University of Alberta

To HAV or not to HAV: Novel hepatitis A virus (HAV) infection in a chimeric mouse model

by

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ABSTRACT

Chimpanzees have been previously used to study HAV infection *in vivo* but they are expensive and difficult to maintain and thus, there is a need for a small animal model. Our aim is to test the susceptibility of the SCID-beige/Alb-uPA mice with chimeric human/mouse livers to HAV infection. Chimeric mice were IV inoculated with HAV and HAV produced fully infectious particles within the serum and feces, by one week post-infection. eHAV circulated the blood of infected chimeric mice consistent with human infections. Due to the lack of an adaptive immune system, HAV caused a persistent infection with a lack of inflammation within the liver. Pre-exposure to neutralizing antibody was fully protective while IFN treatment was able to suppress HAV infection. This study shows that the chimeric mice are permissive to HAV infection and represent a valuable small animal model for future studies.

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LIST OF ABBREVIATIONS

Alb	albumin
ALT	alanine transaminase
AST	aspartate transaminase
Amp	ampicillin
BSA	bovine serum albumin
cDNA	complementary deoxyribonucleic acid
CK-18	cytokeratin-18
Ct	cycle threshold
DCt	delta CT
DMEM	Dulbecco's Modified Eagle Medium
dsRNA	double stranded ribonucleic acid
DTT	dithiothreitol
E.coli	*Escherichia coli*
eHAV	enveloped HAV
ELISA	enzyme-linked immunosorbent assay
FAM	6-carboxyfluorescein
FBS	fetal bovine serum
GE	genome equivalents
GP	glycoprotein
H&E	hematoxylin and eosin
HAI	Hepatic Activity Index

hAlb	human albumin
HAV	hepatitis A virus
HAVCR1	hepatitis A virus cellular receptor 1
HBV	hepatitis B virus
HCC	hepatocellular carcinoma
HCV	hepatitis C virus
HPRT	hypoxanthine-guanine phosphoribosyltransferase
IF	immunofluorescence
IFN	interferon
IFN-α	interferon-alpha
IFN-β	interferon-beta
IgA	immunoglobulin A
IgG	immunoglobulin G
IgM	immunoglobulin M
IL-1	interleukin 1
IP	intraperitoneal
IRES	internal ribosome entry site
IRF-3	interferon regulatory factor 3
ISG	interferon stimulated genes
IV	intravenous
kb	kilobase
LB	Luria broth
LGP2	laboratory of genetics and physiology 2

M cells	microfold cells
MAVS	mitochondrial antiviral signaling protein
MDA5	melanoma differentiation associated gene 5
MMLV RT	Moloney murine leukemia virus reverse transcriptase
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NK cells	natural killer cells
OCT	Optimal Cutting Temperature compound
PAMP	pathogen associated molecular pattern
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pegylated IFN-α2a	Polyethylene glycol conjugated to IFN-α2a
PRR	pattern recognition receptor
qPCR	quantitative (real-time) polymerase chain reaction
qRT-PCR	quantitative reverse transcription polymerase chain reaction
RdRP	RNA-dependent RNA polymerase
RIG-I	retinoic acid inducible gene I
RIP-1	receptor-interacting protein 1
RLRs	RIG-I-like receptors
RNA	ribonucleic acid
rpm	revolutions per minute
SCID	severe combined immunodeficiency disorder
TAMRA	tetramethylrhodamine

TBST	tris buffered saline with tween 20
TIR	Toll/IL-1 receptor homology
TLR3	Toll-like receptor 3
TP	triphosphate
un-eHAV	unenveloped HAV
uPA	urokinase plasminogen activator
UV	ultraviolet
VPg	genome-linked polypeptide
w/v	weight/volume

CHAPTER ONE: INTRODUCTION

1.1 HAV epidemiology

Hepatitis A virus (HAV) is one of the world's most common infections, with exposure to it causing acute liver disease in humans and typically conferring lifelong immunity.[1,2] Although HAV incidence has decreased significantly in recent years due to extended immunization practices, HAV-induced disease remains a public health concern in sporadic cases and epidemics of hepatitis worldwide.[3] There are an estimated 1.4 million cases of HAV-induced hepatitis worldwide annually, with the health cost ranging between 1.5 and 3 billion dollars each year.[4] One challenge is trying to define global HAV risk. A difficulty of trying to map HAV risk is that disease severity increases with age at the time of infection.[5] As a result, HAV infection is a growing health concern in high-income countries where the rate of infection is low and people are typically infected at an older age when disease severity and the risk of death is increased (Figure 1.1).[2] The incidence of HAV infection is higher in low-income areas where access to clean water and sanitation is limited. Paradoxically, the disease burden in these areas is minimal as infections typically occur at an early age when symptoms and disease severity are limited.

1.2 HAV clinical course

HAV is shed in the feces and transmitted primarily via the fecal-oral route in humans.[1] This commonly occurs from ingestion of contaminated food or water

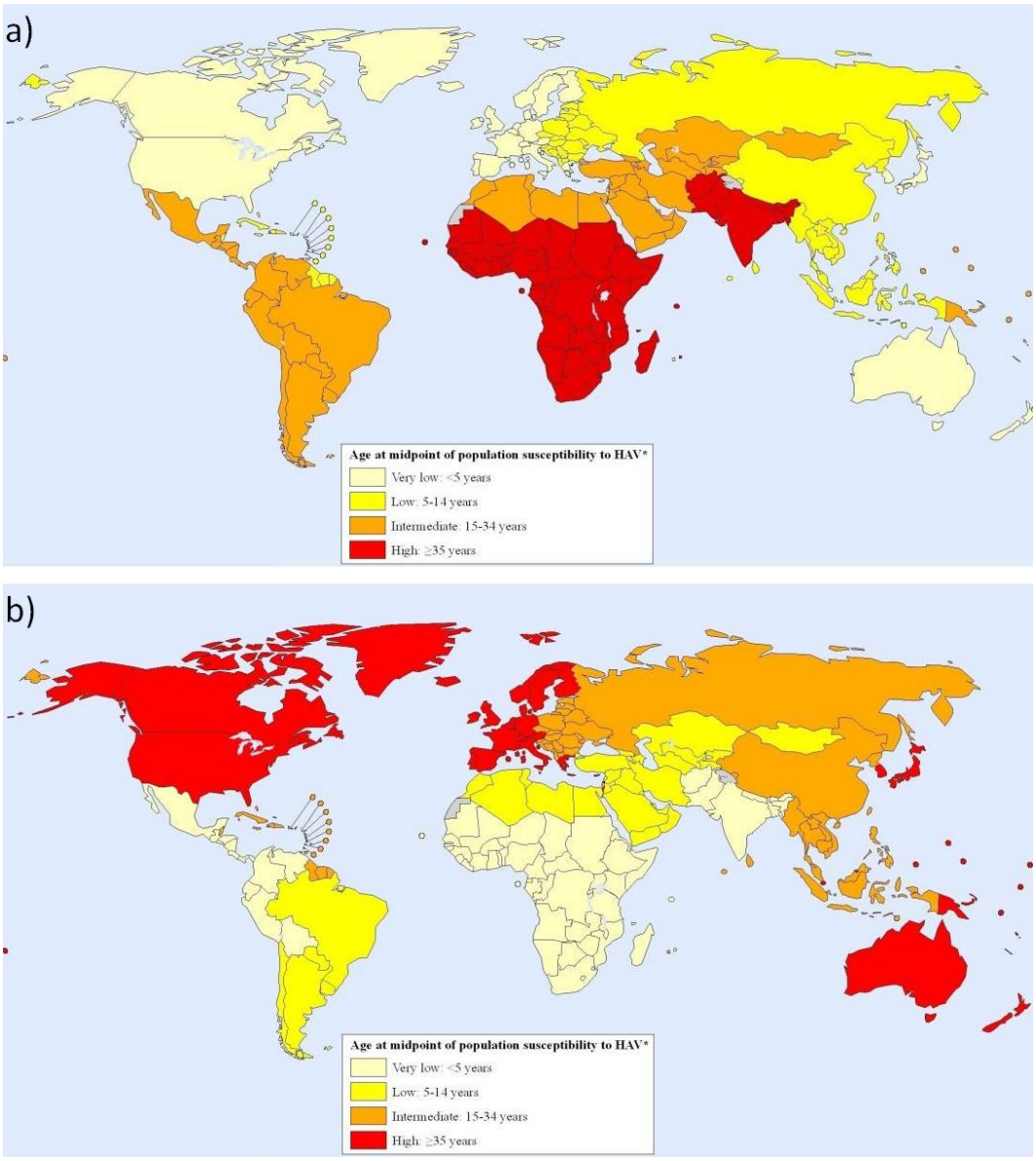


Figure 1.1. Defining HAV risk.
(a) Global risk map of HAV immunity in 2005. High risk areas refer to a high incidence and seroprevalence rate, which correlates with low-income areas where infection occurs in early childhood with little disease. *Age at midpoint of population immunity to HAV is defined as the age at which at least half of the population in that age group has anti-HAV IgG antibodies indicating past exposure to the virus. Age-specific seroprevalence estimates for 2005 for each of 21 world regions were derived from curves fit to pooled data collected between 1995 and 2008 as reported in Jacobsen *et al*., 2010.[2] **(b)** Global risk map of HAV susceptibility in 2005. Risk, in this case, refers to the proportion of susceptible adults to severe disease, which is highest in areas where the incidence of infection is lowest and the age of infection is higher. *Age at midpoint of popoulation susceptibility to HAV is defined as the age at which half of the popoulation in that age group does not have anti-HAV IgG antibodies, indicating no past exposure to the virus. Figure and legend adapted from Mohd Hanafiah et al., (2011).[5]

through contact with fecal contamination from an infected person. Clinical manifestations range on a spectrum from asymptomatic to fulminant hepatitis requiring liver transplantation, depending on the age of the host and other risk factors.[6] Severe disease is rare though. Children under the age of six who acquire the infection are asymptomatic in 70% of cases while approximately 80% of infected adults display acute hepatitis with jaundice and elevated levels of serum aminotransferases. Following an incubation period of about 28 days (range: two to seven weeks) in infected adults, typical symptoms include malaise, fever, nausea, vomiting, diarrhea, dark urine and jaundice.[7] Nevertheless, illness is normally acute and the virus is cleared by a robust adaptive immune response to HAV with the presence of neutralizing antibodies.

Laboratory tests in infected individuals show elevated total bilirubin, alkaline phosphatase and serum transaminases, namely alanine transaminase (ALT) and aspartate transaminase (AST), which coincides with the onset of clinical symptoms (Figure 1.2).[8] Serum immunoglobulin M (IgM) anti-HAV antibodies can be detected within five to ten days post-infection with more than 95 percent sensitivity and specificity.[1] Detection of IgM anti-HAV allows for accurate diagnosis of HAV and distinguishes acute HAV infection from other causes of viral hepatitis. Anti-HAV IgM peaks around five weeks post-infection and begins to decline while anti-HAV immunoglobulin G (IgG) is detectable by week three post-infection and remains positive for the patient's lifetime, offering protection from subsequent exposure.[9] HAV has been shown to be extensively

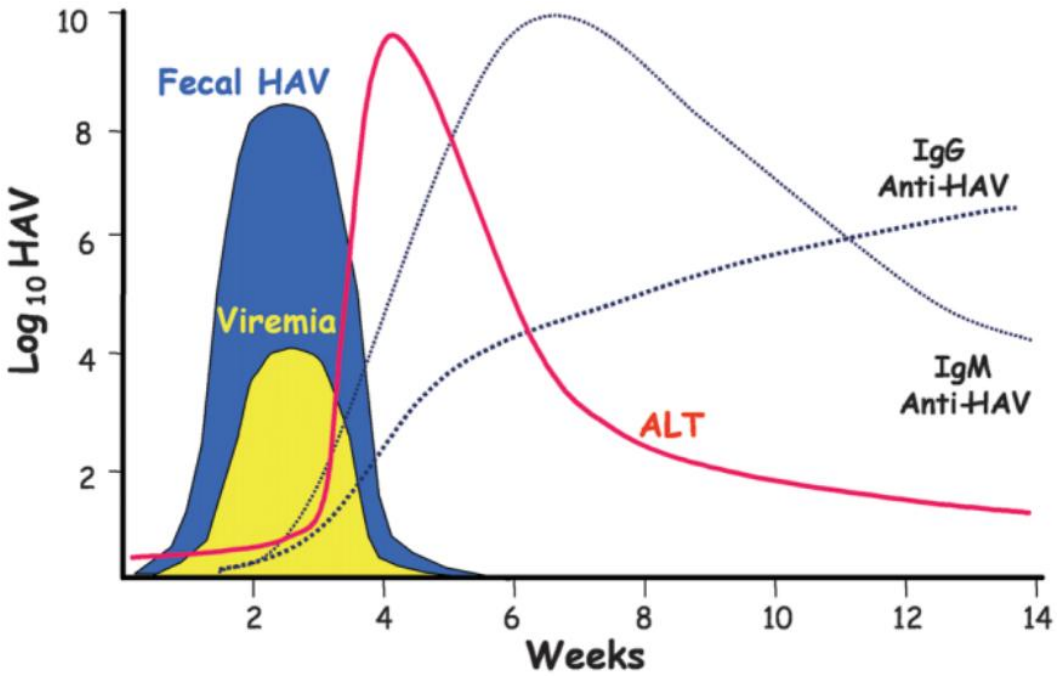


Figure 1.2. Clinical course of acute HAV infection
The infection is typically acute in nature, with symptoms and signs usually occurring within three to five weeks of exposure. The sequence of events includes HAV viremia (yellow), shedding of infectious HAV in feces (blue), followed by increases in serum alanine aminotransferase (ALT) activity (red line), and the appearance of IgM and IgG (typically measured as total) anti-HAV antibody responses (blue lines). IgM antibody is typically short lived but can be detected in some patients as late as 6 to 12 months post- infection with very sensitive assays. Figure reproduced from Martin et al. (2006)[8] with permission from publisher.

shed into the feces during the incubation period prior to clinical illness and thus, HAV is spread before symptoms occur.[8]

Acute illness does not normally persist longer than two months.[1] However, there are reports of relapse in the first six months after infection (10 to 15 percent of cases) during which the patient becomes infectious once again.[10-12] Despite this, there is no evidence for a persistent infection with HAV. In contrast, other major hepatotropic viruses such hepatitis B & C viruses (HBV & HCV) cause chronic infection of the liver.[13] A rare complication of HAV infection is fulminant hepatitis or acute liver failure, which occurs in an estimated 0.015 to 0.5 percent of cases and can be fatal without emergency liver transplantation.[6] Risk for fulminant hepatitis is increased in older patients with pre-existing chronic liver disease such as hepatitis B or C or in the event of co-infection with more than one HAV genotype simultaneously.[14,15] The most recent classification of HAV genotypes describes six different genotypes: three of human origin (I-III) and three of simian origin (IV-VI).[16,17]

1.3 HAV prophylaxis and treatment

Passive immunity via immune globulin is protective against exposure to HAV for up to three months after administration.[18] Of the different human HAV genotypes, only a single serotype has been identified.[19,20] Consistent with this, antibodies against human HAV do not distinguish between individual strains of HAV after either natural or experimental infection.[21-23] Long-term protection

came with the licensure of the HAV vaccine in 1992 in China[4] and 1995 in the United States[24] (Figure 1.3). The vaccine provides lifelong protection and significantly reduces the incidence of infection and/or disease. The vaccine consists of inactivated virus produced and purified from cell culture. Inactivated HAV vaccines are given intramuscularly in two doses and are highly immunogenic, providing excellent protection.[18,25] Protection is broadly acting against all human strains worldwide and the primary mechanism is most likely antibody-based.[26] Protective anti-HAV levels are present in 94-100 percent of adults after one month of receiving the first dose and in 100 percent of adults following the second dose.[27] The vaccine is recommended for all high risk persons, which includes travellers to countries where hepatitis A is of high or intermediate endemicity, persons with chronic liver disease, men who have sex with men, and illicit drug users.[18,28-30] Immunoglobulin may be considered with the vaccine if the patient is immunocompromised, is 40 years or older, has chronic liver disease, or has plans to travel in less than two weeks.

Post-exposure prophylaxis can be effective if the non-immune patient has been recently exposed (within two weeks) to HAV.[32] Based on the results of a randomized trial, the use of HAV vaccine is preferred over administration of immunoglobulin for post-exposure prophylaxis since the vaccine provides long-term protection and is easy to administer.[33] Specific antiviral therapy is currently unavailable.[32] For routine cases of hepatitis A past two weeks of exposure, only supportive therapy is provided. This primarily includes avoidance of

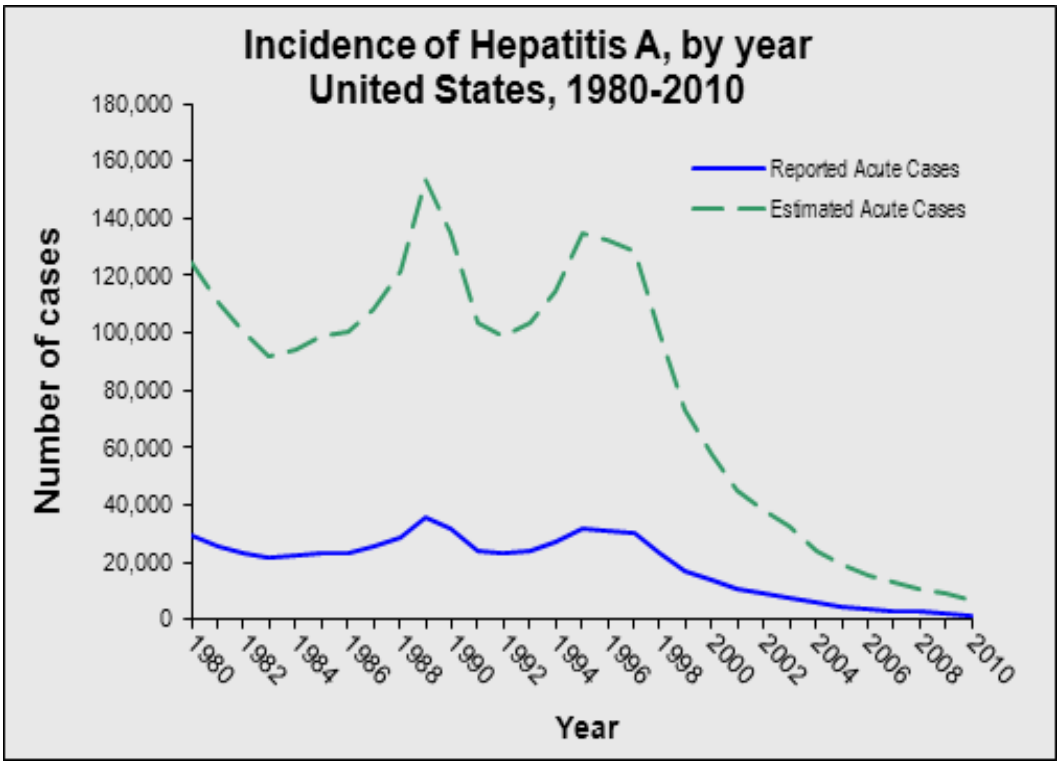


Figure 1.3. Incidence of HAV infection in the United States.
The rates of HAV infection in the United States has decreased by 92% since the HAV vaccine first became available in 1995. The lowest rate ever recorded was in 2009 with 1,987 acute symptomatic cases of HAV infection and an incidence of 1/100,000. The estimated number of new infections was 21,000 after adjusting for underreporting and asymptomatic infections. Figure reproduced with permission from Centers of Disease Control and Prevention.[31]

hepatotoxins, a balanced nutrition and adequate rest until fever and jaundice have subsided.[34,35]

