX platform (Biotechne R&D Systems, Minneapolis, MN, USA, Kit name: LXSAHM-22), results were analyzed using the Luminex xPONENT for MAGPIX software (version 4.3). EDTA plasma samples were run in duplicate alongside a calibration curve. Samples with intraplate coefficient of variance (CV) above 10% were repeated. Samples with variable CV after two repeats were excluded from further analysis.

2.5 Statistical analysis

We used Hardy-Weinberg analysis to test the equilibrium of the population genetics and Chi-Square χ^2 tests with the goodness of fit with two degrees of freedom to investigate the observed and expected allele frequencies. The association of COVID-19 disease severity outcome with SNP genotypes of rs368234815 (TT/TT, TT/ Δ G, and Δ G/ Δ G), and between group demographic differences were analyzed using a Chi-Square χ^2 test to compare counts of categorical variables and Kruskal-Wallis test to compare distributions of continuous variables. Measured circulating interferon concentrations were natural log transformed and we employed a stepwise multinomial logistic regression to explore the impact of clinical covariates on the relationship between IFN levels and COVID-19 disease severity, correcting for age, sex, Caucasian ethnicity, comorbidities (obesity (BMI $\geq$ 30 kg/m²), metabolic disease, and respiratory disease), and other significant IFNs associated with disease severity in univariate analysis. A P-value <0.05 was considered significant. Data are presented as median (interquartile range) unless stated. All statistical analyses were performed with IBM® SPSS® Statistics (version 27), RRID: SCR_016479, GraphPad Prism Software, LLC. (version 9.5.1), RRID: SCR_002798, and R (R version 2024.04.0 + 735, <http://www.r-project.org>), RRID: SCR_001905, using ggplot2 package version 3.1.1 (23), RRID: SCR_014601.

3 Results

3.1 Cohort demographics

Of 886 laboratory-confirmed SARS-CoV-2, AIID participants that were eligible for inclusion in the analysis, 853 had available data. Characteristics of the study population are shown in Table 1. Median (IQR) age was 53.8 (39.6, 66.7), 486 (56.9%) were female and the majority (78.5%) of the cohort were of Caucasian ethnicity. Participants were categorized as having mild [n=500 (58.62%)], moderate [n=164 (19.2%)], and severe [n=189 (22.2%)] COVID-19.

Of the full cohort, 321 (37.6%) participants had samples collected within 10 days of symptom onset and were included in the sub-group analysis of IFN biomarkers (Table 2). Within this sub-group, median (IQR) age was 62.1 (47.8, 76.7), with a majority male [n=174 (54.2%)] and of Caucasian ethnicity [n=252 (78.5%)]. Most of the sub-cohort (81.5%) reported underlying comorbidities including but not limited to hypertension [n=135 (42.2%)], diabetes [n=47 (14.7%)], obesity [n=253 (29.7%)], and respiratory disease [n=81 (25.3%)]. Median (IQR) BMI was 27.4 (24.2, 31.8) kg/m².

TABLE 1 Characteristics of overall study population.

WHO COVID-19 Severity Category	Total [n=853]	Mild [n=500 (58.62%)]	Moderate [n=164 (19.2%)]	Severe [n=189 (22.2%)]	P
Age (years) - Median (IQR)	53.8 (39.6, 66.7)	45.9 (32.6, 61.0)	58.7 (50.0, 70.5)	63.8 (53.4, 75.4)	<0.0001
Sex at Birth - N (%)					
Female	486 (56.9)	328 (65.6)	75 (45.7)	83 (43.9)	<0.0001
Male	367 (43.1)	172 (34.4)	89 (54.3)	106 (56.1)	
Ethnicity - N (%)					
Caucasian	670 (78.5)	421 (84.2)	116 (70.7)	136 (72.0)	<0.0001
Other	183 (21.5)	79 (15.8)	48 (29.3)	53 (28.0)	
BMI (kg/m ²) - Median (IQR)	27.7 (24.2, 32.3)	25.9 (23.2, 31.1)	28.9 (25.4, 32.5)	30.0 (26.5, 34.3)	<0.0001

Characteristics of Overall Study Population. IQR, interquartile range; BMI, body mass index; WHO, World Health Organization. Chi-Square χ^2 test to compare counts of categorical variables and Kruskal-Wallis test to compare distributions of continuous variables.

Bold means significant P values.

The majority (84.7%) of the cohort were admitted to hospital primarily due to SARS-CoV-2 infection.

3.2 Interferon λ SNPs genotype characteristics and association with disease severity

Allelic discrimination data for the cohort (n=853) yielded rs368234815 SNP TT/TT major homozygous genotype [n=471 (55.2%)], TT/ Δ G heterozygotes [n=310 (36.3%)], and Δ G/ Δ G minor homozygotes [n=72 (8.4%)] respectively, with 382 (44.8%) of the cohort expressing IFN λ 4 (TT/ Δ G, Δ G/ Δ G). The rs117648444 SNP showed 88.3% (n=753), 11.25% (n=96), and 0.47% (n=4) allelic frequencies for the three genotypes G/G, G/A, and A/A, respectively. Of those expressing IFN λ 4, 312 (81.7%) had a strong IFN λ 4 outcome. Hardy-Weinberg tests showed no significant departure from equilibrium for both investigated SNPs in this cohort [rs368234815 (P=0.127) and rs117648444 (P=0.884)]. The distribution of genotype allele frequencies of rs368234815 and rs117648444 SNPs according to COVID-19 disease severity group are shown in **Supplementary Table 1**. There were no significant differences in genotype frequencies between COVID-19 severity groups.

3.3 Circulating plasma levels of type I, II, and III interferons

We next investigated whether type I (IFN α 2a, and IFN β) or type II (IFN γ) IFN concentrations in plasma varied with the expression of IFN λ 4-rs368234815. Only IFN α 2a concentrations differed, with significantly higher concentrations in those not expressing IFN λ 4 (rs368234815-TT/TT) [0.819 (-0.544, 2.422) pg/mL] compared to expressing IFN λ 4 rs368234815-TT/ Δ G and Δ G/ Δ G genotypes [0.214 (-1.278, 2.027) pg/mL] (P=0.041). Concentrations of IFN λ 1, IFN λ 2, and IFN λ 3 did not vary between those who express and do not express IFN λ 4 (**Figure 1A**).

Concentrations of type I (IFN α 2a, and IFN β) and type II (IFN γ) IFNs were not different between COVID-19 disease severity groups (**Figure 1B**). For type III IFNs (IFN λ 1, IFN λ 2, and IFN λ 3), IFN λ 1 levels were higher in those with severe COVID-19 [3.789 (3.031, 4.277) pg/mL] compared to mild [3.269 (2.747, 3.842) pg/mL] (P=0.0028), and moderate [3.288 (2.675, 3.858) pg/mL] (P=0.0096) groups. Similarly, IFN λ 2 levels were also significantly higher in the severe group [3.799 (3.694, 3.932) pg/mL] compared to the mild [3.698 (3.540, 3.838) pg/mL] (P<0.001), but not different from the moderate group [3.739 (3.656, 3.872) pg/mL]. Circulating IFN λ 3 levels did not vary between COVID-19 severity groups (**Figure 1B**).

3.4 Impact of obesity on IFN lambda and COVID-19 disease severity

We next investigated the association between obesity (BMI $\geq$ 30 kg/m²) and the induction of IFN responses across different COVID-19 severity categories, given its strong link to severe disease outcomes and related comorbidities, which may influence immune responses. The median (IQR) BMI in the mild [25.1 (23.0, 30.9)] COVID-19 group was significantly lower than the moderate [29.4 (26.1, 32.5) kg/m²] and the severe [29.2 (25.7, 32.3) kg/m²] groups (P=0.0006). Between IFN λ 1 and IFN λ 2, only IFN λ 2 levels were significantly higher in those with obesity (**Figure 2A**), while IFN λ 1 levels did not significantly differ between those with and without obesity. However, exploring associations between IFN λ 2 and COVID-19 severity according to obesity group, revealed a significantly higher IFN λ 2 in those with severe disease only in the people without obesity [3.808 (3.670, 3.945) pg/mL] group (P=0.0017) (**Figure 2B**), as opposed to people with obesity group [3.799 (3.696, 3.910) pg/mL] (**Figure 2C**).

In a forward, stepwise, multinomial logistic regression exploring factors associated with COVID-19 severity, including demographics (age, sex at birth, and ethnicity), and comorbidities (obesity, metabolic disease, and respiratory disease) (**Figure 3**), IFN λ 2 was the only biomarker that remained significantly associated with

TABLE 2 Clinicopathological features of early sampling study population.

WHO COVID-19 Sever- ity Category	Total [N=321]	Mild [n=156 (48.6%)]	Moderate [n=70 (21.8%)]	Severe [n=95 (29.6%)]	P
Age (years) - Median (IQR)	62.1 (47.8, 76.7)	58.8 (33.3, 73.5)	58.7 (51.5, 73.7)	70.7 (56.0, 80.4)	<0.0001
Sex at Birth - N (%)					
Female	147 (45.8)	77 (49.4)	33 (47.1)	37 (38.9)	0.2666
Male	174 (54.2)	79 (50.6)	37 (52.9)	58 (61.1)	
Ethnicity - N (%)					
Caucasian	252 (78.5)	134 (85.9)	51 (72.9)	67 (70.5)	0.0069
Other	69 (21.5)	22 (14.1)	19 (27.1)	28 (29.5)	
Underlying Comorbidities - N (%)	260 (81.5)	116 (74.4)	60 (87.0)	84 (89.4)	0.0118
Hypertension	135 (42.2)	58 (37.2)	27 (38.6)	50 (53.2)	0.0461
Diabetes	47 (14.7)	19 (12.2)	13 (18.6)	15 (16.0)	0.3909
Obesity	253 (29.7)	34 (21.8)	23 (32.9)	32 (33.7)	0.0692
Respiratory Disease	81 (25.3)	25 (16.0)	25 (35.7)	31 (33.0)	0.0010
Renal Disease	2 (0.6)	1 (0.6)	0 (0.0)	1 (1.1)	0.6967
Immunosuppressive Conditions	14 (4.4)	6 (3.8)	1 (1.4)	7 (7.4)	0.1651
BMI (kg/m²) - Median (IQR)	27.4 (24.2, 31.8)	25.1 (23.0, 30.9)	29.4 (26.1, 32.5)	29.2 (25.7, 32.3)	0.0006
Hospitalization Status - N (%)					
Admitted	272 (84.7)	107 (68.6)	70 (100)	95 (100)	<0.0001
Outpatient	49 (25.3)	49 (31.4)	0 (0.0)	0 (0.0)	
COVID-19 Complications - N (%)					
Invasive Ventilation	15 (4.6)	1 (0.6)	0 (0.0)	14 (14.7)	<0.0001
Viral Pneumonia	107 (33.0)	12 (7.7)	37 (53.9)	58 (61.1)	<0.0001
Bacterial Pneumonia	32 (9.9)	6 (3.8)	10 (14.3)	16 (16.8)	0.0015
ARDS	52 (16.0)	0 (0.0)	2 (2.9)	50 (52.6)	<0.0001
Disease Outcome - N (%)					
Discharged	235 (73.2)	105 (67.3)	70 (100)	60 (63.2)	<0.0001
Death	36 (11.2)	1 (0.6)	0 (0.0)	35 (36.8)	
Outpatient	50 (15.6)	50 (32.1)	0 (0.0)	0 (0.0)	

Clinicopathological Features of Early Sampling Study Population. IQR, interquartile range; BMI, body mass index; WHO, World Health Organization; ARDS, Acute Respiratory Distress Syndrome. Chi-Square χ^2 test to compare counts of categorical variables and Kruskal-Wallis test to compare distributions of continuous variables. Bold means significant P values.

severe COVID-19 in fully adjusted analyses, with higher levels of IFN λ 2 associated with a higher likelihood of severe COVID-19 [odds ratio [EXP(B)] (95% confidence interval)] [8.165 (1.850, 36.04)]. In addition, a history of respiratory disease, older age and non-Caucasian ethnicity were also independently associated with severe COVID-19 in fully adjusted analyses.

4 Discussion

This study investigated the association of early host IFN responses with COVID-19 disease severity. We demonstrate that

type III IFNs, and particularly IFN λ 2, may play a role in COVID-19 disease outcome. Additionally, we found higher IFN λ 2 levels in those with obesity and a relationship between IFN λ 2 and COVID-19 disease severity that was only observed in those without obesity, suggesting an interaction between obesity and IFN λ 2 responses that could help explain differing clinical outcomes to COVID-19 in people with obesity. However, when investigating the expression of two common IFN λ 4 SNP genotypes of rs368234815 and rs117648444, we found that the expression of IFN λ 4 had no impact on clinical disease severity in COVID-19 in this study.

Type I and III IFNs demonstrate a paradoxical role in mediating host immune responses to SARS-CoV-2 infection, as their role in

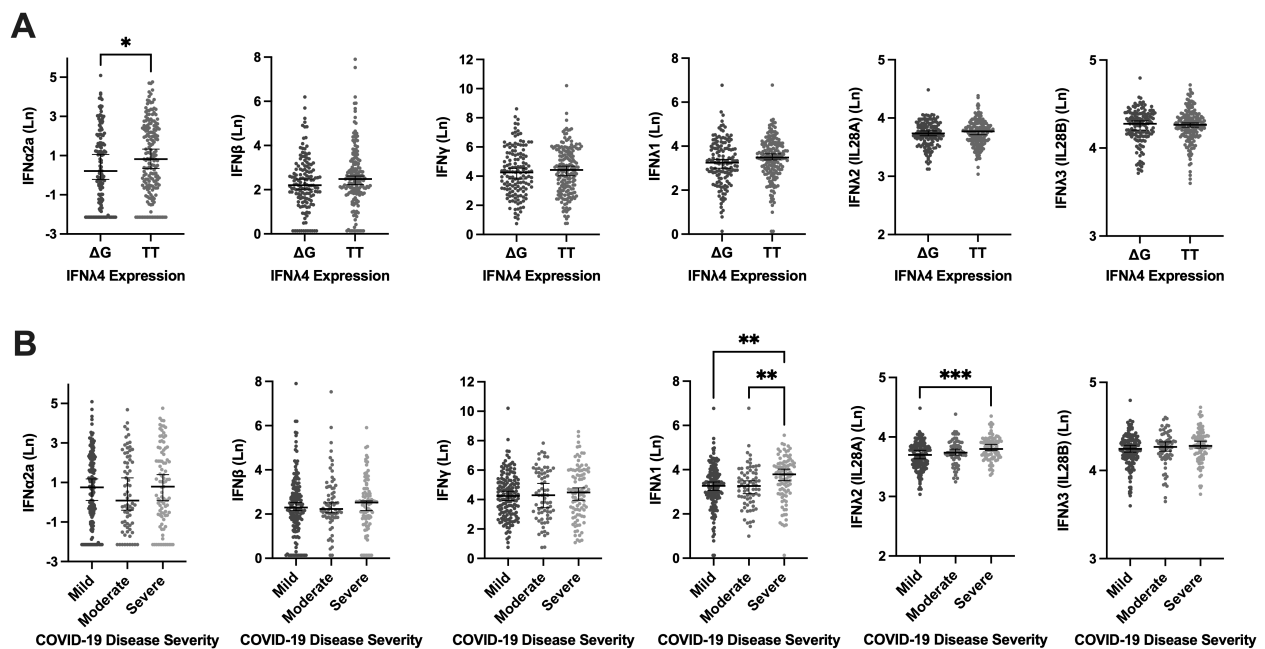


FIGURE 1

Circulating Levels of Type I (IFNα2a, and IFNβ), Type II (IFNγ), and Type III [IFNλ1, IFNλ2 (IL28A), IFNλ3 (IL28B)] Interferons Stratified by IFNλ4 Expression and COVID-19 Severity Groups in the Sub-cohort (n=321). (A) Assessment of the expressing (TT/ΔG, and ΔG/ΔG) and non-expressing genotypes (TT/TT) of IFNλ4 effects on the concentration of Type I, Type II, and Type III Interferons. (B) WHO COVID-19 disease severity criteria association with plasma concentration of Interferons. (Ln) Natural log. Error bars represent the median and interquartile range. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001.

early infection is not well elucidated, with early increased levels shown to be protective against severe disease in some studies, while others have shown the opposite (24, 25). Zaleska et al. found lower levels of IFNλ2 in severe COVID-19 compared to the moderate group, where the majority of the severe group had undetectable levels of IFNλ2 (26). In contrast, our unadjusted analyses showed higher IFNλ1 and IFNλ2 levels in those with moderate and severe COVID-19, possibly driven by the high prevalence of comorbidities in our cohort, which may have amplified the type III IFN response.

Nonetheless, our findings align with Ruytinx et al. who demonstrated higher IFNλ1 in severe and critically ill individuals with COVID-19, with increased IFNλ1 also associated with a higher odds ratio of ICU mortality (27). However, after adjustment, only higher IFNλ2 remained associated with more severe COVID-19 in our cohort, with the opposite observed in the cohort of Rutinx et al. As we collected our samples within a range of 10 days of symptom onset, they collected their samples within 8 days of a positive SARS-CoV-2 PCR test. Additionally, there could be intrinsic differences of

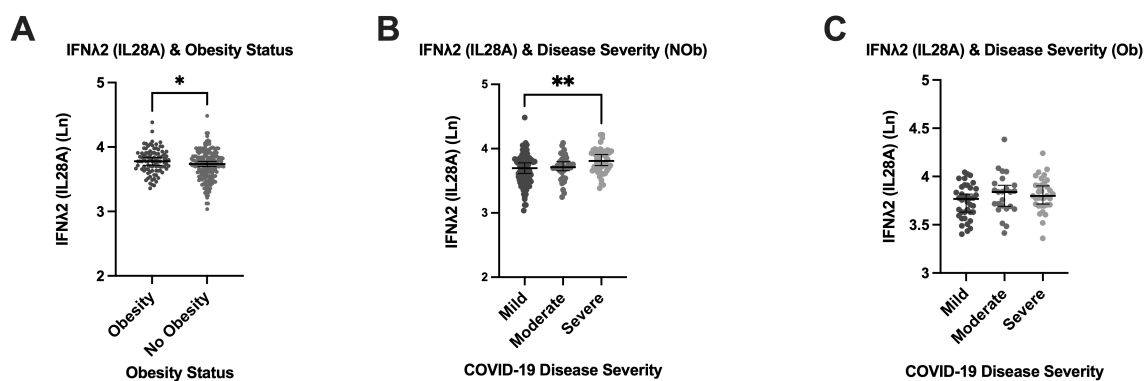


FIGURE 2

Relationship between Type III IFNλ2 (IL28A), Obesity and COVID-19 Disease Severity. Relationship between Type III IFNλ2 (IL28A), Obesity and COVID-19 Disease Severity in the Sub-cohort (n=321). (A) Association of obesity status with plasma concentration of type III IFNλ2 (IL28A). (B) Levels of type III IFNλ2 (IL28A) in the non-obese. (C) Levels of type III IFNλ2 (IL28A) in the obese. Obesity defined as BMI ≥ 30 Kg/m². (NOB) non obese; (Ob) obese; (Ln) Natural log. Error bars represent the median and interquartile range. *P ≤ 0.05, **P ≤ 0.01.

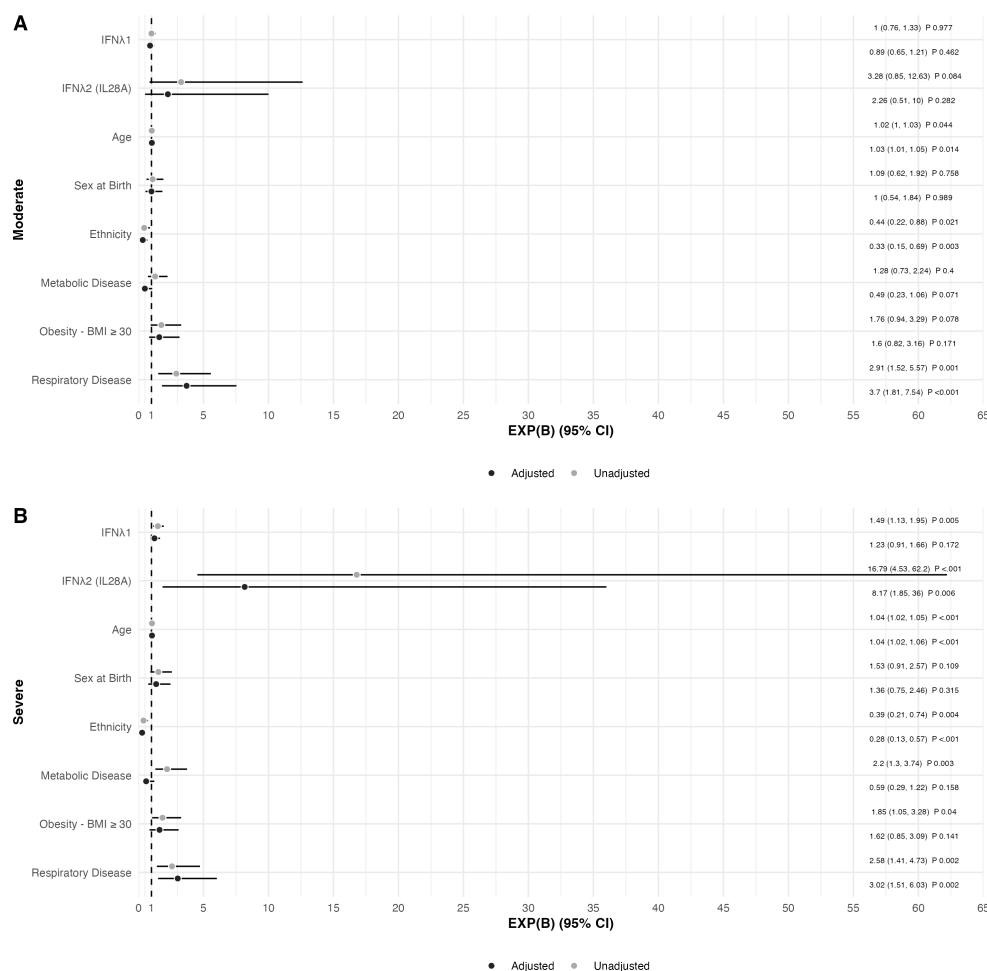


FIGURE 3

Forest Plot of Factors Associated with COVID-19 Disease Severity in the Sub-cohort (n=321) in (A) Moderate and (B) Severe COVID-19 disease adjusted for age, sex at birth, Caucasian ethnicity, obesity (BMI ≥ 30 Kg/m²), metabolic disease, and respiratory disease. Mild COVID-19 disease was used as reference value. Data presented as the odds ratio [EXP(B)] with the 95% confidence interval for EXP(B).

the analytical methods between both studies, although this may be a small difference between methods of time of sampling or cytokine measurement, it could still expound the differences in cytokine levels between our cohort and theirs.

The relative roles of IFNs in mediating host immune responses to respiratory viruses are complex. Our data supports a role for heightened type III IFNs, precisely IFNλ1 and IFNλ2, in driving more severe COVID-19. Type III IFNs are known to be potent cytokines provoked by ISGs (28), several cohorts have found increased transcriptional ISG signatures in individuals with severe COVID-19, where these transcriptional signatures have been linked back to higher SARS-CoV-2 viral loads which may also trigger a more intense cytokine storm (29). Taken together, these data support a scenario where increased expression of ISGs drive expression of type III IFNs which then contribute to the cytokine storm characteristic of severe COVID-19.

Interestingly we also observed differential associations of IFN levels and disease severity based on subjects' BMI. Although we observed significantly higher IFNλ2 in subjects with obesity, the

relationship between elevated IFNλ2 and severe disease was only observed in people without obesity. Various cohorts have shown that obesity is a major predictor of severe COVID-19 disease (30, 31). These studies have focused largely on epidemiological data without the consideration of inflammatory markers in their analytical models. As obesity is considered to be low-grade inflammation, it is not surprising that people with obesity experience an altered expression of pro-inflammatory cytokines, with the adipose tissue being an important producer of TNFα, IL1β, and IL6 (32). Our data would support a scenario whereby individuals with obesity have pre-existing elevations of inflammatory cytokines and interferons, including type III IFNλ2 placing them at higher risk of severe disease, whilst individuals without obesity who do not mount excessive IFNλ2 responses are relatively protected against severe COVID-19. This is confirmed by several studies that found elevated levels of type III IFNs, particularly IFNλ1 in people with obesity (33, 34).

Although, the different subtypes of the IFNλ family share highly homologous amino acid (aa) sequences which likely arose through

gene duplication giving rise to paralogous sequences. IFN λ 1 and IFN λ 2 share 81% aa, IFN λ 2 and IFN λ 3 share 96% aa, while IFN λ 4 may be more distantly related and possesses only 28% aa identity with other IFN λ types (7). Our outcomes showed IFN λ 1 and IFN λ 2 were significantly associated with severe COVID-19, whereas IFN λ 4 expression was not. Our findings on IFN λ 4 are consistent with previous data from a smaller Spanish cohort (N=177) of mainly Caucasian subjects (35). However, our outcomes contrast with another study that analyzed IFN λ 4 expression between survivors and non-survivors in an Iranian population (N=750), which observed associations between IFN λ 4- Δ G/ Δ G genotype and COVID-19 mortality (36). This discrepancy between these outcomes could partially be explained by IFN λ 4 allele frequencies within different ethnicities, as our cohort, although larger, consisted of a primarily Caucasian population, similar to the Spanish cohort, with lower frequencies of the Δ G allele in a European population.

These data build on the growing evidence surrounding the role of IFN λ 4 in modifying host responses to viral infections, some beneficial and some less so. In Hepatitis C (HCV), the rs368234815 SNP is associated with differing responses to treatment depending on the HCV genotype (improved responses in HCV genotype 1 and 4 but not genotypes 2 and 3) (37), and the spontaneous clearance of HCV in the absence of treatment (38). In people with HIV, we previously reported on associations between rs368234815- Δ G/ Δ G and increasing likelihood of normalizing CD4⁺:CD8⁺ ratio in response to antiretroviral therapy (39). In contrast, presence of rs368234815- Δ G/ Δ G linked to IFN λ 3-rs12979860 was associated with reduced clearance of respiratory RNA viruses in Rwandan children (40). These results suggest that the impact of host expression of IFN λ 4 may have differing impacts on clinical outcomes depending on the virus and type of infection. Whether IFN λ 4 polymorphisms impact upon COVID-19 severity and, most importantly, the ultimate outcome following SARS-CoV-2 infection requires further investigation.

Our study does have limitations. The high proportion of Caucasians likely resulted in an under ascertainment of the Δ G allele. A larger, more diverse cohort could provide additional insights into the impact of IFN λ 4 expression and COVID-19 severity and outcome. As our samples were collected between March 2020 and June 2021, we were unable to explore the impact of COVID-19 vaccination on the interaction between type III IFN expression and COVID-19 disease severity, which may change following SARS-CoV-2 vaccination. Additionally, we did not measure neutralizing type I IFN autoantibodies nor inborn errors of type I IFN, which have also been implicated in contributing to COVID-19 disease severity (41). Furthermore, during this time period, a number of distinct SARS-CoV-2 variants of concern were in circulation with differing impacts on clinical severity which may also have impacted the analyses (42). Additionally, obesity was defined as BMI $\geq$ 30 kg/m², which may not fully capture other measures such as percentiles or obesity grades. However, given that most participants were Caucasian, this is unlikely to have significantly impacted our findings. Our ventilation data was limited to a binary variable, with only 15 of 321 participants requiring

ventilation. Similarly, as most participants were either discharged or treated as outpatients, further analysis of ventilation duration and interferon levels between survivors and non-survivors was limited. Lastly, we did not assess the predictive value of IFN levels and IFN λ 4 expression. Future studies with longitudinal data are needed.

In conclusion, common IFN λ 4 genotypes were not associated with COVID-19 disease severity or expression of circulating IFNs. However, both higher IFN λ 1 and IFN λ 2 were associated with more severe COVID-19 disease. IFN λ 2 was also higher in individuals with obesity, although associations between IFN λ 2 and COVID-19 disease severity were only observed in individuals without obesity, suggesting that obesity may contribute to increased risk of severe COVID-19 through increased expression of IFN λ 2, potentially impacting disease progression. These findings suggest that assessing IFN λ 2 could help to identify those at a greater risk of severe disease, enabling earlier interventions and improved management strategies. Future work is required to validate these results and to study the impact of vaccination on the relationships between type III IFNs and COVID-19 severity.

Data availability statement

The data that support the findings of this study manuscript can be requested from the All-Ireland Infectious Diseases Cohort Study group. However, the data can be made available on request subject to approval by a local ethics committee.

Ethics statement

The study was approved by the National Research Ethics Committee as part of The AIID Cohort (20-NREC-COV-056), and all subjects provided written informed consent for the collection of samples and clinical data for further research and publication. The studies were conducted in accordance with the local legislation and institutional requirements.

Author contributions

DA: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing – review & editing. AG: Investigation, Methodology, Writing – review & editing. GS: Investigation, Methodology, Writing – review & editing. CG: Investigation, Methodology, Writing – review & editing. RN: Investigation, Methodology, Writing – review & editing. CH: Investigation, Writing – review & editing. GK: Conceptualization, Data curation, Investigation, Methodology, Writing – review & editing. EF: Conceptualization, Data curation, Investigation, Writing – review & editing. AC: Conceptualization, Data curation, Investigation, Writing – review & editing. CK: Conceptualization, Data curation, Investigation, Writing – review & editing. MC: Conceptualization, Investigation,

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Funding

The author(s) declare that financial support was received for the research and/or publication of this article. This work was supported by Science Foundation Ireland (grant number 20/COV/8549), and a philanthropic donation from Smurfit Kappa. DA is funded by National Irish COVID-19 Biobank funded through the Health Research Board in Ireland (grant number NCov19BB-2021-1).

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Acknowledgments

The authors wish to thank all study participants and their families for their participation and support in the conduct of the All Ireland Infectious Diseases Cohort Study.

Conflict of interest

PM has received honoraria/speaker fees from Gilead Sciences, AstraZeneca, Janssen-Cilag and MSD.

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2025.1516756/full#supplementary-material>

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OPEN ACCESS

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RECEIVED 14 March 2025

ACCEPTED 22 May 2025

PUBLISHED 11 June 2025

CITATION

Yuan J, Wang H, Sun X and Huan C (2025)
The emerging roles of ubiquitin-like
modifications in regulating HIV
replication and host defense.
Front. Cell. Infect. Microbiol. 15:1593445.
doi: 10.3389/fcimb.2025.1593445

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The emerging roles of ubiquitin-like modifications in regulating HIV replication and host defense

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As a post-translational modification (PTM) mechanism analogous to ubiquitination, ubiquitin-like (UBL) modification plays a crucial regulatory role in virus-host interactions. With the increasing discovery of UBL modification types, their roles in diverse biological process, including HIV infection, have gained growing attention. Rather than merely serving as anti-HIV defenses or being exploited by the virus, UBLs often exert dual roles by modulating both host restriction factors and viral proteins, thereby impacting key steps of HIV life cycle, immune evasion, and intracellular signaling. This article summarizes recent advances on the contribution of UBLs in regulating HIV replication and host defense, highlighting their indispensable roles in arms races between HIV and host, aiming to provide a theoretical framework for developing novel therapeutic strategies against HIV-1 targeting virus-host interactions.

KEYWORDS

UBLs, HIV-1, SUMOylation, ISGylation, NEDDylation, ATG8ylation, FAT10ylation

1 Introduction

Acquired immunodeficiency syndrome (AIDS) is an immune deficiency disorder caused by human immunodeficiency virus (HIV) infection (Saunders et al., 2024; Haynes et al., 2016; Chen et al., 2022; Guo et al., 2021b). HIV impairs immune defense by infecting CD4⁺ T cells, leading to severe immune dysfunction. According to UNAIDS estimates, approximately 39 million people worldwide were living with HIV by the end of 2022. Despite the widespread use of antiretroviral therapy (ART), HIV remains a major global public health challenge, particularly in resource-limited regions (Board et al., 2022; Matsui and Miura, 2023; Sheykhhasan et al., 2021; Ouyang et al., 2024). In the evolutionary arms race between HIV and host, the host employs various defense strategies. Restriction factors function by directly blocking specific steps of the viral life cycle, whereas immune recognition mechanisms detect viral signatures and trigger both innate and adaptive immune responses. In return, HIV-1 employs strategies such as high mutation rates, hijacking PTM systems to antagonize host factors and evade restriction. This persistent

conflict drives HIV's adaptive evolution while compelling the host to refine defense strategies to suppress viral persistence. Both HIV-1 and host continuously optimize their strategy to achieve evolutionary advantages (Oswald et al., 2023).

Ubiquitin is a 76-amino-acid small protein ubiquitously expressed in all eukaryotic cells, with nearly all cellular proteins being subject to ubiquitination (Swatek and Komander, 2016). As a prevalent PTM, ubiquitination relies on a cascade of enzymatic catalysis mediated by E1 ubiquitin-activating enzymes (Schulman and Harper, 2009), E2 ubiquitin-conjugating enzymes (Ye and Rape, 2009), and E3 ubiquitin ligases (Deshaies and Joazeiro, 2009; Smit and Sixma, 2014; Rotin and Kumar, 2009). Mechanistically, the E1 enzyme catalyzes ATP-dependent Ub activation, forming a Ub-AMP intermediate that is subsequently transferred to E2 via a thioester bond. Then E2 cooperates with E3 ligases, which specifically recognize and bind substrate proteins, enabling Ub transfer from E2 to lysine residues on target substrates. Canonical ubiquitination regulates fundamental biological processes including protein degradation, signal transduction, cell cycle control, and immune responses (Reichard et al., 2016; Smith et al., 2017; Dang et al., 2021; Liu et al., 2024). Notably, this modification machinery serves not only as a core cellular regulatory mechanism but also as a critical battlefield in virus-

host arms races. During co-evolution with hosts, multiple viruses have developed strategies to hijack the host ubiquitination system, thereby remodeling the cellular microenvironment and achieving immune evasion. A classic example is HIV-1 Vif protein, which recruits host ubiquitination components to mediate degradation of the antiviral restriction factor APOBEC3G, effectively escape from host restriction (Albin and Harris, 2010; Wolf and Goff, 2008).

Recent studies have revealed a series of UBLs including small ubiquitin-like modifiers (SUMOs), interferon-stimulated gene 15 (ISG15), developmentally down-regulated 8 (NEDD8), autophagy-related protein 8 (ATG8), and human leukocyte antigen (HLA)-F adjacent transcript 10 (FAT10) that share structural homology with Ub. Both Ub and UBLs utilize exposed C-terminal glycine residues for substrate conjugation. Sequence alignment (Figure 1) categorizes UBLs into two classes: Type I UBLs, which are precursor forms requiring proteolytic activation to expose the reactive glycine (e.g., SUMOs, ISG15, NEDD8, and ATG8/LC3-GABARAP family), and Type II UBLs, which are constitutively active forms with intrinsically exposed glycine (e.g., FAT10). With the exception of FAT10ylation (which lacks identified E3 ligases), all UBLs employ the conserved E1-E2-E3 enzymatic cascade. Crucially, each UBL system orchestrates specialized regulatory functions through unique enzymatic networks: SUMOylation

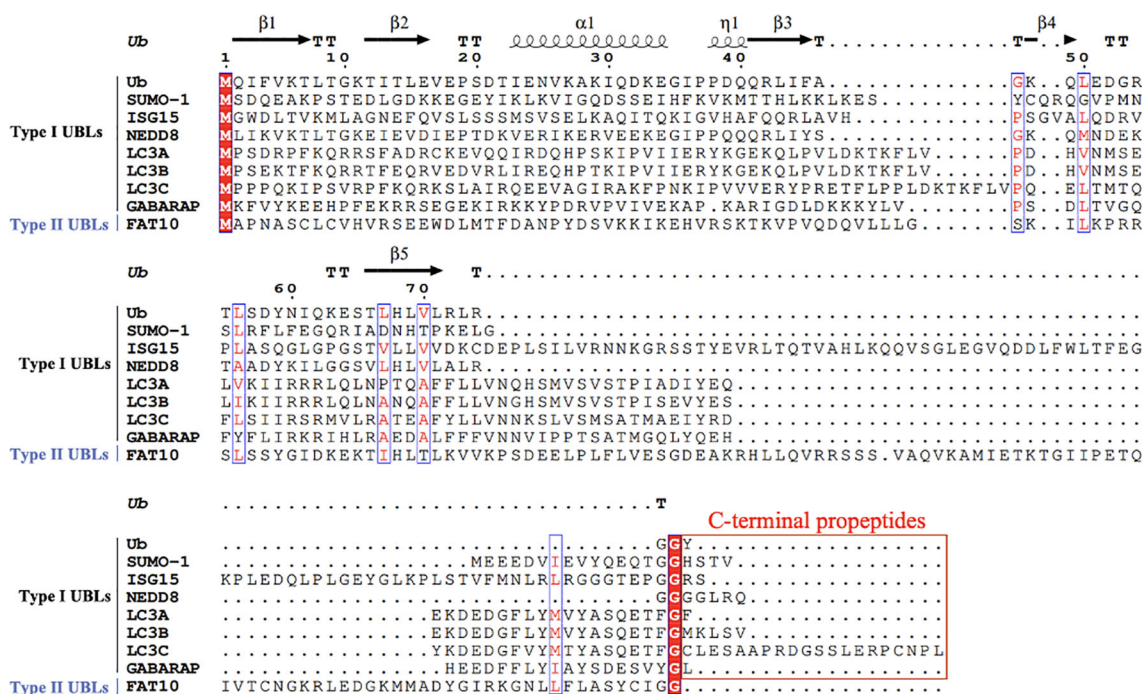


FIGURE 1

Multiple sequence alignment of Ub and UBLs. A multiple sequence alignment of Ub and various UBLs was performed using ClustalW. The residue numbering is based on Ub's first methionine (M1). UBLs are classified into two types: Type I UBLs (e.g., SUMO-1, ISG15, NEDD8, LC3A, LC3B, LC3C, GABARAP), which require the removal of C-terminal propeptides prior to functional activation, and Type II UBLs (e.g., FAT10), which do not require such processing. Conserved residues are shown in red font and enclosed in blue boxes. Functionally important C-terminal glycine residues are also indicated. C-terminal propeptides regions in Type I UBLs are enclosed in red boxes to highlight their required removal before activation. Gaps introduced for optimal alignment are represented by dots. Ub residues are numbered every 10 amino acids along the alignment to assist in identifying conserved and functionally relevant regions. Secondary structure elements of Ub (β -strands, α -helix, and the η -helix) are annotated above the alignment, based on the crystal structure of ubiquitin.

dynamically modulates protein interaction and subcellular trafficking (Han et al., 2018); ISG15ylation exhibits dual roles as covalent modifiers or free molecules to antagonize ubiquitination during antiviral responses (Okumura et al., 2008; Lenschow et al., 2007); NEDDylation regulates substrate stability, conformational dynamics, and functional activation (Zhao et al., 2014); ATG8ylation engages its N-terminal α -helical domain to mediate selective autophagic degradation of membrane-associated targets (Zhang et al., 2023; Deretic et al., 2024); while FAT10ylation coordinates immune homeostasis through inducing proteasomal degradation of substrate (Schmidtke et al., 2009; Bialas et al., 2015).

Compared to classical PTMs such as phosphorylation, acetylation, and methylation (Bilbrough et al., 2022; Ferrante et al., 2020), UBLs can form mono- or polymeric chains through diverse linkage types, generating highly complex signaling codes that enable more precise regulation of cellular responses. During HIV infection, UBL modifications play dual roles by regulating both viral protein functions and host immune responses, exhibiting greater specificity and immune evasion capabilities in the interactions between the virus and the host. This review summarizes current research advancements on UBLs, with particular focus on their functions during HIV infection. Our synthesis aims to provide a theoretical basis for developing potential targeted therapeutics and offers predictive insights into future research directions.

2 SUMOylation

2.1 Introduction of SUMOylation

The SUMO molecule has a molecular weight of approximately 11 kDa and contains a characteristic $\beta\beta\alpha\beta\alpha\beta$ fold with a C-terminal diglycine motif (Wang and Dasso, 2009). Its three-dimensional structure resembles Ub, yet distinct surface charge distribution and amino acid sequences confer divergent functional properties. SUMO requires specific cleavage of the C-terminal propeptides by ubiquitin-like specific protease 1 (UPL1) or sentrin-specific protease 1 (SENP) (Nayak and Müller, 2014). SUMOylation covalently conjugates SUMO molecules to lysine residues on substrates through an enzymatic cascade involving E1 (SAE1/SAE2), E2 (Ubc9), and E3 ligases (PIAS family, RanBP2, etc.). Notably, the SUMOylation not only mediates covalent modification of SUMO molecule to the lysine residues of substrates via isopeptide bonds, but also facilitates non-covalent interactions with effector proteins through SUMO-interacting motifs (SIMs) (Minty et al., 2000). SIMs, primarily composed of four hydrophobic amino acid residues, recognize and bind SUMOylated proteins to regulate the function and stability of substrates (Lascorz et al., 2022).

Distinct from ubiquitination, the E2 enzyme Ubc9 in SUMOylation can directly engage in substrate recognition through non-covalent interaction with SUMO molecules (Jaber et al., 2009) and may bypass E3 ligases under specific conditions (Talamillo et al., 2020; Koidl et al., 2016). Mammals encode five

SUMO paralogs (SUMO1-5) that exhibit antagonistic roles in certain biological processes (Levitskaya et al., 1995; Leight and Sugden, 2000; Falk et al., 1995; Dergai et al., 2013; Zeng et al., 2020). For instance, SUMO-1 enhances transcriptional activity by promoting the interaction between specificity protein-1 (Sp1) and histone acetyltransferase p300, whereas SUMO-2 disrupts this complex and destabilizes Sp1 to exert negative regulation (Gong et al., 2014). This paralogue-dependent bidirectional regulatory paradigm not only expands the versatility of SUMOylation in gene expression control but also establishes a highly plastic host-virus interface through dynamic modulation of protein stability and interaction networks during viral infection.

2.2 SUMOylation enhances host antiviral capacity and maintains cellular homeostasis

During HIV-1 infection, SUMOylation of host restriction factors (TRIM5 α , SAMHD1) enhances host antiviral capacity by increasing their protein stability and recruiting effector proteins via SIMs. Simultaneously, SUMO modifications contribute to maintaining cellular homeostasis through dynamic regulation of stress response pathways.

Host restriction factors serve as the primary defense against HIV-1 by targeting critical steps in the viral life cycle. Recent studies demonstrate that SUMOylation and SIMs orchestrate dual mechanisms to significantly enhance the antiviral potency of multiple host factors. For instance, the host factor TRIM5 α inhibits cross-species transmission by recognizing the HIV-1 capsid protein (CA) and mediating ubiquitination-dependent degradation (Ganser-Pornillos and Pornillos, 2019; Brandariz-Núñez et al., 2013). Mechanistically, SUMOylation of TRIM5 α reduces autoubiquitination-mediated degradation while modulating its E3 ligase activity, thereby promoting TRIM5 α -driven activation of NF- κ B and AP-1 to trigger innate immune responses (Nepveu-Traversy and Berthou, 2014). In addition, SUMOylation mediates nuclear translocation of TRIM5 α in dendritic cells (DCs), facilitating its enrichment in nucleoli and Cajal bodies to amplify antiviral efficacy (Portilho et al., 2016). Another restriction factor, SAMHD1, suppresses HIV-1 replication by depleting dNTP pools (Laguette et al., 2011; Hrecka et al., 2011), with its activity regulated by PTMs. Phosphorylation at T592 residue of SAMHD1 markedly diminishes its antiviral capacity, whereas SUMOylation at K595 and T592 dephosphorylation restores SAMHD1 activity (Martinat et al., 2021).

Beyond covalent modification, the antiviral potency of numerous host factors critically depends on SIM-mediated interactions with SUMOylated proteins. TRIM5 α harbors three SIM domains (SIM1-3) (Dutrieux et al., 2015b). It has been demonstrated that SUMO1 overexpression significantly enhances anti-HIV activity of TRIM5 α , whereas knockdown of SUMO1 or E2 Ubc9 abolishes this function. Intriguingly, the TRIM5 α K10R substitution, deficient in being SUMOylated, does not compromise its antiviral potency (Dutrieux et al., 2015b), suggesting its activity primarily relies on SIM-dependent

protein interaction networks rather than direct covalent modification. A parallel mechanism operates in SAMHD1, which possesses three SIM domains (SIM1-3). Structural analysis reveals that SIM2 integrity is indispensable for SAMHD1's anti-HIV-1 activity, likely through recruiting host or viral cofactors to assemble functional antiviral complexes (Martinat et al., 2021). Besides, the death domain-associated protein (Daxx), a novel antiretroviral factor, inhibits HIV-1 reverse transcription predominantly via its C-terminal SIM domain despite being susceptible to SUMOylation (Dutrieux et al., 2015a). Daxx employs SIM-dependent binding to cyclophilin A (CypA) and CA proteins, thereby orchestrating nuclear recruitment of TNPO3, TRIM5 α , TRIM34, and other potential cofactors. This recruitment enhances complex stability to block HIV-1 uncoating and reverse transcription (Maillet et al., 2020).

PLK1 (Polo-like kinase 1), a core serine/threonine protein kinase governing cell cycle regulation, plays essential roles in maintaining CD4⁺ T cell homeostasis (Zhou et al., 2020). HIV-1 infection induces SUMOylation of PLK1, which enhances its protein stability by antagonizing Ub-proteasome mediated degradation. The SUMO-modified PLK1 exhibits specific nuclear accumulation, and this aberrant nuclear localization activates downstream survival signaling pathways to block programmed cell death in infected cells. This pro-survival mechanism creates a critical microenvironment for establishing HIV-1 latency, thereby contributing to viral reservoir formation (Zhou et al., 2020).

2.3 SUMOylation-mediated host antagonism of HIV-1

The transcriptional regulation of HIV-1 primarily relies on its long terminal repeat (LTR). HIV-1 Tat protein binds to the transactivation response (TAR) RNA element within the LTR and recruits the host cyclin-dependent kinase 9 (CDK9)-cyclin T1 (CycT1) complex (positive transcription elongation factor b, p-TEFb) to activate RNA polymerase II (RNAPII)-mediated transcriptional elongation (Ali et al., 2016). The LTR region harbors binding sites for multiple transcription factors and co-regulators, forming a dynamic regulatory network to achieve high-efficiency viral transcription (El Kharroubi et al., 1998). Mounting evidence indicates that SUMOylation modulates HIV-1 transcription through multilayered regulatory mechanisms.

At the transcription factor level, STAT5 (Signal Transducer and Activator of Transcription 5), a key regulator of the JAK-STAT signaling pathway, is critically involved in HIV-1 reactivation (Dempsey, 2023). Upon stimulation by cytokines such as IL-2, STAT5 undergoes JAK-mediated tyrosine phosphorylation, leading to dimerization and nuclear translocation (Dempsey, 2023). The phosphorylated STAT5 binds to the HIV-1 LTR to promote viral reactivation (Crotti et al., 2007; Selliah et al., 2006). SUMOylation suppresses STAT5 transcriptional activity (Van Nguyen et al., 2012), while latency-reversing agents (LRAs) like benzotriazoles inhibit STAT5 SUMOylation, thereby enhancing its phosphorylation and

nuclear accumulation to significantly boost viral promoter activity (Sorensen et al., 2020; Bosque et al., 2017). Within the NF- κ B pathway, I κ B α inhibits NF- κ B nuclear translocation by binding to NF- κ B dimers (Arenzana-Seisdedos et al., 1995). Competitive regulation occurs at I κ B α K21, where SUMOylation stabilizes the protein by blocking ubiquitination-mediated degradation, thereby suppressing NF- κ B-dependent HIV-1 gene expression (Desterro et al., 1998). Additionally, CTIP2, a chromatin-modifying complex recruitment factor at the HIV-1 LTR (Zhang et al., 2012), undergoes phosphorylation-activated SUMOylation at K679/K877, triggering proteasomal degradation and relieving transcriptional suppression (Imbert and Langford, 2021). It is worthy noted that the SUMO E3 ligase TRIM28 is recruited to the LTR during HIV-1 latency. TRIM28-mediated SUMOylation of CDK9 at K44/K56/K68 residues inhibits its kinase activity, disrupts CDK9-Cyclin T1 complex assembly, and inactivates p-TEFb to suppress transcriptional elongation (Ma et al., 2019).

At the epigenetic regulation level, Suv39h1 (a histone methyltransferase) interacts with the SUMOylation E2 enzyme Ubc9 to catalyze SUMOylation of heterochromatin protein 1 α (HP1 α). This modification enhances HP1 α stability at heterochromatic regions, thereby maintaining LTR silencing (Maison et al., 2016). Additionally, the SUMO E3 ligase CBX4 recruits EZH2 to the HIV-1 LTR and catalyzes its SUMOylation, which reinforces EZH2-mediated deposition of repressive H3K27me3 histone marks to promote viral latency (Wu et al., 2022). Notably, the SMC5/6 complex directly modifies unintegrated HIV-1 DNA through its SUMO E3 ligase subunit NSMCE2, inducing epigenetic silencing (Irwan et al., 2022). It has been demonstrated that either TAK-981 (a SUMOylation inhibitor) or NSMCE2 loss-of-function mutations abolish this silencing effect, thereby promoting viral replication (Irwan et al., 2022). In addition, promyelocytic leukemia (PML) nuclear bodies (NBs) exhibit hyper-SUMOylation, a hallmark of aberrantly elevated SUMO modification levels. These SUMO-enriched PML NBs enhance structural stability and facilitate interactions with transcriptional repressors to cooperatively sustain viral latency (Turelli et al., 2001; Dutrieux et al., 2015a; Kahle et al., 2015; Shytaj et al., 2020).

2.4 SUMOylation-driven viral evasion of host defenses

In the arms race between HIV-1 and host, SUMOylation serves as a defensive strategy deployed by the host to counteract viral infection, while HIV-1 also retaliates through modulation of modification sites.

The HIV-1 Gag p6 protein, a key budding factor (Göttlinger et al., 1991), exhibits competitive interplay between SUMO-1 and Ubiquitination at K27. Monoubiquitination at K27 enhances p6 interaction with TSG101 to promote viral budding efficiency (Gurer et al., 2005). Conversely, Ubc9-mediated K27 SUMOylation markedly reduces HIV-1 infectivity, though the K27R mutation

does not compromise viral replication capacity (Gurer et al., 2005). These findings underscore a complex balance between ubiquitination and SUMOylation in regulating p6 functionality.

