Hepatocyte-Hepatic Stellate Cell Axis in Potentiation of Alcohol and HIV-Induced Liver Injury

Moses O. New-Aaron
University of Nebraska Medical Center

Tell us how you used this information in this short survey.
Follow this and additional works at: https://digitalcommons.unmc.edu/etd

Part of the Toxicology Commons

Recommended Citation
https://digitalcommons.unmc.edu/etd/691

This Dissertation is brought to you for free and open access by the Graduate Studies at DigitalCommons@UNMC. It has been accepted for inclusion in Theses & Dissertations by an authorized administrator of DigitalCommons@UNMC. For more information, please contact digitalcommons@unmc.edu.
HEPATOCYTE-HEPATIC STELLATE CELL AXIS IN POTENTIATION OF ALCOHOL AND HIV-INDUCED LIVER INJURY

By

Moses O. New-Aaron

A DISSERTATION
Presented to the Faculty of
the University of Nebraska Graduate College
in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy
Environmental Health, Occupational Health & Toxicology
Graduate Program
Under the Supervision of Professors Natalia Osna
and Larisa Poluektova
University of Nebraska Medical Center
Omaha, Nebraska
October 2022

Supervisory Committee Members:
Eleanor Rogan, Ph.D.
Todd Wyatt, Ph.D
Murali Ganesan, Ph.D
ACKNOWLEDGMENT

First, my acknowledgment goes to the Almighty God and my Savior, Jesus Christ, the giver of life and inspiration required to complete this project.

I am eternally grateful to the numerous individuals who supported the initiation and completion of this work. I profoundly appreciate my mentor, Dr. Natalia Osna who worked tirelessly over the past four years to see me become the best version of myself. As this project focused on the combined effects of two hepatotoxic agents, I enjoyed adequate tutelage on HIV pathogenesis and designs of mouse-human chimeric models from Dr. Larisa Poluektova. She served as my co-mentor over the past four years.

Completion of this research and training would not have been realistic without the teachings and instructions of Dr. Murali Ganesan, who equipped me with adequate technical skills to perform this work.

As an awardee of the Ruth L. Kirschstein National Research Service Award (NIH F31 Predoctoral Award), I extend my gratitude to NIAAA for funding my research over the past two years.

Moreover, I recognize the American College of Toxicology for the 2-year North American Graduate Fellowship and Support during the research/training period.

I acknowledge my doctoral supervisory committee members, Drs. Eleanor Rogan and Todd Wyatt stood firmly by me before enrolling in the Ph.D. program. They selflessly
recommended me to the proper laboratory and mentors. Without these two great professors, my career path may not be as smooth as it is today.

My special thanks go to Drs. Carol Casey, Kusum K. Kharbanda, Paul G. Thomes, Raghu S. Dagur, Madan Kumar, and Terrence Donohue. Most of my experimental models worked efficiently because I stood on the shoulders of these scientific giants.

I enjoyed a tranquil and friendly work environment because of the fantastic laboratory members (Ms. Moji Ogunnaike, Ms. Grace Bybee, Ms. Haritha Chava, and Dr. Siva S. Koganti) I worked with over the past four years.

This acknowledgment will be incomplete if I fail to recognize Mr. Edward Makarov, Dr. Saumi Matthew, and Dr. Weimin Wang. They provided immense help and support for studies on mouse-human chimeric models.

Finally, I appreciate my family for their unalloyed support. My wife, Temitope New-Aaron, was consistently available to look after our three boys and the home despite her busy schedule as a clinical laboratory scientist. I say thank you to our children, Ivan, Haven, and Bezalel, who endured many hardships because of this training!

My late Dad, Wesley Aaron, whose words inspired me to be the best I can ever be, and my mum, Comfort Aaron, and my sister, Oluwabunmi Adeleke, who never ceased to pray for me, I say thank you!
My mother-in-law, Mrs. Folasade Akintan; my sister-in-law and her husband, Dr. and Dr. Olubiya; and my brothers-in-law (Mr. Olumide Akintan and Mr. Oluwabunmi Akintan), your cheers were always the trigger to keep going!
HEPATOCLYTE-HEPATIC STELLATE CELL AXIS IN POTENTIATION OF
ALCOHOL AND HIV-INDUCED LIVER INJURY

Moses O. New-Aaron

University of Nebraska Medical Center, 2022

Supervisors: Natalia A. Osna, Ph.D. and Larisa Y. Poluektova, Ph.D.

Human Immunodeficiency Virus (HIV) remains a global threat, with approximately 38.4 million active infections and 33 million HIV-related deaths since the first case. While many may be tempted to think of HIV as a relic of the past, emerging data suggests otherwise. By the end of 2021, approximately 1.5 million HIV incidences and 650 000 mortalities were reported globally. The etiologies of HIV-related mortality are numerous. Liver disease is one of the leading etiologies of HIV-related mortality, especially in recent times of antiretroviral therapy (ART)-induced longevity among people living with HIV (PLWH). While co-infections with hepatotropic viruses notoriously contribute to the frequently observed liver disease among HIV-infected individuals, alcohol abuse is another significant trigger of liver disease. In fact, alcohol hepatitis is among the knottiest liver disease etiologies because the liver is not just a significant target for alcohol. Still, alcohol abuse is twice more frequent among PLWH than in HIV-uninfected individuals. Recent work from our group has shown that in the absence of ART, alcohol metabolites enhance HIV accumulation in hepatocytes, which consequently induces massive oxidative hepatocyte apoptosis. Internalization of HIV-and-malonaldehyde (MDA)-containing
hepatocyte apoptotic bodies (AB) by hepatic stellate cells (HSC) induces profibrotic activation which leads to a progression of liver fibrosis.

The pathomechanisms of alcohol-induced liver injury among PLWH are yet to be understood. Hence, this dissertation was focused on deciphering the mechanisms of alcohol and HIV-induced hepatocyte apoptosis (Chapter 2) and the subsequent HSC activation after the internalization of MDA-and HIV-containing AB (Chapter 3). To ameliorate alcohol and HIV-induced liver injury, an anti-fibrotic drug, obeticholic acid (OCA) was employed (Chapter 4). Our findings indicate that metabolically generated acetaldehyde damages lysosomes and likely prevents their repair and restoration, thereby leading to hepatocyte apoptosis (Chapter 2). Hence, this results in the formation of HIV-and MDA-containing hepatocyte apoptotic bodies internalized by HSC through the AXL receptor. The consequent HSC profibrotic activation was mediated by ROS-dependent JNK-ERK1/2 and IL6 triggering of JAK-STAT3 pathways (Chapter 3). While these mechanisms explained liver fibrosis frequently observed among alcohol-abusing PLWH, in an *in vitro* model, OCA attenuated profibrotic activation of HSC via the JAK-STAT3 pathway (Chapter 4).
Contents

HEPATOCYTE-HEPATIC STELLATE CELL AXIS IN POTENTIATION OF ALCOHOL AND HIV-INDUCED LIVER INJURY .............................................................................................................................. i

ACKNOWLEDGMENT ........................................................................................................ ii

HEPATOCYTE-HEPATIC STELLATE CELL AXIS IN POTENTIATION OF ALCOHOL AND HIV-INDUCED LIVER INJURY .............................................................................................................................. v

LIST OF FIGURES ............................................................................................................... xv

LIST OF TABLES ................................................................................................................ xx

LIST OF ABBREVIATIONS .................................................................................................. xxi

LIST OF PUBLICATIONS RELATED TO THIS WORK ........................................... xxiv

CHAPTER 1: INTRODUCTION ......................................................................................... 1

Epidemiology of Liver Injury Among People Living with HIV .............. 1

Risk Factors of Liver Disease Among HIV-Infected Individuals ......... 3

HIV co-infections with hepatotropic viruses .............................................. 5
ART-induced liver damage.................................................................10

Alcohol-induced liver damage among HIV-infected individuals 11

Project Goals and Specific Aims..........................................................15

Project Significance...........................................................................22

CHAPTER 2: ALCOHOL-INDUCED LYSOSOMAL DAMAGE AND
SUPPRESSION OF LYSOSOME BIOGENESIS CONTRIBUTE TO
HEPATOTOXICITY IN HIV-EXPOSED LIVER CELLS .........................26

Introduction.......................................................................................26

Materials and Methods......................................................................28

Reagents and Antibodies....................................................................28

In Vitro Studies....................................................................................29

RNA Isolation and Real-Time PCR (RT-PCR) .................................30

Proteasome Activities.......................................................................30

Cathepsin B and L Activities..............................................................31

Whole-Cell Lysates and Nuclear/Cytosolic Fractionations ..........31
Immunoblotting ........................................................................................................... 31

Immunofluorescence ................................................................................................... 31

Statistical Analyses ...................................................................................................... 32

Results ......................................................................................................................... 33

CCR5 mediates HIV entry into hepatocytes ................................................................. 33

AGS and HIV Exposure Lowers Cathepsin Activities and Lysosome Numbers ............ 35

Both AGS and HIV Enhance Lysosome Leakage ......................................................... 37

AGS and HIV Enhance Intracellular Oxidant Stress .................................................... 39

AGS and HIV Enhance Cathepsin Leakage and Intrinsic Apoptosis ......................... 40

BAX interaction with DRAM1 contributes to AGS+HIV-induced lysosome permeabilization ................................................................. 42

AGS and HIV Exposure Suppress TFEB Protein Expressions in Cytosolic and Nuclear Fractions of RLW Cells ................................................................. 43
AGS and HIV Exposures Suppress Proteasome Activity and Enhance ZKSCAN3 Expression .................................................46

Chronic Ethanol feeding and HIV Infection Damage Lysosomes in Livers of Humanized Mice.............................................48

Discussion ........................................................................................................................................................................50

Conclusion ...........................................................................................................................................................................54

CHAPTER 3: ALCOHOL AND HIV- DERIVED HEPATOCYTE APOPTOTIC BODIES INDUCE HEPATIC STELLATE CELL ACTIVATION ................................................................................................................56

Introduction ........................................................................................................................................................................56

Materials and Methods ........................................................................................................................................................58

Reagents and Antibodies ......................................................................................................................................................58

In Vitro Studies ....................................................................................................................................................................59

Isolation of ABs from RLW Cells .....................................................................................................................................61

Generation of TAMRA-Labeled RLW ABs by Prolonged Incubation ...............................................................................61
siRNA Transfection of LX2 Cells .................................................62

Imaging of ABs by Transmission Electron Microscopy ..................62

In Vivo Studies .............................................................................63

RNA Isolation and RT-PCR ..........................................................64

Immunoblotting ............................................................................65

Immunofluorescence .................................................................65

Statistical Analyses ......................................................................66

Results ........................................................................................66

HIV RNA, HIV Proteins and Malondialdehyde Were Expressed
by RLW AB_{AGS+HIV} ................................................................66

Cytochrome p450 2E1 was expressed by RLW AB_{AGS+HIV} ........69

Engulfment of RLW ABs by LX2 Cells .........................................69

Pharmacological Inhibition of Axl Blocks LX2 Engulfment of RLW
ABs and Attenuates Activation of Profibrotic Genes ....................72

Engulfment of HIV- and MDA-Containing ABs Induces LX2
Profibrotic Activation ..................................................................74
Pharmacological Inhibition of LX2 Cells Exposed to AB\textsubscript{AGS+HIV} Attenuates Profibrotic Activation via JNK and ERK1/2 Pathway 78

Oxidative Stress from AB\textsubscript{AGS+HIV} Activates JNK and ERK1/2 Pathway in LX2 Cells ........................................................................................................... 81

AB\textsubscript{AGS+HIV} Upregulates IL6 mRNA in LX2 Cells ........................................ 83

siRNA STAT3 Transfection Inhibits STAT3 Protein Expressions in LX2 Cells ................................................................................................................................. 84

Silencing STAT3 in LX2 Cells Attenuates AB\textsubscript{AGS+HIV} Induced Profibrotic Activation .................................................................................................................. 87

In Vivo Effects of HIV Containing-Apoptotic Bodies on Ethanol-Fed Mice ................................................................................................................................. 88

Discussion .......................................................................................................................... 90

Conclusion .......................................................................................................................... 93

CHAPTER 4: OBETICHOLIC ACID ATTENUATES HUMAN IMMUNODEFICIENCY VIRUS/ALCOHOL METABOLISM-INDUCED PROFIBROTIC ACTIVATION IN LIVER CELLS ........... 95
Introduction .......................................................................................................................... 95
Materials and Methods ........................................................................................................ 97
Reagents and media .............................................................................................................. 97
Cells and treatments ............................................................................................................ 98
HIV RNA, HIV DNA, integrated HIV DNA, HIV proteins and reactive oxygen species ................................................................................................................................. 98
Apoptosis measurements .................................................................................................... 98
Activities of proteasome and cathepsins ............................................................................. 99
Hepatic stellate cells and treatments with AB ........................................................................ 99
Statistical analyses .............................................................................................................. 99
Results .................................................................................................................................. 99
OCA attenuates AGS-HIV-induced apoptotic cell death and oxidative stress .......................................................... 100
OCA suppresses accumulation of HIV in hepatocytes ....................................................... 101
OCA reduces pro-fibrotic activation of HSC by engulfment of HIV-containing apoptotic hepatocytes .......................................................... 105
OCA reduces profibrotic activation of HSC partly via the JAK-STAT3 pathway ......................................................................................106

Discussion ................................................................................................................. 108

Conclusion ................................................................................................................... 110

CHAPTER 5: DISCUSSION ......................................................................................... 111

Findings Relative to Specific Aim 1 (Chapter 2) ............................................. 111

Findings Relative to Specific Aim 2 (From Chapter 3) ............................... 117

Findings Relative to Specific Aim 3 (From Chapter 4) ............................... 125

Future directions ...................................................................................................... 127

Conclusion ................................................................................................................... 130

BIBLIOGRAPHY ...................................................................................................... 130
LIST OF FIGURES

Figure 1: Risk factors of liver disease among HIV-infected individuals .......................................................... 4

Figure 2: Schematics of alcohol metabolism in hepatocytes. EtOH:
Ethanol, Ach: .................................................................................................................................................. 15

Figure 3: Scheme of crosstalk between hepatocyte and hepatic stellate cells.................................................................................................................................................. 21

Figure 4: Effects of AGS on CCR5 co-receptor.................................................................................................. 34

Figure 5: Acetaldehyde Generating System (AGS) exposure lowered cathepsin activities and lysosome numbers in HIV-infected and -uninfected RLW cells ........................................................................................................................................... 37

Figure 6: AGS and HIV enhance lysosomal leakage in RLW cells treated as indicated in Materials and Methods ............................................................................................................................................... 38
Figure 7: N-acetyl cysteine treatment prevents/reverses AGS and HIV-induced lysosomal leakage. .................................................................39

Figure 8: AGS and HIV enhance cathepsin B-triggered caspase 3-dependent apoptosis. ..................................................................................41

Figure 9: AGS and HIV enhanced the interaction of DRAM1 and BAX. ........................................................................................................43

Figure 10: Suppression by AGS and HIV of transcription factor EB (TFEB) protein expression in cytosolic and nuclear fractions of RLW cells...........................................................................................................45

Figure 11: AGS and HIV enhance ZKSCAN3 but suppress TFEB target genes. ..................................................................................................47

Figure 12: Effect of acute-on chronic ethanol feeding on LAMP1 protein expression, oxidative stress, cathepsin and proteasome peptidase activities in livers of HIV-infected and uninfected humanized mice ........................................................................49
Figure 13: Role of lysosomal rupture/dysfunction in HIV- and ethanol-metabolism-induced apoptosis in hepatocytes .................................................. 54

Figure 14: Characterization of ABs derived from acetaldehyde and HIV-exposed RLW .............................................................................................................. 68

Figure 15: ABs derived from acetaldehyde and HIV-exposed RLW cells ................................................................................................................................. 69

Figure 16: HSC do not express TIM family protein receptors. .............. 71

Figure 17: Gas6 and Protein S mediated engulfment of RLW ABs by the AXL-expressing LX2 cells .................................................................................................................. 72

Figure 18: AXL inhibitor attenuates HIV RNA expression and profibrotic genes in LX2 cells exposed to ABAGS+HIV. ................................. 73
Figure 19:Engulfment of ABAGS+HIV enhances HIV RNA and ROS in LX2 cells leading to their profibrotic activation. ...............................76

Figure 20:ABs derived from acetaldehyde and HIV-exposed RLW cells..............................................................................................................77

Figure 21:ABs derived from acetaldehyde and HIV-infected Jurkat cells did not activate HSCs ........................................................................................................78

Figure 22:Attenuation of profibrotic markers in LX2 cells exposed to ABAGS+HIV by pharmacological inhibition of JNK pathway..............80

Figure 23:Pharmacological inhibition of p38 MAPK induces ERK activation followed by SMA deposition: RT-PCR quantification of...81

Figure 24:NAC treatment reverses or prevents profibrotic activation of LX2 cells by inhibition of ERK1/2 pathway .................................82
Figure 25: engulfment of ABAGS+HIV attenuates STAT1 activation and concurrently upregulates IL6 mRNA, leading to profibrotic activation. 

Figure 26: SiRNA transfection of LX2 cells.

Figure 27: STAT3-deficient LX2 cells show downregulation of profibrotic genes and α-SMA after exposure to ABAGS+HIV.

Figure 28: Ethanol potentiates collagen deposition and liver damage in the liver of mice treated with HIV-containing apoptotic bodies.

Figure 29: HSC internalization of ABs generated from alcohol- and HIV-exposed hepatocytes induced ROS and IL6-mediated HSC activation.

Figure 30: Obeticholic acid attenuates apoptosis in acetaldehyde-generating system-human immunodeficiency virus-exposed Huh7.5-CYP cells.
Figure 31: Obeticholic acid suppresses human immunodeficiency virus expression in Huh7.5-CYP cells exposed to AGS+HIV.

Figure 32: Restorative effects of Obeticholic acid on proteasomal and lysosomal activities in AGS+HIV-exposed Huh7.5-CYP cells.

Figure 33: Anti-fibrotic effects of Obeticholic acid on hepatic stellate cells activated by engulfment of ABAGS+HIV.

Figure 34: Obeticholic acid suppressed pSTAT3 in LX2 cells exposed to ABAGS+HIV.

LIST OF TABLES

Table 1. Endpoints of the current study are regulated by AGS only or by the combination of AGS/ethanol with HIV.
LIST OF ABBREVIATIONS

PLWH- People Living with HIV

HIV-Human Immunodeficiency Virus

ART-Antiretroviral therapy

MDA-Malondialdehyde

AB-Apoptotic Bodies

HSC-Hepatic Stellate Cells

OCA-Obeticholic Acid

JNK- c-Jun N-terminal Kinase

ERK1/2- Extracellular signal-regulated kinase ½

JAK-Janus Kinase

STAT3-Signal Transducer and Activator of Transcription 3

IL6-Interleukin-6

ROS-Reactive Oxygen Species

AIDS-Acquired immune Deficiency Syndrome

HAV-Hepatitis A Virus

CCR5-C-C Chemokine Receptor Type 5
CXCR4-C-X-C Chemokine Receptor Type 4

CD4- Cluster Differentiation 4

HBV- Hepatitis B Virus

HCV- Hepatitis C Virus

DAA- Direct Antiviral Agent

PAMPs- Pathogen Associated Membrane Patterns

DAMPs- Damage Associated Membrane Patterns

LAMP1- Lysosome Associated Membrane Protein 1

TFEB- Transcription Factor EB

GAL3- Galectin 3

TAMRA- 5-carboxy-tetramethylrhodamine

AB\textsubscript{AGS+HIV} – Apoptotic bodies derived from hepatocytes exposed to AGS and HIV

RT PCR- Real-Time Polymerase Chain Reaction

RNA- Ribonucleic Acid

DNA- Deoxyribonucleic Acid

AGS- Acetaldehyde Generating System

DRAM1- Damage Regulated Autophagy Modulator 1

ZKSCAN3- Zinc Finger With KRAB And SCAN Domain
TP53- Tumor Suppressor Protein 53

COL1A1- Collagen Type I Alpha 1 Chain

TGFβ- Transformation Growth Factor Beta

TIMP- Tissue Inhibitors of Metalloproteinases

MMP- Matrix Metalloproteinase

SOCS - Suppressor of Cytokine Signaling

CYP2E1- Cytochrome P450 2E1

ADH- Alcohol Dehydrogenase

NAD- Nicotinamide Adenine Dinucleotide

PBS- Phosphate Buffered Saline

ELISA- Enzyme Linked Immunosorbent Assay

DMEM - High-glucose Dulbecco’s modified eagle medium

PCI- Pan caspase Inhibitor

TBP- Tata Binding Proteins
LIST OF PUBLICATIONS RELATED TO THIS WORK


CHAPTER 1: INTRODUCTION

Epidemiology of Liver Injury Among People Living with HIV

The human immunodeficiency virus (HIV)/acquired immune deficiency syndrome (AIDS) epidemic was first reported in 1981 (Times, 1981), and it became the deadliest epidemic in the history of humanity. At the early stage of the epidemic, an increasing number of young homosexual males who became immunodeficient and developed pneumocystis pneumonia and Kaposi sarcoma were observed (Gottlieb et al., 1981). Consequently, the disease was called gay-related immune deficiency syndrome, GRIDS (Sanford, 1997). However, the name of the disease, which was characterized by immunosuppression was later changed to AIDS when cases began emerging among heterosexual males and females, injection drug users, hemophiliacs, and children (Platt and Platt, 2013). One salient observation among the early AIDS cases was the elevated mortality rates. For example, the number of deaths due to AIDS increased from 451 to 50628 between 1981 and 1991 (Center for Disease Control and Prevention, 2011). This observation heralded the AIDS pandemic, which has engendered approximately 33 million deaths (Lancet, 2021). A retrovirus called HIV was identified as AIDS’ causative agent after three years of the first case.

Meanwhile, AIDS remained without a cure or vaccine despite the audacious proclamation of Dr. Robert Gallo of the National Cancer Institute (NCI) about the promise of an HIV vaccine within five years of identifying the virus. While no HIV
vaccine or cure was discovered, antiretroviral therapy (ART) which has effectively reduced HIV transmission and enhanced survival emerged in 1996. ART availability and accessibility have completely decimated AIDS in many parts of the world. Meanwhile, the paradox in the potency and effectiveness of ART is the dramatic decline in AIDS-related mortality and sudden elevation of non-AIDS related mortality among people living with HIV (PLWH). This is possible because ART increases the survival of PLWH, hence, allowing the incidence of non-AIDS related comorbidities. In fact, the positive impact of ART for the survival of PLWH has transitioned HIV from an infectious disease to a chronic disease (Perazzo and Luz, 2017). Commonly observed non-AIDS-related chronic comorbidities among PLWH are liver disease (Turner et al., 2010, Sherman et al., 2015), cardiovascular disease (Triant et al., 2007, Sudano et al., 2006, Currier, 2021), and pulmonary disease (Crothers et al., 2006, Crothers et al., 2011).

In addition, abnormal liver enzymes indicative of liver injury were reported among PLWH (Sterling et al., 2008). Moreover, liver disease in PLWH accounts for 18% of all-cause mortality, the second leading cause of death among PLWH (Kaspar and Sterling, 2017a, Huerta et al., 2019). A hospital-based cross-sectional study conducted among Nepalese HIV seropositive patients observed elevated liver enzymes in more than 56% of its patients (Jha and Sah, 2017). Another study in an HIV endemic area found 49% elevated liver enzymes among PLWH after 24 weeks of follow-up (Abongwa et al., 2022). This was supported by another study which observed elevation of at least one of the liver enzymes among 45% of HIV-positive participants after 35 months of follow-up.
(Mugusi et al., 2019). A study in Ethiopia observed 32% liver injury among PLWH (Mulu et al., 2013). Due to the commonly observed elevated prevalence of liver injury among PLWH in different settings, it is imperative to explore its risk factors.

**Risk Factors of Liver Disease Among HIV-Infected Individuals**

While HIV by itself is a known etiology for liver disease, the mechanism for HIV-induced liver injury is not fully understood. Meanwhile, cluster differentiation (CD)4-negative hepatocytes are susceptible to HIV due to their C-C chemokine receptor type (CCR) 5 or C-X-C chemokine receptor type (CXCR)4 (HIV entry co-receptor) expressions (Saracino et al., 2018). In fact, HIV proteins and nucleic acids (RNA and DNA) have been detected in hepatocytes (Cao et al., 1992, Ganesan et al., 2019b). Although, information on HIV replication in hepatocytes is limited. Kong et al. recently provided evidence of low-level replication of HIV in hepatocytes (Kong et al., 2012a). HIV entry and replication in hepatocytes were characterized by another study (Xiao et al., 2008b). Ganesan et al. detected low-level HIV proteins in hepatocytes, which was described as HIV accumulation rather than replication (Ganesan et al., 2019b).

