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REVIEW

Engineered Livers for Infectious Diseases

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SUMMARY

This article discusses existing 2-dimensional and 3-dimensional liver models and their applications toward studying hepatotropic pathogens. The importance of selecting the most appropriate models and readout modalities to answer specific scientific questions is emphasized.

Engineered liver systems come in a variety of platform models, from 2-dimensional cocultures of primary human hepatocytes and stem cell–derived progeny, to 3-dimensional organoids and humanized mice. Because of the species-specificity of many human hepatropic pathogens, these engineered systems have been essential tools for biologic discovery and therapeutic agent development in the context of liver-dependent infectious diseases. Although improvement of existing models is always beneficial, and the addition of a robust immune component is a particular need, at present, considerable progress has been made using this combination of research platforms. We highlight advances in the study of hepatitis B and C viruses and malaria-causing *Plasmodium falciparum* and *Plasmodium vivax* parasites, and underscore the importance of pairing the most appropriate model system and readout modality with the particular experimental question at hand, without always requiring a platform that recapitulates human physiology in its entirety. (*Cell Mol Gastroenterol Hepatol 2018;5:131–144; https://doi.org/10.1016/j.jcmgh.2017.11.005*)

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The liver is the largest internal organ in the body, and performs vital and diverse functions in metabolism of carbohydrates, proteins, and lipids; bioproduct synthesis; immunologic processes; and detoxification. Although many viruses, parasites, and bacteria specifically target the cells of the liver, the liver is also exposed to blood-borne pathogens that circulate systemically, or that are derived from the gut,[1] because of its location at the convergence of the hepatic artery and portal vein.

Most liver pathogens specifically target the most abundant cell type in the liver, the hepatocyte, for completion of their life cycles or developmental stages. These include hepatitis viruses and *Plasmodium* protozoan parasites (Figure 1), which together account for an enormous burden on human health. Hepatitis B virus (HBV) and hepatitis C virus (HCV) infect the livers of more than 350 million people worldwide, and are the main causes for chronic liver diseases, such as liver cirrhosis and hepatocellular carcinoma.[2] *Plasmodium* parasites, which cause malaria, result in more than 200 million infections annually[3] and require asymptomatic development in the liver before initiating fevers associated with blood stage infection. Other hepatropic pathogens, including several viruses and bacteria that cause systemic infection, can also target the liver and cause severe liver damage (Table 1).

In addition to hepatocytes, the liver is also populated by other cell types, such as Kupffer cells, liver sinusoidal endothelial cells, cholangiocytes, and stellate cells, some of which can be targeted by pathogens. For example, human cytomegalovirus can infect bile duct epithelia cells and stromal cells,[4] whereas dengue virus can replicate in Kupffer cells[5] and hepatocytes.[6] Aside from being a site for massive pathogen amplification, the liver also hosts such pathogens as *Entamoeba hystolytica*, a protozoan parasite that travels from the gut via portal vein, invades the liver parenchyma, and remains extracellularly, forming amoebic liver abscesses.

To study the mechanisms of pathogen-host cell interactions and to develop novel therapeutics against liver pathogens, robust model systems that can faithfully replicate human hepatotropic infections are needed. Human hepatoma-derived cell lines have been widely used to study the biology of hepatotropic pathogens and to test candidate drugs and vaccines. However, because of their uncontrolled proliferation, abnormal liver-specific functions,[7,8] or the stringent host dependence of some human

Abbreviations used in this paper: 2D, 2-dimensional; 3D, 3-dimensional; EBOV, Ebola virus; HBV, hepatitis B virus; HBC, hepatitis C virus; HLC, hepatocyte-like cells; iHLC, induced pluripotent stem cell–derived hepatocyte-like cells; LASV, Lassa virus; MPCC, micropatterned coculture system; PCR, polymerase chain reaction; SACC, self-assembling coculture.