During viral integration, SUMOylation of HIV-1 integrase (IN) at lysine residues K46/K136/K244 is critical for early replication stages, that SUMOylation-deficient mutants exhibit post-reverse transcription defects despite retaining catalytic activity and LEDGF/p75 cofactor interactions (Shinohara et al., 2002; Turlure et al., 2006; Zamborlini et al., 2011). Intriguingly, IN SUMOylation occurs not during synthesis but in subsequent infection cycles, particularly during reverse transcription and nuclear import of the preintegration complex (PIC) (Zheng and Yao, 2013). This temporal pattern suggests SUMOylation may facilitate PIC nuclear entry by modulating IN's affinity for nuclear transport factors. Notably, IN harbors two functional SIM domains: SIM2 (200IVDI204) and SIM3 (257IKVV260) (Cherepanov et al., 2004; Shun et al., 2007), which mediate SUMO-2/3 binding. Mutations in these domains severely impair viral infectivity, reverse transcription, and integration efficiency (Zheng et al., 2019). Furthermore, Ubc9 overexpression suppresses genomic integration by enhancing IN SUMOylation, with SUMO-1/2 upregulation potentiating this inhibitory effect (Li et al., 2012). Paradoxically, SUMOylation at K364 of LEDGF/p75, while dispensable for IN binding, critically regulates reverse transcription and integration kinetics, indicating that LEDGF/p75 SUMOylation is essential for efficient HIV-1 replication (Bueno et al., 2010).

Emerging evidence suggests functional modulation of HIV-1 Rev by SUMOylation. Specifically, Rev-mediated viral replication is suppressed upon its interaction with the SUMO-1 hexapeptide repeat (SHPR) domain (Roisin et al., 2004). The viral accessory protein Vpu subverts antiviral defenses by targeting the RanBP2/RanGAP1-SUMO1/Ubc9 E3 ligase complex. This manipulation alters SUMOylation states of PML NBs and DNA repair factors, facilitating immune evasion through nuclear clearance of antiviral signaling complexes and residual viral DNA (Volcic et al., 2020).

Collectively, these findings reveal the dual roles of SUMOylation in HIV-1 infection. On one hand, SUMOylation enhances host antiviral defenses by stabilizing restriction factors (e.g., TRIM5 α , SAMHD1) and organizing SIM-mediated protein interaction networks. On the other hand, HIV-1 subverts the SUMOylation machinery to promote its own replication and persistence (e.g., CTIP2, PLK1). These opposing roles highlight SUMOylation as a dynamic regulatory node in the virus-host interplay, offering both opportunities and challenges for therapeutic intervention (Figure 2).

3 ISGylation

3.1 Introduction of ISGylation

ISG15, a protein encoded by the interferon-stimulated gene *ISG15*, exerts multifaceted regulatory roles in antiviral immunity. The precursor of ISG15, Pro-ISG15 (~17 kDa), undergoes proteolytic processing to generate mature ISG15 (~15 kDa)

(Potter et al., 1999). Although ISG15 exhibits low sequence conservation across species (e.g., 66% homology between human and murine orthologs) (Dzimianski et al., 2019), its C-terminal diglycine motif, the catalytic domain mediating ISGylation, remains highly conserved. The ISGylation cascade requires sequential action of E1 (UBE1L), E2 (UBE2L6), and E3 ligases (HERC5, TRIM25) to conjugate ISG15 to specific lysine residues on target proteins. Notably, ISG15 operates through three functional modalities: 1) intracellular free form, 2) covalent conjugates with substrates, and 3) secreted soluble form (Lenschow, 2010). Distinct from K48-linked ubiquitination that triggers proteasomal degradation, ISGylation exerts antiviral effects via non-degradative mechanisms. These include modulating viral protein subcellular localization (e.g., restricting virion assembly) and stabilizing host antiviral proteins to amplify innate immune responses (Woods et al., 2014). It is reported that ISGylation-mediated cellular outcomes depend on both infection stage and substrate specificity (Mathieu et al., 2021; Liu et al., 2003). For instance, late-stage infection may undergo ISGylation-mediated functional inhibition or degradation of host proteins, attenuating cellular immunity (Minakawa et al., 2008).

3.2 ISGylation inhibits HIV-1 replication

ISG15 functions as a host factor against HIV-1, exerting multidimensional antiviral effects through ISGylation. As a key antiviral factor, p53 inhibits HIV replication and induces apoptosis of infected cells, thereby limiting HIV-1 propagations (Xia and Jiang, 2024). Osei Kuffour et al. found that ISGylation suppresses HIV-1 replication via dual regulation of p53: stabilizing p53 by counteracting ubiquitination-mediated degradation while promoting misfolded p53 degradation (Osei Kuffour et al., 2019). Overexpression of ISG15 or its E1 enzyme UBE1L significantly inhibits both early integration and late budding stages of HIV-1 (Shirazi and Pitha, 1992; Pincetic et al., 2010; Okumura et al., 2006). Mechanistically, ISG15 disrupts Ub-dependent interaction between HIV-1 Gag and Tsg101 via ISGylation of cellular targets, thereby blocking virion release (Gómez et al., 2020; Pincetic et al., 2010). The E3 ligase HERC5 employs two distinct antiviral mechanisms: ISGylation-mediated modification of Gag to impair viral assembly at the plasma membrane and RCC1-like domain-dependent suppression of Rev/RRE-mediated RNA nuclear export (Dastur et al., 2006). Notably, HERC5's antiviral activity is also regulated by its own ISGylation, suggesting an autoregulatory loop (Woods et al., 2014). In addition, while early studies indicated ISG15 deficiency inhibits HIV-1 infection (Bosque and Planelles, 2009), subsequent mechanistic analyses revealed that this is due to free cellular ISG15 aids the stabilization of the ubiquitin-specific peptidase 18 (USP18), a negative regulator of type I interferon (IFN-I) signaling. USP18 binds to the IFN-I receptor subunit IFNAR2 and completes with JAK1 (Speer et al., 2016), thereby attenuating JAK-STAT pathway activation. Consequently, ISG15 deficiency leads to USP18 destabilization, resulting in sustained IFN-I signaling and enhanced expression of interferon-stimulated genes (ISGs) (Zhang

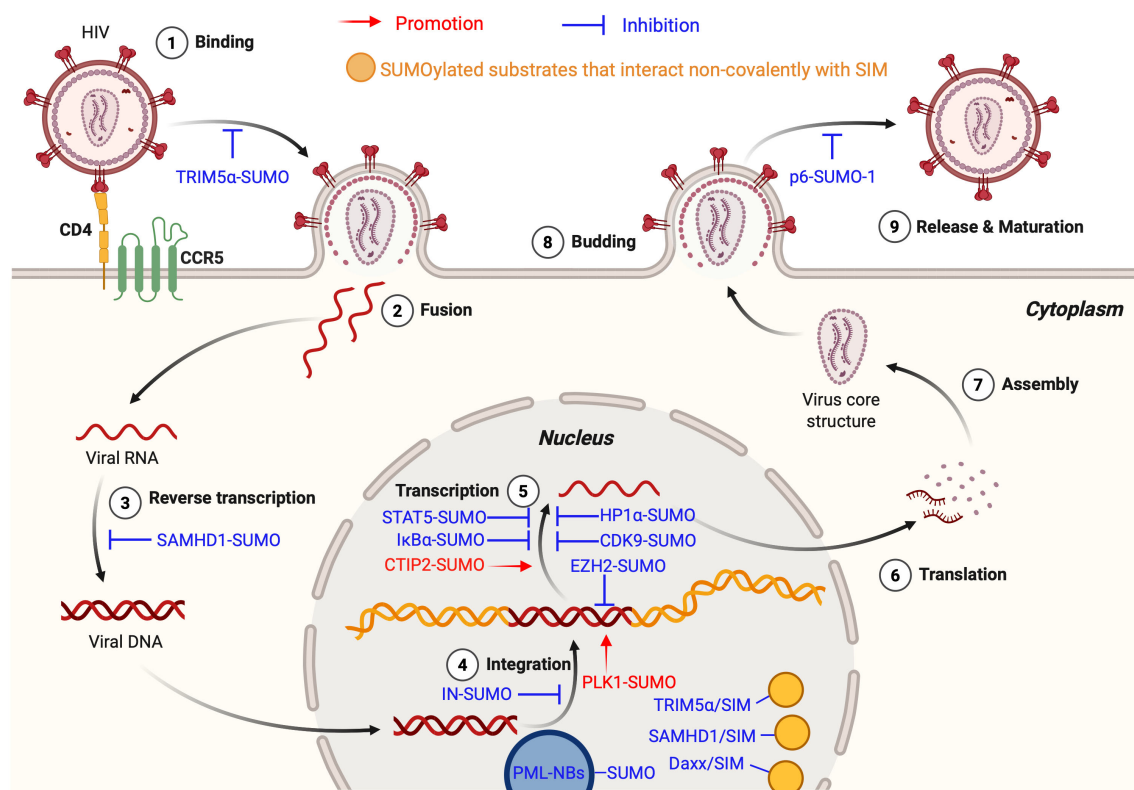


FIGURE 2

SUMOylation in the HIV life cycle. The figure illustrates the dual roles of SUMOylation in the HIV-1 life cycle, encompassing both HIV-promoting (shown in red text and arrows) and anti-HIV (shown in blue text and inhibitory symbols) effects. Yellow circles denote SUMOylated substrates that interact non-covalently with SIM. During the Binding Stage, SUMOylated TRIM5 α recognizes the CA, preventing cross-species transmission. In the Reverse Transcription Stage, SUMOylated SAMHD1 inhibits replication by depleting the intracellular dNTP pool. At the Integration Stage, SUMOylated IN impairs integration, SUMOylated PLK1 supports latency maintenance, and SUMOylated EZH2 promotes epigenetic silencing. At the Transcription Stage, SUMOylated STAT5, I κ B α , HP1 α , and CDK9 suppress HIV-1 transcription, whereas SUMOylated CTIP2 enhances it. During Budding Stage, SUMOylated p6 reduces viral infectivity. In the nucleus, hyper-SUMOylated PML NBs facilitate the establishment and maintenance of viral latency. Additionally, SUMOylated substrates interact non-covalently with SIMs on TRIM5 α , SAMHD1, and Daxx, contributing to inhibition of HIV-1 replication. This figure is generated using BioRender (<http://biorender.com/>).

et al., 2015), which can exert antiviral effects. This explains the paradoxical observation that loss of ISG15, while eliminating its direct antiviral functions via ISGylation, can indirectly inhibit HIV-1 replication by amplifying the IFN-I response and promoting an antiviral state. These findings highlight the conflicting role of ISG15 in host-virus interactions (Figure 3).

These findings not only unravel the multifaceted regulatory networks of ISGylation throughout the HIV-1 life cycle but also highlight the therapeutic potential of targeting HERC5's autoregulatory ISGylation for precise modulation of antiviral activity.

3.3 Immune evasion mechanism of HIV-1 via Vpu-mediated regulation of ISGylation

HIV-1 employs its viral accessory protein Vpu to establish an immune evasion that selectively targets core components of the host ISGylation system, thereby sustaining infection advantage. Okumura et al. reported that Vpu suppresses ISGylation through diverse regulatory strategy: (1) degrading the ISG15-conjugating enzyme UBE2L2 via a Ub-proteasome-independent pathway, (2) impairing

the functional activity of UBE2L6, and (3) modulating subcellular distribution of the transmembrane protein PLP2. The non-canonical degradation of UBE2L2 directly disrupts ISG15-substrate binding capacity, resulting in downregulation of intracellular ISGylation (Jain et al., 2018; Okumura et al., 2006). This unique immune evasion strategy effectively compromises ISGylation-dependent antiviral responses, thereby promoting viral replication and accelerating infection-associated disease progression.

4 NEDDylation

4.1 Introduction of NEDDylation

NEDDylation is a reversible PTM mediated by the UBL molecule NEDD8 through an enzymatic cascade involving E1 (NAE, NEDD8-activating enzyme), E2 (UBE2M/UBE2F), and E3 ligases (Rbx1/Rbx2). This process regulates substrate function through covalent conjugation to lysine residues (Kamitani et al., 1997; Xirodimas et al., 2004; Xirodimas, 2008). The most well-characterized targets of NEDDylation are Cullin family proteins. All

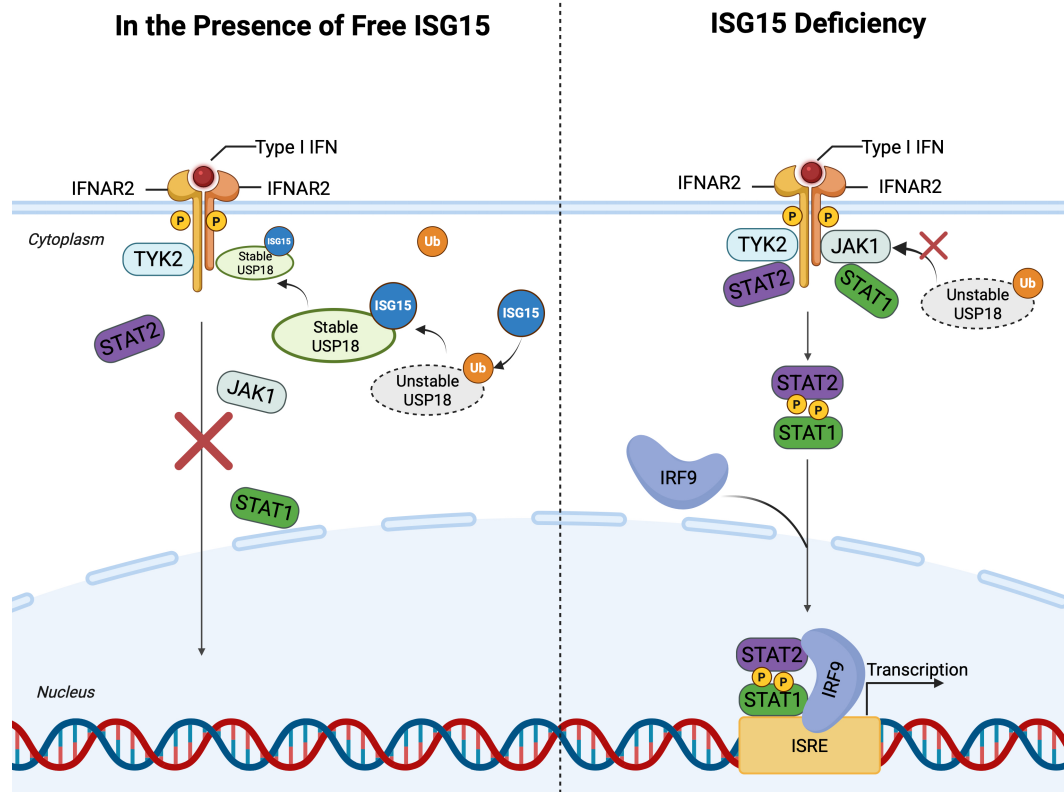


FIGURE 3

ISG15-Mediated stabilization of USP18 and negative regulation of IFN-I signaling. This figure illustrates the interplay between ISG15 and USP18 in regulating the IFN-I signaling. Left panel: In the presence of free ISG15, USP18 is stabilized by competing with ubiquitin, thereby enhancing its association with IFNAR2 and preventing JAK1 binding. This inhibits activation of the JAK-STAT pathway and downregulating ISG expression. Right panel: In the absence of ISG15, USP18 becomes unstable, leading to sustained IFN-I signaling. Upon IFN-I binding to its receptor complex, JAK1 and TYK2 are activated, resulting in the formation of the ISGF3 complex (STAT1, STAT2, and IRF9). ISGF3 translocates into the nucleus and binds to ISREs in the promoters of ISGs. This figure is generated using BioRender (<http://biorender.com/>).

Cullin-associated ubiquitin ligases require NEDDylation for functional activation (Embade et al., 2012; Enchev et al., 2015). NEDDylation induces conformational changes in Cullins to expose substrate-binding sites, thereby activating Cullin-RING E3 ubiquitin ligase complexes (CRLs) and enhancing their ubiquitination efficiency. It has been reported that substrate specificity exists within this system: UBE2M exclusively mediates NEDDylation of Cullin1-4, while UBE2F specifically modifies Cullin5.

4.2 HIV exploits NEDDylation to degrade antiviral factors and enhance infection

Emerging studies reveal that HIV exploits NEDDylation-mediated ubiquitination pathways to potentiate its infectious capacity (Nekorchuk et al., 2013). Specifically, HIV hijacks host CRL complex to degrade antiviral restriction factors. In HIV-2 infection, the viral accessory protein Vpx, via its evolutionarily conserved HHCC motif and zinc-binding domain (Guo et al., 2019;

Wang et al., 2017), interacts with the CRL4 complex (comprising DDB1, RBX1, Cullin4A, and DCAF1) to induce NEDDylation of the Cullin4 subunit. This covalent NEDD8 modification activates CRL4's E3 ubiquitin ligase activity, driving K48-linked ubiquitination and subsequent proteasomal degradation of SAMHD1 (Hrecka et al., 2011; Laguette et al., 2011; Hofmann et al., 2013).

The host restriction factor APOBEC3G exerts antiviral effects by deaminating cytosine (C) to uracil (U) in viral single-stranded DNA during reverse transcription, impairing viral genome integrity and progeny infectivity (Iwatani et al., 2007; Bishop et al., 2006). Counteractively, HIV-1 Vif recruits UBE2F to mediate Cullin5 NEDDylation, activating the CRL5Vif-CBF β complex. This machinery catalyzes K48 ubiquitination of APOBEC3G, marking it for proteasomal destruction (Yu et al., 2003; Liu et al., 2005; Stanley et al., 2012).

Furthermore, HIV-1 Vpr targets uracil-N-glycosylase (UNG2), which exhibits dual regulatory roles in HIV-1 replication: on one hand, it enhances the fidelity of reverse transcription (Chen et al., 2004); on the other hand, its nuclear-localized isoform participates in cDNA degradation and maintains host genomic stability by

repairing uracil mismatches (Akbari et al., 2004). Notably, the HIV-1 viral protein Vpr targets UNG2 to promote NEDD8 modification of the CRL4 complex, thereby inducing CRL4-mediated ubiquitination and degradation of UNG2 (Nekorchuk et al., 2013). This mechanism not only compromises host DNA repair capacity but also enhances HIV-1 replication, thereby promoting viral dissemination.

In summary, host CRL complexes collaborate with viral proteins to orchestrate ubiquitination-dependent elimination of antiviral factors. Given that CRL activity strictly depends on NEDD8 modification of conserved lysine residues within the C-terminal domains of Cullin proteins, this PTM regulatory strategy becomes indispensable for HIV infection. These findings not only elucidate novel viral strategies to subvert immune surveillance but also provide a mechanistic foundation for developing NEDDylation-targeted antiviral therapies.

4.3 NEDDylation inhibitors block HIV infection

Given that HIV and other viruses exploit NEDDylation to enhance infectivity (Abounouh et al., 2022; Lee et al., 2022; Zhang et al., 2021; Nekorchuk et al., 2013), inhibition of this modification has emerged as a promising therapeutic strategy. The NEDD8-activating enzyme (NAE) inhibitor MLN4924, a broad-spectrum antiviral candidate, specifically blocks NEDD8 conjugation to CRLs, effectively perturbing ubiquitination cascades (Xie et al., 2021; Xu et al., 2018). Recent studies demonstrate that MLN4924 suppresses Cullin-mediated degradation pathways, thereby counteracting HIV accessory protein functions, including Vif-driven APOBEC3G elimination, Vpr-mediated UNG2 depletion, and Vpx-dependent SAMHD1 degradation (Stanley et al., 2012; Nekorchuk et al., 2013; Wei et al., 2014).

The antiparasitic agent suramin has garnered significant attention for its potential to disrupt pathogen proliferation (Steverding and Troeberg, 2023). Recent investigations identify suramin as a novel HIV inhibitor that synergizes with MLN4924 to enhance therapeutic efficacy (Zhang et al., 2020). Mechanistically, suramin interferes with biosynthesis of inositol hexakisphosphate (IP6), a critical cofactor for viral capsid assembly. Suramin and its analog NF449 suppress IP6 production by targeting IP5 kinase (IP5K), inhibiting ATP-binding activity and IP5 phosphorylation. Depleted IP6 levels impair COP9 signalosome (a CRL-deneddylase enzyme complex) recruitment to CRLs, thereby disrupting the dynamic equilibrium required for CRL activity cycling (Dick et al., 2018; Mallery et al., 2019; Zhang et al., 2020). This regulatory mechanism, simultaneously inhibiting CRL activation via NEDDylation blockade and preventing CRL inactivation through deneddylase impairment, positions the combinatorial regimen as a promising approach for achieving comprehensive suppression of HIV replication.

Although NEDDylation inhibitors hold promise as antiviral agents, their clinical translation is impeded by substantial challenges. MLN4924 is currently in phase II clinical trials for

various hematological malignancies and solid tumors (Zhou et al., 2018). In addition to its antitumor activity, studies have demonstrated that MLN4924 exhibits significant antiviral effects against a broad spectrum of viruses, including Human Cytomegalovirus (HCMV), Herpes simplex virus (HSV)-1, HSV-2, adenovirus 5 (AdV5), influenza virus PR8 and HIV (Le-Trilling et al., 2016). However, there remains a paucity of clinical studies to substantiate its anti-virus applications. Suramin, initially developed for parasitic infections, has also demonstrated anti-HIV properties and can synergize with MLN4924 to enhance therapeutic efficacy. However, suramin's low therapeutic index and frequent off-target effects, attributable to its interaction with multiple cellular targets, pose substantial limitations for clinical use (Guo et al., 2021a). Moreover, given that NEDDylation is essential for maintaining normal cellular homeostasis, systemic inhibition may lead to concerns regarding toxicity and specificity. To address these challenges, future strategies should focus on enhancing selectivity, such as by targeting virus-specific NEDDylation events or employing cell-type-specific delivery systems, thereby minimizing host toxicity while maximize therapeutic efficacy.

5 ATG8ylation

5.1 Introduction of ATG8ylation

ATG8ylation, a PTM closely associated with autophagy, involves the synthesis of its core molecule ATG8 as an inactive precursor containing a C-terminal non-functional amino acid extension. This modification is initiated by E1 enzyme ATG7-mediated proteolytic processing of the ATG8 precursor, which cleaves the C-terminal extension to expose a glycine residue, generating the reactive mature ATG8. Subsequently, through the coordinated action of E2 (ATG3) and E3 (ATG12), mature ATG8 covalently conjugates to substrate proteins or membranes. Notably, the ATG8 family comprises multiple homologs, with LC3 and GABARAPL2 being the predominant forms in humans (Kumar et al., 2020b).

5.2 Host maintains homeostasis through the autophagy pathway

The tripartite motif (TRIM) protein family serves as critical host antiviral factors by modulating autophagy pathways. Research demonstrates that TRIM5 overexpression significantly activates cellular autophagy processes (Mandell et al., 2014). Furthermore, TRIM5 functions as a selective autophagy receptor through direct interaction with core autophagy proteins of the ATG8 family, particularly GABARAP subtypes, enabling specific recognition of retroviral particles. Notably, this recognition mechanism operates independently of the canonical ubiquitination system. All TRIM family members directly interact with at least one GABARAP subtype, establishing a universal interaction paradigm. This unique molecular strategy allows TRIM5 to direct autophagosomes toward HIV-1 viral particles for degradation, thereby effectively protecting

host cells from viral infection (Mandell et al., 2014). These findings emphasize the critical role of GABARAP, rather than LC3, in mediating selective autophagic immune responses against HIV-1.

5.3 HIV-1 enhances infection through ATG8ylation

ATG8ylation exerts multidimensional regulatory effects throughout the HIV-1 infection cycle. During viral entry, this modification enhances infectivity primarily by facilitating membrane fusion between the virus and host. Specifically, upon HIV-1 exposure to CD4⁺ T lymphocytes, the ATG8 family member LC3B rapidly accumulates at the plasma membrane, even before direct virion contact, suggesting a priming role for membrane remodeling. This recruitment is dependent on Env-CD4 binding and requires key ATG8ylation enzymes such as ATG7 and BECN1, but notably bypasses canonical autophagy initiation factors like ATG13. Such findings demonstrate that LC3B lipidation (i.e., ATG8ylation) serves as a distinct, autophagy-independent mechanism facilitating the membrane curvature or fluidity changes required for viral fusion. Thus, LC3-mediated membrane dynamics serve as a critical molecular switch for HIV-1 entry, although further studies are needed to delineate its coordination with cytoskeletal remodeling and lipid signaling pathways (Pradel et al., 2024).

Furthermore, during HIV-1 infection, mammalian ATG8 homologs (mAtg8s), particularly GABARAP, orchestrate a finely tuned regulatory network involving lysosomal biogenesis and immune restriction. Mechanistically, GABARAP directly binds TFEB and facilitates its nuclear translocation independent of canonical LIR-LDS interactions. This process is modulated by IRGM, which promotes TFEB dephosphorylation both by inhibiting mTOR and activating the phosphatase PPP3CB. Knockout studies further demonstrate that GABARAPs, but not LC3s, are indispensable for starvation- or stress-induced TFEB activation and downstream lysosomal gene expression. In the context of HIV-1, GABARAP also interacts with TRIM5, a cytosolic restriction factor that recognizes the viral capsid and recruits the autophagic machinery to degrade it. These dual functions, enhancing lysosomal capacity via TFEB and mediating innate immune recognition via TRIM5, highlight GABARAP as a key node bridging autophagy, immune defense, and viral modulation (Kumar et al., 2020a).

Notably, HIV-1 Nef protein hijacks the IRGM-TFEB pathway to suppress lysosomal degradation of viral components, establishing a unique “virus-protective autophagic inhibition” mechanism (Kyei et al., 2009). This strategy prevents HIV components from lysosomal destruction, effectively shielding the virus from autophagic clearance during host cell defense. Saribas et al. reported that Nef induces aberrant accumulation of ATG8/LC3 and p62 (SQSTM1), mimicking the action of the autophagy inhibitor Bafilomycin A1 (BAFA1). By blocking autophagosome-lysosome fusion, Nef enables HIV-1 to evade autophagic elimination in human astrocytes (Saribas et al., 2015).

During viral release, HIV-1 Vpu exploits its L₆₃VEM₆₆ motif to specifically bind the ATG8 family member LC3C. In concert with ATG6 (Beclin1) and LC3C, this interaction enhances Vpu’s ability to counteract BST2-mediated restriction (Madjo et al., 2016). These findings collectively demonstrate that the ATG8ylation system not only modulates viral entry but also facilitates virion maturation and release by remodeling host membrane dynamics.

Collectively, these findings demonstrate that the ATG8ylation system involves distinct functions of LC3 and GABARAP subfamilies, with LC3 primarily mediating membrane fusion events facilitating viral entry and release, while GABARAP mainly governs selective autophagic immune responses and lysosomal regulation, highlighting the dual roles within the ATG8 family in HIV-1 regulation.

6 FAT10ylation

6.1 Introduction of FAT10ylation

FAT10, a multifunctional UBL modifier, serves dual roles in cellular immune surveillance and dynamic protein homeostasis by mediating targeted degradation of substrates (Raasi et al., 1999; Mah et al., 2019). The FAT10ylation process involves covalent conjugation of FAT10 to substrates or membranes through a cascade comprising the E1 (UBA6), E2 (USE1), and putative E3 ligases. Unlike canonical ubiquitin and other UBLs, however, FAT10 appears to function with minimal or undefined involvement of dedicated E3 ligases. To date, no FAT10-specific E3 ligase has been conclusively identified, suggesting a potentially non-canonical or more substrate-intrinsic mechanism of substrate selection and modification (Chiu et al., 2007; Aichem et al., 2010, 2014). This apparent absence represents a major gap in our understanding of the FAT10ylation machinery and raises questions about how substrate specificity and regulation are achieved in different cellular contexts. Another unique feature of FAT10 is its C-terminal diglycine motif, which, although functionally analogous to that of ubiquitin, is inherently exposed in its primary sequence and does not require proteolytic processing to achieve conjugation competence. This contrasts with ubiquitin and several other UBLs that are synthesized as precursors requiring cleavage to expose the C-terminal Gly-Gly motif. Despite increasing interest in FAT10 biology, many aspects of its substrate landscape remain obscure. In particular, the identities and functional consequences of FAT10-specific substrates in the context of HIV-1 infection remain largely uncharacterized. Given the virus’s dependence on host proteostasis and immune modulation, elucidating how FAT10-mediated degradation affects viral replication or host antiviral responses could uncover novel therapeutic opportunities. Further studies are urgently required to define substrate repertoires, ascertain the existence of virus-encoded regulators of FAT10ylation, and characterize how HIV-1 might hijack or evade this PTM system.

6.2 HIV-1 induces FAT10 overexpression to promote apoptosis

FAT10ylation exerts multidimensional biological effects within the HIV-1 infection. In HIV-associated nephropathy (HIVAN) models, studies have delineated that FAT10ylation is involved in disease progression through pro-apoptotic pathways: HIV-1-infected renal tubular epithelial cells (RTECs) exhibit significant FAT10 upregulation, which activates intrinsic apoptotic cascades to drive RTEC apoptosis (Ross et al., 2006). RNA interference-mediated silencing of endogenous FAT10 expression effectively abrogates HIV-1-induced RTEC apoptosis, confirming FAT10's central regulatory role in this pathological cascade (Ross et al., 2006).

HIV-1 Vpr has been identified as a critical inducer of FAT10 expression in RTECs, promoting apoptosis by elevating FAT10 levels in these cells (Snyder et al., 2009). Snyder et al. found that FAT10 directly interacts with Vpr and serves as a key mediator of Vpr-driven RTEC apoptosis. However, the precise mechanism by which FAT10 facilitates Vpr-induced apoptosis remains unresolved. Furthermore, it remains unclear whether FAT10-mediated apoptosis is pathogenic to the host or represents an adaptive antiviral response to limit viral spread through covalent modification of target proteins (Snyder et al., 2009).

Similar to ubiquitination, FAT10ylation can trigger substrate degradation (Yi et al., 2020). Emerging evidence suggests FAT10ylation may inhibit HIV-1 by degrading viral proteins essential for infection (Kubo et al., 2022). Nevertheless, the mechanistic basis of FAT10ylation's antiretroviral activity requires further investigation.

7 Therapeutic targeting of UBL pathways: opportunities and challenges

The maintenance of intracellular protein homeostasis is essential for organismal health, and its disruption has been implicated in numerous pathological conditions (Dowell et al., 2007). The ubiquitin–proteasome system (UPS) plays a central role in regulating protein degradation, where the 26S proteasome recognizes and hydrolyzes polyubiquitinated proteins to control various cellular processes (Coll-Martínez and Crosas, 2019). Many viruses, including HIV, exploit the UPS pathway to regulate the stability of their own proteins or to induce the degradation of host restriction factors, therefore enhancing viral replication (Barry and Früh, 2006; Gustin et al., 2011). Thus, inhibition of the UPS pathway is an effective strategy to suppress viral replication. Compounds targeting the ubiquitin-conjugation system (e.g., PYR-41) and the proteasome (e.g., MG-132, Bortezomib) have been shown to interfere with viral replication (Kaspari et al., 2008; Luo et al., 2003; Satheshkumar et al., 2009; Schubert et al., 2000). However, the toxicity of these agents can lead to severe side effects in the host: upon proteasome inhibition, the half-life of more

than 80% of cellular proteins is significantly increased (Meierhofer et al., 2008; Yen et al., 2008). So far, no clinical studies have reported the use of PYR-41, MG132, or Bortezomib in HIV therapy, highlighting the need for further investigation into their potential antiviral effects.

As outlined in Section 2.3, the anticancer drug TAK-981 is a specific inhibitor of the SUMOylation E1 enzyme (Langston et al., 2021). The SUMOylation of the SMC5/6 complex triggers epigenetic silencing of unintegrated HIV-1 DNA. TAK-981 disrupts the structural stability of PML NBs, functioning as the “shock” component in the “shock and kill” strategy, and demonstrating potential therapeutic value in HIV treatment (Irwan et al., 2022). Although TAK-981 has entered Phase I clinical trials for the treatment of solid tumors and lymphomas (Lightcap et al., 2021), there are no clinical reports available concerning its application in HIV treatment.

What's more, as mentioned in Section 4.3, MLN4924 is a selective inhibitor of NAE, which prevents the activation of CRLs and consequently blocks the NEDDylation process (Xie et al., 2021; Xu et al., 2018). MLN4924 has advanced to phase II clinical trials and demonstrated promising antitumor activity in preclinical and clinical settings (Zhou et al., 2018). Its toxicity profile has been extensively studied. Although some adverse effects have been observed, they are generally milder, and to date, no grade 4 adverse events and treatment-related deaths have been reported (Shah et al., 2016). However, UBL-specific inhibitors beyond the NEDDylation pathway remain largely unexplored. High-throughput virtual screening based on compound libraries, combined with structural modeling of key enzymes involved in UBL cascades, represents a viable approach for identifying promising molecules. Although such strategies may yield numerous candidate compounds, significant challenges persist in drug development. Key concerns include the efficient intracellular delivery of compounds to targets, minimizing off-target toxicity, and ensuring sufficient therapeutic efficacy. For instance, suramin has demonstrated inhibitory activity against NEDDylation, but its clinical application has been limited by considerable side effects and suboptimal efficacy (Guo et al., 2021a). Future efforts may focus on chemical modification of existing compounds or combination therapies to enhance binding affinity to UBL-specific targets while reducing adverse effects. Overcoming these pharmacological limitations will be essential for translating UBL-targeted therapies into clinical effective treatments.

8 Discussion

HIV remains a major global health threat, with its infection process highly dependent on the precise regulation of the protein PTM system. PTMs orchestrate HIV infection by reversibly modifying both viral and host proteins, thereby altering their structure, function, and interactions to influence viral replication cycles, immune evasion, and host defense responses. Among diverse PTM types, the canonical regulatory mechanism ubiquitination

governs HIV infection through substrate degradation, localization control, and signaling modulation. Recent advances have unveiled expanding roles of UBLs, including SUMOylation, ISGylation, NEDDylation, ATG8ylation, and FAT10ylation, in regulating HIV-host interactions. This review systematically summarizes the multidimensional functions of UBL networks in HIV infection, highlighting their spatiotemporal regulation of viral entry, replication, latency, and budding (Table 1) (Figure 4). The bidirectional dynamic regulation of PTM is particularly prominent in HIV infection. Deubiquitinating enzymes (DUBs), as key regulatory factors, reverse ubiquitination to participate in viral replication and serve as critical mechanisms for intracellular regulation of protein function, stability, and interactions. Different types of PTMs dynamically add or remove modifying molecules to precisely regulate protein biological functions. During HIV infection, the regulatory role of PTMs is especially crucial, particularly the impact of ubiquitination and UBL modifications on the viral life cycle (Teh et al., 2022). For example, USP21 inhibits HIV-1 replication by downregulating Tat expression (Gao et al., 2021a), while USP7 stabilizes Tat to exert the opposite effect (Ali et al., 2017). USP37 enhances SAMHD1's anti-HIV-2/SIV activity (Cui et al., 2025), and USP8 counteracts Vif-mediated A3G degradation (Gao

et al., 2021b). Notably, UBL systems also possess corresponding deconjugating enzymes (DUBLs). SUMOylation is regulated by the SENP family (Li and Hochstrasser, 1999, 2000), where SENP-mediated deSUMOylation of integrase IN significantly suppresses viral infectivity (Madu et al., 2015). Additionally, the CSN complex (containing CSN5/JAB1) promotes HIV-1 replication in CD4⁺ T cells through deneddylation (Kinoshita et al., 2012). USP18 enhances HIV-1 replication by inhibiting ISG15 synthesis (Lin et al., 2024). These studies suggest that DUBs/DUBLs constitute critical regulators in the host-virus interplay. We propose that other UBLs may harbor undiscovered DUBLs, and systematic characterization of their functional networks will provide novel targets for intervening in HIV infection.

Breakthroughs in ubiquitination research have established critical paradigms for exploring UBL mechanisms. Mabbitt et al. found that the human E3 ubiquitin ligase Myc binding protein 2 (MYCBP2) modifies threonine (Thr) residues via a "RING-Cys-relay" (RCR) mechanism, extending UBL modifications even to non-amino acid molecules such as glycerol (Mabbitt et al., 2020). Furthermore, PTMs of Ub itself, exemplified by serine 65 (Ser65) phosphorylation, dynamically regulate its functionality through

TABLE 1 The effects of UBL modifications on host factors and HIV proteins.

Ubiquitin-like Modifications	E1	E2	E3	Effects on Host Factors	Effects on HIV Proteins
SUMOylation	SAE1/SAE2	Ubc9	PIAS Family and RanBP2, etc	1. Enhancing the Antiviral Activity of Host Restriction Factors TRIM5α (Dutrieux et al., 2015b; Nepveu-Traversy and Berthou, 2014; Portillo et al., 2016), SAMHD1 (Martinat et al., 2021), Daxx (Maillet et al., 2020) 2. Maintaining PLK1 Stability to Support Cell Survival (Zhou et al., 2020) 3. SUMOylation of STAT5 Inhibits HIV Transcription (Sorensen et al., 2020; Bosque et al., 2017) 4. SUMOylation of IκBα Inhibits HIV Transcription (Desterro et al., 1998) 5. SUMOylation of CDK9 Inhibits HIV Transcription (Ma et al., 2019) 6. SUMOylation of HP1α Mediates HIV Silencing (Maison et al., 2016) 7. SUMOylation of HP1α Mediates HIV Silencing (Wu et al., 2022) 8. SUMOylation of the SMC5/6 Complex Mediates Epigenetic Silencing of HIV-1 DNA (Irwan et al., 2022) 9. Hyper-SUMOylation of PML NBs Inhibits HIV Replication (Turelli et al., 2001; Dutrieux et al., 2015a; Kahle et al., 2015; Shytaj et al., 2020)	1. SUMOylation of p6 Reduces Infectivity (Göttlinger et al., 1991; Gurer et al., 2005) 2. SUMOylation of IN Inhibits Intergration Activity (Zamborlini et al., 2011) 3. Rev binds to SHPR to mediate HIV-1 replication inhibition (Roisin et al., 2004).
ISGylation	UBE1L	UBE2L6, etc	HERC5 TRIM28	1. ISGylation of p53 Inhibits HIV-1 Replication (Osei Kuffour et al., 2019)	1. ISGylation of Gag Inhibits Early Assembly of HIV-1 (Dastur et al., 2006)
NEDDylation	NAE	UBE2M or UBE2F	Rbx1, Rbx2, etc	1. Mediating the Degradation of SAMHD1 to Enhance Infection (Hrecka et al., 2011; Laguette et al., 2011; Zhang et al., 2021; Hofmann et al., 2013) 2. Mediating the Degradation of APOBEC3G to Enhance Infection (Yu et al., 2003; Liu et al., 2005) 3. Mediating the Degradation of UNG2 to Enhance Infection (Nekorchuk et al., 2013)	Unidentified
ATG8ylation	ATG7	ATG3	ATG12-ATG5-ATG16L1 complex	1. The Interaction Between TRIM5 and GABARAP Restricts HIV-1 Infection (Mandell et al., 2014) 2. The Interaction Between LC3B and the Plasma Membrane Promotes HIV-1 Entry into Cells (Mandell et al., 2014) 3. The Interaction Between IGRM and GABARAP Promotes HIV-1 Entry into Cells (Kumar et al., 2020a)	1. The Interaction Between HIV-1 Vpu and LC3C Antagonizes the Restriction of BST2 (Madjo et al., 2016)
FAT10ylation	UBA6	USE1	Unknown	1. Apoptosis-Related Proteins Induce the Death of Infected Cells (Ross et al., 2006; Snyder et al., 2009)	Unidentified

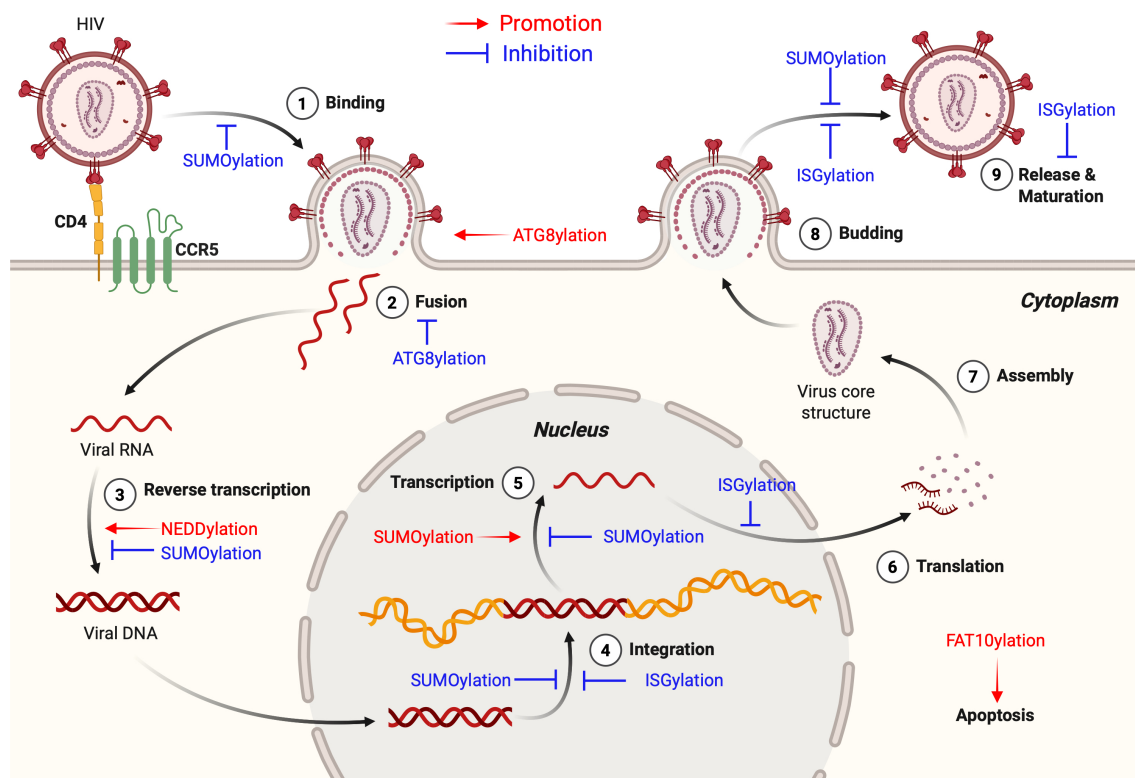


FIGURE 4

Roles of UBL modifications in the HIV-1 life cycle. This figure highlights the roles of various UBL modifications in the HIV-1 life cycle, demonstrating both HIV-promoting (shown in red text and arrows) and anti-HIV (shown in blue text and inhibitory symbols) functions. SUMOylation related regulatory mechanisms are described in detail in Figure 2. ISGylation stabilizes the tumor suppressor protein p53 to limit HIV-1 replication, inhibits early post-entry steps including viral DNA integration, and interferes with viral budding and release. NEDDylation promotes viral replication by facilitating the formation of CRL complexes that target host restriction factors such as A3G, SAMHD1, and UNG2 for degradation. ATG8ylation contributes to viral entry through LC3 lipidation at the plasma membrane, which drives membrane remodeling, and also mediates selective autophagic degradation of HIV-1 virions via TRIM5-GABARAP interactions. FAT10ylation plays a HIV-promoting role as the HIV-1 accessory protein Vpr induces FAT10 expression in RTECs, activating intrinsic apoptotic pathways and promoting cell death. This Figure is drawn using the BioRender website. This figure is generated using BioRender (<http://biorender.com/>).

multiple mechanisms: impairing E2-E3 pairing (Wauer et al., 2015), altering binding domain affinities (Swaney et al., 2015), and modulating Ub chain topology (Gersch et al., 2017). These findings illuminate potential unconventional UBL modification modes, suggesting that UBL modifiers may similarly target non-lysine residues (e.g., Ser/Thr) or non-protein substrates, while their own PTMs (e.g., phosphorylation) could spatiotemporally regulate conjugation dynamics. Systematic investigation of these atypical UBL modification paradigms will profoundly expand our understanding of multidimensional virus-host interplay.

Emerging studies have proved multilevel crosstalk among SUMOylation, ubiquitination, and ISGylation. Interferon (IFN) signaling coordinately upregulates SUMO3, ubiquitination, and ISGylation pathways by stabilizing enzymes like UBE2L6 and HERC5, thereby enhancing expression of antiviral proteins including IFITMs and SAMHD1 (Kubota et al., 2008; Nakagawa and Yokosawa, 2002; Ran et al., 2011). Key regulatory paradigms include: (1) Competitive modification, SUMOylation of TRIM5 α antagonizes its ubiquitination to maintain protein stability

(Nepveu-Traversy and Berthou, 2014); (2) Functional interference, ISG15 conjugation to Ubc13 impairs its Ub-binding capacity (Takeuchi and Yokosawa, 2005; Zou et al., 2005); (3) Synergistic enhancement, SUMOylation stabilizes MDM2 to promote Ub-dependent p53 degradation (Buschmann et al., 2001), while NEDDylation activates CRLs to amplify substrate ubiquitination (Hofmann et al., 2013; Yu et al., 2003; Liu et al., 2005; Stanley et al., 2012; Nekorchuk et al., 2013). These interactions demonstrate that UBLs can independently or cooperatively regulate pivotal biological processes. Systematic elucidation of their interaction networks will provide a conceptual framework for designing multi-target therapeutic strategies.

In summary, the UBL system orchestrates HIV infection through multilayered, dynamically reversible regulatory networks. Mechanistic dissection and therapeutic exploitation of this system, particularly through discovery of novel modification types, exploration of non-canonical modification paradigms, and elucidation of DUB/DUBL regulatory circuits, hold transformative potential for developing novel strategies against HIV.

Author contributions

CH: Supervision, Writing – review & editing, Conceptualization. XS: Supervision, Writing – review & editing. JY: Writing – original draft. HW: Writing – original draft.

Funding

The author(s) declare that financial support was received for the research and/or publication of this article. This work was supported by funding from the National Natural Science Foundation of China (82372239), the Science and Technology Department of Jilin Province (YDZJ202501ZYTS762), and the Key Laboratory of Molecular Virology, Jilin Province (20102209).

Acknowledgments

We thank Biorender (www.biorender.com) for figure drawing.

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OPEN ACCESS

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RECEIVED 20 December 2024

ACCEPTED 27 June 2025

PUBLISHED 09 July 2025

CITATION

Wu X-J, Oppelt KA, Fan M, Marie MA, Swyers MM, Williams AJ, Lemasson IM, Roper RL, Bolin P and Yang LV (2025) The GPR4 antagonist NE-52-QQ57 increases survival, mitigates the hyperinflammatory response and reduces viral load in SARS-CoV-2-infected K18-hACE2 transgenic mice. *Front. Pharmacol.* 16:1549296. doi: 10.3389/fphar.2025.1549296

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The GPR4 antagonist NE-52-QQ57 increases survival, mitigates the hyperinflammatory response and reduces viral load in SARS-CoV-2-infected K18-hACE2 transgenic mice

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COVID-19 (Coronavirus disease 19) is caused by infection with SARS-CoV-2 (severe acute respiratory syndrome coronavirus 2) in the respiratory system and other organ systems. Tissue injuries resulting from viral infection and host hyperinflammatory responses may lead to moderate to severe pneumonia, systemic complications, and even death. While anti-inflammatory agents have been used to treat patients with severe COVID-19, their therapeutic effects are limited. GPR4 (G protein-coupled receptor 4) is a pro-inflammatory receptor expressed on vascular endothelial cells, regulating leukocyte infiltration and inflammatory responses. In this study, we evaluated the effects of a GPR4 antagonist, NE-52-QQ57, in the SARS-CoV-2-infected K18-hACE2 transgenic mouse model. Our results demonstrated that GPR4 antagonist treatment increased the survival rate in this severe COVID-19 mouse model. The inflammatory response, characterized by proinflammatory cytokines and chemokines, was reduced in the GPR4 antagonist group compared with the vehicle group. Additionally, both SARS-CoV-2 RNA copy numbers and infectious viral titers in the mouse lung were decreased in the GPR4 antagonist group. The percentage of SARS-CoV-2 antigen-positive mouse brains was also decreased in the GPR4 antagonist group compared to the vehicle group. Furthermore, the GPR4 antagonist inhibited SARS-CoV-2 propagation in Vero E6 and Caco-2 cells. Together, these results suggest that GPR4 antagonism may be explored as a novel approach for the treatment of COVID-19 and other similar viral diseases.

KEYWORDS

GPR4 antagonist, SARS-CoV-2, COVID-19, mouse model, anti-inflammation, antiviral

Introduction

Infection with SARS-CoV-2 (severe acute respiratory syndrome coronavirus 2) can cause injuries to the lung and other organs, resulting in the development of COVID-19 (Coronavirus disease 19) disease symptoms in patients. The clinical features of COVID-19 vary widely, ranging from asymptomatic infection, mild illness, to severe disease with respiratory failure and death (Guan et al., 2020; Yung-Fang et al., 2020). Inflammatory cell infiltration, diffuse alveolar damage, edema, and thromboembolism in the lung are major pulmonary pathological findings associated with severe COVID-19. Additionally, extrapulmonary manifestations and multiple organ dysfunctions have been observed in COVID-19 patients (Sarkesh et al., 2020; Louis et al., 2022; Gupta et al., 2020). Autopsy studies on patients succumbing to COVID-19 demonstrate that in addition to the respiratory system, SARS-CoV-2 is widely distributed in multiple anatomic sites including the brain, heart, lymph node, gastrointestinal tract, and other tissues (Stein et al., 2022).

To date, several therapeutic modalities have been developed, including antivirals, anti-inflammatory agents, and immunomodulators, to treat COVID-19 patients (Murakami et al., 2023; Chan et al., 2024). As shown in a randomized clinical trial, treatment with the antiviral drug, remdesivir, accelerated recovery of hospitalized COVID-19 patients and demonstrated a trend of survival benefit (Beigel et al., 2020). Another randomized clinical trial, however, did not observe significant therapeutic benefits of remdesivir in patients with severe COVID-19 (Wang et al., 2020). Administering PAXLOVID™ (a combination of nirmatrelvir and ritonavir) to symptomatic COVID-19 patients within 5 days of symptoms reduced the risk of hospitalization or death by 89% compared to a placebo (Hammond et al., 2022). Another study showed that PAXLOVID™ treatment, versus placebo, did not further alleviate symptoms in patients at standard risk for severe COVID-19 (Hammond et al., 2024).

In addition to antiviral drugs, anti-inflammatory agents have been evaluated for the treatment of COVID-19, as hyperinflammatory responses (“cytokine storm”) are observed in some COVID-19 patients and contribute to poor outcomes (Merad and Martin, 2020; Coperchini et al., 2020). SARS-CoV-2 triggered elevated levels of pro-inflammatory cytokines, including interleukin-6 (IL-6) and tumor necrosis factor- α (TNF- α) (Zaim et al., 2020), which are associated with pulmonary inflammation and lung damage (Huang et al., 2020). As shown by the RECOVERY trial, the anti-inflammatory drug, dexamethasone, decreased the mortality rate of hospitalized COVID-19 patients within 28 days (22.9% in the dexamethasone group vs 25.7% in the usual care group, $p < 0.001$) (Group, 2021). Treatment with tocilizumab, a monoclonal antibody targeting the IL-6 receptor, reduced hyperinflammatory responses and provided therapeutic benefits in a subset of COVID-19 patients, but some patients were refractory to tocilizumab treatment (Huang et al., 2020; Xu et al., 2020; Toniati et al., 2020). Baricitinib is an orally administered inhibitor of Janus kinase 1 (JAK1) and JAK2, exhibiting anti-inflammatory properties. Hospitalized patients were administered a daily dosage of baricitinib, which reduced deaths of COVID-19 by approximately 20% as compared to standard care or placebo (Richardson et al., 2020; Marconi et al.,

2021; Ely et al., 2022; Abani et al., 2022). It remains a significant challenge to effectively treat COVID-19 patients with severe disease to reduce mortality and morbidity.