Given that hepatocytes are canonically non-permissive to HIV, it is not unexpected that hepatocytes would only support low-level HIV expression. However, the low-level HIV in hepatocytes may trigger liver injury in the presence of a second hit. These second hits are classified as pathological risk factors for the incidence and progression of liver injury among PLWH. Identifying these risk factors is essential to understand the mechanisms
of liver damage among PLWH. Moreover, sociodemographic characteristics may contribute to the incidence of liver injury among PLWH. For example, being male and having low income have been identified as the demographic risk factors for liver injury (Abongwa et al., 2022, Androutsakos et al., 2020, Wekesa et al., 2020, Nkhoma et al., 2010). However, the sociodemographic factors' effects are less severe compared to the pathological risk factors described in Figure 1.

Figure 1: Risk factors of liver disease among HIV-infected individuals
HIV co-infections with hepatotropic viruses

HIV/HAV co-infections

Hepatitis A virus (HAV) is a highly infectious foodborne virus that targets the liver (Wang et al., 2015). While HAV infection may not result in chronic liver disease, it results in acute liver failure (World Health Organization, 2022a). Since HAV is known to cause mild illnesses (Previsani et al., 2003), many cases go unreported. In spite of this, approximately 150 million infections have been reported annually around the globe (Jefferies et al., 2018). The transmission mode for HAV is basically via the fecal-oral route. However, sexual activities, especially among homosexual men who also have higher rates of HIV infections, account for some cases of HAV transmission. Hepatitis A, which is quickly resolved in immunocompetent individuals, becomes very detrimental to the liver of PLWH. Therefore, hepatitis A infections are very significant among PLWH.

The prevalence of hepatitis A infections among PLWH varies across different settings. A study in the Middle East detected HAV antibodies in 96% of PLWH (Davoudi et al., 2010). Another study in South America found HAV antibodies in 72% of PLWH (Fainboim et al., 1999). Moreover, a European multicenter study detected HAV antibodies in 74% of PLWH (González-García et al., 2005). While the aforementioned studies represent the HIV-infected adult population, the lower prevalence (34%) of hepatitis A infections has been reported among the population of HIV-infected children (Gouvêa et al., 2005). Hepatitis A-induced liver injury is rare due to the availability of
the hepatitis A vaccine. While reduced vaccine-elicited immune response may provide setbacks for the efficiency of the hepatitis A vaccine, immunoglobulin therapy is available for treatment (Ojeda-Martinez et al., 2021, Center for Disease Control and Prevention, 2020b).

**HIV/HBV co-infections**

The Hepatitis B virus (HBV) is a significant member of hepatotropic viruses commonly identified among PLWH (Ganesan et al., 2019c). Approximately 296 million people are HBV infected, with 1.5 million annual new infections globally (Center for Disease Control and Prevention, 2021b). HBV infections often result in complicated liver damage. In fact, more than 50% of hepatocellular carcinoma (HCC) is due to HBV (Venook et al., 2010, El–Serag and Rudolph, 2007). Overlapping transmission routes between HIV and HBV may account for co-infections (Sharma et al., 2018). More than 10% of PLWH are coinfected with HBV globally (Utsumi and Lusida, 2015, Kaspar and Sterling, 2017b, Kourtis et al., 2012). However, this may differ across various regions of the world. For example, more than 15% of PLWH are coinfected with HBV in Africa (World Health Organization, 2011), 5% in Europe and North America, and more than 20% in Asia (Soriano et al., 2006). While HBV mono-infection typically damages the liver, this effect is more severe when co-infected with HIV. This may be due to HIV-induced immunosuppression (Parvez, 2015, Lewin et al., 2009, Thio, 2003, Hadler et al., 1991). For example, a study conducted in Tanzania reported that HIV/HBV-coinfected
patients were approximately four times more likely than HIV mono-infected patients to show an indication of liver injury (Hawkins et al., 2017). In addition, HBV is known to potentiate the progression of liver damage from other assaults. For example, HBV has been shown to increase ART-induced hepatotoxicity among PLWH (Hoffmann et al., 2008, Chun et al., 2012, Sulkowski et al., 2002, van den Berg et al., 2009). Hepatitis Delta Virus (HDV, associated with HBV) co-infections are another example. Although HDV is a hepatotropic virus, it occurs only when there is an active HBV infection (Center for Disease Control and Prevention, 2020a). HBV/HDV co-infection induces more liver damage than HBV mono-infection (Smedile et al., 1982, Pasetti et al., 1988, Raimondo et al., 1982). Moreover, triple infections (HIV/HBV/HDV) cause even more severe liver damage (Motamedifar et al., 2015b). HIV/HBV/HDV is uncommon in the US as HDV is not even one of the notifiable diseases (Center for Disease Control and Prevention, 2021a), and the prevalence of triple infections in the US is unknown. However, its prevalence in Asia and Africa ranges from 2 to 19% (Motamedifar et al., 2015a, Chambal et al., 2017, Salpini et al., 2016, Coffie et al., 2017). HBV may undergo gradual resolution as an etiologic factor for hepatotoxicity among PLWH. A decrease in the prevalence of HBV or HIV/HBV co-infection have been observed over time. As reported, the prevalence of HBV during 1999-2000 is 5.7% and 4.3% during 2015-2018 (Center for Disease Control and Prevention, 2020c). Interestingly, in a Korean HIV cohort, the rate of HBV co-infection among HIV patients born during the pre-HBV vaccine era was 8.8%. In comparison, HBV co-infection among HIV patients born during the HBV vaccine era
was 0% (Kim et al., 2020). This linear HBV decline among HIV patients and a consequent decrease in HBV-induced hepatotoxicity may be supported by increased HBV vaccination among PLWH (Catherine and Piroth, 2017b). Like the HAV vaccine, the HBV vaccine may not elicit the desired immune response in PLWH. However, 2-months post-serologic immune analysis is required to monitor vaccine-elicited immune responses (Schillie et al., 2018). Meanwhile, ART's restorative effects can resolve the problem of vaccine-elicited immune response (Wilson and Sereti, 2013). In fact, a previous study showed that vaccine-elicited immune response was better for patients who were consistent with ART use, and this resulted in elevated CD4 and suppressed HIV viral loads (Overton et al., 2010). While vaccines effectively protect against hepatotropic viruses, they may not offer long-lasting protection. Strategies implemented to increase the effects of vaccines include addition of adjuvant and increasing vaccine doses. Although addition of adjuvant to Hepatitis B vaccine was observed to be unsuccessful among PLWH (Catherine and Piroth, 2017a), increasing the vaccine doses was found to increase immune response (Fonseca et al., 2005, Launay et al., 2011, Sherman et al., 2017).

**HIV/HCV co-infections**

Approximately 58 million people and 1.5 million annual new infections of HCV are reported globally (Center for Disease Control and Prevention, 2021b). Moreover, 6.2% are HIV/HCV coinfected (World Health Organization, 2022b). Like HIV/HBV co-infection, overlapping transmission routes partly contribute to a high prevalence of
HIV/HCV coinfections. The common transmission routes reported are sexual transmission or injection drug use (Sharma et al., 2018). While the aforementioned transmission routes may be valid for less HIV-endemic areas, mother-to-child transmission via close contact (Kourtis et al., 2012) and transmission through cultural practices are more prevalent in HIV-endemic regions (Modi and Feld, 2007). The hepatotoxic effects of HCV in PLWH are well established. In fact, a recent cohort study identified the risk of liver-related mortality among HIV: coinfected HCV patients to be twice as higher as among HIV or HCV mono-infected patients (Thornton et al., 2017, De Ledinghen et al., 2008). While the direct hepatotoxic effects of HCV is known, HCV replication and progression is potentiated by the immunosuppressive effects of HIV (Sohrab et al., 2018). Without adequate intervention, HCV can result in chronic liver injury, covering the whole liver disease spectrum until it reaches end-stage liver disease. While vaccines may not be available for HCV, anti-viral treatment is available for HCV cure. Direct-acting antiviral (DAA) not only restore a sustained virological response but are very efficient for HCV cure (Naggie et al., 2015, Rockstroh et al., 2015, Waziry et al., 2017). While the results of the cure of HCV by DAA are exciting, drug-drug interaction between ART and DAA may be hepatotoxic for PLWH (Arora et al., 2021). However, there is evidence that recent modifications of DAA and ART have attenuated the incidence of liver decompensation due to HCV among PLWH (Neukam et al., 2016).
ART-induced liver damage

Approximately half of the 38 million people infected with HIV have been administered ART globally (Global, 2017). The emergence of ART has undoubtedly improved survival among PLWH by reducing HIV replication and restoring the numbers of immune cells and their functions (Wada et al., 2013, Kilby et al., 1998). Despite the apparent benefits of ART, chronic ART exposure was associated with the incidence of liver injury among PLWH (Chwiki et al., 2017). The burden of ART-induced liver injury varies across different settings. The prevalence of ART-induced liver injury was recently reported as 15% in a four-year African prospective cohort study (Gebremicael et al., 2021). Another 11-year cohort study in Asia reported the prevalence of ART-induced liver injury as 12% (Qin et al., 2019). While the aforementioned ART-induced liver injury prevalence in the African and Asian studies are similar, an American study found 7% ART-induced liver injury among PLWH in a ten-year interventional study (Stern et al., 2003). The higher prevalence of liver injury in Asia and Africa may be due to the higher incidence of HIV/tuberculosis co-infections that are managed by anti-tuberculosis drugs, hence, increasing the risk of hepatotoxicity from anti-tuberculosis and ART drug interaction (Wang et al., 2020, Tostmann et al., 2010, Hoffmann et al., 2007). Moreover, other factors that increase the risk of ART-induced liver injury include ART types (Ouyang et al., 2010), length of ART use (Mulu et al., 2013), and age of ART onset (Abongwa et al., 2022), among others.
Given the liver’s role in the biotransformation of xenobiotics, the liver becomes a significant target for the potentially detrimental effects of ART (Neff et al., 2006). Meanwhile, the underlying mechanisms for ART-induced liver injury are multifaceted. Mitochondria toxicity is commonly described (Neff et al., 2006). It has been shown that ART triggers mitochondria dysfunction by inhibition of the electron transport chain (ETC), ROS production, fatty acid oxidation, calcium accumulation, and mitochondria network fragmentation (Pérez-Matute et al., 2013). Despite ART-induced liver injury, modern ART has been highly modified to ameliorate ART toxicity. For example, thymine or riboflavin is a good improvement regimen for ART-induced mitochondria dysfunction (Kontorinis and Dieterich, 2003).

Moreover, HIV management in the era of numerous ART types (Eggleton and Nagalli, 2020) includes frequent liver function tests, especially after starting a new ART regimen. The implicated ART is immediately modified to a non-hepatotoxic ART regimen if liver injury is indicated. Consequentially, this is known to allay the liver injury associated with chronic exposure to ART (Qin et al., 2019).

**Alcohol-induced liver damage among HIV-infected individuals**

Alcohol is among the common risk factors for liver injury among PLWH (Chaudhry et al., 2009). The onset of liver disease due to excessive alcohol abuse results in steatosis progressing to steatohepatitis, hepatic fibrosis, cirrhosis, and hepatocellular carcinoma if the alcohol intake continues (Szabo and Zakhari, 2011, O'Shea et al., 2010). Alcohol has been demonstrated as a second hit for the progression of liver damage by HBV and
HCV (Dolganiuc, 2015). Alcohol-induced suppression of the host immune system is significant for describing the mechanism of HBV and HCV-induced liver damage (Dolganiuc, 2015). Similarly, alcohol has also been observed as the second hit for liver injury progression in the context of HIV. While it is debatable to consider HIV as a hepatotropic agent, the presence of HIV in hepatocytes has been reported (Kong et al., 2012a, Ganesan et al., 2019b). Several factors can explain HIV translocation to the liver. First, the liver serves as the "graveyard" for activated lymphocytes, which contains actively replicating HIV (Crispe et al., 2000). Hence, activated lymphocytes may deliver HIV to the liver. Secondly, the gut-liver axis is paramount for HIV entry into the liver (Tripathi et al., 2018). The gut, which contains approximately 90% of lymphocytes in the body, becomes the primary target for HIV infection (Cerf-Bensussan and Guy-Grand, 1991). To substantiate this, experimental evidence revealed that the gut mucosa lymphocytes are susceptible to HIV (Lapenta et al., 1999, Anton et al., 2000). HIV tropism in the gut was also confirmed by another study observing the reduced HIV RNA in the rectum, which correlates with serum HIV RNA (Tincati et al., 2009). While HIV depletes mucosa CD4+ T lymphocytes and enterocytes consequently leading to leaky gut (Sim et al., 2021, Lackner et al., 2009), the disruptive effects of alcohol permeabilizes the gut even far greater, and this contributes significantly to the leakage of HIV to the liver (Bishehsari et al., 2017, Yan et al., 2021). Given that hepatic portal vein transports blood carrying oxygen and nutrient from the gut into the liver, the hepatic
portal vein may serve as another route of HIV translocation from the gut to the liver (Zhang et al., 2022).

While alcohol and HIV do not synergize or interact because of the sterilization effects of alcohol, this effect is entirely different in a physiological system. In fact, HIV is known to “conspire” with alcohol to potentiate HIV pathogenesis (Yan et al., 2021). Clinical studies have demonstrated the facilitation of HIV from asymptomatic to symptomatic AIDS in alcohol-consuming individuals (Knox et al., Molina et al., 2018). This is possible since alcohol potentiates viremia, enhances ART non-adherence, suppresses immune response (Dagur et al., 2021), and increases promiscuity (New-Aaron et al., 2021c). In addition to the enhancement of HIV pathogenesis, alcohol targets the liver as the liver is the primary site for alcohol metabolism (Osna and Kharbanda, 2016). Therefore, it becomes reasonable to explore alcohol in the context of HIV, which could be also considered as a hepatotropic virus (Ganesan et al., 2019c). In a cohort of PLWH, 18% have liver-associated mortalities and 48% have a history of excessive alcohol consumption (Szabo and Zakhari, 2011). This underscores that alcohol partly contributes to the severity of liver damage among PLWH. While the mechanisms of HIV co-infection with other hepatotropic viruses are well studied, the mechanism of alcohol/HIV induced hepatotoxicity is unknown. Hence, this work will be focused on exploring the mechanisms of alcohol/HIV-induced liver injury.
**Hepatic Alcohol Metabolism**

Since the toxic effects of alcohol on HIV-containing hepatocytes is from its oxidative metabolites (acetaldehyde/ROS), it therefore reiterates the significance of hepatic alcohol metabolism.

The liver is generally known for its specialized function of alcohol metabolism. Hence, a typical hepatocyte sufficiently expresses alcohol metabolizing enzymes, such as, alcohol dehydrogenase (ADH), Cytochrome P450 2E1 (CYP2E1), and catalase, required for the oxidative metabolism of alcohol to toxic metabolites, acetaldehyde and ROS. Another enzyme, aldehyde dehydrogenase (ALDH)2, then oxidizes acetaldehyde to a non-toxic metabolite, acetate, which is eventually degraded into water and carbon dioxide for easy excretion (Figure 2). In addition to the formation of acetaldehyde as a toxic product of alcohol metabolism, NADH, and NAD+ ratio is altered by alcohol metabolism, which dysregulates lipid metabolism(Day and Yeaman, 1994). Although 90% of alcohol in the liver are metabolized oxidatively, constituting phase I of alcohol biotransformation, alcohol may further exploit the phase II reactions for its metabolism. Alcohol conjugates glucuronic acid under the catalytic actions of glucuronyltransferase to yield ethyl glucuronide as metabolites(Arthur, 2012). This metabolite persists longer in the body, making it a suitable biomarker for ethanol detection in the tissues, especially for forensic purposes (Heier et al., 2016). However, non-oxidative alcohol metabolism may not be significant for this study because alcohol and HIV-induced hepatotoxicity have only been explained within the context of alcohol oxidative metabolism.
Figure 2: Schematics of alcohol metabolism in hepatocytes. EtOH: Ethanol, Ach: Acetaldehyde, ADH: Alcohol dehydrogenase, ALDH2: aldehyde dehydrogenase, CYP2E1: Cytochrome p450 2E1.

Project Goals and Specific Aims

Given the history of HIV described in the first paragraph of this Chapter, it is not unseemly to consider HIV as a critical public health issue. Although ART has increased longevity (Wandeler et al., 2016), the liver disease remains one of the leading causes of non-AIDS-related mortality among PLWH (Group*, 2006). The liver disease accounts for
18% of all-cause mortality, and approximately 50% of mortality from HIV-related liver disease in the United States is attributed to alcohol (Szabo and Zakhari, 2011, Debes et al., 2016b). While alcohol consumption is about 12% in the USA (Galvan et al., 2002), twice more is observed in PLWH (New-Aaron et al., 2021a). It is not quite clear yet whether HIV by itself is hepatotoxic or hepatotoxicity is associated with prolonged ART usage. This underlines the critical need for exploring the mechanisms of alcohol-related hepatotoxicity, particularly, in the absence of ART, as the first step toward developing a therapy for alcohol-related liver disease among PLWH. Recent work from our laboratory showed that in the absence of ART, alcohol metabolites (acetaldehyde and ROS) enhances HIV accumulation in hepatocytes, which consequently induces massive hepatocyte death (Ganesan et al., 2019b). The progression of liver injury to fibrosis/cirrhosis is ultimately related to the activation of non-parenchymal liver cells, namely, hepatic stellate cells (HSC) (Ganesan et al., 2019b). The role of pathogen-and damage-associated molecular patterns (PAMPs and DAMPs) in the development of liver injury has already been demonstrated in other studies (Alisi et al., 2011). Here, we will test the hypothesis that apoptotic hepatocytes generated by exposure to HIV and alcohol metabolites provide the source of PAMPs and DAMPs recognized by HSC to promote their profibrotic activation. In this regard, first, we must unravel the mechanisms of HIV- and alcohol metabolite-induced hepatocyte apoptosis. The proposed study will explore the mechanisms involved in the crosstalk between HIV-infected hepatocytes and
HSC after exposure to alcohol metabolites. Our hypothesis will be tested with the following specific aims:

1. Determine the role of HIV- and ethanol metabolism-induced lysosomal dysfunction in the induction of apoptotic cell death in hepatocytes

2. Define how engulfment of hepatocyte apoptotic bodies by HSC activates profibrotic changes in these cells

3. To study the potential therapeutic regimen that prevents HIV/alcohol metabolite-induced hepatotoxicity and subsequent activation of HSC by HIV+ hepatocyte apoptotic bodies' engulfment

In Aim 1 (chapter 2), the role of lysosomal dysfunction in the induction of hepatocyte apoptosis in HIV and alcohol exposure settings was studied. The background which led us to the formulation of this Aim is based on the fact that HIV induces hepatotoxicity upregulated by exposure to alcohol metabolites (Ganesan et al., 2019b, Babu et al., 2009), but the exact mechanisms for HIV and acetaldehyde-induced hepatotoxicity are unknown (Szabo and Zakhari, 2011). Meanwhile, HIV permissiveness by hepatocytes remains a subject of debate (Xiao et al., 2008a, Park et al., 2014). However, previous data from our laboratory demonstrated that alcohol metabolites, acetaldehyde/ROS, enhance the accumulation of HIV in Huh7.5-CYP2E1 (RLW) cells, which induces oxidative stress followed by massive hepatocyte apoptosis (Ganesan et al., 2019b). Since HIV requires internalization to become hepatotoxic (Yasen et al., 2018, Kong et al., 2012a), the entry
mechanism of HIV into CD4-negative hepatocytes should be clarified (Ganesan et al., 2018c). To this end, the expression of HIV co-receptors, CCR5 (Blackard et al., 2019) and CXCR4 (Babu et al., 2009) on hepatocytes has been identified by previous studies. In our experiments, we used HIV 1\textsubscript{ADA} to infect the cells, and this sub-type of virus is CCR5-tropic. To study the role of the CCR5 co-receptors for the entry of HIV into hepatocytes, the expression of the CCR5 co-receptor was blocked by a pharmacological inhibitor, maraviroc. Since the induction of HIV gag RNA by exposure to alcohol metabolites was observed in hepatocytes treated with HIV (Ganesan et al., 2019b), the effects of alcohol metabolites on CCR5 was determined.

We were also unable to document that alcohol metabolites upregulated the complete cycle of HIV replication. In fact, there was an abortive HIV gag RNA replication in response to acetaldehyde, which was blocked at the level of reverse transcription from HIV RNA to HIV DNA (Ganesan et al., 2019b). One of the mechanisms of how alcohol metabolism affects HIV RNA and protein increase is the changing endosomal/lysosomal pH. Previous data from our laboratory showed that chloroquine increases lysosomal pH leading to better HIV survival and enhanced HIV RNA accumulation in hepatocytes (Ganesan et al., 2019b). This was mimicked by the treatment with alcohol metabolites when lysosome alkalization was observed in hepatocytes exposed to alcohol metabolites (Dagur et al., 2021). Given that lysosome is involved in the degradation of HIV proteins and it requires acidic pH for its functioning, we hypothesized that acetaldehyde-induced lysosome alkalization/damage leads to HIV accumulation and a
consequent reactive oxygen species (ROS) generation, hence, triggering hepatocyte apoptosis. These aspects were explored in Chapter 2, which covers Aim 1. In this Aim, lysosomal damage and dysfunction, which leads to lysosomal leakage, and the enzymes, cathepsins, and their co-localization with mitochondrial membrane proteins was examined. Since the interaction between cross-talk between lysosome and mitochondria typically induces apoptosis (Moles et al., 2018), we examined the type of apoptotic pathway involved.

Since damaged lysosomes are compensated for by lysosome biogenesis, only alcohol and HIV-induced lysosome damage may not provide significant damage to trigger hepatocyte apoptosis (Eriksson et al., 2020). Therefore, the effects of alcohol metabolites on lysosome biogenesis were also evaluated by the comprehensive analysis of Transcription Factor (TF)EB, a lysosome biogenesis transcription factor.

To learn the detrimental consequences of apoptotic bodies (ABs) formation from HIV and ethanol metabolism-exposed hepatocytes, at the next step, we focused on profibrotic activation of hepatic stellate cells (HSC) by engulfment of these ABs (Aim 2, studies are presented in Chapter 3). Meanwhile, to comprehend the role of HSC in liver injury, the cellular component of liver must be understood.

The liver consists of parenchymal and non-parenchymal cells. The parenchymal cells, hepatocytes comprise about 70-80% of liver cells, while the rest are non-parenchymal cells, consisting of HSC, Kupffer cells, and liver sinusoidal endothelial cells (Ding et al.,
Physiologically, all cellular components of the liver are coordinated. Meanwhile, the crosstalk between hepatocytes and HSC has been implicated in the progression of alcohol and HIV-induced liver fibrosis (Yang et al., 2021). Recently, we have shown that HIV-exposed apoptotic hepatocytes activated HSC inducing profibrotic changes (Ganesan et al., 2019b). Therefore, we investigated if HSC profibrotic activation was due to the engulfment of hepatocyte apoptotic bodies (AB). Here, hepatocytes were labeled with 5-carboxy-tetramethylRhodamine (TAMRA), and their engulfment was determined by confocal microscopy.

Given the limited knowledge of AB derived from HIV and alcohol metabolite-exposed hepatocytes (ABAGS-HIV), the size, contents, and surface receptors of ABAGS-HIV were investigated.

The next step was to determine the ABAGS-HIV internalization receptors on HSC. Since ABAGS-HIV express phosphatidylserine as indicated from the characterization of ABAGS-HIV, phosphatidylserine-recognizing receptors from TIM and TAM protein family were measured on HSC. Since ABAGS-HIV-induced profibrotic activation, the mechanistic pathways for the activation were determined. The diagrammatic illustration of Aims 1 and 2 are described in Figure 3.
In Aim 3, we addressed the therapeutic regimens that prevent HIV/ethanol metabolite-induced hepatotoxicity and subsequent activation of hepatic stellate cells (HSC) by HIV+ hepatocyte apoptotic bodies’ engulfment. In the details, this Aim is covered by Chapter 4.