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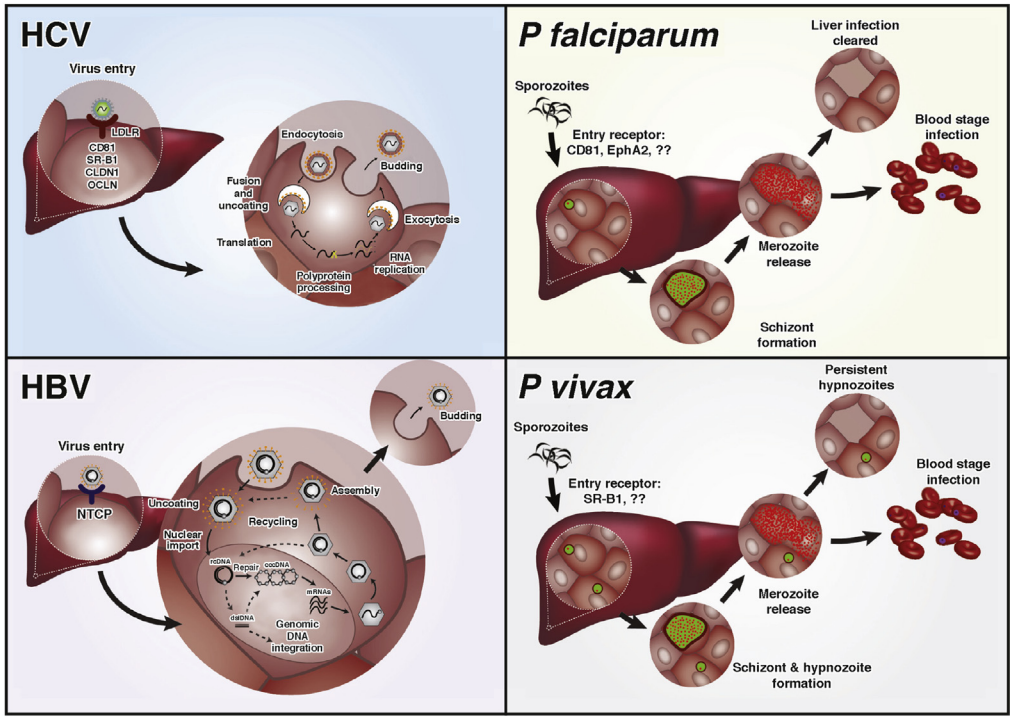

Figure 1. Life cycles of 4 major human hepatotropic pathogens. HCV is a single-stranded, positive-sense RNA virus that belongs to the Flaviviridae family. Initial viral attachment to the hepatocyte membrane is mediated through glycosaminoglycans and the LDL receptor. Interactions with other host factors CD81, scavenger receptor class B member 1 (SRB1), claudin 1 (CLDN1), occludin (OCLDN), and possibly other molecules, such as CLDN9, CLDN6, EphA2, and epidermal growth factor receptor, are required for cell entry. Clathrin-mediated endocytosis of the virus is followed by fusion of the viral and endosomal membranes, resulting in the release of nucleocapsid into the cytoplasm. Positive-strand genomic RNA is released into the cytosol on uncoating of the viral nucleocapsid, which initiates synthesis of the HCV polyprotein. Host cell lipid synthesis pathways are tightly linked to the later stages of assembly and virus release. HBV is a DNA virus that belongs to the family Hepadnaviridae. HBV enters the hepatocyte via the sodium/bile acid cotransporter NTCP.[23] After uncoating, the partially relaxed double-stranded circular viral DNA (rcDNA) is directed to the nucleus where viral DNA lesions are repaired by the host machinery, converting into covalently closed circular DNA (cccDNA), which serves as a template for viral RNA production. Five transcripts are made that encode envelope, core and X antigens, viral polymerase, and pregenomic RNA (pgRNA). pgRNA can be reverse transcribed into rcDNA, which is assembled with the viral capsids and released from the host cell. During reverse transcription of pgRNA double-stranded linear (dsl) DNA can be formed and are capable of integration into human chromosomes. *Plasmodium falciparum* and *Plasmodium vivax* are apicomplexan parasites. *Plasmodium* sporozoites are deposited into the human skin via bite of an infected Anopheles mosquito and travel to the liver where they invade hepatocytes. CD81[38,148] and EphA2[106] for *P falciparum*, and more recently SR-B1[134] for *P vivax*, have been implicated as required entry factors. On invasion of hepatocytes, parasites differentiate and divide by schizogony to form thousands of progeny, merozoites, which are released into the bloodstream where they can cyclically invade red blood cells, initiating the blood stage of the disease. *P vivax* has an additional, unique aspect of its liver development where a subset of the parasites, called hypnozoites, remain dormant and can reactivate weeks to years after the initial infection to reinitiate disease.

hepatotropic pathogens, immortalized cell lines do not always fully recapitulate the entire pathogen life cycle. Animal models are also not alternatives because many human-tropic species can only infect hepatocytes of human origin. Human hepatocytes are thus considered the gold standard cell type to study the biology of human hepatotropic pathogens and pathogen-host interactions, yet conservation of the polarized morphology and functions of hepatocytes *ex vivo* is challenging.