1.4 Molecular virology of HAV

HAV is a 7.5 kilobase (kb) positive-sense ribonucleic acid (RNA) virus classified in the genus *Hepatovirus* of the *Picornaviradae* family.[8] The genome is enclosed in an icosahedral protein capsid made up three major structural proteins, VP1, VP2 and VP3 (Figure 1.4). The virus gains entry into hepatocytes via interaction with a cellular receptor, hepatitis A virus cellular receptor 1 (HAVCR1).[36] During entry, the viral particle uncoats and releases the viral RNA genome into the cell cytoplasm. The viral genome encodes a large polyprotein with a single open-reading frame.[37] The 5'-terminal third of the genome codes for the structural proteins (VP1, VP2, VP3) while the remainder of the genome codes for the non-structural proteins (2B, 2C, 3A, 3B, $3C^{pro}$ and $3D^{pol}$) needed for viral replication.[38] The 5' terminus of the genome also encodes for the putative fourth capsid protein VP4, although it has never been identified directly in purified virus preparations and its function is still under controversy.[39,40] The 2A segment is the carboxy-terminal extension of VP1 and cleavage at the VP1-2A junction is an important step during virion morphogenesis.[41-43] Cap-independent translation of the viral polyprotein within the cytoplasm is directed by an internal ribosome entry site (IRES) located in the 5' nontranslated region of the viral genome.[44] Co- and post-translational proteolytic processing of the polyprotein is mediated by the viral protease, $3C^{pro}$, and an unidentified cellular protease. Cleavage releases

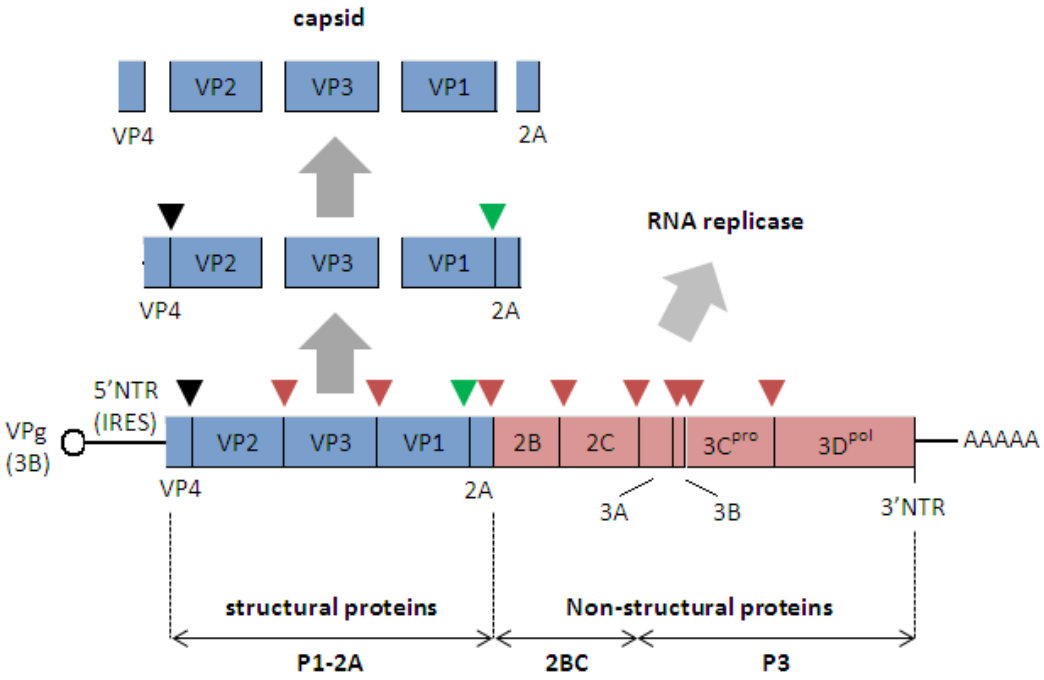


Figure 1.4. Organization and polyprotein processing of the HAV genome. The viral protease $3C^{pro}$ cleaves the viral polyprotein (shown by red triangles) encoded by a single open reading frame within the positive-strand (messenger-sense) RNA genome. The polyprotein is also proteolytically processed by an unknown cellular protease (green triangle) and an unknown proteolytic activity (black triangle) that releases the mature nonstructural (red) and structural (blue) proteins. Figure adapted from Martin et al. (2006).[8]

mature structural and nonstructural proteins (Figure 1.4).[45-47] The initial cleavage products are P1-2A, P2, and P3. These are further cleaved into individual mature proteins. The structural proteins (VP1, VP2, VP3) are derived from the P1-2A peptide segment while the P2 and P3 segments encode the nonstructural proteins required for assembly of the HAV replicase. Negative-strand RNA synthesis is initiated by the nonstructural proteins (spanning 2B-3D^{pol}) with the assembly of a large replicase complex on membranes derived from the cellular endoplasmic reticulum.[48] 3B and associated downstream proteins are anchored to the membrane by 3A, which contains a transmembrane domain.[49,50] Uridylylated VPg (protein 3B) is covalently linked to the 5' end of the genome and likely acts as the protein primer for synthesis of negative-strand RNA similar to poliovirus.[8] 3D^{pol} is the RNA-dependent RNA polymerase.[51,52] Positive-strand RNA genomes are amplified from a negative-strand template. In addition to being used as a template for further replication and translation, newly synthesized positive-sense RNA is packaged into new virions.[41] The formation of new viral particles requires the assembly of the structural proteins, cleavage of the VP1-2A precursor by an unidentified cellular protease and cleavage of the VP4/VP2 junction.[42,43] Newly assembled virions are thought to be secreted mainly across the basolateral side of the cellular membrane of hepatocytes.[53] From here, virus is able to enter the hepatic sinusoids and result in serum viremia. However, this may not be the only egress pathway from hepatocytes since a significant amount of virus found in bile and in the feces.[54]

HAV is highly conserved at both the nucleotide and amino acid level. Viral isolates show greater than 90 percent identity at the nucleotide level and 98 percent in predicted amino acid sequences.[16] HAV was traditionally classified into seven genotypes (designated I to VII) from the analysis of the genetic variability of 152 HAV strains recovered around the world.[55] This classification was based on the criteria applied to poliovirus, another virus within the *Picornaviradae* family. It was based on the percentage sequence identity around the VP1/2A junction (168 nucleotides).[56] However, a comprehensive analysis of South American HAV strains showed that the VP1 amino terminus contains more information to establish genetic relationships within the *Picornaviridae* family compared to the VP1/2A junction.[57] VP1 is the main surface protein in the mature picornavirus virion accessible to antibody binding.[58,59] Based on monoclonal antibody-resistant mutants, the major immunodominant site of HAV is found within the VP1 protein, making it a very informative variable segment for molecular evolution and phylogeny studies within the family *Picornaviridae*.[60-62] Examination of virus strains recovered from six different countries from 1983 to 2001 based on the complete VP1 protein sequence (900 nucleotides) suggested six different genotypes because two genotypes of the old classification (genotypes II and VII) may be one or two subgenotypes of the same type.[17] In this novel classification, three are of simian origin (IV-VI) and three genotypes were found in humans (I-III). Genotypes I and III are the most prevalent in humans.

1.5 Enveloped HAV (eHAV)

A recent study by Feng *et al.*, (2013) revealed that HAV is able to acquire an envelope-like structure encapsulating the capsid through hijacking of cellular membranes.[63] This population of virus was referred to as enveloped HAV (eHAV) and was shown to predominate in the blood of infected humans and chimpanzees whereas it was nearly absent within the feces from which virus has historically been isolated. The two virus populations are distinguished by their buoyant densities determined by density gradient centrifugation. The low density population (1.06–1.10 g cm^{-3}) is eHAV and the high density population (1.22–1.28 g cm^{-3}) is unenveloped HAV (un-eHAV) expected for picornaviruses. In addition, the presence of virus-like particles enclosed in membranes was visualized by electron microscopy of the light fractions. The membrane-bound structures contained up to four virus-like particles and ranged from 50 to 110 nm in diameter. Aside from the membrane, the morphology of the virus-like particles was indistinguishable from HAV particles of the dense fractions (27nm).[63] In contrast to the dense fraction, the light fraction was undetectable by capsid antigen enzyme-linked immunosorbent assay (ELISA).[63] Treatment of the light fraction with detergent (1% NP-40) reversed this effect and detection was observed by ELISA, suggesting the envelope might protect eHAV against antibody neutralization. Extraction with chloroform, which disrupts membranous envelope, had no effect on the infectivity of standard HAV but significantly reduced the infectivity of eHAV suggesting a critical role for the envelope in a distinct entry pathway for eHAV.[63] Despite these findings, the authors showed

that eHAV could be neutralized by antibody up to six hours post-infection in cultured cells, suggesting that antibody binding to capsid may occur following entry into the cell and loss of the envelope. These results suggest a novel mechanism for how post-exposure therapy with neutralizing antibodies is effective for the control of HAV infection despite the evasion of antibody binding by eHAV observed by ELISA.[63]

1.6 *In vitro* studies with HAV

Similar to HCV, wild-type HAV propagates poorly in cell culture and the virus requires a process of adaptation before efficient replication is observed.[13,64] After adaptation, HAV can replicate in many types of cultured mammalian cells including human & simian fibroblasts and human hepatoma (Huh7, Huh7.5) cells. These adaptations have been reported to result from mutations within the internal ribosome entry site (IRES) that promotes cap-independent viral translation, or mutations in 2B that enhance viral RNA replication.[65,66] Unlike poliovirus and other member of the *Picornaviridae* family, HAV is typically noncytopathic and produces a slow and persistent infection in cell culture with low virus yields.[8] Infection is self-limiting *in vitro* and the virus down-regulates its replication in common cell lines (FRhK-4 and MRC-5 cells) used for its propagation.[67] This effect, in addition to a lack of an adaptive immune response *in vitro,* may contribute to the establishment of a persistent infection. This is in contrast to *in vivo* conditions where infection and illness are acute in nature.

1.7 Similarities between HAV with HCV

HCV is also a positive-sense RNA virus that targets the liver in humans.[13] It is an enveloped virus categorized within the genus *Hepacivirus* of the *Flaviviridae* family whereas HAV is in the *Picornaviradae* family. However, both HAV and HCV are similar in many aspects of their genome structure, replication and immune evasion strategies. For example, the organization of the HCV genome also contains an IRES near the 5' terminus followed by genome segments that represent the structural proteins and the nonstructural proteins at the 3' terminus (Figure 1.5). The HCV genome also encodes a single large polyprotein that is processed into different structural and nonstructural proteins by viral and host proteases. The HCV protease, NS3, along with its cofactor, NS4A, triggers *cis*- and *trans*-cleavage of the polyprotein during HCV replication, similar to the functions of 3Cpro of HAV.[8] Because both are positive-sense RNA viruses, they also share exclusively cytoplasmic replication cycles and generate double stranded RNA (dsRNA) replication intermediates.[13] Moreover, both viruses replicate their genomes within membrane-bound replicase complexes composed of intracellular host membranes recruited by viral nonstructural proteins: 2C for HAV and NS4B for HCV.[48] Within these complexes, functionally related but distinct sets of viral enzymes are found such as RNA helicases (2C for HAV and NS3 for HCV) and RNA-dependent RNA polymerases (3Dpol for HAV and NS5B for HCV).[13]

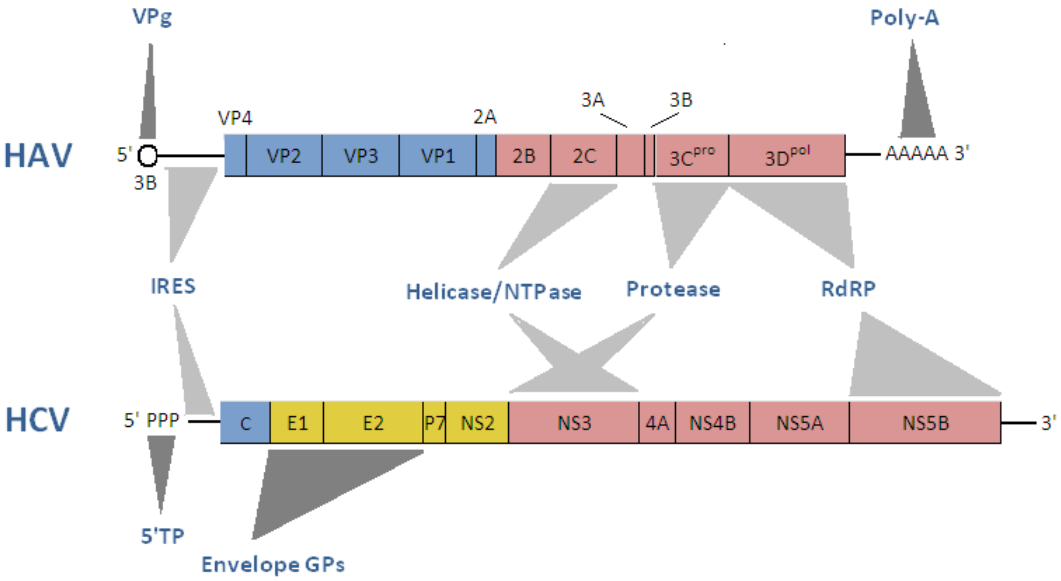


Figure 1.5. Similar genome organization for HAV and HCV.
Both HAV and HCV are positive-strand RNA viruses and organize their genomes in a similar fashion. The structural proteins are arranged near the 5' end in the HAV (extending from VP4 to 2A) and in the HCV (extending from C to E2) genome. The remainder of the genome encodes for nonstructural proteins that are needed for viral replication. IRES, internal ribosome entry site; RdRP, RNA-dependent RNA polymerase; VPg, genome-linked polypeptide; GPs, glycoproteins; TP, triphosphate. Figure adapted from Qu et al. (2010).[13]

In terms of the immune response, both viruses produce a dsRNA replication intermediate that is a pathogen associated molecular pattern (PAMP) recognized by the innate immune sysetm.[13] Toll-like receptor 3 (TLR3) is one of the pattern recognition receptors (PRR) that senses dsRNA.[68,69] TLR3 is found primarily in early endosomal compartments where it detects extracellular ligands (Figure 1.6).[70] Upon binding to dsRNA, TLR3 dimerizes and interacts with Toll/IL-1 receptor (TIR) homology domains on a recruited adaptor protein named TIR-domain-containing adaptor-inducing interferon-β (TRIF).[71-73] This interaction is critical for the downstream signaling leading to the activation (by phosphorylation) and transit of IFN regulatory factor 3 (IRF-3) into the nucleus.[74,75] TLR signaling also induces nuclear factor-κB (NF-κB) activity by TRIF interaction with receptor-interacting protein 1 (RIP-1) kinase.[76] IRF-3 and NF-κB are highly regulated transcription factors that act in coordination to form an enhanceosome complex on the interferon-β (IFN-β) promoter. This mechanisms leads to IFN-β transcription and translation.[77] HCV has also been reported to disrupt TLR3 signaling and IFN-β transcription via cleavage of TRIF by the chymotrypsin-like protease, NS3/4A.[78,79] HAV appears to share a remarkably similar immune evasion mechanism by also targeting TRIF for degradation through its cleavage by the 3CD protease-polymerase precursor (a 3ABCD processing intermediate).[80]

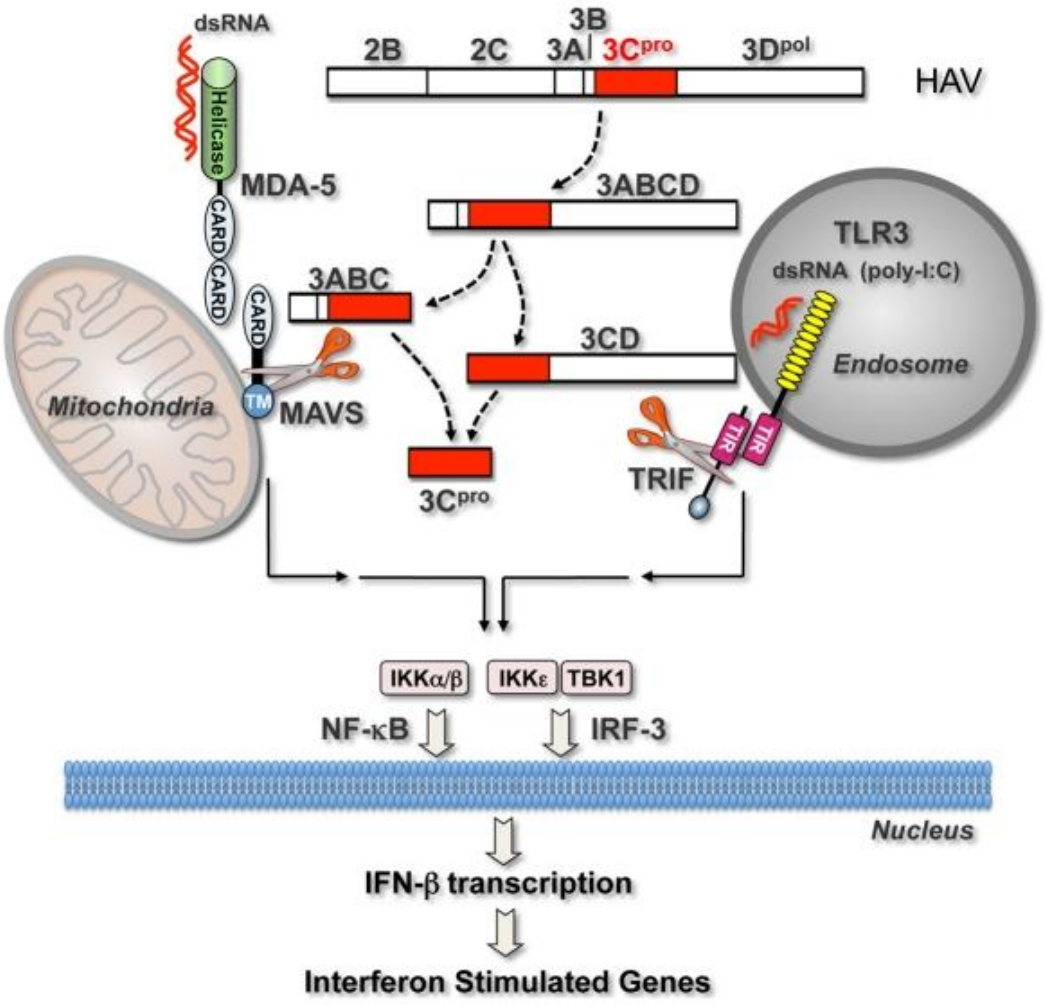


Figure 1.6. Disruption of IFNβ expression by HAV.
HAV dsRNA intermediate is sensed by two parallel signaling pathways: the RLR pathway (most likely by MDA-5) and by the TLR3 pathway. Both pathways lead to the activation and translocation of transcription factors, IRF-3 and NF-κB, that act to induce the transcription of IFN-β mRNA. Both pathways are disrupted by precursors of the HAV $3C^{pro}$ cysteine protease. 3ABC cleaves the adaptor protein MAVS, which is critical for MDA-5 signaling, and 3CD cleaves the adaptor protein TRIF, which is essential for TLR3 signaling. Both 3ABC and 3CD are processing intermediates of $3C^{pro}$ derived from the P2P3 polyprotein fragment (2B to $3D^{pol}$). HCV has also been shown to similarly target MAVS and TRIF for degradation via cleavage by the chymotrypsin-like protease, NS3/4A. Figure reproduced with permission from Qu et al. (2011).[13] Copyright © 2011 Qu et al.