As a background information, ART may be potent for immune restoration and attenuation of viremia. However, it may not adequately resolve HIV-induced liver damage since HIV and alcohol-induced hepatotoxicity mechanisms do not involve viral replication (New-Aaron et al., 2021a). Therefore, ART must be supplemented by another regimen to resolve liver damage and prevent liver fibrosis among PLWH. To develop a
functional regimen for this purpose, the mechanisms/targets for HIV and alcohol-induced hepatotoxicity as explored in aims 1 and 2, were studied. Therefore, a therapeutic agent with ROS-scavenging potentials, such as antioxidants, may be used as a potential therapeutic regimen for reversing the hepatotoxic effects of alcohol and HIV. However, this may result in dire consequences for HIV retention since hepatocyte apoptosis is known as a mechanism for HIV clearance from the liver. Hence, HIV and alcohol-induced apoptosis may be beneficial for decreasing HIV liver reservoirs. A potential therapeutic regimen for ameliorating HIV and alcohol-induced liver damage should be antifibrotic as HIV, and alcohol-induced apoptosis leads to fibrotic activation of HSC. Therefore, obeticholic acid (OCA), an antifibrotic therapeutic drug that is currently in phase III clinical trial for non-alcohol steatohepatitis (Shah and Kowdley, 2020b), was used in this study to prevent fibrotic progression due to activation of HSC exposed to ABAGS+HIV. To achieve this, HSCs were pre-treated with OCA and exposed to ABAGS+HIV, followed by measuring pro-fibrotic gene activation in HSCs. Moreover, alcohol and HIV-exposed hepatocytes were pretreated with OCA to determine the effects of OCA on hepatocytes. Enhanced HIV degradation and anti-apoptotic activities of OCA were measured as described in Chapter 4.

**Project Significance**

HIV is a major global health problem of epic proportions, with approximately 38 million people living globally with HIV/AIDS (Duflo et al., 2019, New-Aaron et al., 2021b), and
about three percent of these cases are in the United States (Fauci et al., 2019, New-Aaron et al., 2021a). ART has led to a significant decline in AIDS-related mortality (Brown et al., 2018). Still, this success is impeded by an alarming rise in non-AIDS-related mortality (Pettit et al., 2018). With liver disease as the most common non-AIDS-related mortality (Smith et al., 2014, Ford et al., 2015, Price et al., 2018, Althoff et al., 2019), which accounts for approximately 18% of all mortality, liver disease has become a significant concern among PLWH (Debes et al., 2016a). As previously described, alcohol, antiretroviral therapy, and hepatotropic viruses (Hepatitis B and C) are associated with liver disease among HIV-infected individuals (Ganesan et al., 2018d). Although the mechanisms of hepatotoxicity by ART (Sastry et al., 2018, Gwag et al., 2019) and hepatotropic viruses (Ganesan et al., 2018b, Nevola et al., 2018) have been extensively studied, the explanation of the role of alcohol in HIV-infection and pathogenesis of liver disease development remain elusive despite the emerging burden of liver disease in HIV management (Kaspar and Sterling, 2017b). This highlights the need for adequate attention to decipher the mechanisms of liver damage in the context of HIV. Moreover, excessive alcohol consumption is currently a social dilemma in many parts of the world. In the United States, it has been estimated that about 1.2 million emergency visits per year were attributed to excessive alcohol consumption (National Institute on Alcohol Abuse and Alcoholism and 2013). Despite high alcohol abuse in the general US population, alcohol abuse among PLWH is estimated to be twice the general population (Madhombiro et al., 2019, Cook et al., 2018, Petoumenos and Law, 2016,
Williams et al., 2016). This undoubtedly makes alcohol abuse among PLWH a serious public health issue since the liver is a known target for both alcohol and HIV.

Furthermore, many ART alternatives are available and easily accessible in cases of ART hepatotoxicity (World Health Organization, 2021), and HAV/HBV vaccines (Catherine and Piroth, 2017b) and DAA now exist (Bhattacharya et al., 2017, Rockstroh et al., 2018) in cases of hepatotoxicity due to hepatotropic viruses. However, nothing is currently available to ameliorate alcohol-related hepatotoxicity among PLWH (Crane et al., 2017).

Liver fibrosis is the preliminary stage preceding liver cirrhosis and alcohol was previously reported in a population study to be associated with liver fibrosis (Barve et al., 2010). Clinical studies supported alcohol as a risk factor for developing liver fibrosis among PLWH (Bonacini, 2016). Furthermore, in vitro studies supported the data on alcohol-induced hepatotoxicity in hepatocytes exposed to HIV (Han et al., 2017), and we confirmed it with the data from our lab (Ganesan et al., 2019b). Although clinical data and in vitro studies demonstrated alcohol-induced hepatotoxicity in the context of HIV, the underlying mechanisms are not fully characterized. The liver was previously described as a silent reservoir for HIV (Ganesan et al., 2018c). Still, recent data shows that a second hit such as alcohol might enhance HIV accumulation in hepatocytes, which subsequently causes liver cell death (Ganesan et al., 2019b). Given that hepatocytes comprise 70% of the liver, which is approximately 1.5kg organ, it is extremely sensitive to hepatotoxic effects of alcohol due to their high alcohol-metabolizing capacity. Therefore, the combined effects of alcohol and HIV are of paramount importance for
liver injury. The crosstalk between hepatocytes and hepatic stellate cells (HSC) is an essential part of pathogenesis for liver fibrosis development, and the effective treatment approaches are not clear yet.
CHAPTER 2: ALCOHOL-INDUCED LYSOSOMAL DAMAGE AND SUPPRESSION OF LYSOSOME BIOGENESIS CONTRIBUTE TO HEPATOTOXICITY IN HIV-EXPOSED LIVER CELLS


Introduction

Typically, hepatocyte’s lysosomes degrade HIV proteins, thereby preventing HIV intracellular accumulation and a subsequent oxidant stress (Ganesan et al., 2019b, Ivanov et al., 2016). However, this may not be true for hepatocytes exposed to alcohol metabolites. Recent studies reported alcohol-induced lysosome impairment which led to HIV accumulation (Donohue Jr et al., 2019, Dagur et al., 2021), but the involved mechanism is not fully understood. In this Chapter, we explored the mechanisms of alcohol and HIV-induced hepatocyte apoptosis by characterizing the contribution of alcohol metabolites to lysosome impairment in HIV-exposed liver cells. First, we investigated whether alcohol metabolites decreased lysosome functions. Second, we
determined whether the induced-lysosome dysfunction was due to lysosome leakage. Moreover, lysosome leakage/rupture causes the release of cathepsins and other hydrolases into the cytosol. While the etiology of lysosome damage and subsequent leakage are diverse, ROS production induces such damage (Boya and Kroemer, 2008).

Here, we used an in vitro acetaldehyde-generating system (AGS) that continuously generates physiologically relevant amounts of acetaldehyde and mimics ethanol metabolism in HIV-infected Huh 7.5 (also known as RLW) cells that overexpress cytochrome P450 2E1 (CYP2E1), but not alcohol dehydrogenase (ADH). AGS generates acetaldehyde by using ethanol as the primary substrate of yeast alcohol dehydrogenase in the presence of nicotinamide adenine dinucleotide (NAD+) as a co-factor. Exposure of cells to the AGS elevates ROS levels in RLW cells since ethanol is part of AGS, and RLW cells generate ROS by CYP2E1 upon ethanol exposure (Ganesan et al., 2019b). Hence, we postulated that in HIV-exposed RLW cells, the decrease in lysosome function is due to lysosome leakage triggered by enzymatically generated acetaldehyde and oxidant stress, which mimics ethanol metabolism in primary hepatocytes. Such leakage is detrimental if lysosome repair/replacement is inhibited as well. The activity and intracellular localization of Transcription Factor EB (TFEB), which activates genes that encode lysosomal proteins and lysosome biogenesis (Sardiello et al., 2009), is disrupted by chronic ethanol administration (Chao et al., 2018b, Bala and Szabo, 2018). Here, we sought to determine whether HIV and AGS prevent TFEB translocation from cytosol to the nucleus of HIV-infected RLW cells. TFEB nuclear translocation activates genes
involved in lysosome biogenesis. TFEB nuclear translocation/gene activation is prevented by its phosphorylation (Napolitano et al., 2018), by reduced proteasomal degradation in the cytosol (Sha et al., 2017b), and by impaired TFEB trafficking; an increased expression of ZKSCAN3, a transcriptional repressor of autophagy (Chauhan et al., 2013), also affects lysosomal gene activation. We hypothesized that exposure of hepatocytes to HIV and ethanol and its metabolite, acetaldehyde, induces oxidant stress to trigger lysosome leakage and lysosome biogenesis suppression. The latter suppression blocks intracellular replacement of damaged lysosomes causing cytotoxicity during combined HIV and alcohol/acetaldehyde exposure to liver cells.

**Materials and Methods**

**Reagents and Antibodies**

High-glucose Dulbecco’s modified eagle medium (DMEM) and fetal bovine serum were purchased from Invitrogen (Waltham, MA, USA); Trizol was from Life Technologies (Carlsbad, CA, USA); primer probes, high-capacity reverse transcription kit, and real-time polymerase chain reaction (RT-PCR) reagents were from Applied Biosystems by Thermo Fisher Scientific, Carlsbad, CA, USA. Pan-caspase inhibitor (PCI) was obtained from Ubiquitin-Proteasome Biotechnologies (UBPBio) Inc. (Cat#F7110, Aurora, CO, USA), Nocodazole was from Sigma Aldrich (St. Louis, MO, USA, M1404). Primary antibodies were: (a) mouse monoclonal: anti-TFEB (Santa Cruz Biotechnology, Dallas, TX, USA, sc-166736), anti-cathepsin B (H-5) (sc-365558), anti-TATA binding protein
(Millipore Sigma, Burlington, MA, USA, MAB3658), anti-beta-actin (Santa Cruz Biotechnology, Dallas, TX, USA, sc-69879), anti-LAMP1 (Santa Cruz Biotechnology, Dallas, TX, USA, sc-20011), anti-Acetylated α-tubulin (Millipore Sigma, Burlington, MA, USA, T6793), anti-α-tubulin (Millipore Sigma, Burlington, MA, USA, T9026); (b) rabbit monoclonal and polyclonal anti-TFEB, 42405S; anti-Phospho-TFEB (ser211), 37681S; anti-Tom20 (D8T4N), 42406; anti-Galectin-3/LGALS3 (D4I2R), #87985; anti-ZKSCAN3 (LSBio, Seattle, WA, USA, LS-C501696); (c) secondary antibodies: (I) IRDye 680RD Goat anti-Rabbit, C50317-02; IRDye 680RD Donkey anti-Mouse, C50520-02; (II) Goat anti-Mouse IgG (H + L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 555, Carlsbad, CA, USA, A21422; and Donkey anti-Rabbit IgG (H + L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 488.

In Vitro Studies

Due to the paucity of primary human hepatocytes, we performed all experiments on hepatocyte-like RLW cells. These cells have reduced innate immunity and do not sufficiently oxidize (metabolize) ethanol because they lack ADH. To circumvent this limitation, we exposed RLW cells to an exogenous constant source of acetaldehyde (acetaldehyde-generating system, AGS), which consists of 0.02 EU yeast alcohol dehydrogenase (ADH), 2 mM nicotinamide adenine dinucleotide (NAD), and 50 mM ethanol. AGS is known to continuously generate a physiologically relevant amount of acetaldehyde without unwarranted toxicities (Ganesan et al., 2019b). HIV-1ADA propagated on primary human macrophages was purified at University of Nebraska.
Medical Center (UNMC) (Snapper et al., 1988). RLW cells were pre-treated for 24 h with AGS, infected with HIV-1 Ada, and then exposed again to AGS for another 48 h. HIV was removed after overnight treatment, and the cells were washed thoroughly with 1× phosphate-buffered saline (PBS) to remove extracellular HIV.

**RNA Isolation and Real-Time PCR (RT-PCR)**

RNAs encoding p53, cathepsin D, and LAMP1 were quantified by RT-PCR as previously described (Ganesan et al., 2019b). Briefly, total cellular RNA was isolated from cells using Trizol reagent. A two-step procedure was used, in which 200 ng RNA was reverse-transcribed to cDNA using the high-capacity reverse transcription kit. In the second step, the cDNA was amplified using TaqMan Universal Master Mix II with fluorescent-labeled primers (TaqMan gene expression systems). These were incubated in a Model 7500 qRT-PCR thermal cycler. The relative quantity of each RNA transcript was calculated by its threshold cycle (Ct) after subtracting that of the reference (GAPDH).

**Proteasome Activities**

Chymotrypsin (Cht-L) and trypsin-like (T-L) peptidase activities were detected by in vitro fluorometric assay as previously reported by our laboratory (Ganesan et al., 2019a). Fluorogenic peptide substrates N-succinyl-leu-leu-val-tyr-7-amido-4-methlycoumarin (suc-LLVY-AMC; UBPBio, Inc., Aurora, CO, USA) and boc-leu-ser-thr-arg-7-amido-4-methlycoumarin (boc-LSTR-AMC; UBPBio, Inc., Aurora, CO, USA) were used to measure chymotrypsin- and trypsin-like activities of the proteasome.
Cathepsin B and L Activities

Cathepsin B and L activities were measured as previously described (Thomes et al., 2013), using Z-arg-arg-7-amido-4-methylcoumarin hydrochloride (cathepsin B) and L-phe-arg-7-amido-4-methylcoumarin hydrochloride (cathepsin L).

Whole-Cell Lysates and Nuclear/Cytosolic Fractionations

Whole-cell lysates were prepared in phosphorylation capture buffer (0.5 M EDTA, 2 M Tris, 20 mM Na3VO4, 200 mM Na4P2O7, 100 mM PMSF, 1 M NaF, 20% Triton X-100, and aprotinin, pH 7). The extraction of the nuclear and cytosolic components from RLW cells was performed using the NE-PER Nuclear and Cytoplasmic Extraction Kit (ThermoFisher Scientific, Carlsbad, CA, USA) based on the manufacturer’s protocol.

Immunoblotting

Immunoblotting was performed as described (Ganesan et al., 2016a, Ganesan et al., 2016b); blots were developed using Odyssey infrared imaging system, which was also used to quantify the protein bands (Ganesan et al., 2018a). Equal (20 µg) amounts of protein were loaded in each lane. Beta-Actin or TATA-binding proteins (TBP) were used as loading controls to normalize the protein band densities.

Immunofluorescence

RLW Cells (130,000/well) were seeded onto coverslips, which were inserted into each well of a 12-well plate. Cells were infected with the HIV-1 virus (Multiplicity of infection, MOI = 0.1). After overnight incubation of cells with HIV, the virus-containing
medium was removed and replenished with fresh media. Cells were incubated for another 48 h of AGS treatment; cells were washed with 1× PBS, fixed with 4% paraformaldehyde for 12 min at 37 °C, permeabilized with 0.1% Triton X-100 for 3 min at room temperature, and blocked for 30 min with 1% (g/vol) bovine serum albumin (BSA) in PBS. Cells were stained to study the colocalization of LAMP1:Galectin 3, Tom20:Cathepsin-B, and quantification of LAMP1. First, cells were incubated with primary antibodies for 1 h. The cells were then washed and incubated with the mixture of Alexa-Fluor-labeled secondary antibodies for 30 min. Nuclei were stained with DAPI. The coverslips were transferred to microscope slides for imaging by using LSM 710 confocal microscope. Immunostainings and colocalization were quantified for intensity using National Institute of Health (NIH) Image J program.

**Statistical Analyses**

Data were analyzed using GraphPad Prism v7.03 software (GraphPad, La Jolla, CA, USA). Data from at least three duplicate independent experiments were expressed as mean ± SEM. Comparisons among multiple groups were performed by one-way ANOVA, using a Tukey post hoc test. For comparisons between two groups, we used Student’s t-test. A p-value of 0.05 or less was considered significant.
Results

CCR5 mediates HIV entry into hepatocytes

We observed the induction of HIV gag RNA in RLW cells treated with both HIV-1\textsubscript{ADA} and AGS compared to RLW cells treated with only HIV-1\textsubscript{ADA} (Ganesan et al., 2019b). Our sub-hypothesis here is that alcohol metabolites enhance HIV co-receptors expression on RLW cells to allow the massive HIV entry into RLW cells. Previously, we elaborated the optimal scheme of infected RLW cells treatment with AGS: the highest HIV RNA expression was achieved in RLW cells exposed to AGS one day before exposing the cells to HIV and 2 days after exposing the cells to HIV (AGS double treatment) (Ganesan et al., 2019b). Here, HIV-infected RLW cells were pre- and post-treated with AGS and compared to HIV-infected RLW cells (not treated with AGS) to determine the expression of CCR5 by flow cytometry. Furthermore, CCR5 were blocked with maraviroc, a CCR5 pharmacological inhibitor, in HIV-infected RLW cells treated or not with AGS to determine if HIV RNA accumulation inside hepatocyte is prevented. While HIV entry into RLW cells was inhibited by 2\textmu M maraviroc (Figure 4A), AGS did not significantly increase CCR5 expression on RLW cells (Figure 4B&C). Hence, AGS-induced HIV accumulation in hepatocytes cannot be explained by AGS effects on CCR5.
Figure 4: Effects of AGS on CCR5 co-receptor (A) HIV gag RNA expression in RLW cells exposed to maraviroc (B) C-C-chemokine receptor type 5 (CCR5) expression in untreated RLW cells (C) CCR5 expression in AGS-treated RLW cells. Data are from 3 independent experiments presented as mean ± SEM. Bars marked with the same letter are not significantly different; bars with different letters are significantly different from each other (p ≤ 0.05).
AGS and HIV Exposure Lowers Cathepsin Activities and Lysosome Numbers

While CCR5 mediates HIV entry into RLW cells, it did not explain AGS-induced HIV accumulation in RLW cells. To understand the mechanism(s) by which HIV and acetaldehyde induce accumulation of HIV proteins in hepatocytes, we measured cathepsin B and L activities as indices of lysosomal enzyme function. The combination of AGS and HIV treatment (AGS + HIV) decreased \( p < 0.05 \) cathepsin B and L activities (Figure 5A, B). To determine whether lower cathepsin B and L activities by AGS + HIV exposure reflected lower lysosome numbers, we measured LAMP1 immunofluorescence intensity. Images were normalized to the number of cells in each field. LAMP1 intensity was 42\% lower in AGS-treated uninfected cells, and this was further suppressed in HIV-infected cells (Figure 5C, D). Similarly, AGS decreased LAMP1 protein expression of HIV-infected cells as demonstrated on Western blots (Figure 5E, F). AGS exposure suppressed lysosome numbers in both HIV-infected and -uninfected RLW cells, and AGS + HIV suppressed lysosome functions in RLW cells.
Both AGS and HIV Enhance Lysosome Leakage

We investigated whether AGS-induced LAMP1 suppression affected lysosome membrane permeabilization and lysosomal leakage (LL) in HIV-infected and -uninfected cells. To this end, we quantified the levels of damage-regulatory autophagy modulator (DRAM)-1, which induces lysosome leakage, thereby triggering p53-dependent apoptosis (Laforge et al., 2013). Since we found that AGS treatment increased p53 mRNA levels in both infected and uninfected RLW cells (Figure 6A), we postulated that DRAM1 expressing cells would be cleared by apoptosis. In fact, it was observed that AGS and AGS + HIV exposed cells expressed a very low amount of DRAM1 (Figure 6B, C) because DRAM1-expressing cells were unaccounted for due to apoptosis. To prevent/slow the clearance of DRAM-positive cells, we measured DRAM expression after blocking apoptosis by a pan-caspase inhibitor (PCI). In the presence of PCI, we observed 64% higher levels of intracellular DRAM in AGS-exposed HIV-infected cells (Figure 6D, E). To further confirm these findings, we measured the co-localization of
galectin 3 and LAMP1 puncta and found that AGS exposure exhibited greater co-localization of galectin 3 and LAMP1 puncta in HIV-infected cells than in uninfected cells (Figure 6F, G), indicating that both HIV and acetaldehyde exposure contributes to lysosome leakage.

Figure 6: AGS and HIV enhance lysosomal leakage in RLW cells treated as indicated in Materials and Methods. (A) p53 mRNA levels were measured by RT-PCR with GAPDH mRNA as the internal standard. (B,C) DRAM1 protein expression in the absence of pan-caspase inhibitor (PCI) was detected by immunoblotting. Quantification of protein bands from 3 independent experiments is presented. Equal (20 µg) amounts of protein were loaded in each lane (as stated in Materials and Methods). β-actin was used as the internal standard. (D,E) DRAM1 protein expression in the presence of PCI was detected by immunoblotting. Quantification of protein
bands from 3 independent experiments is presented. (F) Co-localization of galectin 3 and LAMP1 was measured by immunofluorescence. Proteins were visualized using a 40× lens LSM 710 confocal microscope. (G) Co-localization of galectin 3 and LAMP1 was quantified using NIH Image J. Data are from 3 independent experiments presented as mean ± SEM. Bars marked with the same letter are not significantly different from each other; bars with different letters are significantly different from each other (p ≤ 0.05).

AGS and HIV Enhance Intracellular Oxidant Stress

We investigated the contribution of oxidant stress to AGS-induced decline of cathepsin activities in control and HIV-exposed RLW cells as well as downstream apoptosis.

Combined AGS and HIV exposure suppressed cathepsin B and L activities and caused an increase in apoptosis, detected by ELISA as elevated levels of M30 (cleaved cytokeratin 18) in cell supernatants. M30 detection was significantly lower (p < 0.05) after treatment of cells with N-acetyl cysteine (NAC), which restored cathepsin B and L activities (Figure 7A–C).

Figure 7: N-acetyl cysteine treatment prevents/reverses AGS and HIV-induced lysosomal leakage (RLW cells were treated as indicated in Materials and Methods). (A,B) Cathepsin B and Cathepsin L activities were measured in cell lysates (in the presence or absence of NAC) by fluorometric assay, using fluorimetric substrates. (C) Apoptosis was measured by M30 levels in cell culture supernatants. Data are from 3 independent experiments. Bars marked with the same
letter are not significantly different from each other; bars with different letters are significantly different from each other ($p \leq 0.05$).

AGS and HIV Enhance Cathepsin Leakage and Intrinsic Apoptosis

We measured the leakage of cathepsin B from lysosomes and its translocation to the outer mitochondrial membrane, which likely damages the mitochondrial membrane. In AGS + HIV-treated cells, cathepsin B was co-localized with mitochondria, as indicated by immunofluorescent co-localization of cathepsin B and Tom20. Tom20 is a protein on the mitochondrial outer membrane that facilitates the import of mitochondrial precursor proteins into the mitochondrion (Chacinska et al., 2009, Yamamoto et al., 2011) (Figure 8A,B). Furthermore, caspase 3 cleavage in AGS-exposed HIV-infected liver cells (the condition with the highest cathepsin B–Tom20 co-localization and apoptotic effects) was suppressed nearly three-fold in the presence of 20 µM caspase 9 inhibitor, while the caspase 8 inhibitor had a minimal effect on the observed apoptosis (Figure 8C, D).
Figure 8: AGS and HIV enhance cathepsin B-triggered caspase 3-dependent apoptosis (RLW cells were treated as described in Materials and Methods) (A,B) Immunofluorescence detection of co-localization of Cathepsin B and Tom20 and quantification of staining. Staining was visualized using a 40× lens in LSM 710 confocal microscope, and staining intensity was quantified using NIH ImageJ. (C,D) Cleaved caspase-3 was measured by immunoblot analysis and quantification of immunoreactive bands from 3 independent experiments. Data are from 3 independent experiments presented as mean values ± SEM. Bars marked with the same letter are not significantly different from each other; bars with different letters are significantly different from each other (p ≤ 0.05).
BAX interaction with DRAM1 contributes to AGS+HIV-induced lysosome permeabilization

Since lysosome leakage triggered intrinsic hepatocyte apoptosis, the involvement of BAX, a proapoptotic mitochondria protein was investigated. Meanwhile, BAX interaction with DRAM1 as a mediator of lysosome permeabilization and p53-dependent apoptosis was already investigated in Hela cells (Guan et al., 2015b). While we observed upregulation of p53 genes when RLW cells were treated with the combination of AGS and HIV it is not clear if this was mediated by DRAM1 and BAX interaction. Therefore, we measured the colocalization of DRAM1 and BAX when hepatocytes were exposed to the combined treatment of AGS and HIV. We found that AGS+HIV which induced the highest lysosome leakage, and the highest intrinsic apoptosis expressed the highest interaction with DRAM1 and BAX (Figure 9).
Figure 9: AGS and HIV enhanced the interaction of DRAM1 and BAX. Immunofluorescence detection of co-localization of DRAM1 and BAX and quantification of staining. Staining was visualized using a 40× lens in LSM 710 confocal microscope, and staining intensity was quantified using NIH ImageJ. Data are from 3 independent experiments presented as mean values ± SEM. Bars marked with the same letter are not significantly different from each other; bars with different letters are significantly different from each other (p ≤ 0.05).