GPR4 is a pro-inflammatory receptor predominantly expressed in vascular endothelial cells and regulates leukocyte infiltration and inflammatory responses (Chen et al., 2011; Dong et al., 2013; Dong et al., 2017; Sanderlin et al., 2017; Sanderlin et al., 2019; Tobo et al., 2015; Velicky et al., 2017; Miltz et al., 2017; Wang et al., 2018; Krewson et al., 2020; Justus et al., 2024). Biochemically, GPR4 is partially active at physiological pH and fully activated by extracellular acidic pH (acidosis), which commonly exists in inflamed and hypoxic tissues (Justus et al., 2013; Lardner, 2001; Okajima, 2013). Notably, acidosis is a common complication observed in COVID-19 patients with severe disease (Zhou et al., 2020). Activation of GPR4 increases the expression of inflammatory cytokines, chemokines, and adhesion molecules on endothelial cells to facilitate the adhesion and extravasation of leukocytes (Chen et al., 2011; Dong et al., 2013; Sanderlin et al., 2017; Tobo et al., 2015). GPR4 also regulates vascular permeability and exudate formation under inflammatory conditions (Krewson et al., 2020). GPR4 is expressed in various tissues, with high expression in the lung, heart, and kidney (An et al., 1995; Mahadevan et al., 1995). The gene expression of GPR4 is upregulated in COVID-19 patient lung and colon tissues (Wu and McGoogan, 2020; Yang et al., 2021). Therefore, we hypothesized that GPR4 plays an integral role in COVID-19 pathophysiology (Yang et al., 2021). GPR4 antagonists have been shown to reduce inflammation, vessel permeability, exudate formation, angiogenesis, and pain in several preclinical models (Dong et al., 2013; Dong et al., 2017; Sanderlin et al., 2019; Tobo et al., 2015; Velicky et al., 2017; Miltz et al., 2017; Krewson et al., 2020).

In this study, we evaluated the effects of a GPR4 antagonist, NE-52-QQ57, in the SARS-CoV-2-infected K18-hACE2 transgenic mouse model. Our results demonstrated that the GPR4 antagonist reduced the inflammatory response and SARS-CoV-2 viral load and increased the survival rate in this severe COVID-19 murine model.

Materials and methods

Animals and ethics statement

The K18-hACE2 transgenic mice, expressing the human angiotensin-converting enzyme 2 (hACE2) under the control of the epithelial cytokeratin 18 (K18) promoter, were purchased from the Jackson Laboratory (JAX, strain # 034860). The K18-hACE2 hemizygous transgenic mice were bred with wild-type (WT) mice in the animal facility of East Carolina University (ECU). The progenies were genotyped using the protocol provided by the Jackson Laboratory. Male and female hemizygous K18-hACE2 mice (approximately 10 months old) were used in the experiments to model COVID-19 pathology, as SARS-CoV-2 causes a higher rate of severe illness and mortality in older patients (Guan et al., 2020; Yung-Fang et al., 2020). All SARS-CoV-2 infectivity studies were performed in the ECU animal biosafety level 3 (ABSL-3) laboratory in accordance with a protocol approved by the ECU Institutional Animal Care and Use Committee.

The SARS-CoV-2 infected K18-hACE2 mouse model and GPR4 antagonist treatment

SARS-CoV-2 was obtained from the Biodefense and Emerging Infections Research Resources Repository (BEI Resources, NR-52281, SARS-Related Coronavirus 2, Isolate USA-WA1/2020). We propagated the SARS-CoV-2 virus in Vero E6 cells and determined viral titer (plaque-forming units, PFU) by plaque assay (Hassan et al., 2020; Sun et al., 2020).

The K18-hACE2 transgenic mice were fed standard chow diets and housed individually in ventilated cages to minimize the risk of SARS-CoV-2 cross-infection. To induce COVID-19 in mice, K18-hACE2 transgenic mice were anesthetized with isoflurane and challenged with 1000 PFU SARS-CoV-2 virus in a final volume of 50 μ L by pipetting via the intranasal route (Winkler et al., 2020). At 4 dpi (days post-infection), mice showed some body weight loss with respiratory symptoms and were randomly assigned to orally receive either the GPR4 antagonist (NE-52-QQ57, provided by Novartis, 30 mg/kg, q. d.) or the vehicle control (0.5% methylcellulose, 0.5% Tween 80, and 99% water) (12 mice per group, 6 males and 6 females, from a total of 3 independent experiments). The effects on COVID-19 disease severity and body weight were monitored daily. Animals were weighed daily throughout the study period. The mice were euthanized either upon meeting the criteria for humane endpoints (weight loss exceeding 20% of their initial body weight or displaying severe clinical signs such as lethargy, inactivity, hunched posture, and/or markedly increased or decreased respirations) or at the end of the experiment, which occurred at 10 dpi. Additionally, age and sex-matched K18-hACE2 mice were anesthetized with isoflurane and intranasally administered 50 μ L phosphate-buffered saline (PBS) and euthanized 10 days after the PBS administration as the baseline control.

Sample processing

Mice were euthanized via intraperitoneal (IP) injection of 250 mg/kg of TBE (tribromoethanol). Blood was collected from the mouse heart and left at room temperature for 20 min to form a clot. Then, the tube was centrifuged at 2000g for 10 min to separate the serum from the cellular components. The serum was immediately frozen at -80°C for later cytokine analysis. Mouse right lungs were harvested and weighed. The right lungs were homogenized in 1 mL of FBS (fetal bovine serum)-free Dulbecco's Modified Eagle Medium (DMEM) using ceramic beads (MagNA Lyser Green Beads, Roche, #03358941001) in a MagNA Lyser instrument (Roche Life Science) for 30s. The homogenates were stored at -80°C . The left lungs and brains were fixed in 10% neutral-buffered formalin for histology.

Histology and immunohistochemistry (IHC)

The tissues were fixed in 10% neutral-buffered formalin for at least 1 week to inactivate SARS-CoV-2 before further processing.

They were then embedded in paraffin and sectioned at a thickness of 5 μ m before being stained with hematoxylin and eosin (H&E). Lung slides were scored histopathologically based on edema, hemorrhage, alveoli collapse and alveolar wall thickness, graded as 0, 1, 2, 3 which corresponded to healthy, slight, moderate, and severe levels of lung histopathology, respectively.

Additionally, lung and brain slides were immunostained with anti-CD4 (Abcam, #ab183685, dilution 1:500), anti-CD8 (Abcam, #ab217344, dilution 1:500), or SARS-CoV-2 specific anti-nucleocapsid antibody (Novus Biologicals, #NB100-56683, dilution 1:500) and horseradish peroxidase (HRP) conjugated anti-rabbit secondary antibody following the manufacturer's instructions. Briefly, sections were deparaffinized, and antigen retrieval was performed. Endogenous peroxidase blocking was carried out by treating slides with H_2O_2 . Endogenous biotin, biotin receptors, and avidin binding sites were blocked with an Avidin-biotin blocking kit (Life Technologies, #004303), followed by a subsequent normal serum blocking step. Slides were incubated with the primary antibody overnight at 4°C and processed using the VECTASTAIN[®] Elite ABC-HRP Rabbit IgG Kit (Vector Laboratories, #PK-6101) according to the manufacturer's instructions. HRP detection was achieved through incubation with the secondary antibody followed by DAB (3,3'-diaminobenzidine, Vector Laboratories, #SK-4105) development for 5 min. Tissues were counterstained with Gill's hematoxylin. As a negative control, lung and brain slides from three uninfected mice treated with PBS were evaluated for comparison. The slides were then scanned using an optical microscope (Zeiss Axio Imager M2 microscope) with a $\times 10$ objective to check the existence of positive staining of SARS-CoV-2 antigens.

Plaque assay

To compare viral titers of the lung homogenates, plaque assays in Vero E6 cells were performed. Lung lysate samples were serially diluted ten-fold with serum-free DMEM medium and used to infect Vero E6 cells seeded in a 12-well plate (1.5×10^5 cells per well). The plate was then incubated at 37°C in a 5% CO_2 atmosphere for 1 h with intermittent rocking every 10 min to prevent cellular desiccation and facilitate viral binding. Following this viral adsorption, the medium was removed and cells were overlaid with 1 mL of a mixture containing DMEM, 2% fetal bovine serum (FBS), 1% Penicillin/Streptomycin, and 0.4% methylcellulose. After 3 days of incubation, the methylcellulose overlays were gently removed, and the cells were fixed with 10% neutral-buffered formalin for 20 min. Subsequently, the 10% neutral-buffered formalin solution was removed, and 0.05% (w/v) crystal violet stain solution in distilled water was added for 10 min. Viral plaques were counted, and viral titers were calculated as plaques/mg lung.

Quantitative reverse transcription-polymerase chain reaction (RT-qPCR)

Total RNA was isolated using the DNA/RNA/Protein extraction kit (IBI Scientific, #IB47702), and cDNA was synthesized using 1 μ g

of RNA (SuperScript IV First Strand cDNA synthesis Reaction, Invitrogen, #18091050). Subsequently, RT-qPCR was performed using a Quantstudio 3 Flex real-time PCR machine with 2× TaqMan Universal Master Mix II (Applied Biosystems, #2704844). Commercial primers/probe sets specific for mouse *Gpr4* (Mm01322176_s1), *Il-1β* (Mm00434228_m1), *Il-6* (Mm00446190_m1), *Il-10* (Mm01288386_m1), *Il-18* (Mm00434226_m1), *Tnf-α* (Mm00443258_m1), *Atf3* (Mm00476033_m1), *Cox2/Ptgs2* (Mm00478374_m1), *Cxcl2* (Mm00436450_m1), *E-selectin* (Mm00441278_m1), *Icam1* (Mm00516023_m1), *Vcam1* (Mm01320970_m1), human GPR4 (Hs00270999_s1), mouse *Ace2* (Mm01159006_m1), human *ACE2* (Hs01085333_m1), mouse *Tmprss2* (Mm00443687_m1), and human *TMPRSS2* (Hs01122322_m1) were purchased from ThermoFisher and the expressions were normalized to 18S rRNA (HS99999901_s1) levels.

Additionally, RT-qPCR of the SARS-CoV-2 RNA copy number was performed to test the viral load of lung tissue using the primer kit from Biosearch Technologies (2019-nCoV CDC probe and primer kit for SARS-CoV-2, KIT-nCoV-PP1-1000) targeting the nucleocapsid gene (2019-nCoV_N1): forward primer 5' GACCCCAAAATCAGCGAAAT 3', reverse primer 5' TCTGGTTACTGCCAGTTGAATCTG 3', and probe 5' FAM-ACCCCGCATTACGTTTGGTGGACC-BHQ-1 3'. An inactivated SARS-CoV-2 genomic RNA from BEI Resources (#NR-52347) was serially diluted and used as a standard curve control. SARS-CoV-2 levels were determined utilizing a standard curve and expressed as quantified viral RNA copy numbers/μg of RNA.

Lung and serum cytokines

The lung lysates and serum samples were analyzed using a Luminex 200™ system with a ProcartaPlex Mouse Cytokine/Chemokine Panel 1 26plex platform (ThermoFisher, #EPX260-26088-901), following the manufacturer's instructions. Cytokine and chemokine concentrations were determined based on standard curves. Acquired data were analyzed using ProcartaPlex Analysis APP (ThermoFisher). Changes in cytokine levels were analyzed using GraphPad Prism 9 (N = 12 for the vehicle group; N = 10 for the GPR4 antagonist group as two mice died at night between health checks and their lung and serum samples were not suitable or available for collection for Luminex or RNA analyses).

In vitro assay of the GPR4 antagonist incubated with SARS-CoV-2

GPR4 antagonist (20 μM, 10 μM, 1 μM, and 0.1 μM) or DMSO (dimethyl sulfoxide) vehicle control in 100 μL of DMEM medium were incubated with SARS-CoV-2 (100 PFU, 100 μL) for 1 h at room temperature, and then the mixture was used to infect Vero E6 cells for 1 h at 37°C in a 5% CO₂ atmosphere. Following this incubation, the medium was removed and 1 mL of a mixture containing DMEM, 2% FBS, 1% Penicillin/Streptomycin, and 0.4% methylcellulose was added and incubated for 3 days before plaque counting. For each

condition, three biological replicates were used with two experimental repeats.

Anti-SARS-CoV-2 effects of the GPR4 antagonist post-infection in Vero E6 and Caco-2 cells

Confluent Vero E6 cells in 12-well plates were infected with 100 PFU (for RT-qPCR) or 30 PFU (for plaque assay) for 1 h at 37°C in a 5% CO₂ incubator. Confluent Caco-2 cells in 12-well plates were infected with 1000 PFU (for RT-qPCR) for 1 h at 37°C in a 5% CO₂ incubator. To assess SARS-CoV-2 RNA levels, after 1 h of virus infection, the culture medium containing the virus was removed. Fresh complete DMEM medium with the GPR4 antagonist or the DMSO vehicle control was added to the cells for a 24-h treatment. After the treatment, RNA was harvested from Vero E6 and Caco-2 cells and RT-qPCR assay was performed to measure the SARS-CoV-2 RNA copy numbers. For the plaque assay, after 1 h of virus infection, the culture medium containing the virus was removed. Cell medium was changed to DMEM, 2% FBS, 1% Penicillin/Streptomycin, 0.4% methylcellulose, and various concentrations of GPR4 antagonist or the DMSO vehicle control. After 3 days of incubation, the medium was removed, cells were fixed with 10% neutral-buffered formalin, and then stained with crystal violet. For each condition, three biological replicates were used with two experimental repeats.

Cell viability assay

Cell viability was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Roche, #11465007001) and CellTiter-Glo Luminescent Cell Viability (CTG) Assay (Promega, #G7570). Vero E6 cells cultured in 96-well plates (5 × 10⁴ per well) were incubated with the GPR4 antagonist or the DMSO vehicle control for 24 h. For the MTT assay, 20 μL of MTT solution was added per well and incubated at 37 °C for 4 h. After this, the cells were lysed using 200 μL of the solubilization solution and absorbance was measured at 570 nm. For CTG assay, 100 μL of CellTiter-Glo reagent (Promega, #G7573) was added into each well and luminescence intensities were measured using a SpectraMax ID5 plate reader (Molecular Devices). For each condition, three biological replicates were used with two experimental repeats.

Statistical analysis

Statistical analysis and graph preparation were conducted using GraphPad Prism 9. Results are presented as mean ± SEM (standard error of the mean). The normality of the data was assessed using the Shapiro-Wilk test prior to hypothesis testing. In cases where the data followed a normal distribution, differences between two groups were evaluated using the unpaired t-test. Alternatively, for datasets exhibiting a non-normal distribution, differences between groups were assessed using the Mann-Whitney test. Survival significance

was examined using the log-rank (Mantel-Cox) test. The Chi-square test was used to compare the SARS-CoV-2 positive infection rate in the mouse brain. A P value ≤ 0.05 was considered statistically significant.

Results

The GPR4 antagonist NE-52-QQ57 increases the survival rate of the K18-hACE2 mice infected with SARS-CoV-2

We intranasally inoculated 1000 PFU of SARS-CoV-2 (strain 2019n-CoV/USA_WA1/2020) into 10-month-old male and female hemizygous K18-hACE2 transgenic mice. K18-hACE2 mice infected with SARS-CoV-2 developed severe disease. These mice exhibited weight loss of 10%–20%, lethargy, inactivity, ruffled fur, markedly increased or decreased respirations, and hunched posture, with most mice reaching the humane endpoint or dying by 8 dpi. Starting from 4 dpi, we administered either vehicle or the GPR4 antagonist NE-52-QQ57 to assess its effects on SARS-CoV-2-induced inflammatory responses and disease severity in the K18-hACE2 mouse model. Our results demonstrated that 25% of the mice in the vehicle control group (group size equals 6 males and 6 females) survived at 10 dpi. In comparison to vehicle controls, 66.7% of the mice in the GPR4 antagonist-treated group (group size equals 6 males and 6 females) survived at 10 dpi. The results indicate that the GPR4 antagonist treatment increased the survival rate in this severe COVID-19 mouse model ($p = 0.03$, by log-rank (Mantel-Cox) test, [Figure 1A](#)). All infected mice were weighed daily throughout the study period. Beginning at 5 dpi, all SARS-CoV-2-infected K18-hACE2 mice demonstrated marked weight loss. GPR4 antagonist treatment decreased the amount of weight loss by 7 dpi, but differences between vehicle and treatment groups were not statistically significant ([Figure 1B](#)). Notably, all surviving mice started regaining weight after 7–8 dpi ([Figure 1B](#)) and their overall health condition and activity were gradually improved by 10 dpi.

SARS-CoV-2 induced GPR4 expression in the mouse lungs

Quantitative RT-PCR was performed to examine GPR4 RNA expression in the mouse lungs infected with SARS-CoV-2 and the control mouse lungs. GPR4 RNA expression levels were significantly increased by about 7-fold in the lungs of K18-hACE2 mice infected with SARS-CoV-2 when compared to the lungs of control mice intranasally administrated with PBS ([Figure 1C](#)). Within the mice infected with SARS-CoV-2, a trend of reduced GPR4 expression was observed in the GPR4 antagonist group compared to the vehicle group ([Figure 1C](#)). For lung histological analysis, there was a trend (not statistically significant) of reduced histopathological scores of SARS-CoV-2 infected mouse lung tissues in the GPR4 antagonist group compared with the vehicle control group ([Figures 1D,E](#)). Additionally, 1 out of 12 mice in the

vehicle control SARS-CoV-2 infected group had brain hemorrhage which was not observed in the GPR4 antagonist group ([Supplementary Figure S1](#)).

Reduced pro-inflammatory cytokine and chemokine levels in the GPR4 antagonist-treated K18-hACE2 mice infected with SARS-CoV-2

COVID-19 disease progression is correlated with significant alterations in cytokine profiles ([Huang et al., 2020; Mehta et al., 2020](#)). To evaluate the impact of GPR4 antagonist treatment on the inflammatory response to SARS-CoV-2 infection, RT-qPCR was employed to analyze the RNA levels of inflammatory genes in the lung tissues of SARS-CoV-2-infected K18-hACE2 mice. Compared with the lungs of control mice (PBS), the expression of inflammatory genes was increased in the lungs of SARS-CoV-2-infected mice ([Figure 2A](#)). Compared with the lungs of vehicle control mice, GPR4 antagonist treatment significantly reduced the expression of the inflammatory genes *Cxcl2*, *Ptgs2*, and *Icam1* in the lungs of K18-hACE2 mice infected with SARS-CoV-2 ([Figure 2A](#)). Moreover, other inflammatory genes (e.g., *Il-1 β* , *Il-6*, *Il-10*, *Il-18*, *Tnf- α* , *E-selectin*, *Atf3*, and *Vcam1*) also showed a strong trend of reduced expression, although not statistically significant ([Figure 2A](#)). In addition, cytokine and chemokine protein levels in the lung tissue and serum were measured using a mouse 26 plex Luminex panel ([Figures 2B,C; Supplementary Figures S2 and S3](#)). IL-6, IL-10, IL-17A, and CXCL2 proteins showed a trend of reduced expression in the mouse lung in the GPR4 antagonist group ([Figure 2B](#)). Regarding serum cytokines and chemokines, the T cell-associated cytokine IFN γ showed a significant decrease in the GPR4 antagonist group ([Figure 2C](#)). Several other serum cytokines (e.g., IL-1 β , IL-6, IL-10, IL-17A, and TNF α) had a trend of lower expression in the GPR4 antagonist treated mice compared with the vehicle group ([Figure 2C](#)). IL-4 and IL-13 were under detection limits in the serum samples ([Supplementary Figure S3](#)). Overall, our data suggest that the GPR4 antagonist treatment dampens the expression levels of cytokines and chemokines induced by SARS-CoV-2 infection.

GPR4 antagonist treatment decreases the viral loads in the lungs and brains of the K18-hACE2 mice infected with SARS-CoV-2

To assess the viral load, mouse lung lysates were analyzed by RT-qPCR and plaque assays. Treatment with the GPR4 antagonist reduced SARS-CoV-2 viral loads in the mouse lung tissues. RT-qPCR revealed that SARS-CoV-2 viral RNA copy numbers were reduced about 11.4-fold in the lungs of GPR4 antagonist-treated mice compared to the vehicle treatment group ($6.28 \times 10^8 \pm 3.99 \times 10^8$ copies/ μ g RNA vs $7.18 \times 10^9 \pm 3.20 \times 10^9$ copies/ μ g RNA, $p = 0.08$; [Figure 3A](#)). Likewise, plaque assays demonstrated a statistically significant reduction in infectious SARS-CoV-2 in the mouse lung tissues of the GPR4 antagonist group compared to the vehicle-control group (73.83 ± 63.50 pfu/mg vs 397.6 ± 213.0 pfu/mg, $p = 0.02$; [Figure 3B](#)).

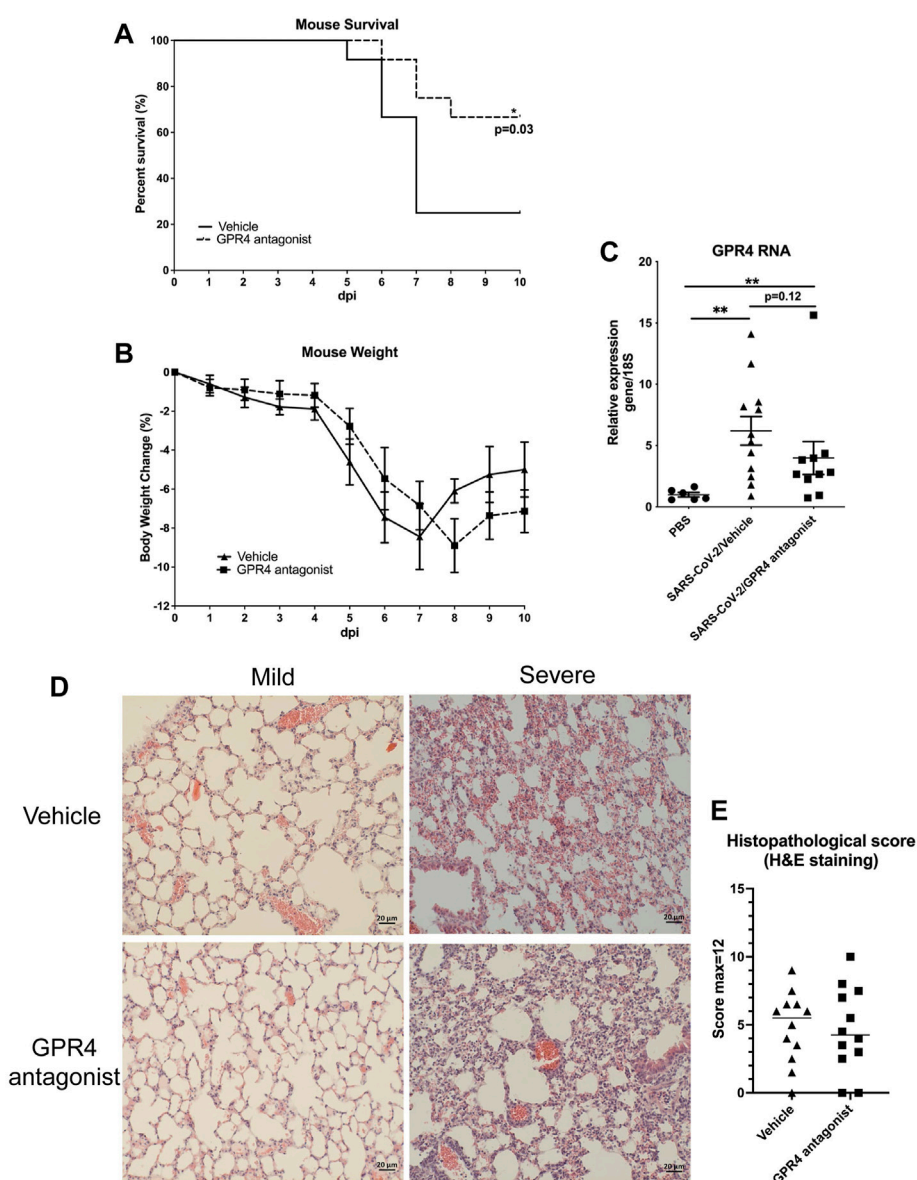


FIGURE 1

Treatment with GPR4 antagonist NE-52-QQ57 improves survival of SARS-CoV-2-infected K18-hACE2 mice. Mice were treated with GPR4 antagonist NE-52-QQ57 or vehicle control for up to 6 days starting from 4 dpi. **(A)** The survival rate of SARS-CoV-2-infected K18-hACE2 mice is increased by the administration of the GPR4 antagonist. Ten-month-old male and female K18-hACE2 transgenic mice were intranasally inoculated with 1000 PFU of SARS-CoV-2 ($N = 12$). Survival analysis was performed using the Kaplan-Meier method with a log-rank (Mantel-Cox) test, $*p < 0.05$. **(B)** Daily body weight changes in GPR4 antagonist-treated or vehicle control mice were recorded up to 10 dpi or until the mice reached the humane endpoint. The difference in body weight change was analyzed using multiple unpaired t-tests. **(C)** RT-qPCR was conducted to quantify the expression of GPR4 in non-infected (PBS) and SARS-CoV-2-infected mouse lung tissues (compared using two-tailed Mann-Whitney test) ($N = 6$ for PBS no virus inoculation; $N = 12$ for vehicle; $N = 10$ for GPR4 antagonist). Error bars indicate means $\pm$ SEM. $**p < 0.01$. **(D)** Representative pictures of mouse lung histology (H&E staining) with mild or severe histopathology in vehicle or GPR4 antagonist-treated mice. Scale bar = 20 μ m. **(E)** Mouse lung histopathological score. Two-tailed Student's t-test did not indicate significance.

To evaluate the effects of GPR4 antagonist treatment on viral burden in mouse brains, the presence of the SARS-CoV-2 virus in the brains was assessed through IHC staining with an antibody detecting the viral nucleocapsid protein. Subsequently, the percentage of brain tissue positive for the viral antigen was determined. IHC staining demonstrated that 66.67% of GPR4 antagonist-treated mice (8 out of 12) showed no detection of SARS-CoV-2 in the brain. In contrast, only 25% of the vehicle-

treated mice (3 out of 12) showed no detection of SARS-CoV-2 in the brain (Figure 3C). The GPR4 antagonist group of mice exhibited significantly lower levels of the virus in the brain compared to the vehicle control group (Figure 3D, Chi-Square test, $p = 0.04$). All the mice without detectable SARS-CoV-2 by IHC in their brains survived to 10 dpi. Together, these findings demonstrate that GPR4 antagonist treatment systemically reduces SARS-CoV-2 viral loads in K18-hACE2 mice.

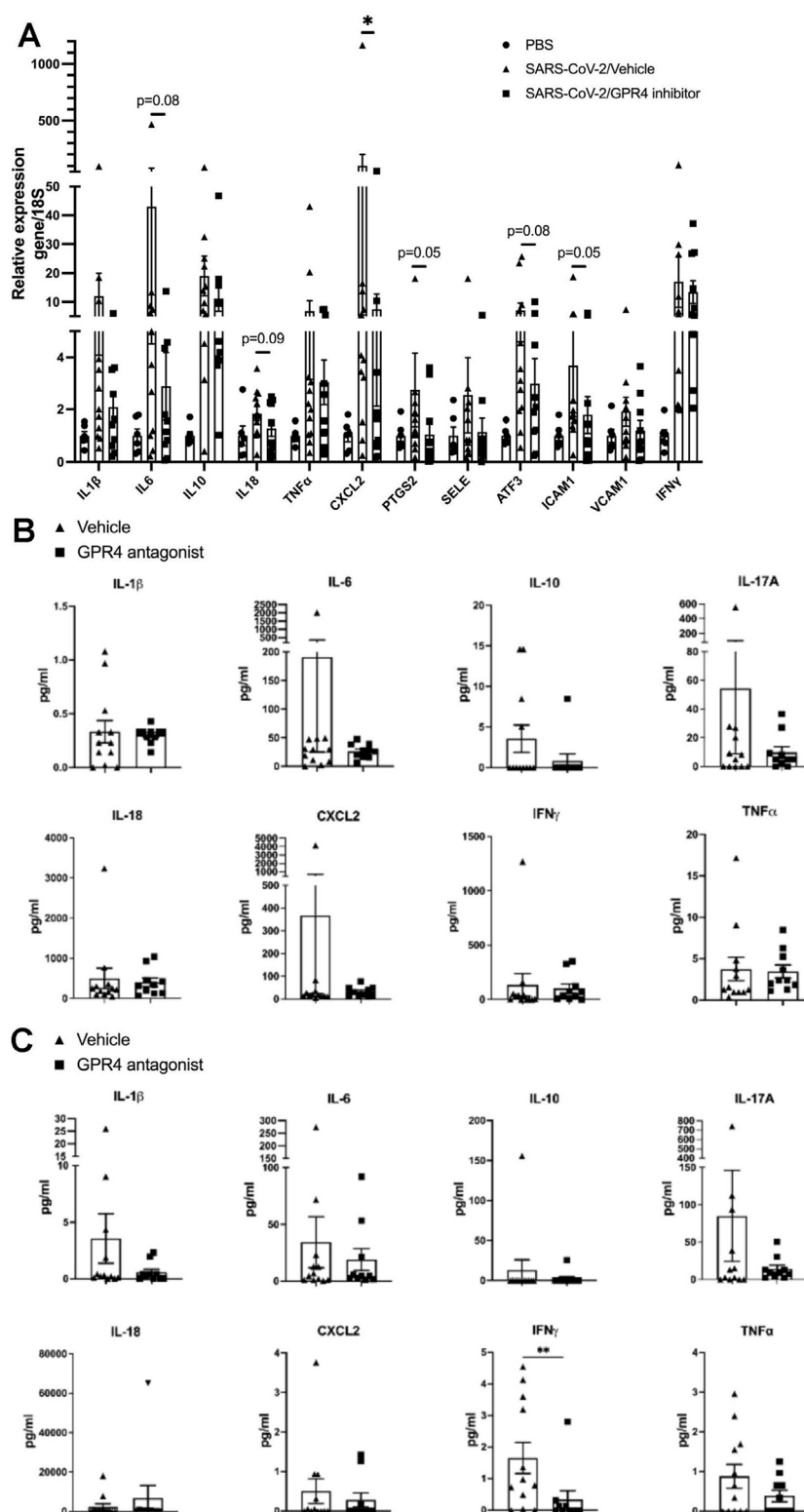


FIGURE 2

GPR4 antagonist treatment reduces cytokine and chemokine levels in K18-hACE2 mice infected with SARS-CoV-2. (A) Fold change in gene expression levels of specified cytokines, chemokines, and other inflammatory genes assessed via RT-qPCR and normalized to 18S rRNA, compared with vehicle controls in mouse lung homogenates (N = 12 for vehicle, N = 10 for GPR4 antagonist). * $p < 0.05$. Gene expression in the lung of control mice (PBS) without SARS-CoV-2 infection was set as 1. (B) Cytokine/chemokine protein levels in mouse lung tissues measured by the Luminex multiplex platform. (C) Cytokine/chemokine protein levels in mouse serum measured by the Luminex multiplex platform. Statistical differences in cytokine/chemokine levels were analyzed using the one-tailed Mann-Whitney test (N = 12 for vehicle, N = 10 for GPR4 antagonist). Error bars indicate mean $\pm$ SEM.

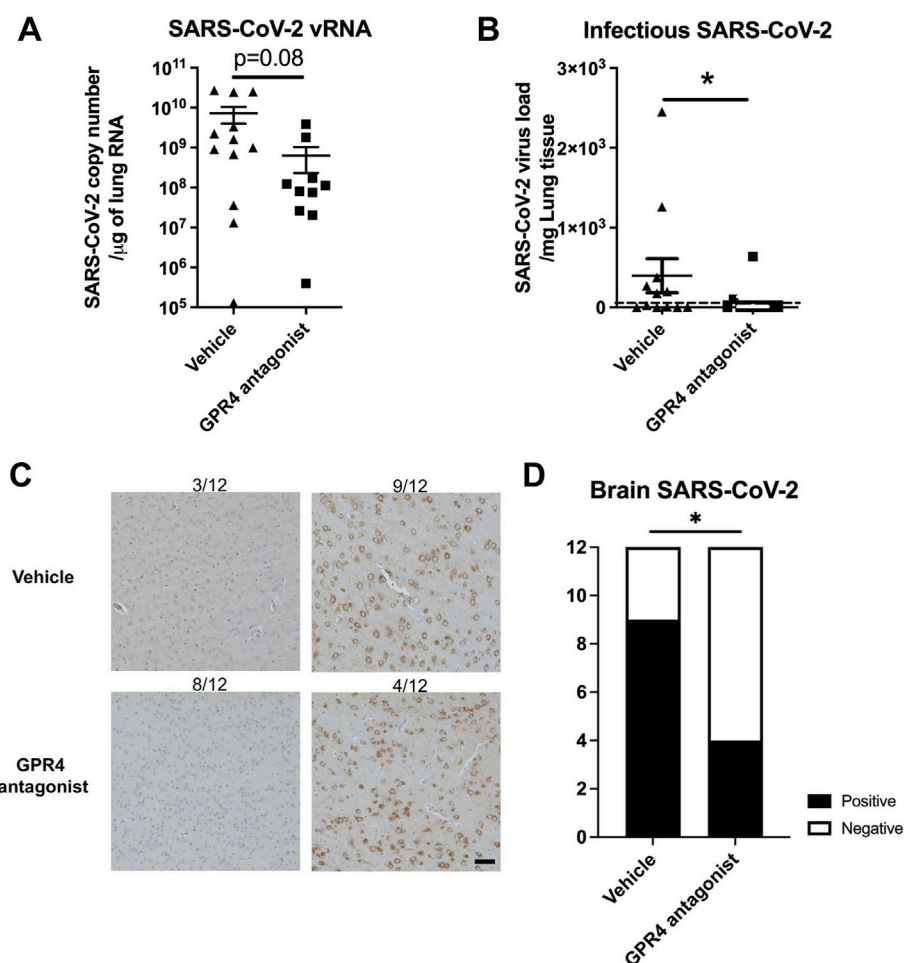


FIGURE 3

SARS-CoV-2 viral load in the lungs and brains from K18-hACE2 mice that received either GPR4 antagonist or vehicle. **(A)** RT-qPCR to quantify viral RNA levels in mouse lung tissues (RNA copies/ μ g lung RNA). The data were analyzed using the two-tailed unpaired t-test and shown in mean $\pm$ SEM (N = 12 for vehicle, N = 10 for GPR4 antagonist). **(B)** Plaque assays were analyzed to determine the infectious viral titers (PFU/mg lung) in the lungs of vehicle- and GPR4 antagonist-treated mice infected with SARS-CoV-2. The limit of detection (LOD = 5 PFU/mg lung) is indicated by the dotted horizontal line. * $p < 0.05$. **(C)** Analysis of SARS-CoV-2 virus nucleocapsid distribution in mouse brain through IHC. The percentage of SARS-CoV-2 positive viral staining in the mouse brain was assessed using a microscope (N = 12 for vehicle, N = 12 for GPR4 antagonist). In the vehicle group, 3 out of 12 mouse brains (3/12) are negative for SARS-CoV-2 and 9 out of 12 are positive. In comparison, 8 out of 12 are negative and 4 out of 12 mouse brains are positive for SARS-CoV-2 in the GPR4 antagonist group. Scale bar = 20 μ m. Error bars indicate mean $\pm$ SEM. **(D)** SARS-CoV-2 positive ratio in the brains of mice treated with GPR4 antagonist or vehicle. Analyzed using the Chi-square test, * $p < 0.05$.

Reduction in CD4⁺ and CD8⁺ immune cell clusters in the brains and lungs of SARS-CoV-2-infected mice treated with the GPR4 antagonist

We further assessed the presence of CD4⁺ and CD8⁺ T cells in the brains and lungs of SARS-CoV-2-infected mice by IHC, as these cells are essential for the inflammatory and immune responses. CD4⁺ and CD8⁺ T cell infiltration was observed on stained sections as clusters or scatterings of cells in the brains of SARS-CoV-2-infected mice (Figures 4A,C). Compared to the vehicle group, GPR4 antagonist-treated mice exhibited fewer CD4⁺ and CD8⁺ immune cell clusters in the mouse brain sections (Figures 4B,D). Moreover, fewer CD4⁺ and CD8⁺ T cell clusters were detected in the lung sections of SARS-CoV-2-infected mice treated with the GPR4 antagonist (Supplementary Figure S4).

GPR4 antagonist inhibits SARS-CoV-2 propagation *in vitro*

As the GPR4 antagonist treatment reduced the viral load in the lungs and brains of K18-hACE2 mice infected with SARS-CoV-2 (Figure 3), we then used cell cultures to assess the potential anti-viral effects of the GPR4 antagonist in pharmacologically relevant concentrations as previously demonstrated in mouse models (Velcicky et al., 2017). To investigate if the GPR4 antagonist could inactivate SARS-CoV-2 directly, we incubated SARS-CoV-2 (100 PFU) with the GPR4 antagonist (20 μ M, 10 μ M, 1 μ M, and 0.1 μ M) or the DMSO vehicle control for 1 h at room temperature before infecting Vero E6 cells. Plaque assays demonstrated that the GPR4 antagonist could not directly inactivate SARS-CoV-2, as similar plaque numbers were observed in the GPR4 antagonist

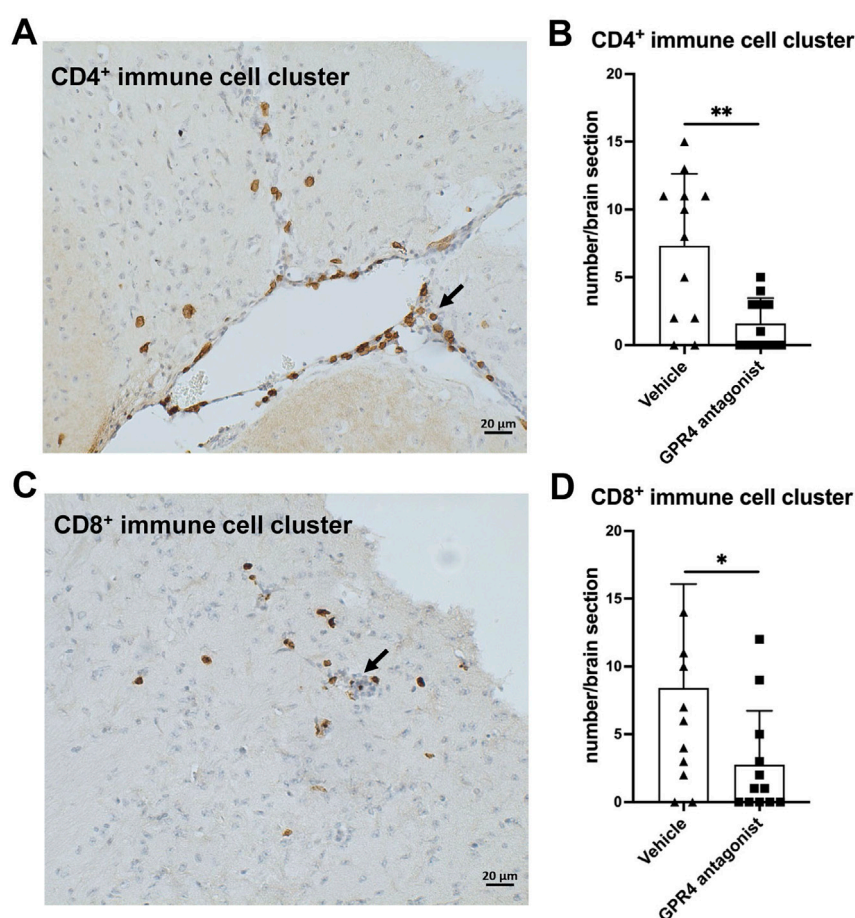


FIGURE 4 GPR4 antagonist treatment reduces CD4⁺ and CD8⁺ immune cell clusters in the brains of SARS-CoV-2-infected K18-hACE2 mice. **(A)** A representative image of a CD4⁺ immune cell cluster (indicated by the arrow) in the mouse brain, visualized using IHC with an antibody detecting CD4. Scale bar = 20 μ m. **(B)** Quantification of the number of CD4⁺ immune cell clusters in the brain using microscopy. Analyzed using the two-tailed Student's t-test, ** $p < 0.01$. Error bars represent mean $\pm$ SEM. $N = 12$ for the vehicle group, and $N = 12$ for the GPR4 antagonist group. **(C)** A representative image of a CD8⁺ immune cell cluster (indicated by the arrow) in the mouse brain, visualized using IHC with an antibody detecting CD8. Scale bar = 20 μ m. **(D)** Quantification of the number of CD8⁺ immune cell clusters in the brain using microscopy. Analyzed by the two-tailed Student's t-test, * $p < 0.05$. Error bars represent mean $\pm$ SEM. $N = 12$ for the vehicle group, and $N = 12$ for the GPR4 antagonist group.

groups compared with the DMSO group (Figure 5A). To determine if the GPR4 antagonist had anti-SARS-CoV-2 effects when administered post-infection, we infected Vero E6 cells with virus for a 1-h adsorption period and then treated the cells with the GPR4 antagonist for 24 h (Figure 5B). The GPR4 antagonist significantly decreased the RNA copy numbers of SARS-CoV-2 in Vero E6 cells in a dose-dependent manner as assessed by RT-qPCR (Figure 5B). GPR4 antagonist (20 μ M) reduced the genomic RNA levels of SARS-CoV-2 by 8.3-fold (Figure 5B). Similarly, plaque assays confirmed a decrease in infectious virus after 72 h of GPR4 antagonist treatment (Figure 5C). Both CTG assay and MTT assay showed that the GPR4 antagonist (20 μ M, 10 μ M, 1 μ M, 0.1 μ M) had no cytotoxic effects on Vero E6 cells (Supplementary Figure S5). Moreover, the GPR4 antagonist (20 μ M) significantly inhibited SARS-CoV-2 replication by 3.6-fold when compared to the DMSO vehicle control in Caco-2 human colon epithelial cells as assessed by RT-qPCR (Supplementary Figure S6).

Discussion

Following the onset of the COVID-19 pandemic, vaccines and therapeutic interventions have been developed to address the spectrum of disease severity associated with the SARS-CoV-2 virus. COVID-19 vaccines have been shown to reduce the risk of severe illness, hospitalization, death, and long COVID symptoms (Scobie et al., 2021; Link-Gelles et al., 2023; Català et al., 2024). Antivirals, such as PAXLOVIDTM, can also reduce the risk of hospitalization and death in COVID-19 patients (Hammond et al., 2022). Despite the remarkable development of SARS-CoV-2 vaccines and antivirals, some COVID-19 patients may still progress to severe illness, including hospitalization and death. Hyperinflammation and cytokine storm have been demonstrated to play a pivotal role in the pathophysiology of severe COVID-19 and have consistently been linked to an elevated risk of mortality among patients afflicted with the disease (Mehta et al., 2020; Del Valle et al., 2020; Short et al., 2021). Anti-inflammatory and

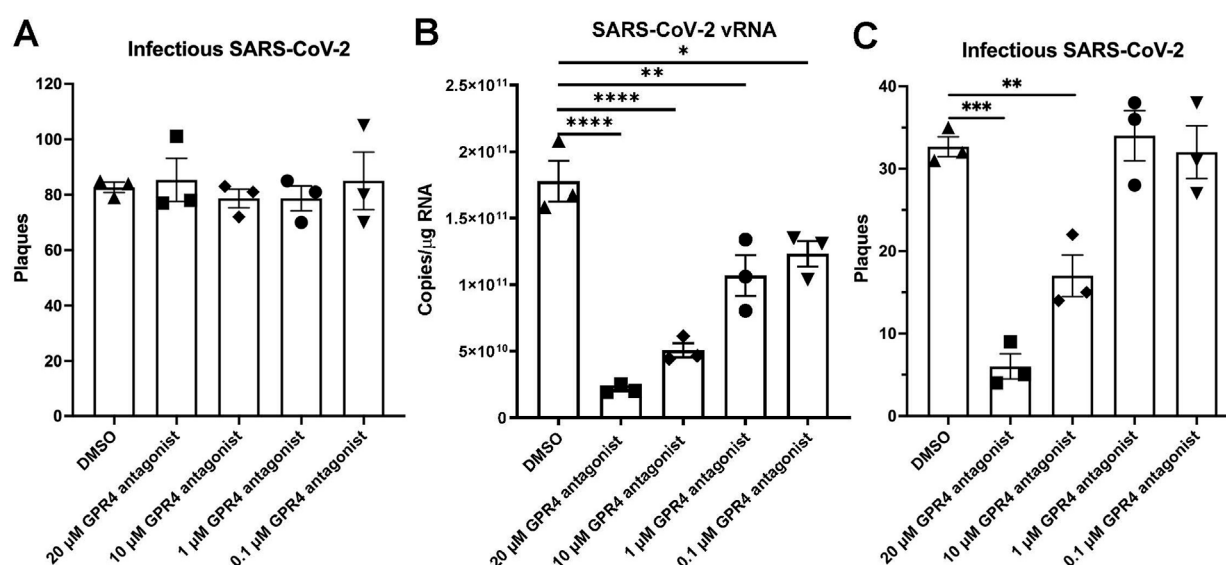


FIGURE 5
Anti-SARS-CoV-2 effects of GPR4 antagonist *in vitro*. **(A)** GPR4 antagonist incubated with SARS-CoV-2 (100 PFU) for 1 h before infecting Vero E6 cells. GPR4 antagonist-containing medium was removed after infection. Plaque formation was measured to determine the infectious SARS-CoV-2 viral titer. N = 3 samples. **(B)** Viral RNA in the SARS-CoV-2-infected Vero E6 cells (100 PFU inoculum) treated with various concentrations of GPR4 antagonist was determined 24 h post-infection. The GPR4 antagonist was maintained in the medium until cell assessment 24 h after treatment. Viral RNA isolated from Vero E6 cells was quantified by RT-qPCR targeting the nucleocapsid gene. N = 3 samples. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$. **(C)** The infectious viral load by the plaque assay in SARS-CoV-2-infected Vero E6 cells (30 PFU inoculum) in response to treatment of vehicle DMSO or GPR4 antagonist at 72 h post-infection. The GPR4 antagonist was maintained in the medium for 72 h until cell assessment. N = 3 samples. ** $p < 0.01$, *** $p < 0.001$. Comparisons between groups were analyzed by one-way ANOVA followed by *post hoc* Dunnett's test. Error bars indicate mean $\pm$ SEM.

immunosuppressant agents can bring therapeutic benefits to COVID-19 patients with severe disease (Murakami et al., 2023). It has been reported that anti-inflammatory and immunosuppressant agents, such as dexamethasone, tocilizumab, and baricitinib, moderately reduce the mortality rate in hospitalized COVID-19 patients by decreasing the late-phase hyperinflammatory responses (Huang et al., 2020; Group, 2021; Xu et al., 2020; Toniati et al., 2020; Richardson et al., 2020; Marconi et al., 2021; Ely et al., 2022; Abani et al., 2022).

In this study, we demonstrated the anti-inflammatory and anti-viral effects of the GPR4 antagonist NE-52-QQ57 in the SARS-CoV-2-infected K18-hACE2 mouse model. Animals administered with the GPR4 antagonist exhibited an increased survival rate compared to those in the vehicle control group in this severe COVID-19 mouse model (Figure 1A). Also, GPR4 antagonist treatment reduced the expression of inflammatory cytokines, chemokines, adhesion molecules, and PTGS2 (COX2) in the SARS-CoV-2-infected mouse lungs and serum samples (Figure 2). GPR4 is a pro-inflammatory receptor overexpressed in inflamed tissues (Chen et al., 2011; Dong et al., 2013; Dong et al., 2017; Sanderlin et al., 2017; Sanderlin et al., 2019; Tobo et al., 2015; Velicky et al., 2017; Miltz et al., 2017; Wang et al., 2018; Krewson et al., 2020; Justus et al., 2024). In line with previous findings that GPR4 expression is upregulated in lung and colon tissues of COVID-19 patients (Wu and McGoogan, 2020; Yang et al., 2021), we observed an increase in GPR4 expression in the K18-hACE2 mouse lung following SARS-CoV-2 infection (Figure 1C). Altogether, the findings suggest that GPR4 is a pro-inflammatory receptor involved in COVID-19 and GPR4 antagonism may be exploited as a potential therapeutic

approach to dampen the hyperinflammatory response of COVID-19 to alleviate disease severity.

In addition to its anti-inflammatory effects, we demonstrated the novel anti-viral effects of the GPR4 antagonist NE-52-QQ57 against SARS-CoV-2. The animals treated with the GPR4 antagonist showed significantly lower viral titers in both lung and brain tissues compared with higher titers in the vehicle control group. These results demonstrate a protective effect of the GPR4 antagonist in the SARS-CoV-2 infection, even though the underlying mechanisms remain unclear. We found that the GPR4 antagonist had no direct effects on inactivating SARS-CoV-2 and did not block SARS-CoV-2 virus entry into Vero E6 cells (Figure 5A). However, the GPR4 antagonist can inhibit SARS-CoV-2 propagation in Vero E6 cells after the virus enters the cells (Figures 5B,C). Furthermore, the GPR4 antagonist also inhibits SARS-CoV-2 propagation in Caco-2 cells (Supplementary Figure S6). In an attempt to further evaluate potential mechanisms by which the GPR4 antagonist inhibits SARS-CoV-2 propagation, we assessed its effects on the gene expression of ACE2 and TMPRSS2 which are important for viral cellular entry. We found no difference in Vero E6 cells using the TaqMan primer specific for human ACE2 (Supplementary Figure S7A); however, the TaqMan primer specific for human TMPRSS2 did not work with Vero E6 (data not shown). Similarly, no differences in ACE2 or TMPRSS2 expressions were found in A549 human lung epithelial cancer cells, Caco-2 human colon epithelial cancer cells, and mouse Lewis lung carcinoma cells after a 24-h GPR4 antagonist treatment (Supplementary Figures S7B-D). These results further support that the GPR4 antagonist does not affect the entry of SARS-CoV-2 into

cells (Figure 5A). Moreover, neither SARS-CoV-2 infection nor GPR4 antagonist treatment significantly changed GPR4 mRNA expression in Vero E6 and Caco-2 cells (Supplementary Figure S8), which suggests that the antagonist exerts its effects by inhibiting the activity rather than expression of GPR4. As SARS-CoV-2 propagation involves multiple steps such as viral entry, replication, release, and host immune response, further research is needed to elucidate the precise mechanisms by which the GPR4 antagonist reduces SARS-CoV-2 propagation in cells and viral burden in animals. Interestingly, previous studies have demonstrated that the pH homeostasis of host cells is critical for SARS-CoV-2 replication (Fares et al., 2025; Aganovic, 2023). Since GPR4 functions as a pH sensor, it is plausible to investigate in future studies whether the GPR4 antagonist inhibits SARS-CoV-2 propagation via modulating host cell pH homeostasis.