The retinoic acid inducible gene I (RIG-I)-like receptors (RLRs) are another set of PRRs that detects viral dsRNA.[81,82] RLRs represent a family of intracellular PRRs comprised of three members: RIG-I, melanoma differentiation associated gene 5 (MDA5), and laboratory of genetics and physiology 2 (LGP2) protein.[83,84] The binding of these members to dsRNA triggers a signaling cascade that causes the production of type I interferons (IFN-α & IFN-β) via IRF-3 and NF-κB activation similar to in the TLR3 pathway.[85] HAV RNA is most likely sensed by MDA-5, which requires the binding to an essential signaling adaptor protein known as mitochondrial antiviral signaling protein (MAVS) (Figure 1.6).[13,86] As the name suggests, MAVS localizes to the mitochondrial outer membrane. Its discovery in 2005 provided evidence for the importance of the mitochondria in the assembly of virus-triggered signaling complexes that lead to IFN production.[87-89] Interestingly, the cleavage of MAVS as an immune evasion strategy has been reported in both HAV and HCV infection via distinct cleavage sites. 3ABC, an intermediate precursor protein of HAV, cleaves MAVS at a site 80 amino acid residues upstream of the cleavage site for HCV NS3/4A (Figure 1.6).[13,80,87] Nonetheless, both mechanisms results in the release of MAVS from the mitochondria and the disruption of its signaling ability. With no clear phylogenetic relationship between 3ABC and NS3/4A, these results represent a unique example of convergent evolution when similar innate immune responses are encountered within hepatocytes.[13]

Despite the many similarities in genome structure, replication and immune evasion strategies, HAV and HCV infections produce very different clinical outcomes.[13] HAV infection is self-limiting and chronic infection has never been reported, whereas HCV infection causes chronic infection in the majority of infected immunocompetent adults.[90] The long-term persistence of HCV infection makes HCV one of the main causes of fibrosis of the liver, cirrhosis, as well as hepatocellular carcinoma (HCC).[91] Unfortunately, HAV research has declined since the licensure of the HAV vaccine nearly two decades ago.[54] A better understanding of HAV pathogenesis may provide valuable comparisons with HCV infection.[13] Further research will help to uncover the mechanisms in pathogenesis and immune response that accounts for their differences in viral persistence and disease outcomes in humans.

1.8 Animal models for HAV infection

A reliable and accessible animal model is very useful to study virus pathogenesis. *In vivo* studies with human HAV have been hampered by its strict tropism for humans and higher level primates.[92] Despite the susceptibility to natural HAV infection being reduced in non-human primates compared to humans, the course of infection resembles that in humans making them valuable animal models.[93] The primary animal models used to study HAV infection are chimpanzees (Pan troglodytes),[94] marmosets (Saguinus mystax),[95] and owl monkeys (Aotus trivirgatus)[96]. Infection by IV or oral inoculation has been successful in these animal models. Studies in marmosets have shown the coincidence of HAV-

associated pathology with peak antibody titres in the absence of any cytopathic effect from HAV, suggesting an immune-mediated mechanism of liver damage.[97] Positive fluorescent staining of virus within cells of the small intestine was demonstrated in marmosets suggesting the potential for replication at these sites. However, the evidence is conflicting and this still remains to be proven.[96,98] More recently, studies in chimpanzees have shown a surprising lack of intrahepatic type I (IFN-α/β) interferon stimulated gene (ISG) responses in infected animals.[54] These studies also revealed the importance of a strong and sustained $CD4^+$ T cell response in the control of HAV infection.[99] In contrast, there was no obvious temporal correlation between the $CD8^+$ T cell response and control of the virus and liver damage. The use of non-human primates for the study of HAV has allowed for a comprehensive and integrative analysis of HAV infection in a good animal model.

Although non-human primates have provided a wealth of information, further animal studies are required to answer questions of HAV pathogenesis. For instance, the mechanisms underlying HAV transit through the gut and potential tropism for intestinal cells is still not well understood. Chimpanzees and other non-human primates provide good physiological and immunological models for studying HAV infection *in vivo*. However, ethical concerns have reduced use of chimpanzees for invasive research experiments.[100] In addition, chimpanzees are not routine laboratory animals and are limited by cost and maintenance challenges. Chimpanzees have been used for many years for large-scale research

in the United States but the Great Ape Protection Act, which is currently under review, is aiming to eliminate invasive research on chimpanzees.[101] For these reasons, chimpanzees are not easily accessible to researchers who are interested in studying HAV infection. Thus, there is a need for a small animal model for future studies looking to investigate HAV pathogenesis.

1.9 Chimeric mouse model

The severe combined immunodeficiency disorder (SCID)-beige/albumin (Alb)-urokinase plasminogen activator (uPA) mouse model with transplanted human hepatocytes provided the first small animal model to effectively study HCV infection *in vivo*.[102,103] Developed in 1990 to study neonatal bleeding disorders[104], the Alb-uPA transgenic mouse overproduces urokinase in the liver as a result of a tandem array of four murine urokinase genes under control by an albumin promoter. As a result, these transgenic mice enter a state of hypofibrinogenemia (abnormally low levels of serum fibrinogen) that increases the rate of hepatocyte death. This loss of murine hepatocytes creates a niche allowing transplanted human hepatocytes to repopulate the liver (Figure 1.7).[105,106] The SCID and beige mutations result in a lack of T and B cells and a deficiency in natural killer (NK) cell function, respectively. The lack of an adaptive immune system prevents immune rejection of the transplanted human hepatocytes within the chimeric mouse model.[102] The crossing of mice transgenic for Alb-uPA with SCID/beige mice provided mice that were homozygous for all three traits. By supporting the survival and growth of primary human hepatocytes, the chimeric SCID-

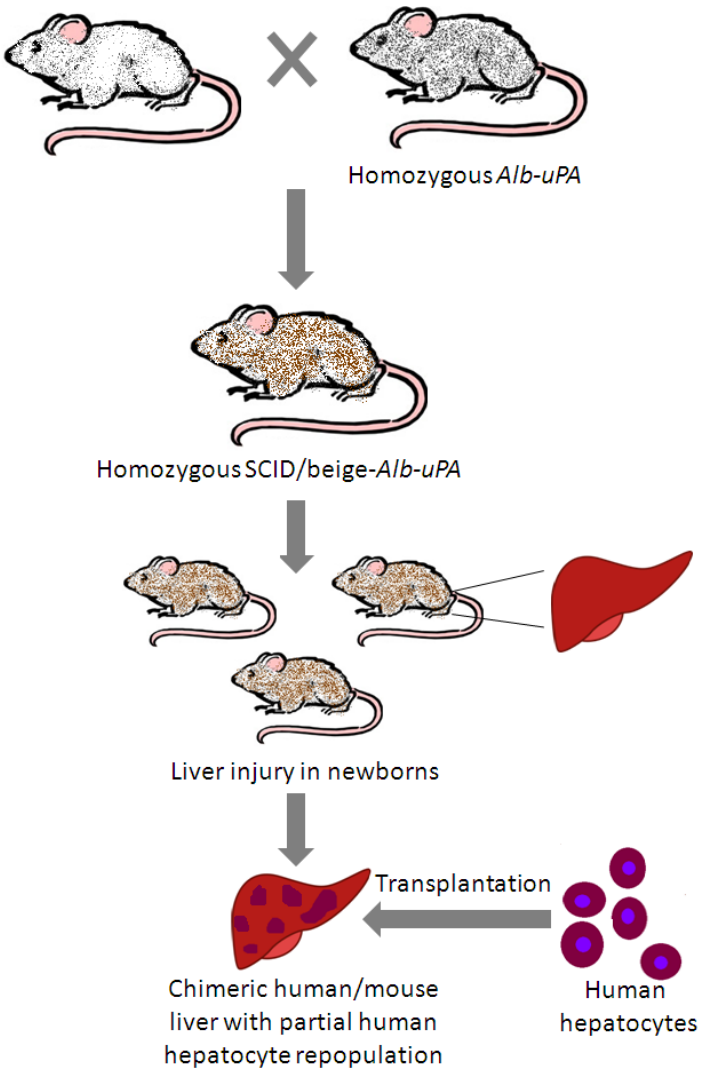


Figure 1.7. Generation of immunodeficient mice with chimeric human/mouse livers.
Mercer et al. (2001)[102] crossed mice transgenic for Alb-uPA with SCID/beige mice, which provided mice that were homozygous for both traits. The Alb-uPA transgene results in urokinase overproduction (under the albumin promoter) in the liver causing transgenic mice to enter a state of hypofibrinogenemia (abnormally low levels of serum fibrinogen) that accelerates the rate of hepatocyte death. Human hepatocytes were transplanted into newborn transgenic mice with liver injury, resulting in a chimeric human/mouse liver through the partial repopulation with human hepatocytes. Figure adapted from Fausto (2001).[103]

beige/Alb-uPA mice has chimeric human/mouse livers containing the natural host cells for HBV and HCV replication. As predicted, the chimeric mice have been shown to be permissive to both persistent HBV[107,108] and HCV[102,109-111] infections. Based on these results, we predicted this chimeric mouse model would be permissive to HAV infection, which also replicates in human hepatocytes.

1.10 Hypothesis and purpose

We hypothesize that the chimeric SCID-beige/Alb-uPA mice will be permissive to HAV infection. Our objective is to develop a more accessible small animal model to study HAV infection *in vivo*. We want to test the susceptibility of the chimeric mice to different routes of infection with HAV. Viral replication will be measured by quantitative reverse-transcription polymerase chain reaction (qRT-PCR) for HAV RNA in sera, feces, bile and liver tissue samples. In addition, we will study the innate immune response to HAV infection. We will also study the effect of exogenous IFN treatment on HAV infection as well as the ability of neutralizing antibody to provide passive immune protection in this model.

CHAPTER TWO: MATERIALS AND METHODS

2.1 Animals and housing

Homozygous SCID-beige/Alb-uPA mice were obtained from the Tyrrell laboratory breeding colony (Figure 1.7).[102] Transgenic mice were housed and cared for in a virus-free/antigen-free environment according to Canadian Council on Animal Care guidelines. Mice were fed a standard rodent diet. Cages were changed on a weekly basis.

2.2 Generation of chimeric mice with human/mouse livers

The transplantation procedure was done on recipient transgenic mice between 10-21 days of age (with the help of Ran Chen from the Tyrrell laboratory). Animals were anesthetized with isoflurane/O_2 followed by a laparotomy and exposure of the spleen. We injected $1x10^6$ human hepatocytes into the inferior splenic pole for repopulation of the mouse liver by primary human hepatocytes. Previous studies in the Kneteman laboratory (by David Mercer) have shown that human hepatocytes injected into spleen repopulate the mouse liver within one hour. All animal experiments were approved by the University of Alberta Animal Welfare Committee and informed consent was obtained from all donors of human hepatocytes in accordance with the University of Alberta Faculty of Medicine Research Ethics Board guidelines. However, for most of these studies we used commercially purchased primary human hepatocytes from a female Caucasian donor (CellzDirect; Lot Hu8085) and a male donor (Celsis, M00995). The level

of human engraftment was estimated by ELISA analysis of serum human albumin (hAlb) levels (produced exclusively by human hepatocytes) at six and eight weeks post-transplantation. Mice with hAlb levels between 1000 and 10000µg/mL at eight weeks post-transplantation were chosen for experimental studies with HAV infection.

2.3 Virus used for inoculation of chimeric mice with HAV

The wild type HM175 strain of HAV (provided by Dr. Stanley Lemon, University of North Carolina) was used to infect chimeric mice. This virus was clarified and filtered from a fecal suspension of HAV-infected chimpanzees. Eight weeks following transplantation, mice with appropriate human engraftment were infected with $5x10^3$ genomic equivalents (GE) HAV via a 50µL intraperitoneal (IP) or tail intravenous (IV) injection. Intragastric inoculation (100µL volume) of HAV was also performed using an oral gavage. Mice inoculated orally were housed separately from mice inoculated IV or IP to prevent any potential blood transfer between animals.

2.4 Serial passage of virus

Blood (100µL) drawn from HAV-infected chimeric mice was left to clot at room temperature and centrifuged at 1500 x *g* (gravity) for 10 minutes for collection of the supernatant containing the sera. Sera (50µl; $5x10^3$GE) were used to inoculate naïve chimeric mice via IV administration. The dose was adjusted to $5x10^3$ GE HAV in a 50µL inoculum following quantification of viral RNA by quantitative

reverse-transcription polymerase chain reaction (qRT-PCR). Mice inoculated with infectious sera were bled at four weeks post-infection and sera (50µl) were used to inoculate a subsequent group of naïve chimeric mice to test serial passage of the virus.

2.5 Subcutaneous injection of IFN

IFN treatment of mice used the commercial PEGASYS preparation, which contains polyethylene glycol conjugated to IFN-α2a (pegylated IFN-α2a) and is used clinically to treat HCV infection in humans. We used a dosage of 35 and 350ng/g mouse administered once per week based on previous studies (by Ran Chen, Tyrrell laboratory) using pegylated IFN-α2a in HCV infected chimeric mice. The injection was prepared in phosphate buffered saline (PBS) to a final volume of 30µL and administered subcutaneously to the mice.

2.6 Feces and blood collection

Feces were collected from each mouse individually on a weekly basis and stored at -80^{o}C for future processing. Blood (100µL) was collected via the jugular vein on a bi-weekly basis. Blood was left to clot at room temperature and centrifuged at 1500 x *g* for 10 minutes and the serum (supernatant) was collected and stored in a -80 ^{o}C freezer for future processing.

2.7 Tissue harvest from chimeric mice

Infected chimeric mice were euthanized for organ harvesting at pre-determined endpoints or when animals looked ill as indicated by ruffled fur (mouse looked unkempt), a hunched back, lethargy and/or a reluctance to move. Mice were first anesthetized by isoflurane administration. Ethanol (70%) was used to sanitize the mouse abdomen followed by a laparotomy using surgical scissors to gain access into the abdominal cavity. The diaphragm was penetrated and blood was collected through an apical puncture of the heart by a 25-gauge needle and syringe. Bile was drawn by puncture of the gallbladder using a 30-gauge needle and syringe. The liver was dissected, rinsed in cold PBS and dissected by a scalpel into eight equal pieces: six pieces were snap-frozen in liquid nitrogen, one piece was placed in a cassette and fixed in 10% buffered formalin phosphate for paraffin embedding and sectioning, and one piece was placed into a labeled mold with Tissue-Tek Optimal Cutting Temperature compound (OCT) (Sakura; #4583) and snap-frozen in liquid nitrogen for cryostat sectioning. The spleen was excised and dissected into three pieces for frozen storage, paraffin embedding and cryostat sectioning, similar to the liver tissue. The gastrointestinal tract was dissected for harvest of the distal ileum and proximal duodenum. Both sections were divided into three pieces for snap-freezing in liquid nitrogen and subsequent paraffin embedding and cryostat sectioning. Lung, heart and kidney tissue was also harvested in a cassette and placed in formalin for paraffin embedding. Snap-frozen tissue was transferred for long-term storage in a -80^{o}C freezer. Blood was centrifuged at 1500 x *g* for 10 minutes and the serum (supernatant) was stored in a

-80^{o}C freezer. Tissue for paraffin embedding was fixed in 10% buffered formalin phosphate overnight on a shaker. Tissues were then incubated on a shaker in 90% ethanol for 90 minutes, followed by two incubations in 100% ethanol for 90 minutes each. The tissues were incubated in butanol on a shaker overnight and this was repeated twice. The cassettes containing the tissues were put into melted paraffin wax (Paraplast X-TRA for tissue embedding; Sigma-Aldrich, P38808) for five hours under negative pressure at 60^{o}C. Tissues were placed into molds and cut onto four micron sections using a microtome.