AGS and HIV Exposure Suppress TFEB Protein Expressions in Cytosolic and Nuclear Fractions of RLW Cells

Lysosome damage reportedly activates lysosome biogenesis as a compensatory mechanism to replace damaged lysosomes (Wang et al., 2018a) via enhanced TFEB transcription (Napolitano et al., 2018). After we separated nuclear and cytosolic fractions from the control and treated RLW cells, we observed greater amounts of immunoreactive TFEB in the cytosolic fractions of HIV- and AGS-treated cells. Both treatments significantly decreased the nuclear to cytosolic TFEB ratios compared with
untreated cells (Figure 10A, B). The purities of our nuclear and cytosolic fractions were confirmed by Western blotting by exposing both fractions to anti-α-tubulin (a cytosolic marker) and to the anti-TATA binding protein (a nuclear marker) (Figure 10C).

Suppression by AGS of nuclear TFEB expression in HIV-infected and -uninfected cells prompted us to analyze the role of ethanol metabolism on the factors that regulate TFEB cytosolic-nuclear shuttling. As reported, mTORC1-mediated TFEB phosphorylation is an upstream event, which causes TFEB retention in the cytosol (Napolitano et al., 2018). Surprisingly, we observed the downregulation of TFEB phosphorylation (pTFEB) at the mTOR-regulated serine sites (S211) in both AGS-exposed uninfected and HIV-infected cells, which decreased the pTFEB/TFEB ratio (Figure 10D, E). To test whether microtubules regulate TFEB trafficking to the nucleus, RLW cells were exposed for 24 h to 10 μM nocodazole, a microtubule-disrupting drug. We found that TFEB nuclear translocation was attenuated by nocodazole, indicating that translocation is microtubule-dependent (Figure 10F, G). We also found that exposure of HIV-infected and -uninfected RLW cells to AGS enhanced α-tubulin acetylation (Figure 10H, I).
Figure 10: Suppression by AGS and HIV of transcription factor EB (TFEB) protein expression in cytosolic and nuclear fractions of RLW cells. (A) Nuclear and cytosolic TFEB protein expressions were measured by immunoblot analysis. Equal (20 µg) amounts of protein were loaded in each lane. β-actin and TATA-binding proteins (TBP) were used as internal controls. (B) Quantification of nuclear and cytosolic TFEB ratios from 3 independent experiments. (C) Immunoblot analysis to confirm the purity of cytosolic and nuclear fractions. Equal amounts of cytosolic and nuclear proteins were loaded onto gels and blotted onto nitrocellulose. α-tubulin was checked in nuclear fractions and TBP in cytosolic fractions. (D) Immunoblotting analysis of phospho TFEB serine 211 protein expressions. Equal (20 µg) amounts of protein were loaded in each lane. β-actin and total TFEB were used as an internal control. (E) Quantification of serine 211 phosphorylation/total TFEB ratios. Data are from 3 independent experiments, presented as the mean ± SEM. Bars marked with the same letter are not significantly different from each other; bars with different letters are significantly different (p ≤ 0.05). (F) Nuclear and cytosolic TFEB was measured by immunoblotting analysis. Equal (20 µg) amounts of protein were loaded in each lane. β-actin and TBP were used as an internal control (G) Quantification of nuclear to cytosolic TFEB ratios from 3 independent experiments. (H,I) Acetylated tubulin was measured by immunoblot analysis and quantification of protein bands from 3 independent experiments. Data are from 3 independent experiments presented as mean ± SEM. Bars marked with the same letter.
are not significantly different from each other; bars with different letters are significantly different \( (p \leq 0.05) \).

**AGS and HIV Exposures Suppress Proteasome Activity and Enhance ZKSCAN3 Expression**

Because TFEB is degraded by the proteasome, we examined whether HIV or AGS influences 20S proteasome peptidase activities. HIV infection suppressed chymotrypsin and trypsin-like activities (Figure 11A, B). However, this effect was exacerbated in AGS-exposed HIV-infected and -uninfected RLW cells. We explored whether acetaldehyde influenced TFEB induced activation of lysosomal genes. To this end, we measured the levels of ZKSCAN3 in RLW nuclear fractions as ZKSCANS represses TFEB-activated lysosome biogenesis. We observed a 40% rise in ZKSCAN3 expression after AGS treatment in both HIV-exposed and -unexposed RLW cells (Figure 11C, D). To corroborate these findings, we quantified the mRNAs encoding LAMP1 and cathepsin D, two genes activated by TFEB. Both LAMP1 and cathepsin D mRNAs were lower after cells were treated with AGS. This suppression effect was even greater in AGS + HIV-infected cells (Figure 11E).
Figure 11: AGS and HIV enhance ZKSCAN3 but suppress TFEB target genes. (A,B) Chit-L, and T-L proteasome peptidase activities were detected in cell lysates by in vitro assay using fluorimetric substrates (C) Immunoblot analysis of ZKSCAN3 protein expression in RLW nuclear fraction. Equal (20 µg) amounts of protein were loaded in each lane. β-actin was used as an internal control. (D) Quantification of immunoreactive bands of ZKSCAN3 from 3 independent experiments. (E) LAMP1 and cathepsin D mRNA expression detected by RT-PCR analysis. GAPDH mRNA was used as an internal control. Data are from 3 independent experiments, presented as the mean ± SEM. Bars marked with the same letter are not significantly different from each other; bars with different letters are significantly different (p ≤ 0.05).
Chronic Ethanol feeding and HIV Infection Damage Lysosomes in Livers of Humanized Mice

To validate our in vitro findings in hepatocyte-like RLW cells, we conducted in vivo experiments with HIV-infected humanized mice transplanted with human hepatocytes. We found that acute on chronic ethanol-fed HIV-positive mice exhibited 25% lower LAMP1 protein levels than the pair-fed control group (Figure 12A & B). Acute on chronic, ethanol feeding also suppressed liver cathepsin B and L activities by 45% and 33%, respectively, in HIV-infected humanized mice compared with mice fed control diet (Figure 12C&D). Also, livers of ethanol-fed mice exhibited 37.5% and 29% lower chymotrypsin- and trypsin-like peptidase activities, respectively in homogenates of livers from HIV-infected mice (Figure 12E&F). Finally, we found that the levels of the oxidative stress marker and lipid peroxidation product, malondialdehyde (measured by TBARS) was significantly higher in both ethanol-fed uninfected and HIV-infected humanized mice than in pair-fed control mice (Figure 12G).
Figure 12: Effect of acute-on chronic ethanol feeding on LAMP1 protein expression, oxidative stress, cathepsin and proteasome peptidase activities in livers of HIV-infected and uninfected humanized mice: Livers from control- and ethanol-fed either HIV-infected or uninfected mice were used for (A, B) Immunoblotting analysis of LAMP 1 protein expression and quantification of immunoreactive bands. (C, D) Cathepsins B and L activities were measured in liver homogenates by in vitro fluorometric assay using fluorometric substrates. (E, F) Chymotrypsin-Like Activity-Cht-L, and Trypsin Like Activity-T-L were detected in liver homogenates by in vitro fluorometric assay using fluorometric substrates. (G) Lipid peroxidation product, malondialdehyde (MDA) was measured by thiobarbituric acid reactive substances (TBARS) assay. Data are presented as Mean ± SEM. (n=3 mice/group) Bars marked with the same letter are not significantly different from each other; bars with different letters are significantly different (P ≤0.05).
Discussion

Alcohol-impaired lysosomal function in HIV-infected hepatocytes is the mechanism for HIV and alcohol-induced hepatocyte apoptosis. However, HIV, which may not be generally considered hepatotropic enters hepatocytes to induce apoptosis. Previous studies have identified the gut-liver axis as the biggest portal for HIV entry to the liver (Sherman et al., 2019, Khan et al., 2017). While the latter may support HIV presence in the liver, HIV entry into CD4-negative hepatocyte is required for HIV-induced pathogenicity in the liver. Unlike CD4 positive immune cells, hepatocytes rely on either CCR5/CXCR4 or direct endocytosis for access into hepatocytes (Miyauchi et al., 2009, Tavares et al., 2021, Yasen et al., 2017). Hence, we treated RLW cells with maraviroc to identify the receptor of HIV entry into hepatocytes. HIV entry was completely attenuated by maraviroc in the presence and absence of alcohol metabolites. This was not surprising as CCR5 was already reported as HIV entry receptor in hepatocytes (Blackard et al., 2019). Since HIV accumulation was potentiated by alcohol metabolites, upregulation of CCR5 expression was expected in RLW cells exposed to alcohol metabolites. However, only minimal effects of AGS were observed on CCR5 expression in hepatocytes. Hence, this does not explain AGS-induced HIV accumulation in RLW cells. Moreover, HIV endocytosis have also been described as alternative pathway for HIV entry for CD4-negative cells. While the endocytic HIV entry pathway may be disputable, substantial supporting evidence for this pathway exists. Marechal et al. demonstrated receptor-mediated endocytosis of HIV in monocyte-derived macrophages
(Maréchal et al., 2001). Similarly, another study suggested endocytosis as the mechanism for HIV entry into T cells and monocytes after blocking CD4 receptors with antibodies (Pauza and Price, 1988). A more recent study also validated endocytosis as the HIV entry mechanism for CD4 negative cells (Miyauchi et al., 2009). Hepatocytes that are CD4 negative are therefore not exempted from endocytic internalization of HIV. Antigens that are internalized through the endocytic pathways are canonically fated for degradation by the pH-dependent lysosome (Pu et al., 2016). This may explain why only low-level HIV is detected in hepatocytes (Ganesan et al., 2019b, Kong et al., 2012b). However, HIV survival in the endosome may be sustained when lysosome degradation function is impaired. In fact, bafilomycin-mediated lysosome alkalinization, which impaired lysosome functions increased HIV infectivity in HeLa Magi cells (Fredericksen et al., 2002). Similarly, alcohol is known to alkalinize lysosomes (Kharbanda et al., 1997). This will prolong HIV survival in the endosomal-lysosomal system, where they were fated for degradation (Dagur et al., 2021). While this partly explains HIV accumulation in hepatocytes, it does not completely explain the mechanism of alcohol metabolite-induced apoptosis in HIV-containing hepatocytes. Meanwhile, previous data from our lab indicated suppressed lysosome function, which correlates with ROS generation and apoptosis in alcohol and HIV-treated hepatocytes. Therefore, alcohol-induced lysosomal dysfunction in HIV-infected hepatocytes was studied. Here, ROS generation due to alcohol metabolites and HIV accumulation was observed. The ROS-induced DRAM1 interacted with BAX to disrupt lysosomal membranes. Hence, co-localization of
cathepsins that leaked out of lysosome co-localized with mitochondria to permeabilize the outer membrane and to induce intrinsic hepatocyte apoptosis. Along with aforementioned effect strongly impairing lysosome functions in RLW cells exposed to both alcohol metabolites and HIV, alcohol metabolites also impair lysosome biogenesis. The latter may be attributed to decreased degradation of non-ubiquitylated TFEB by 20S proteasome in cytosol combined with alcohol metabolites- impaired TFEB trafficking to the nucleus. Additionally, exposure of RLW cells to AGS increases the expression of master repressor ZKSCAN3 that blocks TFEB target gene activation, thereby suppressing lysosomal genes. To our knowledge, this transcriptional repressor of lysosome genes has never been explored in the context of the combined treatment of hepatocytes with acetaldehyde and HIV.

It became clear from our results that some downstream events we addressed in this study were a result of synergistic/additive effects of HIV and acetaldehyde. To sort out the events which are “specific” for HIV-infected hepatocytes only and/or are of more general character in ethanol-metabolite-exposed liver cells was one of the purposes of this study, and the results are summarized in Table 1.

**Table 1.** Endpoints of the current study regulated by AGS only or by the combination of AGS/ethanol with HIV.
<table>
<thead>
<tr>
<th>Parameters</th>
<th>Effect due to:</th>
<th>Figures</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cathepsin B activity</td>
<td>AGS + HIV</td>
<td>5A</td>
</tr>
<tr>
<td>Cathepsin L activity</td>
<td>AGS + HIV</td>
<td>5B</td>
</tr>
<tr>
<td>LAMP1 immunostaining</td>
<td>AGS</td>
<td>5C,D</td>
</tr>
<tr>
<td>LAMP1 Western blot</td>
<td>AGS + HIV</td>
<td>5E,F</td>
</tr>
<tr>
<td>p53 mRNA</td>
<td>AGS</td>
<td>6A</td>
</tr>
<tr>
<td>DRAM1</td>
<td>AGS + HIV</td>
<td>6D,E</td>
</tr>
<tr>
<td>LAMP1 + Galectin 3 immunostaining</td>
<td>AGS + HIV</td>
<td>6F,G</td>
</tr>
<tr>
<td>Cathepsin B restoration by NAC</td>
<td>AGS + HIV—higher</td>
<td>7A</td>
</tr>
<tr>
<td></td>
<td>magnitude</td>
<td></td>
</tr>
<tr>
<td>Cathepsin L restoration by NAC</td>
<td>AGS + HIV—higher</td>
<td>7B</td>
</tr>
<tr>
<td></td>
<td>magnitude</td>
<td></td>
</tr>
<tr>
<td>M30</td>
<td>AGS + HIV</td>
<td>7C</td>
</tr>
<tr>
<td>Tom 20 + Cathepsin B immunostaining</td>
<td>AGS + HIV</td>
<td>8A,B</td>
</tr>
<tr>
<td>Cleaved caspase 3</td>
<td>AGS + HIV</td>
<td>8C,D</td>
</tr>
<tr>
<td>DRAM1+BAX immunostaining</td>
<td>AGS+HIV</td>
<td>9</td>
</tr>
<tr>
<td>Nuclear TFEB/Cyto TFEB</td>
<td>AGS + HIV</td>
<td>10A,B</td>
</tr>
<tr>
<td>PTFEB (S211)</td>
<td>AGS</td>
<td>10D,E</td>
</tr>
<tr>
<td>Acetylated Tubulin</td>
<td>AGS</td>
<td>10H,I</td>
</tr>
<tr>
<td>Chymotrypsin-like activity</td>
<td>AGS</td>
<td>11A</td>
</tr>
<tr>
<td>Trypsin-like activity</td>
<td>AGS</td>
<td>11B</td>
</tr>
<tr>
<td>ZKSCAN3</td>
<td>AGS</td>
<td>11C,D</td>
</tr>
<tr>
<td>LAMP1 mRNA</td>
<td>AGS + HIV</td>
<td>11E</td>
</tr>
<tr>
<td>Cathepsin D</td>
<td>AGS + HIV</td>
<td>11E</td>
</tr>
</tbody>
</table>
Importantly, the changes in cathepsin activities, lysosome leakage-related parameters (DRAM expression, DRAM1-BAX colocalization and LAMP1–Galectin 3 co-localization) as well as caspase 3 cleavage and TFEB translocation to nucleus were higher in AGS-exposed HIV-infected than in non-infected liver cells and are the results of synergistic or additive effects from both components—HIV and ethanol metabolites.

Conclusion

The summary of the events leading to alcohol-induced apoptosis due to lysosomal dysfunction in HIV-expressing hepatocytes is highlighted in Figure 13.

Figure 13: Role of lysosomal rupture/dysfunction in HIV- and ethanol-metabolism-induced apoptosis in hepatocytes: (1) Combined treatment with HIV and AGS triggers ROS and acetaldehyde release, which mediates lysosome leakage; (2) HIV/ethanol metabolism triggers the release of DRAM1 and BAX from mitochondria. Interaction between DRAM1 and BAX induces lysosome leakage; (3) Cathepsin B leaked out from damaged lysosome and diffused into the mitochondrion to initiate the intrinsic apoptotic pathway; (4) Caspases 9 and 3 become cleaved,
leading to hepatocyte apoptosis; (5) Alcohol metabolites inhibit lysosome biogenesis factor TFEB, hence impairing the compensation of damaged lysosomes; Both lysosome damage and impaired lysosome biogenesis lead to HIV–ethanol-metabolism-induced lysosome dysfunction, which triggers apoptosis.
CHAPTER 3: ALCOHOL AND HIV-DERIVED HEPATOCYTE APOPTOTIC BODIES INDUCE HEPATIC STELLATE CELL ACTIVATION


Introduction

Recently, we demonstrated a “double-edged sword” phenomenon where alcohol metabolites favorably induced massive apoptosis in HIV-expressing hepatocytes. While clearance of hepatocytes bearing HIV RNA and HIV proteins prevents the accumulation of HIV DNA and its integration in the hepatocyte genome, the generated hepatocyte apoptotic bodies (AB) adversely activate profibrotic genes when engulfed by HSC (Ganesan et al., 2019b). In the previous Chapter, we uncovered oxidative stress-mediated lysosome impairment as the basis for alcohol metabolite-induced apoptosis in HIV-expressing hepatocytes (New-Aaron et al., 2021d). However, the mechanisms of AB engulfment and profibrotic activation of HSC in the context of HIV and alcohol are not yet understood.
Hepatic fibrosis is characterized by two dynamic and contrasting events, i.e., excessive extracellular matrix (ECM) deposition accompanied by its decreased degradation. Activation of hepatic perisinusoidal resident non-parenchymal cells, HSC, is a principal event in liver fibrotic development and progression. Quiescent HSC typically contain retinoids. Upon activation, HSC undergo a morphological and functional transformation into α-smooth muscle actin (SMA)-expressing myofibroblasts. Several triggers, including bacterial lipopolysaccharide (Chen et al., 2017), reactive oxygen species (Ramos-Tovar and Muriel, 2020), and viruses (Cheng et al., 2017), are known to activate HSC. In addition, multiple studies have identified CpG motifs in DNA as a potent stimulant of HSC through the toll-like receptor (TLR)-9, localized in the endosomal compartment of HSC (Gäbele et al., 2008, Kiziltas, 2016, Nakamoto and Kanai, 2014). Since hepatocyte apoptosis often accompanies liver fibrosis, it can be attributed to the direct effects of Damage Associated Molecular Patterns, DAMPs, from hepatocyte ABs (Watanabe et al., 2007).

Although hepatocyte ABs are known to canonically activate HSC via TLR9, ABs from acetaldehyde and HIV exposed hepatocytes (AB\textsubscript{AGS+HIV}), in our hands, surprisingly, did not activate HSC through TLR9. This prompted us to look for other mechanistic explanations for AB\textsubscript{AGS+HIV}-induced HSC activation. Therefore, we first characterized AB exposed to acetaldehyde and HIV to explore their content. Furthermore, HSCs were characterized to identify the candidate receptors involved in AB engulfment. Given that AB\textsubscript{AGS+HIV} is a product of HIV particle accumulation and the subsequent oxidative stress,
mitogen activated protein kinases (MAPK) (Zhang et al., 2016, Watanabe et al., 2007, Son et al., 2011, Ichijo, 1999), and the JAK-STAT3 pathway (Simon et al., 1998, Butturini et al., 2020, Charras et al., 2019), which notoriously respond to signals from reactive oxygen species (ROS), endothelial cell products and viral particles were explored as potential mechanisms for ABAGS+HIV-induced HSC activation.

**Materials and Methods**

**Reagents and Antibodies**

Reagents were purchased from the following commercial suppliers: high-glucose Dulbecco’s Modified Eagle Medium (DMEM) and fetal bovine serum (FBS) were obtained from Invitrogen (Waltham, MA, USA); Trizol was purchased from Life Technologies (Carlsbad, CA, USA); primer probes, high-capacity reverse transcription kit, and real-time polymerase chain reaction (RT-PCR) reagents were from Applied Biosystems by Thermo Fisher Scientific, Carlsbad, CA, USA. Pharmacological Inhibitors: TAM Family protein inhibitor, LDC1267, was obtained from MedChemExpress (Monmouth Junction, NJ, USA, HY-12494), JNK inhibitor, SP600125, was obtained from Selleckchem (Houston, TX, USA, S1460), SB202190 for MAPK p38 was obtained from Selleckchem (Houston, TX, USA, S1077). Primary antibodies used were: (a) mouse monoclonal: Anti-HIV-1 p24 Antibody: sc-69728, Anti-Stat3 Antibody (F-2): sc-8019, Anti-β-Actin Antibody (C4): sc-47778 (Santa Cruz Biotechnology, Dallas, TX, USA), anti-smooth muscle actin: MAB1420-SP, anti-human proteinS: AMB4036-SP (R&D Systems,
Minneapolis, MN, USA). Anti-Nef antibody: ARP-3689, Anti-HIV1 Tat antibody: ARP7377 (American Type Culture Collection, Manassas, VA, USA) (b) rabbit monoclonal and polyclonal: anti-HIV-1 Vpr (American Type Culture Collection, Manassas, VA, USA, ARP-11836); anti-Stat1 antibody (Santa Cruz Biotechnology, Dallas, TX, USA, Sc-592), anti-phospho-ERK1/2: 9101, anti-ERK1/2: 4694, phosphor-STAT1:9177 (Cell Signaling Technology, Danvers, MA, USA), Anti-Cytochrome P450 Enzyme CYP2E1 Antibody: AB1252 (Millipore Sigma, Burlington, MA, USA) (c) Goat polyclonal: anti human AXL antibody: AF154-SP, anti-human Gas6 antibody: AF885-SP (R&D Systems, Minneapolis, MN, USA) (d) secondary antibodies: (I) IRDye 680RD Goat anti-Rabbit, C50317-02; IRDye 680RD Donkey anti-Mouse, C50520-02; (II) Goat anti-Mouse IgG (H + L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 555, Carlsbad, CA, USA, A21422; and Donkey anti-Rabbit IgG (H + L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 488.

In Vitro Studies

Since our experiments required cell-to-cell communication, we performed most experiments on two major human cell lines: hepatocyte-like RLW cells (Huh7.5 cells stably transfected with CYP2E1) and HSC-like LX2 cells. We used these cell lines because primary human hepatocytes (PHH) and HSC are not readily available. RLW cells were characterized by attenuated innate immunity, making them suitable for demonstrating the HIV-infection effects. However, RLW cells do not express alcohol dehydrogenase (ADH) and only metabolize ethanol by CYP2E1, generating very low
amount of acetaldehyde. Therefore, to mimic ethanol metabolism in RLW cells as observed in primary human hepatocytes, we developed an exogenous, continuous source of acetaldehyde because the observed pro-apoptotic effect of ethanol comes from ethanol metabolism. The exogenous supply of acetaldehyde, called the acetaldehyde-generating system (AGS), was introduced to the cell culture medium. AGS continuously produces physiological amounts of acetaldehyde when 0.02 EU yeast ADH metabolizes 50 mM ethanol in the presence of 90mM nicotinamide adenine dinucleotide (NAD) as a co-factor (New-Aaron et al., 2020).

As a source of HIV, primary human macrophage-propagated HIV-1\textsubscript{ADA} was purified at University of Nebraska Medical Center (UNMC) [14]. The LX2 cell line, derived from human HSC, was generously provided by Dr. Laura Schrum (Carolinas Healthcare System, Charlotte, NC, USA), who originally obtained the cell line from Dr. Scott Friedman (Icahn School of Medicine at Mount Sinai, NY, USA).

To generate the required ABs, RLW cells were pretreated or not for 24 h with AGS, infected or not with 0.1 MOI HIV-1\textsubscript{ADA} overnight, and then exposed to AGS or not for 96 h. HIV in culture medium and cell membrane-bound HIV were removed from the culture system by copiously washing the HIV-exposed RLW cells with DMEM at least three times. This 96-h exposure to AGS after the treatment of cells with HIV induces robust apoptosis in RLW cells.
Isolated RLW ABs were introduced to LX2 cells grown in 2% FBS-containing DMEM. RLW Abs, quantified by nanoparticle tracking analysis (NTA), were introduced to LX2 cells at the ratio of 3:1 and incubated for either 2 h or 48 h depending on the endpoint measurement. mRNA measurements were obtained from LX2 cells exposed to RLW ABs for 2 h, while proteins were measured in LX2 cells exposed to RLW ABs for 48 h.