As described above, our results demonstrate that the GPR4 antagonist NE-52-QQ57 has anti-inflammatory and anti-viral dual effects revealed by studying the SARS-CoV-2-infected mouse model and cell culture models (Figures 2–5; Supplementary Figure S6). This pharmacological feature of the GPR4 antagonist can be advantageous for the treatment of COVID-19, as the reduction in both viral load and hyperinflammatory response is necessary for optimal management of COVID-19 related complications (Sasaki et al., 2024; Li et al., 2023). Notably, certain pharmacological agents with anti-inflammatory and anti-viral effects have been evaluated in preclinical studies (Khanna et al., 2022; Ma et al., 2020).

In addition to the lungs, SARS-CoV-2 can infect the brain and other organs and cause systemic effects (Sarkesh et al., 2020; Louis et al., 2022; Gupta et al., 2020). Neurological complications associated with COVID-19 have the potential to be debilitating and even life-threatening (Schwablenland et al., 2021; Villadiego et al., 2023; Pang et al., 2024). In the SARS-CoV-2-infected K18-hACE2 mouse model, viral neuroinvasion and encephalitis are commonly observed in the brain and are associated with severe disease and mortality of the infected animals (Kumari et al., 2021). Concordantly, our study also detected SARS-CoV-2 in the brains of all the mice that succumbed to the viral infection, but not the mice that survived (Figures 3C,D). The results suggest that SARS-CoV-2 neuroinvasion in the brain can be a biomarker for mortality and disease severity in the K18-hACE2 mouse model, which appears to be an even stronger indicator than the histopathology score of the lung (Figure 1E). Reduced viral burden in mouse brain samples may contribute to the higher survival rate of animals in the GPR4 antagonist-treated group (Figures 3C,D). Moreover, neurological symptoms are commonly reported in patients with Long Covid (Antar and Cox, 2024). SARS-CoV-2 infection in patients is also found to be associated with the rapid progression of pre-existing dementia (Dubey et al., 2023). Future studies are warranted to evaluate the effects of GPR4 antagonism on neurological and other complications related to Long Covid.

In summary, this study highlights the effectiveness of the GPR4 antagonist NE-52-QQ57 in reducing mortality from SARS-CoV-2 infection in K18-hACE2 mice. Animals treated with the GPR4 antagonist exhibited markedly enhanced survival rates, reduced lung and brain SARS-CoV-2 viral burden, and mitigated inflammatory responses. Our results demonstrate that the GPR4 antagonist NE-52-QQ57 has both anti-inflammatory and anti-viral effects against SARS-CoV-2 infection. While the anti-

inflammatory effects of GPR4 antagonists have been shown in other models of inflammatory diseases, such as arthritis and colitis (Dong et al., 2013; Dong et al., 2017; Sanderlin et al., 2019; Tobo et al., 2015; Velcicky et al., 2017; Miltz et al., 2017; Krewson et al., 2020), the anti-viral effects were first uncovered in this study. Further research is needed to elucidate the mechanisms by which the GPR4 antagonist inhibits SARS-CoV-2 propagation in cells and tissues. It is also crucial to investigate whether the GPR4 antagonist exhibits anti-inflammatory and anti-viral effects in other similar viral diseases in addition to COVID-19.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

Ethics statement

Ethical approval was not required for the studies on humans in accordance with the local legislation and institutional requirements because only commercially available established cell lines were used. The animal study was approved by Institutional Animal Care and Use Committee of East Carolina University. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

X-JW: Data curation, Formal Analysis, Investigation, Methodology, Resources, Validation, Visualization, Writing – original draft, Writing – review and editing. KO: Investigation, Methodology, Writing – review and editing, Funding acquisition. MF: Investigation, Writing – review and editing. MM: Investigation, Writing – review and editing. MS: Investigation, Writing – review and editing. AW: Investigation, Writing – review and editing. IL: Investigation, Writing – review and editing, Methodology. RR: Methodology, Writing – review and editing, Resources. PB: Methodology, Resources, Writing – review and editing, Funding acquisition. LY: Funding acquisition, Resources, Writing – review and editing, Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Project administration, Supervision, Validation, Visualization, Writing – original draft.

Funding

The author(s) declare that financial support was received for the research and/or publication of this article. This study was supported by research grants from the North Carolina COVID-19 Special State Appropriations (grant # 112115, LY and PB), the Brody Brothers Endowment Fund (grant # 217222, LY and KO), and the East Carolina University Research & Creative Activity Awards (grant # 111110, LY).

Acknowledgments

We thank the ABSL-3 facility staff at East Carolina University (ECU) for technical support, Shayan Nik Akhtar for initial assistance in genotyping K18-hACE2 mice, and Cindy Kukoly in the Department of Internal Medicine and Drs. Gabriel Abuna and Ramiro Murata in the School of Dental Medicine at ECU for their assistance with the Luminex instrument. We also thank Drs. Pius Loetscher and Juraj Velicky at the Novartis Institutes for BioMedical Research for providing the GPR4 antagonist NE-52-QQ57, and the BEI Resources for providing the SARS-Related Coronavirus 2 (SARS-CoV-2, Isolate USA-WA1/2020).

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fphar.2025.1549296/full#supplementary-material>

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SUPPLEMENTARY FIGURE 1

Representative images of H&E staining of mouse brains and their associated hemorrhage rates. Black arrows indicate hemorrhagic areas in the brain of a SARS-CoV-2-infected mouse treated with vehicle (1 out of 12 mice or 1/12). Note: no hemorrhagic areas in the brains of mice treated with GPR4 antagonist (0 out of 12 mice or 0/12). Scale bar = 20 μ m.

SUPPLEMENTARY FIGURE 2

Other cytokines and chemokines in the lung tissues of SARS-CoV-2-infected mice treated with GPR4 antagonist or vehicle. Cytokine/chemokine protein levels in mouse lung tissues were measured by the Luminex multiplex platform.

SUPPLEMENTARY FIGURE 3

Other cytokines and chemokines in the serum of SARS-CoV-2-infected mice treated with GPR4 antagonist or vehicle. Cytokine/chemokine protein levels in mouse serum were measured by the Luminex multiplex platform.

SUPPLEMENTARY FIGURE 4

CD4⁺ and CD8⁺ T cell clusters in the mouse lung. GPR4 antagonist treatment reduced CD4⁺ and CD8⁺ immune cell clusters in the lungs of SARS-CoV-2-infected K18-hACE2 mice. Black arrows indicate immune cell clusters. Scale bar = 20 μ m.

SUPPLEMENTARY FIGURE 5

Viability of Vero E6 cells treated with the GPR4 antagonist. The viability of Vero E6 cells was approximately 100% relative to the DMSO control in the presence of 20 μ M, 10 μ M, 1 μ M, and 0.1 μ M GPR4 antagonist for 24h. (A) CellTiter-Glo (CTG) assay. (B) MTT assay.

SUPPLEMENTARY FIGURE 6

Anti-SARS-CoV-2 effects of GPR4 antagonist in Caco-2 cells. Caco-2 cells were incubated with SARS-CoV-2 (1,000 PFU inoculum) for 1 h. Then, the virus-containing media were removed, and SARS-CoV-2-infected Caco-2 cells were treated with various concentrations of GPR4 antagonist NE-52-QQ57 or the DMSO vehicle control. Viral RNA was determined 24 h post-infection. The GPR4 antagonist was maintained in the medium until cell assessment 24 h after treatment. Viral RNA isolated from Caco-2 cells was quantified by RT-qPCR targeting the nucleocapsid gene. N = 5 samples. *p < 0.05, compared to the DMSO vehicle control, non-parametric ANOVA (Kruskal-Wallis test) followed by Dunn's test.

SUPPLEMENTARY FIGURE 7

ACE2 and TMPRSS2 RNA expressions in cells treated with the GPR4 antagonists *in vitro*. Quantitative RT-PCR was performed to assess ACE2 or TMPRSS2 RNA expressions in Vero E6, A549, Caco-2, and Lewis lung carcinoma cells after a 24 h GPR4 antagonist treatment. Two-tailed Student's t-tests were used to compare ACE2 and TMPRSS2 expression between vehicle and GPR4 antagonist treatment in cell lines.

SUPPLEMENTARY FIGURE 8

GPR4 RNA expression in cells infected with SARS-CoV-2 and treated with the GPR4 antagonist NE-52-QQ57. Quantitative RT-PCR was performed to assess GPR4 RNA expression in Vero E6 and Caco-2 cells infected with SARS-CoV-2 after a 24 h GPR4 antagonist treatment. One-way ANOVA was used to compare GPR4 expressions between treatment groups, and no statistically significant differences were detected. No virus: cells without SARS-CoV-2 infection; DMEM: cells infected with SARS-CoV-2 and treated with the DMEM complete medium; DMSO: cells infected with SARS-CoV-2 and treated with the DMEM complete medium containing DMSO vehicle control; GPR4 antagonist: cells infected with SARS-CoV-2 and treated with the DMEM complete medium containing the GPR4 antagonist.

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OPEN ACCESS

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RECEIVED 13 May 2025

ACCEPTED 30 June 2025

PUBLISHED 17 July 2025

CITATION

Freiberger RN, López CAM, Sviercz FA,
Jarmoluk P, Palma MB, García MN,
Quarleri J and Delpino MV (2025) HIV
infection drives proinflammatory adipocyte
differentiation in an *in vitro* model and
reveals a new inflammatory pathway.
Front. Cell. Infect. Microbiol. 15:1627963.
doi: 10.3389/fcimb.2025.1627963

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HIV infection drives proinflammatory adipocyte differentiation in an *in vitro* model and reveals a new inflammatory pathway

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Introduction: Adipose tissue regulates metabolic homeostasis and serves as a reservoir for mesenchymal stem cells (MSCs), which differentiate into osteoblasts and adipocytes, balancing bone and lipid metabolism. Bone loss and fat accumulation are common in individuals living with HIV, prompting us to investigate how R5- and X4-tropic HIV modulates adipocyte differentiation and tissue homeostasis using an *in vitro* model of MSC-derived adipogenesis.

Methods: The study used an *in vitro* model of MSCs to examine how R5- and X4-tropic HIV strains affect adipocyte differentiation and function. Researchers assessed adipogenesis by analyzing lipid droplet formation, expression of adipogenic transcription factors (C/EBP α , C/EBP β , PPAR- γ), lipogenic/lipolytic enzymes, SREBPs, cytokine secretion, and the effects of CXCR4 and CCR5 with specific inhibitors.

Results: HIV exposure influences adipogenesis, increasing lipid droplet size in a tropism dependent manner and upregulating key adipogenic factors such as C/EBP α , C/EBP β , and PPAR- γ . This process involves the regulation of lipogenic and lipolytic enzymes, lipid droplet-lysosome interactions, and potential lipid droplet mitochondria cross-talk to fuel lipid accumulation. Additionally, HIV modulates sterol regulatory element-binding proteins (SREBPs), which control fatty acid, triacylglycerol, and cholesterol synthesis. Notably, SREBP2 downregulation correlates with increased type I interferons (IFN α 2, IFN β 1), linking lipid metabolism to immune responses in HIV infection. HIV-infected adipocytes also exhibit an increased leptin/adiponectin ratio and enhanced IL-1 β and IL-6 secretion, contributing to the inflammatory state observed in people with HIV. CXCR4 plays a key role in adipocyte differentiation, as its inhibition with AMD3100 reduces adipocyte number, size, and lipid droplet accumulation under X4-tropic HIV exposure. In contrast, CCR5 does not appear to be significantly involved in adipose tissue homeostasis under R5-tropic HIV exposure.

Discussion: These findings, derived from an *in vitro* model, suggest that HIV alters MSC differentiation into adipocytes, impacting adipose tissue homeostasis and function.

KEYWORDS

HIV, adipocyte, MSCs, lipid droplets (LD), adipogenesis

Introduction

Previous studies have shown that a pathological decline in bone mineral density is often accompanied by an increase in fat accumulation in bone tissue (Meunier et al., 1971; Verma et al., 2002; Yeung et al., 2005). Even with highly active antiretroviral therapy (HAART), many individuals living with HIV-1 experience metabolic abnormalities, including lipodystrophy, dyslipidemia, and hematological changes, alongside reduced bone mineral density (Mccomsey et al., 2010; Feeney and Mallon, 2011; Durandt et al., 2019). Initially attributed to antiretroviral therapy (ART) toxicity, the persistence of these abnormalities in ART-naïve HIV-infected individuals suggests that the virus itself plays a direct role in their development (Bruera et al., 2003; Koethe et al., 2020).

Adipogenesis is regulated by key transcription factors, including peroxisome proliferator-activated receptor gamma (PPAR- γ) and CCAAT enhancer-binding protein alpha (C/EBP α) (Rosen and Macdougald, 2006). Interestingly, PPAR- γ drives adipocyte differentiation and suppresses osteoblastogenesis by inhibiting Runt-related transcription factor 2 (Runx2), a master regulator of osteoblast commitment (Jeon et al., 2003). This interplay highlights the complex balance between these lineages and the potential for their disruption in pathological conditions (Bennett et al., 1991; Beresford et al., 1992; Muruganandan et al., 2009). Adipocytes and osteoblasts also share several genetic, hormonal, and environmental regulatory factors, and they exhibit a remarkable degree of plasticity, allowing for interconversion under certain conditions. Furthermore, increased adipocyte accumulation in bone marrow can disrupt bone remodeling by altering osteoblast function and regulating the development and activity of osteoclasts, which contribute to bone resorption (Muruganandan et al., 2020).

In response to hypoxia, cellular stress, and hypertrophy, adipocytes produce various cytokines and adipokines (e.g., TNF- α , IL-6, leptin, and adiponectin) that regulate immune responses and inflammation (Trayhurn and Wood, 2004).

Although productive infection of mesenchymal stem cells (MSCs) has not been conclusively demonstrated, these cells are believed to be susceptible to HIV infection (Couturier et al., 2015; Damouche et al., 2015). Adipose tissue has been proposed as a potential reservoir for HIV-1, as adipocytes express CD4, CCR5, and CXCR4 receptors necessary for viral entry (Hazan et al., 2002; Nazari-Shafti et al., 2011). *In vitro* studies have shown that

adipocytes can harbor HIV-1 (Maurin et al., 2005). However, the presence of HIV-1 within adipocytes has not been conclusively reported (Dupin et al., 2002; Munier et al., 2003). Beyond its role in metabolic regulation, adipose tissue contains MSCs that are critical for maintaining lipid homeostasis, with the ability to differentiate into adipocytes (Hu et al., 2018; Souza-Moreira et al., 2019).

In this study, we examine whether HIV exposure influences MSC differentiation into adipocytes and how this affects lipid metabolism and adipose tissue homeostasis. By elucidating the mechanisms underlying adipose tissue dysfunction in people living with HIV, our findings provide insight into the metabolic and inflammatory complications associated with the virus.

Materials and methods

Isolation and expansion of MSCs

The umbilical cords were preserved in α -Minimal Essential Medium (α -MEM, Gibco) and handled following previously outlined procedures (Palma et al., 2021).

In summary, each umbilical cord was cut into 5 mm fragments, with a sagittal incision made to expose Wharton's jelly. Careful removal of umbilical blood vessels was performed using clamps. The fragments were then washed 2 or 3 times with Dulbecco's phosphate-buffered saline from Sigma-Aldrich to eliminate residual blood. The section with the exposed jelly was placed face down at the bottom of a culture plate, and α -MEM supplemented with 10% platelet lysate was added. The plates were then incubated at 37°C in a humid atmosphere containing 5% CO₂, with the cell culture medium replenished every 2 to 3 days. Expansion of umbilical cord-derived MSCs was typically observed between 10 to 14 days after explantation, and cells were further amplified until reaching passage 2/3. MSCs were characterized by the expression of CD105, CD73, and CD90, and the absence of CD45, CD34, CD14, CD19, and HLA-DR molecules (Dominici et al., 2006). For experiments, MSCs were cultured in α -MEM supplemented with 10% heat-inactivated fetal bovine serum (Gibco-BRL, Life Technologies, Grand Island, NY), 100 U/ml of penicillin, and 100 mg/ml of streptomycin (complete medium) and were utilized until passage 5. The cells were routinely tested for mycoplasma contamination using the MycoAlert[®] Mycoplasma detection kit (LT07-318, Lonza, Tampa, FL, USA).

This study received approval from the Comité de Ética de la Facultad de Ciencias Médicas de la Universidad de Buenos Aires, Argentina (RESCD-2023-1291). Written informed consent was obtained from each mother before normal cesarean birth, and human umbilical cords were collected from discarded placentas.

Cell-free wild-type HIV infection of MSCs and adipocytes

The full-length infectious clones of wild-type (WT)-HIV AD8 and NL43 strains were obtained from the NIH AIDS Reagent Program (Division of AIDS, NIAID, NIH, USA).

The quantification of HIV capsid (p24 antigen) in viral stocks was conducted using a commercial ELISA assay (INNOTEST® HIV Antigen mAb).

Adipocyte differentiation

MSCs were seeded at a density of 5×10^4 cells per well in 24-well plates and allowed to reach confluence. Subsequently, the culture medium was replaced with an adipocyte differentiation medium, consisting of DMEM, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), 0.1 μ M dexamethasone, 50 μ M indomethacin, and 10 μ g/ml human insulin, all obtained from Sigma Aldrich, St. Louis, MO, USA (Crivaro et al., 2020, 2023). Complete differentiation was attained within the timeframe of days 7 to 10.

Surface expression of CD4, CXCR4, and CCR5 during adipocyte differentiation

At 0, 3, and 7 days after initiating the adipocyte differentiation, the cells were washed and subjected to staining for surface antigens. This staining procedure involved incubation with the following antibodies all used at a 1:100 dilution for 30 minutes at 4°C: FITC Mouse anti-human CD195 (561747) (BD Pharmingen™, United States), PE Mouse Anti-Human CD184 (555974) (BD Pharmingen™, United States), and PerCP anti-human CD4 antibody (344624) (Biolegend, United States). Data were acquired using a Full Spectrum Flow Cytometry Cytex® Northern Lights 3000™ (Cytex Biosciences Inc., USA) and analyzed with FlowJo.v10.6.2 (Ashland, USA).

HIV-infections using X4- and R5-tropic strains

MSCs and differentiated adipocytes (at 7 days) were infected with an inoculum of 1 pg of p24/cell using the CCR5-tropic HIV strain AD8 and the CXCR4-tropic HIV strain NL43, following the previously described procedure (Freiberger et al., 2024; Lopez et al., 2024). Infection efficiency and replication were evaluated by

measuring intracellular p24 expression using the KC57 monoclonal antibody labeled with phycoerythrin against p24 (PE-KC57 [FH190-1-1] (6604667) protein (Beckman Coulter, United States) used at a 1:250 dilution via confocal microscopy using a Zeiss LSM 800 confocal microscope (Zeiss, Jena, Germany).

The detection of HIV proviral DNA was performed using the Alu-PCR technique, following the protocol described by Kumar et al. (2002). MSCs and adipocytes cells exposed to HIV, along with ACH-2 cells (used as a positive control), were centrifuged in 1.5-ml microtubes at 16,000 g for 5 minutes. After carefully removing and discarding the supernatants, cell pellets were resuspended in lysis buffer (10 mM Tris-HCl, pH 8.0, 50 mM KCl, 400 μ g/ml proteinase K; Invitrogen) at appropriate concentrations (20×10^6 cells/ml for ACH-2 cells; 5×10^6 to 10×10^6 cells/ml for LX-2) and digested for 12 to 16 hours at 55°C in a heating shaker. Proteinase K was then inactivated by heating the digested samples at 95°C for 5 minutes. The cell lysates were either immediately used for HIV DNA quantification or stored at -70°C until further analysis.

Integrated HIV DNA determination was carried out in a 50 μ l reaction mixture containing Taq polymerase buffer (Invitrogen), 3 mM MgCl₂, 300 mM deoxynucleoside triphosphates (Invitrogen), 300 nM of each of the 4 primers, and 2.5 U Taq polymerase (Invitrogen).

The primers used for preamplification were:

ULF1: 5'-ATG CCA CGT AAG CGA AAC TCT GGG TCT CTC
TDG TTA GAC-3'

Alu1: 5'- TCC CAG CTA CTG GGG AGG CTG AGG-3'

Alu2: 5'- GCC TCC CAA AGT GCT GGG ATT ACA G-3'

For Real-time PCR, the following primers were used:

Lambda T: 5'-ATG CCA CGT AAG CGA AAC T-3'

UR2: 5'-CTG AGG GAT CTC TAG TTA CC-3'

UHIV TaqMan LC640: 5'-CAC TCA AGG CAA GCT TTA TTG
AGG C-3'.

Co-receptor antagonists

We treated MSCs with the CXCR4 antagonist AMD3100 (20 μ M), or the CCR5 antagonist TAK-779 (20 μ M) (all from Sigma-Aldrich, Argentina). Each inhibitor was applied separately, and the cells were incubated at 37°C for 1 hour. Following this pre-incubation, the cells were exposed to HIV.

Determination of cell death

The percentage of cellular death was assessed by staining with 7-AAD apoptosis detection kit (BD Biosciences, United States). Staurosporine stimulation was used as a positive control of cell death. Data were acquired using a Full Spectrum Flow Cytometry Cytex® Northern Lights 3000™ (Cytex Biosciences Inc., USA) and analyzed with FlowJo.v10.6.2 (Ashland, USA).

Assessment of mitochondrial reactive oxygen species generation

mROS, which includes superoxide, was measured using flow cytometry. The cells were stained with 5 μ M MitoSOXTM (catalog number M36008; Thermo Fisher Scientific) for 30 minutes. This fluorescence-based assay employs a positively charged probe that swiftly accumulates within mitochondria, where it becomes susceptible to oxidation by ROS. Rotenone stimulation was used as the positive control. Data were acquired using a Full Spectrum Flow Cytometry Cytex[®] Northern Lights 3000TM (Cytex Biosciences Inc., USA) and analyzed with FlowJo.v10.6.2 (Ashland, USA).

Cellular mRNA preparation and RT-qPCR

Total cellular mRNA was extracted using Quick-RNA MiniPrep Kit (Zymo Research) and 1 μ g of RNA was employed to perform the reverse transcription by means Improm-II Reverse Transcriptase (Promega). Quantitative reverse transcription-polymerase chain reaction (RT-qPCR) analysis was achieved by run on a StepOne real-time PCR detection system (Life Technology) using SYBR Green as fluorescent DNA binding dye. Primers sequences used for amplification were the following: β -actin sense 5'-CCTGGCACCCAGCACAAT-3', antisense 5'-CGGGATCCACACGGAGTACT-3'; PPAR- γ sense 5'-GGCCGCAGATTGAAAGAAG-3', antisense 5'-GTTTGAGAAAATGGCCTTGTGT-3'; C-EBP α sense 5'-CCAAGAAGTCGGTGGACAAGA-3', antisense 5'-ATTGTCACTGGTCAGCTCCA-3'; C-EBP β sense 5'-TACTACGAGGCGGACTGCTT-3', antisense 5'-CTGGTAGCCGAGGTAAGCG-3'; hormone-sensitive lipase (HSL) sense 5'-CATCTCCATTGGGCTGGTGT-3', antisense 5'-ATCTCAAAGGCTTCGGGTGG-3'; lipoprotein lipase (LPL) sense 5'-ATCCGCGTGATTGCAGAGAG-3', antisense 5'-GATGAATGGAGCGCTCGTGG-3'; adipose triglyceride lipase (ATGL) sense 5'-CAAGCGGAGGATTACTCGCA-3', antisense 5'-CAAGCGGATGGTAAGGACA-3'; diglyceride acyltransferase (DGAT)1 sense 5'-CCGACAATCTGACCTACCG-3', antisense 5'-GGGATGTTCCAGTTCTGCCA-3'; DGAT2 sense 5'-GCCTGTGTTGAGGGAGTACC-3', antisense 5'-CAGGGCCAGTTTCACAAAGC-3'; sterol regulatory element-binding proteins (SREBP)1 sense 5'-GGGACCACTGTCACTTCCAG-3', antisense 5'-TTCAAAGCTTCGACGCAGG-3'; SREBP2 sense 5'-ATGGGCAGCAGAGTTCCTTC-3', antisense 5'-CGACAGTAGCAGGTCACAGG-3'; IFN β 1 sense 5'-ACGCCGATTGACCATCTAT-3', antisense 5'-GTCTCATTCAGCCAGTGCT-3'; IFN α 2 sense 5'-CTTGTGCCTGGGAGGTTGTC-3', antisense 5'-GGTGAGCTGGCA TACGAATCAA-3'. Lysosomal acid lipase (LIPA) sense 5'-GTGGGTCATTCTCAAGG-CACCA-3', antisense 5'-CCATAGGGCTAGTACAGAAGGC-3'; PPAR- α sense 5'-AAGCAAACT-GAAAGCAGAA-3', antisense 5'-GTCTTCTCAGCCATACACAG3'.

The amplification cycle for PPAR- γ , C-EBP α , C-EBP β , HSL, LPL, ATGL, DGAT, DGAT2, SREBP1, SREBP2, IFN β 1, IFN α 2 was

95°C for 15s, 59°C for 30s and 72°C for 60s. For β -actin, PPAR- α and LIPA was 95 °C for 15 s, 57 °C for 30 s, and 72 °C for 60 s.

All primer sets yielded a single product of the correct size. Relative transcript levels were calculated using the $2^{-\Delta\Delta C_t}$ method using β -actin as a normalizer gene. The C_t values for β -actin showed no significant variation between infected and non-infected samples or across different time points ($\Delta C_t < 0.5$), confirming its stability and suitability for normalization.

Glycerol determination

Glycerol release from differentiated adipocytes was quantified using a modified version of the method of Garland and Randle (Garland and Randle, 1962). Briefly, culture medium was aspirated and cells were washed once with phosphate-buffered saline (PBS). Thereafter, adipocytes were incubated in PBS supplemented with 2% (w/v) fatty-acid-free bovine serum albumin (BSA) for 6 h at 37°C in a humidified 5% CO₂ atmosphere. Following incubation, aliquots of the extracellular medium were collected and centrifuged at 1,000 $\times$ g for 5 min to remove any cellular debris. Glycerol concentration in the cleared supernatant was determined using the TG Color GPO/PAP AA enzymatic assay kit (Wiener, Buenos Aires, Argentina), according to the manufacturer's protocol. Absorbance was read at 505 nm on a microplate reader.

Intracellular triglyceride and cholesterol quantification

Differentiated adipocytes were lysed in PBS containing 1% (v/v) Triton X-100, and the resulting lysates were used for both triglyceride and cholesterol determinations. For triglycerides, intracellular lipids were extracted directly in the Triton X-100 buffer and enzymatically hydrolyzed to glycerol and free fatty acids using lipase as specified in the TG Color GPO/PAP AA kit protocol (Wiener, Buenos Aires, Argentina), after which released glycerol was quantified by measuring absorbance at 505 nm on a microplate reader. Total cholesterol was measured in the same lysates using the Colestat enzymatic assay kit (Wiener, Buenos Aires, Argentina) according to the manufacturer's instructions, with absorbance also read at 505 nm. All lipid values were normalized to total protein content, which was determined by the Bradford assay (Bio-Rad Protein Assay Kit) using bovine serum albumin as the standard.

Assessment of adipocyte differentiation measuring lipid droplet accumulation

Adipocyte differentiation was examined using Bodipy 493/503 (Life Technologies). Cells cultured in 24-well plates were fixed with 10% formalin for one hour, followed by permeabilization using 0.3% Triton X100. Subsequently, lipid droplets were stained with 1 μ g/mL of Bodipy 493/503 from Invitrogen. For nuclear visualization, DAPI (Thermo Scientific) was used for

counterstaining. The prepared coverslips were mounted in a PBS-glycerin solution (9:1 v/v) and analyzed using a Zeiss LSM 800 confocal microscope (Zeiss, Jena, Germany) with 10× and 40× objectives. This method involved quantifying five microscopic fields per well, within two wells per condition, for each experimental set.

Adipocyte morphology

Adipocyte morphology was further evaluated using Differential Interference Contrast (DIC) microscopy, acquired in parallel with the confocal fluorescence images. DIC and fluorescence channels were superposed to enable precise identification of individual adipocytes based on their characteristic round shape and the presence of intracellular lipid droplets. Cell borders were manually delineated using Fiji software (ImageJ, National Institutes of Health, Bethesda, MD, USA), and the cross-sectional area of each adipocyte was measured. Only clearly defined and isolated cells were included in the analysis.

Lysosome and mitochondria staining

For lysosomal staining, cells were treated with 0.5 mmol/L LysoTracker Red (Life Technologies) in full medium for 45 minutes before fixation. Following this, cells were fixed with 4% paraformaldehyde (PFA) in PBS for 10 minutes at room temperature.

To identify mitochondria, cells were stained with MitoTracker Deep Red (500 nM, Life Technologies) for 30 minutes at 37°C and then fixed as described.

Nuclei were visualized using DAPI from Thermo Scientific for counterstaining. Confocal images were examined using a Zeiss LSM 800 confocal microscope (Zeiss, Jena, Germany). To quantify the degree of colocalization between Bodipy 493/503-labeled LD and the LysoTracker probe, as well as between Bodipy 493/503-labeled LD and the MitoTracker probe, we calculated the Manders' correlation coefficient from the corresponding confocal images using FIJI software (ImageJ, National Institutes of Health, Bethesda, MD, USA) (Manders et al., 1993).

Statistical analysis

Where applicable, statistical analysis was performed with one-way ANOVA. Multiple comparisons between all pairs of groups were made with Tukey's post-test, and those against two groups were made with the Mann-Whitney U test. To determine normality, the Shapiro-Wilk normality test was used. Graphical and statistical analyses were performed with GraphPad Prism 5.0 software (San Diego, CA, USA). Each experiment was performed in duplicate with different culture preparations in at least three independent occasions. Data were represented as mean $\pm$ SD. A $p < 0.05$ is represented as *, $p < 0.01$ as **, $p < 0.001$ as ***, and $p < 0.0001$ as ****; $p < 0.05$ was the minimum level regarded as a statistically significant difference between groups.

Results

Surface expression of HIV receptors and co-receptors on MSCs and adipocytes

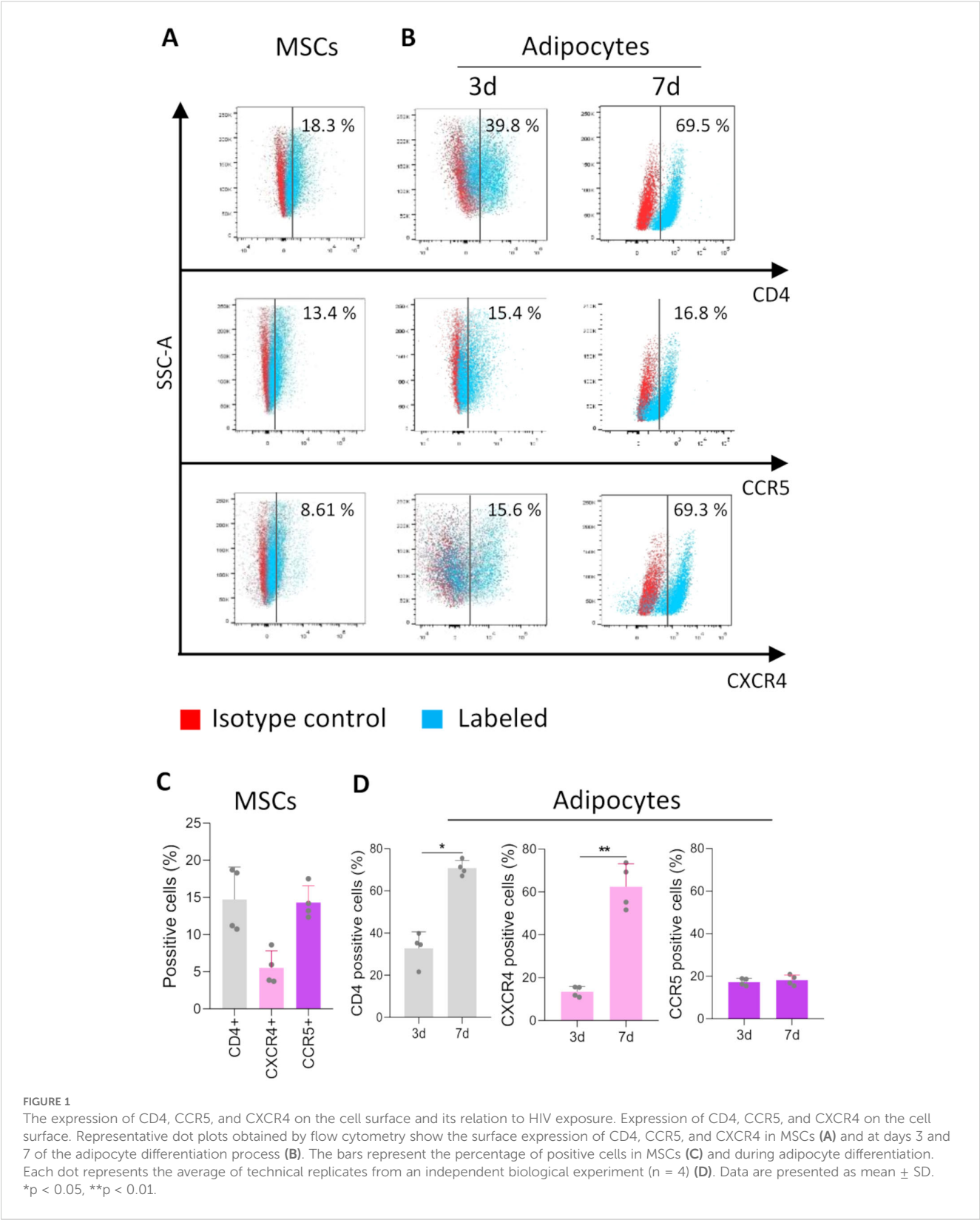
The attachment and entry of HIV into susceptible cells depend on the presence of chemokine receptors CCR5 and CXCR4, along with CD4. In our study, we assessed the expression of these receptors on the surface of MSCs derived from the umbilical cord. Our findings revealed that $18.3 \pm 3.18\%$ of MSCs expressed CD4, $8.61 \pm 0.22\%$ expressed CXCR4, and $13.4 \pm 0.63\%$ expressed CCR5 receptors (Figures 1A, C).

MSCs exhibit versatility and pluripotency, enabling differentiation into various cell types, including adipocytes. Subsequent experiments aimed to evaluate the potential modulation of HIV receptor expression during the differentiation of adipocytes. During the differentiation process, CXCR4, CCR5, and CD4 expression levels were measured on days 3 and 7. Our findings revealed a significant increase in CXCR4 expression throughout the differentiation, peaking at 7 days ($69.3 \pm 2.24\%$ of positive cells). In contrast, CCR5 expression remained relatively steady until 7 days of the differentiation process ($16.8 \pm 2.47\%$ of positive cells). When analyzing CD4 expression, our results indicated a gradual significant increase during differentiation, reaching its maximum levels at 7 days post-differentiation ($69.5 \pm 1.76\%$) (Figures 1B, D).

Together, these results indicate that the HIV-receptor/coreceptors are expressed differentially among the study cell types, and their expression is temporally dissimilar during adipocyte differentiation and maturation.

MSCs and adipocytes are susceptible to HIV infection

Experiments were conducted to determine whether MSCs and adipocytes were susceptible and permissive to HIV infection. To assess the susceptibility, the cells were exposed to R5-tropic HIV (AD8) or X4-tropic HIV (NL43) at an inoculum of 1 pg of p24 per cell for 24 hours. Unbound viral particles were extensively washed with the culture medium. At 3-, 5-, and 7-days post-infection, the presence of infected cells was evaluated by confocal microscopy using immunodetection with an antibody targeting the p24 antigen. Our results indicated that MSCs and adipocytes were susceptible to HIV infection (Figure 2). However non-significant increase in the percentage of infected cells was observed between 3-, 5- and 7-days post-infection in MSC (Figure 2B). In adipocytes, a low increase in the percentage of infected cells was observed at 5 days, albeit with statistical significance, followed by a decrease on day 7 (Figure 2C). These results suggest that MSCs and adipocytes were unable to support productive replication of R5- and X4- tropic HIV. However, HIV proviral genome was detected by Alu-qPCR in MSCs and adipocytes (Not shown). These results suggest that although MSCs and adipocytes express HIV receptors and co-receptors and allow viral genome



integration, they are unable to support productive replication of both R5- and X4-tropic HIV. This indicates that additional cellular restrictions may prevent efficient viral replication in these cell types.

HIV modulates adipocyte differentiation

To assess whether infection affects adipocyte differentiation, MSCs were exposed to X4- and R5-tropic HIV in the presence of a

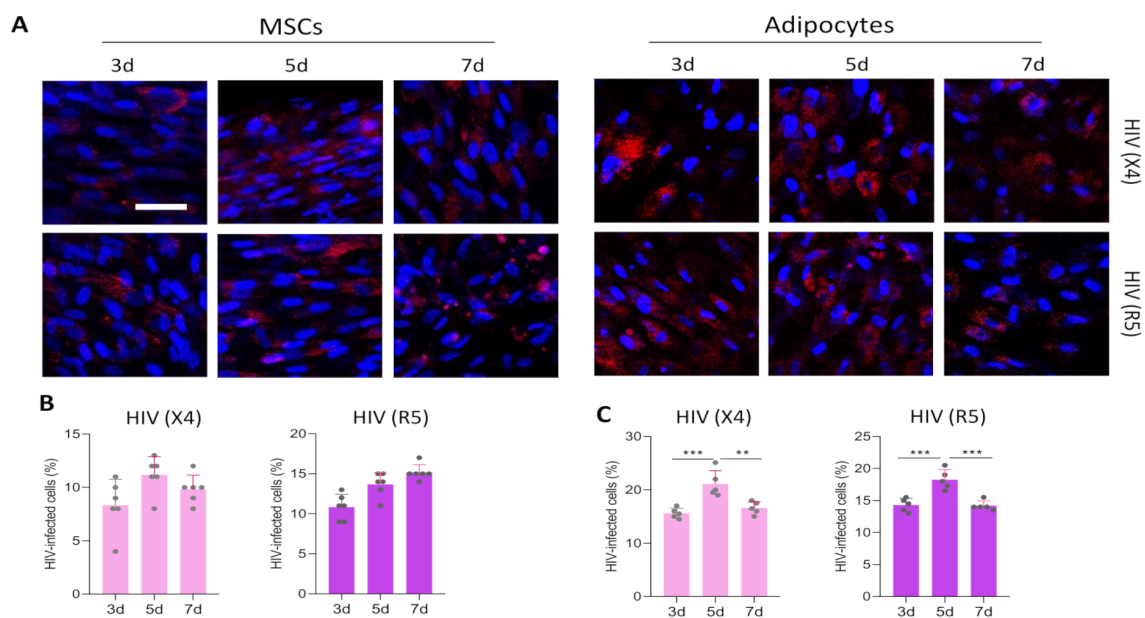


FIGURE 2

Exposure of MSCs and adipocytes to X4- and R5-tropic HIV. MSCs and 7-day differentiated adipocytes were infected with an inoculum of 1 pg of p24 per cell using CXCR4-tropic HIV (HIV (X4)) and CCR5-tropic HIV (HIV (R5)). Representative microscopy images show the kinetics of HIV replication at 3-, 5-, and 7-days post-infection (d), visualized by immunostaining of the HIV-p24 capsid antigen (A). Bars indicate the percentage of HIV-infected MSCs. Each dot represents the average of technical replicates from an independent biological experiment ($n = 6$) (B) and adipocytes (C). Scale bar: 50 μm . Data are presented as mean $\pm$ SD. ** $p < 0.01$, *** $p < 0.001$.

differentiation medium. At 7- and 10-days post-differentiation, the presence of adipocytes was revealed by staining lipid droplets with Bodipy 493/503.

At 7 days post-differentiation, our results indicated that R5-tropic HIV increased adipocyte differentiation, evidenced by a significant rise in the number and size of differentiated adipocytes and an increase in both the number and size of lipid droplets. This occurred without affecting the total cell count compared with uninfected cells. In contrast, X4-tropic HIV did not modulate adipocyte differentiation compared to uninfected controls (Figures 3A–G).

At 10 days post-differentiation, both viral tropisms induced an increase in adipocyte differentiation, accompanied by an increase in the number and size of lipid droplets, without affecting the total cell count compared to uninfected cells (Figures 3H–N).

Given the ability of HIV to stimulate adipocyte differentiation, we conducted subsequent experiments to determine whether infection could also modulate the transcription of essential adipogenic factors, namely *PPAR- γ* , *C/EBP α* , and *C/EBP β* (Darlington et al., 1998). MSCs were infected, incubated with adipocyte differentiation medium, and mRNA levels of the mentioned factors were measured at 1-, 3-, 7- and 10-days post-differentiation.

Our results (Figure 4; Supplementary Figure 1) indicated that both HIV tropisms can induce *PPAR- γ* upregulation at 1- 7- and 10-days post-differentiation compared to uninfected controls (Figure 4A). The analysis of *C/EBP α* levels revealed that R5-tropic HIV induces its increase only at 1- and 10-days post-

infection, whereas X4-tropic virus induces the increase at 3- and 7-days post-differentiation respect to uninfected controls (Figure 4B). In contrast, the analysis of *C/EBP β* showed that R5-tropic HIV induces a rise in mRNA levels at 1-, 3-, and 7-days post-differentiation, while X4-tropic HIV induces an increase in *C/EBP β* only at 3- and 7-days post-differentiation in comparison with uninfected cells (Figure 4C). These findings suggest that HIV infection can modulate adipocyte differentiation in a tropism-dependent manner. While R5-tropic HIV promotes early and sustained adipocyte differentiation, X4-tropic HIV appears to exert a delayed effect. The upregulation of key adipogenic transcription factors, particularly *PPAR- γ* , *C/EBP α* , and *C/EBP β* , may underlie these differences. The distinct temporal patterns of *C/EBP α* and *C/EBP β* expression induced by each viral tropism could contribute to the differential regulation of adipogenesis, highlighting a potential role for HIV in altering adipose tissue homeostasis.

Lipogenesis and Lipolysis upregulation are related to increased lipid droplets size during HIV-induced adipocyte differentiation

To investigate whether the increase in lipid droplet number and size was linked to altered lipogenesis and adipogenesis, we examined the mRNA expression of key lipogenic and lipolytic enzymes—

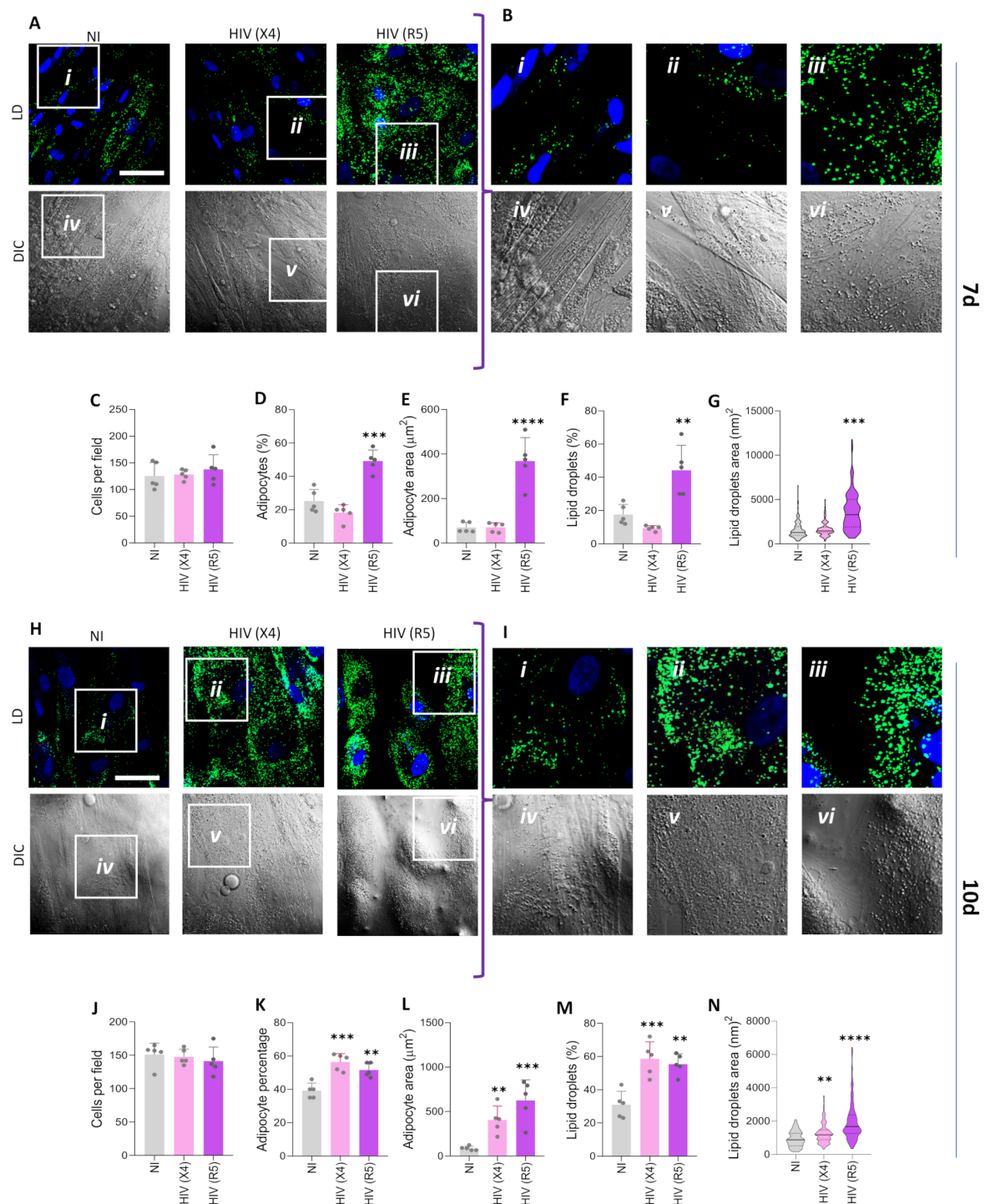


FIGURE 3

HIV modulates adipocyte differentiation. Effect of CXCR4-tropic HIV [HIV (X4)] and CCR5-tropic HIV [HIV (R5)] exposure on adipocyte differentiation. Representative microscopy images show the presence of lipid droplets stained with Bodipy 493/503 (LD) and Differential Interference Contrast (DIC) at 7 (A) and 10 (H) days post-differentiation. (B, I) Magnified insets from panels A and H, respectively. Quantification of the experiments shown in panels A and H includes the number of cells per field (C, J), percentage of adipocytes per field (D, K), adipocyte area (E, L), percentage of lipid droplets per field (F, M) Scale bar: 50 μm . Data are presented as mean $\pm$ SD. Each dot represents the average of technical replicates from an independent biological experiment ($n = 5$). ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$ compared to non-infected cells (NI).

including DGAT1 and DGAT2, which are critical for triglyceride synthesis and lipid droplet expansion—during adipocyte differentiation in the presence of HIV. We assessed the mRNA transcription of lipolytic and lipogenic enzymes during adipocyte

differentiation in the presence of HIV. Our results revealed that *HSL* was significantly upregulated three days after infection with both X4 and R5 tropic HIV (Figure 4D). In contrast, *LPL* expression was significantly downregulated in MSCs differentiated in the presence of

X4-tropic HIV at 1, 3, 7, and 10 days post-differentiation. While, R5-tropic HIV induced *LPL* downregulation only at 10 days post-differentiation, compared to uninfected cells (Figure 4E). Additionally, *ATGL* was upregulated at days 1, 7, and 10 post-differentiation in MSCs exposed to R5-tropic HIV, while X4-tropic HIV only upregulated *ATGL* expression at day 10 (Figure 4F).

DGAT1 and DGAT2, key enzymes involved in triglyceride synthesis, were not modulated by X4- or R5-tropic HIV at 1- and 3-days post-differentiation, according to our results. However, at 7- and 10-days post-differentiation, R5-tropic HIV induced a significant increase in *DGAT1* whereas X4-tropic HIV upregulate *DGAT1* only at 10 days post differentiation. Both viral tropisms significantly upregulated *DGAT2* at days 7 and 10, coinciding with the increase in the size of lipid droplets compared to uninfected controls (Figures 4G, H). *SREBP-1*, a transcription factor critical for fatty acid and triglyceride synthesis, was significantly upregulated at days 1 and 7 in cells exposed to both viral strains (Figure 4I), indicating early activation of the lipogenic program.

SREBP-2, primarily associated with cholesterol metabolism, was also upregulated by R5-HIV at days 1 and 7, whereas X4-HIV induced a moderate increase only at day 7 (Figure 4J). The timing of *SREBP-2* upregulation also coincided with lipid droplet hypertrophy.

Collectively, these results indicate that HIV infection modulates lipid homeostasis by upregulating lipolytic and lipogenic pathways in a tropism- and time-dependent manner.

As a result, at 7 days post-differentiation, HIV (R5- and X4- tropic) induces an increase in intracellular cholesterol compared to uninfected controls, and it remains elevated only in comparison to uninfected controls when exposed to HIV-X4 (Figure 4K). Additionally, an increase in intracellular triglycerides was observed in cell lysates from cells infected with HIV (X4 and R5), along with a concomitant reduction in glycerol presence in culture supernatants (Figures 4L, M).

Adipocyte hypertrophy is linked to an enhanced inflammatory profile (Gao et al., 2025). In line with this our findings show that both HIV tropisms increase the *LEPTIN/ADIPONECTIN* ratio (Figures 4N–P) and stimulate IL-1 β secretion at 10 days post-infection (Figure 4Q). Additionally, both viral strains induce elevated IL-6 secretion at 7 and 10 days post-infection (Figure 4R); although IL-6 is commonly associated with inflammation, it also plays a role in metabolic regulation and can promote adipocyte browning under specific conditions (Radvanyi and Roszer, 2024).

Notably, TNF- α production was not stimulated by HIV infection (not shown). Collectively, our results suggest that HIV induces adipocyte hypertrophy in a tropism-dependent manner, characterized by increased lipid accumulation and a proinflammatory profile.

X4- or R5- tropic HIV infected adipocytes induce IFN- α 2 and IFN- β 1

Previous studies have shown that downregulation of *SREBP2*, a key regulator of cholesterol biosynthesis, enhances the expression of type I interferons and interferon-stimulated genes (ISGs) (York et al., 2015). Conversely, activation of the cholesterol biosynthetic pathway may dampen the type I IFN response, thereby hindering the

elimination of infectious viruses. Given the observed modulation of *SREBP2* during HIV-induced adipocyte differentiation, we next investigated whether HIV infection alters the expression of type I IFNs. We quantified the mRNA levels of *IFN- α 2* and *IFN- β 1* at various time points during adipocyte differentiation in the presence or absence of X4- or R5-tropic HIV. Our findings revealed that *IFN α 2* and *IFN β 1* mRNA transcription fluctuated following HIV exposure. Specifically, their expression increased at 3 days post-differentiation in the presence of both X4- and R5-tropic HIV compared to uninfected controls. By 7- and 10-days post-differentiation, *IFN α 2* and *IFN β 1* levels decreased but remained significantly elevated in cells differentiated with X4-tropic HIV compared to uninfected controls (Figures 4S, T). In contrast, *IFN* expression in R5-HIV-infected cells returned to baseline by day 10.