To develop systems that closely recapitulate human liver biology and support hepatotropic infections, tissue engineering tools have been applied to create 2-dimensional (2D), 3-dimensional (3D), and humanized mouse systems by using a combination of cell lines, primary human hepatocytes, or stem cell–derived cells with various extracellular matrix manipulations (Table 1). The available systems are capable of modeling some, but not all, aspects of the shared pathogen-host interaction, thus researchers should carefully select a model that is best suited to the specific question being investigated. In this review, we summarize key aspects of each platform, their advantages and disadvantages, and discuss biologic insights gained using models of liver infections, with a focus on HBV and HCV viruses and the major species of human malaria parasites, *Plasmodium falciparum* and *Plasmodium vivax* (Figure 1). For more technical details on the assembly of various engineered liver model systems we recommend a collection of recent review articles.[9–12]

Table 1. Summary of Liver Models Applied to Human Hepatotropic Pathogens

		Plasmodium falciparum	*Plasmodium vivax*	HBV	HCV	Other hepatotropic pathogens
2D cultures	Cancer cell lines	HepG2-A16[104,105] HC04[106]	HepG2-A16[20,21] HC04[107]	Rat Q7[108] HepaRG[109] Hep3B[110] HuS-E/2[111] HLCZ01[112] HepG2-NTCP[22,23] Huh7-NTCP[22]	HLCZ01[112] Huh7/Huh7.5 [113–115] Huh6[116] Hep3B[117] JHH-4[118] HepG2+IMY-N9[119] LH86[120]	Brucella: HepG2[121] Listeria: HepG2[122,123] HEV: PLC/PRF/5, A549[124–126] HEV: HepaRG, PICM-19[127] DenV: Huh7,[31] HepG1,[128] HepG2[129] YF: HepG2,[130] PH5CH8[131] EBOV: Huh7,[27] HepG2[28] LASV: Huh7[29] HCMV: HepG2[30]
	Primary cells	Adult PHH[132–134]	Adult PHH[134]	Adult PHH[135] Fetal PHH[136] HepCHLine-7[137]	Adult PHH[138]	HCMV: adult PHH[4] DenV: adult PHH[128]
	iPS cells	iHLC[50]	iHLC[50]	iHLC[39] iPS-HPCs[139] iPS-Heps[139]	iPS-iHLC[41,47–49]	
	Co-cultures	MPCC[38]	MPCC	Adult PHH + mouse fibroblasts[39] Fetal PHH + nonparenchymal cells[35]	Adult PHH + mouse fibroblasts[40]	
3D cultures	Cocultures			Huh7.5-NTCP + LSEC[62] PHH + BAECs[66]	Rotating wall vessel[53] 3D radial flow bioreactor[51] Hollow fiber system[52] Polyethylene glycol hydrogel[54] Gelatin polymer[55] Alginate beads[58] Matrigel[60] Huh7 spheroids[57] spheroids[59]	Entamoeba: Huh7 + LSEC[62,63] HEV: Rotating wall vessel[140]
Animal models	Rodent models	Alb-UPA/SCID[70,71] FRG[74] TK-NOG[76] DRAG[91] Ectopic artificial livers[81]	FRG[75]	NOD/SCID[141] Trimera[142] uPA/RAG2[143]	Alb-uPA/SCID[77,144] FRG[72] TK-NOG[73] AFC8-Hu HSC/Hep[92,145] Genetically humanized mice[80]	HDV: NOD/SCID[141] HCMV: HuNSG[146] HCMV: Alb-uPA/SCID[147]

2D, 2-dimensional; 3D, 3-dimensional; BAEC, bovine aortic endothelial cells; DenV, dengue virus; HBV, hepatitis B virus; HCMV, human cytomegalovirus; HCV, hepatitis C virus; HDV, hepatitis D virus; HLC, hepatocyte-like cells; iHLC, induced pluripotent stem cells–derived hepatocyte-like cells; HPC, hepatic progenitor like cells; iPS, induced pluripotent stem; LASV, Lassa virus; LSEC, liver sinusoidal endothelial cells; MPCC, micropatterned coculture system; NTCP, liver bile acid transporter; PHH, primary human hepatocyte.

2-Dimensional Monolayer Cell Culture Systems

Despite their regenerative potential in the human body, isolated human primary hepatocytes are difficult to maintain *in vitro*, requiring adherence to extracellular matrices to survive for more than a few hours.[13] Even in adherent formats, fully confluent monolayers of primary hepatocytes are only appropriate for short-term studies because of a rapid decline in hepatic functions after 48 hours.[14,15] Hepatocytes cultured between collagen layers[16] retain metabolic stability over longer periods of time; however, the batch-to-batch variation in collagen, combined with limitations in drug access caused by collagen's barrier effect, limit their use. Ongoing work is focused on immortalizing mature hepatocytes to address cell source limitations.[17,18] In one example, transduction of viral genes E6 and E7 led to expansion of primary hepatocytes.[19] In these cells, infectivity of cell culture–derived HCV was similar to that observed in Huh7 hepatoma cells 9 days postinfection. However, it should be noted that although such approaches provide the ability to capture patient-to-patient variability in response to pathogen infection, these cells have not yet been evaluated for long-term culture and hold the risk of oncogenic transformation.