2.8 HAV standard for absolute viral RNA quantification

To develop a standard for absolute viral RNA quantification by qRT-PCR, we used the HM175/18f plasmid (Figure 2.1), which contains the HAV genome and ampicillin resistance gene sequences (provided by Dr. Stanley Lemon, University of North Carolina). The HM175/18f plasmid was used to transform competent TOP10 *Escherichia coli* (*E.coli*) cells by mixing 1µL of HM175/18f with 1mL of TOP10 cells. The samples were incubated on ice for 30 minutes before being incubated at 42^{o}C for 45 seconds and then back on ice. Luria broth (LB) media (900µL) was added to the cells and mixed on a roller at 37^{o}C for one hour. From this LB solution, 50µL was seeded onto a LB agar plate containing ampicillin (Amp) using glass rods under sterile conditions by a running flame. The remaining solution was centrifuged at 6000 revolutions per minute (rpm) for five minutes to remove excess LB media to a final volume of 50µL. The pellet was resuspended and seeded onto another LB/Amp plate and incubated at 37^{o}C

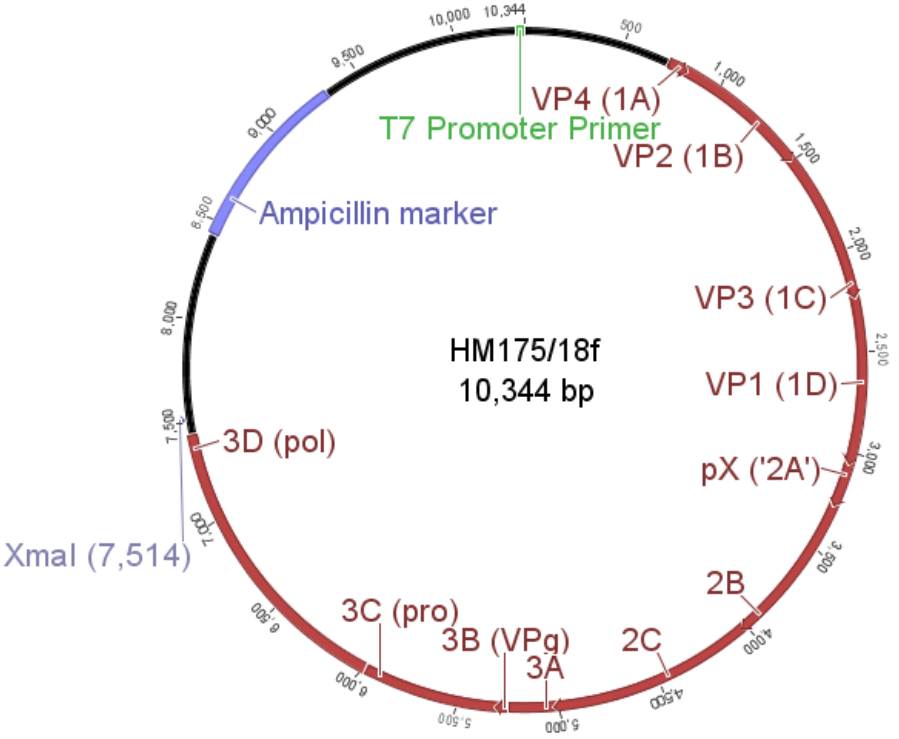


Figure 2.1. HM175/18f plasmid containing HAV genome.
The HM175/18f plasmid is 10344 base pairs in length and contains the DNA encoding the entire HAV genome. The plasmid contains a T7 promoter primer at position 10326 and an ampicillin resistance marker at position 8449. The plasmid contains an XmaI restriction site at position 7514, which was used to linearize the plasmid. Figure produced by Geneious: *Drummond AJ, Ashton B, Buxton S, Cheung M, Heled J, Kearse M, Moir R, Stones-Havas S, Thierer T, Wilson A (2013) Geneious v4.8, Available from http://www.geneious.com.*

overnight. The following day, single transformed colonies on the plates were picked with sterile toothpicks and placed into culture tubes containing 10mL of LB media containing ampicillin. The ampicillin stock was made at a concentration of 100mg/mL in H_20 and syringe filtered through a 0.2µm membrane and stored in -20^oC. The working LB/Amp media was made by mixing 250µL of the stock ampicillin solution with 500µL of LB for a final concentration of 50µg/mL. Culture tubes containing picked colonies were incubated on a shaker overnight at 37^oC.

Plasmid DNA was purified from cells in the culture tubes using the QIAprep Spin Miniprep Kit (Qiagen; 27104) according to the manufacturer's protocol. The DNA concentration was measured using the NanoDrop 1000 spectrophotometer (Thermo Scientific). The presence of the HM175/18f plasmid containing the HAV genome was tested by PCR using HAV specific primers: forward primer GGT AGG CTA CGG GTG AAA C, reverse primer AAC AAC TCA CCA ATA TCC GC. The products were electrophoresed on an agarose gel and visualized under ultraviolet (UV) light. Plasmid DNA was linearlized using XmaI restriction enzymes (New England BioLabs; R0180S) as per the manufacturer's instructions. A mixture containing 1µg plasmid DNA, 0.5µL (10U/µL) XmaI enzyme, 0.5µL bovine serum albumin (BSA) and 5µL of Buffer 4 was diluted in H_20 to a final volume of 50µL and incubated overnight at 37^oC.

The treated DNA was electrophoresed on an agarose gel to visualize linearized plasmid (10344 base pairs) under UV light. DNA of correct size was sliced from the gel using a scalpel and the containing DNA was purified using the QIAquick Gel Extraction Kit (Qiagen; 28704) according to the manufacturer's protocol. The quality and concentration of linearized plasmid was examined by the NanoDrop spectrophotometer. The number of copies of the template plasmid was determined by inputting the DNA concentration and the length of template into an online calculator by the URI Genomics & Sequencing Center (http://www.uri.edu/research/gsc/resources/cndna.html).[112] The calculation assumes that the average weight of a base pair is 650 Daltons. Based on the calculation, the template plasmid was diluted to make a $1x10^8$ genome equivalent (GE)/mL stock. This was then serially diluted 10-fold down to $1x10^1$ GE/mL for use as a working stock to generate a standard curve for subsequent absolute viral RNA quantifications by qPCR.

2.9 HAV RNA isolation

Viral RNA was isolated from serum, feces and bile by a guanidine purification method using the High Pure Viral Nucleic Acid Kit (Roche; #11 858 874 001) according to manufacturer's directions. Sera and bile (30µL) were added directly to the viral RNA isolation protocol for purification. Feces were diluted 10 times in PBS to make a 10% weight/volume (w/v) mixture. The resultant mixture was homogenized using the PT 1200 handheld homogenizer (Polytron; #11010026) and then centrifuged at 1500 x *g* for 10 minutes. The PT homogenizer was

sequentially washed in bleach, water and 100% ethanol for 10 seconds each between samples. The supernatant containing virus was collected and 200µL of fecal suspension was used for RNA purification by the High Pure Viral Nucleic Acid Kit as per the manufacturer's instructions. The same volume of tissue culture supernatant from the culture adapted p16 HAV strain (provided by Dr. Stanley Lemon) was processed with every run and served as a positive control. Pooled sera from uninfected individuals were used as a negative control in each assay. Purified viral nucleic acid was eluted in 50µL of the provided elution buffer.

2.10 HAV RNA quantification by qRT-PCR

Extracted RNA was dried in a vacuum centrifuge for 90 minutes at 2000rpm and prepared for first strand complementary DNA (cDNA) synthesis using random primers (Invitrogen; #48190-011) and SuperScript™ III Reverse Transcriptase (Invitrogen; #18080-044) according to manufacturer's instructions. Briefly, we mixed 300ng of random primers, 1µl of 10mM dNTP mix and 13µl of distilled water to the dried pellet containing viral RNA. The reaction was performed in a PTC-100 Thermal Cycler (MJ Research) for cDNA synthesis.

qPCR was performed on each sample in duplicate using the Applied Biosystems 7300 Real Time PCR System with TaqMan probes.[113] The reaction conditions were 50^{o}C for two minutes, 95^{o}C for 10 minutes, 95^{o}C for 15 seconds and 60^{o}C for one minute and was repeated 40 times. The HAV primer/probe sequences

cover nucleotides 392 to 480 within the 5' untranslated RNA segment of the genome and the following primers were used: forward primer GGT AGG CTA CGG GTG AAA C and reverse primer AAC AAC TCA CCA ATA TCC GC. The 6-carboxyfluorescein/tetramethylrhodamine (FAM/TAMRA) probe sequence was CTT AGG CTA ATA CTT CTA TGA AGA GAT GC. A final reaction volume of 25µL consisting of 12.5µL TaqMan Universal PCR Master Mix (Applied Biosystems®; #4324018), 2.25µL forward primer (10uM), 2.25µL reverse primer (10uM), 2.5µL probe (2.5uM), 3.5µL distilled water and 2µL of cDNA converted sample. The HM175/18f plasmid described previously, which contains the sequence for HAV, was used to generate a standard curve of known concentrations (from 10^1-10^7 GE). The standard curve was used for absolute quantification of viral RNA copies from serum, feces and bile samples by interpolation based on their cycle threshold (Ct) values. The qPCR threshold was manually set at 0.2 in the middle of the geometric phase of the amplification curve where the precision of the replicates were highest. Calculated values were adjusted for their dilutions and represented as GE/mL serum, bile or GE/g feces. The positive control derived from HAV/p16 was run through the same RNA purification and qRT-PCR protocol. HAV extraction efficiencies were normalized relative to the positive control processed in parallel.

2.11 Relative HAV RNA quantification from liver tissue

One mL of TRIzol reagent (Ambion; #15596-026) was added to a piece of frozen liver tissue (~100mg) and homogenized using the PT 1200 handheld homogenizer

(Polytron; #11010026). The homogenate was put on a test tube shaker for 30 minutes at room temperature to permit the complete dissociation of the nucleoprotein complexes. Chloroform (0.3mL) was added to the homogenate and mixed by vortexing for 15 seconds. The resultant mixture was put on a shaker for 10 minutes followed by centrifugation at 12000 x *g* for 15 minutes at 4^{o}C, which separates the mixture into a lower red phenol-chloroform phase, interphase and clourless upper aqueous phase. The aqueous phase containing RNA was extracted (~60% of volume of TRIzol used) and added to 0.5mL of chloroform to remove the traces of phenol. This was vortexted for 15-20 seconds and put on a shaker for two minutes before centrifugation at 12000 x *g* for 15 minutes at 4^{o}C. The aqueous phase was collected and mixed with 250µL isopropanol and 250µL high salt solution (0.8M sodium citrate, 1.2M NaCl). Samples were incubated at -20^{o}C overnight. Samples were centrifuged at 12000 x g for 30 minutes at 4^{o}C and the supernatant was discarded followed by two washes with 1mL of 75% ethanol. Each wash was followed by centrifugation at 13000rpm for 5-10 minutes at 4^{o}C. The ethanol was removed and the RNA pellet was dried for eight minutes at room temperature before dissolving it in 20-40µL of RNase free water. The RNA concentration was measured by a NanoDrop spectrophotometer. The RNA quality was also tested by measuring the ratio of absorptions at 260nm versus at 280 nm (a 260:280 ratio close to two suggests no protein contamination). RNA was aliquoted into a 4µL volume containing 2µg of RNA. RNA was converted to cDNA using Moloney Murine Leukemia Virus Reverse Transcriptase (MMLV RT) enzyme (Invitrogen; # 28025-013). A 20µL reaction mixture contained of

2µg RNA in 9.85µL H_20, 4µL 5xFirst-Strand Buffer, 0.5µL dNTP (10mM of each), 0.2µL BSA (10mg/mL), 2µL (0.1M) dithiothreitol (DTT), 0.15µL random primer (3µg/µL), 0.8µL RNasOUT, 1µL MMLV RT, and 1.5µL H_20. The mixture was incubated at 22°C for five minutes, 37°C for 60 minutes and then 95°C for 10 minutes. qPCR was performed on the cDNA samples (in duplicates) using the Applied Biosystems 7300 Real Time PCR System. The same qPCR protocol utilizing TaqMan probes as described previously in section 2.10 was used for relative HAV RNA quantification. In addition, intrahepatic hypoxanthine-guanine phosphoribosyltransferase (HPRT) gene expression was measured. HPRT is a commonly used housekeeping gene that is constitutively expressed for its essential role in the maintenance of basic cellular function.[114,115] For each sample, a total reaction volume of 25µL was prepared consisting of 14.4µL SYBR Green PCR Master Mix (Applied Biosystems; #4309155), 0.9µL primer mixture for HPRT (10µM), 7.7µL H_20, and 2µL cDNA. The delta CT (DCt) was calculated by subtracting the HPRT Ct from the HAV Ct for each sample. Relative HAV RNA was represented as a percentage of HPRT by using the formula: 2^{DCt} x 100%.

2.12 Intrahepatic transcriptional analysis by qRT-PCR

RNA isolation and cDNA conversion from liver tissue was performed as described in section 2.11. qPCR was performed on converted cDNA (in duplicates) using the Applied Biosystems 7300 Real Time PCR System to determine the intrahepatic transcriptional expression of five IFN genes (IFNα1,

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')	Probe (5'-56-FAM3'-TAMARA)
HPRT1	CTTGGTCAGGCAGTATAATCCA	CAAATCCAACAAAGTCTGGCT	n/a
IFNα1	AACTCCCCTGATGAATGC	CTGCTCTGACAACCTCCC	n/a
IFNβ1	CAATTTTCAGTGTCAGAAGCTCC	AAAGTTCATCCTGTCCTTGAGG	CTGTGGCAATTGAATGGGAGGCTT
IL28	GACGCTGAAGGTTCTGGAG	ATATGGTGCAGGGTGTGAAG	CCACCGCTGACACTGACCCA
IL29	CTGTCACCTTCAACCTCTTCC	GACGTTCTCAGACACAGGTTC	CCCATCGGCCACATATTTGAGGTCT
IFNγ	GACTTGAATGTCCAACGCAA	GCATCTGACTCCTTTTTCGC	n/a
CXCL9	AGGAACCCCAGTAGTGAGAAAGG	CCTGCATCAGCACCAACCAAGGGA	GGTCTTTCAAGGATTGTAGGTGGAT
IFIT2	AGGAAGATTTCTGAAGAGTGC	CACTGCAACCATGAGTGAGAACAATAAGAA	GTTCCAGGTGAAATGGCA
IRF3	CCCTCACGACCCACATAAAATC	CCCAGTAACTCATCCAGAATGTC	CAGACACCTCTCCGGACACCAATG
ISG15	TGGTGAGGAATAACAAGGGC	CAGATTCATGAACACGGTGC	n/a
MX1	ACCTGATGGCCTATCACCAG	TTCAGGAGCCAGCTGTAGGT	n/a
OAS2	GGTGAACACCATCTGTGACG	TGAACCCATCAAGGGACTTC	n/a
IRF7	GTGAAGCTGGAACCCTGG	CCATAAGGAAGCACTCGATGTC	AGCGCCAACAGCCTCTATGACG
CXCL10	TGAAAAAGAAGGGTGAGAAGAGATG	CTGAATCCAGAATCGAAGGCCATCAAGA	CCTTTCCTTGCTAACTGCTTTCAG
STAT1	GTGGAAAGACAGCCCTGCAT	AACGCACCCTCAGAGGCCGC	ACTGGACCCCTGTCTTCAAGAC
IFI27	GTAGTTTTGCCCCTGGC	TGTGATTGGAGGAGTTGTGGCTGT	GACATCATCTTGGCTGCT
IFI6	AAGGCCCTGACCTTCAT	AGGAGGACTCGCAGTCGCC	ATTCAGGATCGCAGACCA

Table 2.1. Human-specific primers and probes used to identify intrahepatic gene expression by qPCR.

IFNβ1, IFNγ, IL28, IL29) and 11 ISGs (ISG15, MxA, OAS2, CXCL9, IFI27, IFI6, IFIT2, IRF3, IRF7, IP10, STAT1) (Table 2.1). HPRT was also analyzed as a housekeeping gene for normalization. Human specific primers for each gene were designed by Ran Chen (Tyrrell laboratory). A combination of TaqMan and SYBR chemistry was performed depending on whether probes were used for detection. For TaqMan chemistry, reactions consisted of 5µL TaqMan Universal PCR Master Mix, 1µL forward primer (10uM), 1µL reverse primer (10uM), 1µL probe (2.5uM), and 2µL cDNA to be tested. For SYBR chemistry, reactions consisted of 5µL SYBR Green PCR Master Mix, 0.3µL primer mixture (10uM), 2.7µLH_20, and 2µL cDNA to be tested. The DCt was calculated by subtracting the HPRT Ct from the HAV Ct for each sample. Relative HAV RNA was represented as a percentage of HPRT by using the formula: 2^{DCt} x 100%. The statistical significance ($p \leq 0.5$) of the fold change over uninfected samples was determined by the students T-test using the software, GraphPad Prism 5.

2.13 Immunofluorescent (IF) staining and confocal microscopy

Four micron sections were cut from liver tissue samples frozen in OCT compound (by Suellen Lamb, Tyrrell laboratory) and labeled with murine monoclonal antibody to HAV capsid antigen (K32F2; provided by Dr. Stanley Lemon) to visualize infection. Monoclonal antibody against cytokeratin-18 (Abcam, ab32118) was used to visualize human hepatocytes, and DAPI was used to stain cell nuclei. Sections were fixed with 4% paraformaldehyde for 20 minutes at room temperature and then permeabilized using 0.2% Triton X-100 (in PBS) for

15 minutes. Sections were blocked in normal goat serum (Sigma-Aldrich, G9023) for one hour at room temperature, washed with Tris buffered saline with Tween 20 (TBST), and blocked with mouse IgG (1:50 dilution; Sigma-Aldrich, I5381). Sections were washed again in TBST followed by blocking with goat anti-mouse IgG (1:50 dilution; Sigma-Aldrich, M8642) diluted in normal donkey serum block (Sigma-Aldrich, D9663). Tissue sections were then incubated with the primary antibodies overnight at 4^{o}C: murine anti-HAV capsid antigen (1:100 dilution) and rabbit anti-human CK-18 (1:50 dilution). As a negative control, sections were treated similarly, but incubated with isotype-matched control antibodies with no known specificities at the same concentration as the test antibodies. The isotype matched control antibody used for CK-18 was rabbit IgG (Sigma-Aldrich, I5006) and for HAV capsid antigen was mouse IgG2a (Abcam, ab18415). Tissue sections were then incubated with 1:10 dilution DAPI (4′,6-Diamidino-2-phenyindole; Sigma-Aldrich, D9564-10MG) and secondary antibodies for one hour at room temperature: goat anti-mouse IgG Alexa 488 (1:200 dilution; Molecular Probes, A-11001) and donkey anti-rabbit IgG Alexa 555 (1:200 dilution; Molecular Probes, A-11011). Coverslips were applied onto slides and staining was visualized using the Leica TCS SP5 Confocal microscope. Using specific filters, bound Alexa 488 fluorphores on sections were excited at 495nm and emission was observed at 519nm. Bound Alexa 555 fluorphores on sections were excited at 555nm and emission was observed at 565nm. DAPI was excited at 358nm and emission was observed at 461nm. Emission results were converted into digital images with DAPI staining shown in blue, CK-18 staining

shown in red and HAV capsid antigen staining shown in green. Pictures were taken at an objective magnification of 40X and 100X for each slide. A scale of 50µm (40X) and 100µm (100X) was used in captured pictures.

2.14 Hematoxylin and eosin (H&E) staining

Sections were deparaffinized in xylene and rehydrated in 100% alcohol (two minutes), 95% alcohol (two minutes), 70% alcohol and with distilled water. They were subsequently stained in Harris' (alum) hematoxylin for 15 minutes and rinsed under running water. To remove loosely bound hematoxylin dye, 1% acid alcohol was applied to sections (5-10 drops) and sections were rinsed under running water again. Sections were rinsed in Scott's Tap Water substitute (blueing reagent) for one minute, which blues hematoxylin gently, and then rinsed under running water to remove excess reagent. To counterstain, 1% aqueous (acidified) eosin was added for two to three minutes followed by rinsing under running water. Sections were dehydrated through a series of graded alcohol treatments with gentle agitation by shaking: 70% alcohol for 10-15 seconds, 95% alcohol for 10-15 seconds, and 100% alcohol for 30 seconds. A second change in 100% alcohol was performed for one minute. Sections were treated with xylene for one minute each to make sections miscible with the mounting agent. Another xylene treatment for two minutes was performed before mounting in synthetic resin (DPX mountant). Sections were viewed under a light microscope for photography and analysis. Sections were analyzed by a pathologist, Dr. Jason Doyle, for inflammation based on a modified Hepatic Activity Index (HAI)

scoring.[116,117] Selected slides were scanned for further analysis and image capture using the ImageScope Viewer software by Aperio.