These findings suggest that HIV infection triggers a transient activation of type I IFN responses during early adipocyte differentiation, with a more sustained effect observed in X4-HIV-infected cells.

Lipid droplets-lysosome interaction during adipocyte differentiation under X4- and R5- tropic HIV infection

Organelle interactions have emerged as critical regulators of intracellular lipid trafficking and metabolism. To evaluate lysosomal dynamics during HIV infection, we used LysoTracker, a fluorescent probe, to monitor lysosomal activity via confocal microscopy (Figure 5). Our analysis revealed that both X4- and R5-tropic HIV infections increased lysosomal activity in differentiating adipocytes, notably at 7- and 10-days post-infection (Figures 5B, D). To investigate whether HIV infection promotes lipid droplet-lysosome interaction, we performed colocalization analysis using fluorescent labeling of lipid droplets and lysosomes. Confocal imaging showed evident colocalization in infected adipocytes, with temporal differences depending on HIV tropism. R5-tropic HIV induced peak colocalization at 7 days, whereas X4-tropic HIV showed maximal interaction at 10 days post-infection (Figures 5C, E). This suggests that acid lipolysis is dynamically regulated during HIV infection and may contribute to lipid remodeling in a tropism-specific manner.

Lysosomal acid lipase (LAL), encoded by the *LIPA* gene, is the sole enzyme identified to function at acidic pH within the lysosome. Consistently, we observed significant upregulation of *LIPA* mRNA expression in adipocytes infected with either HIV strain at 10 days post-differentiation (Figure 5F), further supporting the activation of lysosomal lipid degradation pathways.

LIPA is regulated by *PPAR- α* which was significantly upregulated at both day 1 and day 10 post-differentiation in adipocytes infected with either X4- or R5-tropic HIV (Figure 5G), indicating a coordinated transcriptional program promoting lysosomal lipolysis and mitochondrial fatty acid oxidation.

Together, these findings suggest that HIV infection enhances lysosomal function and promotes lipid droplet-lysosome interaction in differentiating adipocytes, potentially facilitating lipid mobilization through acid lipolysis.

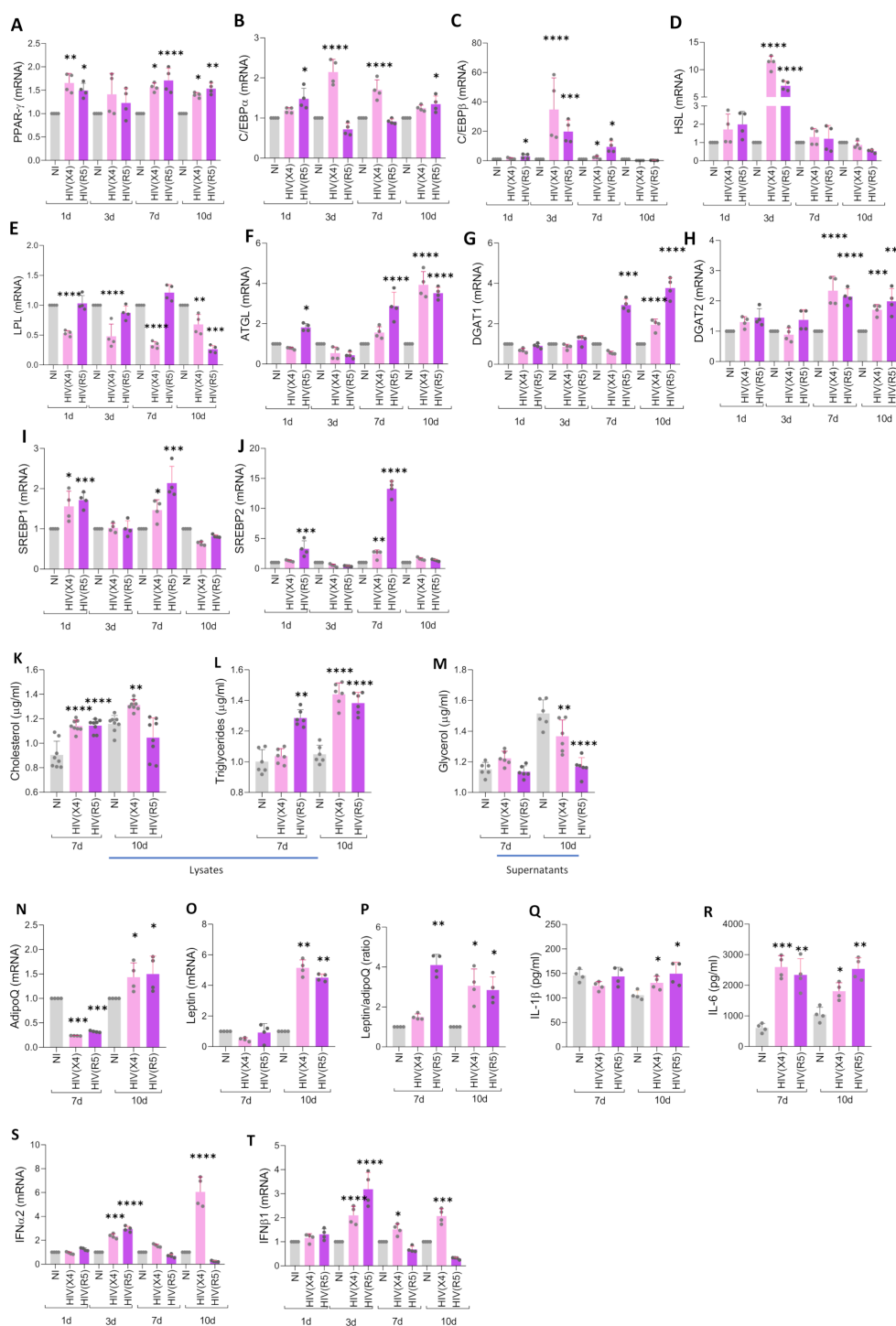


FIGURE 4

HIV modulates adipocyte mediators during its differentiation. Effect of HIV infection on the expression of *PPAR-γ* (A), *C/EBPα* (B), *C/EBPβ* (C), *HSL* (D), *LPL* (E), *ATGL* (F), *DGAT1* (G), *DGAT2* (H), *SREBP1* (I), and *SREBP2* (J), as determined by RT-qPCR at days 1, 3, 7, and 10 post-differentiation. Cholesterol (K) and triglycerides (L) were measured in cell lysates, while glycerol release was measured in culture supernatants (M). *ADIPOQ* (N) and *LEPTIN* (O) transcription levels were assessed by RT-qPCR, and the *LEPTIN/ADIPOQ* ratio was calculated (P). *IL-1β* (Q) and *IL-6* (R) secretion levels were measured in culture supernatants using ELISA. The analyses in panels K–R were performed at days 7 and 10 post-infection. *IFN-α2* (S) and *IFN-β1* (T) transcription was determined by RT-qPCR at days 1, 3, 7, and 10 post-differentiation. Data are presented as mean ± SD. Each dot represents the average of technical replicates from an independent biological experiment (n = 4). *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 compared to non-infected cells (NI).

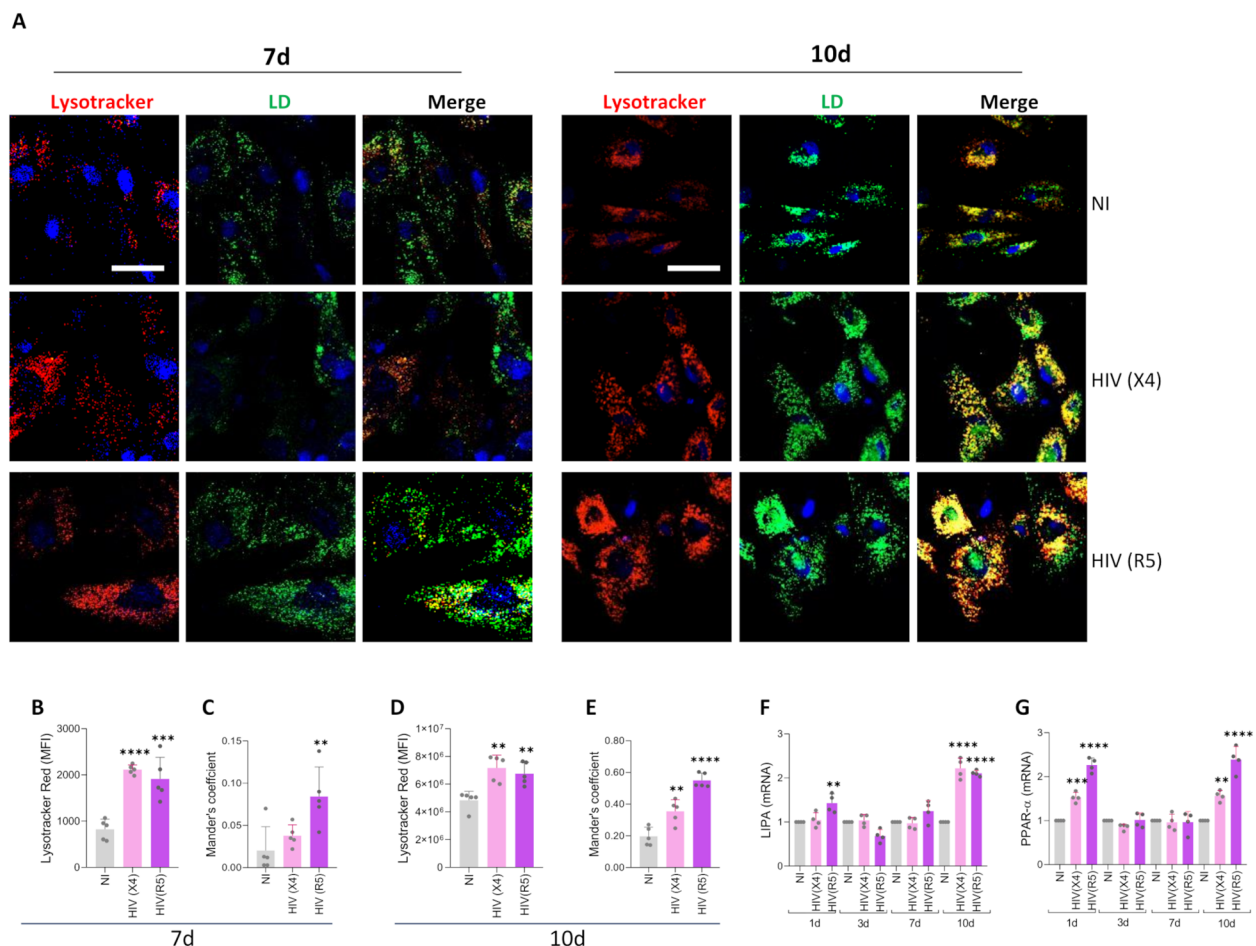


FIGURE 5

Lipid droplets-lysosome interaction during adipocyte differentiation under X4- and R5- tropic HIV infection. Representative images showing lysosomes stained with LysoTracker Red and lipid droplets (LD) stained with Bodipy 493/503 at 7- and 10-days post-differentiation in cells exposed to HIV (X4 and R5 strains) (A). Quantification of median fluorescence intensity (MFI) for lysosomal content was performed using ImageJ from the images in panel A at 7 (B) and 10 (D) days post-differentiation. Lipid droplet-lysosome colocalization was analyzed using Mander's overlap coefficient at 7 (C) and 10 (E) days post-differentiation. *LIPA* (F) and *PPAR-α* (G) transcription were measured by RT-qPCR at 1-, 3-, 7- and 10-days post-differentiation. Scale bar: 50 μ m. Data are presented as mean $\pm$ SD. Each dot represents the average of technical replicates from an independent biological experiment ($n = 5$). ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$ compared to non-infected cells (NI).

Interaction between lipid droplets and mitochondria during adipocyte differentiation under X4- and R5-tropic HIV infection

Mitochondria are central regulators of cellular metabolism and play a key role in lipid catabolism. In turn, mitochondrial function supports cellular energy demands and may contribute indirectly to lipid droplet growth by fueling lipogenic processes.

To investigate the relationship between mitochondria and lipid droplets during HIV-induced adipocyte differentiation, we used MitoTracker to assess mitochondrial mass and colocalization with lipid droplets at days 7 and 10 post-differentiation time points that coincided with the observed increase in lipid droplet size.

At day 7, R5-tropic HIV significantly increased mitochondrial mass, whereas X4-tropic HIV did not (Figures 6A, B). However, both tropisms promoted enhanced colocalization between mitochondria and lipid droplets, suggesting early activation of lipid trafficking between these organelles (Figures 6A, C).

By day 10, both viral tropisms induced a significant increase in mitochondrial mass and also in mitochondria and lipid droplets colocalization compared to uninfected controls (Figures 6A, D, E). These findings indicate that HIV infection promotes mitochondrial biogenesis and enhances physical interactions between mitochondria and lipid droplets during adipocyte differentiation.

HIV was not able to modulate mROS production during adipocyte differentiation

To determine whether the observed increase in mitochondrial mass corresponded to enhanced oxidative metabolism or stress, we measured mROS production during adipocyte differentiation in the presence or absence of X4- or R5-tropic HIV.

Our results showed that neither X4- nor R5-tropic HIV infection significantly increased mROS levels compared to uninfected controls at 3-, 7-, or 10-days post-differentiation

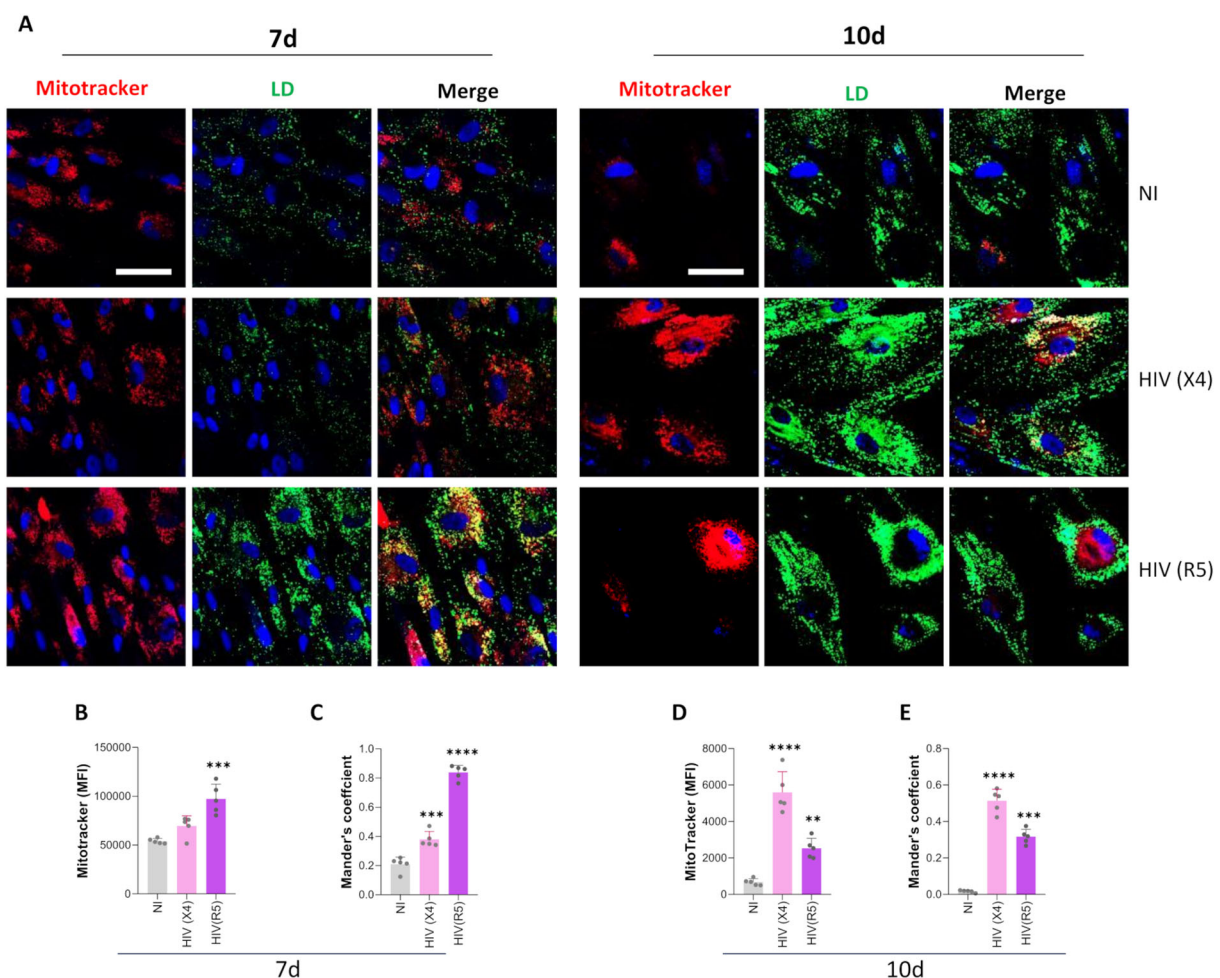


FIGURE 6

Interaction between lipid droplets and mitochondria during adipocyte differentiation under X4- and R5-tropic HIV infection. Representative images showing mitochondria stained with Mitotracker Deep Red and lipid droplets (LD) stained with Bodipy 493/503 at 7- and 10-days post-differentiation in cells exposed to HIV (X4 and R5 strains) (A). Quantification of median fluorescence intensity (MFI) for mitochondrial content was performed using ImageJ from the images in panel A at 7 (B) and 10 (D) days post-differentiation. Lipid droplet-mitochondria colocalization was analyzed using Mander's overlap coefficient at 7 (C) and 10 (E) days post-differentiation. Scale bar: 50 μ m. Data are presented as mean $\pm$ SD. Each dot represents the average of technical replicates from an independent biological experiment ($n = 5$). ** $p < 0.005$; *** $p < 0.001$; **** $p < 0.0001$ compared to non-infected cells (NI).

(Figures 7A, C). Additionally, no significant changes in cell viability were observed under any condition (Figures 7B, D).

These findings indicate that while HIV infection promotes mitochondrial biogenesis and organelle interaction with lipid droplets, it does not induce oxidative stress or compromise cell viability during adipocyte differentiation. This suggests that mitochondrial remodeling under these conditions may support metabolic adaptation rather than reflecting mitochondrial dysfunction.

Role of CXCR4 and CCR5 in the increase of lipid droplets size induced by X4- and R5-tropic HIV infection

CXCR4 and CCR5 have been associated with adipogenesis and adipose tissue remodeling, suggesting roles in differentiation

and inflammation during adipocyte development. To assess the specific contributions of CXCR4 and CCR5 in HIV-mediated modulation of adipocyte differentiation, we treated MSCs with X4- or R5-tropic HIV during differentiation in the presence or absence of the CXCR4 antagonist AMD3100 or the CCR5 antagonist TAK799.

In the presence of X4-tropic HIV, CXCR4 inhibition by AMD3100 significantly reduced adipocyte number, cell size, and lipid droplet accumulation to levels comparable to uninfected controls at 10 days post-differentiation. No significant effects were observed at 7 days post-differentiation (Figures 8A–L). Conversely, CCR5 inhibition with TAK799 did not affect adipocyte number or lipid droplet accumulation in cells exposed to R5-tropic HIV. However, TAK799 treatment significantly altered lipid droplet size in these cells at 7 and 10 days post-differentiation (Figures 8M–X). Importantly, treatment with AMD3100 or

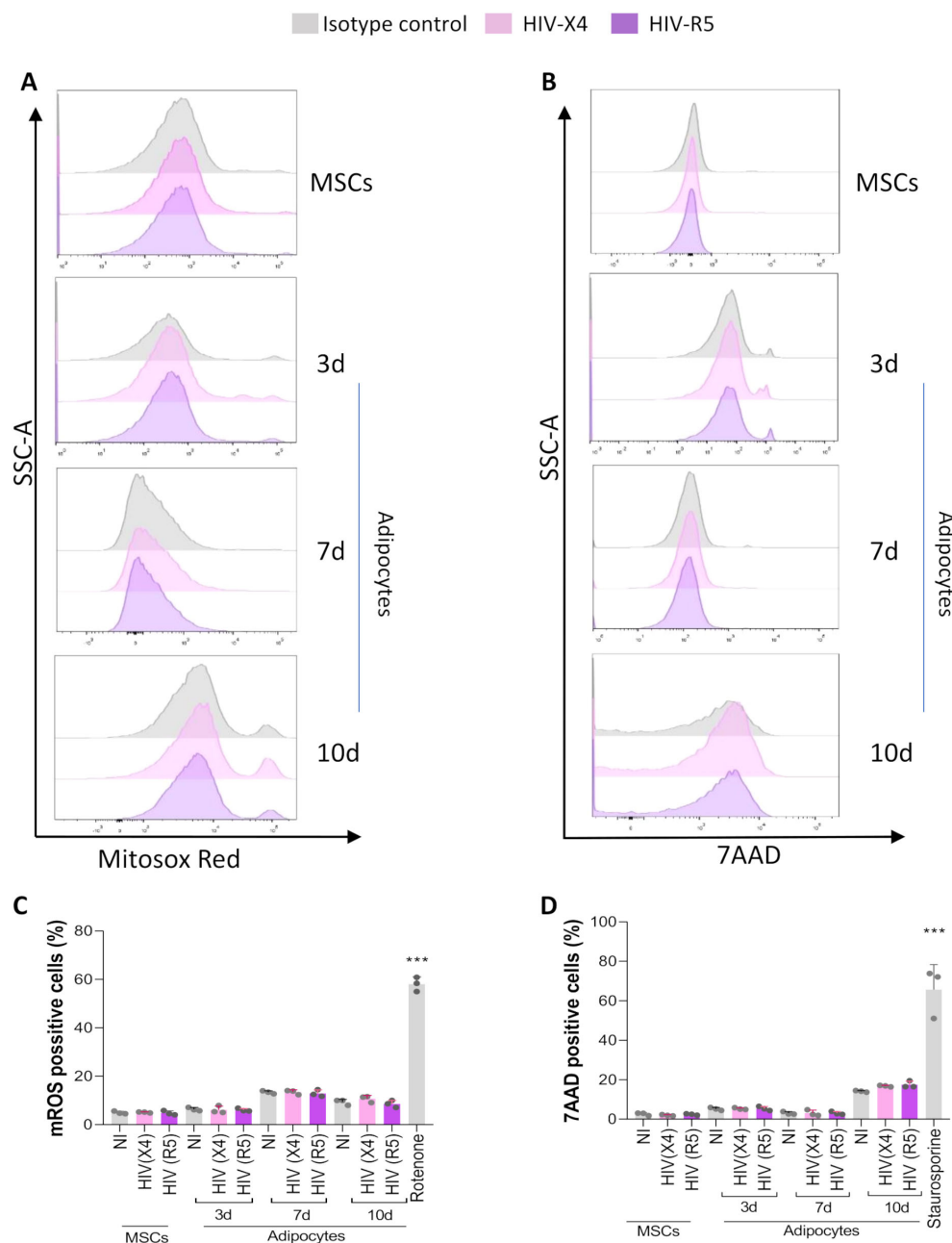


FIGURE 7

Effects of HIV on cell viability and mROS production. Representative flow cytometry histograms of MitoSOX staining, illustrating mROS un MSCs and at 1,3 and 7 days (d) post-adipocyte differentiation, in cells exposed to HIV (X4 and R5 strains) (A). Representative flow cytometry histograms of 7AAD staining, illustrating cell death un MSCs and at 3, 7 and 10 days (d) post-adipocyte differentiation (B). Quantification of mROS production presented in panel A, expressed as the percentage of cells stained with MitoSOX Red (C). Assessment of cell viability, shown as the percentage of cells stained with 7AAD at the conditions at time points presented in panel B (D). NI: non-infected; Rotenone and staurosporine were used as positive control of mROS production and cell death, respectively. Data are presented as mean $\pm$ SD. Each dot represents the average of technical replicates from an independent biological experiment ($n = 3$). *** $p < 0.001$ compared to non-infected cells (NI).

TAK799 alone did not affect adipocyte differentiation, highlighting the specificity of the CXCR4-dependent effect in the context of X4-HIV exposure.

These findings identify CXCR4 as a critical mediator of HIV-induced adipocyte hypertrophy and lipid droplet enlargement during X4-tropic HIV infection, whereas CCR5 does not appear to play a direct role in modulating these processes during R5-tropic HIV exposure.

Discussion

People living with HIV (PLWH) frequently exhibit reduced bone mineral density (BMD), predisposing individuals to conditions like osteopenia and osteoporosis. This heightened susceptibility to bone fragility increases the likelihood of fractures compared to those in the general population (Cotter and Mallon, 2014).

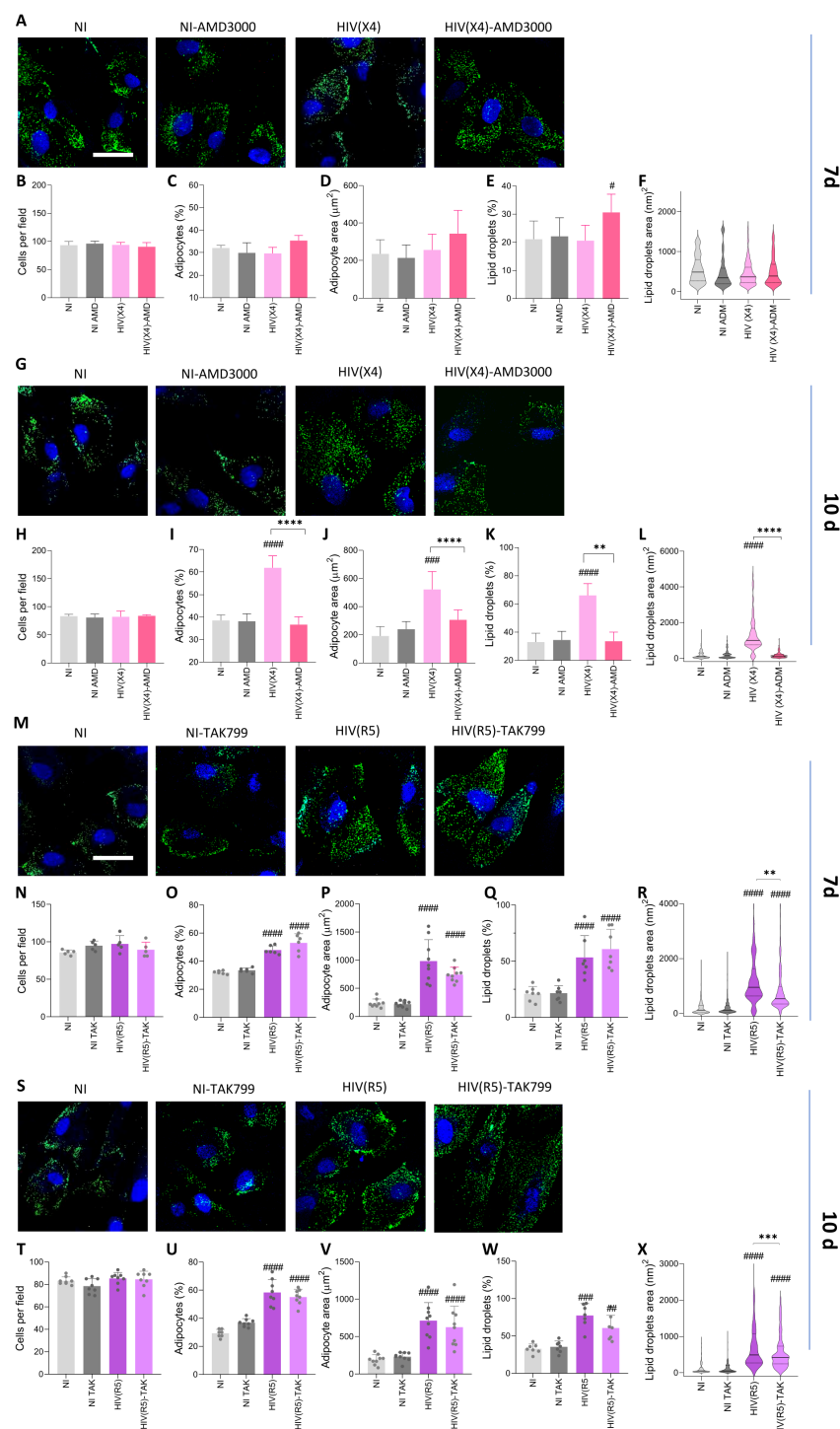


FIGURE 8

Role of CCR5 and CXCR4 in adipocyte differentiation. Effect of CXCR4-tropic HIV (HIV (X4)), HIV-X4 + AMD3100, CCR5-tropic HIV (HIV (R5)), and HIV-R5 + TAK-779 exposure on adipocyte differentiation. Representative microscopy images show the presence of lipid droplets stained with Bodipy 493/503 (LD) at 7 (A, M) and 10 (G, S) days post-differentiation. Quantification of the experiments shown in panels (A, F, M, S) includes the number of cells per field (B, H, N, T), percentage of adipocytes per field (C, I, O, U), adipocyte area (D, J, P, V), percentage of lipid droplets per field (E, K, Q, W) and lipid droplets size (F, L, R, X). Scale bar: 50 μm . Data are presented as mean $\pm$ SD. Each dot represents the average of technical replicates from an independent biological experiment ($n = 6$). ## $p < 0.01$, ### $p < 0.001$ and #### $p < 0.0001$ compared to non-infected cells (NI). * $p < 0.05$ ** $p < 0.01$, and **** $p < 0.0001$.

In bone diseases, a pathological decline in BMD often occurs alongside increased fat deposition within the marrow and adjacent tissues (Meunier et al., 1971; Verma et al., 2002; Yeung et al., 2005).

In our laboratory, we have shown that HIV exposure during MSC-to-osteoblast differentiation does not impair matrix deposition; however, it markedly elevates RANKL production and

vitronectin accumulation, implying a potential shift toward enhanced osteoclastogenesis and altered bone homeostasis (Freiberger et al., 2024). Building on this inverse relationship between osteogenesis and marrow adiposity, we therefore set out to determine whether HIV also influences MSC differentiation into adipocytes.

Notably, although adipocytes derived from MSCs permitted viral entry, they were non-permissive to productive HIV infection. This result is consistent with previous studies showing that, while HIV can enter adipocytes, these cells are unable to support productive viral replication (Munier et al., 2003; Maurin et al., 2005). In those studies, a peak in viral protein production was observed at three days post-infection, followed by a gradual decline. This phenomenon was not prevented by HIV inhibitors, suggesting passive release of viral antigens rather than active replication (Maurin et al., 2005). Furthermore, Munier et al. demonstrated using VSV-G pseudotyped viral particles that viral entry is the limiting step in these cells (Munier et al., 2003). In contrast, we found that adipocytes differentiated from MSCs were not productively infected, even by X4-tropic HIV, despite expressing high surface levels of CD4 and CXCR4. This indicates a non-permissive replication environment, possibly due to the activation of intrinsic restriction factors upon CD4 and co-receptor engagement during viral entry.

Nonetheless, both R5- and X4-tropic HIV strains induced a significant increase in adipocyte number, lipid droplet count, and size. Notably, R5-tropic HIV induced these changes earlier than the X4-tropic strain.

Differences in adipocyte differentiation dynamics may be linked to receptor expression patterns. While CCR5 remained stable, CXCR4 was markedly upregulated by day 7, potentially driving the increased adipocyte number and lipid accumulation by day 10.

Multiple transcription factors, including *PPAR-γ*, *C/EBPα*, and *C/EBPβ*, are key regulators of adipogenesis. *PPAR-γ* plays a central role, and its upregulation in response to both viral tropisms aligns with findings from *in vitro* models of infectious diseases (Ayyappan et al., 2019; Gonzalez et al., 2019; Pesce Viglietti et al., 2020). *C/EBPα* is expressed in the late phase of adipocyte differentiation, while *C/EBPβ* is expressed very early during adipogenesis. Additionally, it is necessary for sustaining the expression of *PPAR-γ* and *C/EBPα* (Darlington et al., 1998). Both transcription factors were upregulated in response to HIV exposure, though with temporal and tropism-specific differences. These variations may be attributed to post-transcriptional modifications that regulate their activity (Hattori et al., 2003; Cesena et al., 2007; Nerlov, 2008), and potentially to compensatory mechanisms between *C/EBPα* and *C/EBPβ* (Lefterova et al., 2008).

Adipocyte enlargement is associated with increased lipid droplet size, necessitating enhanced protein and membrane synthesis, as well as coordinated lipogenesis and lipolysis (Laurencikienė et al., 2011).

ATGL and *HSL* are the major rate-determining enzymes for lipolysis in adipocytes. They are involved in the hydrolysis of triglycerides, releasing fatty acids essential as energy substrates, precursors for membrane lipid synthesis, and ligands for nuclear

receptors (Miyoshi et al., 2008; Schreiber et al., 2019). In our study, *ATGL* expression increased in adipocytes derived from R5-HIV-infected MSCs, corresponding with larger lipid droplets. Conversely, *HSL* expression decreased at day 10 post-differentiation in cells exposed to both viral tropisms. *LPL*, which facilitates the uptake of fatty acids from circulating triglycerides (Weinstock et al., 1997; Zechner et al., 2000), was downregulated at early stages of differentiation in X4-HIV-infected cells. This reduction may have limited lipid accumulation at early time points in these cells.

In more recent findings, a notable function of acid lipolysis has emerged, implicating the involvement of lysosomal lipases in the breakdown of lipid droplets, operating alongside cytoplasmic lipases (Yu and Li, 2017). Here, we have found that X4 and R5 tropic HIV induces an increase in lipid droplet-lysosome interaction, suggesting that acid lipolysis could play a role in the degradation of lipid droplets. The *LIPA* gene, which encodes LAL, was upregulated in cells exposed to X4- and R5-tropic HIV (Li and Zhang, 2019).

LIPA (LAL) is *PPARα*-regulated (Bougarne et al., 2018), and HIV (X4/R5) induced its upregulation alongside elevated *PPARα* at days 1 and 10 post-differentiation, suggesting that *PPARγ* activation, more than mere overexpression, may underlie this increase.

Triglyceride synthesis is catalyzed by the *DGAT* family of enzymes (Liu et al., 2012; Chitraju et al., 2017). *DGAT1*, localized to the ER, prevents lipid-induced ER stress (Chitraju et al., 2017), and its expression was increased in R5-HIV-infected cells at day 7 and in both tropisms by day 10. On the other hand, *DGAT2* is implicated in increasing lipid droplet size (Zhang et al., 2014), was upregulated at all time points post differentiation in response to both HIV strains. In X4-HIV-infected cells, *DGAT2* upregulation may act as a compensatory mechanism in response to reduced *LPL* expression.

Lipid biosynthesis is also regulated by *SREBPs*, which control fatty acid (*SREBP1a/c*) and cholesterol (*SREBP2*) synthesis (Horton et al., 2002a, 2002b; Iizuka et al., 2004). Moreover, *SREBP-2* transcription factor is a common feature of hypertrophied adipocytes (Bauer et al., 2011). The observed increase in triglyceride and cholesterol production in infected cells suggests the active involvement of both *SREBP* pathways.

As previously reported, type I interferons are linked to cholesterol metabolism (York et al., 2015). Accordingly, we observed that increases in *IFNα2* and *IFNβ1* transcription in infected adipocytes coincided with periods when *SREBP2* was not upregulated.

Lipid droplet-mitochondria contact sites are critical for lipid storage and β -oxidation (Liao et al., 2022). HIV exposure increased lipid droplet-mitochondria contacts and mitochondrial mass in adipocytes. Although mROS function as adipogenic signaling molecules (Orava et al., 2011), their levels remained unchanged in infected cells, implying that mitochondrial expansion occurred without increased oxidative stress.

Our results also highlight the distinct roles of CXCR4 and CCR5 in adipocyte differentiation. CXCR4 expression increased during adipogenesis and was associated with *PPAR-γ* expression

(Tontonoz et al., 1994; Steiner et al., 2024). Blocking CXCR4 with AMD3100 during differentiation in the presence of X4-tropic HIV significantly reduced adipocyte number, size, and lipid droplet accumulation, reinforcing its role in this process. Conversely, CCR5, primarily linked to adipose tissue inflammation (Mello Coelho et al., 2009; Kitade et al., 2012), did not appear to influence adipocyte differentiation under R5-tropic HIV exposure, as TAK799 had no observable effect. These results suggest that CXCR4 is essential for adipocyte differentiation, whereas CCR5 may be more involved in adipose tissue homeostasis and inflammation.

In summary, our findings demonstrate that exposure of MSCs to HIV can modulate adipocyte differentiation—a process essential for maintaining bone-fat homeostasis. These alterations may underlie the metabolic disturbances and bone loss commonly observed in people living with HIV. In line with this, early metabolic changes seen in untreated patients suggest that HIV-1 proteins may directly contribute to adipocyte dysfunction, as reported in cases of HIV-associated lipodystrophy (Giralt et al., 2011). Fat redistribution and metabolic abnormalities in PLWH are known to be complex and multifactorial, involving not only direct effects of viral proteins and antiretroviral therapy on adipocyte health, but also genetic factors, microbial translocation, immune dysregulation, chronic inflammation, and accelerated fibrosis (Koethe et al., 2020). Further research is needed to better understand how HIV influences inflammatory responses, metabolic regulation, and the development of dyslipidemia.

This study has limitations. The experiments were conducted *in vitro* using umbilical cord-derived MSCs, which, although a useful model for studying adipocyte differentiation, might not fully recapitulate the complexity of adult adipose tissue or the *in vivo* environment. Furthermore, the study focused on a limited set of viral strains (R5- and X4-tropic HIV) and did not address how other viral variants or the presence of antiretroviral therapy might modify adipocyte differentiation and function. Although the investigation provided insights into changes in key adipogenic and lipolytic factors and highlighted the role of CXCR4, the precise molecular mechanisms underlying these alterations and their broader implications for metabolic dysregulation are not completely defined.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

This study received approval from the Comité de Ética de la Facultad de Ciencias Médicas de la Universidad de Buenos Aires, Argentina (RESCD-2023-1291). The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

RF: Data curation, Investigation, Writing – review & editing, Conceptualization, Formal Analysis, Methodology, Validation. CL: Data curation, Formal Analysis, Investigation, Methodology, Writing – review & editing. FS: Formal Analysis, Investigation, Methodology, Writing – review & editing. PJ: Formal Analysis, Investigation, Methodology, Writing – review & editing. MP: Methodology, Writing – review & editing, Resources. MG: Methodology, Resources, Writing – review & editing. JQ: Writing – review & editing, Funding acquisition, Investigation. MD: Funding acquisition, Investigation, Writing – review & editing, Data curation, Project administration, Supervision, Writing – original draft.

Funding

The author(s) declare that financial support was received for the research and/or publication of this article. This work was supported by grants from Agencia Nacional de Promoción Científica y Tecnológica (ANPCYT, Argentina), PICT 2020-0691 to MD. Funding agencies had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fcimb.2025.1627963/full#supplementary-material>

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OPEN ACCESS

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RECEIVED 09 June 2025

ACCEPTED 08 July 2025

PUBLISHED 21 July 2025

CITATION

Gao X, Wu X, Li Y, Dai X and Cai B (2025)
Tryptophan metabolic reprogramming
modulates cytokine networks in
nucleos(t)ide analogue-treated
chronic hepatitis B patients.
Front. Cell. Infect. Microbiol. 15:1643636.
doi: 10.3389/fcimb.2025.1643636

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Tryptophan metabolic reprogramming modulates cytokine networks in nucleos(t)ide analogue-treated chronic hepatitis B patients

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Background & aims: Hepatitis B virus (HBV) infection remains a global health challenge. Tryptophan metabolism influences immune regulation, but its interplay with cytokines during antiviral therapy is unclear. We investigated associations between tryptophan pathways and cytokine profiles in the chronic hepatitis B (CHB) patients with varying treatment outcomes.

Methods: This retrospective study included 106 CHB patients (including 29 functional cure cases) receiving nucleos(t)ide analogues (NAs) and 29 healthy controls. Plasma levels of 20 tryptophan metabolites (kynurenine, serotonin, and bacterial pathways) were quantified by HPLC-MS/MS, and 12 cytokines were measured via flow cytometry. Multivariate analyses were performed.

Results: Functional cure patients showed unique metabolic patterns. Indole-3-carboxaldehyde (IAld) levels increased progressively from HBsAg positive groups (HBeAg-: 63.324 nmol/L; HBeAg+: 65.938 nmol/L) to functional cure (91.44 nmol/L) and healthy controls (130.634 nmol/L) ($P < 0.01$), exhibiting negative correlations with HBsAg ($r = -0.31$) and IFN- γ ($r = -0.53$) but positive correlation with IL-1 β ($r = 0.47$). Picolinic acid (PA) was significantly elevated in the functional cure group ($P < 0.001$), associated with reduced HBsAg, IL-2 and increased IL-1 β , IL-10 levels, indicating potential antiviral effects. Serotonin (5-HT) levels were higher in cured patients and correlated with IL-1 β and IFN- α ($P < 0.05$). HBeAg-positive patients displayed increased kynurenine-to-tryptophan (Kyn/Trp) ratios ($P < 0.05$), while non-cured patients showed metabolic blockade downstream of 3-hydroxykynurenine (elevated 3-HK/Kyn ratios and reduced KA, XA/3-HK, 3-HAA/3-HK, and NAA levels; $P < 0.05$).

Conclusions: The tryptophan metabolites (IAld, PA, 5-HT) were found to correlate with cytokine levels (IL-1 β , IL-10), potentially implicating their involvement in immune regulation and antiviral responses. These observations delineate a metabolic-immune framework that may inform future therapeutic development for HBV.

KEYWORDS

tryptophan metabolism, cytokine profiles, chronic hepatitis B, bacterial degradation pathway, functional cure, indole-3-carboxaldehyde, picolinic acid, 5-hydroxytryptamine

1 Introduction

Hepatitis B Virus (HBV) infection remains a major global public health challenge and one of the leading causes of liver disease-related deaths worldwide. According to the Global Burden of Disease (GBD) 2019 study, the global prevalence of chronic HBV infection across all age groups was estimated at 4.1% (95% uncertainty interval: 3.7–4.5), corresponding to 316 million infected individuals (GBD 2019 Hepatitis B Collaborators, 2022). The World Health Organization (WHO) estimated in 2024 that about 1.23 million new HBV infections occur annually globally, and about 1.1 million people die from diseases associated with HBV infection (World Health Organization, 2024). Although the current first-line antiviral therapies, Nucleos(t)ide Analogues (NAs) and Pegylated Interferon-alpha (PEG-IFN α), are effective in suppressing viral replication and slowing the progression of cirrhosis and hepatocellular carcinoma (HCC), functional cure rates (defined as sustained HBsAg clearance) remain below 10%, and most patients require lifelong treatment or experience poor tolerability (Wu et al., 2025; Yuen et al., 2018). The key to achieving a functional cure for chronic hepatitis B (CHB) lies in suppressing HBV-DNA replication and clearance of viral antigens (HBsAg and HBeAg). However, the relationship between this process and metabolic reprogramming remains unclear.

In recent years, the application of metabolomics in liver disease research has become increasingly widespread. Li Hai's team identified numerous potential prognostic and diagnostic biomarkers for acute-on-chronic liver failure (ACLF) through untargeted metabolite analysis, including membrane lipid metabolism, steroid hormones, oxidative stress pathways, and energy metabolism, and developed targeted liquid chromatography tandem mass spectrometry (LC-MS/MS) assays for four metabolites for clinical laboratories use (Zhang et al., 2023). Additionally, metabolic comorbidities (such as obesity and diabetes) have been shown to exacerbate liver fibrosis in HBV patients and reduce fibrosis regression rates after antiviral therapy (van Velsen et al., 2025). Tryptophan is metabolized through the kynurenine pathway, the serotonin (5-HT) pathway, and the bacterial degradation pathway, with its metabolites (such as

kynurenine and indole derivatives) participating in the regulation of various pathophysiological processes, including protein synthesis, inflammation, oxidative stress, and immune responses (Xue et al., 2023). Numerous studies have shown that tryptophan metabolites modulate systemic inflammation. For instance, indoleamine 2,3-dioxygenase (IDO), an interferon- γ -induced enzyme in the tryptophan pathway, catalyzes the conversion of tryptophan to kynurenine (Werner et al., 1987) and exhibits a dual role in infectious diseases—both promoting inflammation and regulating acute and chronic infections (Mehraj and Routy, 2015). Previous studies have found that platelet-derived 5-HT exacerbates viral hepatitis (Lang et al., 2008). However, other studies suggest that 5-HT-mediated DDX37 agonists (AS-19) increase IFN- β expression and inhibit HBV replication (Kang et al., 2019).

Numerous studies have demonstrated that cytokine networks dictate the immunopathology of hepatitis B virus infection. For instance, Th1 cytokines (e.g., IFN- γ , IL-2) promote viral clearance (Li et al., 2016), whereas in chronic viral infections, IL-10 frequently induces T cell exhaustion and inactivates antiviral T-cell immunity by modulating T cells and antigen-presenting cells (APCs), thereby facilitating immune evasion and persistent/latent infection (Rojas et al., 2017). Recent evidence indicates that tryptophan metabolites modulate cytokine responses. For example, IDO increases populations of T lymphocytes producing IFN- γ and IL-17, thereby exerting inhibitory effects (Huang et al., 2020). Indole-3-carboxaldehyde (IAld) restores virus-induced pro-inflammatory features by binding to the aryl hydrocarbon receptor (AhR), reducing IL-1 β production and increasing IL-10 (Pariano et al., 2024). Additionally, serotonin stimulates secretion of pro-inflammatory cytokines (IL-1 and IL-6) while enhancing the cytotoxicity of IFN- γ , playing a significant role in antiviral defense (Kanova and Kohout, 2021).

However, research on the tryptophan metabolic pathway related to HBV remains limited. As a key immunoregulatory metabolic pathway, its role in viral antigen clearance during chronic HBV infection has yet to be elucidated. Therefore, this study focuses on patients with different CHB disease status, aiming to investigate the relationship between tryptophan metabolites and viral antigen clearance. The findings may reveal the immune

regulatory function of tryptophan metabolism in HBV and provide novel therapeutic targets for optimizing clinical cure strategies.

2 Materials and methods

2.1 Study population

This retrospective analysis included 135 participants: 106 CHB patients (including functional cure cases) who received NAs therapy for 48 weeks (without prior interferon treatment) and were followed at our outpatient clinic from July 2023 to August 2024, along with 29 healthy controls. All patients met the diagnostic criteria before treatment outlined in the Chinese *Guidelines for the Prevention and Treatment of Chronic Hepatitis B (2022 Edition)* (Chinese Society of Hepatology, C.M.A and Chinese Society of Infectious Diseases, Chinese Medical Association, 2022). And functional cure was defined according to the *Expert Consensus on Clinical Cure (Functional Cure) of Chronic Hepatitis B* (Chinese Society of Infectious Disease Chinese Society of Hepatology, C.M.A, 2019), characterized as sustained, undetectable serum HBsAg, HBeAg and HBV DNA with or without seroconversion to anti-HBs, but with the persistence of residual cccDNA, accompanied by resolution of liver injury after the completion of a finite course of treatment. Healthy controls were selected from individuals undergoing routine health examinations at our center. Exclusion criteria included acute hepatitis B, severe hepatitis B, overlapping-infections (other viruses/bacteria), autoimmune diseases, liver cirrhosis, and malignancies. Baseline clinical and laboratory characteristics are summarized in Table 1. This study was approved by the Ethics Committee of West China Hospital of Sichuan University (No: 2021-140). The sample used in this study were residual blood samples from clinical testing, and informed consent from patients was waived.

2.2 HPLC-MS/MS analysis of tryptophan and its metabolites

High-performance liquid chromatography-mass spectrometry (HPLC-MS/MS) was used to quantitatively detect tryptophan and its metabolites in plasma, covering 20 metabolites across three pathways (Supplementary Figure 1):

- Kynurenine pathway: Tryptophan (Trp), Kynurenine (Kyn), 3-Hydroxykynurenine (3-HK), Kynurenic acid (KA), Xanthurenic acid (XA), 3-Hydroxyanthranilic acid (3-HAA), Quinolinic acid (QA), Picolinic acid (PA), Nicotinic acid (NA), Nicotinamide (NAA).
- Serotonin (5-HT) pathway: 5-Hydroxytryptophan (5-HTP), 5-Hydroxytryptamine (5-HT) (Serotonin), 5-Hydroxyindole-3-acetic acid (5-HIAA), N-Acetylserotonin (NAS), Melatonin (M).
- Bacterial degradation pathway (Indole pathway): Tryptamine (TA), IAlD, indole-3-acetic acid (IAA), Indole-3-lactic acid (ILA), Indolepropionic acid (IPA).

The analytical methods were referenced from previous studies by our research group (Miao et al., 2025). Additionally, this study incorporated several calculated values to enhance the analytical strategy, such as Sum-5-HT, Sum-Indoles, and Sum-Kyn, which represent the overall levels of all metabolites in the 5-HT pathway, bacterial degradation pathway, and kynurenine pathway, respectively. Furthermore, metabolite-to-precursor ratios were used, such as Kyn/Trp, KA/Kyn, 3-HK/Kyn, XA/3-HK, 3-HAA/3-HK, QA/3-HAA, and PA/3-HAA, which reflect the activity of key enzymes in metabolic processes (Moulin et al., 2024).

2.3 Flow cytometry assay for cytokines

Plasma cytokine profiling was performed using FACS Canto II™ flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA) with a multiplex cytokine assay kit (immunofluorescence method) (Jiangxi Saiji Biotechnology Co., Ltd., Nanchang, China) according to the manufacturer's protocol. Twelve cytokines were simultaneously quantified: IL-2, IL-4, IL-6, IL-10, TNF- α , IFN- γ , IL-17, IL-1 β , IL-5, IL-12p70, IFN- α , and IL-8.

2.4 Statistical analysis

Statistical analyses were performed using SPSS 27.0 (IBM SPSS Software Inc., Armonk, NY, USA) and figures were produced with Origin 2024 (OriginLab Corporation, Northampton, MA, USA) including principal component analysis, heatmaps, and violin plots. Figure of tryptophan metabolic pathway was designed using Microsoft PowerPoint (Microsoft Corporation, Redmond, WA, USA). All clinical characteristics of patients were analyzed using descriptive statistics. For continuous variables with a normal distribution, data were expressed as mean $\pm$ standard deviation (Mean $\pm$ SD). For continuous variables with a non-normal distribution, data were expressed as median (interquartile range) (Median (P25, P75)). For categorical variables, data were expressed as frequency (percentage) (n (%)). Group comparisons were conducted using one-way ANOVA for normally distributed continuous variables, Kruskal-Wallis H test for non-normally distributed continuous variables, and chi-square test for categorical variables. Both univariate and multivariate logistic regression analyses were conducted to control for potential confounding factors. Spearman's rank correlation was used to evaluate relationships between metabolite levels and cytokine levels. A *P*-value < 0.05 was considered statistically significant.