**Isolation of ABs from RLW Cells**

Isolation of ABs from RLW cells was performed by a differential centrifugation technique as previously reported [30]. Briefly, AB-containing HIV-exposed and AGS-pretreated RLW cell supernatants were collected in a 50 mL conical tube and then centrifuged at 300× g for 10 min to pellet the cell debris. The cell debris-free supernatants were collected into a new 50 mL conical tube and then centrifuged at 3000× g for 20 min to pellet RLW ABs. The pelleted RLW ABs were separated from the exosome and microvesicle-rich supernatant. To avoid exosome or microvesicle contamination, the pelleted ABs were washed three times with FBS-free DMEM.

**Generation of TAMRA-Labeled RLW ABs by Prolonged Incubation**

RLW cells were cultured as described in Section 2.2. Before the final 96 h incubation of RLW cells with AGS, 10 µM TAMRA-succinimide ester was added to the RLW media and incubated at 37 °C for 30 min. Then excess TAMRA dye was removed by washing the cells thrice with copious media, and the TAMRA-labeled cells were left to incubate.
After 96 h of incubation, TAMRA-labeled AB-containing supernatants were collected from the culture flask or dish and processed as indicated in Section 2.3

**siRNA Transfection of LX2 Cells**

Inhibition of STAT3 expression in LX2 cells was achieved by STAT3 siRNA (sc-29493, Santa Cruz, Dallas, TX, USA) transfection according to the manufacturer’s instructions. The siRNA transfection efficiency was evaluated using scrambled siRNA-FITC Conjugate-A (sc-36869, Santa Cruz, Dallas, TX, USA), and immunostaining was conducted to detect signal-positive cells. Four hours before the siRNA (STAT3 or scrambled) application, FBS-containing media (DMEM, 2% FBS and 1% Penicillin streptomycin) were replaced with FBS-free media (DMEM and 1% Penicillin streptomycin) in the LX2 culture system. Thereafter, the cells were incubated with siRNA in transfection media (sc-36868, Santa Cruz, Dallas, TX, USA) for 6 h. To evaluate the effects of AB_{AGS+HIV} from RLW cells on LX2 cells after STAT3 genes silencing, AB_{AGS+HIV} were introduced to LX2 cells at the ratio of 3:1 for 2 h or 48 h to measure changes in profibrotic genes and proteins. Control siRNA (sc-37007, Santa Cruz, Dallas, TX, USA) was included in the experiment as a negative control.

**Imaging of ABs by Transmission Electron Microscopy**

ABs derived from RLW cells were isolated as described in Section 2.3. To study the size and morphology of the ABs, an isolated sample was submitted to the Electron
Microscopy Core Facility at the UNMC. A FEI Technai G2 Spirit transmission electron microscope was used to evaluate AB sizes and morphologies.

**In Vivo Studies**

For validation purposes, immunodeficient NSG mice (NOD.Cg-Prkdcsid Il2rgtm1Wjl/SzJ, NSGTM Stock No: 005557, The Jackson Laboratories) were used for evaluating the ability of HIV-infected ABs to promote profibrotic changes when engulfed by LX2 cells. Immunodeficient NSG mice were used because (I) the exclusion of a species-specific immune response elicited between mouse HSC and human hepatocyte-derived ABs is necessary, (II) previous experience working with NSG mice and evidence from already published works showed that NSG mice have a high tolerance for alcohol administration (Powers et al., 2012), and (III) NSG mice are natural killer (NK) cell deficient, which is beneficial for our model because NK cells in the presence of ABs can interfere with liver fibrosis (Melhem et al., 2006).

Immunodeficient NSG male mice, 8–10 weeks old, were fed with a nutritionally adequate liquid diet containing 5% ethanol or a pair fed diet in which ethanol was isocalorically substituted with dextran maltose (BioServ, Frenchtown, NJ, USA). For the ethanol group, ethanol was introduced gradually by increasing ethanol content by 1% (v/v) every day until the mice attained a consumption of 5% ethanol (v/v). This feeding was continued for ten weeks. To study the role of HIV-containing ABs and alcohol in fibrosis development, mice were subdivided into the following groups:
Control-fed mice exposed to (A) uninfected and (B) HIV-infected ABs generated from RLW cells.

Ethanol-fed mice exposed to (A) uninfected and (B) HIV-infected ABs from RLW cells. Each subgroup contains 3 mice.

Given that previous fibrosis studies supplemented ethanol diet with carbon tetrachloride, CCl4, injections to attain liver fibrosis in NSG mice (Muthiah et al., 2019), 2.5 mL/kg body weight of 10% CCl4 (Sigma, St. Louis, MO, USA) dissolved in olive oil was injected intraperitoneally three times per week during the last two weeks of ethanol/control feeding. This corresponded to the period of RLW ABs (0.5–1 × 10^7/mouse) intraperitoneal injection (i.p). In the 10th week of the experiment, the mice were sacrificed. Liver damage was measured by ALT/AST in serum. Liver tissue was stained by Sirius Red for fibrosis. Profibrotic genes were measured by real time polymerase chain reaction (RT-PCR).

**RNA Isolation and RT-PCR**

HIV gag, TIMP1, TGFβ1, TGFβ2, COL1A1, MMP2, SOCS1, ACTA2, APOBEC, OAS1, ISG15, and IL6 mRNAs were measured by RT-PCR as previously described (New-Aaron et al., 2021d). Total cellular RNA was isolated from cells using Trizol reagent. This involved a two-step procedure in which at least 200 ng of RNA was transcribed to cDNA using a high-capacity reverse transcription kit (Applied Biosystems, ThermoFisher Scientific, Waltham, MA USA), and the cDNA transcript was amplified
using TaqMan Universal Master Mix II with fluorescent-labeled primers (TaqMan gene expression systems) using a Model 7500 qRT-PCR thermal cycler. The relative quantity of each RNA transcript was calculated by its threshold cycle (Ct) after subtracting the reference, GAPDH.

**Immunoblotting**

Immunoblotting was performed as described (Ganesan et al., 2019b); however, in this study, blots were developed using 27444 Bio-Rad Imaging System Chemidoc Touch Imaging System and protein band densities were quantified using the National Institute of Health (NIH) Image J software program. Equal amounts of protein (20 µg) were loaded in each lane. β-actin was used as the loading control for normalization.

**Immunofluorescence**

LX2 cells (15,000/well) were seeded onto a 16-well chamber slide. RLW ABs were introduced to LX2 cells at the ratio of 3:1. After overnight incubation of cells with RLW ABs, the RLW ABs containing medium were removed and replaced with fresh media. LX2 cells were incubated for another 48 h, then were washed with 1× PBS, fixed with 4% paraformaldehyde for 12 min at 37 °C, permeabilized with 0.1% Triton X-100 for 3 min at room temperature, and blocked for 30 min with 1% (g/vol) bovine serum albumin (BSA) in PBS. LX2 cells were stained to study α-SMA and STAT3. First, cells were incubated with primary antibodies for 1 h, then with Alexa-Fluor-labeled secondary antibodies for 30 min. Nuclei were stained with DAPI. The coverslips were transferred
to microscope slides for imaging with a Keyence BZ-X810 fluorescence microscope.

Immunostainings were quantified for intensity using the Image J V1.8.0 software program, NIH (Bethesda, MD, USA).

**Statistical Analyses**

Data were analyzed using GraphPad Prism v7.03 software (GraphPad, La Jolla, CA, USA). Data from at least three duplicate, independent experiments were expressed as mean ± SEM. Comparisons among multiple groups were performed by non-parametric one-way ANOVA and Kruskal–Wallis test, accompanied by a post hoc test. For comparisons between two groups, we used the Mann–Whitney U test. A p-value of 0.05 or less was considered statistically significant.

**Results**

**HIV RNA, HIV Proteins and Malondialdehyde Were Expressed by RLW ABs**

Since extracellular vesicles (EVs) are established carriers of molecular cargoes known to mediate intercellular communications (Maacha et al., 2019, Maas et al., 2017, Yoon et al., 2014), it became expedient to uncover the contents of RLW ABs. First, the sizes of the isolated RLW ABs were determined to be 800–1700 nm (Figure 14A) via electron microscopy. This corresponds to previously reported sizes of ABs (Battistelli and Falcieri, 2020). Then, we observed through NTA that the combined treatment of acetaldehyde and HIV engendered the highest concentration of RLW ABs (Figure 14B). Since
previously observed intense hepatocyte apoptosis was due to acetaldehyde-induced HIV RNA and protein accumulation in hepatocytes, the HIV RNA content of RLW ABs was investigated. We found the highest content of HIV gag RNA in RLW ABs generated from cells treated with both acetaldehyde and HIV (Figure 14C). This was accompanied by upregulated HIV protein (p24, Nef, and Tat, but not Vpr) expressions in RLW AB_{AGS+HIV} (Figure 14D–G). Moreover, we measured the oxidative stress marker, malondialdehyde, MDA by immunoblotting analysis and this was upregulated by 62.5% in AB_{AGS+HIV} as compared to AB_{Control} (Figure 14H,I). The purities of the isolated RLW ABs were confirmed by probing the ABs lysate for exosome marker CD63. While CD63 was not expressed by RLW ABs, other apoptotic markers, namely, calnexin, calreticulin (Figure 14J) and phosphatidylserine (PtylS) (Figure 14K), were sufficiently expressed. CYP2E1 (Figure 15A,B) in AB_{AGS+HIV} was expressed, and this validate that ROS are one of the triggers of RLW apoptosis under the combined treatment with acetaldehyde and HIV as indicated previously (Ganesan et al., 2019b, New-Aaron et al., 2021d).
Figure 14: Characterization of ABs derived from acetaldehyde and HIV-exposed RLW: (A) Sizes of RLW ABs visualized by transmission electron microscopy. (B) Quantification of RLW ABs by NTA. (C) HIV gag RNA expression in RLW ABs quantified by RT-PCR analysis. (D) HIV proteins: p24, Nef, and Tat in RLW ABs were measured by immunoblotting analysis. Equal amounts of protein (20 µg) were loaded in each lane. β-actin was used as an internal control. (E–G) Quantification of the immunoreactive bands of HIV proteins: p24, Nef, and Tat in RLW ABs. (H,I) Malondialdehyde (MDA) content in RLW ABs was measured by immunoblotting analysis and quantification of immunoreactive protein bands was provided. Equal amounts of protein (20 µg) were loaded in each lane. β-actin was used as an internal control. (J) Exosome marker (CD63) and AB markers (calnexin and calreticulin) were measured by immunoblotting analysis of RLW AB lysate. Equal amounts of protein (20 µg) were loaded in each lane. (K) Quantification of phosphatidylserine surface expression on RLW ABs measured by flow cytometry. Western blot detected bands were from the representative experiments. Statistically processed data are from 3 independent experiments presented as mean ± SEM. Bars marked with the same letter are not significantly different; bars with different letters are significantly different from each other (p ≤ 0.05).
Cytochrome p450 2E1 was expressed by RLW AB\textsubscript{AGS+HIV}

![Image of gel Electrophoresis showing expression levels of CYP2E1 and β-actin](image)

**Figure 15**: ABs derived from acetaldehyde and HIV-exposed RLW cells. (A) Upregulation of CYP2E1 (B) Quantification of the immunoreactive bands of CYP2E1. Data are from 3 independent experiments presented as mean ± SEM. Bars marked with the same letter are not significantly different and bars with different letter are different from each other (p ≤ 0.05).

**Engulfment of RLW ABs by LX2 Cells**

During efferocytosis of ABs, PtylS expressed on ABs are recognized by phosphatidylserine recognition receptors (PtylSRR) on HSC (Naeini et al., 2020). These PtylSRR are either TIM family receptors or TAM family receptors (Miyanishi et al., 2007, Lemke, 2017). While LX2 cells do not express TIM 1 and TIM3 (Figure 16A&B), they express TAM receptors. The TAM family receptors consist of Axl, Tyro3 and Mer, sub-family members of receptor tyrosine kinases (RTKs) (Lemke, 2017). As previously reported, Mer is predominantly expressed on macrophages of specific organs but not in
the liver (Shao et al., 2009, Raggi et al., 2017). Therefore, only Axl and Tyro3 were tested as potential receptor candidates for LX2 cell engulfment of RLW ABs.

While immunofluorescence staining revealed the expression of both Axl and Tyro3 in LX2 cells, Axl was predominantly localized extranuclearly (Figure 17A), while Tyro3 was localized intranuclearly (Figure 17B). This excludes Tyro3 as the potential TAM receptor candidate on cell surface responsible for the engulfment of RLW ABs by LX2 cells. Furthermore, effective engulfment via TAM receptors requires intracellularly generated ligands, Gas 6 and Protein S, to act as inserts between the PtdIns on ABs and TAM receptors on LX2 cells (Chua et al., 2018). To this end, the expressions of these ligands were confirmed by the visualization of Gas 6 and Protein S in LX2 cells (Figure 17C, D). Since LX2 cells express Axl and Gas 6/Protein S, the LX2 engulfment of RLW ABs was demonstrated by exposing TAMRA-labeled ABs to LX2 cells (Figure 17E).
Figure 16: HSC do not express TIM family protein receptors. Flow cytometric measurement of (A) TIM1, (B) TIM3.
Figure 17: Gas6 and Protein S mediated engulfment of RLW ABs by the AXL-expressing LX2 cells: (A) Axl receptors, (B) Tyro3 receptors, (C) Gas 6, (D) Protein S, (E) Immunofluorescence staining to demonstrate the engulfment of RLW ABs by LX2 cells. RLW ABs were labeled with TAMRA dye and LX2 membranes were stained with cytoplasmic dye (CellBrite® Cytoplasmic Membrane Dyes, San Francisco, USA). Staining was visualized using 20× lens of a Keyence BZ-X810 fluorescence microscope. Pictures are of representative data from three independent experiments.

Pharmacological Inhibition of Axl Blocks LX2 Engulfment of RLW ABs and Attenuates Activation of Profibrotic Genes

Given that Axl is the potential TAM receptor for LX2 engulfment of RLW ABs, pharmacological inhibition of Axl with 1µM LDC1267 (Axl inhibitor) reduced the
number of engulfed TAMRA-labeled RLW ABs by 6-fold (Figure 18A, B). As an additional confirmation, this inhibitor showed attenuated expression of TAMRA-labeled ABs in LX2 cells, as indicated by flow cytometry (Figure 18C). Consequentially, this inhibitor downregulated HIV gag RNA expression (Figure 18D), SOCS 1 genes (mRNA) (Figure 18E) and suppressed profibrotic genes (Figure 18F–I) in LX2 cells exposed to RLW AB\textsubscript{AGS-HIV}.

**Figure 18:** AXL inhibitor attenuates HIV RNA expression and profibrotic genes in LX2 cells exposed to AB\textsubscript{AGS-HIV}: (A) Immunofluorescence staining demonstrates the attenuation of TAMRA-labeled AB entry into LX2 cells pretreated with Axl-inhibitor. Staining was visualized
using a 20× BZ-X810 fluorescence microscope. Pictures are the representative data from three independent experiments. (B) Quantification of engulfed TAMRA-labelled RLW ABs by LX2 cells. Engulfment of TAMRA-labelled RLW ABs by LX2 cells was counted manually in 3 fields. (C) Intracellular quantification of TAMRA-labelled RLW ABs exposed to LX2 cells (flow cytometry). RT PCR analysis of: (D) HIV gag RNA, (E) SOCS1 mRNA, (F) TIMP1 mRNA, (G) COL1A1 mRNA, (H) TGFβ1 mRNA, and (I) ACTA2 mRNA. Data are from 3 independent experiments presented as mean ± SEM. * Indicates significant difference (p ≤ 0.05).

Engulfment of HIV- and MDA-Containing ABs Induces LX2 Profibrotic Activation

We explored the possible effects of RLW ABs engulfment by LX2 cells. First, we checked if the HIV gag RNA or HIV proteins were delivered to recipient LX2 cells with RLW ABs. It appeared that, in LX2 cells, the expression of HIV gag RNA was 5.5-fold more after exposure to AB_{AGS+HIV} compared with exposure to RLW AB_{HIV} (Figure 19A). To determine if engulfed RLW AB_{AGS+HIV} can elicit productive infection in recipient LX2 cells, we exposed AB_{AGS+HIV} at the ratio of 3:1 to permissive-macrophage-like cell THP1 (as a positive control). While we observed upregulation of HIV RNA in THP1 cells exposed to increasing amount of HIV_{ADA}, there was no such effect when AB generated from AGS+HIV-exposed hepatocytes were used to infect THP1 cells (Figure 19B); at days 1 and 3 post-AB_{AGS+HIV} exposure, using ELISA techniques, we also could not detect the release of p24 to the culture media from these AB macrophages (Figure 19C).

Furthermore, a two-time point (Day 1 and 3) kinetic experiment in THP1 cells incubated with AB_{AGS+HIV} did not significantly change reverse transcriptase (RT), an HIV-replication enzyme, activity (Figure 19D). However, the level of ROS generated in LX2 cells after
internalization of RLW AB\textsubscript{AGS+HIV} was 45\% higher than in AB-non-exposed LX2 cells (Figure 19E). Moreover, significant profibrotic activation was observed in LX2 cells exposed to AB\textsubscript{AGS+HIV} (Figure 19F–J). Also, RLW AB\textsubscript{AGS+HIV} expressed no profibrotic genes up-regulated in HSCs due to AB internalization (Figure 20A&B). Interestingly, when we used AB\textsubscript{AGS+HIV} generated from lymphocyte-like Jurkat cells replicating HIV and treated with AGS and HIV as RLW cells, we observed no profibrotic activation of LX2 cells after internalization of these ABs even though they transferred HIV gag RNA to LX2 cells (Figure 21A-D).
Figure 19: Engagement of ABAGS+HIV enhances HIV RNA and ROS in LX2 cells leading to their profibrotic activation: (A) RT-PCR analysis of HIV gag RNA expression in LX2 cells after engulfment of ABAGS+HIV. (B) RT-PCR analysis of HIV gag RNA expression in THP-1 cells after 48 h exposure to RLW ABAGS+HIV. (C) Quantification of HIV p24 levels in the culture media from THP1 exposed to RLW ABAGS+HIV for 48 h by ELISA. (D) RT activity in THP-1 cells exposed to RLW ABAGS+HIV. Open dots represent THP1 cells exposed to HIV1ADA; closed dots represent THP1 cells exposed to Abs. (E) ROS generation after 48 h exposure of LX2 cells to RLW ABAGS+HIV (2’,7’-dichlorodihydrofluorescein diacetate assay). RT-PCR analysis of (F) COL1A1 mRNA, (G) TIMP1 mRNA, and (H) TGFβ2 mRNA of LX2 cells exposed to RLW ABAGS+HIV for 2 h. (I,J) α-Smooth muscle actin (SMA) of LX2 cells exposed to RLW ABAGS+HIV for 48 h was measured by immunoblot analysis and the quantification of immunoreactive protein bands. Equal amounts of protein (20 µg) were loaded in each lane. β-actin was used as an internal control. Data are from 3 independent experiments presented as mean ± SEM. Bars marked with the same letter are not significantly different; bars with different letters are significantly different from each other. Bars with * are significantly different from each other (p ≤ 0.05).
Figure 20: ABs derived from acetaldehyde and HIV-exposed RLW cells. (A) TGFβ mRNA (B) COL1A1 mRNA. Data are from 3 independent experiments presented as mean ± SEM. Bars with * indicate significant difference (p ≤ 0.05).
Figure 21: ABs derived from acetaldehyde and HIV-infected Jurkat cells did not activate HSCs:
RT-PCR quantification of: (A) HIV gag RNA, (B) COL1A1 mRNA, (C) TGFβ1 mRNA, and (D) MMP2 mRNA. Data are from 3 independent experiments presented as mean ± SEM. Bars marked with the same letter are not significantly different; bars with different letters are significantly different from each other (p ≤ 0.05).

Pharmacological Inhibition of LX2 Cells Exposed to AB<sub>AGS-HIV</sub> Attenuates

Profibrotic Activation via JNK and ERK1/2 Pathway

Since MDA and HIV-containing AB<sub>AGS-HIV</sub> triggered ROS generation in LX2 cells and correlates positively with profibrotic gene activation, we hypothesize that c-jun N-
terminal Kinase (JNK) and extracellular signal-regulated kinase 1 and 2 (ERK1/2), members of the four distinct mammalian MAPK known to respond to ROS (McCubrey et al., 2006), are involved in the profibrotic activation of LX2 cells after engulfment of MDA- and HIV-containing RLW ABAGS-HIV. To test our hypothesis, LX2 cells exposed to ABAGS-HIV were pretreated with 10μM JNK inhibitor, SP600125. In this case, TGFβ1, ACTA2, and COL1A1 mRNAs were attenuated by approximately 25% when compared to LX2 cells without the inhibitor (Figure 22A–C). Since JNK is upstream to ERK1/2, JNK inhibitor attenuated phospho-ERK1/2, which is required for nuclear translocation of ERK1/2 to the nucleus to control proliferation and growth of LX2 cells. This consequentially led to the attenuation of α-SMA, Col1A1 and TGFβ genes in LX2 cells (Figure 22D–F). Immunostaining analysis corroborated the findings by the attenuation of α-SMA when LX2 cells were treated with JNK inhibitor (Figure 22G &H). To test if other MAPK family members provided similar effects, LX2 cells exposed to ABAGS-HIV were pretreated with 10μM p38 MAPK inhibitor, SB202190. Surprisingly, phosphoERK1/2, ACTA2, TGFβ1 mRNAs and α-SMA were upregulated in LX2 cells exposed to ABAGS-HIV (Figure 23A–E).
Figure 22: Attenuation of profibrotic markers in LX2 cells exposed to ABAGS+HIV by pharmacological inhibition of JNK pathway: RT-PCR analysis of (A) TGFβ1 mRNA, (B) ACTA2 mRNA, and (C) COL1A1 mRNA. (D–F) Phospho-ERK1/2 and α-smooth muscle actin were measured by immunoblot analysis and immunoreactive blots were quantified. Equal amounts of protein (20 µg) were loaded in each lane. β-actin was used as an internal control. (G) Immunofluorescent staining of α-smooth muscle actin. Staining was visualized using a Keyence BZ-X810 fluorescence microscope. Pictures are representative data from three independent experiments. (H) Quantification of SMA was measured using NIH ImageJ. Data are from 3 independent experiments presented as mean ± SEM. Bars marked with the same letter are not significantly different. Bars with * are significantly different from each other (p ≤ 0.05).
Figure 23: Pharmacological inhibition of p38 MAPK induces ERK activation followed by SMA deposition: RT-PCR quantification of: (A) ACTA2 and (B) TGFβ1. (C–E) Phopho-ERK1/2 and α-smooth muscle actin were measured by immunoblot analysis and the quantification of immunoreactive blots. Equal amounts of protein (20 µg) were loaded in each lane. β-actin was used as an internal control. Data are from 3 independent experiments presented as mean ± SEM. Bars with * indicate significant difference (p ≤ 0.05).

Oxidative Stress from ABAGS-HIV Activates JNK and ERK1/2 Pathway in LX2 Cells

Oxidative stresses are established signals for the activation of JNK and ERK1/2 pathways to activate critical cellular processes, such as differentiation, growth, cell proliferation, and cell survival (McCubrey et al., 2006). Given that oxidative products are
a vital cargo of RLW \textsubscript{ABAGS-HIV}, it becomes evident that oxidative stress contributes to the observed profibrotic activation. This was confirmed when pretreatment with 5 mM NAC, a known antioxidant, attenuated TGFβ2 mRNA and TIMP1 mRNA by 50% and 20%, respectively, in RLW \textsubscript{ABAGS-HIV}-treated LX2 cells (Figure 24A &B). NAC further attenuated ERK1/2 phosphorylation in a similar fashion as the JNK inhibitor, thereby resulting in the downregulation of α-SMA (Figure 24C–G).