2.15 Isopycnic gradient centrifugation of virus

Isopycnic gradient centrifugations were performed by our collaborators Dr. Zongdi Feng and Dr. Stanley Lemon (University of North Carolina).[63] To remove cells and debris, serum, fecal suspension and bile fluids were centrifuged at 1,000 x *g* for 10 minutes at 4°C and then further clarified by centrifugation twice at 10,000 x *g* for 30 minutes, and concentrated by ultracentrifugation at 100,000 x *g* for one hour at 4°C. PBS was used to resuspend the pellet before being loaded onto an 8–40% iodixanol (Opti-Prep) step gradient, and centrifuged in a Superspin 630 rotor at 141,000 x *g* for 48 hours at 4°C in a Sorvall Ultra-80 ultracentrifuge. Approximately 20 fractions were collected from the top of the gradient and density was determined using a refractometer.

2.16 HAV isolation from iodixanol gradient fractions

HAV isolations from iodixanol gradient fractions were performed by our collaborators Dr. Zongdi Feng and Dr. Stanley Lemon (University of North Carolina).[63] 10–100µL of peak iodixanol gradient fractions containing virus recovered from chimeric mice sera, feces and bile were used were used to inoculate Huh7.5 cells in 12-well culture plates. Cells were re-fed on a weekly basis and passaged 1:3 at two weeks. At 8, 23 and 30 days post-inoculation, RNA was extracted from the supernatant culture fluid with the QiaAmp viral RNA

Isolation Kit (Qiagen). A synthetic RNA standard containing the HAV genome was used to quantify HAV RNA by real-time qRT-PCR as described previously.[54] To confirm viral isolates, purified RNA was transcribed to cDNA with random primers (Superscript III First-Strand Synthesis System; Invitrogen) and amplified by nested RT–PCR using primer sets around the VP1pX-2B junction (nucleotides 2908–3306 in wild-type HM175) (PrimeSTAR; TaKaRa). Products from second-round PCR were purified from agarose gels (Mini-Elute Gel Extraction Kit; Qiagen) for direct sequencing.

2.17 Purification of human IgG from blood for subcutaneous injection

Blood was drawn from two groups: those who have received the HAV vaccine and have adequate antibody titres, and those who have not been vaccinated and have no anti-HAV antibodies. Serum was collected by centrifugation at 1500 x *g* for 10 minutes and sera from the same group were then pooled together. Sera were then clarified at 21000 x *g* for five minutes at room temperature. The soluble material was then diluted with nine mL of binding buffer (1.5 M Glycine/NaOH, 3M NaCl, pH 8.9) and filtered through a 0.22 um filter prior to use with HiTrap Protein G column (GE Healthcare; 17-0404-01) (performed by Karl Fisher, Tyrrell laboratory). The column was washed with 5-10 column volumes of binding buffer (5-10mL) at 1mL/minute. The sample was then applied at an optimal flow rate of 0.5mL/minute followed by washing with 10 column volumes of binding buffer at 1mL/minute). Antibody was eluted with 10 column volumes of elution buffer 1 (0.1M Sodium citrate buffer, pH 5.5)

followed by 10 column volumes of elution buffer 2 (0.2M glycine/HCl buffer pH 2.5) at a flow rate of 1mL/minute. Fractions (0.9mL) of the elution were collected into 100µL of neutralization buffer (1M Tris-HCl, pH 9).

The DC Protein Assay kit (Bio-Rad; 500-0111) was used to determine the IgG concentration according to the manufacturer's instructions. Briefly, five dilutions of the protein standard, bovine serum albumin (BSA), containing from 0.2mg/mL to 1.5mg/mL protein was prepared for generation of a standard curve. Samples and standards (5µL) were pipetted into a 96-well plate with 25µLof reagent A from the kit. After 15 minutes of incubation at room temperature, the absorbance of the calorimetric reaction was read at 750nm and the protein concentrations were calculated based on the generated standard curve. Based on these results, 50µL of purified IgG (50µg/g mouse) was used for subcutaneous injection of naïve chimeric mice 24 hours prior to IV HAV infection for the passive immunity experiments.

CHAPTER THREE: RESULTS - CHARACTERIZATION OF A SMALL ANIMAL MODEL FOR HAV INFECTION

3.1 HAV causes a persistent infection in chimeric mice

To determine whether mice with chimeric human/mouse livers are susceptible to HAV infection, we inoculated four mice IV (B1151, B1153, B1154, B1167) and two mice IP (B1148, B1170) with the HM175 strain of HAV ($5x10^3$GE) derived from the fecal suspension of HAV-infected chimpanzees. HAV RNA could be detected in the sera of all six mice within one week of inoculation (Figure 3.1a,b). Average viral titres were detected at $2.9x10^4$ GE/mL sera and $4.4x10^6$ GE/g feces at week one post-infection, 1000 times higher than the inocula. Viral RNA peaked at $1.6x10^6$ GE/mL sera and $3.4x10^9$ GE/g feces by week three post-infection (Figure 3.1c). There was no difference in the kinetics of infection between the IV and IP virus inoculation routes. In contrast to human infections, HAV caused a persistent infection in the chimeric mice with HAV RNA detectable in the serum and feces at peak levels for 10 weeks post-infection at which time animals were euthanized. Viral titres in the feces were approximately 1000-fold higher compared to levels observed in sera at the respective time points.

HAV RNA was detected in liver tissue harvested from all euthanized HAV-infected chimeric mice (Figure 3.1a). Moreover, viral RNA was found in the bile (Figure 3.1b) at concentrations comparable to corresponding levels found in

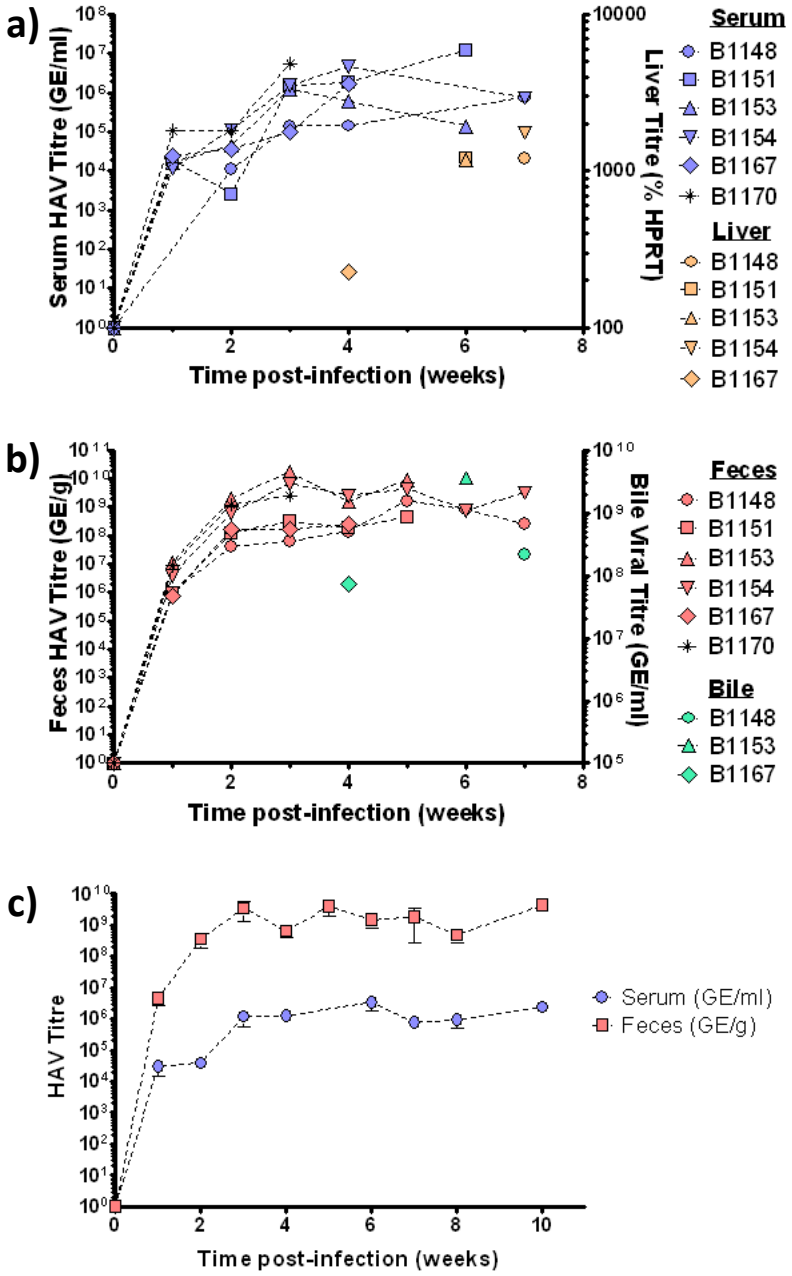


Figure 3.1. Persistence of viral RNA in HAV-infected chimeric mice.
Six chimeric mice were infected with the HM175 strain of HAV via IV (B1151, B1153, B1154, B1167) and IP (B1148, B1170) administration. Blood and feces were collected from infected mice and RNA was purified for HAV RNA detection by qRT-PCR. HAV RNA was detected in the serum **(a)** and feces **(b)** of all infected animals by the first week post-infection. Viral RNA was also detected within liver tissue **(a)** and bile **(b)** harvested from the gallbladder collected at the time of sacrifice. Intrahepatic viral titres are displayed as relative quantifications as a percentage of HPRT (housekeeping gene). The average weekly titres from 17 HAV-infected mice within the sera and feces are shown **(c)**.

feces, suggesting that the virus reaches the feces via the biliary tract. These results demonstrate the chimeric mice are susceptible to persistent HAV infection with detectable viral RNA within the serum, feces, liver and bile compartments.

3.2 HAV infects human hepatocytes in chimeric livers

To visualize HAV by confocal microscopy, we performed IF staining on five HAV infected (between four to seven weeks of infection) frozen liver tissue sections with a monoclonal antibody (K32F2) against the capsid antigen. Monoclonal antibody against human cytokeratin-18 (CK-18) was used to stain human hepatocytes in liver sections from chimeric mice. No staining was observed using an isotype-specific control antibody on the liver sections (Figure 3.2b). As expected, sections taken from uninfected chimeric mice showed staining against CK-18 only and not against HAV capsid antigen (Figure 3.2a). Tissue from HAV-infected mice showed capsid staining that localized with staining of human hepatocytes by CK-18 suggesting that HAV replication occurs primarily within the human hepatocytes of chimeric livers (Figure 3.2c,d). HAV staining was localized in the cytoplasm of human hepatocytes, consistent with the virology and replication mechanism of the virus. The predominant staining pattern for HAV capsid within human hepatocytes is evidence for the selective tropism HAV has for human hepatocytes, which allows for the chimeric mice to be permissive to HAV infection.

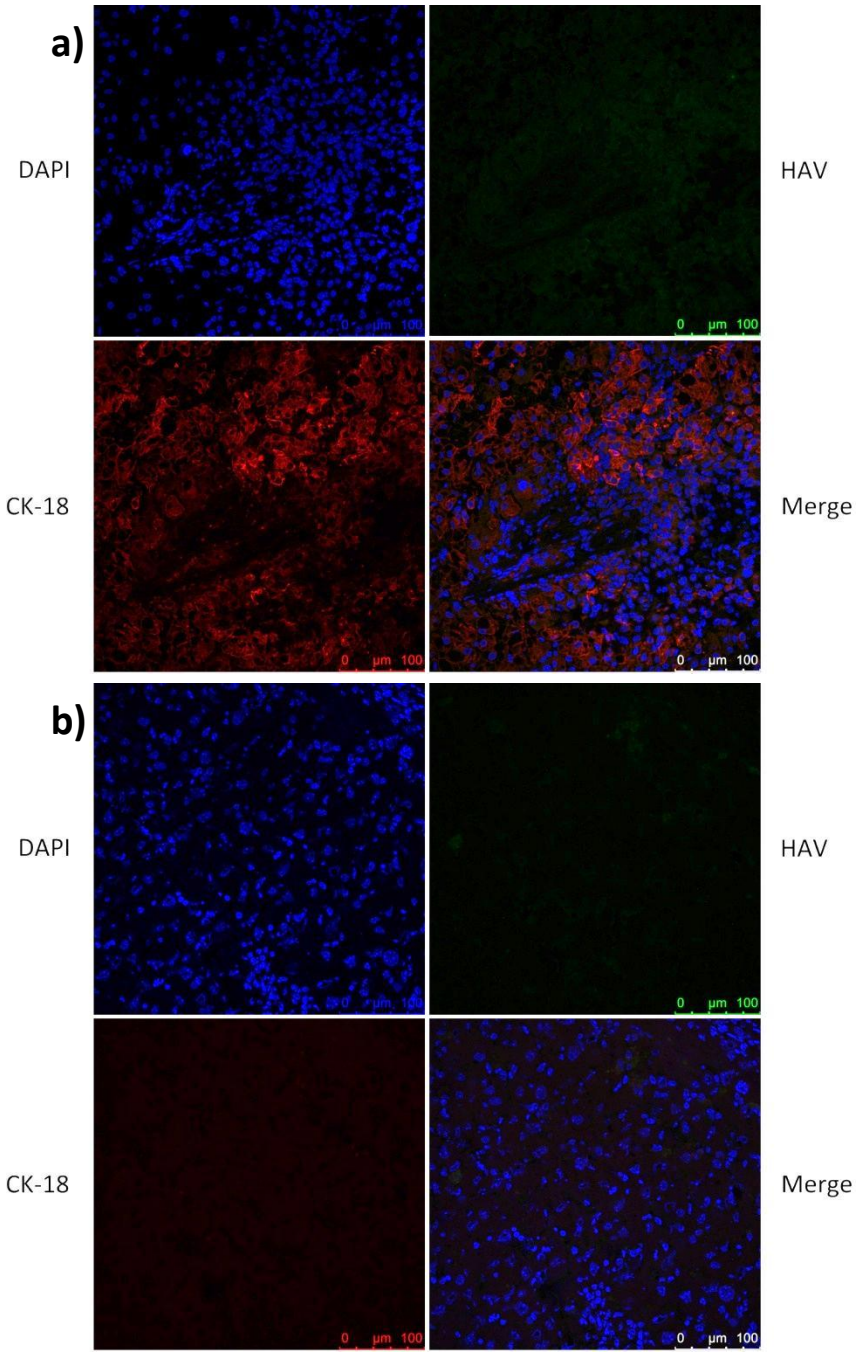
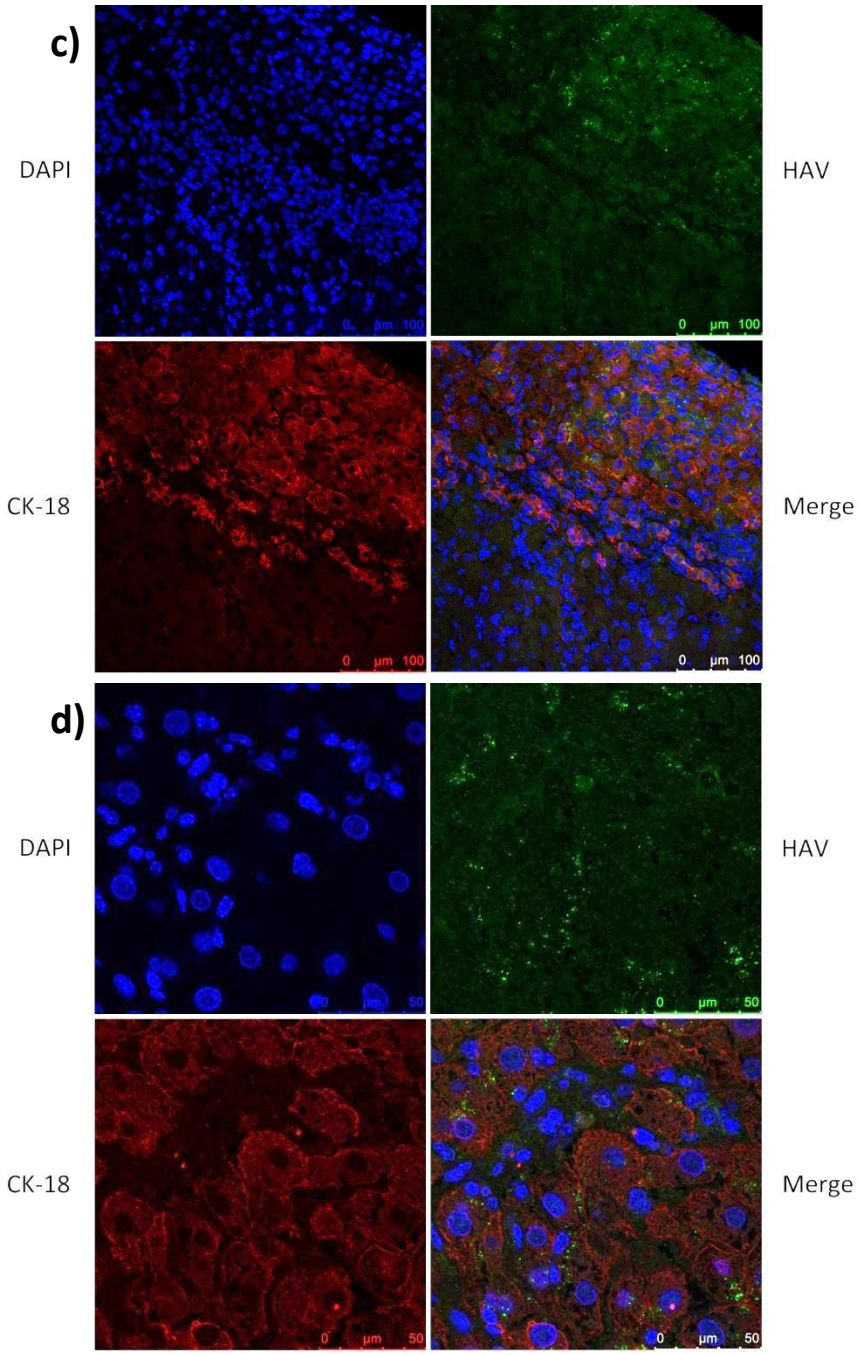


Figure 3.2. Confocal microscopy of HAV capsid in livers of HAV-infected chimeric mice.
IF staining for HAV capsid and human hepatocytes was performed on frozen liver tissue from uninfected and HAV-infected chimeric mice and analyzed by confocal microscopy. Sections were doubly stained using mouse monoclonal antibody against HAV capsid antigen and with rabbit anti-human CK-18 antibody for staining of human hepatocytes. Cell nuclei were stained using DAPI (blue). A secondary fluorescent goat anti-mouse IgG Alexa 488 (green) was used to stain capsid and donkey anti-rabbit IgG Alexa 548 (red) was used to stain CK-18. CK-18 staining was observed in the absence of capsid staining in all samples from uninfected mice **(a)**. Capsid staining was evident within the liver of all HAV-infected mice and localized in human hepatocytes observed at 40X **(c)** and 100X **(d)** objective magnification. Isotype controls **(b)** showed no staining of capsid antigen and CK-18. The images shown represent staining of liver tissue from uninfected mouse B707 **(a)** and infected mouse B1151 **(b-d)**.