3 Results

3.1 Clinical characteristics of the study cohort

This study enrolled 106 patients with CHB who received NAs therapy for over 48 weeks, stratified into four groups based on

TABLE 1 Baseline clinical characteristics and laboratory parameters.

Group ^a	Functional cure (n 29)	HBeAg-negative (n 40)	HBeAg-positive (n 37)	Healthy Control (n 29)	Total (n 135)	<i>P value</i> ^c
Age (y)	48.38 ± 12.77	45.88 ± 9.49	39.54 ± 9.03	43.38 ± 10.92	44.14 ± 10.86	0.006
Sex (n (%))						
Male	26 (89.7%)	20 (50%)	22 (59.5%)	11 (37.9%)	79 (58.5%)	< 0.001
Famale	3 (10.3%)	20 (50%)	15 (40.5%)	18 (62.1%)	56 (41.5%)	
Disease duration (y) ^b						
<5	1 (4.2%)	5 (15.2%)	4 (13.3%)	/	10 (11.5%)	0.382
5~10	2 (8.3%)	7 (21.2%)	3 (10%)	/	12 (13.8%)	
10~20	11 (45.8%)	12 (36.4%)	16 (53.3%)	/	39 (44.8%)	
>20	10 (41.7%)	9 (27.3%)	7 (23.3%)	/	26 (29.9%)	
Duration of treatment (y)	5 (3, 10)	3 (1, 7)	5 (2.25, 8)	/	5 (2, 8)	0.085
HBsAg (IU/mL)	/	1091.5 (383.75, 2901)	1805 (777, 8898)	/	/	/
HBV DNA (n (%))						
<100 copies/mL	29 (100%)	40 (100%)	33 (89.2%)	/	102 (96.2%)	0.017
≥100 copies/mL	/	/	4 (10.8%)	/	4 (3.8%)	
ALT (U/L)	26 (16, 34)	23 (17, 31)	22 (15.5, 34.5)	15.5 (9, 21.25)	22 (15, 29)	0.002
AST (U/L)	24 (21, 25)	23 (21, 27)	23 (20, 26)	17.5 (15, 21)	22 (18, 25)	< 0.001
ALP (IU/L)	83.81 ± 16.38	83.36 ± 25.16	79.08 ± 20.30	63.24 ± 15.86	77.79 ± 21.66	< 0.001
GGT (IU/L)	22 (14, 29)	18 (14, 28)	20 (12, 32)	15 (10, 19.25)	17 (12, 26)	0.045
ALB (g/L)	48.50 ± 2.39	47.97 ± 2.42	47.25 ± 2.24	46.10 ± 2.54	47.46 ± 2.51	0.001
WBC (×10 ⁹ /L)	5.84 ± 1.62	5.62 ± 1.26	6.22 ± 1.43	5.23 ± 1.11	5.74 ± 1.38	0.031
AFP (ng/mL)	2.44 (1.65, 3.18)	2.51 (1.78, 3.53)	2.49 (1.92, 3.52)	2.35 (2.04, 4.01)	2.46 (1.87, 3.44)	0.638

^aFunctional cure: HBV-infected patients with sustained HBsAg loss and undetectable HBV DNA; HBeAg-negative: patients with HBsAg+HBeAg-HBcAb+; HBeAg-positive: patients with HBsAg+HBeAg+HBcAb+.

^bRelevant data were missing for 19 patients.

^c*P* value < 0.05 indicates statistical significance.

ALT, Alanine Aminotransferase; AST, Aspartate Aminotransferase; ALP, Alkaline Phosphatase; GGT, Gamma-Glutamyl Transferase; ALB, Albumin; WBC, White Blood Cell Count; AFP, Alpha-Fetoprotein.

treatment outcomes: Functional cure group (n = 29), HBeAg-negative (HBsAg+HBeAg-HBcAb+) group (n = 40), HBeAg-positive (HBsAg+HBeAg+HBcAb+) group (n = 37), and Healthy control group (n = 29). As summarized in [Table 1](#), the overall age of participants was 44.14 ± 10.86 years, with the functional cure group demonstrating the highest mean age (48.38 ± 12.77 years), while the HBeAg-negative group and HBeAg-positive group had relatively lower mean ages (45.88 ± 9.49 years and 39.54 ± 9.03 years, respectively). The proportion of males in the functional cure group (89.7%) were significantly higher than that in the HBeAg-positive group (50%) and HBeAg-negative group (59.5%). While disease duration and treatment length varied considerably across patient groups, no significant intergroup differences were observed. Laboratory analyses showed that liver function parameters (Alanine aminotransferase (ALT), Aspartate aminotransferase (AST), Alkaline phosphatase (ALP), Gamma-glutamyl transferase (GGT), Albumin (ALB)) were

higher in all disease groups than in the healthy control group. White blood cell count (WBC) was highest in the HBeAg-positive group and lowest in controls. And there were no statistically significant differences in alpha-fetoprotein (AFP) levels between groups.

The baseline imbalances in laboratory indicators were attributed to differences between the disease group and healthy controls, while no significant differences were observed between functional cure and non-cured patients within the disease group ([Supplementary Table 1](#)). Therefore, we adjusted only for confounding factors including age and sex in both univariate and multivariate regression analyses ([Table 2](#)). Univariate logistic regression analysis revealed that multiple tryptophan metabolic pathway markers and cytokines were significantly associated with functional cure in chronic hepatitis B (CHB) patients. Subsequently, multivariate logistic regression analysis using the forward likelihood ratio (Forward: LR) method identified IAI, PA, PA/3-HAA ratio, and TNF-α as independent predictors.

TABLE 2 Logistic regression analysis of tryptophan metabolites and cytokines on the functional cure of hepatitis B^a.

Variables	Univariate analysis		Multivariate analysis ^b	
	OR(95% CI)	<i>P</i> value ^c	OR(95% CI)	<i>P</i> value ^c
Age (y)	0.954 (0.916, 0.993)	0.023	NA	0.144
Sex (male)	1.131 (0.037, 0.471)	0.002	NA	0.084
Sum-5HT (nmol/L)	0.994 (0.99, 0.999)	0.010	NA	0.208
5-HT (nmol/L)	0.994 (0.989, 0.999)	0.011	NA	0.207
IAld (nmol/L)	0.98 (0.968, 0.993)	0.002	0.94 (0.906, 0.975)	0.001
Sum-Kyn (nmol/L)	0.999 (0.999, 1)	0.036	NA	0.867
Kyn (nmol/L)	0.999 (0.998, 1)	0.024	NA	0.619
PA (nmol/L)	0.878 (0.83, 0.929)	0.000	0.909 (0.835, 0.991)	0.030
Kyn/Trp	0.919 (0.859, 0.984)	0.015	NA	0.977
XA/3-HK	0.053 (0.003, 0.972)	0.048	NA	0.713
PA/3-HAA	0.412 (0.277, 0.615)	0.000	0.371 (0.171, 0.806)	0.012
IL-2 (pg/mL)	2.545 (1.446, 4.479)	0.001	NA	0.171
IL-10 (pg/mL)	0.586 (0.451, 0.761)	0.000	NA	0.988
TNF- α (pg/mL)	0.58 (0.453, 0.744)	0.000	0.361 (0.205, 0.638)	< 0.001
IL-8 (pg/mL)	0.913 (0.839, 0.993)	0.034	NA	0.190
IL-6/IL-10	7.697 (1.379, 42.951)	0.020	NA	0.962

^aThe dependent variable is a binary classification variable (functional cure (*n* = 29) and non-cured (HBeAg-negative and HBeAg-positive (*n* = 77)).

^bThe final model was determined through multivariate analysis using the Forward: LR method, with collinearity verified by VIF (all variables had VIF < 5). NA, ORs cannot be calculated for variables not retained in the final model equation.

^c*P* value < 0.05 indicates statistical significance.

OR, Odds Ratio; CI, confidence interval; Sum-5-HT, Collective metabolites in the serotonin pathway; 5-HT, 5-Hydroxytryptamine (Serotonin); NAS, N-Acetylserotonin; IAld, Indole-3-carboxaldehyde; Sum-Kyn, Collective metabolites in the kynurenine pathway; Kyn, Kynurenine; PA, Picolinic acid; Kyn/Trp, ratio of Kynurenine to Tryptophan; XA/3-HK, ratio of Xanthurenic acid to 3-Hydroxykynurenine; PA/3-HAA, ratio of Picolinic acid to 3-Hydroxyanthranilic acid; IL, Interleukin; TNF, Tumor Necrosis Factor.

3.2 Analysis of tryptophan and its metabolites levels

Comprehensive profiling of all metabolites and calculated indices was performed, with NA and TA metabolites excluded from detailed analysis due to their consistently low concentrations across all groups. The principal component analysis (PCA) was performed on the combined dataset of tryptophan metabolites and cytokines from all four study groups (Supplementary Figure 2). Principal components 1 and 2 accounted for 20.8% and 12.3% of the total variance, respectively. Distinct clustering was observed between all patient groups and healthy controls, whereas the HBeAg-negative and -positive groups demonstrated overlapping distributions. Additional PCA analyses stratifying the functional cure group and non-cured subgroups are presented in Supplementary Figure 3 (which includes separate PCA analyses for metabolites and cytokines).

Our findings demonstrated statistically significant differences (*P* < 0.05) in multiple metabolic pathways among groups (Supplementary Table 2), including Trp and Sum-5-HT, 5-HT, NAS, M in the 5-HT pathway, IAld and ILA in the bacterial degradation pathway, and Sum-Kyn, KA, PA, NAA, Kyn/Trp, 3-HK/Kyn, XA/3-HK, 3-HAA/3-HK, and PA/3-HAA in the kynurenine pathway.

For metabolites and calculated values with significant differences, we further conducted intergroup differential analysis (Figure 1). The

results showed significantly elevated tryptophan levels in healthy controls compared to all patient groups, with the HBeAg-positive cohort exhibiting obvious reduction in tryptophan concentration. Which indicating that tryptophan metabolism may be inhibited in HBV patients and is closely related to disease efficacy.

In the downstream bacterial degradation pathway of tryptophan, IAld and ILA were also significantly higher in the healthy control than in the disease groups, while IAld exhibited a progressively higher levels from HBsAg-positive (median 63.324 nmol/L in HBeAg-negative and 65.938 nmol/L in HBeAg-positive) to functional cure groups (91.44 nmol/L) and finally to the healthy control groups (130.634 nmol/L, *P* < 0.01), showing a gradual increase as HBV antigen levels decreased.

Similar patterns were observed in the 5-HT pathway, with Sum-5-HT and 5-HT exhibiting comparable variations across groups, with no significant differences between the healthy control group and the functional cure group but were markedly higher than non-cured HBV groups, particularly the HBeAg-positive cohort. Although there were no significant differences between the functional cure group and the HBeAg-negative group, both were significantly higher than the HBeAg-positive group. The two metabolites NAS and M in this pathway also showed higher levels in the healthy control group than in the disease group.

The kynurenine pathway revealed the most differential metabolites and indices, with the overall metabolite level Sum-Kyn showing no

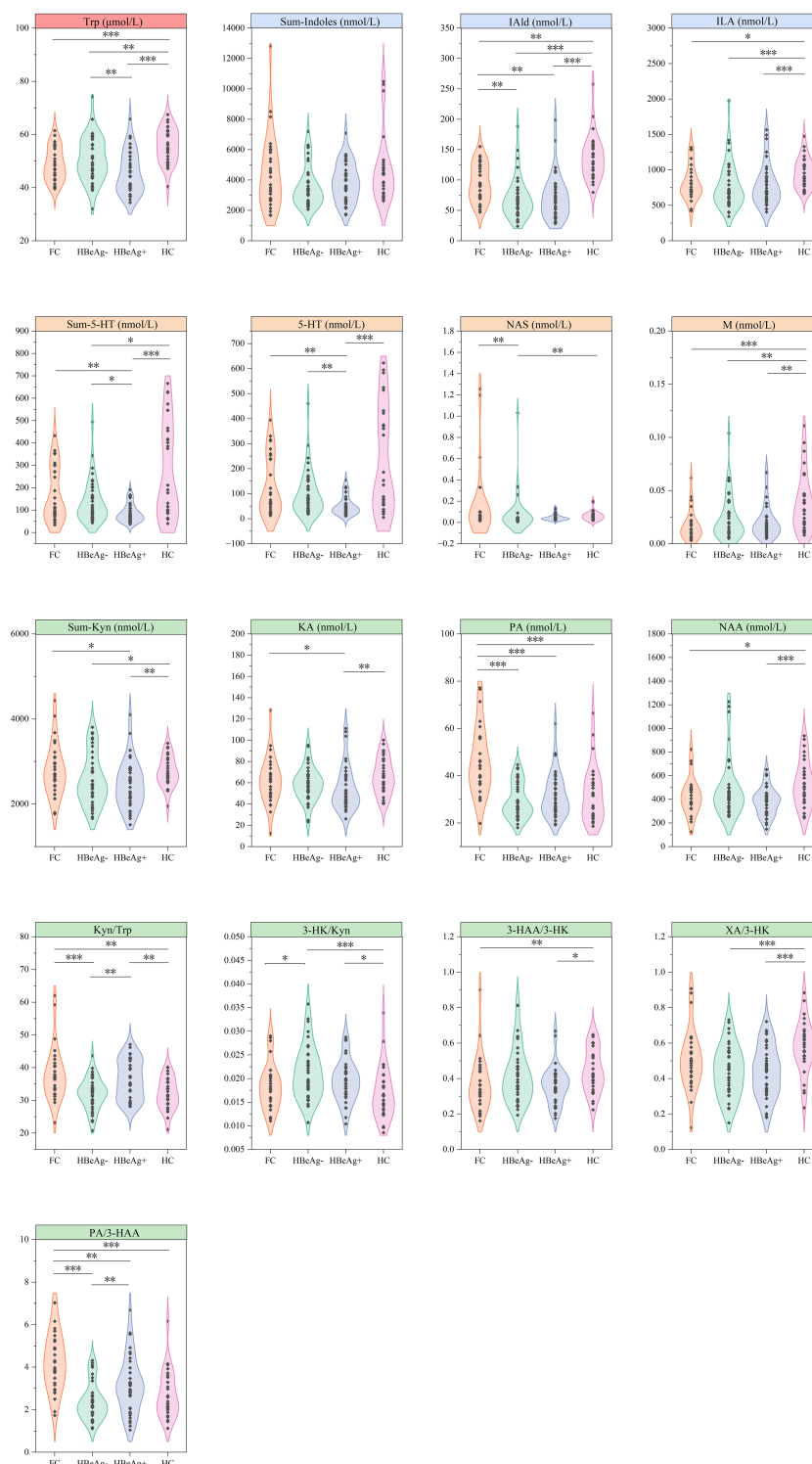


FIGURE 1

Differential analysis of tryptophan metabolites across groups. FC, Functional cure group ($n = 29$); HBeAg⁻, HBeAg-negative group ($n = 40$); HBeAg⁺, HBeAg-positive group ($n = 37$); HC, Healthy Control group ($n = 29$). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

significant difference between healthy controls and the functional cure group, but both were significantly higher than non-cured groups. While the functional cure and HBeAg-negative groups showed comparable levels, they differed significantly from the HBeAg-positive group. Analysis of downstream kynurenine metabolites and

metabolic enzymes revealed that while Kyn/Trp (reflecting Indoleamine 2,3-dioxygenase (IDO) activity) exhibited unclear variations across groups, the remaining differential metabolites consistently demonstrated significant differences between patient groups and healthy controls. Specifically, 3-HK/Kyn (indicating

kynurenine 3-monooxygenase (KMO) activity) was elevated in non-cured groups, whereas KA (the metabolic product of Kyn and kynurenine aminotransferase (KAT)), XA/3-HK (representing KAT II activity), 3-HAA/3-HK (reflecting kynureninase (KYNU) activity), and NAA (the terminal product of kynurenine pathway) were all reduced in patient groups. These observations suggest that NAs-treated HBV patients, particularly those who failed to achieve functional cure, may experience metabolic inhibition downstream of 3-HK in the kynurenine pathway. Interestingly, PA, products of another metabolic pathway of 3-HAA, and PA/3-HAA, were significantly elevated only in the cured group.

These findings suggests that all three tryptophan metabolic pathways exhibit meaningful changes following NAs therapy in HBV patients.

3.3 Cytokine profile analysis

In the intergroup comparison of cytokines, all cytokines except IL-5 showed significant differences between different groups (Figure 2), demonstrating persistent immune dysregulation in both HBV-infected patients and those achieving clinical cure. Specifically, healthy controls

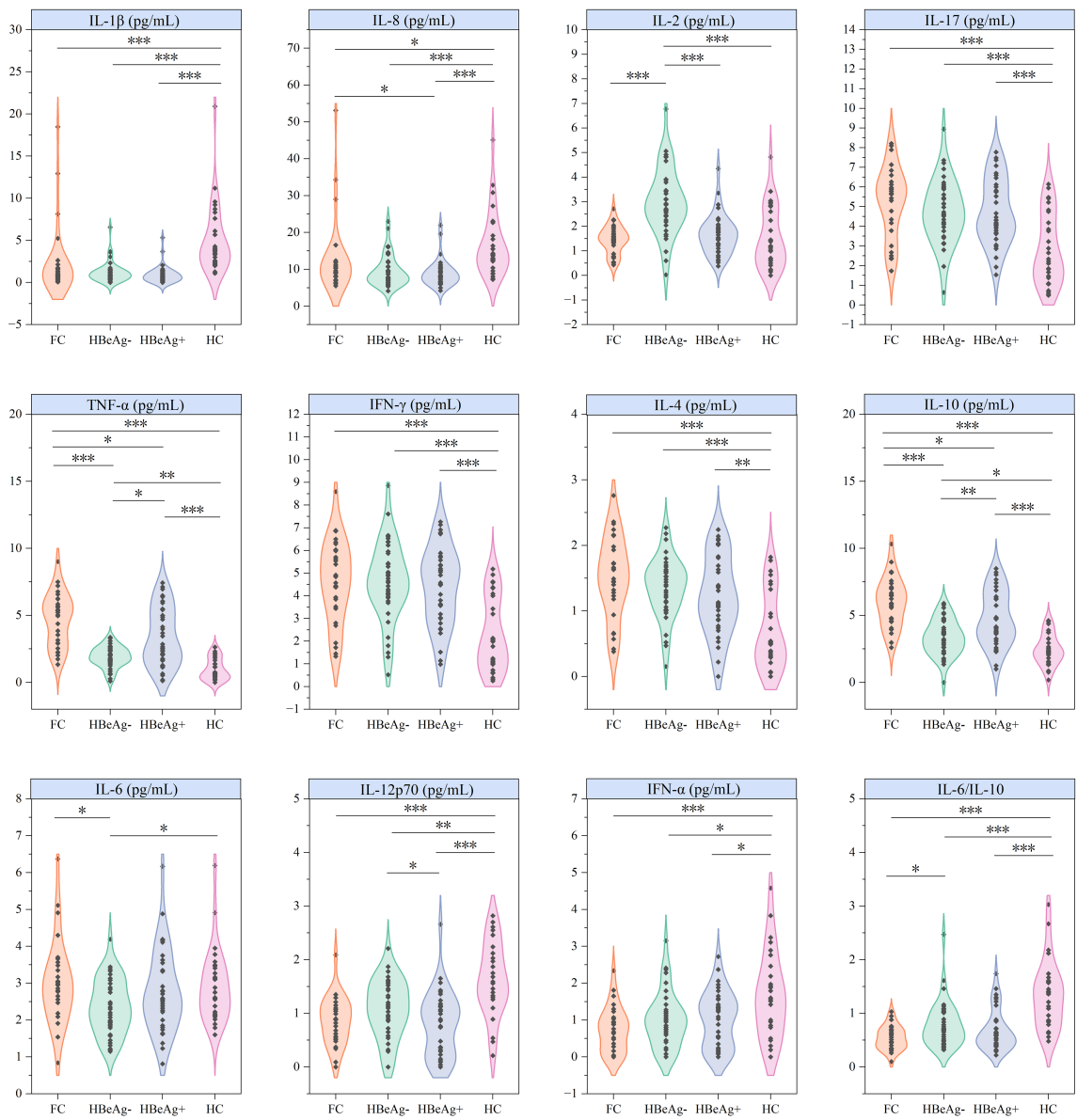


FIGURE 2
Differential analysis of cytokines across groups. FC, Functional cure group (n = 29); HBeAg-, HBeAg-negative group (n = 40); HBeAg+, HBeAg-positive group (n = 37); HC, Healthy Control group (n = 29). *P < 0.05, **P < 0.01, ***P < 0.001.

showed markedly higher levels of IL-1 β , IL-8, IL-12p70, IFN- α , and IL-6/IL-10 ratio compared to patient groups, while exhibiting significantly lower concentrations of IL-17, TNF- α , IFN- γ , IL-4, and IL-10. Notably, the HBeAg-negative group displayed distinct cytokine patterns characterized by elevated IL-2 levels, reduced TNF- α and IL-10 concentrations, and lower IL-6 levels compared to both functional cure patients and healthy controls.

3.4 Correlation analysis between tryptophan metabolites and cytokine levels

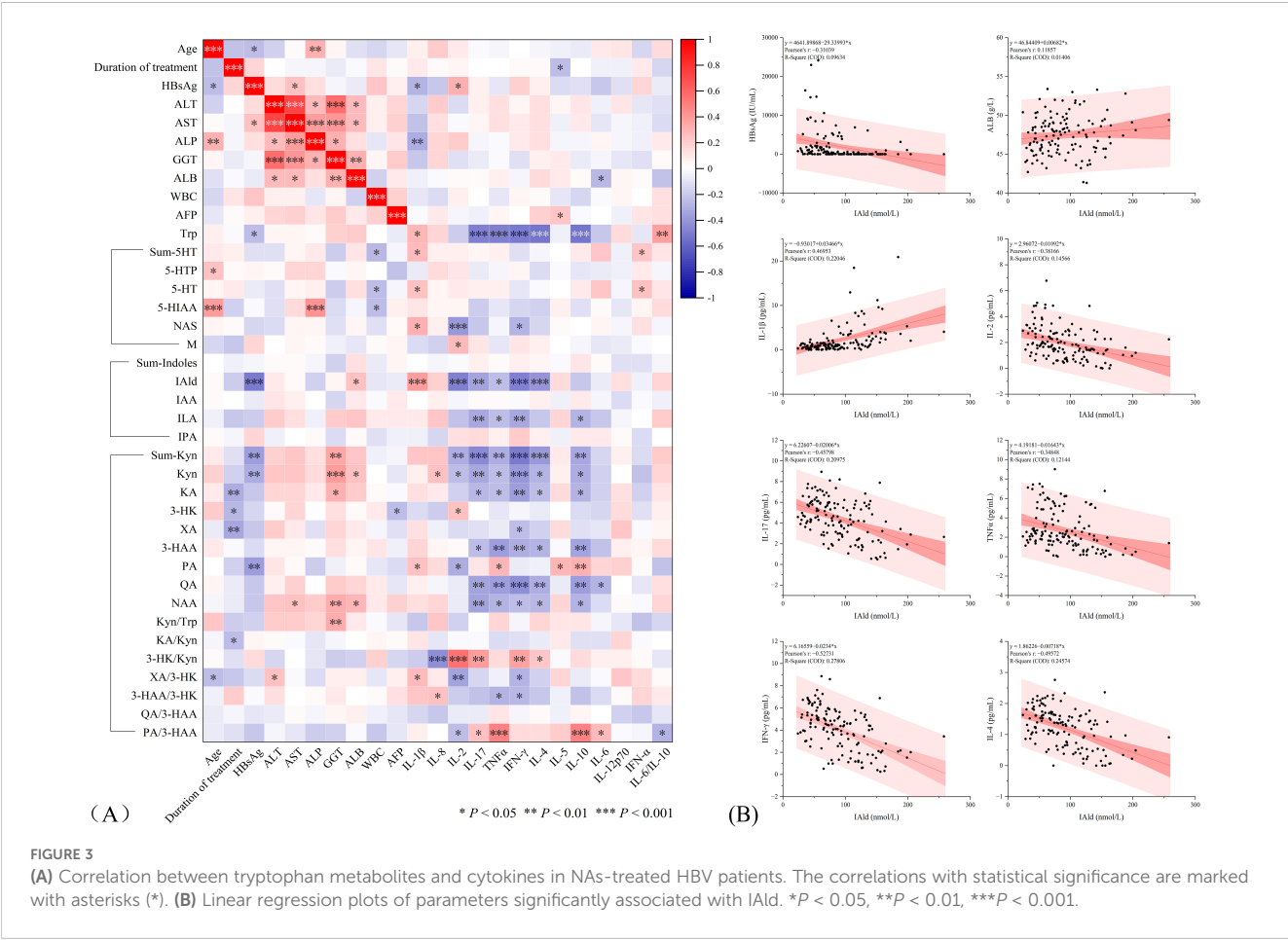
We examined correlations between patient basic information and important laboratory parameters with tryptophan metabolites and cytokines. The heatmap shows the strength of correlation between each indicator, with statistically significant correlations indicated by *P* values (Figure 3A). The results showed significant correlations between multiple tryptophan metabolites and cytokines. Overall, Trp, bacterial degradation pathway metabolites (IAld and ILA), and kynurenine pathway metabolites (Sum-Kyn, Kyn, KA, 3-HAA, QA, and NAA) were significantly negatively correlated with IL-2, IL-17, TNF- α , IFN- γ , IL-4, and IL-10. Sum-5-HT, 5-HT, and NAS in the 5-HT pathway were positively correlated with IL-1 β and IFN- α . As mentioned earlier, we observed that IAld exhibited a satisfactory gradient across all subgroups, and IAld also

showed a statistically significant correlation with HBsAg. The observed patterns indicate this substance could be clinically significant in treatment response. We therefore generated linear regression plots specifically for IAld (Figure 3B), revealing a weak positive correlation with ALB ($r = 0.11$), moderate positive correlation with IL-1 β ($r = 0.47$), weak negative correlation with HBsAg ($r = -0.31$), and the strongest negative correlation with IFN- γ ($r = -0.53$).

4 Discussion

In recent years, the role of the tryptophan metabolic pathway in disease regulation has garnered increasing attention (Chen et al., 2024), with research primarily focused on neuropsychiatric disorders, digestive diseases, immune regulation, and oncology, particularly investigating depression, inflammatory bowel disease, metabolic disorders, and malignancies (Correia and Vale, 2022; Davidson et al., 2022; Peyraud et al., 2022). However, its implications in viral infections, especially HBV infection, remain relatively underexplored.

This study conducted a retrospective analysis of 106 CHB patients who received NAs therapy for over 48 weeks and 29 healthy controls, aiming to explore the relationship between tryptophan metabolites and cytokines with HBV treatment



outcomes. The study found that multiple tryptophan metabolic pathways were closely associated with HBV treatment efficacy, particularly IALd in the bacterial degradation pathway, which showed a remarkable significant gradient correlation with treatment response and a negative correlation with HBsAg titer, a finding not previously reported in previous studies. Additionally, several metabolites in the 5-HT pathway and the kynurenine pathway also exhibited significant intergroup variations. These findings not only reveal the potential role of tryptophan metabolism in HBV treatment but also provide new perspectives and potential targets for future research and clinical applications.

In this study, the bacterial degradation pathway metabolite IALd was significantly higher in the cured group than in the non-cured group, and showed significant correlations with HBsAg levels, IFN- γ , IL-4, IL-1 β , IL-17, IL-2, and TNF- α , suggesting that IALd may play an important role in the immune regulation of HBV antigen clearance. The bacterial degradation pathway is mediated by the gut microbiota, with some commensal microbiota producing AhR ligands, which generate IAA and IPA through oxidative and reductive pathways, thereby influencing intestinal permeability and host immunity (Dodd et al., 2017). AhR is a ligand-dependent transcription factor activated by various synthetic or biomolecular compounds, playing a crucial role in immune responses and inflammation suppression (Stockinger et al., 2014). IALd has recently been identified as an AhR (Zelante et al., 2013), with studies demonstrating that microbiota-derived Trp metabolites like IALd play protective roles against mucosal candidiasis (Zelante et al., 2013). In dextran sodium sulfate (DSS)-induced mouse colitis, dietary Trp supplementation restores AhR ligands produced by the gut microbiota, thereby alleviating the severity of colitis (Islam et al., 2017). Similarly, in experimental autoimmune encephalomyelitis models, supplementation with Trp or Trp-derived AhR agonists enhanced IFN suppression and limit central nervous system (CNS) inflammation in an AhR-dependent mechanisms (Rothhammer et al., 2016). Recent studies have also found that IALd can ameliorate Coronavirus disease 2019 (COVID-19)-associated pulmonary aspergillosis (CAPA) during Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) and *A. fumigatus* co-infection by multiple mechanisms, including protecting the epithelial barrier, restoring virus-induced pro-inflammatory features by reducing IL-1 β production and increasing IL-10 to promote antiviral responses and limit viral replication, and IALd works better in a prophylactic rather than therapeutic protocol (Pariano et al., 2024). Unlike the aforementioned study, all disease groups in this study exhibited decreased IL-1 β and increased IL-10 levels, which is consistent with the conclusions of other studies. The studies have found that in CHB patients non-responsive to IFN- α therapy, IL-1 β levels may be significantly suppressed, possibly due to inhibition of NF- κ B signaling and activation of the inflammasome/caspase-1 pathway (Lei et al., 2019; Wang et al., 2019). Additional research has found that stimulation of HBV-infected cells with IL-1 β *in vitro* can rapidly reduce intracellular HBV RNA levels (Delphin et al., 2021). These studies suggest that HBV may maintain persistent infection by inhibiting the antiviral effects of IL-1 β , while IL-1 β may serve as a potential therapeutic candidate molecule against HBV. Based on these studies and the results of this study, we may also

hypothesize that supplementation or stimulation of IALd and IL-1 β could regulate the HBV immune response and promote the clearance of HBsAg, though the conclusions and potential mechanisms require further investigation.

Our study found that multiple 5-HT pathway metabolites were significantly lower in non-cured patients compared to both cured patients and healthy controls, suggesting a potential association with 5-HT's anti-inflammatory role in immune regulation. 5-HT, also known as serotonin, is produced in the central nervous system, gastrointestinal tract and platelets (Brenner et al., 2007). It functions as both a neurotransmitter and peripheral hormone, regulating diverse physiological and psychological processes including appetite, sleep, pain, and mood. Through binding to 5-HT receptors on immune cells, it also serves as an effective regulator of both the innate and adaptive immune systems. A recent study on post-acute sequelae of COVID-19 (PASC, or "Long COVID") found that PASC is associated with reduced serotonin levels, and other acute viral infections also cause a significant serotonin depletion. Although serotonin levels can rebound to baseline after acute infection resolution, chronic viral infections may cause persistent serotonin deficiency—consistent with our findings. The study also suggests that viral infections and type I interferon-driven inflammation reduce serotonin levels through diminished intestinal absorption of the serotonin precursor tryptophan, platelet overactivation and thrombocytopenia and monoamine oxidase (MAO)-mediated serotonin turnover (Wong et al., 2023). Similarly, Wu et al. also found that serotonin levels were significantly lower in decompensated CHB patients than in compensated CHB patients, and serotonin levels were lower in the HCC group than in the CHB group regardless of HBV DNA levels, suggesting serotonin may be a good prognostic marker (Wu et al., 2022). However, increasing evidence also suggests 5-HT's contradictory role in immunomodulatory, exerting both stimulatory and inhibitory activities (Mawe and Hoffman, 2013; Renga et al., 2023). Earlier studies have also suggested that serotonin acts as a chemokine to increase the secretion of pro-inflammatory cytokines (IL-1, IL-6, NF- κ B) and enhance phagocytosis (Kanova and Kohout, 2021). In patients with inflammatory bowel disease (IBD), 5-HT also increases Nicotinamide adenine dinucleotide phosphate (NADPH) -dependent reactive oxygen species (ROS) production and upregulates IL-6 and IL-8 (Regmi et al., 2014). And serotonin stored in platelets has been shown to supports viral persistence in the liver and exacerbates cytotoxic T lymphocyte (CTL) -mediated liver immunopathology in viral hepatitis (Lang et al., 2008). Although these studies suggest that 5-HT may have pro-inflammatory effects in certain inflammatory diseases, the causal relationship and specific mechanisms between 5-HT and treatment outcomes in HBV patients require further exploration.

IDO, which catalyzes the conversion of tryptophan to kynurenine, has emerged as a prominent research focus due to its dual immunoregulatory roles. This enzyme can be induced by pro-inflammatory cytokines including IFN- γ and IL-6 (Werner et al., 1987; Xie et al., 2023), promotes immune tolerance by T-cell suppression, and simultaneously inhibiting replication of intracellular pathogens such as human immunodeficiency virus

(HIV), herpes simplex virus (HSV), or cytomegalovirus (CMV) (Adams et al., 2004; Boasso and Shearer, 2007; Cassoux et al., 1999). Studies suggest IDO may exert anti-HBV effects through reciprocal activation of NK cells and pDCs, mediated by IFN- γ and IFN- α release. In patients with acute hepatitis B, early strong activation of IDO is a marker of successful clearance of HBV (Yoshio et al., 2016). Mao et al. also found that IFN- γ -induced IDO can inhibit HBV replication in HepG2 cells transfected with the HBV genome (Mao et al., 2011). Our findings of elevated Kyn/Trp ratios in cured patients align with previous reports, though we observed particularly high levels in HBeAg-positive group. Through a comprehensive literature review, we identified that Kyn, produced by IDO1 through the metabolism of Trp, serves as an endogenous ligand for the AhR. Binding of Kyn to AhR induces the differentiation of immature CD4⁺ T cells into regulatory T cells. Additionally, Kyn binding to AhR can also induce IDO1 expression, further suppressing T cell immune responses (Proietti et al., 2020). Previous studies have also suggested that kynurenine produced by IDO exacerbates liver damage in HBV specific CTL-induced fulminant hepatitis (Ohtaki et al., 2014). Similar inhibitory effects have been observed in HIV, the serum kynurenine-to-tryptophan ratio in HIV-infected patients increases with disease progression and immune stimulation (Huengsborg et al., 1998). Additionally, studies have shown that the proportion of granulocytic myeloid-derived suppressor cells (gMDSCs) in peripheral blood and liver tissue of HBV-ACLF patients significantly increases and inhibits T cell proliferation and IFN- γ production via IDO/IL-10 pathway (Yu et al., 2022). HBeAg-positive patients exhibit higher circulating MDSC frequencies than HBeAg-negative individuals, as HBeAg induce the expansion of MDSCs by upregulating IDO, thereby impairing T-cell function and maintaining HBV persistent infection, which is consistent with our findings (Yang et al., 2019). Increased IDO activity directly or indirectly influences the production of various cytokines, including IL-10, IFN- γ , and TNF- α , thereby participating in the regulation of inflammatory responses and immune tolerance (de Araújo et al., 2017; Huang et al., 2020). IL-10, as a potent anti-inflammatory cytokine, may also suppress the activity of Thelper type 1 (Th1) cells, Natural Killer (NK) cells, and macrophages to impede pathogen clearance (Couper et al., 2008). While our data show no significant correlation between Kyn/Trp and cytokines like TNF- α , IL-10, and IL-6, their coordinated directional changes suggest IDO may influence multiple cytokine networks in HBeAg-positive patients, potentially hindering HBeAg clearance. These findings collectively position IDO as a double-edged sword in HBV-related inflammation (Munn and Mellor, 2013; Xu et al., 2008), warranting cautious consideration of IDO as a potential therapeutic target for HBV infection.

In the kynurenine pathway, we also found that 3-HK/Kyn ratios (reflecting KMO activity), was elevated in non-cured groups, while downstream metabolites including KA (KAT product), XA/3-HK (KAT II activity), 3-HAA/3-HK (KYNU activity), and the terminal metabolite NAA were all reduced across patient groups. These findings suggest a potential metabolic blockade downstream of 3-HK in nucleos(t)ide analogue-treated HBV patients, particularly those failing to achieve functional cure. This metabolic profile is

similar to the tryptophan metabolite patterns observed by Moulin et al. in rheumatoid arthritis (Moulin et al., 2024), while the use of exogenous catalysts conversion of Kyn and 3-HK to produce XA and KA via the recombinant enzyme aminoadipate aminotransferase (AADAT) directly regulates endogenous tryptophan metabolism through AhR activation and cellular metabolic reprogramming, thereby reducing the severity of inflammatory bowel disease and rheumatoid arthritis (Michaudel et al., 2023; Moulin et al., 2024). Therefore, regulating the kynurenine pathway may also become a new therapeutic strategy for HBV infection.

There were also some interesting findings in this study. The PA concentration and PA/3-HAA ratio were exclusively elevated in the cured group, showing significant increases compared to both non-cured patients and healthy controls. And these measurements demonstrated negative correlations with HBsAg and IL-2 levels, while exhibiting positive correlations with cytokines including IL-1 β , IL-5, IL-10, and TNF- α , potentially reflecting unique regulatory mechanisms in the terminal stages of tryptophan metabolism among cured individuals. PA is considered a naturally toxic pyridine derivative and an important intermediate widely used in the chemical industry (Shi et al., 2024; Xu et al., 2022; Yan et al., 2023). In the field of biological research, picolinic acid and its derivatives have been shown to possess antibacterial and antifungal properties (Tamer et al., 2018). Research indicates PA may be involved in macrophage activation in the human body (Melillo et al., 1996), and exhibit neuroprotective and osteoprotective effects (Duque et al., 2020; Knapskog et al., 2023). Notably, recent studies have also revealed PA's broad-spectrum antiviral activity against enveloped viruses - including SARS-CoV-2, influenza A virus (IAV), flaviviruses, HSV, and paramyxoviruses - through mechanisms involving viral membrane integrity disruption, fusion inhibition of virus-cell membrane, and cellular endocytosis interference, while it is ineffective against non-enveloped viruses and bacteriophages. Therefore, PA is also an important target for broad-spectrum antiviral drugs (Narayan et al., 2023). However, high-dose PA (≥ 500 mg/kg/d) may induce neurovascular toxicity (Knapskog et al., 2023). We reasonably hypothesize that elevated PA levels in cured group may contribute significantly to HBV clearance. Whether PA also exhibits antiviral properties in the enveloped virus HBV is a question worthy of further exploration, which also provides a new perspective on PA as a therapeutic target for HBV.

This study still has some limitations. First, the relatively small sample size may have affected the statistical power of our findings. Second, none of the participants in this study had received interferon therapy. With the WHO's strategy to eliminate hepatitis B as a public health threat by 2030 in 2016 (World Health Organization, 2016), the application of interferon therapy in the treatment of HBV infection has gradually expanded. The complex regulatory effects of IFN- α on tryptophan metabolism further underscore this limitation. Additionally, while this study did not delve into the specific mechanisms of tryptophan metabolic pathway in HBV treatment regulation, our preliminary findings may provide direction for future research. Subsequent studies

should incorporate larger cohorts including interferon-treated patients to validate the role of tryptophan metabolism across different therapeutic regimens. Furthermore, in-depth research into the interaction mechanisms between the tryptophan metabolic pathway and the immune system, along with clinical prospective studies, such as the specific role of IAld in HBV immune regulation, could help elucidate the potential application of tryptophan metabolites as biomarkers or therapeutic targets in HBV therapy or for identifying patient populations with higher likelihood of functional cure.

In summary, this study represents the first systematic analysis of tryptophan metabolic pathway alterations in chronic HBV infection, establishing a correlation map across tryptophan metabolic profiles, cytokines and HBV functional cure under NAS therapy. The findings revealed that functional cure patients exhibited distinct metabolic characteristics: IAld levels showed negative correlations with HBsAg titer and pro-inflammatory cytokine IFN- γ , while demonstrating positive correlations with pro-inflammatory cytokine IL-1 β . Elevated PA levels were associated with decreased IL-2 and increased IL-1 β and IL-10, and higher 5-HT levels were accompanied by increased IL-1 β and IFN- α . These results not only uncover the coordinated regulatory network between tryptophan metabolites (IAld, PA, 5-HT, etc.) and key cytokines (IL-1 β , IL-10, IFN- γ , etc.), providing new perspectives for immunometabolic research in viral infections, but also establish a theoretical foundation for developing biomarkers for HBV treatment by identifying characteristic association patterns between metabolites and cytokines, offering important insights for designing therapeutic strategies targeting immunometabolic pathways. Targeting Trp metabolites represents a novel and promising strategy (Xue et al., 2023). The strong associations of IAld and PA with functional cure, alongside their established immunomodulatory roles in viral infections, suggest these metabolites as candidate therapeutic nodes. A comprehensive investigation of the role and regulatory mechanisms of Trp metabolites and immune in HBV treatment is warranted to facilitate their clinical application.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

Ethics statement

The studies involving humans were approved by Ethics Committee of West China Hospital of Sichuan University. The studies were conducted in accordance with the local legislation and institutional requirements. The samples used in this study were residual blood samples from clinical testing, and informed consent

from patients was waived by the Ethics Committee of West China Hospital of Sichuan University.

Author contributions

XG: Formal Analysis, Writing – original draft, Data curation. XW: Data curation, Writing – review & editing, Formal Analysis. YL: Data curation, Writing – review & editing. XD: Writing – review & editing, Methodology, Visualization. BC: Conceptualization, Writing – review & editing, Funding acquisition.

Funding

The author(s) declare that financial support was received for the research and/or publication of this article. This work was funded by grants from the National Natural Science Foundation (No. 81702002), Sichuan Science and Technology Program (2020YFS0137) and Qimingxing Research Fund for Young Talents of West China Hospital (HXQMX0007).

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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The author(s) declare that no Generative AI was used in the creation of this manuscript.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fcimb.2025.1643636/full#supplementary-material>

SUPPLEMENTARY FIGURE 1
Tryptophan metabolic pathway.

SUPPLEMENTARY FIGURE 2

Principal component analysis of tryptophan metabolites and cytokines.

SUPPLEMENTARY FIGURE 3

Principal component analysis of tryptophan metabolites and cytokines across different groups.

SUPPLEMENTARY TABLE 1

Comparison of clinical laboratory indicators between functional cure group and non-cured groups.

SUPPLEMENTARY TABLE 2

The levels of tryptophan metabolites and cytokines in each group.

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OPEN ACCESS

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RECEIVED 28 March 2025

ACCEPTED 20 June 2025

PUBLISHED 29 July 2025

CITATION

Gandhi P, Peter K, Varshney S, Atlani MK and Maudar KK (2025) Detecting early kidney injury due to host–virus interaction response in treatment-naïve CHB—a pilot study. *Front. Cell. Infect. Microbiol.* 15:1601678. doi: 10.3389/fcimb.2025.1601678

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Detecting early kidney injury due to host–virus interaction response in treatment-naïve CHB—a pilot study

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Purpose: Clinical evidence suggests that patients with chronic hepatitis B (CHB) have an increased risk of renal impairment due to inflammation induced by virus–host interaction. We aimed to evaluate and validate a set of protein biomarkers singularly and in combination for the early detection of subclinical kidney injury in patients with CHB naïve to antiretroviral therapy.

Methods: This work is part of a prospective cross-sectional study for which 69 HBsAg-positive, treatment-naïve patients with CHB with an equal number of age-matched healthy volunteers were considered. At diagnosis, serum creatinine (sCr), urea, alanine transaminase, aspartate transaminase, serum cystatin-C (sCys-C), serum neutrophil gelatinase-associated lipocalin (sNGAL), serum Fetuin-A (sFet-A), urinary interleukin-18 binding protein (uIL-18BP), and urinary kidney injury molecule-1 (uKIM-1) levels were determined.

Results: There was a significant elevation in the concentrations of three proteins in our CHB cohort (sCys-C, sNGAL, and uIL-18BP; $p < 0.0001$) while sFet-A was down-regulated ($p < 0.01$) as compared to the control group. A receiver operating characteristic curve analysis revealed an Area under the curve of 0.935 for sCys-C and 0.811 for sNGAL, which improved to 0.984 when all four indicators were combined in a panel to discriminate the onset of renal injury incited by inflammatory response in CHB with 97.1% sensitivity at 91.3% specificity. Additionally, only sCys-C and sNGAL differed significantly among the phases of CHB infection ($p < 0.05$).

Conclusions: This novel noninvasive diagnostic screen is expedient in detecting inflammation and early kidney injury before a rise in sCr and can aid in predicting renal outcomes in patients with CHB.

KEYWORDS

biomarker, kidney injury, hepatitis B virus, chronic hepatitis B, inflammation

Introduction

Hepatitis B infection has become a global health concern due to its adverse outcomes, including liver cirrhosis, hepatocellular carcinoma, and renal impairment. According to the latest World Health Organization (WHO) estimates, approximately 1.1 million people died due to hepatitis B-related complications in 2022 (World Health Organization, 2024). India presented the second highest viral hepatitis cases, which account for 11.7% of the total global burden. Also, India, together with China and Indonesia, showed 50% of the global viral hepatitis load (World Health Organization, 2024).

Chronic hepatitis B virus (HBV) infection is a major etiological factor in an array of renal diseases, with co-existent clinical comorbidities, which altogether leads to a worse prognosis (Liu et al., 2025; Cacoub and Asselah, 2022). The underlying mechanism of HBV evasion remains controversial, but different groups suggest that the virus dodges recognition and escapes the host's innate and adaptive immune responses (Zhao et al., 2022; Kuiper et al., 2020). HBV mediates kidney damage in the host via immune-complex deposition in the glomerulus, activating complement-mediated inflammatory response leading to apoptosis in tubular epithelial cells (Yu et al., 2022). The ability to improve clinical outcomes in chronic hepatitis B (CHB) is hindered by insufficient lab support to diagnose renal involvement at a reversible stage early in the disease process. To date, numerous biochemical markers have been assessed in cohorts with defined extrahepatic kidney ailments. They have changed our outlook towards serum creatinine (sCr) and serum urea (sUr), as these conventional parameters take weeks to escalate after a renal insult, especially in cases where the primary diagnosis is a virus-induced liver ailment. The current developments in molecular proteomics have greatly augmented the discovery of novel noninvasively evaluated markers that promptly fluctuate in body fluids in response to viral stimuli.

Kidney diseases are multifactorial and heterogeneous in origin; therefore, it is likely that not one single biomarker but a panel of biomarkers representing all aspects of renal involvement may better detect kidney injury during early disease onset. Cystatin-C (Cys-C; 13 kDa) is an inhibitor of the cysteine protease and a marker of renal function. It is produced by nucleated cells and catabolized by tubular epithelial cells (Fiseha, 2015). Circulating neutrophil gelatinase-associated lipocalin (NGAL; 25 kDa), a protein predominantly associated with human neutrophil gelatinase, is a promising early diagnostic biomarker for acute kidney injury (AKI) (Romejko et al., 2023). KIM-1 is a type I transmembrane glycoprotein (90 kDa), a marker of proximal tubular injury. It is undetectable in healthy kidney tissue but upregulated in various human kidney diseases (Anandkumar et al., 2023). Fet-A is a negative acute-phase reactant glycoprotein (64 kDa) exclusively synthesized by the liver and is downregulated in systemic inflammation (Ricken et al., 2022). IL-18BP is an anti-inflammatory secretory protein with a high affinity for IL-18 (a marker for renal damage), which inhibits the production of

interferon- γ by neutralizing IL-18 (Hirooka and Nozaki, 2021). In particular, NGAL, Fetuin-A, and IL-18BP are known to induce the expression of pro-inflammatory molecules and activation of various cytokine cascades (Hennige et al., 2008; Jung and Ryu, 2023; Wang, 2024) as presented in Figure 1.

Therefore, the purpose of the current investigation was to evaluate the diagnostic potential of the above-described renal injury markers singularly and in combination for the detection of hepatitis B-associated inflammation and very early kidney injury in treatment-naïve patients with CHB in various phases of HBV infection.

Materials and methods

This work is a part of a prospective cross-sectional single-center study. All patients visiting outpatient or inpatient wards of the Department of Gastrointestinal Medicine and Surgical Gastroenterology during the tenure of the study, with complaints like flatulence, fatigue, indigestion, nausea, edema, and a metallic taste in the mouth, were tested for HBsAg positivity. Among these, 109 were found to be HBsAg-positive. Furthermore, 71 patients were confirmed as treatment-naïve HBsAg-positive and were considered for this study. The CHB cohort was defined as serum HBsAg positivity for more than 6 months, presence of HBV-DNA, absence of anti-HBc IgM, HCV co-infection, and any associated liver disease. Two patients were excluded due to the serological presence of anti-HBc IgM. A total of 69 age-matched healthy volunteers were considered to determine the normal levels of the biomarkers. Patients with CHB plus HCC, diabetes, or intrinsic renal disease were excluded.

Sample collection and conventional marker analysis

Overnight fasting blood by venipuncture and mid-stream urine specimens were collected from all participants. The serum was separated from blood samples within 30 min of collection by centrifuging at 3,000 rpm for 15 min. Urine samples were centrifuged at 2,500 rpm for 10 min. Each sample was coded, aliquoted, and stored at -80°C until used for biomarker determination. HBsAg testing was performed using qualitative enzyme-linked immunosorbent assay (ELISA) (Diasorin S.p.A., Saluggia, Vercelli, Italy). At the same time, anti-HBc IgM, HBeAg, and anti-HBeAg were checked using ELISA kits according to the manufacturer's instructions (Immucheck, Genomix Molecular Diagnostics Pvt. Ltd, India).

HBV DNA quantitation was performed using FRET probes, HBV core-region-specific primers, quantitative standards, and internal controls on real-time PCR (LightCycler 2.0, Roche Diagnostics, Germany; minimum detection limit 10 copies/mL) and recorded from the lab log (Punde et al., 2011).