**Figure 24:** NAC treatment reverses or prevents profibrotic activation of LX2 cells by inhibition of ERK1/2 pathway: RT-PCR analysis of (A) TGFβ2 mRNA and (B) TIMP1 mRNA. (C–E) Phospho-ERK1/2 and α-SMA were measured by immunoblot analysis and the quantification of immunoreactive blots. Equal amounts of protein (20 μg) were loaded in each lane. β-actin was
used as an internal control. (F) Immunofluorescent staining of α-SMA. Staining was visualized using a Keyence BZ-X810 fluorescence microscope. Pictures are representative data from three independent experiments. (G) Quantification of α-SMA by NIH ImageJ. Data are from 3 independent experiments presented as mean ± SEM. Bars with * are significantly different from each other (p ≤ 0.05).

**ABAGS-HIV Upregulates IL6 mRNA in LX2 Cells**

Since HIV gag RNA contained in AB AGS+HIV is deposited in LX2 cells after engulfment, it is necessary to investigate the JAK-STAT1 (antiviral) pathway which is required for Interferon Stimulating Genes (ISGs) activation. To induce JAK-STAT1 signaling, LX2 cells were treated with 1000 U/mL interferon IFNα for 30 min in the presence or absence of RLW ABAGS-HIV. Here, RLW ABAGS-HIV downregulated the STAT1 phosphorylation induced by IFNα as indicated by the immunoblots (Figure 25A &B). Next, to determine the effects of RLW ABAGS-HIV on ISGs, LX2 cells were treated with 400 U/mL IFNα for 6 h in the presence or absence of RLW ABAGS-HIV. RLW ABAGS-HIV attenuated APOBEC-3G (Figure 25C), OAS1 (Figure 25D) and ISG15 (Figure 25E) mRNAs. Moreover, RLW ABAGS-HIV induced the activation of IL6 mRNA (Figure 25F) suggesting up-regulation of IL6 cytokine, an inducer of the JAK-STAT3 activation.
Figure 25: Engulfment of ABAGS+HIV attenuates STAT1 activation and concurrently upregulates IL6 mRNA, leading to profibrotic activation. (A,B) STAT1 was measured by immunoblot analysis and the quantification of immunoreactive protein bands. Equal amounts of protein (20 µg) were loaded in each lane. β-actin was used as an internal control. RT-PCR analysis of (C) APOBEC-3G mRNA, (D) OAS1 mRNA, (E) ISG15 mRNA, and (F) IL6 mRNA. Data are from 3 independent experiments presented as mean ± SEM. Bars marked with the same letter are not significantly different. Bars with different letters are significantly different from each other (p ≤ 0.05).

siRNA STAT3 Transfection Inhibits STAT3 Protein Expressions in LX2 Cells

Since ABAGS+HIV induced the upregulation of IL6 mRNA, a STAT3 ligand gene, it became expedient to explore the role of ABAGS+HIV in the activation of the STAT3 pathway in LX2.
cells. To examine the input of the STAT3 pathway in the profibrotic activation of LX2 cells exposed to RLW ABAGS-HIV, we inhibited STAT3 genes by STAT3 siRNA transfection. The siRNA transfection efficiency was evaluated with scrambled FITC-labeled siRNA, resulting in 90.25% siRNA FITC-positive cells (Figure 26A&B). Control siRNA transfection was used as a negative control. Then, we cultured LX2 cells with STAT3 siRNA and control siRNA as described in the Section 2. An approximately 11-fold reduction of STAT3 puncta was observed in immunostained LX2 cells transfected with STAT3 siRNA compared to those transfected with control siRNA (Figure 26C&D). Additional analysis from immunoblots revealed the attenuation of STAT3 protein expression by 60% in LX2 STAT3 siRNA transfected cells compared to the cells transfected with control siRNA (Figure 26E&F).
Figure 26: SiRNA transfection of LX2 cells: (A) Transfection of LX2 cells with scrambled siRNA-FITC (green). Visualized using a 20× Keyence microscope. Pictures are representative data from three independent experiments. (B) Quantification of transfection efficiency. (C) Immunofluorescent staining of STAT3. Staining was visualized using a 20× lens in Keyence BZ-X810 fluorescence microscope. Pictures are representative data from three independent experiments. (D) STAT3 immunofluorescence intensity was measured using NIH ImageJ. (E,F) STAT3 was measured by immunoblot analysis and the quantification of immunoreactive protein bands. Equal amounts of protein (20 µg) were loaded in each lane. β-actin was used as an internal control. Data are from 3 independent experiments presented as mean ± SEM. Bars with * are significantly different from each other (p ≤ 0.05).
Silencing STAT3 in LX2 Cells Attenuates ABAGS+HIV Induced Profibrotic Activation

Since ABAGS+HIV induced upregulation of IL6 mRNA after 6 h of incubation with LX2 cells, STAT3 may be involved in the observed ABAGS+HIV-induced LX2 activation (Xiang et al., 2018). As a result, STAT3 was inhibited in LX2 cells by STAT3 siRNA transfection to evaluate the direct influence of STAT3 on ABAGS+HIV-induced LX2 activation. We found the attenuation of profibrotic genes: ACTA2 (Figure 27A), COL1A1 (Figure 27B), and TGFβ1 (Figure 27D). Furthermore, MMP2 mRNA, which encodes for the synthesis of a matrix-degrading enzyme, was upregulated by 50% (Figure 27C). These findings were paralleled by immunoblotting analysis of α-SMA in STAT3-silenced LX2 cells exposed to ABAGS+HIV. α-SMA in these LX2 cells was reduced by approximately 43% when compared to not transfected LX2 cells (Figure 27E &F).
Figure 27: STAT3-deficient LX2 cells show downregulation of profibrotic genes and α-SMA after exposure to ABAGS+HIV: RT-PCR analysis of: (A) ACTA2 mRNA, (B) COL1A1 mRNA, (C) MMP2 mRNA, and (D) TGFβ1 mRNA. (E,F) α-SMA was measured by immunoblot analysis and the quantification of immunoreactive protein bands. Equal amounts of protein (20 µg) were loaded in each lane. β-actin was used as an internal control. Data are from 3 independent experiments presented as mean ± SEM. Bars with * are significantly different from each other (p < 0.05).

In Vivo Effects of HIV Containing-Apoptotic Bodies on Ethanol-Fed Mice

To validate the in vitro effects of HIV-derived ABs in vivo, we used immunodeficient NSG mice. The NSG mice were fed ethanol or control diet for 10 weeks and
intravenously injected with HIV-containing human hepatocyte (RLW) ABs (0.5–1.0 × 10⁷ ABs/mouse) during the last two weeks of ethanol feeding. To induce liver fibrosis, the mice were injected with CCl₄ (i.p twice a week for 2 weeks). At week 10, mice were euthanized. As shown in Figure 28A & B, ethanol-fed mice injected with HIV-containing RLW ABs showed elevation of profibrotic genes (TGFβ2 and COL1A1) in liver tissue. Further analysis revealed elevation of liver enzymes, AST and ALT, in the same mice (Figure 28C&D). Deposition of ECM component (collagen) was also observed in ethanol-fed mice injected with HIV-containing ABs (Figure 28E).
Figure 28: Ethanol potentiates collagen deposition and liver damage in the liver of mice treated with HIV-containing apoptotic bodies: RT-PCR analysis of mouse tissues for (A) TGFβ2 mRNA and (B) COL1A1 mRNA. (C) ALT levels (U/mL); (D) AST levels (U/mL). (E) Histological examination of mouse liver tissues using Sirius red staining. Bars marked with the same letter are not significantly different. Bars with different letters are significantly different from each other (p ≤ 0.05).

Discussion

According to liver homeostasis, hepatocyte apoptosis usually correlates with HSC profibrotic activation (Chakraborty et al., 2012b). This phenomenon is profoundly expected given the high regenerative ability of the liver. Meanwhile, HSC profibrotic
activation may not lead to any major liver impairment if only few hepatocytes undergo apoptosis. However, this may be different for massive acetaldehyde production in HIV-infected hepatocytes. As described in Chapter 2, massive hepatocyte intrinsic apoptosis from the combined treatment of hepatocytes with acetaldehyde and HIV was observed (New-Aaron et al., 2021d, Ganesan et al., 2019b). Although, hepatocyte apoptosis serves as alcohol metabolite-mediated HIV clearance from the liver. However, profibrotic genes were activated in HSC when HIV-bearing hepatocyte apoptotic bodies (AB) were internalized by HSC (Ganesan et al., 2019b). Thus, the hepatocyte apoptosis clears HIV infection in ethanol-metabolizing hepatocytes, but it leads to HSC activation and fibrosis development. While the mechanisms of alcohol metabolite and HIV-induced hepatocyte apoptosis have been explicitly enumerated in Chapter 2, the mechanism of HSC activation after internalization of hepatocyte apoptotic bodies is required for developing therapeutic intervention. Activation of HSC after AB engulfment has been observed by many investigators (Canbay et al., 2003, Zhan et al., 2006, Jiang et al., 2009, Watanabe et al., 2007). Moreover, only ABs of hepatocyte origin could activate HSC (Ganesan et al., 2019b). Beyond AB cell origin, the mechanisms of HSC activation may depend on the type of apoptotic agent. For example, studies that utilized UV as an apoptotic trigger indicate toll-like receptor (TLR) -9 as the mechanistic pathway for HSC activation (Watanabe et al., 2007). This may be attributed to the ability of UV to efficiently disintegrate DNA into the CpG motifs required to activate the TLR9 (Vollmer, 2006, Gentile et al., 2003). However, this is not the case when alcohol
metabolites and HIV induce apoptosis of hepatocytes as it is expected in vivo (Ganesan et al., 2019b). Therefore, it becomes paramount to decipher the cargo of hepatocyte ABAGS+HIV. First, we observed enormous amount of HIV proteins, HIV RNA and oxidative products, malondialdehyde (MDA), as the part of ABAGS+HIV cargo. Second, ABAGS+HIV expressed phosphatidylinerine, which acts as the "eat me" signal for HSC (Fadok et al., 2001).

To complement these ABAGS+HIV characteristics, HSCs were found to express ligand bridge proteins, Gas 6 and ProS, as well as AXL, a phosphatidylinerine recognition receptor. Interaction between the ligand bridge proteins and AXL mediates ABAGS+HIV entry into HSC. Since internalized ABAGS+HIV contains HIV proteins and MDA, the HSC activation should highlight the pathways that are mediated by oxidative stress and/or HIV proteins. As reported by other studies JNK mediated HSC profibrotic activation (Cheng et al., 2015, Hu et al., 2013) although this was achieved in RLW ABAGS+HIV-independent manner. Similarly, JNK inhibitor attenuated HSC profibrotic activation via ERK1/2 pathway in this study. Likewise, N-acetyl cysteine attenuated ERK1/2 and HSC profibrotic activation. This suggest that profibrotic activation in HSC is partly triggered by oxidative stress through the JNK-ERK1/2 pathway. Given an oxidative product, 4-Hydroxy-2, 3-nonenal, triggered the JNK pathway in the study of Parola et al. (Parola et al., 1998), MDA from ABAGS+HIV may have triggered the observed JNK-ERK1/2 pathway. In addition, the JAK-STAT3 pathway are another ROS-dependent pathway for HSC profibrotic activation (Jiang et al., 2009). Our data observed
attenuation of HSC activation by hepatic AB in STAT3-silenced HSC. This confirms the involvement of the JAK-STAT3 pathway after AB_{AGS-HIV} engulfment by HSC.

While hepatocyte apoptosis provides the premises for HIV and acetaldehyde/ROS-induced hepatic fibrosis, the inhibition of hepatocyte apoptosis may seem like the best target for clinical intervention. However, this may have detrimental consequences since alcohol metabolite-induced hepatocyte apoptosis are avenue for HIV clearance from these liver cells leading to increased HIV DNA expression and even HIV DNA integration into human genome. Moreover, the profibrotic effects of the MDA and HIV-containing AB_{AGS-HIV} can be attenuated by augmenting antiretroviral therapy with liver-targeted antioxidants

**Conclusion**

In summary, we observed a massive generation of HIV and MDA-containing ABs when hepatocytes were treated with the combination of acetaldehyde and HIV. When these ABs were exposed to HSCs, no productive HIV infection was elicited; however, massive ROS release was generated, leading to HSC activation through the JNK-ERK1/2 and JAK-STAT3 pathways (Details are highlighted in Figure 29). We believe that these results provide a mechanistic insight on alcohol- and HIV-induced liver fibrosis development. Therefore, a therapeutic regimen with antioxidant drugs should be effective in ameliorating the progression of liver fibrosis among alcohol abusing PLWH.
Figure 29: HSC internalization of ABs generated from alcohol- and HIV-exposed hepatocytes induced ROS and IL6-mediated HSC activation. The mechanistic steps include: 1. Both the alcohol metabolite, acetaldehyde, and HIV trigger hepatocyte apoptosis. This leads to the generation of HIV and MDA-containing hepatocyte ABs which express PtylS. 2. HSC internalizes HIV and MDA-containing ABs through Axl, a PtylSRR, with the help of the two bridging molecules, Gas 6 and protein S. 3. The release of HIV proteins, HIV gag RNA and MDA into HSC by internalized ABs triggers intracellular ROS generation. This ROS mediates JNK activation, which leads to the phosphorylation of the ERK1/2 pathway. 4. Nuclear translocation of phosphorylated ERK1/2 induced the biosynthesis of profibrotic genes. 5. The delivery of HIV and MDA into HSC by internalized ABs induced IL-6 synthesis required to activate the JAK-STAT3 pathway. The activated STAT3 leads to the biosynthesis of profibrotic genes. 6. Activation of profibrotic genes in HSC promotes liver fibrosis development.
CHAPTER 4: OBETICHOLIC ACID ATTENUATES HUMAN IMMUNODEFICIENCY VIRUS/ALCOHOL METABOLISM-INDUCED PROFIBROTIC ACTIVATION IN LIVER CELLS


Introduction

We have shown that alcohol metabolites promote accumulation of HIV proteins in hepatocytes, inducing oxidative stress and hepatocyte apoptosis (Ganesan et al., 2019b). Furthermore, the engulfment of these HIV-containing hepatocytes AB by HSC causes pro-fibrotic activation of HSC (Ganesan et al., 2019b). This indicates that massive apoptosis of infected hepatocytes may induce continuous activation of HSC leading to liver fibrosis development (New-Aaron et al., 2022). Since alcohol metabolite-triggered HIV replication in hepatocytes is abortive, HIV accumulation cannot be fully controlled by ART, and detrimental consequences of AB internalization by HSC requires the treatment with additional anti-fibrotic drugs.
One of the promising drugs with anti-fibrotic activity is Obeticholic acid (OCA). In 2016, OCA became the United States Food and Drug Administration-approved to treat primary biliary cholangitis and currently is at phase 3 trial (Regenerate) to treat fibrosis caused by non-alcoholic steatohepatitis (NASH) (Shah and Kowdley, 2020a). OCA is a farnesoid-X receptor (FXR) agonist, which binds to the FXR in the nucleus of liver and intestinal cells. Multiple genes are activated by this pathway, including the control of metabolism of bile acids, lipids, glucose, and amino acids. FXR is highly expressed on hepatocytes and HSC, and is involved in pathogenesis of viral hepatitis, alcohol-and non-alcohol-induced liver disease (Wang et al., 2018b). In NASH fibrosis, OCA regulates liver injury progression via targeting of gut microbiota (Zhang et al., 2019). Currently, it is not quite clear whether OCA directly modulates hepatocyte apoptosis: While some studies reported the lack of anti-apoptotic effects of OCA on these cells (Zhou et al., 2019a), other studies demonstrated the reduction of hepatocyte apoptosis by OCA-mediated suppression of metabolic stress and prevention of subsequent p53 activation, with further anti-fibrotic downstream effects (Goto et al., 2018). These studies were mainly performed on experimental in vivo models, which makes it difficult to exclude the effects of OCA on gut microbiota, narrowing down the mechanisms to only OCA-regulated hepatocyte apoptosis. None of published in vivo or in vitro studies have been performed in the context of the effects of OCA on HIV- and alcohol-induced liver injury. However, activation of HSC by engulfment of HIV and MDA-expressing hepatocyte AB generated as a downstream event in HIV and alcohol metabolism-induced oxidative
stress is one of the reasons for liver fibrosis progression, which serves as an important target to prevent end-stage liver disease development. Thus, based on already characterized mechanisms of liver injury progression triggered by the combination of HIV with acetaldehyde in Chapter 2, we aimed to investigate whether OCA protects from apoptotic hepatocyte death and from activation of HSC by engulfment of apoptotic HIV-infected hepatocytes exposed to acetaldehyde as described in Chapter 3.

Materials and Methods

This is the original (basic) study performed at University of Nebraska Medical Center, Omaha, NE, United States. Here, for the first time, in vitro approach is used to characterize the ability of OCA to reverse the pathology induced by HIV and ethanol metabolism in liver parenchymal and non-parenchymal cells. In our study, OCA has been tested as an anti-fibrotic drug, which affects the pathogenesis of HIV-alcohol-induced liver fibrosis development.

Reagents and media

High glucose Dulbecco’s modified eagle medium and fetal bovine serum were purchased from Invitrogen (Carlsbad, CA, United States), Trizol was from Life Technologies, primer probes and real-time polymerase chain reaction (RT-PCR) reagents were from Applied Biosystems by Thermo Fisher Scientific, CA, United States. Other reagents, all analytical grade quality, were from Sigma (St. Louis, MO, United States).
**Cells and treatments**

As experimental prototype of human primary hepatocytes, we used Huh7.5-CYP (RLW) cells. These cells have reduced innate immunity and can be infected with HIV. We have characterized and successfully used these cells and AGS for HCV-based ethanol liver studies (Ganesan et al., 2018a, Ganesan et al., 2016a, Ganesan et al., 2018e). Cells were pre-treated for 24 h with AGS and then exposed to HIV\_ADA (MOI 0.1) for 48 h. To investigate the effects of OCA, the cells were pre-treated with OCA (50 μmol/L) for 4 h before experiment.

**HIV RNA, HIV DNA, integrated HIV DNA, HIV proteins and reactive oxygen species**

HIV RNA was detected by RT-PCR; HIV DNA was detected by semi-nested PCR; integrated HIV DNA was measured by digital droplet PCR; reactive oxygen species (ROS) were quantified by DCF (2’,7’–dichlorofluorescein fluorescence method); western blot was performed as described before. The details of all these methods were already published (Ganesan et al., 2019b).

**Apoptosis measurements**

Apoptosis in RLW cells was measured by caspase 3 and PARP cleavage in cell lysates (western blot) and M30 Apoptosense ELISA (Duapharma group, Inc. West Chester, OH, United States) in cell supernatants.


**Activities of proteasome and cathepsins**

Proteasome activities and cathepsin B and L activities were assayed fluorometrically as described previously (Ganesan et al., 2019a, Thomes et al., 2013).

**Hepatic stellate cells and treatments with AB**

Apoptotic bodies (AB) were generated from HIV-infected RLW cells exposed to AGS as shown in Chapter 3. AB_{AGS-HIV} were incubated with LX2 cells for 2-48h at 1:3 ratio and then pro-fibrotic markers [Col1A1, transforming growth factor (TGF)-β mRNAs] were measured (New-Aaron et al., 2022).

**Statistical analyses**

Data were analyzed using GraphPad Prism v7.03 software (GraphPad, La Jolla, CA, United States). Data from at least three duplicate independent experiments were expressed as mean ± SEM. Comparisons among multiple groups were performed by one-way ANOVA, using a Tukey post-hoc test. For comparisons between two groups, we used Student’s t-test. A P value of 0.05 or less was considered significant.

**Results**

Our previous studies on primary human hepatocytes exposed to ethanol and RLW cells treated with AGS demonstrated, first, that incubation of ADH-non-expressing RLW cells with AGS recapitulated the effects of ethanol on ethanol-metabolizing hepatocytes and second, that the highest levels of apoptotic hepatocyte death were observed when cells were exposed to both insults (ethanol/AGS and HIV) (Ganesan et al., 2019b). That is why
in this paper, we presented the data on ability of OCA to protect cells from the harmful effects of AGS on HIV-infected hepatocyte-like RLW cells to mimic ethanol metabolism observed in primary human hepatocytes. For OCA screening, we did not use primary human hepatocytes since their supply was limited and they quickly (in 24 h) de-differentiate to lose ethanol-metabolizing capacity.

**OCA attenuates AGS-HIV-induced apoptotic cell death and oxidative stress**

We found that while AGS + HIV induced caspase 3 cleavage almost three-fold, pre-treatment of RLW cells with OCA suppresses these effects (Figure 30A &B). The same trend (but with lower magnitude of response to AGS) was observed on cleaved PARP, and OCA also suppressed it (Figure30 C& D). Since apoptosis may be induced by elevated p53, we measured the induction of p53 mRNA by AGS+HIV, in the presence or absence of pan-caspase inhibitor or OCA. As shown on Figure 30E, while AGS and HIV induced p53 mRNA level two-fold, both OCA and pan-caspase inhibitor reduced p53 expression only by 14%. In addition, OCA attenuated AGS-HIV-induced ROS production (Figure30F), thereby protecting liver cells from oxidative stress.
Figure 30: Obeticholic acid attenuates apoptosis in acetaldehyde-generating system-human immunodeficiency virus-exposed Huh7.5-CYP cells. A: Cleaved caspase 3 (western blot, representative blot); B: Cleaved caspase 3 cleavage, quantification; C: Cleaved PARP (western blot, representative blot); D: Cleaved PARP, quantification; E: RT PCR quantification of p53 mRNA. F. Reactive oxygen species. All data were obtained from 3 independent experiments and presented as mean ± SEM. Bars with different letters are significantly different at P ≤ 0.05. AGS: Acetaldehyde-generating system; HIV: Human immunodeficiency virus; OCA: Obeticholic acid.

OCA suppresses accumulation of HIV in hepatocytes

Since there is always a risk that by suppressing hepatocyte apoptosis, we may increase expression of HIV markers in hepatocytes, we measured the effects of OCA on accumulation of HIV gag RNA (RT-PCR) and p24 (western blot) induced by AGS + HIV
exposure to RLW cells. However, in these experiments, OCA suppressed the treatment-induced by 24% (Figure 31A&B) and HIV gag RNA levels by 50% (Figure31C). OCA also decreased the level of HIV DNA in RLW cells by about 60% (Figure31D), with no integration of HIV DNA in cell genome. As has been shown by us before, the accumulation of HIV components in RLW cells is controlled by HIV-AGS-decreased lysosomal and proteasomal activities (Ganesan et al., 2019b). In fact, we observed partial restoration of chymotrypsin-like proteasome and cathepsin B and L lysosomal activities by OCA; however, OCA did not restore AGS-HIV-affected trypsin-like proteasome activity (Figure32A-D).
Figure 31: Obeticholic acid suppresses human immunodeficiency virus expression in Huh7.5-CYP cells exposed to AGS+HIV. A, B: p24; B. HIV gag RNA; C: HIV DNA. All data were obtained from 3 independent experiments and presented as mean ± SEM. Bars with different letters are significantly different at P ≤ 0.05. AGS: Acetaldehyde-generating system; HIV: Human immunodeficiency virus; OCA: Obeticholic acid.
Figure 32: Restorative effects of Obeticholic acid on proteasomal and lysosomal activities in AGS+HIV-exposed Huh7.5-CYP cells. A: Chymotrypsin-like activity, proteasome; B: Trypsin-like activity, proteasome; C: Cathepsin B activity, lysosome; D: Cathepsin L activity, lysosome. All data were obtained from 3 independent experiments and presented as mean ± SEM. Bars with different letters are significantly different at P ≤ 0.05. AGS: Acetaldehyde-generating system; HIV: Human immunodeficiency virus; OCA: Obeticholic acid.
OCA reduces pro-fibrotic activation of HSC by engulfment of HIV-containing apoptotic hepatocytes

RLW ABAGS-HIV were exposed to LX2 cells, followed by measurement of Col1A1, TGF-β2, TIMP1, ACTA2 and TP53 mRNAs by RT-PCR. We found that OCA attenuated profibrotic activation by HIV and MDA-containing apoptotic bodies in HSC after engulfment (Figure 33A-D). Moreover, the observed OCA mediated profibrotic attenuation was not triggered by cell death as OCA also suppressed TP53 genes in HSC exposed to RLW ABAGS-HIV (Figure 33E).
Figure 33: Anti-fibrotic effects of Obeticholic acid on hepatic stellate cells activated by engulfment of ABAGS+HIV. A: Col1A1 mRNA; Col1A1 mRNA; B: TGF-β mRNA; C TIMP1 mRNA D. ACTA2 mRNA E. TP53 mRNA. All data were obtained from 3 independent experiments and presented as mean ± SEM. Bars with different letters are significantly different at P ≤ 0.05. TGF: Transforming growth factor; HIV: Human immunodeficiency virus; OCA: Obeticholic acid.