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3.3 HAV-infected chimeric mice produce fully infectious virions

To determine whether a true infection was established with production of fully infectious particles within the serum, we tested the ability for HAV infection to be serially passaged in mice. Two naïve chimeric mice (B1271 and B1293) were IV inoculated ($5x10^3$GE) using sera taken from mouse B1148, which was previously infected with HAV. An additional naïve chimeric mouse (B1273) was IV inoculated ($5x10^3$GE) with sera taken from another HAV infected chimeric mouse (B1148). Sera and feces from these mice were analyzed for viral RNA. By week four, all three inoculated mice showed viral RNA in the feces (~10^7 GE/g) and sera (~10^4 GE/mL) at 10 times the amount of the initial inocula (Table 3.1). Sera taken from one of the three first-passage mice (B1293) were injected IV ($5x10^3$GE) into two additional naïve chimeric mice (B1443 & B1446). Viral RNA was detected in the sera and feces (~10^4GE/mL & 10^7GE/g, respectively) of both recipient mice at week four post-passage. The ability to passage HAV at least twice indicates the chimeric mice produce fully infectious virions.

3.4 Buoyant density analysis of HAV

It was recently shown that HAV acquires an envelope by hijacking cellular membranes and circulates in the blood of infected humans and chimpanzees in this enveloped form (eHAV).[63] In contrast, virus that has historically been isolated from feces is not enveloped (un-eHAV). The difference between the two versions can be detected by their buoyant densities: a low density population (1.06–1.10 g cm^{-3}) representing eHAV and a high density population (1.22–1.28 g

	Inoculum source ($5x10^3$GE)	Recipient Mouse	Week 4 Viral Titres	
			Sera (GE/mL)	Feces (GE/g)
First passage	B1154 sera	B1271	$2.0x10^6$	$1.8x10^9$
	B1154 sera	B1293	$2.9x10^4$	$3.7x10^7$
	B1148 sera	B1273	$9.0x10^4$	$1.9x10^7$
Second passage	B1293 sera	B1443	$6.2x10^4$	$3.6x10^7$
	B1293 sera	B1446	$2.1x10^3$	$8.1x10^6$

Table 3.1. Serial passage of sera from HAV infected chimeric mice.
Sera from a previously HAV-infected chimeric mouse (B1154) was used to IV inoculate ($5x10^3$GE) two naïve chimeric mice (B1271 and B1293). An additional naïve chimeric mouse (B1273) was inoculated with sera taken from another HAV infected chimeric mouse (B1148). Viral RNA was detected by qRT-PCR within the serum and feces of the first passage mice by four weeks post-infection at virus levels higher than the initial inoculum. A second passage was performed by transfer of sera ($5x10^3$GE) from B1293 into two naïve chimeric mice (B1443 & B1146) by IV inoculation. High HAV RNA levels were also detected in the serum and feces of these mice by four weeks post-infection.

cm^{-3}) that is expected for picornaviruses that lack an envelope membrane. We wanted to determine if these enveloped and un-enveloped forms of the virus would be detected in the chimeric mice.

Sera, feces and bile samples from two HAV-infected chimeric mice (B1153, B1167) were sent to our collaborator Dr. Stanley Lemon for buoyant density analysis on isopycnic iodixanol gradients. Two populations of virus particles were detected in both mice: a predominantly low density population (eHAV) in the serum and a high density population (un-eHAV) in the feces (Figure 3.3). Interestingly, bile fluid harvested from the gallbladder of both infected mice contained a population of high density virus particles similar to that found in feces. These results illustrate that the differences in virus populations observed in human infection in sera, feces and bile are replicated in the HAV-infected chimeric mouse model system.

3.5 Investigating oral transmission of HAV in chimeric mice

HAV is generally transmitted through the fecal-oral route in humans. We were interested to test whether the chimeric mice would be permissive to infection by this natural route. As a preliminary experiment, we co-housed a naïve chimeric mouse with three IV infected chimeric mice. The three IV infected chimeric mice showed HAV RNA within the feces by week two (Figure 3.4a). HAV RNA was also detected in the co-housed naïve chimeric mouse (B1288) by week two in the feces ($1.2x10^7$ GE/g) and by week four within the serum ($4.2x10^5$ GE/mL) (Figure

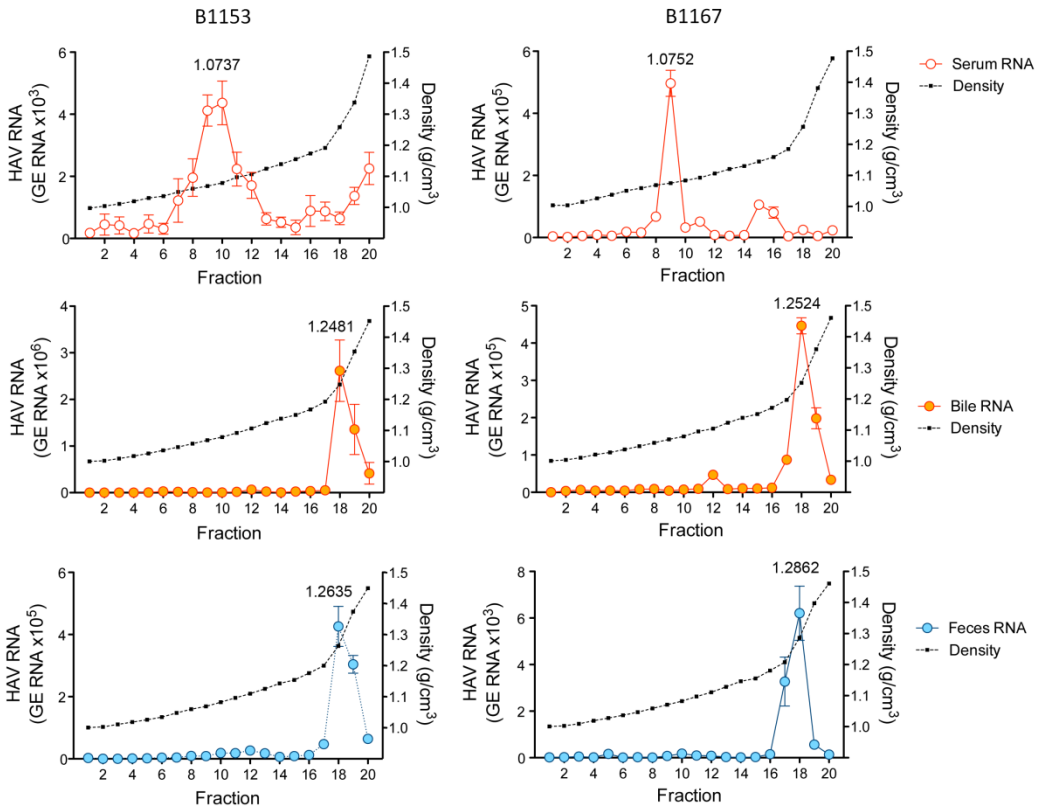


Figure 3.3. Enveloped HAV (eHAV) circulates in the blood of HAV infected chimeric mice.

Buoyant density analysis was performed on serum (red open circles), feces (blue closed circles) and bile (orange closed circles) samples from B1153 & B1167 by isopycnic gradient centrifugation. Twenty fractions were collected from the top of the gradient and density was determined using a refractometer. HAV was isolated from gradient fractions and viral RNA was measured by qRT-PCR. Low buoyant density virus (eHAV; 1.06–1.10 g cm^{-3}) was identified in the serum while high buoyant density virus (non-enveloped; 1.22–1.28 g cm^{-3}) was most prevalent within the feces and bile compartments.

3.4a). However, there was the possibility for virus transmission through blood exchange when housed with other infected mice (from biting and scratching).

To reduce the possibility of transmission by blood, we housed naïve chimeric mice in used cages containing infectious bedding and water from previous housing of IV infected chimeric mice in which peak viral titres had been reached. Animals were housed in groups (B1412 & B1417) or individually (B1449 & B1492). In all cases, viral RNA was detected in the feces ($8.0x10^5$ GE/g) by week two (Figure 3.4b). A noticeable difference is the slower viral RNA rise in titre in the sera and feces observed in chimeric mice that were housed in an infectious environment compared to mice infected IV (Figure 3.4b), which may suggest a separate transmission pathway or a lower infectious inoculum titre. The housing of an individual mouse in an infectious environment reduces the likelihood for virus transfer through the blood from biting and fighting with infected mice but it does not exclude the possibility for potential breaks in the skin from other reasons such as scratching.

To address this possibility, we directly inoculated chimeric mice via the oral route by oral gavage. Nine chimeric mice were inoculated (100µL oral gavage) using various sources of HAV. None of the mice produced detectable viral titres at four weeks post-inoculation (Table 3.2). Infection by the oral route was unsuccessful, which is in contrast to the data from the co-housing and infectious environment

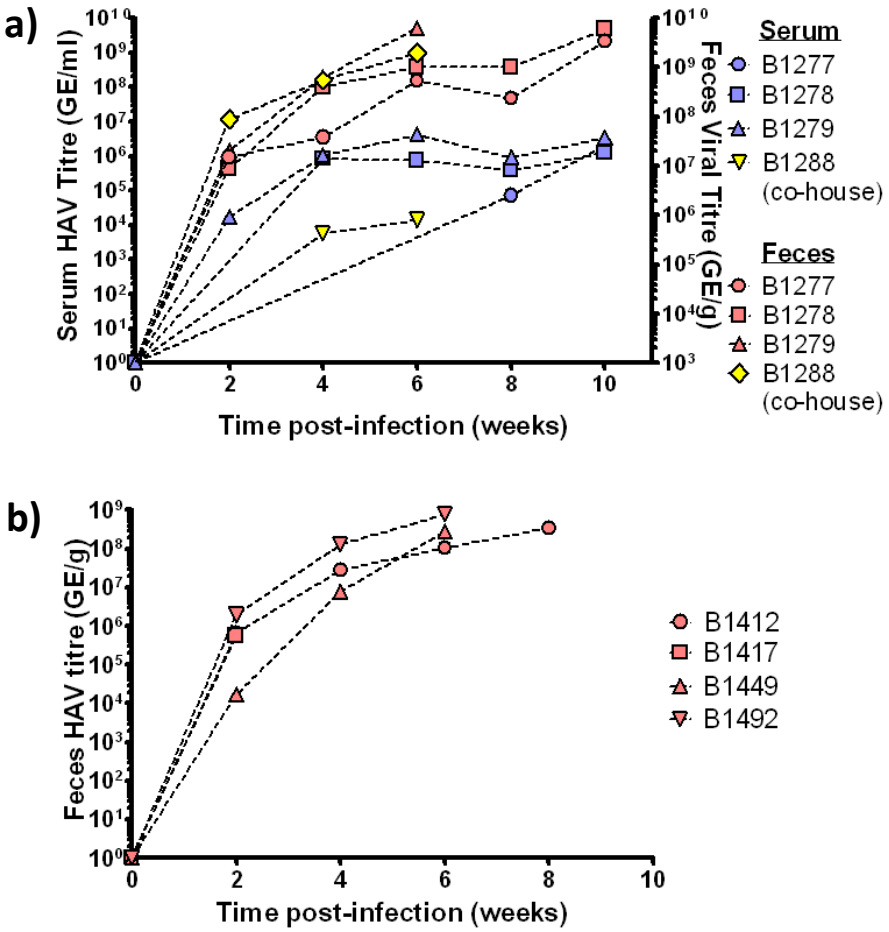


Figure 3.4. Testing oral transmission in chimeric mice.
(a) A naïve chimeric mouse (B1288) was co-housed in the same cage with three IV HAV-infected mice. Blood and feces were harvested for RNA purification for viral RNA detection by qRT-PCR. HAV RNA was detected in all IV infected mice by week two post-infection in the feces and serum. Viral RNA was also detected in the co-housed naïve chimeric mouse in the feces by week two post-infection and in the serum by week four post-inoculation. **(b)** Four naïve chimeric mouse were housed in dirty cages, which previously housed HAV-infected mice that had reached peak viral titres within the serum and feces. Animals were housed in groups (B1412 & B1417) or individually (B1449 & B1492). HAV RNA was detected in the feces by two weeks in all naïve chimeric mice housed in dirty cages containing an infectious environment.

experiments and suggests that transmission by the oral route is more complex. The ability for the chimeric mice to transmit the virus fecal-orally is still inconclusive and further tests will need to be done.

3.6 Discussion

With an infection rate of 100% via IV and IP inoculation, the chimeric mice were permissive to HAV infection with HAV RNA production in the serum, feces, liver and bile at levels. These results demonstrate that the chimeric mice represent a new small animal model for HAV infection. We hypothesized that this would be the case with the stable integration of human hepatocytes within the chimeric mouse liver, which would provide HAV with the natural host cells for replication and proliferation of the virus. The microscopy analysis on infected chimeric livers supported this assumption by showing the selective localization of virus within the human hepatocytes and not within surrounding hepatocytes of murine origin.

A unique aspect of the chimeric mouse model is the SCID/beige mutation which means they lack an adaptive immune system (no T and B cells).[102] This allows us to examine the innate and adaptive arms of the immune system and their influence on HAV infection. The chimeric mice sustained a persistent infection similar to that seen *in vitro*.[8] However, this is uncommon in humans or chimpanzees with intact immune systems[54], suggesting the importance of the

Mouse Number	Source of inoculum	Inoculum	HAV RNA in feces at four weeks post-inoculation
B1444	original stock (chimpanzee fecal suspension)	$1x10^4$ GE	undetectable
B1447			undetectable
B1450			undetectable
B1528	fecal suspension from B1278	$1x10^6$ GE	undetectable
B1530			undetectable
B1532			undetectable
B1531	sera from B1279	$1x10^5$ GE	undetectable
B1537			undetectable
B1540			undetectable

Table 3.2. Testing oral transmission of HAV by oral gavage.
Naïve chimeric mice were directly inoculated with HAV via the oral route by oral gavage (100µL). Different viral sources were used for inoculation that included virus from infected chimpanzee fecal suspension, infected mouse fecal suspension and infected mouse sera. Viral titres in the inoculum ranged from $1x10^4$ GE to $1x10^6$ GE. HAV RNA was undetectable in the feces of all mice inoculated by oral gavage measured at week four post-inoculation.

adaptive immune system in HAV control and clearance. The HAV RNA peaked during the third week post-infection and remained stable throughout the study. The level in the feces stabilized at 1000-fold higher than in the sera. HAV-infected owl monkeys[96], chimpanzees[54], and humans[8] also reached peak viral titres by three weeks after infection and produced higher viral titres in the feces compared to the sera. Viral RNA was also detected in bile fluid of HAV-infected chimeric mice at high concentrations comparable to levels found in the feces, which is consistent with the current understanding that HAV reaches the feces via transit through biliary system into the gastrointestinal tract.

We have shown that the infection can be serially passaged without loss of viral transmissibility. If by the null hypothesis it is assumed that the initial inoculum (from infected chimpanzee fecal suspension) simply persists and viral replication did not occur, each passage would have diluted the concentration 1:33 assuming the volume of mouse serum is approximately 1000µL. Instead, the concentration at four weeks post-infection in each passage was often 10 times the initial inoculum within the sera and 1000 times the initial inoculum within the feces (Table 3.1). These results demonstrate the active replication of the HAV genome in the chimeric mice rather than persistence of the initial inoculum. We have also shown the virus produced in the chimeric is fully infectious.

Consistent with results observed in humans and chimpanzees[63], we have shown that HAV-infected chimeric mice produced distinct populations of eHAV within

the serum and un-eHAV in the feces. Because most of the virus in feces originates from the liver[118], one hypothesis is that eHAV may be stripped of its envelope through passage in bile fluid and through the gastrointestinal tract and into the feces. However, experiments suspending eHAV in bile did not appear to have this effect as this treatment only minimally changed the buoyant density of eHAV particles.[63] Moreover, we have shown that virus isolated from bile fluid from HAV-infected mice consisted primarily of high density (un-eHAV) similar to the virus found in feces. These results suggest that virus may be entering bile in its un-enveloped form. One hypothesis is there may be different egress pathways for eHAV and un-eHAV from hepatocytes such that virus entering into the bile canaliculi may be released in an un-enveloped form. In support of this model, HAV infection in Huh7.5 cells (a human hepatoma cell line) released both populations of virus particles into the supernatant fluid, with eHAV representing the majority (~80%).[63] Another study *in vitro* using a clone of HepG2 (human hepatoma cell line) demonstrated that HAV was released almost exclusively through the basolateral side (facing hepatic sinusoids) in polarized hepatocytes.[53] These results may explain how the high percentage of eHAV released from hepatocytes contributed to its predominance in the blood during the significant viremia observed during acute infection. However, this conclusion does not account for the high concentration of virus found in the bile and feces, which may be explained by an alternate egress mechanism through the apical side that faces the bile canaliculi. One alternate egress pathway that has been proposed is that of the natural entry of IgA into bile by a receptor-mediated vesicular pathway with

HAV carried as an immunocomplex with anti-HAV IgA.[12,53,119] However, this pathway is unlikely for HAV since infected chimeric mice secrete HAV to high viral titres into the bile and feces despite the absence of a humoral immune system. Regardless, these results shows that distinct egress pathways may exist for virus entering the hepatic sinusoids (eHAV) and virus entering the bile canaliculi (un-eHAV) from polarized hepatocytes. Future studies will aim at testing for the presence of eHAV and un-eHAV being released from the basolateral and/or apical domains of polarized hepatocytes.

Transmission of HAV generally occurs via the fecal-oral route.[8] However, the mechanism underlying how HAV transits through the intestinal lining to reach the bloodstream is still controversial. We were curious to examine whether the chimeric mice would support infection via the fecal-oral route despite the lack of human cells within the intestinal lining. Interestingly, our results show that both co-housing infected animals with uninfected animals as well as housing uninfected mice in infectious environments was able to transmit the infection. Even mice housed individually in infectious environments, which limits the potential for blood transmission from fighting between animals, were shown to acquire the infection. The course of infection was somewhat delayed in these mice compared to mice infected by the IV route. This delay could have resulted from a lower inoculum titre or may suggest a potentially distinct route of transmission that requires transit through the gastrointestinal tract prior to infecting the liver. Similar results are observed in orally infected tamarins

(subspecies of marmoset, *Saguinus labiatus*) that show longer incubation periods.[97] However, there are also reports that IV inoculation does not decrease the incubation time for infection in tamarins and chimpanzees.[120]

We hypothesized that the confirmation of the oral transmission would be shown by transmission through direct oral administration of HAV. In contrast to the hypothesis based on our previous results, oral gavage of multiple chimeric mice did not produce infection. It may be possible that the administration mechanism via oral gavage may be bypassing an important site of replication/transmission upstream of the stomach such as the perioral or nasal cavity. However, intragastric administration of HAV via a feeding tube has been shown to produce infection in marmosets[95] and owl monkeys[96] while our studies did not show similar results. Alternatively the HAV inoculum used for oral gavage may not have been sufficient to produce infection in comparison to the constant exposure to higher titres within the feces in animals housed in used cages (infectious environment). Studies in tamarins and chimpanzees showed that approximately $10^{4.5}$ times more virus was needed to cause infection by the oral route than by the IV route. [120] Marmosets and owl monkeys that acquired the infection from intragastric administration received 1 mL of a 15-20% w/v fecal suspension[95,96], which is substantially more virus than we administered to the chimeric mice in a 100µL volume. The highest inoculum given orally to the chimeric mice, $1x10^6$ GE, was only 200-fold greater than what was given IV ($5x10^3$ GE) to produce

infection. Future studies will address these questions by administering a more concentrated inoculum in an attempt to produce infection by oral gavage.