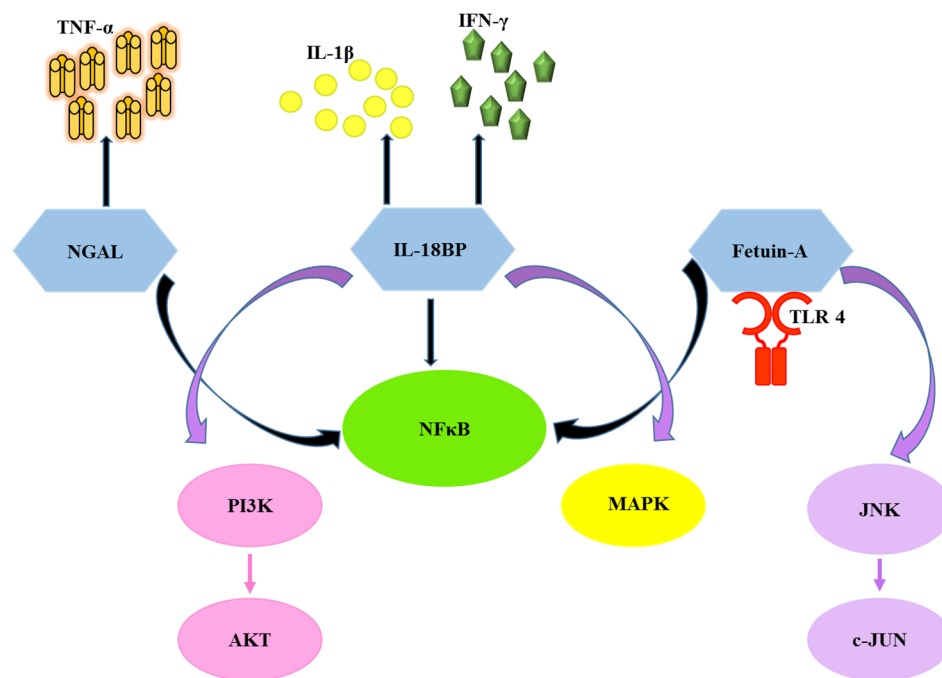


FIGURE 1

Diagram illustrates the activation of pro-inflammatory cytokines and related pathways by the chosen biomarkers. NGAL, neutrophil gelatinase associated lipocalin; Fet-A, fetuin-A; IL-18BP, interleukin-18 binding protein; TLR4, toll-like receptor 4; NFκB, nuclear factor kappa-light-chain-enhancer of activated B cells; JNK, c-Jun N-terminal kinase; c-JUN, c-Jun proto-oncogene; MAPK, mitogen-activated protein kinase; PI3K, phosphatidylinositol 3-kinase; AKT, protein kinase B (PKB); IL-1β, interleukin-1 beta; IFN-γ, interferon gamma; TNF-α, tumor necrosis factor alpha.

Serum ALT and AST (Reitman and Frankel, 1957), sCr (Jaffe, 1886), and sUr (Rosenthal, 1955) were evaluated by the enzymatic colorimetric method. The estimated glomerulus filtration rate (eGFR) was calculated using the MDRD equation (modification of diet in renal disease) in mL/min per 1.73 m² (Levey et al., 1999). $eGFR = 186 \times (sCr)^{-1.154} \times (\text{age})^{-0.203} \times 0.742$ (if the subject is female). Categorization of the patients with CHB was done in accordance with EASL guidelines, 2017 (European Association for the Study of the Liver, 2017), based on HBeAg status, ALT levels, and viral load.

1. HBeAg-positive chronic HBV infection (EPI), previously referred to as immune-tolerant—nine cases with viral load $>10^7$ IU/mL and normal ALT.
2. HBeAg-positive CHB (EPB), previously called immune clearance—11 cases with viral load 10^4 – 10^7 IU/mL, moderate to elevated ALT.
3. HBeAg-negative chronic HBV infection (ENI), previously termed as inactive carrier—25 HBeAg-negative and anti-HBe +ve patients with viral titer $\leq 2 \times 10^3$ IU/mL, normal ALT.
4. HBeAg-negative CHB (ENB), previously termed as pre-core mutant—19 HBeAg-negative and anti-HBe positive patients with viral load $>2 \times 10^3$ IU/mL, moderate to high ALT.

*(Five HBeAg and anti-HBe negative cases were taken to be in the early seroconversion phase and omitted from analysis).

Biomarker panel for assessing renal dysfunction

Serum levels of Cys-C, NGAL, serum Fetuin-A (sFet-A), and urine content of KIM-1 were analyzed using commercially available ELISAs (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Also, urine levels of IL-18BP were determined using the Raybiotech ELISA kit as per insert (Norcross, GA, USA). The reference range for sCys-C, sNGAL, sFet-A, urinary kidney injury molecule-1 (uKIM-1), and urinary interleukin-18 binding protein (uIL-18BP) was 0–100, 0–10, 0–500, 0–10, and 0–6,000 ng/mL, respectively. The mean minimum detectable dose for each biomarker measured was 0.102 ng/mL for sCys-C, 0.012 ng/mL for sNGAL, 0.062 ng/mL for sFet-A, 0.009 ng/mL for uKIM-1, and less than 20 pg/mL for uIL-18BP. Calibration curves were generated with standards provided for each biomarker using Microplate Manager software 6.1, on iMark, Microplate Reader, and Bio-Rad, and were fit by four-parameter logistic regression.

Statistical analysis

Descriptive statistics were reported as the median (range) for continuous variables. The analyses were performed using GraphPad Prism software 9.0. Since the data were non-normally distributed, differences between two groups and more than two groups were analyzed using the Mann–Whitney *U* and Kruskal–Wallis tests, respectively. Bivariate correlation analysis was assessed using Spearman’s nonparametric rank correlation coefficients. The receiver operating characteristic (ROC) curve analysis was used to determine the predictive and diagnostic value of the biomarkers. The interactive web tool CombiROC was employed to analyze the diagnostic performance of candidate biomarker panels for the best combinations (Mazzara et al., 2017). Statistical significance was defined by a two-tailed value of $p < 0.05$.

Ethics statement

The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki (6th revision, 2008). The research procedures were reviewed and approved by the institutional ethics committee (IRB/14/Res/10). Written informed consent was

obtained from all participants before inclusion. Permission for publication was received from DG-ICMR.

Results

Cohort characteristics

The final analysis for the study was performed on 69 treatment-naïve patients with CHB (48 men and 21 women) with a median age of 36 years (19–68) and 69 age-matched healthy individuals (40 men and 29 women). The baseline characteristics of the studied subjects are summarized in Table 1. No statistically significant differences between the subjects and controls were observed for conventional renal parameters, sCr and sUr, indicating that they cannot assess the early onset of kidney injury in patients with CHB naïve to antiretroviral therapy, thus justifying the selection of our kidney injury marker panel.

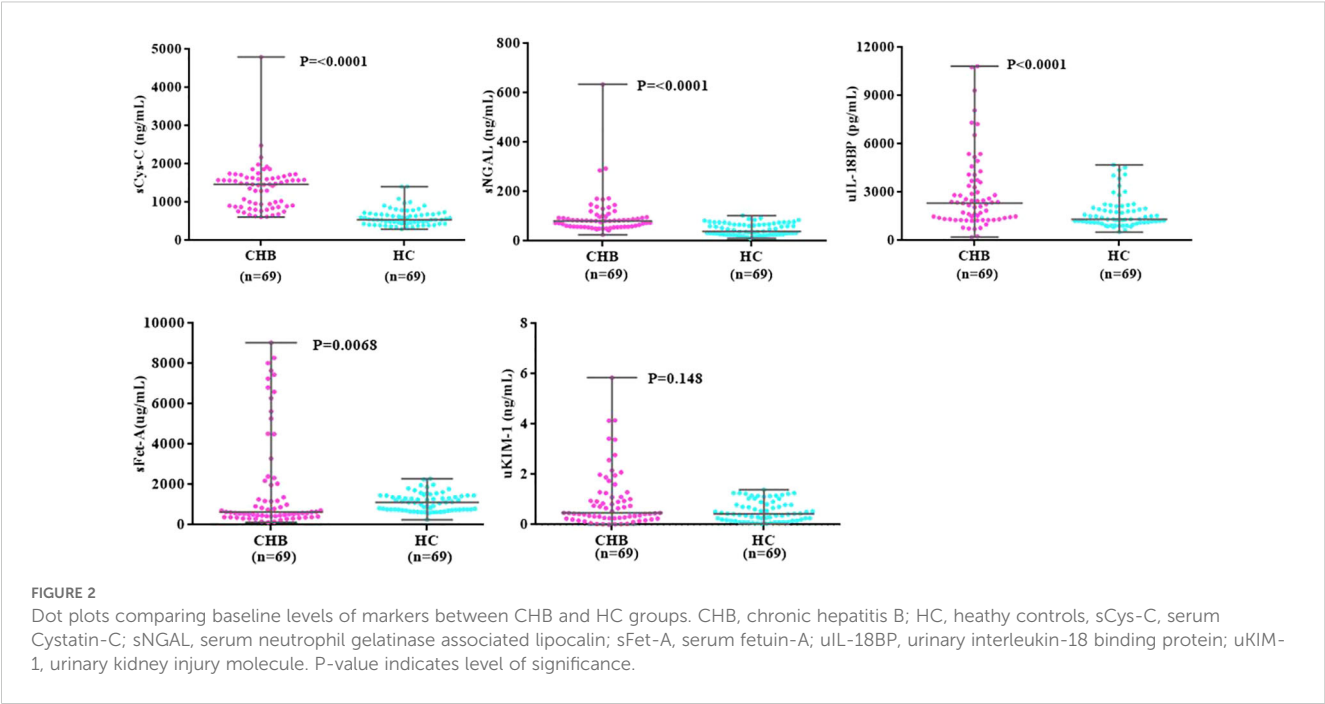
Markers at baseline

As shown in Table 1; Figure 2, the Mann–Whitney test established the baseline levels of sCys-C, sNGAL, and uIL-18BP

TABLE 1 Baseline clinical and biochemical characteristics of subjects under investigation.

Parameters	Cohort	Therapy-naïve CHB ($n = 69$)	HC ($n = 69$)	p -value
Median age (range)		36 (19–68)	35 (19–68)	
Male: Female ratio		48:21	40:29	
BMI (kg/m^2)		22.17 (16.13–30.14)	22.71 (16.4–28.38)	
HbsAg		Positive	–	
Anti-HBcIgM		Negative	–	
HBeAg		20 positive/49 negative	–	
Anti-HBeAg		63 positive/6 negative	–	
Hypertension		6	–	
ALT (normal range: ≤ 49 IU/L)		38.5 (17–139)	27 (10–48)	$<0.0001^{****}$
HBV DNA load $\log_{10}$ (IU/mL)		4.1 (0.52–9.43)	–	–
sCr (mg/dL) (normal range: 0.6–1.4 mg/dL)		0.93 (0.4–1.47)	0.83 (0.58–1.24)	0.56
eGFR $\text{mL}/\text{min}/1.73 \text{ m}^2$ (normal range: ≥ 90 $\text{mL}/\text{min}/1.73 \text{ m}^2$)		90.87 (65.42–145.41)	90.75 (85.31–157.66)	0.54
sUr (mg/dL) (normal range: 10–50 mg/dL)		21 (11–50)	20 (10–38)	0.23
sCys-C (ng/mL)		1,468 (614.6–4,801)	549.1 (297.3–1,409)	$<0.0001^{****}$
sNGAL (ng/mL)		81.9 (25.64–635)	39.85 (11.78–103.7)	$<0.0001^{****}$
uIL-18BP (pg/mL)		2,329 (218.5–10,832)	1,326 (536.4–4,700)	$<0.0001^{****}$
sFetuin A ($\mu\text{g}/\text{mL}$)		642.2 (130.3–9,046)	1,121 (256.6–2,297)	$<0.01^{**}$
uKIM-1 (ng/mL)		0.468 (0.006–5.85)	0.436 (0.035–1.4)	0.148

Data expressed as median (range). CHB, chronic hepatitis B; healthy controls, HC; BMI, body mass index; HbsAg, hepatitis B surface antigen; anti-HBcIgM, IgM antibody to hepatitis B core antigen; HBeAg, hepatitis B e-antigen; anti-HBeAg, IgM antibody to hepatitis B e-antigen; eGFR, estimated glomerulus filtration rate; sCr, serum creatinine; sUr, serum urea; sCys-C, serum cystatin-C; sNGAL, serum neutrophil gelatinase-associated lipocalin; sFet-A, serum Fetuin-A; uIL-18BP, urinary interleukin-18 binding protein; uKIM-1, urinary kidney injury molecule-1. $p < 0.01^{**}$, $p < 0.0001^{****}$.



to be significantly upregulated in the CHB group compared to the healthy controls ($p < 0.0001$, $p < 0.0001$, and $p = 0.0001$, respectively). At the same time, sFet-A was found to be significantly downregulated ($p = 0.0068$). No significant differences were detected in baseline values of uKIM-1 between the analyzed groups.

Next, we deduced the association of sCys, sNGAL, sFet-A, and uIL-18BP in terms of other markers in the panel with conventional renal parameters and viral load using the Spearman rank correlation test. sCys-C showed a very weak positive correlation with uKIM-1 ($r = 0.2607$, $p = 0.0305$) and sCr levels ($r = 0.24$, $p = 0.047$). No correlations emerged for other clinical parameters and kidney biomarkers.

Sensitivity analyses

ROC analysis was applied to define the diagnostic performance of sCys C, sNGAL, sFet-A, and uIL-18BP using optimal cutoff points to delineate early kidney injury in patients with CHB

(Table 2; Figure 3a). sCys-C turned out to be the most robust and precise stand-alone indicator, as evident from an AUROC of 0.935 (95% CI, 0.8867–0.9632). At an optimal cutoff value of ≥ 729.98 ng/mL, the sensitivity, specificity, and accuracy were 91.3%, 79.7%, and 85.51%, respectively, indicating accurate differentiation. Next in the line was sNGAL with an AUC of 0.81, 95% CI, 0.7281–0.8704), sensitivity of 95.59% at 56.52% specificity, and accuracy of 75.91%.

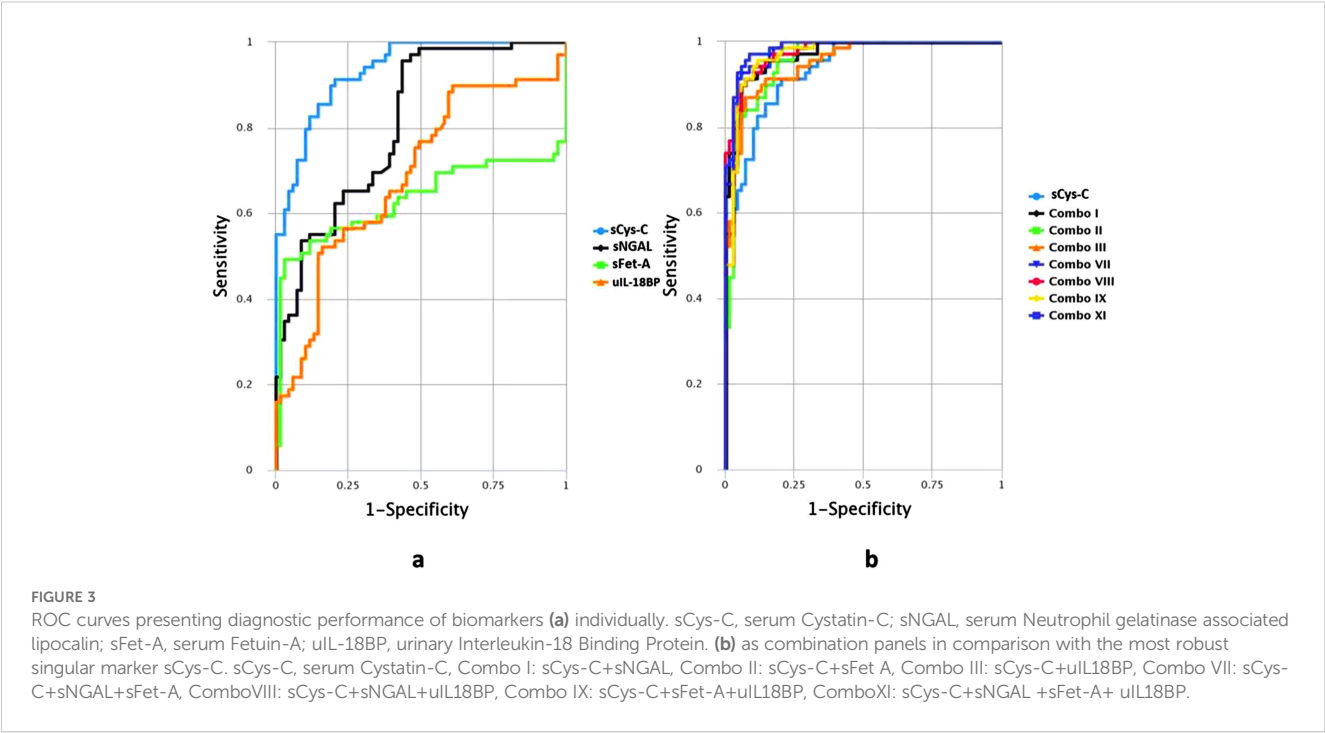
Biomarker panel analysis

Subsequently, the performance of the above biomarkers together as a panel was evaluated for diagnosing early renal impairment with greater sensitivity and specificity in patients with CHB asymptomatic for any kidney dysfunction. Potential candidate markers were combined in various combinations (two to four proteins) to serve as a panel. Among the 11 possible combinations, 7 outperformed the stand-alone single marker sCys-C in distinguishing HBV-induced kidney dysfunction. The best panel, composed of four proteins (combo XI), presented an

TABLE 2 Receiver operating characteristics and cutoff values of biomarkers for detecting presence of kidney injury in CHB.

Parameter	AUC (95% CI)	<i>p</i> -value	Youden index	Cutoffs	Sensitivity	Specificity	Accuracy
sCys-C (ng/mL)	0.935 (0.8867–0.9632)	<0.0001	0.7101	≥ 729.98	91.30%	79.7%	85.51%
sNGAL (ng/mL)	0.810 (0.7281–0.8704)	<0.0001	0.5211	≥ 50.10	95.59%	56.52%	75.91%
sFet-A (μg/mL)	0.633 (0.5177–0.7255)	0.0060	0.4638	≤ 617.89	49.28%	97.1%	73.19%
uIL-18BP (pg/mL)	0.689 (0.5890–0.7686)	0.0001	0.3623	$\geq 2,329.16$	50.2%	85.5%	66.89%

*The optimal cutoff value determined by Youden’s index. sCys-C, serum cystatin-C; sNGAL, serum neutrophil gelatinase-associated lipocalin; sFet-A, serum Fetuin-A; uIL-18BP, urinary interleukin-18 binding protein; AUC, area under the curve.



AUC value of 0.984 and 97.1% sensitivity at 91.3% specificity. It was followed by combo VII consisting of trio sCys-C+ sNGAL+ sFet-A (AUC=0.980) (Table 3 and Figure 3b).

Evaluation of different phases of CHB infection

To assess the interplay of viral replication and host immune response in different states of HBV infection within the CHB cohort, detailed analyses between HBeAg-positive and HBeAg-negative subjects were conducted for our markers, but no statistical difference was

recorded. However, among various phases of chronic HBV infection (EPI, EPB, ENI, and ENB), a moderately significant difference ($p = 0.048$) was found only in levels of sCys-C as an indicator of kidney dysfunction (Table 4), signifying that all HBV infection phases are vulnerable to kidney damage irrespective of replication status. On combining both HBeAg-positive groups (EPI and EPB) as EP, sNGAL levels were seen to differ significantly among EP, ENI, and ENB groups ($p = 0.039$), pointing to an alteration in inflammation status among the patients with active viral replication, carriers, and pre-core mutants. This result indicated a viral infection-initiated host immune response, which may have manifested as inflammation leading to kidney injury during the HBV course of infection.

TABLE 3 Area under the curve for receiver operating characteristics of various combinations for predicting kidney injury in patients with CHB.

Combinations	Markers	AUC	Sensitivity	Specificity
Combo I	sCys C + sNGAL	0.970	0.913	0.928
Combo II	sCys C + sFet A	0.957	0.826	0.957
Combo III	sCys C + uIL-18BP	0.951	0.870	0.928
Combo IV	sNGAL + sFet A	0.813	0.957	0.565
Combo V	sNGAL + IL-18BP	0.823	0.971	0.551
Combo VI	sFet A + IL-18BP	0.689	0.522	0.855
Combo VII	sCys C + sNGAL + sFet A	0.980	0.928	0.957
Combo VIII	sCys C + sNGAL + uIL-18BP	0.976	0.913	0.928
Combo IX	sCys C +sFet A + uIL-18BP	0.969	0.957	0.884
Combo X	sCys C + sFet A + uIL-18BP	0.825	0.971	0.551
Combo XI	sCys C + sNGAL + sFet A + uIL-18BP	0.984	0.971	0.913

sCys-C, serum cystatin-C; sNGAL, serum neutrophil gelatinase-associated lipocalin; sFet-A, serum Fetuin-A; uIL-18BP, urinary interleukin-18 binding protein; AUC, area under the curve.

TABLE 4 Clinical and biochemical characteristics of patients in various phases of HBV infection.

Parameters	Phases	EPI (<i>n</i> = 9)	EPB (<i>n</i> = 11)	ENB (<i>n</i> = 25)	ENI (<i>n</i> = 19)	<i>p</i> -value
Median age (range)		47 (19–74)	49 (23–60)	35 (19–71)	36 (19–67)	
Male: Female ratio		7:2	10:1	18:7	10:9	
BMI (kg/m ²)		22.58 (18.50–27.754)	22.77 (17.98–29.29)	21.35 (16.13–29.29)	22.18 (16.60–30.14)	
HbsAg		Positive	Positive	Positive	Positive	
Anti-HBcIgM		Negative	Negative	Negative	Negative	
HbeAg		Positive	Positive	Negative	Negative	
Anti-HbeAg		Negative	Negative	Positive	Positive	
HBV DNA load log10 (IU/mL)		8.034 (7.2–9.43)	6.22 (4.63–7.0)	4.1 (3.5–7.75)	2.6 (0.52–3.3)	<0.0001****
ALT (normal range: ≤49 IU/L)		26 (17–44)	51 (39–139)	49 (31–130)	22 (16–45)	<0.0001****
sCr (mg/dL) (normal range: 0.6–1.4 mg/dL)		1.12 (0.51–1.46)	0.89 (0.54–1.2)	0.96 (0.5–1.47)	0.91 (0.5–1.45)	0.24
eGFR mL/min/1.73 m ² (normal range: ≥90 mL/min/1.73 m ²)		91.8 (65.45–128.89)	93.67 (87.05–106.89)	92.34 (63.38–121.77)	90.22 (71.27–127.73)	0.71
sUr (mg/dL) (normal range: 10–50 mg/dL)		2.72 (0.03–11.66)	21 (18–48)	22 (17–50)	19 (11–28)	0.102
sCys-C (ng/mL)		1,579.31 (754.65–2,175.93)	1,081.31 (624.8–1,752.08)	1,296.64 (614.55–1,930.34)	1,572.62 (771.73–4,800.57)	<0.05*
sNGAL (ng/mL)		83.363 (43.10–635.05)	81.39 (50.76–340)	70.25 (48.01–148.45)	96.74 (51.087–286.17)	0.65
uIL-18BP (pg/mL)		3,715.7 (263.739–10,832)	1,458.33 (218.48–10,772.3)	2,365.14 (724.86–6,557.78)	2,409.73 (753.28–9,328.57)	0.83
sFetuin A (μg/mL)		753.18 (416.02–6,288.05)	474.745 (130.34–6,606.62)	642.229 (294.87–9,045.87)	617.89 (309.07–8,289.09)	0.23
uKIM-1 (ng/mL)		0.425 (0.02–4.136)	0.7 (0.016–5.85)	0.54 (0.03–3.43)	0.46 (0.006–2.77)	0.4

Data expressed as median (range); EPI, HBeAg-positive chronic HBV infection; EPB, HBeAg-positive chronic hepatitis B; ENI, HBeAg-negative chronic HBV infection; ENB HBeAg-negative chronic hepatitis B; BMI, body mass index; HBsAg, hepatitis B surface antigen; anti-HBcIgM, IgM antibody to hepatitis B core antigen; HBeAg, hepatitis B e-antigen; anti-HBeAg, antibody to hepatitis B e-antigen; sALT, serum alanine transaminase; eGFR, estimated glomerulus filtration rate; sCr, serum creatinine; sCys-C, serum cystatin-C; sNGAL, serum neutrophil gelatinase-associated lipocalin; sFet-A, serum Fetuin-A; uIL-18BP, urinary interleukin-18 binding protein; uKIM-1, urinary kidney injury molecule-1.

p < 0.05*, *p* < 0.0001****.

When we tried to differentiate within the HBeAg-positive groups (EPB and EPI) as they have similar HBeAg-related pathophysiology, borderline but insignificantly higher sCys-C values (*p* = 0.056) were observed in the EPI group (titer ≥ 10⁷ IU/mL) as compared to EPB, demonstrating increased predisposition for kidney injury in the immune-tolerant phase than those in the immune clearance phase. Correlation analysis also supported the results, pointing to a positive association of sCys-C with viral load (*r* = 0.25, *p* = 0.049) in the EP group.

Similarly, when we analyzed the HBeAg-negative groups (ENB and ENI), significantly elevated levels of sNGAL (*p* = 0.0088), along with borderline insignificance for sCys-C (*p* = 0.052), were observed in the ENI (inactive carrier) phase as compared to the levels in the ENB (pre-core mutant) phase. The analysis was suggestive of a predilection of chronic inactive HBV carriers to develop renal injury, which may be due to their prolonged exposure to HBV

infection status without treatment with anti-viral and/or steroids. However, no statistical difference was observed in the baseline values of sFet-A and uKIM-1 in the two-, three-, and four-group comparisons.

Outcome analysis

At the end of the study on contact follow-up, nine cases out of 69 patients with CHB reported to have developed kidney injury. On tracking back, among these nine cases, five were in an inactive carrier state at baseline, three were in an ENB state, and one was in an EPI state during sampling. All nine cases had increased levels of sCys-C and sNGAL with a median (range) of 1,638.6 (1,004.5–4,800.6) and 99.48 (73.81–171.47), respectively, at baseline. An ROC analysis unveiled an AUC of 0.73 (95% CI, 0.5865 to

0.8820) with 77.78.3% sensitivity and 60% specificity at a cutoff value of $\geq 1,491$ ng/mL for sCys-C, while sNGAL with an AUC of 0.8, 95% CI (0.6773 to 0.9208), and a sensitivity of 77.78% at 76.67% specificity also showed optimal differentiation. Thus, patients with higher baseline sCys-C and sNGAL levels have been observed to present an increased risk of decline in renal function within the 5-year study period compared to subjects with lower baseline sCys-C and sNGAL levels. Log-rank test for disease outcome (early kidney injury) is depicted in [Supplementary Figure 1](#).

Discussion

The inflammatory response generated in renal cells due to host-virus interaction manifests as renal injury in individuals with CHB, contributing to the overall disease burden and increasing mortality risk ([Hu et al., 2025](#)). Considerable silent kidney deterioration occurs subclinically before a quantifiable decline in renal function is pathologically diagnosed. Therefore, it is important to identify and treat kidney dysfunction in HBV patients at an early juncture before the symptoms of hepato-renal syndrome impinge.

In routine laboratory practice, sCr is a marker for kidney function estimation. However, sCr does not accurately represent GFR in liver maladies ([Griffin et al., 2023](#)) and is also less sensitive in patients with mild renal dysfunction ([Qiu et al., 2017](#)). Studies have also propounded that sCr levels and associated GFR values are noticeably biased by non-renal factors such as age, gender, protein intake, and weight of the subjects ([Qiu et al., 2017](#)). Similarly, sUr is an unreliable indicator of early kidney dysfunction ([Ji et al., 2020](#)). In our present analysis, these traditional renal function parameters were not statistically significant ([Table 1](#)).

Previous experimental research has explored the role of serum Cys-C, NGAL, and KIM-1 in individuals with liver disease ([Kamimura et al., 2018](#)). Nevertheless, as far as we know, this study is the first to investigate the role of these markers in identifying very early asymptomatic renal injury in treatment-naive patients with CHB. When assessed as an indicator of renal dysfunction, circulating sCys-C effectively distinguished between patients with CHB and control subjects ($p < 0.0001$), proving to be a reliable and strong marker, with the highest AUC of 0.935 and a sensitivity of 91.30%. Zheng and his colleagues have also attributed a similar role of sCys-C in renal impairment, specifically in patients with HBV-related fibrosis and cirrhosis ([Zheng et al., 2020](#)). Out of the five biomarkers examined, only sCys-C displayed a significant positive correlation with uKIM-1 and sCr, concomitant with the findings of a prior study involving CLD patients ([Li et al., 2016](#)).

Prior research has demonstrated that sNGAL is an indicator of early kidney damage in HBV patients undergoing antiretroviral therapy, indicating that the toxicity from prolonged treatment with nucleos(t)ide analogs is responsible for the decline in tubular kidney function ([Carey et al., 2018](#)). However, the current evaluation

suggests that renal injury is attributed solely to the host's response to the HBV virus in the absence of any antiretroviral therapy, as the tubular injury protein marker sNGAL (AUC 0.811) was identified to be second most reliable biomarker for detecting renal impairment in HBV patients who have not received antiretroviral treatment.

The third and fourth markers identified were uIL-18BP and sFet-A ([Table 2](#); [Figure 3a](#)). Earlier research has shown that lower levels of sFet-A are associated with early stages of CKD ([Deepa and Sasivathanam, 2016](#)), while a recent study using a CKD rat model suggests that sFet-A enhances renal function in CKD due to its anti-inflammatory effects ([Ashour et al., 2023](#)). These studies align with our current laboratory findings, which, for the first time, reveal significantly reduced levels of sFet-A in a CHB cohort, indicating inflammation-related renal damage.

Over the past two decades, there have been no human studies on uIL-18BP in patients with CHB with kidney issues. However, a single mention of elevated circulatory IL-18BP was found in subjects with reduced kidney function ([Lonnemann et al., 2000](#)). Enhanced expression of uIL-18BP in the present work is plausibly the host's response to neutralize its inflammatory target IL-18, aligning with the former *in vivo* studies that have demonstrated its ability to inhibit IL-18 ([Girard-Guyonvarc'h et al., 2018](#)). The fifth marker, uKIM-1, showed higher baseline levels in the CHB group relative to the control group; however, this difference was statistically insignificant. This finding contradicts earlier research that elucidates increased levels of uKIM-1, attributed to nephrotoxicity caused by antiretroviral therapy in patients with CHB ([Li et al., 2016](#)).

This is the first attempt where a set of markers—sCysC, sNGAL, sFet-A, and uIL-18BP combined in a panel—have significantly improved AUC and have outshone the efficacy of the singular marker sCysC. Prior work by Prowle and his group has indicated that using a combination of Cr 24 h + post-operative π -GST (π glutathione S-transferases) improves AUC (0.86) and is more effective than single markers in identifying renal injury as a comorbidity ([Prowle et al., 2015](#)). Our findings regarding sCys-C, sNGAL, sFet-A, and uIL-18BP suggest that these serum and urine biomarkers can offer a distinctive noninvasive approach to screen and distinguish the onset of CHB-related early kidney injury and thereby help to identify patients with CHB anticipated to develop renal insufficiency in the future ([Figure 4](#)).

The pathogenesis of HBV-mediated nephropathy depends on the complex interplay between the HBV and the host immune response. Regardless of replication status, the serum from patients with CHB can induce apoptotic damage to renal tubular epithelial cells, thereby leading to renal fibrosis ([Deng et al., 2006](#)). This *in vitro* finding is consistent with the current study, as even the non-replicative states are equally vulnerable to renal damage. The presence of viral antigens (HBsAg, HBcAg, and HBeAg) and host antibodies expressed in the tubular epithelial cells in response can

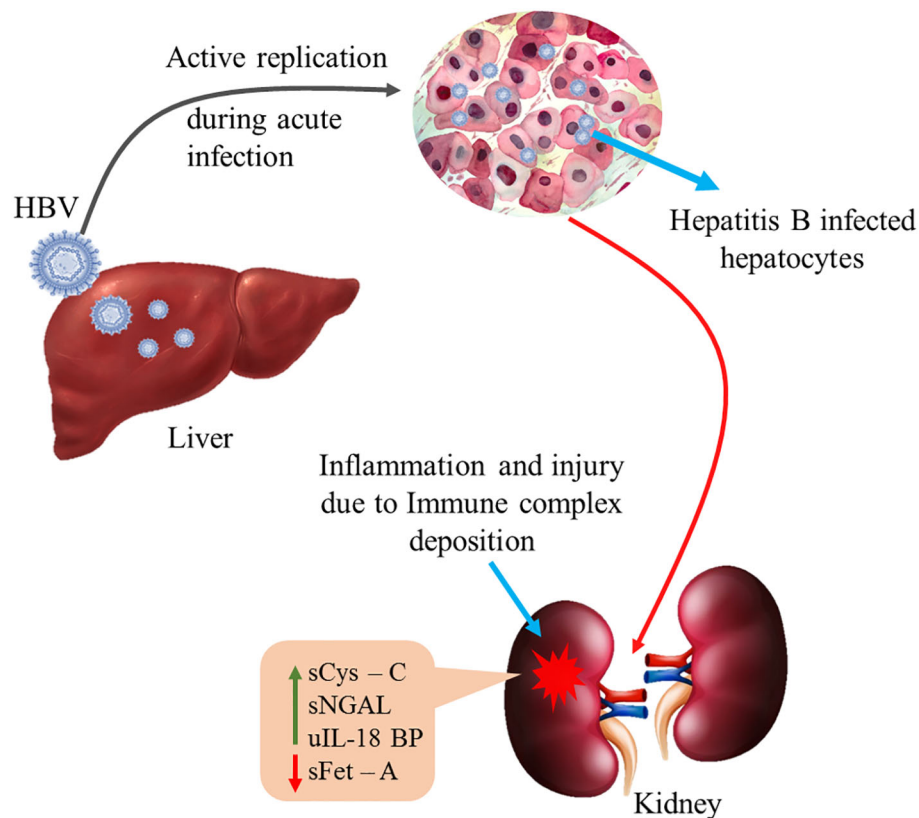


FIGURE 4

Active HBV replication in the hepatocytes elicits immune response resulting in inflammation and kidney injury due to immune complex deposition. sCys-C, serum cystatin-C; sNGAL, serum neutrophil gelatinase associated lipocalin; sFet-A, serum fetuin-A; uIL-18BP, urinary interleukin-18 binding protein. Green arrow indicates up-regulation of sCys-C, sNGAL and uIL18BP while red arrow indicates down-regulation of sFet-A in circulation.

result in complement activation and apoptosis induction, which have a pivotal role in the pathogenesis of renal injury (Shah and Amarapurkar, 2017). Thus, depending on the phase of infection, HBV antigens, host antibodies, and HBV DNA elicit a host immune response leading to chronic inflammation. Our results have established that sCys-C is very sensitive for detecting early kidney injury, as its values were elevated even in immune-tolerant and inactive HBV carrier phases with normal Cr and ALT. Also, its expression was not associated with liver function markers. This is aligned with Hong and the group's observation that active HBV replication is not the only factor responsible for the development of kidney disease (Hong et al., 2018). Based on this analysis, an important role for Cys-C as a potential marker of renal function decline is evident. The present investigation also indicates that kidney dysfunction is frequent in our population moderately endemic for HBV, and it occurs even before the initiation of any anti-viral treatment. It emphasizes the need for a baseline renal assessment in all patients with CHB, followed by kidney function monitoring in every phase of HBV infection, irrespective of therapy.

However, the conclusions drawn in this investigation are preliminary and need to be validated in a large-sized, multi-centric cohort, which is a limitation of this study.

Conclusion

In a nutshell, renal oddity in the study is due to host response in the presence of the virus and is pre-existent even before the commencement of antiretroviral treatment. Also, the clinical expediency of the tested markers is remarkable as they can be used not only to screen early renal damage but also to assess the progression of damage noninvasively during the course of HBV infection. The proposed combination (sCys-C + sNGAL + sFet-A + uIL18BP) is even better than sCys-C, a very good stand-alone superior marker compared to the currently used pathological parameters for detecting renal impairment in patients with CHB. However, a larger sample size and follow-up of patients can substantiate the results of our study.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The studies involving humans were approved by Institutional Ethics Committee, BMHRC. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

PG: Supervision, Investigation, Conceptualization, Funding acquisition, Writing – review & editing, Project administration, Validation, Data curation, Resources, Methodology. KP: Validation, Writing – review & editing, Investigation, Methodology, Visualization, Data curation, Writing – original draft, Formal analysis. SV: Methodology, Investigation, Resources, Writing – review & editing. MA: Methodology, Resources, Writing – review & editing, Investigation. KM: Project administration, Writing – review & editing.

Funding

The author(s) declare that financial support was received for the research and/or publication of this article. Dr. Puneet Gandhi received funding from ICMR, Ref: No.5/8/4-3\Env11\NCD-I, ICMR.

Acknowledgments

This study is a part of project 5/8/4-3\Env11\NCD-I, sanctioned by ICMR. The authors are thankful to ICMR for financial support

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and BMHRC for infrastructural facilities; Dr. KR Pardasani for the calculation of statistical size; and Mr. Jaswant Patel for technical assistance.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fcimb.2025.1601678/full#supplementary-material>

SUPPLEMENTARY FIGURE 1

Kaplan Meier analysis for assessing early kidney injury.

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OPEN ACCESS

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RECEIVED 09 May 2025

ACCEPTED 24 July 2025

PUBLISHED 11 August 2025

CITATION

Cevallos C, Jarmoluk P, Sviercz F,
Freiberger RN, López CAM, Delpino MV and
Quarleri J (2025) Ferroptosis and
mitochondrial ROS are central to
SARS-CoV-2-induced hepatocyte death.
Front. Cell. Infect. Microbiol. 15:1625928.
doi: 10.3389/fcimb.2025.1625928

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Ferroptosis and mitochondrial ROS are central to SARS-CoV-2-induced hepatocyte death

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Background: Although COVID-19 primarily affects the respiratory tract, liver injury has been increasingly reported in infected individuals. The mechanisms by which SARS-CoV-2 induces hepatocyte damage remain poorly understood. Given the role of mitochondrial dysfunction, oxidative stress, and regulated cell death in COVID-19 pathogenesis, we investigated the impact of SARS-CoV-2 infection on hepatocytes using the Huh7.5 cell model.

Methods: Huh7.5 hepatocytes were infected with either the ancestral Wuhan (Wh) or Omicron (BA.5) variant of SARS-CoV-2. Viral replication was quantified via RT-qPCR, nucleocapsid protein detection, and infectious particle titration. Mitochondrial function was assessed through mitochondrial membrane potential ($\Delta\Psi_m$), mROS production, and mitophagy analysis. Lipid metabolism and regulated cell death (apoptosis, pyroptosis, ferroptosis) were evaluated by confocal microscopy and flow cytometry. The role of specific cell death pathways was probed using chemical inhibitors.

Results: Both SARS-CoV-2 variants efficiently infected Huh7.5 cells, with distinct replication kinetics. Infection induced mitochondrial fragmentation, elevated mROS levels, and lipid droplet accumulation. Ferroptosis was identified as a predominant mode of cell death, as evidenced by increased lipid peroxidation and the protective effect of ferrostatin-1. Expression of angiotensin-converting enzyme 2 (ACE2) and transferrin receptor 1 (TfR1), a ferroptosis marker and alternative viral entry receptor, was significantly upregulated post-infection in a variant-dependent manner. Additionally, mROS scavenging with MitoTEMPO impaired viral replication, underscoring the role of oxidative stress in the SARS-CoV-2 life cycle.

Conclusions: SARS-CoV-2 disrupts mitochondrial homeostasis and lipid metabolism in hepatocytes, promoting ferroptosis as a major contributor to virus-induced cytopathology. These findings suggest that ferroptosis may play a central role in COVID-19-related liver injury and identify mitochondrial ROS and iron metabolism as potential therapeutic targets.

KEYWORDS

SARS-CoV-2, hepatocytes, mitochondrial ROS, ferroptosis, lipid metabolism, regulated cell death, transferrin receptor, COVID-19

1 Introduction

COVID-19 is primarily characterized by respiratory tract infection and subsequent pulmonary involvement (Proal et al., 2023). However, extrapulmonary manifestations have also been reported, as SARS-CoV-2 exhibits tropism for various tissues (Wissler Gerdes et al., 2022). Among these, liver damage of varying severity has been observed in patients with SARS-CoV-2 infection, and clinical studies have documented the development of acute liver injury (ALI) (Dufour et al., 2022). However, the mechanisms underlying liver injury during SARS-CoV-2 infection remain unclear. Potential contributors include the virus's direct cytopathic effects, immune response activation, and drug-induced hepatotoxicity (Fratta Pasini et al., 2021; Dufour et al., 2022).

Redox homeostasis is critical for cellular function, and viruses, including SARS-CoV-2, can disrupt mitochondrial dynamics to promote their replication. Specifically, increased mitochondrial reactive oxygen species (mROS) levels can enhance viral replication and regulate cell death pathways (Foo et al., 2022). Additionally, viral infections often interfere with lipid metabolism, and lipid droplet (LD) biogenesis has been implicated in SARS-CoV-2 replication and pathogenesis (Dias et al., 2020; D'Avila et al., 2024).

SARS-CoV-2 modulates cell death pathways, contributing to COVID-19 pathogenesis and progression (Yuan et al., 2023). Iron metabolism is disrupted in SARS-CoV-2 infection (Muhoberac, 2020), and its dysregulation can trigger ferroptosis—a form of programmed cell death driven by iron-dependent lipid peroxidation. The human transferrin receptor (TfR1, CD71) mediates Fe³⁺ uptake into cells, is upregulated during ferroptosis, and serves as a ferroptosis-specific marker. Interestingly, studies in lung cells have shown that TfR1 can mediate SARS-CoV-2 infection in an angiotensin-converting enzyme 2 (ACE2)-independent manner, allowing viral entry into tissues with low or negligible ACE2 expression (Liao et al., 2024). Ferroptosis plays a key role in COVID-19-related lung disease, and increased ROS levels may promote lipid peroxidation and ferroptosis during SARS-CoV-2 infection (Fratta Pasini et al., 2021; Qiu et al., 2024).

This study aims to elucidate the direct effects of SARS-CoV-2 infection on hepatocytes, using the Huh7.5 human hepatocyte cell line as a model. We evaluated two SARS-CoV-2 strains and found that the virus efficiently infects Huh7.5 cells, disrupts mitochondrial homeostasis and lipid metabolism to facilitate replication, and induces hepatocyte death predominantly via the ferroptosis pathway.

2 Materials and methods

2.1 Cell culture

The human Huh7.5 hepatocellular carcinoma-derived cell line was obtained from ATCC. Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal

bovine serum (FBS), 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin at 37°C in a 5% CO₂ atmosphere. All experiments were conducted under biosafety level 3 (BSL-3) conditions at INBIRS. Biological materials were autoclaved and incinerated following institutional guidelines.

2.2 SARS-CoV-2 stocks and viral titration

Viral stocks were obtained, titrated, and maintained as previously described (Svircz et al., 2024). Briefly, the ancestral Wuhan (Wh) strain was provided by Dr. Sandra Gallego (Universidad Nacional de Córdoba, Argentina). In contrast, the Omicron (BA.5) strain was isolated from a nasopharyngeal swab, characterized, propagated, and titrated in Vero cells, yielding a titer of 2.0×10⁶ plaque-forming units (PFU)/mL. Vero E6 cells (ATCC, Rockville, MD) were maintained as monolayers at 37°C in a 5% CO₂ atmosphere in DMEM supplemented with 2 mM L-glutamine, 10% FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin.

2.3 Determination of ACE2 and TfR expression in Huh7.5 cells

The cell surface expression of ACE2 and TfR (CD71) was quantified 72 hours post-infection (hpi) using flow cytometry. Cells were incubated with a rabbit polyclonal anti-ACE2 antibody (#ab272690, Abcam, UK) or a rabbit polyclonal anti-TfR/CD71 antibody (#ab84036, Abcam, UK), followed by a PE-conjugated secondary anti-rabbit IgG antibody (cat#31210, Invitrogen, US). Data were acquired using Full Spectrum Flow Cytometry Cytex[®] Northern Lights 3000[™] (Cytex Biosciences Inc.) and analyzed with FlowJo.v10.6.2 (Ashland).

2.4 Infection of Huh7.5 cells with SARS-CoV-2

Huh7.5 cells were seeded at 5×10⁴ cells/well in 24-well plates and infected with SARS-CoV-2 Wh or BA.5 at a multiplicity of infection (MOI) of 0.05 for 1 hour at 37°C in a 5% CO₂ atmosphere. After infection, monolayers were washed three times with phosphate buffered saline (PBS) and maintained in DMEM supplemented with 2 mM L-glutamine, 10% heat-inactivated FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin. Huh7.5 cells were left untreated for each condition as a negative control (mock).

2.5 Assessment of infection and viral replication

Viral infection and replication efficiency were assessed at 24-, 48-, and 72-hpi using the following approaches:

2.5.1 Intracellular detection of SARS-CoV-2 nucleocapsid protein by flow cytometry

Cells were fixed, permeabilized, and stained with a rabbit anti-nucleocapsid antibody (clone EPR24334-118, #ab271180, Abcam), followed by a PE-conjugated secondary antibody. Fluorescence was analyzed by flow cytometry (Cytek Biosciences Inc.) to quantify infected cells with FlowJo.v10.6.2 (Ashland).

2.5.2 Confocal microscopy

SARS-CoV-2-infected Huh7.5 cells at 72 hpi were fixed, permeabilized, and stained to visualize viral nucleocapsid protein for the qualitative visualization of the SARS-CoV-2 nucleocapsid protein in infected Huh7.5 cells. Images were acquired using confocal microscopy.

2.5.3 Quantification of SARS-CoV-2 genomic RNA in culture supernatants

Viral RNA was extracted using the ChemagicTM Viral DNA/RNA Kit Special H96 on the automated ChemagicTM 360 instrument (PerkinElmer, Germany). RNA concentration was assessed using a NanoDropTM spectrophotometer (Thermo ScientificTM) for quality control. SARS-CoV-2 RNA was quantified by RT-qPCR using the DisCoVery SARS-CoV-2 RT-PCR Detection Kit Rox, targeting the ORF1ab and N genes. Viral load was determined by interpolating Ct values with a standard curve generated from quantified SARS-CoV-2 positive RNA controls (GISAID EPI_ISL_420600). The assay's limit of detection (LOD) was 100 SARS-CoV-2 genomic RNA copies/mL.

2.5.4 Titration of infectious viral particles

Supernatants from infected Huh7.5 cells were used to infect Vero E6 cells. After one hour of adsorption, the inoculum was replaced with DMEM containing 2% FBS. Plates were incubated for 72 hours, after which visible plaques were counted. Infectious viral titers were calculated based on plaque formation and expressed as plaque-forming units (PFU)/mL, using the formula:

$$PFU/mL = \text{Number of plaques} / (\text{dilution factor} \times \text{volume of inoculum}).$$

2.6 Detection and scavenging of mitochondrial reactive oxygen species

mROS levels were quantified by flow cytometry (Cytek Biosciences Inc.) following staining with 5 μ M MitoSOXTM Red (Thermo Fisher Scientific) for 15 minutes at 37°C in PBS. Rotenone (10 μ M) was used as a positive control for mROS induction. To assess mROS scavenging, cells were pretreated with 50 μ M MitoTEMPO (#SML0737, Sigma-Aldrich) for 24 hours before infection. MitoSOXTM fluorescence intensity (excitation: 510 nm, emission: 580 nm) was measured at 24-, 48-, and 72-hpi. Analysis was done with FlowJo.v10.6.2 (Ashland).

2.7 Measurement of mitochondrial mass and membrane potential

The mitochondrial membrane potential was assessed as previously described (Ojeda et al., 2018). At 24, 48, and 72 hpi, cells were incubated with 100 nM MitoTrackerTM Deep Red (MTDR, Thermo Fisher Scientific) for 15 minutes at 37°C in appropriate culture conditions. After incubation, fluorescence intensity was analyzed by flow cytometry. A decrease in MTDR fluorescence intensity indicated mitochondrial depolarization.

Mitochondrial mass and membrane potential ($\Delta\Psi$ m) in Huh7.5 cells were assessed using MitoTrackerTM Green FM (MTG; Thermo Fisher Scientific) and MitoTrackerTM Deep Red FM (MTDR; Thermo Fisher Scientific), respectively. Briefly, cells were incubated with 100 nM MTG and 100 nM MTDR in pre-warmed complete medium at 37°C for 30 minutes, protected from light. After staining, cells were washed twice with PBS, resuspended in fresh medium, and immediately analyzed by flow cytometry (Cytek Biosciences Inc.) and FlowJo.v10.6.2 (Ashland). MTG fluorescence was used to evaluate total mitochondrial content (independent of membrane potential), while MTDR fluorescence reflected mitochondrial membrane potential.

2.8 Colocalization of mitochondria and lysosomes

Huh7.5 cells were infected with SARS-CoV-2 Wh or BA.5, and mitochondria-lysosome colocalization was evaluated at 24-, 48-, and 72-hpi. Mock-infected cells served as controls. Mitochondria were stained with MitoTrackerTM Red CMXRos, lysosomes with LysoTrackerTM Green, and nuclei with DAPI (Invitrogen). Mdivi-1 (mitophagy inhibitor) was added to the medium at a final concentration of 50 μ M/L for 2 h before infection. Cells were fixed with 4% paraformaldehyde and mounted in PBS-glycerol (9:1 v/v) with an antifade reagent for confocal microscopy analysis (Zeiss LSM 800, 63 $\times$ objective). Ten microscopic fields per well were analyzed in triplicate. To quantify the degree of colocalization, we calculated the Manders' correlation coefficient from the corresponding confocal images using FIJI software (ImageJ, National Institutes of Health, Bethesda, MD, USA) (Manders et al., 1993).

2.9 Lipid droplets staining

Lipid content was evaluated using BodipyTM 493/503 staining at 24-, 48-, and 72-hpi, in the presence or absence of 10 μ M Ferrostatin-1 (#SML0583-5MG, Sigma-Aldrich). Mock-infected cells, treated or untreated with Ferrostatin-1, served as controls. Cells were fixed with 4% paraformaldehyde, permeabilized with 0.3% Triton X-100, stained with Bodipy 493/503, and analyzed by confocal microscopy (Zeiss LSM 800, 63 $\times$ objective). Lipid accumulation was quantified based on fluorescence intensity,

analyzing ten microscopic fields per well from three wells per experimental condition.

2.10 Correlation between viral replication and mROS production

To assess the correlation between viral replication and mROS levels, Huh7.5 cells were pretreated with 50 μ M MitoTEMPO for 24 hours, infected with SARS-CoV-2 variants, and analyzed at 24-, 48-, and 72-hpi. mROS production was quantified by flow cytometry (MitoSOXTM staining), and viral replication was assessed by RT-qPCR quantifying SARS-CoV-2 RNA in supernatants. Untreated and mock-infected controls were included for comparison.

2.11 Measurement of regulated cell death (apoptosis, pyroptosis, ferroptosis)

Regulated cell death (RCD) was quantified by flow cytometry using allophycocyanin (APC)-conjugated Annexin V and 7-AAD (BD Biosciences). Staurosporine (1 μ M) was used as a positive control for apoptosis. Caspase-dependent and independent pathways were assessed using specific inhibitors, including the caspase-3 inhibitor Z-DEVD-FMK for apoptosis, the caspase-1 inhibitor Z-YVAD-FMK for pyroptosis, and Ferrostatin-1 (10 μ M) for ferroptosis. Caspase activity was measured using the VybrantTM FAM Caspase-3/-7 Assay Kit and the Caspase-1 Staining Kit, following the manufacturer's protocols. RCD was analyzed at 24, 48, and 72 hpi using Full Spectrum Flow Cytometry Cytex[®] Northern Lights 3000TM (Cytex Biosciences Inc.) and analyzed with FlowJo.v10.6.2 (Ashland).