OCA reduces profibrotic activation of HSC partly via the JAK-STAT3 pathway

Since OCA attenuates profibrotic activation, it became important to identify the involved mechanisms. Meanwhile, the JNK-ERK1/2 and JAK-STAT3 pathways were listed in Chapter 3 as the mediators of ABAGS+HIV-induced HSC activation. Therefore,
OCA-pretreated LX2 cells exposed to AB\textsubscript{AGS+HIV} were probed for pSTAT3 and pERK1/2. It appeared that OCA suppressed pSTAT3 (Figure 34A&B), but had no effect on pERK1/2 (Figure 34C&D).

Figure 34: Obeticholic acid suppressed pSTAT3 in LX2 cells exposed to AB\textsubscript{AGS+HIV}. A, B: STAT3 was measured by immunoblot analysis and the quantification of immunoreactive protein bands; C, D: ERK1/2 was measured by immunoblot analysis and the quantification of immunoreactive bands. Equal (20 μg) amounts of protein were loaded in each lane. β-actin and TATA-binding proteins (TBP) were used as internal controls. All data were obtained from 3
independent experiments and presented as mean ± SEM. Bars with different letters are significantly different at \( P \leq 0.05 \). HIV: Human immunodeficiency virus; OCA: Obeticholic acid.

**Discussion**

Due to frequently observed association of morbidity and mortality of HIV with liver injury, the inclusion of therapeutic regimens with anti-fibrotic activity to the treatment of PLWH is pathogenically important. In addition to alcohol as a risk factor for HIV transmission, alcohol consumption is also known to facilitate liver fibrosis development. Previously, we have shown that the exposure of hepatocytes to HIV and ethanol metabolites causes high oxidative stress, which impairs lysosomal functions and hepatocyte apoptosis (New-Aaron et al., 2021d), and the crosstalk between hepatic stellate cells and apoptotic HIV and MDA-containing hepatocytes promotes progression to liver fibrosis (New-Aaron et al., 2022). Since the hepatocyte apoptosis is the major trigger for HSC activation and liver fibrosis (Guicciardi and Gores, 2005), inhibition of hepatocyte apoptosis seems to be the best target for therapeutic intervention. However, this may result in detrimental consequences for alcohol and HIV-induced hepatocyte apoptosis. This is because hepatocyte apoptosis due to the effects of alcohol metabolites aborts and triggers HIV clearance. Therefore, inhibition of hepatocyte apoptosis may establish HIV reservoir in the liver even if it prevents liver fibrosis development. Hence, a potent therapeutic regimen should be antiapoptotic if it enhances HIV degradation in hepatocyte. Here, we investigated OCA as a potential therapeutic regimen that prevents HIV/ethanol metabolism-induced hepatotoxicity and a subsequent activation of HSC by
the engulfment of HIV and MDA-containing hepatocyte apoptotic bodies. While the
attenuation of profibrotic activation in HSC by OCA is not entirely novel (Zhou et al.,
2019b), anti-apoptotic and antifibrotic properties of OCA are unknown in the context of
HIV and alcohol-induced hepatotoxicity. OCA, which treats cholestasis by attenuating
oxidative stress, mitochondria impairment and ER stress (Pinto et al., 2021) also
attenuated similar effects in RLW cells exposed to alcohol and HIV. In vitro experiments
from the current study demonstrated attenuation of p53 genes as well as indicators of
intrinsic apoptosis in OCA-pretreated RLW cells exposed to alcohol metabolites and
HIV. Given that alcohol-metabolite-induced HIV accumulation is the major trigger of
hepatocyte apoptosis via ROS generation and a consequent lysosome impairment, the
effects of OCA on lysosome and proteasome functions were examined. Here, the
treatment of RLW cells with OCA restored cellular protein degradation systems
(proteasome and lysosome functions) and consequentially suppressed HIV
accumulation induced by alcohol metabolites. Our findings were consistent with other
studies, which reported the restorative effect of lysosome functions by OCA (Pinto et al.,
2021, Tawfiq et al., 2022). This led to the suppression of ROS generated by HIV
accumulation in AGS-exposed hepatocytes. In addition, OCA attenuate profibrotic HSC
activation via the JAK/STAT3 pathway, which was one of the pathways that mediate
HSC profibrotic activation by AB engulfment as discussed in the previous Chapters.
Similarly, OCA attenuated cell proliferation in other studies via the JAK/STAT3
pathway (Li et al., 2020, Attia et al., 2017). Since inhibition of the JAK/STAT3 triggered
apoptosis (Abongwa et al., 2022), p53 genes was examined in HSC to ensure OCA inhibition of the JAK/STAT3 does not result in HSC depletion. Here, p53 genes were suppressed in LX2 cells exposed to OCA.

The JAK/STAT3 pathway is not the only HSC activation pathway triggered by the internalization of ABAGS+HIV. JNK-ERK1/2 pathway also mediated HSC profibrotic activation after ABAGS+HIV internalization. However, OCA provided no effect through the JNK-ERK pathway.

**Conclusion**

In conclusion, in HIV-infected hepatocytes exposed to continuously released acetaldehyde, OCA attenuates apoptotic death of infected cells and pro-fibrotic Jak-STAT3-mediated activation of HSC engulfed apoptotic HIV+ hepatocytes. OCA, thereby, protects the liver from fibrosis development under the conditions of HIV infection and alcohol exposure.
CHAPTER 5: DISCUSSION

Findings Relative to Specific Aim 1 (Chapter 2)

As revealed from our previous studies, pre-exposure of hepatocytes to the alcohol metabolite sensitizes cells to HIV-induced apoptotic cell death. This was partly due to acetaldehyde-induced intracellular accumulation of HIV proteins, which occurs due to suppression of HIV protein degradation by both the proteasome and lysosomes (Ganesan et al., 2019b). In fact, inhibition of protein-degrading enzyme activities with specific inhibitors, MG132 and carfilzomib for proteasomes or bafilomycin and chloroquine for lysosomes, prolongs the persistence of HIV proteins in hepatocytes (Ganesan et al., 2019b). As reported by others, HIV proteins are degraded by proteasomes and lysosomes (Rojas and Park, 2019, Seissler et al., 2017, Fredericksen et al., 2002, Ali et al., 2019). We showed that the accumulation of HIV proteins in hepatocytes was greater after treatment with lysosome inhibitors than with proteasome inhibitors. These findings underscore the importance of ethanol- and HIV-induced lysosomal dysfunction/damage for HIV protein retention, which can cause oxidative stress. In kinetic studies, we indeed observed that the accumulation of HIV proteins after exposure to AGS induces oxidative stress accompanied by ROS release, which leads to apoptotic hepatocyte death (Ganesan et al., 2019b). The latter event results in the formation of apoptotic bodies, which are engulfed by non-parenchymal cells as we observed in some canonic hepatotropic (like HCV) infections. These observations were further confirmed in HIV infection (Ganesan et al., 2016a, Ganesan et al., 2018b).
In Chapter 2, we addressed the contribution of lysosomal impairment to HIV and alcohol-metabolite-induced hepatotoxicity. Our results lead us to suggest that acetaldehyde induces lysosomal damage in HIV-infected hepatocytes and that leakage of lysosomal enzymes (cathepsins) to other cell compartments may damage adjacent organelles, which hastens hepatic cell death. For example, leakage of cathepsins to mitochondria can disrupt the mitochondrial membrane and may induce intrinsic apoptotic events (Wang et al., 2018a).

We focused on the underlying mechanism(s) by which HIV, ethanol metabolite(s), and their combination impaired lysosome activity. To mimic ethanol metabolism in hepatocytes, we exposed CYP2E1-overexpressing RLW cells to AGS. Lysosome leakage (LL) is one mechanism that leads to a decline in lysosome hydrolytic potency. The pathogenic impact of LL in the liver has already been discussed for non-alcoholic (Feldstein et al., 2006, Li et al., 2008) and alcoholic steatohepatitis (Donohue et al., 2007). It was also implicated in the liver injury of ethanol-fed rodents (Donohue et al., 2007, Li et al., 2016). However, this is the first time investigated in non-immune cells (hepatocytes) after HIV infection treated with ethanol metabolites. In our hands, the treatment of both HIV-infected and -uninfected RLW cells with AGS lowered the expression of LAMP1, a lysosomal membrane protein important for maintaining lysosome structural integrity. These findings indicate that acetaldehyde exposure causes lysosomal membrane instability. The decrease in lysosomal numbers and functions were, at least in part, triggered by alcohol metabolite-induced oxidative stress since
lysosome instability was attenuated by treatment with the antioxidant NAC. In fact, lysosomes do not express anti-oxidative enzymes, and they are sensitive to the effects of ROS, which easily destabilize lysosomal membranes (Wang et al., 2018a). Indeed, ROS have been shown to trigger LL in alcoholic steatohepatitis (Li et al., 2008).

In the current study, co-localization of LAMP1 and galectin 3 reported by others as an indication of increased lysosomal permeability (Aits et al., 2015) was highest in AGS-treated HIV-infected RLW cells. While the role of HIV-induced lysosomal damage has not been studied in hepatocytes before, in CD4+ lymphocytes, HIV potentiates LL by activating lysosome-co-localized protein, DRAM1, responsible for lysosome membrane permeabilization (LMP) (Laforge et al., 2013). Furthermore, DRAM1 regulates apoptosis by increasing the lysosomal localization of pro-apoptotic protein BAX (Guan et al., 2015a) and has p53 as its downstream target (Takahashi et al., 2013). Therefore, by measuring p53 gene activation in AGS + HIV-treated RLW cells, we identified a significant increase in p53 mRNA levels. Because p53-mediated apoptosis is caspase-dependent (Garufi et al., 2017), to preserve DRAM1-expressing RLW cells, we tested the effects of HIV and AGS treatments on DRAM1 expression in the presence of pan-caspase inhibitor (PCI) and found the increased levels of DRAM1 in these double-treated cells compared with untreated (control) cells and cells exposed to either HIV or AGS. These changes were not observed in the absence of PCI because DRAM1-expressing cells undergo robust caspase-dependent apoptosis. Thus, it is likely that by activating p53-
mediated genes in hepatocytes, DRAM1 contributes to apoptosis induction, demonstrating the link between LL and apoptotic cell death.

The link between LL and HIV-AGS-induced apoptosis in hepatocytes is still unclear. When RLW cells were exposed to both HIV and AGS, leading to the highest levels of apoptotic cell death based on the results of M30 ELISA, we observed the co-localization of cathepsin B to mitochondria (specifically, to outer membrane protein, Tom20 (Saitoh et al., 2007)), indicating that cathepsin B translocation to mitochondria may be pro-apoptotic. This translocation can trigger caspase 3 cleavage and subsequent apoptotic cell death via upstream activation of caspase 9 by cytochrome C’s release from permeable mitochondria. In our hands, inhibition of caspase 9 by the specific blocker significantly suppressed HIV-AGS-induced caspase 3 cleavage, and this effect was more profound than the contribution of caspase 8 inhibition to caspase 3 cleavage, suggesting that activation of the intrinsic apoptotic pathway plays a major role in HIV + AGS-induced hepatocyte death. This prompts us to suggest that further investigations are warranted to study the interdependence of lysosomal and mitochondrial leakages in the pathogenesis of HIV and ethanol-metabolism-induced hepatotoxicity. However, since this is a highly complex and important area, we did not plan to intensively investigate lysosome–mitochondrial interactions in the frame of this study, but we intend to detail this crosstalk in future experiments. While in other studies, the role of LL and mitochondrial dysfunction for apoptosis induction has already been demonstrated (Boya et al., 2003, Paquet et al., 2005, Johansson et al., 2010), it has never been
implemented into the lysosome–mitochondrion axis driving HIV-ethanol-metabolism-induced hepatotoxicity.

There are multiple compensatory mechanisms that remove damaged lysosomes by autophagy/lysophagy (Papadopoulos and Meyer, 2017, Yoshida et al., 2017, Radulovic et al., 2018, Jia et al., 2020, Fujita et al., 2013, Jia et al., 2018). Major repair mechanisms are usually related to lysosome biogenesis via TFEB (Papadopoulos and Meyer, 2017). Here, we found that TFEB translocation from cytosol to the nucleus is necessary to activate lysosome-biogenesis-regulating genes and that this was impaired in AGS-exposed, HIV-infected hepatic cells. Indeed, the attenuation of TFEB translocation by ethanol metabolism in hepatocytes was reported before (Chao et al., 2018b, Thomes et al., 2015, Chao et al., 2018a, Thomes et al., 2019), but not in the context of HIV liver studies.

To address the mechanisms by which AGS (mainly, acetaldehyde) inhibits TFEB nuclear translocation in hepatocytes in the settings of HIV, we tested the factors regulating TFEB stabilization in the cytosol.

A variety of proteins and kinases control the translocation of TFEB to the nucleus to activate lysosomal biogenesis (Napolitano et al., 2018, Sha et al., 2017b, Jia et al., 2017, Sha et al., 2017a). In this regard, we explored the role of phosphorylation as a post-translational modification that determine TFEB cytosolic accumulation in acetaldehyde-treated cells. Thus, phospho-TFEB dimerizes with TFEB to promote the retention of TFEB in the cytosol (Puertollano et al., 2018), which we observed in AGS-exposed, HIV-
infected hepatocytes. mTOR phosphorylates TFEB at multiple serine residues (S122, S142, and S211). However, despite TFEB retention in cytosol, we even found the downregulation of acetaldehyde-induced TFEB phosphorylation at S211 (Puertollano et al., 2018). The partial explanation for this event might be related to the overexpression of galectins in acetaldehyde-exposed liver cells due to their enhanced damage, leading to suppression of mTOR (Gu et al., 2020). However, while we have no evidence that acetaldehyde increases TFEB phosphorylation, some other downstream acetaldehyde-affected events (e.g., aggregation) might account for TFEB cytosolic retention.

Given that TFEB is a substrate for the proteasome, we measured proteasome activities in HIV-infected and -uninfected RLW cells exposed or not to AGS and found a concomitant downregulation of chymotrypsin and trypsin-like proteasome activities. While there is no evidence of synergistic interactions between HIV and AGS, this is a novel finding because, to our knowledge, there are no studies that demonstrate the effects of acetaldehyde on TFEB in relation to proteasome activity in HIV-infected hepatocytes. The most likely scenario in ethanol-treated hepatic cells is the degradation of non-ubiquitylated TFEB by 20S proteasome because high oxidative stress causes the dissociation of 19S from 20S proteasome, thereby limiting its ability to recognize the ubiquitinated substrates by the 26S proteasome (Donohue Jr et al., 2019). In HIV-noninfected liver cells, we and others (Ganesan et al., 2019b, Osna and Donohue Jr, 2007, Bardag-Gorce, 2010, Bardag-Gorce et al., 2005, Bardag-Gorce et al., 2004) have previously reported that ethanol or acetaldehyde impair chymotrypsin-like and trypsin-
like activities of 20S proteasome catalytic core. We cannot exclude that proteasome dysfunction under HIV–AGS cell exposure attenuates the degradation of total TFEB in the cytosolic compartment. This undegraded TFEB should translocate to the nucleus for gene activation if its trafficking is not blocked.

The trafficking of TFEB by microtubules has not been reported before. However, in hepatocytes, the translocation of some other transcription factors such as STAT3 and STAT5B from cytosol to the nucleus is suppressed by ethanol metabolism, which impairs their trafficking due to microtubule acetylation (Fernandez et al., 2012, Groebner et al., 2019). Here, we observed that nocodazole, an inhibitor of microtubule trafficking, suppressed TFEB translocation to the nucleus in RLW cells. These findings indicate that microtubules are necessary for TFEB translocation from cytosol to nucleus. While based on our findings, HIV by itself does not induce acetylation of microtubules, AGS exposure increases TFEB acetylation, which negatively affects TFEB trafficking to the nucleus, resulting in its cytosolic retention.

**Findings Relative to Specific Aim 2 (From Chapter 3)**

Mortality due to end-stage liver disease is on the rise among people living with HIV (Bica et al., 2001). Clinical studies suggest that alcohol, antiretroviral therapy and co-infection with hepatotropic viruses (Hepatitis B and C) may be associated with liver disease among HIV-infected individuals (Ganesan et al., 2018d). While the mechanisms of hepatotoxicity by antiretroviral therapy (Sastry et al., 2018, Gwag et al., 2019) and
hepatotropic viruses (Ganesan et al., 2018b, Nevola et al., 2018) have been extensively explored, the mechanisms explaining the role of alcohol in HIV-infection and pathogenesis of liver disease development has not received adequate attention despite the emerging burden of liver disease in HIV management (Kaspar and Sterling, 2017b). In response, this study explored preclinical experimental designs to define how engulfment of apoptotic hepatocytes by HSC activates profibrotic changes.

The motivation for addressing the specific aims of this study emanated from the profound relationship between hepatocyte apoptosis and liver fibrosis (Chakraborty et al., 2012a). In fact, previous studies have reversed the progression of liver fibrosis by blocking hepatocyte apoptosis (Witek et al., 2009, Barreyro et al., 2015, Shiffman et al., 2010a, SHIFFMAN et al., 2010b). We cannot do the same for alcohol and HIV-induced liver injury, hence the need for this study. In our hands, profibrotic activation of HSC became even more prominent when hepatocytes, as a source of ABs, expressed HIV and oxidative stress markers. While numerous factors have been identified as triggers for hepatocyte apoptosis, the combination of ethanol and HIV was clearly demonstrated by us as a potent trigger of hepatocyte apoptosis via oxidative stress. This is not surprising because hepatocytes predominantly metabolize ethanol by ADH to generate the most toxic oxidative metabolite, acetaldehyde (Guo and Ren, 2010). Acetaldehyde is the major trigger for HIV accumulation, and the consequent hepatocyte apoptosis as reported in our previous study (Ganesan et al., 2019b). However, ADH is not the only ethanol-metabolizing enzyme potentiating HIV-induced hepatocyte apoptosis. In addition, the
microsomal ethanol oxidizing system (MEOS) consisting of CYP2E1 may also be involved (Teschke et al., 2021, Doody et al., 2017). In fact, we previously showed in primary human hepatocytes that the combined treatment of ethanol and HIV which engendered the highest apoptosis upregulates CYP2E1 expression as well (Ganesan et al., 2019b). Here, as a source of hepatocytes, we used RLW cells, which are Huh7.5 cells stably transfected with CYP2E1. These cells do not express ADH and, thus, do not make a sufficient amount of acetaldehyde. To mimic ethanol metabolism in primary human hepatocytes, we exposed RLW cells to an acetaldehyde-generating system (AGS). Since ethanol is a part of this system, exposure of CYP2E1-overexpressing cells to ethanol allows stabilizing CYP2E1 under ethanol treatment and generates ROS (Koop, 2006). This in a combination with acetaldehyde continuously produced by AGS provides hepatotoxic effects leading to hepatocyte AB formation. Our previous studies on HIV-infected hepatocytes exposed to ethanol demonstrated that these hepatotoxic effects can be reversed by 4-methyl pyrazole, an ADH inhibitor (Ganesan et al., 2019b). Here, we also demonstrated that antioxidant NAC reverses profibrotic activation of HSC after engulfment of HIV+AGS-induced AB, indicating the role of CYP2E1-generated ROS in liver fibrosis development (Urtasun et al., 2008).

Furthermore, our recent studies uncovered how the combined treatment with acetaldehyde and HIV triggered hepatocyte accumulation of p24 and HIV gag RNA, which resulted in oxidative stress-induced apoptosis of HIV-infected hepatocytes via lysosome impairment (Ganesan et al., 2019b, New-Aaron et al., 2021d). Although the
apoptotic death of HIV-infected hepatocytes may be considered beneficial for the reduction of the HIV liver reservoir, it was concurrently attributed to profibrotic changes in the liver. Large EVs, particularly, AB derived from apoptotic hepatocytes, were found to mediate the observed profibrotic changes, which are specific to AB of hepatocyte origin, while lymphocyte-derived AB elicited no profibrotic changes in HSC. To substantiate that the observed profibrotic effects of AB on HSC were attributed to activation of HSC and do not come directly from the internalized AB, AB-induced profibrotic genes in HSC were measured but not determined in the hepatocyte AB. Therefore, the observed profibrotic genes were due to HSC pro-fibrotic activation.

Although AB in other studies were generated by different apoptotic stimuli, such as viral agents (hepatitis C virus (HCV) (Ganesan et al., 2018b, Ganesan et al., 2016a), HIV(Ganesan et al., 2019b)), ultraviolet light (Poon et al., 2019), and toxic substances, namely, CCl4(Johnston and Kroening, 1998) and ethanol (Higuchi et al., 1996), in the current study, AB were generated by the combined treatment of acetaldehyde and HIV thus, closely mimicking the situation in alcohol-abusing PLWH. This provides an appropriate model to study frequently observed liver fibrosis in alcohol-abusing HIV-infected individuals. Therefore, aiming to explore the mechanisms of liver fibrosis in the context of HIV and alcohol, we hypothesize that hepatocyte exposure to the combination of HIV and acetaldehyde ends up with the generation of HIV-containing ABs that activate oxidative stress-triggered profibrotic changes in HSC.
HSCs are specialized cells generating ECM to replace dead hepatocytes. While this process is a well-defined homeostatic process, it becomes pathological when hepatocyte death is excessive. HSC activation to myofibroblast has been demonstrated by HSC engulfment of ABs in this and other studies (Jiang et al., 2009, Xu et al., 2019). Although it may be argued that macrophages are better professional “eaters” of AB than HSC (Depraetere, 2000), the anatomical proximity of HSC to hepatocytes in the liver architecture is favorable for HSCs to encounter hepatocyte ABs. Moreover, the combination of HIV and acetaldehyde induces massive hepatocyte apoptosis (Ganesan et al., 2019b), allowing non-professional (HSC) phagocytosis of hepatocyte ABAGS-HIV. So, it is logical to design an experiment involving the exposure of HSC to ABs to study AB-induced HSC activation. While AB engulfment capacity and the consequential profibrotic activation in HSC is well studied, the AB cargo-dependent mechanism of HSC activation in HIV infection needs to be clarified. Thus, this study focused on characterizing ABAGS-HIV, with a special focus on measuring AB contents. Here, we observed significant expression of HIV gag RNA, HIV proteins and MDA both in AB and in HSC after AB internalization, suggesting an important role of AB in the crosstalk between liver parenchymal and non-parenchymal cells. Although the HIV product transfer did not elicit productive HIV infection in HSC or even permissive immune cells, both the HIV proteins and the oxidative stress products (MDA, ROS) promoted HSC activation as indicated by the upregulation of profibrotic genes in LX2 cells exposed to ABs obtained from both acetaldehyde- and HIV-exposed RLW cells.
Externalized PtdIns, robustly expressed by AB is known as the “eat me” signal for HSC engulfment of ABs (Fadok et al., 2001). While HSC internalization of ABs was previously demonstrated using an array of scavenger receptors of both A and B classes (Canbay et al., 2003), our data confirmed that HSC engulfment of AB is possible through one of the receptor tyrosine kinases (RTK). In fact, HSCs express Gas 6 and Protein S, which are required as the inserts to complete AB internalization and the engulfment of TAMRA-labeled ABs, resulting in profibrotic gene activation in LX2 cells. This activation was significantly attenuated by Axl inhibitor. Given that Axl and Gas6 are notoriously involved in ABs engulfment by liver resident macrophages (Mukherjee et al., 2016, Myers et al., 2019, Ortmayr et al., 2022), our observation of Axl, Gas 6 and Protein S involvement in HSC engulfment of AB_{AGS+HIV} confirms that this mechanism is functional for HSCs exposed to AB.

While many studies confirm that HSC engulfment of AB is an essential trigger of profibrotic activation, the next question is which signaling pathways are responsible for this HSC activation after AB_{AGS+HIV} engulfment. Previous studies identified the involvement of p38 MAPK in the activation of HSC (Canbay et al., 2003). However, our data on the engulfment of AB_{AGS+HIV} conversely observed the profibrotic activation through the ERK1/2 pathway after the inhibition of p38 MAPK. In addition to p38 MAPK, we also tested the role of JNK, another MAPK upstream from ERK1/2. JNK inhibition attenuated profibrotic activation as well as the secretion of ECM component α-SMA in LX2 cells. This finding is in congruence with previous studies that reported
the role of JNK in hepatic fibrogenesis (Kluwe et al., 2010, Hong et al., 2013), even though ABs induced no JNK activation in those studies. While it is not clear why the MDA and HIV-containing AB<sub>AGS-HIV</sub> activate JNK, we are inclined to lean towards MDA as being partly involved in JNK activation. In fact, a previous study identified 4-HNE-protein-adducts, another oxidative product, as a stimulus for JNK activation in HSC (Parola et al., 1998).