The ability for oral transmission of HAV is not only limited to primates and has also been observed in guinea pigs, a species of rodent similar to mice.[121] Thus, the possibility for oral transmission in mice may not be completely surprising. This observation raises more questions on whether replication in intestinal cells is required for passage of the virus through the gastrointestinal epithelium for transit to the liver. Our efforts to try to visualize HAV within the gastrointestinal tract have been challenging due to high background staining and future studies will address this problem. There has been limited and inconsistent evidence of HAV replication in intestinal cells in animal models.[96,97,122,123] *In vitro* experiments using Caco-2 cells (human epithelial colorectal adenocarcinoma cells) showed that HAV exits from polarized intestinal cells almost exclusively via the apical side, back into the lumen of the gastrointestinal tract, as opposed to the basolateral side (basement membrane)[124] suggesting that replication in intestinal cells is not likely to permit spread of the virus beyond the gastrointestinal epithelium into deeper tissues required for transit to the liver. Another proposed mechanism is through translocation of HAV in association with immunoglobulin A (IgA) through the intestinal barrier, which was shown *in vitro* in polarized Madin-Darby Canine Kidney Cells (MDCK) that are not permissive for HAV infection.[125] Some studies have also suggested the transcytosis by specialized microfold (M) cells in the distal ileum, which has been shown for other picornaviruses, including

poliovirus and reovirus.[126,127] Infection of intestinal cells could, however, serve a critical role in propagating the inoculum into the intestinal lumen before the virus reaches the distal M cells.[124] Future studies should address the role of M cells in the transit of HAV from the gastrointestinal tract to the liver.

CHAPTER FOUR: RESULTS - HAV INFECTION AND THE INNATE AND ADAPTIVE IMMUNE RESPONSE

4.1 Limited ISG response in HAV infected chimeric mice

The IFN response is the first line of defense against viral infection and is capable of controlling most infections.[128] As a result of its effectiveness, some viruses have developed specific strategies to circumvent its antiviral effects. One of these viruses is HAV, which has been shown to inhibit IFN-β production in cell culture.[67,129] Interestingly, experiments in chimpanzees showed a limited ISG response, suggesting similar IFN evasion strategies *in vitro* and *in vivo*.[54] However, the limited ISG response observed *in vivo* does not appear to be sufficient to result in a persistent infection, a feature of cell culture infections and the most common result in infection with another hepatotropic virus, HCV.

We were interested to examine whether a limited ISG response would be replicated within the chimeric mouse model. To study the innate immune response to HAV infection, we performed experiments on the intrahepatic transcriptional response of genes commonly upregulated in response to HCV infection. Five mice were euthanized between four to seven weeks post-infection and liver tissue was harvested for RNA isolation and qRT-PCR analysis of IFN and ISG transcript expression. IFN genes were tested, which included type I IFNs (IFNα1 & IFNβ1), type II IFNs (IFNγ), and type III IFNs (IL28 & IL29). We also looked at 11 selected ISGs (Figure 4.1) that have been previously shown to

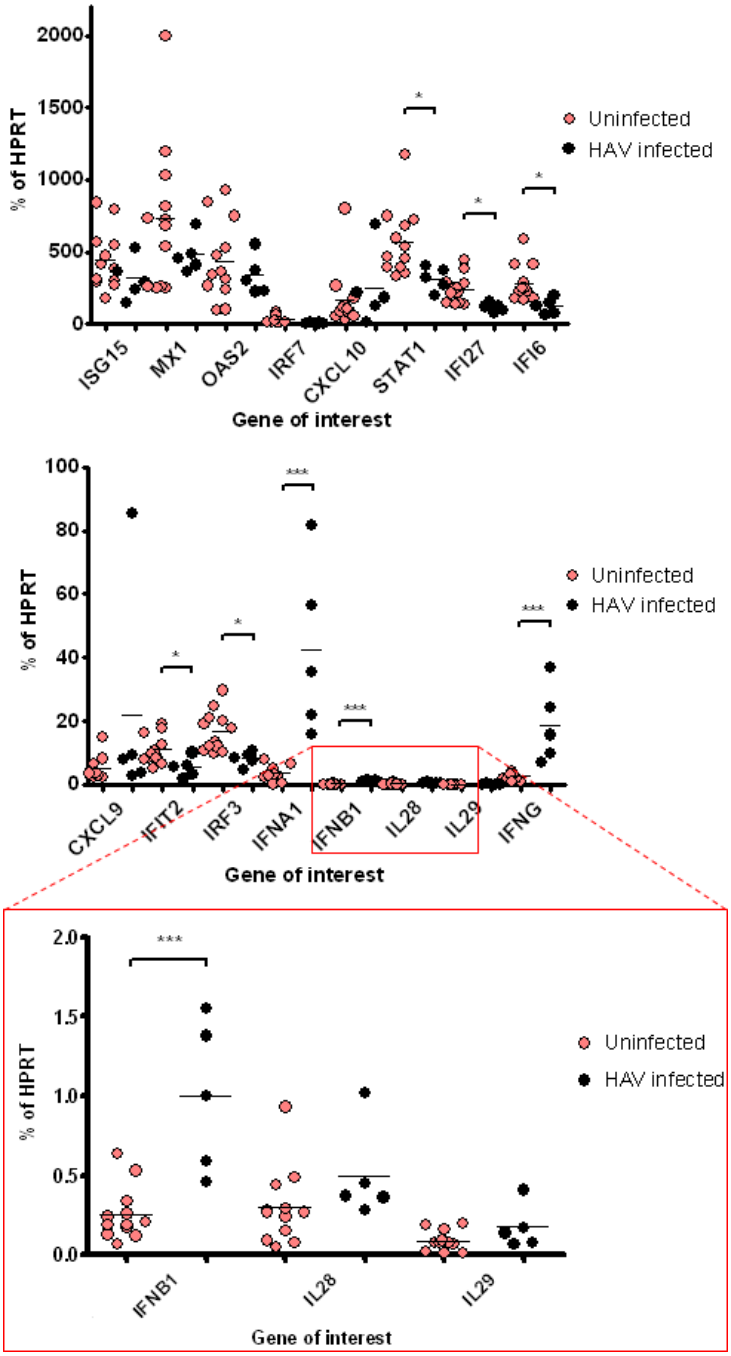


Figure 4.1. Limited ISG response to HAV infection

(a) Chimeric mice were infected with HAV and liver tissue was harvested at different lengths of infection (between four to seven weeks post-infection) for RNA purification. Intrahepatic transcript levels of five IFN genes and 11 ISGs were examined from five HAV-infected mice by qRT-PCR using human-specific primers. Both type I IFNs (IFNα1 & IFNβ1) and type II IFN (IFNγ) expression were significantly upregulated. An enlarged image of IFNβ1, IL28 & IL29 expression is shown within the red box. None of the ISGs tested were significantly upregulated over the level in uninfected chimeric mice. Five ISGs were significantly downregulated with HAV infection: IFIT2, IRF3, STAT1, IFI27, and MX1. Transcript levels are expressed as a percentage of HPRT1 (housekeeping gene) expression and statistical significance was determined by the student's T-test (* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$).

be induced by HCV infection but not by HAV infection in chimpanzees.[54] Human-specific primers for each gene of interest used in the qPCR reaction were designed by Ran Chen to exclude cross hybridization with murine homologs. Transcript levels were normalized to HPRT1 mRNA level, a housekeeping gene that is essential for maintenance of basic cellular function and thus, constitutively expressed.[130]

Both type I IFN (IFNα1 & IFNβ1) and type II IFN (IFNγ) expression was significantly upregulated by HAV compared to uninfected mice. In contrast, expression of type III IFNs (IL28 & IL29) or the ISGs tested were not significantly increased over uninfected animals. Five ISGs were actually significantly downregulated with HAV infection: IFIT2, IRF3, STAT1, IFI27, and MX1. These results suggest an overall dampening of the innate antiviral response, specifically the ISG response, which is consistent with results observed in HAV-infected chimpanzees.[54] However, we used a small sample of infected mice. Future studies should look at earlier time points in a time-course experiment to further confirm and extend these observations.

4.2 IFN-α treatment of HAV-infection in chimeric mice

It is likely that strategies by HAV to inhibit dsRNA-induced transcriptional activation of IFN are of critical importance for viral replication *in vivo* since it is sensitive to IFN-α & IFN-β *in vitro*.[131,132] In contrast to the results observed *in vitro*, we have shown that the intrahepatic transcriptional expression of type I

IFNs was significantly increased in HAV-infected chimeric mice compared to the expression in uninfected mice. However, the ISGs were not increased by HAV infection. We were interested in knowing whether HAV would be sensitive to exogenous IFN treatment similar to that seen in cultured cells. We studied whether treatment with pegylated IFN-α2a (PEGASYS), which is used clinically to treat HCV infection in humans, was able to suppress the infection in HAV-infected chimeric mice. Three chimeric mice (B1363, B1348, B1412) were infected IV with HAV and the animals were treated with pegylated IFN-α2a once peak viral titres were reached in the serum (~10^6GE/mL) and in the feces (~10^9GE/g). A dosage of 35ng/g mouse was injected subcutaneously once a week. Three additional HAV-infected chimeric mice were subcutaneously inoculated with PBS instead of pegylated IFN-α2a as a negative control. Sera were collected biweekly and feces were sampled weekly 24 hours after the pegylated IFN-α2a treatment. HAV RNA in the feces and sera remained unchanged in mice that received PBS treatment. In contrast, we observed a slow but significant reduction in viral RNA within the sera (Figure 4.2a) and feces (Figure 4.2b) of mice treated with pegylated IFN-α2a. Viral load decreased at a rate of approximately $1\log_{10}$ GE per week in the feces (Figure 4.2b). HAV RNA was undetectable in the feces after six weeks of treatment in B1412 & B1363 and after eight weeks in B1348. Viral RNA declined at a slower rate in blood and was undetectable at approximately the same time (after seven to eight weeks of

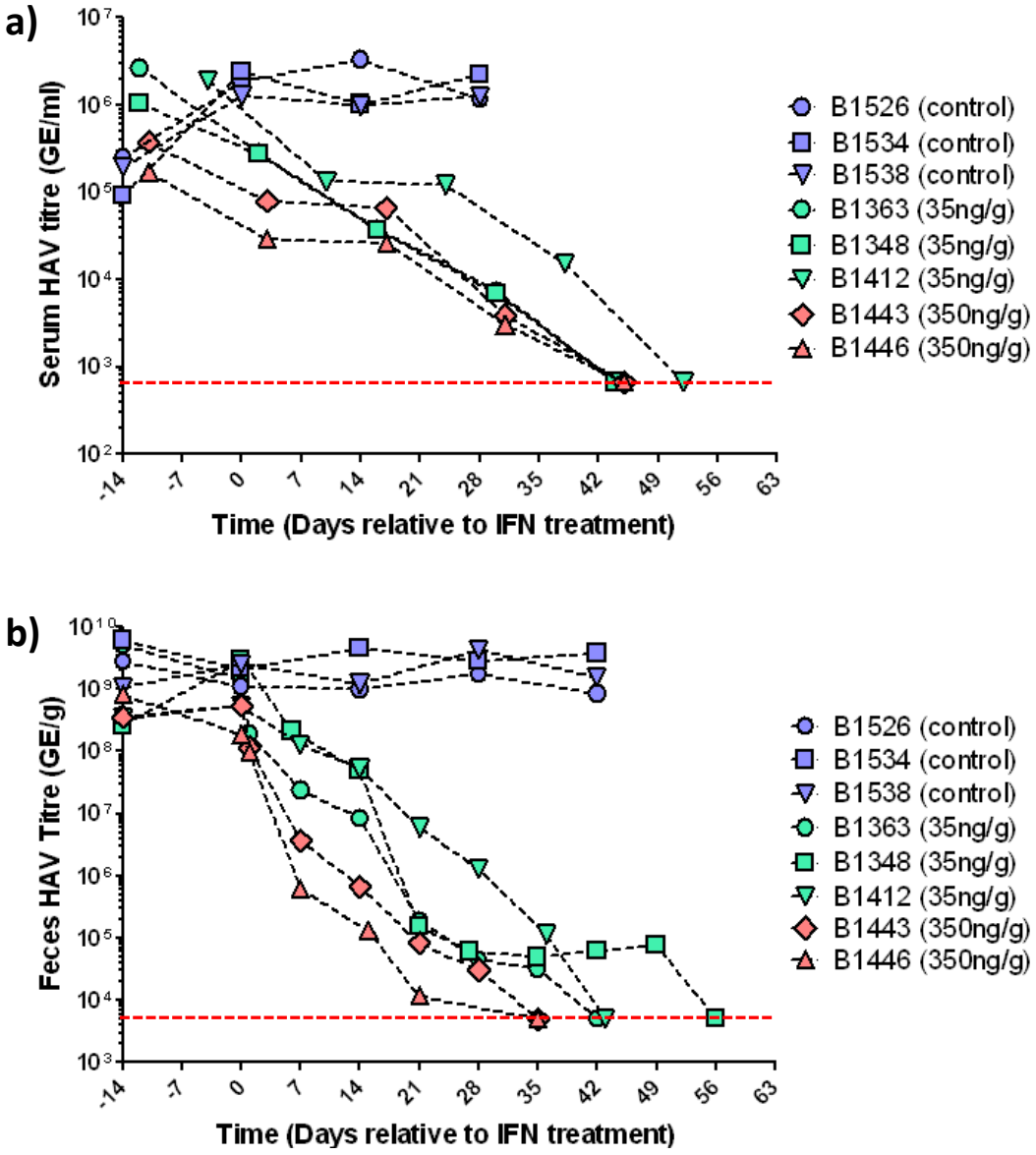


Figure 4.2. IFN treatment suppresses HAV infection in chimeric mice.
Three chimeric mice (B1363, B1348, B1412) were infected IV with HAV. Once peak viral titres were reached in the serum (~10^6 GE/ml) and in the feces (~10^9 GE/g), HAV-infected mice were treated with pegylated IFN-α2a (PEGASYS). A dosage of 35ng/g mouse was injected subcutaneously (30µL) once a week. HAV RNA displayed a slow but significant reduction in the sera **(a)** and feces **(b)** of mice treated with IFN, decreasing at a rate of approximately 1$\log_{10}$ GE per week within the feces. The HAV RNA detection limit is indicated by the dashed red line. Two mice (B1443 & B1446) were treated with 10 times the dosage (350 ng/g mouse per week) and showed a marginal increase in viral RNA suppression in the feces and sera. There was no reduction in HAV RNA in the serum and feces of HAV-infected negative control mice that received PBS (30µL) instead of IFN treatment.

treatment). Two additional mice (B1443 & B1446) were treated with a 10 times the dosage (350ng/g mouse) to test a dose-response relationship. The reduction in viral RNA was slightly faster in the feces with undetectable titres observed during the fourth (B1446) and fifth weeks (B1443) of treatment (Figure 4.2b). There was not a significant difference in the rate of HAV suppression in the sera of treated mice by the increased pegylated IFN-α2a dose. In the absence of an adaptive immune system, these results show that albeit a slow response, HAV infection is sensitive to exogenous IFN treatment *in vivo*.

4.3 Examining liver pathology in HAV-infected chimeric mice

HAV is not thought to be directly cytopathic, as indicated by its propagation and persistence in cell culture.[133] The adaptive immune response is thought be the key contributor of histopathology of the liver, which has been shown to coincide with peak $CD4^+$ T cell responses in chimpanzees[99], and peak antibody titres in tamarins[97] and in humans[134,135] usually by the fourth week of infection. We were interested to examine whether HAV-infected chimeric mice, lacking an adaptive immune system, would show histological signs of liver damage. We performed H&E staining on paraffin-embedded liver tissue sections from uninfected and HAV-infected chimeric mice. Detailed examination by a modified Hepatic Activity Index (HAI)[116] comparing 16 HAV-infected (between 10 days to 10 weeks of infection) and 15 uninfected chimeric liver samples revealed no significant difference in the degree of human hepatocyte necrosis and inflammation, which were all very mild (Figure 4.3a). The length of infection did

not appear to make a difference in the level of pathology observed in livers of HAV-infected chimeric mice. The only differences were in the degree of steatosis and human hepatocyte ballooning (not included in the HAI scoring), which were noticeably less in HAV infected samples (Figure 4.3b,c). Infected chimeric mice appear to have less ballooning and steatosis. These results contrast those from HAV infection in immunocompetent owl monkeys and chimpanzees where hepatocyte ballooning and steatosis are increased. [54,96] Overall, these results provide further support for the hypothesis that the adaptive immune response plays a major role in HAV associated liver damage.