2.12 Cytokine measurement

IL-1 β levels were assessed using anti-IL-1 β -fluorescein isothiocyanate (FITC) (JK1B-1, BioLegend, US) by flow cytometry. In parallel, IL-1 β concentrations in cell culture supernatants were quantified using a commercially available human IL-1 β ELISA kit (R&D Systems), following the manufacturer's instructions. Absorbance was measured at 450 nm using a microplate reader, and cytokine concentrations were interpolated from a standard curve generated with known IL-1 β concentrations.

2.13 Statistical analysis

Statistical analysis was performed wherever applicable. Statistical analysis was performed with one-way ANOVA. Multiple comparisons between all pairs of groups were made using Tukey's test, and those between two groups were made using the Mann-Whitney U test. Graphical and statistical analyses were performed with GraphPad Prism 8.0 software. Each

experiment was performed in triplicate (technical replicates) with different culture preparations on 2–4 independent occasions (biological replicates). Data were represented as mean $\pm$ SD measured in triplicate from three individual experiments. A $p < 0.05$ is represented as *, $p < 0.01$ as **, $p < 0.001$ as ***, and $p < 0.0001$ as ****. A statistically significant difference between groups was accepted at a minimum level of $p < 0.05$.

3 Results

3.1 Huh7.5 cells are permissive to SARS-CoV-2 infection but exhibit variant-dependent viral kinetics

Liver damage severity may vary depending on the SARS-CoV-2 variant involved in the infection, potentially due to differences in replication kinetics and infectivity (Bartolomeo et al., 2022; Zhang et al., 2022). To assess this, viral replication and infection efficiency were evaluated at three post-infection time points -24, 48, and 72 hpi- by quantifying intracellular SARS-CoV-2 nucleocapsid protein (N) expression via flow cytometry and measuring viral load in Huh7.5 cell supernatants using RT-qPCR.

As shown in Figures 1A, B, N protein expression levels increased over time for both variants, but significant differences were observed between the ancestral strain (Wh) and the omicron BA.5 variant at all-time points: 24 hpi (Wh: 4.1% $\pm$ 1.2; BA.5: 9.8% $\pm$ 2.2), 48 hpi (Wh: 38.3% $\pm$ 4.7; BA.5: 24.7% $\pm$ 1.9), and 72 hpi (Wh: 76.9% $\pm$ 4.0; BA.5: 53.9% $\pm$ 5.3). Similarly, viral load levels (RNA copies/mL) in Huh7.5 culture supernatants increased from 24 hpi to 72 hpi for both variants, with significant differences observed at 24 hpi (Wh: $(1.55 \pm 0.30) \times 10^6$; BA.5: $(7.53 \pm 1.94) \times 10^7$), 48 hpi (Wh: $(2.61 \pm 0.82) \times 10^7$; BA.5: $(9.64 \pm 1.54) \times 10^8$), and 72 hpi (Wh: $(5.50 \pm 0.39) \times 10^8$; BA.5: $(1.48 \pm 0.21) \times 10^9$). Confocal microscopy further confirmed SARS-CoV-2 infection in Huh7.5 cells (Figure 1C). These findings demonstrate that SARS-CoV-2 efficiently infects Huh7.5 cells, revealing differences in replication kinetics between the Wh and BA.5 variants.

To assess the infectivity of viral particles released into the Huh7.5 culture supernatants, samples collected at 24, 48, and 72 hpi were used to infect Vero E6 cells, following a previously described protocol. Infectious titers of SARS-CoV-2 in Vero E6 monolayers were determined at 72 hpi and expressed as PFU/mL. Results confirmed the infectivity of viral particles released from Huh7.5 cells and revealed variant-dependent differences in infection titers. Specifically, the viral titer obtained at 72 hpi for Vero E6 monolayers infected with supernatants collected at 24 and 48 hpi was $(1.09 \pm 0.10) \times 10^5$ PFU/mL for Wh and $(1.09 \pm 0.10) \times 10^8$ PFU/mL for BA.5. However, no differences were observed in viral titers when Vero E6 cells were infected with Huh7.5 supernatants collected at 72 hpi, with both variants yielding titers of $(1.09 \pm 0.10) \times 10^8$ PFU/mL (Figure 1D).

In summary, both the Wh and BA.5 variants efficiently infect and replicate in hepatocytes, suggesting that hepatocyte infection may contribute, at least in part, to liver injury observed in COVID-

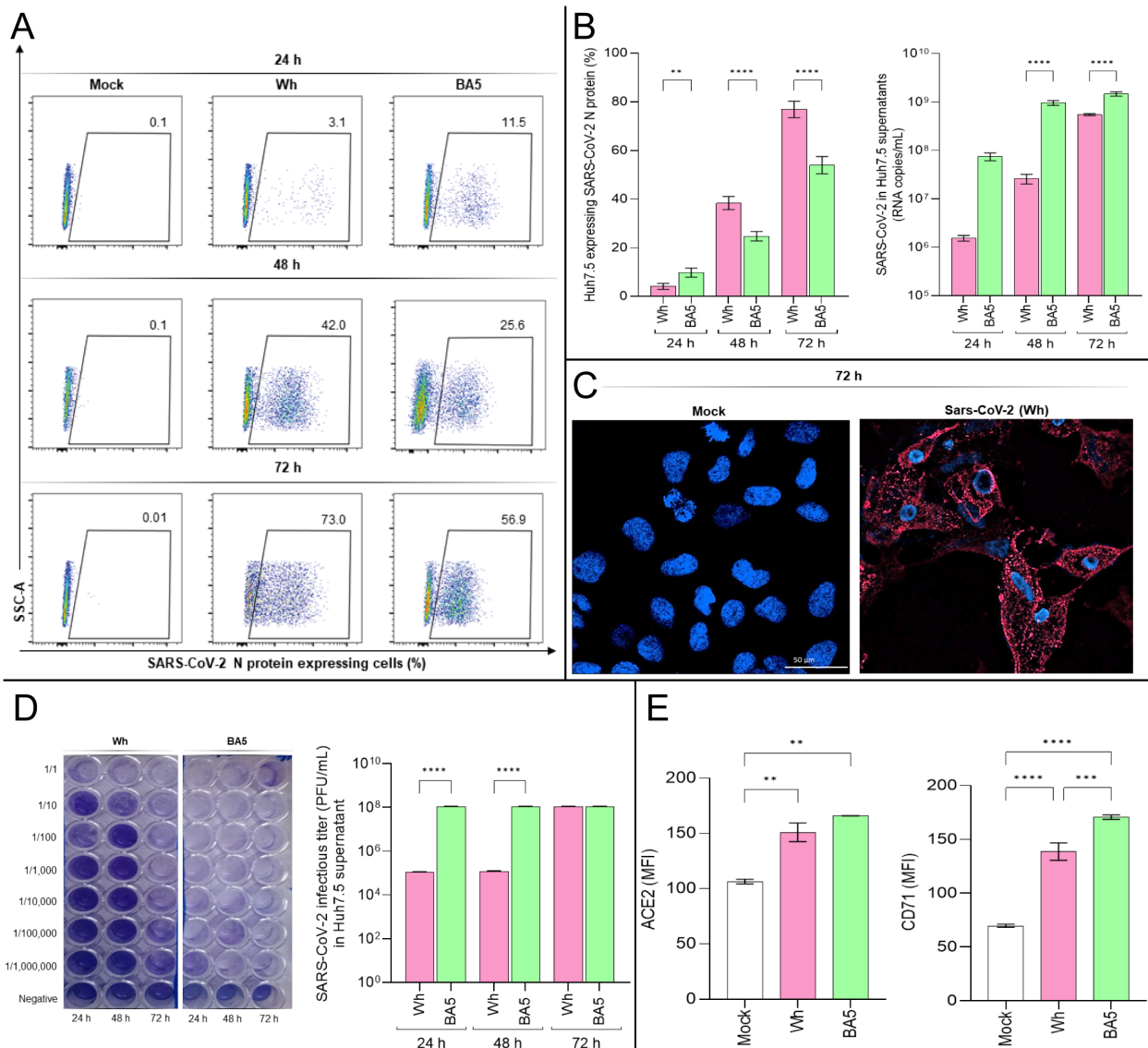


FIGURE 1

SARS-CoV-2 infection dynamics and receptor expression in Huh7.5 cells. (A, B) Flow cytometry analysis of intracellular SARS-CoV-2 nucleocapsid (N) protein expression in Huh7.5 cells infected with the ancestral Wuhan (Wh) or Omicron BA.5 variants at 24-, 48-, and 72-hours post-infection (hpi). (C) Representative confocal images of N protein expression (red) in infected cells at 72 hpi. Nuclei were stained with DAPI (blue). (D) Viral titers in culture supernatants determined by plaque-forming units (PFU/mL) using Vero E6 cells. (E) Surface expression of ACE2 and transferrin receptor 1 (TfR1) was assessed by flow cytometry at 72 hpi. Data represent mean $\pm$ SD of three independent experiments. Statistical significance was determined by one-way ANOVA with Tukey's multiple comparisons test. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

19 patients. Furthermore, the accelerated replication kinetics of the Omicron (BA.5) compared to the ancestral (Wh) variant in hepatocytes may be linked to differences in cellular entry efficiency.

3.2 ACE2 and TfR1 (CD71) expression are differentially modulated in a variant-dependent manner

Although ACE2 is the primary receptor for SARS-CoV-2 entry, viral particles have been detected in multiple organs regardless of ACE2 expression levels (Liao et al., 2024). Other molecules expressed

in extrapulmonary tissues can mediate viral entry into cells. One such molecule, the human transferrin receptor (TfR1), is a ubiquitously expressed membrane protein that interacts with the SARS-CoV-2 Spike protein with high affinity and facilitates viral entry via the endocytic pathway (Luo et al., 2022). Given that Huh7.5 cells are susceptible to SARS-CoV-2 infection, we evaluated the expression levels of ACE2 and TfR1 in these cells before and after infection. Huh7.5 cells were infected with the Wh and BA.5 variants at a multiplicity of infection (MOI) of 0.05. After 72 hpi, ACE2 and TfR1 expression levels were measured using flow cytometry.

The results showed that, following infection with either variant, the expression of ACE2 and TfR1, measured as median

fluorescence intensity (MFI), was significantly higher than in control cells. Specifically, ACE2 expression levels were: Mock: 106.5 ± 2.1 ; Wh: 151.0 ± 8.4 ; BA.5: 166.0 ± 0.1 . Similarly, TfR1 expression levels were: Mock: 69.7 ± 1.5 ; Wh: 138.7 ± 8.1 ; BA.5: 170.7 ± 2.0 (Figure 1E). Moreover, BA.5 induced a significantly greater increase in TfR1 expression in Huh7.5 cells compared to Wh. However, ACE2 upregulation occurred to a similar extent following infection with both variants (Figure 1E).

These findings demonstrate that SARS-CoV-2 infection upregulates ACE2 and TfR1 levels in a variant-dependent manner. Although BA.5 exhibits a high affinity for hACE2, it also harbors multiple Spike (S) protein mutations that may alter its conformation and reduce S1/S2 cleavage efficiency by TMPRSS2. As a result, BA.5 may rely on a late endocytic pathway for cell entry (Erabi et al., 2024). The higher TfR1 expression levels observed at 72 hpi with BA.5 may reflect TfR1's role in mediating viral entry via endocytosis, the primary route used by this variant to infect cells.

3.3 SARS-CoV-2 infection induces cell death in Huh7.5 cells via both caspase-dependent and caspase-independent pathways

The SARS-CoV-2 Wuhan (Wh) and BA.5 variants efficiently infect and replicate in hepatocytes, potentially inducing cytopathic effects that trigger cell death pathways and contribute to tissue damage. Liver abnormalities have been observed in individuals infected with SARS-CoV-2, with postmortem studies revealing histopathological alterations. Annexin V and 7-AAD staining were used to assess apoptosis and necrosis by flow cytometry at 24, 48, and 72 hpi.

As shown in Figure 2A, a significant increase in Huh7.5 cell death was observed at 48 hpi (Mock: $6.5\% \pm 2.6$; Wh: $14.0\% \pm 1.8$; BA.5: $24.6\% \pm 1.7$) and 72 hpi (Mock: $4.6\% \pm 2.0$; Wh: $60.1\% \pm 4.8$; BA.5: $30.0\% \pm 4.5$). At 48 hpi, cell death in Wh-infected cells was significantly lower than in BA.5-infected cells. However, by 72 hpi, this trend reversed, with Wh-infected cells exhibiting significantly higher cell death levels than BA.5-infected cells (Figure 2B). Additionally, IL-1 β production increased during viral replication in Huh7.5 cells. Notably, at 72 hpi, both extracellular and intracellular levels of IL-1 β were significantly higher in Wh-infected cells compared to BA.5-infected cells (Figure 2C).

To investigate the involvement of specific cell death pathways, Huh7.5 cells were pretreated with inhibitors of caspase-3-dependent apoptosis (Z-DEVD), caspase-1-dependent pyroptosis (Z-YVAD), and ferroptosis (ferrostatin-1) before infection. Cell death levels at 72 hpi are shown in Figure 2D. Infected Huh7.5 cells showed $54.3\% \pm 6.9$ (Wh) and $45.0\% \pm 4.7$ (BA.5) cell death. Pretreatment with inhibitors significantly reduced cell death: Wh/ferrostatin-1: $9.8\% \pm 2.6$; Wh/Z-DEVD: $17.1\% \pm 3.4$; Wh/Z-YVAD: $28.2\% \pm 3.5$; BA.5/ferrostatin-1: $14.0\% \pm 1.9$; BA.5/Z-DEVD: $21.0\% \pm 3.4$; BA.5/Z-YVAD: $28.5\% \pm 1.6$.

The roles of caspase-dependent pathways were further explored by measuring caspase-3/7 and caspase-1 activity. Both were

significantly elevated in infected cells compared to mock-infected controls, with caspase-1 activity showing a more pronounced increase (Figure 2E). These findings suggest that SARS-CoV-2 activates multiple cell death pathways in hepatocytes. Notably, ferroptosis, a caspase-independent mechanism, appears to contribute substantially to cell death regardless of the viral variant. The extent of SARS-CoV-2-induced regulated cell death in hepatocytes may vary by variant, likely reflecting differences in viral entry efficiency and replication kinetics.

3.4 SARS-CoV-2 infection induces mitochondrial dysfunction and mROS imbalance

Mitochondria play a crucial role in regulating multiple cell death pathways, including apoptotic and non-apoptotic mechanisms such as necroptosis, ferroptosis, pyroptosis, parthanatos, and paraptosis (Nguyen et al., 2023). Viruses, including SARS-CoV-2, can manipulate mitochondrial function, disrupting reactive oxygen species (ROS) homeostasis and contributing to cellular damage (Ren et al., 2020; Shang et al., 2021). Oxidative stress arises when excessive ROS production overwhelms the cellular antioxidant defenses, with mitochondrial respiration serving as a primary source of these free radicals.

To assess the impact of SARS-CoV-2 infection on mitochondrial function, intracellular mitochondrial ROS (mROS) levels were measured at 24-, 48-, and 72-hpi in Huh7.5 cells infected with the Wh and BA.5 variants. Compared to mock-infected controls, both viral variants significantly increased mROS production at study time points. At 24 hpi, mROS levels were $6.1\% \pm 1.2$ in mock-infected cells, $11.5\% \pm 0.5$ in Wh-infected cells, and $14.2\% \pm 2.1$ in BA.5-infected cells. At 48 hpi, the levels increased to $6.9\% \pm 1.5$, $46.6\% \pm 3.4$, and $62.0\% \pm 3.2$, respectively. By 72 hpi, mROS production reached $6.5\% \pm 1.3$ in mock-infected cells, $90.7\% \pm 3.9$ in Wh-infected cells, and $84.7\% \pm 6.6$ in BA.5-infected cells. At 48 hpi, mROS levels were significantly higher in BA.5-infected cells compared to Wh-infected cells. Still, this difference was no longer significant at 72 hpi, even when both variants substantially increased mROS production (Figures 3A, B).

To assess the impact of SARS-CoV-2 infection on mitochondrial function, Huh7.5 cells were analyzed by flow cytometry to evaluate mitochondrial mass (MitoTrackerTM Green FM (MTG) and mitochondrial membrane potential $-\Delta\Psi_m-$ (MitoTrackerTM Deep Red FM (MTDR) following infection with either the ancestral (Wh) or omicron (BA.5) variants. Infected cells exhibited widespread mitochondrial fragmentation while largely maintaining $\Delta\Psi_m$ (MTG+/MTDR+, indicating fragmented mitochondria with maintained membrane potential). Thus, at 24 hpi, the relative abundance of MTG+/MTDR+ was $10.6 \pm 5.8\%$ in mock-infected controls, $31.7 \pm 10.4\%$ in Wh-infected cells, and $20.1 \pm 14.4\%$ in BA.5-infected cells. The frequency of MTG+/MTDR- single-positive cells was $1.1 \pm 0.8\%$ (mock), $1.8 \pm 0.9\%$ (Wh), and $1.5 \pm 1.3\%$ (BA.5).

At 48 hpi, the abundance of double-positive cells was $4.6 \pm 3.3\%$ (mock), $19.4 \pm 6.1\%$ (Wh), and $11.5 \pm 5.3\%$ (BA.5), while MTG

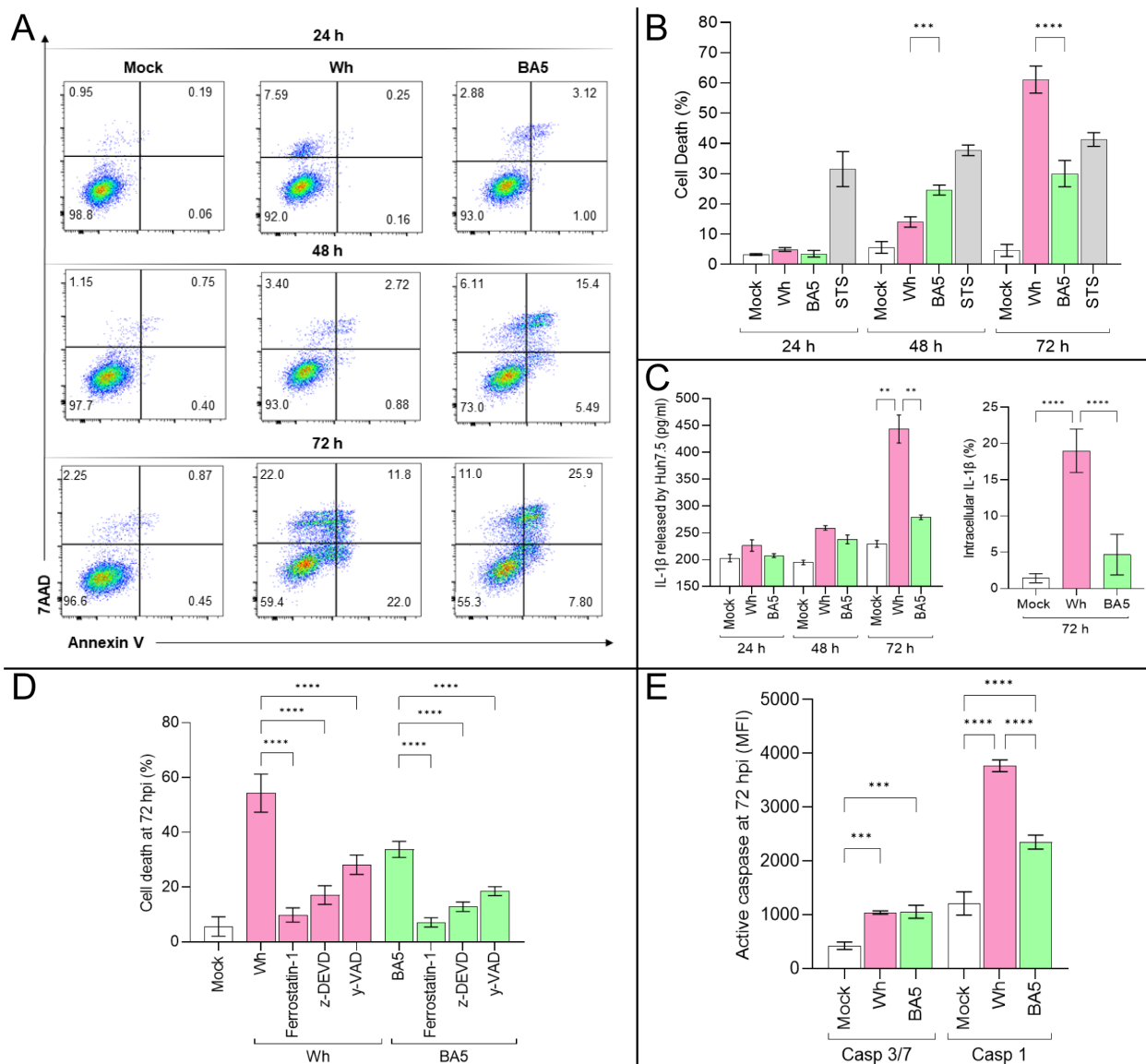


FIGURE 2

SARS-CoV-2 infection triggers regulated cell death pathways in Huh7.5 cells. (A, B) Quantification of cell death by Annexin V and 7-AAD staining at 24, 48, and 72 hpi in mock- and virus-infected cells. (C) IL-1 β levels released to Huh7.5 culture supernatant and intracellular were measured at 72 hpi by ELISA and flow cytometry, respectively. (D) Effects of specific inhibitors of apoptosis (z-DEVD-FMK), pyroptosis (Y-VAD-FMK), and ferroptosis (ferrostatin-1) on virus-induced cell death at 72 hpi. (E) Activity of caspase-3/7 and caspase-1 at 72 hpi in infected cells. Data are presented as mean $\pm$ SD from three independent experiments. Statistical analysis was performed using one-way ANOVA with Tukey's *post-hoc* test. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

+/MTDR- cells increased to $2.1 \pm 1.1\%$ (mock), $7.7 \pm 4.7\%$ (Wh), and $10.3 \pm 7.5\%$ (BA.5).

Lastly, at 72 hpi, the proportion of double-positive cells rose to $5.2 \pm 6.7\%$ (mock), $53.1 \pm 19.4\%$ (Wh), and $30.4 \pm 23.0\%$ (BA.5), whereas MTG+/MTDR- single-positive cells were detected at $1.3 \pm 0.4\%$ (mock), $20.9 \pm 7.0\%$ (Wh), and $17.2 \pm 8.3\%$ (BA.5). These results indicate a time-dependent and variant-specific modulation of mitochondrial dynamics in infected cells. Notably, the accumulation of MTG+/MTDR- cells, indicative of mitochondrial depolarization, coincided with viral replication, suggesting that mitochondrial functionality may be compromised in a variant-dependent manner (Figure 3C).

Despite the marked increase in mROS production, mitochondrial fragmentation occurred without a complete loss of $\Delta\Psi_m$, suggesting the activation of compensatory mechanisms such as mitophagy. To investigate this possibility, the colocalization of mitochondria with lysosomal markers was analyzed by confocal microscopy at 48 hpi. SARS-CoV-2-infected cells exhibited a significant increase in mitochondrial-lysosomal colocalization compared to mock-infected controls, as quantified using Mander's overlap coefficient. Notably, this colocalization was abolished when Huh7.5 cells were pretreated with the mitochondrial division inhibitor Mdivi, further supporting the involvement of mitochondrial fission in the activation of mitophagy (Figure 3D).

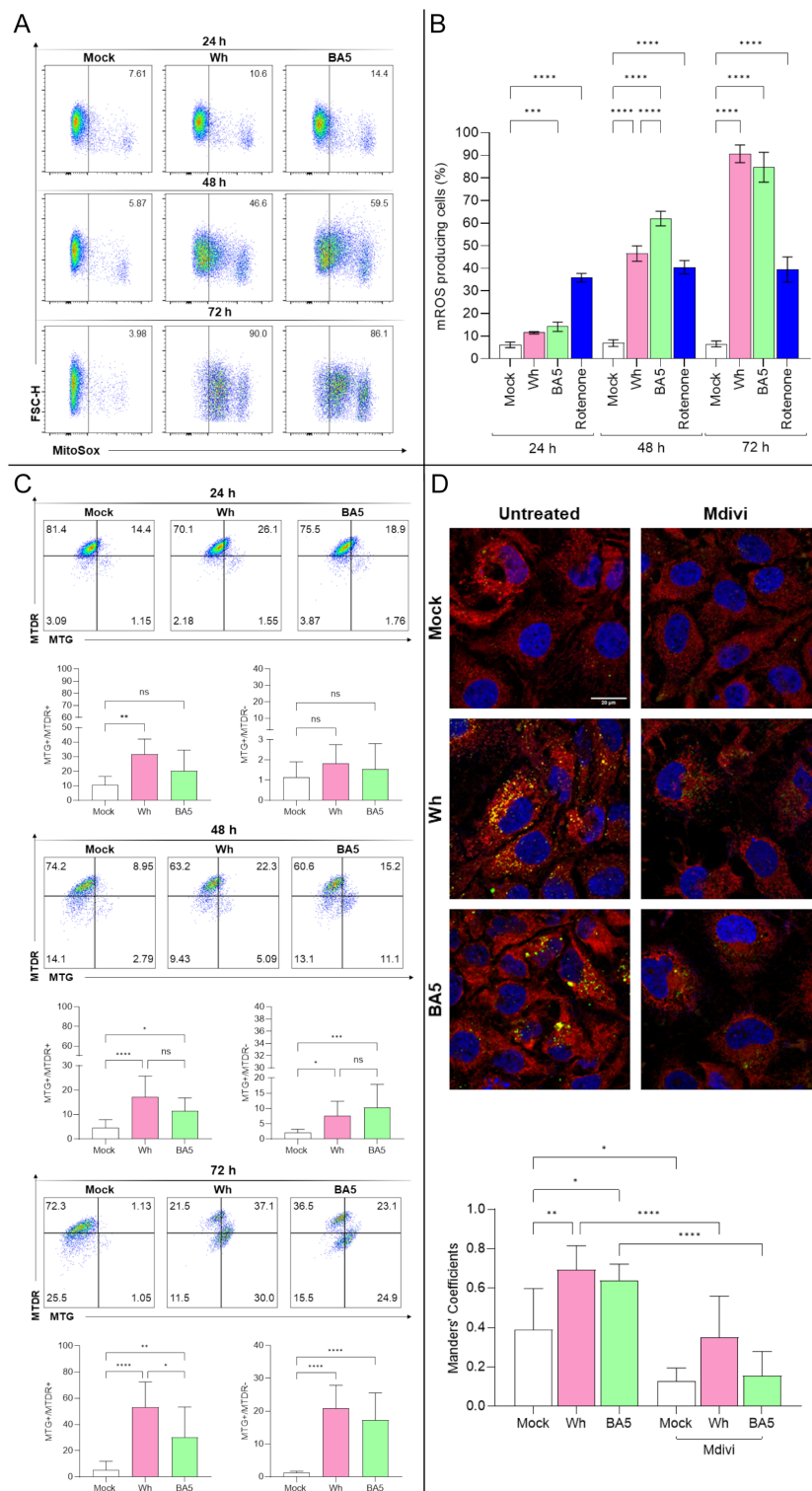


FIGURE 3

Mitochondrial dysfunction and mitophagy in SARS-CoV-2-infected hepatocytes. **(A, B)** Measurement of mitochondrial ROS (mROS) production in Huh7.5 cells infected with SARS-CoV-2 Wh or BA.5 at 24, 48, and 72 hpi using MitoSOX™ and flow cytometry. **(C)** Mitochondrial mass and membrane potential ($\Delta\Psi_m$) were assessed at 24, 48, and 72 hpi with MitoTracker™ Green (MTG) and Deep Red (MTDR) via flow cytometry. Representative dot plots are shown. **(D)** Confocal microscopy analysis of mitochondria-lysosome colocalization at 48 hpi. Mitochondria were stained with MitoTracker™ Red, lysosomes with LysoTracker™ Green, and nuclei with DAPI (blue). Colocalization was quantified using Manders' overlap coefficient. Pretreatment with Mdivi-1 (50 μ M) inhibited colocalization, indicating suppression of mitophagy. Data represent mean $\pm$ SD of three independent experiments. Statistical analysis was performed using one-way ANOVA with Tukey's *post-hoc* test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. ns, not significant.

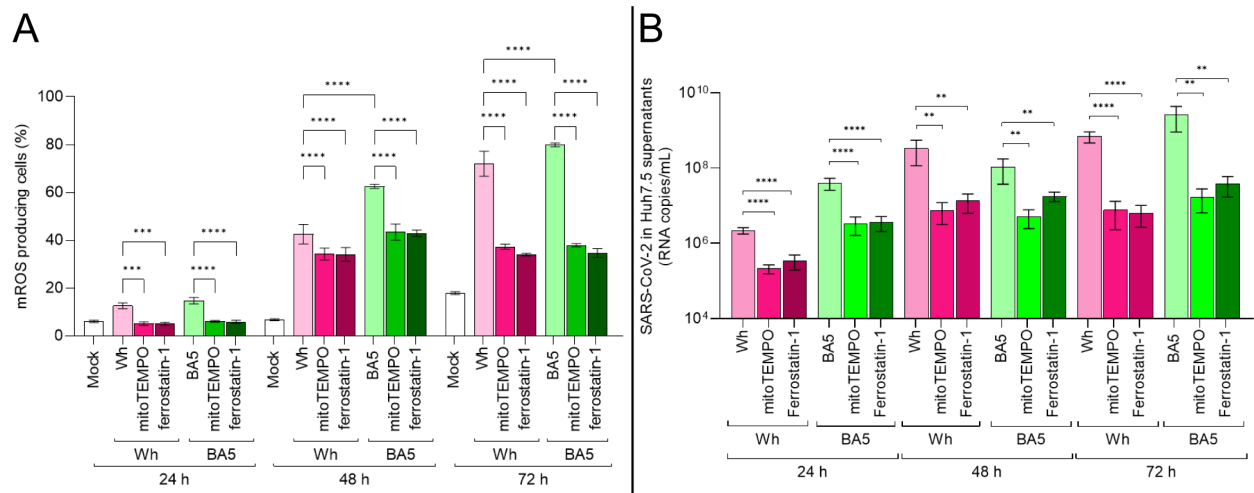


FIGURE 4
mROS scavenging impairs SARS-CoV-2 replication in hepatocytes. **(A)** mROS levels in infected Huh7.5 cells pretreated with MitoTEMPO (50 μ M) or ferrostatin-1 (10 μ M), measured at 24, 48, and 72 hpi by MitoSOXTM using flow cytometry. **(B)** Viral RNA copies in culture supernatants at each time point, quantified by RT-qPCR. Data show that antioxidant pretreatment significantly reduces mROS and viral replication in both Wh and BA.5 infections. Values are mean $\pm$ SD from three biological replicates. Statistical differences were evaluated using one-way ANOVA and Tukey's *post-hoc* test. ** p <0.01, **** p <0.0001.

These findings reveal a dynamic mitochondrial response in Huh7.5 cells infected with SARS-CoV-2, characterized by an early increase in the population of cells with polarized mitochondria and preserved mitochondrial mass. This suggests a transient adaptive phase, possibly supporting viral replication by maintaining cellular bioenergetics and redox balance. However, at 48- and 72-hours post-infection, we observed an accumulation of depolarized mitochondria with preserved mass, accompanied by increased mitochondrial ROS (mROS), indicating progressive mitochondrial dysfunction. By 72 hours, features compatible with mitophagy were evident, suggesting an attempt by the cell to eliminate damaged mitochondria.

3.5 mROS scavenging impairs SARS-CoV-2 replication in Huh7.5 cells

To assess the contribution of mitochondrial reactive oxygen species (mROS) to SARS-CoV-2 replication, Huh7.5 cells were pretreated with either the mROS scavenger mitoTEMPO or the lipid peroxidation inhibitor ferrostatin-1, and subsequently infected with the ancestral Wuhan (Wh) or Omicron BA.5 variants. Viral replication was evaluated by quantifying viral RNA in culture supernatants using real-time PCR at 24, 48, and 72 hpi.

As previously described (Figure 3B), SARS-CoV-2 infection is associated with a progressive increase in mROS levels from 24 to 72 hpi. As shown in Figure 4A, pretreatment with either antioxidant significantly reduced mROS accumulation by approximately twofold in infected cells, with similar effects observed for both viral variants. This reduction in oxidative stress was accompanied by a significant decrease in viral replication, with viral RNA levels in

antioxidant-treated cells reduced by up to 100-fold compared to untreated controls (Figure 4B). This inhibitory effect was observed at study time points and was most pronounced at 48 and 72 hpi, coinciding with peak mROS production in untreated infections. Notably, mitoTEMPO exerted an inhibitory effect on viral replication comparable to that of ferrostatin-1, regardless of the viral variant.

Together, these findings indicate that SARS-CoV-2 replication in Huh7.5 cells is closely linked to mROS generation and that pharmacological scavenging of mitochondrial ROS significantly impairs viral replication in a variant-independent manner.

3.6 SARS-CoV-2 infection modulates the lipid metabolism in Huh7.5 cells

Lipid synthesis, storage, and degradation are tightly regulated processes, with lipid droplets (LDs) functioning as dynamic organelles in infection, inflammation, and cellular homeostasis. To evaluate whether SARS-CoV-2 infection influences LD synthesis in Huh7.5 cells, cells were infected with the Wh and BA.5 variants (MOI=0.05). By using confocal microscopy, the LD accumulation was assessed at 24, 48, and 72 hpi. The results indicate that SARS-CoV-2 infection induces LD formation at 24 hpi, with sustained accumulation up to 72 hpi. The Wh variant induced significantly greater LD accumulation than BA.5 (Figures 5A, B).

Previous results demonstrated that ferroptosis contributes to regulated cell death in SARS-CoV-2-infected Huh7.5 cells. Moreover, disturbances in lipid metabolism are known to promote ferroptosis. Huh7.5 cells were pretreated with ferrostatin-1 for 24 hours before infection with either variant to

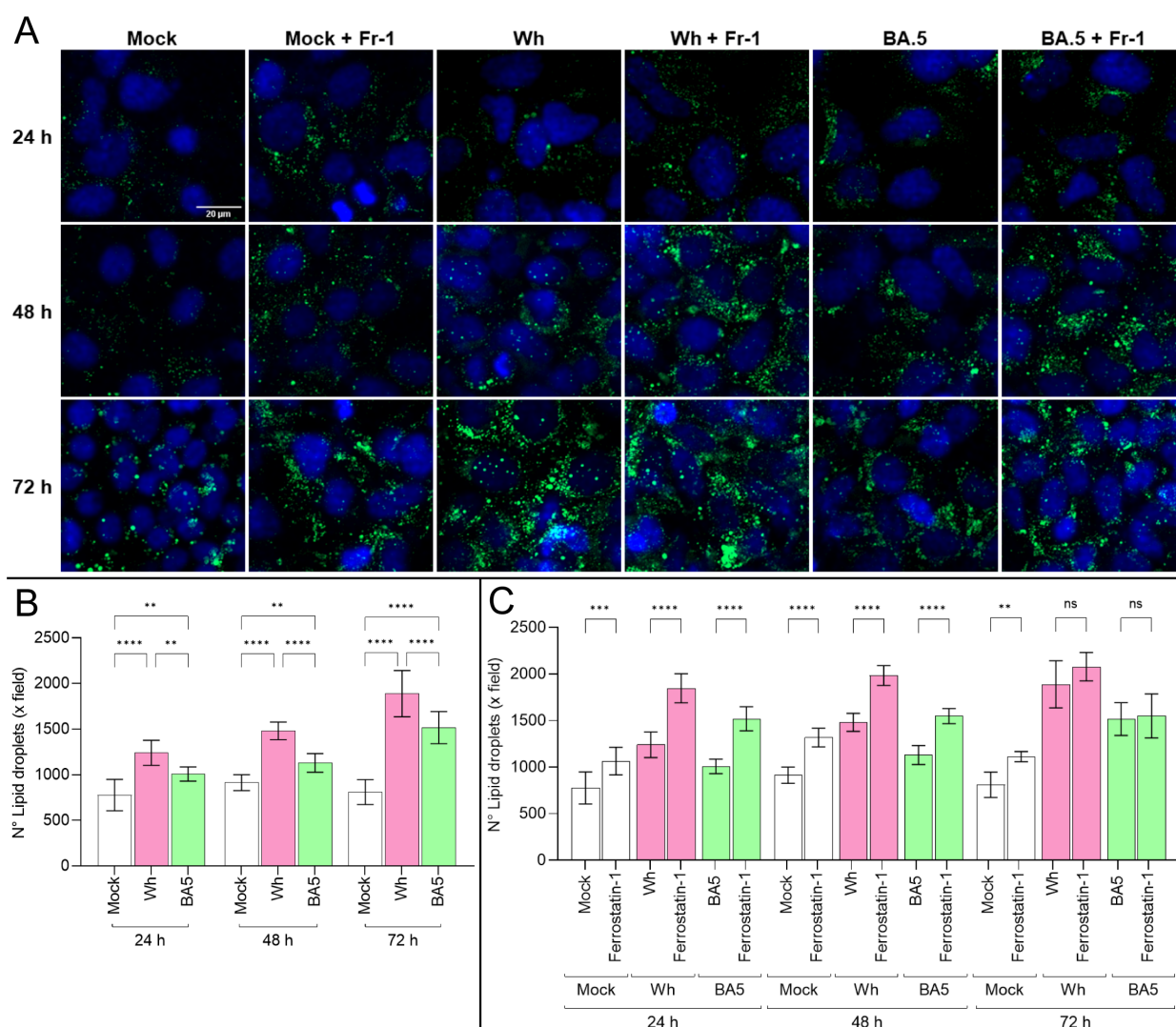


FIGURE 5

SARS-CoV-2 infection induces lipid droplet accumulation modulated by ferroptosis. **(A)** Representative confocal microscopy images of lipid droplet (LD) accumulation in Huh7.5 cells infected with Wh or BA.5 variants at 24, 48, and 72 hpi, stained with BodipyTM 493/503 (green). Nuclei were stained with DAPI (blue). **(B, C)** Quantification of LD accumulation in untreated and ferrostatin-1-treated cells over time. Ferrostatin-1 pretreatment increased LD accumulation at early time points (24 and 48 hpi), but this effect was no longer observed at 72 hpi. Data represent mean $\pm$ SD from three independent experiments. Statistical analysis was performed using one-way ANOVA. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. ns, not significant.

explore a potential link between lipid droplet dynamics and ferroptosis. Under these conditions, LD accumulation was more pronounced in ferrostatin-1-treated cells at both 24 and 48 hpi for both variants. However, by 72 hpi, no differences in LD levels were observed between treated and untreated cells (Figures 5A, C).

These findings suggest that SARS-CoV-2 infection rapidly induces lipid droplet formation in Huh7.5 cells, with a variant-specific temporal profile that may be influenced by ferroptosis-related mechanisms. The transient effect of ferrostatin-1 on LD accumulation highlights a potential interplay between lipid metabolism and ferroptotic pathways during infection. Altogether, these results support the notion that SARS-CoV-2 modulates host lipid homeostasis, potentially contributing to viral replication and pathogenesis.

4 Discussion

The present study provides new insights into the direct effects of SARS-CoV-2 infection on hepatocytes, highlighting the contribution of mitochondrial dysfunction and ferroptosis to virus-induced liver injury. Although COVID-19 is primarily characterized as a respiratory illness, extrapulmonary manifestations—including hepatic dysfunction—are increasingly recognized (Bertolini et al., 2020; Wang et al., 2020; Dufour et al., 2022; Luo et al., 2022; Zhang et al., 2022; Quarleri and Delpino, 2024). Consistent with recent reports (Pradhan et al., 2023; Ko et al., 2025), our findings demonstrate that hepatocytes are permissive to SARS-CoV-2 infection. Histopathological and virological analyses of human liver biopsy and autopsy samples provide strong evidence for hepatic

involvement in the clinical outcome of COVID-19 (Wanner et al., 2022; Maffia-Bizzozero et al., 2023; Oprinca et al., 2024; Rodriguez-Espada et al., 2024; Chen et al., 2025; Pita-Juarez et al., 2025; Sacco et al., 2025). Viral ancestral and Omicron variants modulate host cellular pathways to facilitate replication and promote cell death.

In line with a previous report (Foo et al., 2022), we observed a marked loss of mitochondrial membrane potential ($\Delta\psi_m$) while total mitochondrial mass remained preserved, suggesting an early phase of mitochondrial stress and dysfunction. Then, an increased colocalization of mitochondria with lysosomes further supports the activation of mitophagy as a potential compensatory response to infection-induced mitochondrial stress, indicating that damaged mitochondria were selectively targeted for degradation. This sequence of events—depolarization with maintained mass followed by mitophagy—is consistent with previous studies demonstrating that SARS-CoV-2 disrupts mitochondrial homeostasis to modulate host cell responses. In particular, viral proteins such as ORF9b have been shown to localize to mitochondria and interfere with mitochondrial dynamics and antiviral signaling pathways, promoting mitochondrial depolarization and autophagic clearance (Gordon et al., 2020; Singh et al., 2020; Bhowal et al., 2023; Shoraka et al., 2023). Similar observations of mitophagy following $\Delta\psi_m$ dissipation have been reported in other viral contexts, underscoring the conserved nature of this stress response (Li et al., 2022). These alterations indicate mitochondrial stress and align with prior reports that describe mitochondrial manipulation as a hallmark of SARS-CoV-2 pathogenesis (Shang et al., 2021; Yu et al., 2023). The correlation between mROS levels and viral replication suggests a potential feed-forward loop wherein oxidative stress promotes viral propagation, possibly by altering antiviral signaling or supporting biosynthetic demands (Singh et al., 2020).

In support of this, we found that pharmacologic scavenging of mROS with mitoTEMPO or ferrostatin-1 significantly impaired SARS-CoV-2 replication in Huh7.5 cells, as shown by reduced viral RNA levels in the culture supernatants. These findings underscore the functional relevance of oxidative stress in sustaining efficient viral replication. Notably, mitoTEMPO, by specifically targeting mitochondrial superoxide, produced a slightly greater reduction in viral load than ferrostatin-1, particularly with the BA.5. This suggests that mitochondrial ROS may play a more direct role in viral replication than lipid peroxidation, which are interconnected (Guarnieri et al., 2024; Xie et al., 2024). Importantly, antioxidant pretreatment blunted the progressive increase in mROS observed during infection, supporting the idea that SARS-CoV-2 hijacks mitochondrial redox signaling to support its life cycle. These data support that antioxidant strategies, particularly those targeting mitochondrial ROS, could be therapeutic adjuncts to limit viral replication and protect hepatocellular integrity (Gain et al., 2022; Galli et al., 2022; Guarnieri et al., 2024; Xie et al., 2024).

In addition, infection induced lipid droplet (LD) accumulation and lipid peroxidation—hallmarks of ferroptosis, a regulated cell death (RCD) pathway characterized by iron-dependent lipid damage (Endale et al., 2023; Wang et al., 2023a).

Clinical evidence increasingly supports a role for ferroptosis in the development of COVID-19. Elevated serum ferritin and malondialdehyde (MDA), which are key markers of iron overload and lipid peroxidation, have been consistently observed in hospitalized and critically ill patients (Qiu et al., 2024). This reflects increased oxidative stress and ferroptotic cell death (Chen et al., 2022; Jankauskas et al., 2023; Peleman et al., 2023). At the same time, reduced levels of glutathione (GSH) and glutathione peroxidase 4 (GPX4) have been found in patient plasma and tissues exposed to SARS-CoV-2. These substances are essential for maintaining redox balance and preventing ferroptosis (Li et al., 2023; Zhao et al., 2024). Notably, losing GPX4 has been linked to greater disease severity and a higher risk of long COVID (Qiu et al., 2024). These findings are further supported by studies showing that serum from non-survivors of COVID-19 triggers lipid peroxidation and reduces GPX4 in human endothelial cells (Jankauskas et al., 2023). Together, these results suggest that ferroptosis may lead to multi-organ injury in COVID-19 and point to it as a possible treatment target.

Interestingly, we observed a progressive enlargement of LD in infected Huh7.5, becoming increasingly prominent from 24 to 72 hours post-infection, with the largest droplets correlating with the peak of cell death at 72 hours. This temporal pattern suggests that LD expansion may be linked to the intensification of oxidative stress and ferroptotic cell death. The temporal association between LD accumulation and ferroptosis inhibition with ferrostatin-1 suggests a functional interplay between disturbed lipid metabolism and ferroptotic cell death pathways in hepatocytes (Wu et al., 2020). These observations extend recent findings in lung, heart, and CNS tissues (Dias et al., 2020; Alizadeh Saghati et al., 2024; Jia and Han, 2024; Qiu et al., 2024) and suggest that ferroptosis may also contribute to COVID-19-associated liver pathology (Chen et al., 2022).

Single-cell RNA sequencing studies have shown ACE2 expression in 1–14% of cholangiocytes and 0.3–10% of hepatocytes (Ko et al., 2025). Hepatic ACE2 expression is elevated in patients with nonfibrotic MASH and shows a positive association with age, liver fat content, and fibroinflammatory markers. Similarly, increased hepatic ACE2 levels are observed in COVID-19 patients with liver injury (Cano et al., 2024; Jacobs et al., 2024; Rodriguez-Espada et al., 2024). Here, we demonstrate that ACE2 and TfR1 expression increased following infection, with variant-specific differences. While ACE2 remains the primary entry receptor for SARS-CoV-2, TfR1 has been shown to mediate viral entry independently of ACE2, especially in tissues with low ACE2 expression (Wang et al., 2023b; Liao et al., 2024). The upregulation of TfR1 could enhance viral entry through endocytosis while supporting ferroptosis via disturbed iron homeostasis (Habib et al., 2021). This dual role implies a plausible convergence of entry mechanisms and cell death pathways that may exacerbate liver injury (Wu et al., 2022). Together, in SARS-CoV-2-infected hepatocytes, ferroptosis appears to be triggered by a synergistic interaction between the rise of mROS, TfR1 overexpression, and lipid droplet accumulation, thus playing a role in hepatocellular damage during SARS-CoV-2 infection.

As previously reported (Scheuermann et al., 2023), differences in replication kinetics and cell death between variants were also observed. The ancestral strain showed faster replication and induced greater cell death at later time points than BA.5. These discrepancies may stem from differences in viral entry routes, membrane fusion efficiency, or immune evasion strategies. Importantly, both variants activated caspase-dependent (apoptosis and pyroptosis) and caspase-independent (ferroptosis) pathways, reflecting a multifaceted RCD response shaped by mitochondrial and immunometabolic stress (Vabret et al., 2020).

While pathway-specific chemical inhibitors helped delineate these responses, it is important to recognize the inherent complexity and overlap among regulated cell death (RCD) mechanisms (Eskander et al., 2025). For example, caspase-3 activity (apoptosis), caspase-1 activation, and IL-1 β production (pyroptosis) were significantly elevated in infected cells. Nevertheless, inhibition of ferroptosis provided the most pronounced cytoprotective effect, reducing cell death by more than fivefold compared to untreated infected controls. Consequently, the data indicate that ferroptosis may play a prominent, though not exclusive, role, though additive or synergistic contributions from caspase-dependent pathways cannot be excluded.

Moreover, RCD pathways often share upstream signals, such as mitochondrial stress, ROS, and metabolic imbalance, that create extensive crosstalk (Marchi et al., 2012; Kist and Vucic, 2021). mROS, which was markedly increased during infection, can trigger ferroptosis and modulate caspase activity, illustrating the interdependency of these processes. Compensatory signaling may obscure the inhibition of one pathway when others remain active. Therefore, accurately quantifying the relative contribution of each RCD mechanism in the context of dynamic infection remains methodologically challenging, particularly in the absence of targeted genetic tools or single-cell resolution techniques.

Future studies employing time-resolved analyses, multi-parameter imaging, or integrative omics may better resolve the sequence and hierarchy of cell death events. Understanding this interplay is not only of mechanistic importance but may also inform therapeutic strategies to mitigate tissue damage by selectively modulating host responses.

Overall, this study underscores the relevance of regulated cell death, particularly ferroptosis, in SARS-CoV-2-infected hepatocytes and highlights the central role of mitochondrial dysfunction and oxidative stress in shaping disease outcomes. Considering the frequency of liver abnormalities in patients with COVID-19, our findings have significant implications for understanding the pathophysiology of extrapulmonary SARS-CoV-2 infection and identifying novel therapeutic targets. Pharmacologic modulation of mitochondrial ROS or ferroptosis may offer promising strategies to protect hepatic tissue during acute infection and reduce long-term sequelae in COVID-19 survivors.

Limitations of our study should be acknowledged. First, we employed the Huh7.5 hepatoma-derived cell line, which, while widely accepted as a hepatocyte model, does not fully capture the complexity of primary hepatocytes or the liver microenvironment.

The transformed nature of these cells may alter mitochondrial behavior and stress responses, potentially limiting generalizability. Second, although we demonstrated upregulation of ACE2 and TfR1 and highlighted the functional relevance of mROS and ferroptosis, we did not examine the upstream signaling pathways in depth. Third, the absence of *in vivo* validation limits our ability to assess immune-mediated effects or multicellular interactions that occur in liver tissue during infection. Fourth, Bodipy 493/503 mainly detects neutral lipids and cannot differentiate oxidized lipid species. As a result, it might underestimate the level of lipid peroxidation. Furthermore, measuring GPX4 enzymatic activity directly or quantifying specific lipid peroxidation markers like malondialdehyde (MDA) or 4-hydroxynonenal (4-HNE) would offer stronger evidence for the role of ferroptosis. Finally, while we analyzed two viral variants, expanding the scope to include emerging lineages would enhance the translational value of our findings.

Despite these limitations, our study provides novel insights into the mechanisms underlying SARS-CoV-2-induced liver injury and offers a foundation for future investigations using primary hepatocytes, organoid models, or *in vivo* systems. A deeper understanding of how viral replication intersects with host stress responses and immune signaling will be essential for developing effective interventions to prevent or treat liver damage in COVID-19 and related viral diseases.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

Ethical approval was not required for the studies on humans in accordance with the local legislation and institutional requirements because only commercially available established cell lines were used.

Author contributions

CC: Investigation, Conceptualization, Funding acquisition, Data curation, Formal Analysis, Methodology, Writing – original draft. PJ: Investigation, Formal Analysis, Software, Validation, Data curation, Methodology, Writing – review & editing. FS: Software, Formal Analysis, Data curation, Validation, Methodology, Investigation, Writing – review & editing. RF: Investigation, Validation, Formal Analysis, Writing – original draft, Methodology. CL: Methodology, Writing – original draft, Investigation, Formal Analysis, Software. MD: Funding acquisition, Investigation, Writing – review & editing, Writing – original draft, Formal Analysis, Data curation, Validation, Conceptualization, Methodology, Project administration, Supervision. JQ: Funding acquisition, Writing – review & editing,

Supervision, Investigation, Writing – original draft, Software, Validation, Project administration, Formal Analysis, Data curation, Conceptualization, Methodology.

Funding

The author(s) declare financial support was received for the research and/or publication of this article. This research was funded by Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT), PICT-2021-I-00587 (to C.C.), PICTO-2021-00005-COVID Secuelas (to J.Q. and M.V.D.), and by Fundación Florencio Fiorini (to J.Q., 2024).

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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