Moreover, our data revealed that the engulfment of HIV and MDA-containing AB<sub>AGS-HIV</sub> led to ROS generation in HSC. In addition, HSC activation via the JNK-ERK1/2 pathways after AB<sub>AGS-HIV</sub> engulfment was attenuated by NAC, a known antioxidant. This is counterintuitive because ROS are pro-apoptotic in cells, while, here, ROS are paradoxically anti-apoptotic since they promote cell survival, proliferation, and profibrotic activation in HSC (Novo et al., 2006b, Novo et al., 2006a, Pessayre et al., 2004, Gandhi, 2012, Jiang et al., 2010).

As expected, HSC activation after AB<sub>AGS-HIV</sub> engulfment involved multiple pathways. The JAK-STAT3-mediated HSC activation after AB engulfment was observed through ROS generation (Jiang et al., 2009). Similarly, our data demonstrated HSC activation via the JAK-STAT3 pathway after AB<sub>AGS-HIV</sub> engulfment. In fact, STAT3 gene inhibition significantly attenuated α-SMA and profibrotic genes in HSCs. Moreover, the upregulation of IL6 mRNA in HSC after AB<sub>AGS-HIV</sub> engulfment and the expression of IL6 receptors on HSC (Schmidt-Arras and Rose-John, 2016) confirms that AB<sub>AGS-HIV</sub>-
mediated JAK-STAT3 activation in HSC may be induced by IL6, a canonic ligand for STAT3 activation (Wang et al., 2013). In addition, SOCS1, a suppressor of STAT3 activation (Flowers et al., 2005), was downregulated by AB<sub>AGS-HIV</sub> internalization, which increases the JAK-STAT3 signaling in LX2 cells. This signaling is pro-survival for HSC and is known to interfere with apoptosis-inducing anti-viral IFN type 1 signaling which attenuates the activation of anti-viral ISGs.

A major limitation to our experimental design was the lack of primary human hepatocytes, which were not available in necessary quantities to generate the substantial amount of hepatocyte ABs required for this study. However, HIV and ethanol-induced hepatocyte apoptosis observed in RLW cells and its downstream effects on LX2 cells mimicked previously reported effects of AB induced by ethanol in primary human hepatocytes (Ganesan et al., 2019b). In this study, LX2 cells were grown on plastic plates, a known trigger for LX2 activation; however, this did not affect AB-induced HSC activation because the AB-unexposed LX2 cells were also cultured on plastic plates as LX2 cells exposed to AB.

AB-induced HSC activation within the context of ethanol metabolites and HIV was validated in vivo by injecting ethanol-fed immunodeficient NSG mice with HIV-containing ABs. In this experiment, we observed the upregulation of TGFβ2 and COL1A1 genes which led to the deposition of collagen in the liver of ethanol fed mice.
injected with hepatocyte AB<sub>HIV</sub>, and this was accompanied by elevated liver enzymes.

Our major findings are summarized in Figure 12.

**Findings Relative to Specific Aim 3 (From Chapter 4)**

As previously shown, HIV accumulation induced by pre-exposure of cells to alcohol metabolites, mainly, acetaldehyde produced by AGS triggered oxidative stress and apoptosis in hepatocytes. This is beneficial due to infected hepatocytes clearance before the integration of HIV DNA into human genome occurs. However, intensive hepatocyte apoptosis may have detrimental outcomes since HIV-containing apoptotic hepatocytes induce pro-fibrotic activation of HSC, thereby promoting fibrosis development (Ganesan et al., 2019b). Here, we investigated whether OCA protects from AGS-HIV-induced hepatocyte apoptosis, which causes HSC activation by AB<sub>AGS+HIV</sub> engulfment to drive liver fibrosis development.

In our model, OCA pre-treatment attenuated apoptosis (caspase 3 and PARP cleavage as well as a cleaved cytokeratin 18 expression) in AGS-HIV-exposed liver cells. Unlike suppression of p53 in liver cells by OCA reported in HIV-non-infected cells by others (Goto et al., 2018), here, OCA mildly suppressed AGS-HIV-induced p53 mRNA.

Apoptotic hepatocyte death was triggered by oxidative stress induced by AGS/ROS and HIV in CYP2E1-expressing RLW cells. In our hands, OCA indeed suppressed ROS production, thereby attenuating oxidative stress, which corroborated the data obtained on different models (Zhu et al., 2018, Zhang et al., 2017). Here, the suppression of
oxidative stress by OCA restores proteasome and lysosome functions, which increases the degradation of HIV proteins (Lata et al., 2018, Wei et al., 2005) and thus, diminishes the expression of HIV gag RNA and p24 gag protein in infected RLW cells. However, as we established before, the prevention of HIV and AGS-induced apoptotic hepatocyte death by exposure to pan-caspase inhibitor causes accumulation of cells with integrated HIV DNA (Ganesan et al., 2019b), which is an unwanted event. Importantly, while OCA pre-treatment suppresses apoptosis in HIV-infected hepatocytes, there was no increase in hepatocytes expressing integrated HIV DNA.

In addition to beneficial effects of OCA on attenuation of HIV-AGS-induced hepatocyte death and reduction of HIV markers expression, OCA also reverses pro-fibrotic activation (based on Col1A1, TGF-β2, TIMP1 & ACTA2 mRNA levels) of HSC by engulfment of ABAGS+HIV. Similar event was observed by (Zhou et al., 2019a) in CCl4-injured mice, indicating that OCA controls HSC activation triggered via multiple mechanisms.

In fact, in vivo protection from early alcohol-induced liver damage by OCA has been already demonstrated on alcohol-fed uninfected mice without disclosing the involved mechanisms. (Iracheta-Vellve et al., 2018). However, the OCA-induced attenuation of STAT3 expression is the mechanistic explanation for OCA antifibrotic effects in HSC exposed to ABAGS+HIV. While the efficacy of OCA in HIV-infected alcohol abused patient have never been tested, it might be beneficial to explore a FXR agonists like OCA. Our
experiments provide in vitro evidence for protective effects of OCA from liver fibrosis progression induced by HIV and alcohol metabolism. The major limitation of this innovative study is that the results are currently based only on in vitro, but not in vivo experiments. Nevertheless, these in vitro experiments are necessary to characterize the exact mechanisms, by which OCA prevents HIV/ethanol metabolism-induced liver injury. These mechanisms are difficult to identify by in vivo studies, due to multiple triggers of liver fibrosis progression. We plan to confirm the in vitro effects of OCA in future by in vivo studies on liver humanized mice model since only these mice can be infected with human live HIV and fed the liquid ethanol-containing diet. Thus, our current in vitro study pioneers in justifying OCA inclusion to the treatment scheme of HIV-infected alcohol abusers with a high risk of liver fibrosis development.

**Future directions**

Since HIV is a human virus, the use of animal models to demonstrate HIV pathogenesis presented some difficulties during the early phase of HIV research. However, advancement in HIV research has engendered animal modification to demonstrate HIV pathogenesis in animal models. The use of mouse-human chimeric models (Limoges et al., 2001, Limoges et al., 2000, Persidsky et al., 2001, Dou et al., 2003, Dou et al., 2005, Nukuna et al., 2004) for the study of HIV pathogenesis is well described. In fact, the in vivo confirmation for our in vitro findings were performed using the mouse-human chimeric models (Chapters 2 and 3). However, only downstream events from alcohol
and HIV-induced liver injury could adequately be demonstrated in this model. While this may be considered a limitation, our studies intentionally adopted in vitro models for most of our designs to minimize the confounding effects of other tissue components or endogenous triggers of hepatotoxicity that may be unwarranted for our studies. Although, it is important to demonstrate all the events observed during the in vitro designs by an animal model to achieve a holistic understanding of the mechanisms of HIV and alcohol-induced liver injury. However, the mouse-human chimeric model may inadequately provide the mechanistic pathways of alcohol and HIV-induced liver injury. This is largely due to its inability to naturally retain HIV infection since the mice are continuously fed with ethanol for 6-8 weeks (Ganesan et al., 2019b, Bertola et al., 2013). Such duration of ethanol feeding is necessary for the liver to undergo adequate hepatocyte apoptosis enough to activate HSC just as observed in alcohol abusing PLWH (Bertola et al., 2013). Meanwhile our current mouse-human chimeric model only retains HIV infection by intraperitoneal injection of $3 \times 10^5$ TCID50 HIV-1 every two days (Ganesan et al., 2019b). This is because HIV requires human immune cells to replicate and retain its infection in vivo (Wilén et al., 2012). However, humanization of mouse immune cells may cause unwarranted complications for the mouse already transplanted with human hepatocytes. This is because immunodeficient mouse are mostly preferred during human hepatocyte transplantation to minimize graft-versus-host reaction between mouse tissue and human hepatocytes which occur in 10-20% of animals. (Schroeder and DiPersio, 2011). Moreover, the mouse-human chimeric model
requires longer time for cell differentiation in host mouse and HIV will only directly affects the transplanted human hepatocyte, and sometimes the crosstalk between human and mouse cells is compromised by the lack of species-specific interactions.

While it may be challenging to adequately modify the host (mouse) model then the alternative of modifying the agent (HIV) prevails. EcoHIV mouse model, a model that allowed for the modification of HIV, has been characterized by other groups for the demonstration of HIV pathogenesis in mouse model (Nitkiewicz et al., 2004, Potash et al., 2005, Sindberg et al., 2015, Skowronska et al., 2018, Jones et al., 2016, Jaureguiberry-Bravo et al., 2021). While the EcoHIV is adequate for the purpose of this study, it is not without limitation. For example, the EcoHIV model is limited by lack of gp120, as EcoHIV has been engineered by switching out the HIV gene encoding the surface protein gp120 recognized by human cells and replacing it with a gp80 gene from a mouse leukemia virus (Gu et al., 2018). Therefore, the specific potential pathogenic effects of gp120 on mouse hepatocytes after alcohol exposure will be excluded. Moreover, this may not be an issue since no effect of gp120 on hepatocyte was identified for our studies. Furthermore, EcoHIV replicates in mouse macrophages and lymphocytes, and induces inflammatory reactions similar to HIV responses of human cells (Potash et al., 2005). Therefore, to demonstrate the mechanisms of HIV and alcohol-induced liver injury in future studies, the EcoHIV mouse model, which will ensure natural HIV infection in the mouse will be considered for future works.
Conclusion

The emergence of potent and effective ART has changed the dynamics of mortality for PLWH from AIDS-related mortality to non-AIDS related mortality. Liver disease is among the leading causes of non-AIDS related mortality, which accounts for 18% of all-cause mortality, and approximately 50% of mortality from HIV-related liver disease in the United States is attributed to alcohol. Therefore, this project aimed at unveiling the mechanisms of alcohol and HIV hepatocyte apoptosis and a subsequent profibrotic activation of HSC to develop a therapeutic regimen that will ameliorate the frequently observed liver injury among PLWH. Findings from this project implicated ROS generation from alcohol and HIV exposure as the major trigger of hepatocyte apoptosis, which drove HSC activation in ROS-dependent manner via the JNK-ERK1/2 and JAK-STAT3 pathway. Therefore, therapeutic regimen with ROS scavenging properties or anti-fibrotic tendencies will be valuable for strategic intervention.

BIBLIOGRAPHY

antiretroviral therapy in the Northwest Region of Cameroon. *BMC Gastroenterology,* 22, 286.


BALA, S. & SZABO, G. 2018. TFEB, a master regulator of lysosome biogenesis and autophagy, is a new player in alcoholic liver disease. *Digestive medicine research, 1*.


genotype 1–coinfected patients treated in routine practice. *Clinical Infectious Diseases*, 64, 1711-1720.


CENTER FOR DISEASE CONTROL AND PREVENTION 2020a. Hepatitis D Questions and Answers for Health Professionals.

CENTER FOR DISEASE CONTROL AND PREVENTION 2020b. People Coinfected with HIV and Viral Hepatitis.


CENTER FOR DISEASE CONTROL AND PREVENTION 2021b. Global Viral Hepatitis: Millions of People are Affected.


Role of TLR9 in hepatic stellate cells and experimental liver fibrosis. *Biochemical and biophysical research communications, 376*, 271-276.


GANESAN, M., POLUEKTOVA, L. Y., TUMA, D. J., KHBANDA, K. K. & OSNA, N. A. 2016b. Acetaldehyde Disrupts Interferon Alpha Signaling in Hepatitis C


GONZÁLEZ-GARCÍA, J. J., MAHILLO, B., HERNÁNDEZ, S., PACHECO, R., DIZ, S.,
GARCÍA, P., ESTEBAN, H., ARRIBAS, J. R., QUEREDA, C., RUBIO, R., DÍEZ, J.,

GOTO, T., ITOH, M., SUGANAMI, T., KANAI, S., SHIRAKAWA, I., SAKAI, T.,

GOTTLIEB, M. S., SCHANKER, H. M., FAN, P. T., SAXON, A., WEISMAN, J. D. &

GOUVÊA, A. F., MORAES-PINTO, M. I., MACHADO, D. M., CARMO, F. B., BELTRÃO,

GROEBNER, J. L., GIRÓN-BRAVO, M. T., ROTHBERG, M. L., ADHIKARI, R., TUMA,


GUAN, J., ZHANG, X., SUN, W., QI, L., WU, J. & QIN, Z. 2015a. DRAM1 regulates apoptosis through increasing protein levels and lysosomal localization of BAX. Cell death & disease, 6, e1624-e1624.

GUAN, J. J., ZHANG, X. D., SUN, W., QI, L., WU, J. C. & QIN, Z. H. 2015b. DRAM1 regulates apoptosis through increasing protein levels and lysosomal localization of BAX. Cell Death Dis, 6, e1624.


JIANG, J. X., VENUGOPAL, S., SERIZAWA, N., CHEN, X., SCOTT, F., LI, Y., ADAMSON, R., DEVARAJ, S., SHAH, V. & GERSHWIN, M. E. 2010. Reduced nicotinamide adenine dinucleotide phosphate oxidase 2 plays a key role in


KONG, L., MAYA, W. C., MORENO-FERNANDEZ, M. E., MA, G., SHATA, M. T.,
SHERMAN, K. E., CHOUGNET, C. & BLACKARD, J. T. 2012b. Low-level HIV

AIDS Rev, 5, 36-43.

KOOP, D. R. 2006. Alcohol metabolism’s damaging effects on the cell: a focus on reactive
oxygen generation by the enzyme cytochrome P450 2E1. Alcohol Research &
Health, 29, 274.

KOURTIS, A. P., BULTERYS, M., HU, D. J. & JAMIESON, D. J. 2012. HIV-HBV

LACKNER, A. A., MOHAN, M. & VEAZEY, R. S. 2009. The gastrointestinal tract and

LAFORGE, M., LIMOU, S., HARPER, F., CASARTELLI, N., RODRIGUES, V.,
DRAM triggers lysosomal membrane permeabilization and cell death in CD4+ T
cells infected with HIV. PLoS pathogens, 9, e1003328.


LAPENTA, C., BOIRIVANT, M., MARINI, M., SANTINI, S. M., LOGOZZI, M., VIORA,
lymphocytes are naturally permissive to HIV-1 infection. European journal of
immunology, 29, 1202-1208.


induced hepatic oxidative damage and apoptosis: protective effects of quercetin.

*Oxidative Medicine and Cellular Longevity, 2016.*


*Hepatology, 47, 1495-1503.*


PAROLA, M., ROBINO, G., MARRA, F., PINZANI, M., BELLOMO, G.,
LEONARDUZZI, G., CHIARUGI, P., CAMANDOLA, S., POLI, G. & WAEG, G.
1998. HNE interacts directly with JNK isoforms in human hepatic stellate cells.

PARVEZ, M. K. 2015. HBV and HIV co-infection: Impact on liver pathobiology and
therapeutic approaches. World J Hepatol, 7, 121-6.

PASETTI, G., CALZETTI, C., DEGLI ANTONI, A., FERRARI, C., PENNA, A. &
FIACCADORI, F. 1988. Clinical features of hepatitis delta virus infection in a

PAUZA, C. D. & PRICE, T. M. 1988. Human immunodeficiency virus infection of T cells
and monocytes proceeds via receptor-mediated endocytosis. The Journal of cell
biology, 107, 959-968.

PERAZZO, H. & LUZ, P. M. 2017. Liver disease and healthy life-expectancy with HIV.
The Lancet HIV, 4, e236-e237.

of mitochondria in HIV infection and associated metabolic disorders: focus on
nonalcoholic fatty liver disease and lipodystrophy syndrome. Oxid Med Cell
Longev, 2013, 493413.

PERSIDSKY, Y., LIMOGES, J., RASMUSSEN, J., ZHENG, J., GEARING, A. &
GENDELMAN, H. E. 2001. Reduction in glial immunity and neuropathology by
a PAF antagonist and an MMP and TNFα inhibitor in SCID mice with HIV-1
encephalitis. *Journal of neuroimmunology*, 114, 57-68.


PETOUUMENOS, K. & LAW, M. G. 2016. Smoking, alcohol and illicit drug use effects on

PETTIT, A. C., GIGANTI, M. J., INGLE, S. M., MAY, M. T., SHEPHERD, B. E., GILL, M.
Increased non-AIDS mortality among persons with AIDS-defining events after
antiretroviral therapy initiation. *Journal of the International AIDS Society*, 21,
e25031.


PLATT, M. B. & PLATT, M. O. 2013. From GRID to gridlock: the relationship between
scientific biomedical breakthroughs and HIV/AIDS policy in the US Congress. *J
Int AIDS Soc*, 16, 18446.

POON, I. K., PARKES, M. A., JIANG, L., ATKIN-SMITH, G. K., TIXEIRA, R.,
GREGORY, C. D., OZKOCAK, D. C., RUTTER, S. F., CARUSO, S. &
SANTAVANOND, J. P. 2019. Moving beyond size and phosphatidylserine
exposure: evidence for a diversity of apoptotic cell-derived extracellular vesicles
POTASH, M. J., CHAO, W., BENTSMAN, G., PARIS, N., SAINI, M., NITKIEWICZ, J.,
for study of systemic HIV-1 infection, antiviral immune responses, and
neuroinvasiveness. *Proceedings of the National Academy of Sciences*, 102, 3760-3765.

POWERS, J., ZHANG, H., BATTRELL, L., MEADOWS, G. G. & TROBRIDGE, G. D.
2012. Establishment of an immunodeficient alcohol mouse model to study the
effects of alcohol on human cells in vivo. *Journal of Studies on Alcohol and Drugs*,
73, 933-937.

PREVISANI, N., LAVANCHY, D. & SIEGL, G. 2003. Hepatitis A. *Perspectives in Medical
Virology*. Elsevier.

PRICE, J. C., SEABERG, E. C., STOSOR, V., WITT, M. D., LELLOCK, C. D. & THIO, C. L.
2018. Aspartate aminotransferase-to-platelet ratio index increases significantly 3
years prior to liver-related death in HIV-hepatitis-coinfected men. *AIDS*, 32,
2636-2638.


PUERTOLLANO, R., FERGUSON, S. M., BRUGAROLAS, J. & BALLABIO, A. 2018. The
complex relationship between TFEB transcription factor phosphorylation and

QIN, F., JIANG, J., QIN, C., HUANG, Y., LIANG, B., XU, Y., HUANG, J., XU, Z., NING,
C., LIAO, Y., ZANG, N., LAI, J., WEI, W., YU, J., YE, L., QIN, X. & LIANG, H.


SARACINO, A., COZZI-LEPRI, A., SHANYINDE, M., CECCHERINI SILBERSTEIN, F., NOZZA, S., DI BIAGIO, A., CASSOLA, G., BRUNO, G., CAPOBIANCHI, M.


THOMES, P. G., RASINENI, K., YANG, L., DONOHUE JR, T. M., KUBIK, J. L.,
MCNIVEN, M. A. & CASEY, C. A. 2019. Ethanol withdrawal mitigates fatty liver
by normalizing lipid catabolism. *American Journal of Physiology-Gastrointestinal
and Liver Physiology*, 316, G509-G518.

Acute and chronic ethanol administration differentially modulate hepatic
autophagy and transcription factor EB. *Alcoholism: Clinical and Experimental
Research*, 39, 2354-2363.

THORNTON, A. C., JOSE, S., BHAGANI, S., CHADWICK, D., DUNN, D., GILSON, R.,
MAIN, J., NELSON, M., RODGER, A., TAYLOR, C., YOUSSEF, E., LEEN, C.,
GOMPELS, M., KEGG, S., SCHWENK, A. & SABIN, C. 2017. Hepatitis B,

TIMES, T. N. Y. 1981. RARE CANCER SEEN IN 41 HOMOSEXUALS.

TINCATI, C., BIASIN, M., BANDERA, A., VIOLIN, M., MARCHETTI, G., PIACENTINI,
L., VAGO, G. L., BALOTTA, C., MORONI, M., FRANZETTI, F., CLERICI, M. &
GORI, A. 2009. Early initiation of highly active antiretroviral therapy fails to
reverse immunovirological abnormalities in gut-associated lymphoid tissue
induced by acute HIV infection. *Antivir Ther*, 14, 321-30.

TOSTMANN, A., VAN DEN BOOGAARD, J., SEMVUA, H., KISONGA, R., KIBIKI, G.
hepatotoxicity is uncommon in Tanzanian hospitalized pulmonary TB patients.

*Trop Med Int Health,* 15, 268-72.


induced liver injury. *Journal of International Medical Research, 48*, 0300060518811512.


WANG, Y., VAN BOXEL-DEZAIRE, A. H., CHEON, H., YANG, J. & STARK, G. R. 2013. STAT3 activation in response to IL-6 is prolonged by the binding of IL-6 receptor to EGF receptor. *Proceedings of the National Academy of Sciences, 110*, 16975-16980.


WORLD HEALTH ORGANIZATION 2022a. Hepatitis A.

WORLD HEALTH ORGANIZATION 2022b. Hepatitis C.
XIANG, D.-M., SUN, W., NING, B.-F., ZHOU, T.-F., LI, X.-F., ZHONG, W., CHENG, Z.,
XIA, M.-Y., WANG, X. & DENG, X. 2018. The HLF/IL-6/STAT3 feedforward
circuit drives hepatic stellate cell activation to promote liver fibrosis. *Gut*, 67,
1704-1715.

XIAO, P., USAMI, O., SUZUKI, Y., LING, H., SHIMIZU, N., HOSHINO, H., ZHUANG,
M., ASHINO, Y., GU, H. & HATTORI, T. 2008a. Characterization of a CD4-
independent clinical HIV-1 that can efficiently infect human hepatocytes through

XIAO, P., USAMI, O., SUZUKI, Y., LING, H., SHIMIZU, N., HOSHINO, H., ZHUANG,
M., ASHINO, Y., GU, H. & HATTORI, T. 2008b. Characterization of a CD4-
independent clinical HIV-1 that can efficiently infect human hepatocytes through

XU, X., LAI, Y. & HUA, Z.-C. 2019. Apoptosis and apoptotic body: disease message and

YAMAMOTO, H., ITOH, N., KAWANO, S., YATSUKAWA, Y.-I., MOMOSE, T.,
Dual role of the receptor Tom20 in specificity and efficiency of protein import

YAN, J., OUYANG, J., ISNARD, S., ZHOU, X., HARYPURSAT, V., ROUTY, J. P. &
CHEN, Y. 2021. Alcohol Use and Abuse Conspires With HIV Infection to
Aggravate Intestinal Dysbiosis and Increase Microbial Translocation in People Living With HIV: A Review. Front Immunol, 12, 741658.


induces NADPH oxidase and is associated with liver fibrosis in vivo. *Hepatology*, 43, 435-443.


ZHU, J.-B., XU, S., LI, J., SONG, J., LUO, B., SONG, Y.-P., ZHANG, Z.-H., CHEN, Y.-H.,
XIE, D.-D. & YU, D.-X. 2018. Farnesoid X receptor agonist obeticholic acid
inhibits renal inflammation and oxidative stress during lipopolysaccharide-