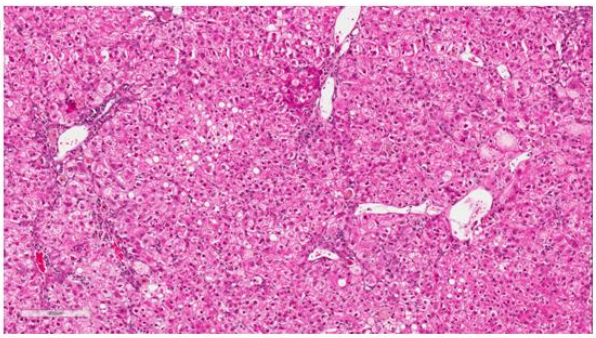
4.4 Passive immunity against HAV infection

The development of neutralizing antibodies coincides with the control of HAV infection in humans.[8] The important role for neutralizing antibodies is further supported by the effectiveness of the HAV vaccine, which is highly immunogenic and provides protection that is primarily antibody-based.[18] Moreover, immune globulin has been known for decades to protect against hepatitis A through passive transfer of antibody. Efficacy is highest when administered as a pre-exposure prophylaxis, providing protection for up to five months. Protection is still conferred even when given up to two weeks post-exposure.[33,136] We wanted to test the ability of pre-exposure treatment with neutralizing antibody to protect against HAV infection in chimeric mice.

a)

Mouse #	Length of infection	Grade					Stage
		PN	CN	SN	PI	TGS	
B707	uninfected	1	0	1	1	3	0
B733	uninfected	1	0	1	1	3	0
B658	uninfected	1	0	1	1	3	0
B721	uninfected	1	0	1	1	3	0
B666	uninfected	1	0	1	1	3	0
B669	uninfected	1	0	1	1	3	0
B739	uninfected	1	0	1	1	3	0
B738	uninfected	1	0	1	1	3	0
B952	uninfected	1	0	1	1	3	0
B863	uninfected	1	0	1	1	3	0
B860	uninfected	1	0	1	1	3	0
B861	uninfected	1	0	1	1	3	0
B862	uninfected	1	0	1	1	3	0
B950	uninfected	1	0	1	1	3	0
B983	uninfected	1	0	1	1	3	0
B1294		1	0	1	1	3	0
B1291	10 days	1	0	1	1	3	0
B1290		1	0	1	1	3	0
B1295		1	0	1	1	3	0
B1274	3 weeks	1	0	1	1	3	0
B1276		1	0	1	1	3	0
B1167	4 weeks	1	0	1	1	3	0
B1151	6 weeks	1	0	1	1	3	0
B1153	6 weeks	1	0	1	1	3	0
B1154	7 weeks	1	0	1	1	3	0
B1148	7 weeks	1	0	1	1	3	0
B1277		1	0	1	1	3	0
B1278		1	0	1	1	3	0
B1279	10 weeks	1	0	1	1	3	0
B1297		1	0	1	1	3	0
B1293		1	0	1	1	3	0

b)



c)

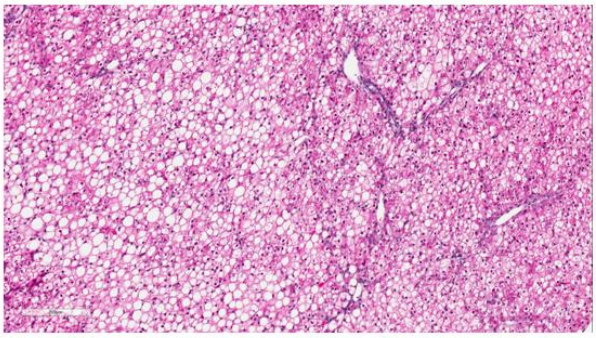


Figure 4.3. Analysis of liver pathology by Modified Hepatic Activity Index (HAI)

H&E staining was performed on paraffin-embedded liver tissue sections from 15 uninfected and 16 HAV-infected chimeric mice. Chimeric mice from both groups were transplanted using the same donor hepatocytes. Staining was analyzed according to a modified Hepatic Activity Index (HAI) which measures the level of piecemeal necrosis (PN; 0-4), confluent necrosis (CN; 0-6), focal (spotty) lytic necrosis (SN; 0-4), portal inflammation (PI; 0-4) and the total grade score (TGS). No significant difference was observed between infected and uninfected livers in the degree of human hepatocyte necrosis and inflammation, which were all very mild **(a)**. There was no apparent difference between the time of infection (between 10 days to 10 weeks of infection) and the level of pathology observed in livers of HAV-infected chimeric mice. The only observed differences were the degree of steatosis and human hepatocyte ballooning (not in the HAI scoring), which were noticeably less in HAV infected samples (**b**; B1290 shown) compared to uninfected livers (**c**; B950).

Human IgG was purified from pooled sera from individuals who had been previously vaccinated and had anti-HAV antibody, and from those who were not previously vaccinated and did not have anti-HAV antibody. Purified IgG from vaccinated individuals was given subcutaneously (50 µg/g mouse) to three naïve chimeric mice (B1521, B1523, B1527) 24 hours prior to exposure to IV HAV inoculation. A separate control group of mice (B1526, B1534, B1538) received purified IgG from non-vaccinated individuals. Infection occurred in the control group, with viral RNA being detected in the feces at similar titres to HAV infection without IgG treatment (Figure 4.4). In contrast, mice that received purified IgG from vaccinated sources showed complete protection from HAV infection with undetectable titres up to six weeks post-inoculation. These results demonstrate that neutralizing antibodies are protective against HAV infection even in the absence of an adaptive immune system.

4.5 Discussion

The lack of an adaptive immune system in the chimeric mouse model permitted us to study the specific responses of the innate immune system to HAV infection *in vivo*. Our results showed a lack of intrahepatic type I (IFN-α/β) ISG induction in HAV-infected chimeric mice. These results are consistent with results obtained during acute HAV infection in chimpanzees.[54] The same ISGs, including ISG15, MX1, IRF7 & OAS2 that showed no response to HAV infection in our study are significantly upregulated in acute resolving HCV infection in chimpanzees.[54,137,138] The intrahepatic expressions of five of the ISGs tested

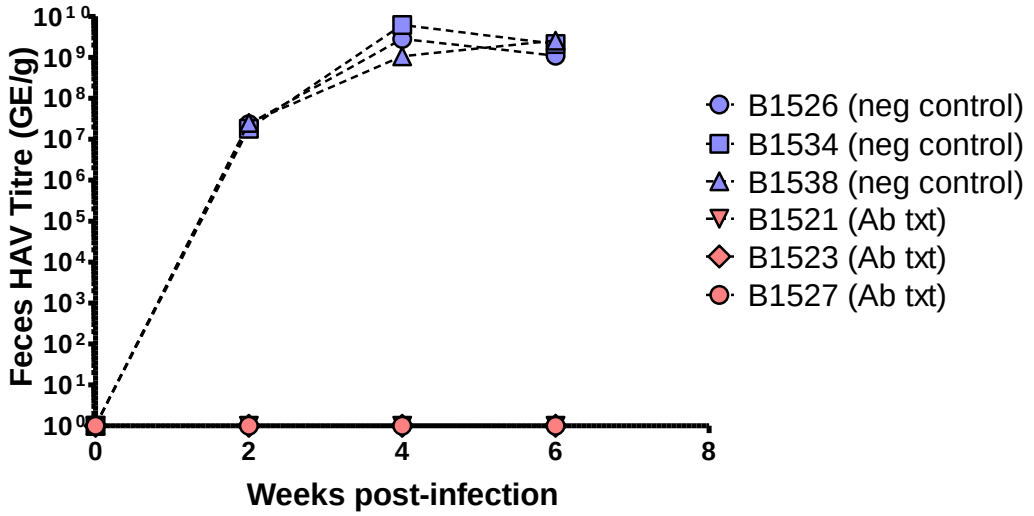


Figure 4.4. Neutralizing antibody treatment protects against infection
Human IgG was purified from pooled sera from immune individuals (vaccinated) who have anti-HAV antibody, and from non-immune volunteers without anti-HAV antibody (negative control). Purified IgG from vaccinated individuals was administered subcutaneously (50 µg/g mouse) to three naïve chimeric mice (B1521, B1523, B1527) 24 hours prior to exposure to IV HAV inoculation ($5x10^3$GE). Antibody treatment derived from immune individuals was fully protective against HAV infection in chimeric mice with undetectable titres up to six weeks post-inoculation with HAV. Negative control mice that received purified IgG from non-vaccinated sources were not protected from infection. HAV RNA was detected in the feces of these mice by week two post-infection at similar titres to HAV infection without IgG. Ab txt, antibody treatment; neg control, negative control.

(IFIT2, IRF3, STAT1, IFI27, and MX1) were significantly downregulated in HAV infected mice. In contrast, these five ISGs were shown to be significantly upregulated in acute resolving HCV infection and remained elevated for 10 weeks post-infection.[54,138] The robust ISG response during HCV infection in chimpanzees occurs despite HCV RNA levels within the liver being 100-fold lower compared to HAV-infection.[54] Moreover, the strong ISG response is insufficient to control HCV infection in most cases while HAV infection is typically cleared by the immune system. This paradox is still not fully understood. A strong ISG response may not be directly correlated to an adaptive immune response that is effective in viral clearance as evident by the difference in clinical outcomes between HAV (acute only) and HCV (often persistent) infections.[99] Future studies should measure the number of infected hepatocytes during HAV and HCV infection of chimeric mice and address whether this plays a role in the ISG response.

Expression of type I IFN (IFN-α and IFN-β) transcripts was upregulated in HAV-infected chimeric mice (at four to seven weeks post-infection) despite the weak ISG response. These results differ from *in vitro* experiments that have shown no production of IFN-β during HAV infection.[67,129] Various studies have described a similar mechanism of action, whereby the HAV protein 3ABC (a stable polyprotein intermediate) cleaves MAVS and inhibits its downstream signaling and activation of IRF-3, which ultimately blocks IFN-β production (Figure 1.6).[13,131,139] In addition, another HAV polyprotein intermediate, 3CD, has been

shown to cleave the adaptor protein TRIF that is critical for TLR3 signaling and IFN-β expression. HCV has also been shown to disrupt IFN-β production by cleavage of MAVS and TRIF by the viral serine protease NS3/4A.[13,80] It is possible that the increase in type I IFNs we observed were not sufficient to limit infection because we have shown that exogenous IFN-α2a treatment at high doses was able to suppress HAV infection in chimeric mice. The decline in HAV RNA in HAV-infected chimeric mice was slow but significant, with titres being undetectable after approximately six weeks of treatment. The increased dosage of pegylated IFN-α2a used did not significantly change the rate of viral decline detected in the feces and sera. Studies *in vitro* have shown that HAV is undetectable after four weeks of treatment with exogenous IFN-β, which is slightly faster than what we saw in HAV-infected chimeric mice treated with pegylated IFN-α2a.[132] The immune evasion mechanisms mentioned above likely contribute to the clinically silent phase during the first three to five weeks of infection before symptoms begin to appear. Due to HAV sensitivity to type I IFNs *in vitro*[132,140] and *in vivo* in the chimeric mice, strategies to inhibit dsRNA-induced transcriptional activation of IFN are likely to be of critical importance for the modulation of the host cell's antiviral response.[129] This ability would prevent the antiviral effects of ISGs and allow slowly replicating HAV to produce sufficient progeny to establish new rounds of infection in neighboring host cells. This strategy may also facilitate the persistent nature of HAV infection observed *in vitro* and in the chimeric mice. Future studies should examine whether the intrahepatic IFN and ISG levels are low early in infection and whether they are

increased following exogenous IFN treatment. If the observed upregulation in type I IFNs is biologically relevant, there may be addition mechanisms by which HAV restricts ISG expression downstream of type I IFN production.

There was no evidence of liver damage or pathology in the HAV-infected chimeric mice when compared to uninfected mice. We suspected these results were due to the lack of an adaptive immune response, which is thought to be the primary cause of HAV-induced liver injury.[97,141] Our results provide further support to the models proposing that acute hepatitis caused by HAV is unlikely due to direct cytotoxicity, but associated with the adaptive immune response in immune competent animals. This is seen in immunocompetent owl monkeys, tamarins and chimpanzees infected with HAV, all of which show substantial inflammation, with severe hepatocyte swelling, necrosis and steatosis.[54,96,97] These changes also coincide with peak $CD4^+$ T cell and peak HAV specific antibody levels around four weeks post-infection.[99,134] Interestingly, there appeared to be a reduction of hepatocyte ballooning and steatosis in HAV-infected chimeric mice compared to uninfected chimeric mice. HAV appears to reduce liver disease in the immunocompromised chimeric mice, opposite to the observations made in immunocompetent animal models. Clearly, the adaptive immune response plays a significant role in liver pathology and in the clearance of HAV infection.

We have shown that the passive transfer of HAV antibody was effective in protecting against HAV infection in the chimeric mice, which is consistent with the protective effect of HAV specific antibodies in humans.[33] The HAV capsid is very immunogenic[18] and thus, the recent discovery of eHAV within the circulating blood[63] may mask HAV epitopes from B cell recognition and antibody neutralization[54]. The hijacking of cellular membranes by eHAV has been shown to protect virus against direct antibody detection by enzyme-linked immunosorbent assay (ELISA) targeting capsid antigen.[63] These results raises some questions regarding the mechanism underlying the effectiveness of neutralizing antibody treatment given post-exposure to HAV.[32] The mechanism of action may not be as simple as the direct neutralization of virus in the blood, which has been shown to be predominantly in the enveloped form. eHAV has been shown to be effectively neutralized with exposure to antibody either before or up to six hours after HAV-infection of cultured cells.[63] Although the capsid is an effective target for neutralizing antibodies, these results suggest that the envelope membrane needs to be bypassed before neutralization can occur. Thus, the neutralization of eHAV shown *in vitro* by antibody treatment up to six hours post-infection may occur after shedding of the membrane following internalization of virions into hepatocytes by endocytosis. Conversely, the authors showed that antibody treatment only protected against un-eHAV infection when given prior to infection and not post-infection.[63] These results suggest a potentially alternate entry pathway for un-eHAV from eHAV that may not facilitate the entry of neutralizing antibody within infected cells. Future studies

should look to answer whether virus specific antibody is able to neutralize eHAV following entry into infected cells. Using the chimeric mouse model, future studies will also look at whether antibody treatment is protective when given post-exposure to HAV.

CHAPTER FIVE: CONCLUSIONS & FUTURE DIRECTIONS

Non-human primates have been the primary animal models used to study HAV infection *in vivo*.[54,96,97] Although they have provided a wealth of knowledge, they are expensive and becoming harder to access and maintain as animal models.[100,101] We have developed a more accessible small animal model for *de novo* HAV infection in the SCID-beige/Alb-uPA mice with chimeric human/mouse livers.

We have shown that mice with chimeric human/mouse livers are permissive to HAV infection, producing infectious viral particles detectable within the serum, feces, liver and bile. HAV capsid antigen can be detected by IF staining and confocal microscopy within human hepatocytes of HAV-infected chimeric livers. Moreover, HAV-infected chimeric mice produce eHAV that circulates in the blood and un-eHAV in the bile and feces, consistent with recent findings that distinguish two distinct populations of HAV during infection in chimpanzees and humans.[63]

Because the chimeric mice lack an adaptive immune system, a persistent infection is observed similar to the persistent infection in cultured cells.[8] This feature also provides a unique perspective to study isolated aspects of the innate and adaptive immune response. Without the adaptive immune response, there was an absence of disease and histopathological changes in the livers of HAV-infected chimeric

mice. In addition, infected chimeric mice displayed a weak type I ISG response consistent with previous observations in infected chimpanzees.[54] The HAV infection in the chimeric mice was slowly responsive to exogenous IFN treatment consistent with the sensitivity of HAV observed *in vitro*.[131,132,140] Pre-exposure to neutralizing antibody was also fully protective against HAV infection, consistent with the ability of HAV-specific antibodies to prevent infection in humans.[33] However, the establishment of a persistent infection in these animals emphasizes the importance of the adaptive immune response for HAV clearance.

One unanswered question from these studies is whether the chimeric mice are susceptible to oral transmission of HAV. Future studies will determine whether a higher viral dose is needed to transmit the virus by oral gavage or not. Alternatively, virus could be added to the food or water sources of chimeric mice to test for oral transmission. The chimeric mice could also be used as an effective tool to study the many unanswered questions regarding the trafficking of HAV throughout the gastrointestinal tract and possibly test egress pathways from hepatocytes into blood (eHAV) and bile (un-eHAV). HAV transit from the gastrointestinal tract to the liver is still unclear. We were unsuccessful in IF staining of virus from intestinal tissue isolated from HAV-infected chimeric mice. Future studies examining the role of the gastrointestinal tract during HAV infection will also be important to understand the mechanisms required for oral transmission of the virus.

The chimeric mice have been previously shown to be permissive to persistent HCV infection.[102,110,111] Thus, the chimeric mouse model represents a valuable tool for *in vivo* comparisons between HAV and HCV, two hepatotropic infections that produce distinct clinical outcomes despite their similarities in tropism, genome structure, replication and immune evasion mechanisms.[13] One mechanism that has been attributed to the failure of the adaptive immune response against HCV infection is the virus ability to evade neutralizing antibodies.[142,143] In one study, persistent HCV infection in patients was associated with a slower development of neutralizing antibodies whereas they were induced much more quickly in acute-resolving HCV infection, which suggests the importance of neutralizing antibodies in HCV clearance.[144] The majority of HCV-specific antibodies produced during infection have no antiviral activity and only a small subset are neutralizing antibodies that can prevent virus binding or inhibit other steps of the viral lifecycle.[143,145] The likely reason for these observations is the high replication and mutation rates of the HCV genome during HCV infection.[146] The HCV RdRP, NS5B, lacks a proof-reading mechanism and is error-prone similar to other RNA viruses, which contributes to the generation of diverse variants of the virus that are genetically related by mutation (quasispecies).[147,148] It is thought that this high mutation rate allows the virus to quickly evade the robust selective pressure of the host immune response and neutralizing antibodies through mutations within targeted epitopes.[149,150] The reported *in vivo* mutation rate of HCV is approximately $1.2\pm0.3x10^{-4}$ substitutions/site/year which is similar to other RNA viruses.[151] The adaptive immune response to HCV infection

is slow with seroconversion occurring six weeks after the onset of viremia in infected patients.[152] The delayed appearance of neutralizing antibodies, if present at all, occurs six to eight months following seroconversion.[152]

In contrast, neutralizing antibodies during HAV infection are detected quickly during the third to fourth week post-infection in parallel with the earliest signs of hepatocellular injury and serum aminotransferase elevation.[8,54,99] HAV is also a positive sense RNA virus with an error-prone RdRP to replicate its genome.[16] The estimated substitution rate of the human HAV genome is approximately 1.61-1.99x10^{-4} substitutions/site/year[153], which is significantly lower than other members of the family *Picornaviridae* (Poliovirus type 1: 3.96x10^{-2} substitutions/site/year[154]; Enterovirus 70: 2.2x10^{-2} substitutions/site/ year[155]), although it is not significantly different from the reported substitution rate for HCV. Two differences between HAV and HCV may explain the more robust neutralizing antibody response and viral clearance in HAV infection. Despite the high mutation rate and the presence of several viral genotypes in HAV, there is low antigenic variability and only one serotype has been identified, allowing for easy antigenic detection of the HAV capsid and virus neutralization. A proposed reason for the low variability of the HAV capsid is attributed to the slow translation rate resulting from the abundance of rare codons clusters in the P1 coding region (encoding the immunodominant site of capsid proteins).[156] These rare codons induce transient pauses of the translational complex, which may slow translation but qualitatively benefits proper protein synthesis and folding.[156] In

addition, HAV is an unenveloped picornavirus whereas HCV encodes envelope glycoproteins that may protect it from neutralizing antibody. Recent evidence has shown that HAV can also hijack cellular membranes to obtain envelop membranes that protect against antibody neutralization.[63] However, a significant amount of virus is also found in the unenveloped form in the feces of HAV-infected chimpanzees and humans and in the bile in HAV-infected chimeric mice. Moreover, despite the presence of an envelope membrane resistant to antibody binding, the authors have shown that HAV-specific antibody added prior to or up to six hours post HAV-infection was able to neutralize the virus in vitro.[63] Without the presence of a humoral and cellular immune response, HAV-infected chimeric mice were not able to clear the infection. The presence of a robust and effective humoral immune response with HAV-specific antibodies capable of effectively neutralizing virus during infection may explain why HAV is normally cleared during infection in humans while HCV typically causes a persistent infection. The ability to establish persistent HAV infection in the chimeric mouse model provides a unique opportunity for future comparison studies with HCV. In addition, future studies will examine the ability of antibody and cellular T cell responses ($CD4^+$ and/or $CD8^+$) to clear the persistent HAV infection in our mouse model. I believe the development of this new model to support HAV infection and replication will be very useful in future studies.

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