For infections lasting a few days, monolayers of human hepatoma-derived cell lines represent a renewable alternative to primary hepatocytes that is relatively simple to maintain and amplify, and amenable to scale up for drug screening. Moreover, stable transfected lines can be produced because these are dividing cells. Historically, hepatoma cells have been invaluable tools for expanding knowledge of hepatic-amplified pathogens and understanding of host-pathogen interactions. For example, *in vitro* infection of CD81-deficient HepG2-A16 cells with different human *Plasmodium* parasites (*P vivax* and *P falciparum*)[20,21] provided insight into species-specific host entry factors required to establish malaria infection. Likewise, comparison of differentiated and naive HepaRG cells confirmed the liver bile acid transporter (NTCP) as the entry receptor used by HBV and hepatitis D virus to infect human hepatocytes.[22,23] Notably, stable transfected hepatoma cell lines remain the primary source for generation of infectious virion stocks of HCV[24] and HBV,[25,26] among others. Human hepatoma cell lines have also proven to be useful for studying other hepatotropic viruses and bacteria that infect the human body systemically, namely Ebola (EBOV),[27,28] Lassa (LASV),[29] human cytomegalovirus,[30] and dengue[31] viruses (Table 1).

2-Dimensional Coculture Systems

Not all aspects of host-pathogen interactions can be recapitulated in immortalized cell lines. In one example, certain clinical isolates of HCV cannot robustly infect hepatoma cell lines and require primary human hepatocytes for propagation.[32] Furthermore, for liver pathogens, such as *P vivax*, where dormant liver forms can remain in culture for weeks, long-term culture is essential but cannot be maintained in cell lines because the infected cells continue to proliferate and might detach from culture. HCV, HBV, and human *Plasmodium* species have been studied in primary human hepatocyte monocultures (Table 1); however, the short lifetime of the cells in culture presents a hurdle for any scientific inquiries that require longer-term analyses. To overcome these problems, tissue-engineering tools have been applied to drive immortalized cell lines toward more polarized or differentiated states, and to maintain primary human hepatocyte functions, such as biosynthesis (often tracked by measuring albumin production) and metabolism (cytochrome P-450 [CYP] enzyme activities) for a longer period. Cell-cell interactions are pivotal to the function of many organ systems, including the liver. Cocultivation of hepatocytes with nonparenchymal cells has been shown to preserve hepatocyte phenotype because of heterotypic interactions between cell types, and via homotypic interactions between parenchymal cells.[33,34] In one example, Zhou et al[35] created a 2D coculture system where irregular patches of human fetal hepatocytes were surrounded by nonparenchymal cells. In this format, hepatocyte hallmarks were maintained for 72 days, and the authors observed successful HBV infection, albeit requiring high viral titers.[35] More recently, a random, self-assembling coculture (SACC) of primary human hepatocytes and mouse fibroblasts was shown to be susceptible to very high HBV genome copies derived from cell culture or purified from patient plasma. Infection in this system was maintained for more than 30 days and, in a proof-of-concept experiment, the authors demonstrated that the system could potentially be used for testing anti-HBV drugs.[36]

The Bhatia laboratory previously developed a micropatterned coculture system (MPCC), in which hepatocytes are positioned in "islands" via photolithographic patterning of collagen, and are subsequently surrounded by mouse embryonic fibroblasts.[37] In this configuration, MPCCs were shown to exhibit induced and stable hepatocyte phenotypes for 4–6 weeks, and remain permissive to *P falciparum*,[38] *P vivax*,[38] HBV,[39] and HCV[40,41] infections. During HBV and HCV infection of MPCCs, persistent viral infection was maintained for nearly 3 weeks, offering the potential for testing antiviral therapeutics.[39,40] Comparison of MPCC infections versus other culture platforms, including random cocultures similar to the SACC method, using the same source of human primary hepatocytes and stromal cells, revealed more robust infection in the MPCC cultures, which could be further enhanced by blocking the hepatocyte innate immune response.[39,40] Testing of several antivirals and antibodies against HCV, including those under preclinical development, in the MPCC system revealed that the system closely mimicked *in vivo* antiviral responses.[40]

In addition, MPCCs support full developmental liver stages of both *P falciparum* and *P vivax*, with the release of merozoites from hepatocytes and their subsequent infection of overlaid human red blood cells.[42] For *P vivax*, hypnozoites are observed in these cultures for up to 21 days, enabling potential reactivation studies in an *in vitro* format (Gural et al, unpublished data, 2018). Hepatocytes cultured in MPCCs exhibit human-specific hepatic drug metabolism and

have been shown to be more predictive of drug metabolites and drug-induced liver injury than previous *in vitro* liver platforms.[37,43] Human hepatic drug metabolism is especially important within the context of *P vivax*, because primaquine, the only clinically available antihypnozoite drug, requires bioactivation by the liver. In the MPCC model, this phenomenon has been used to observe an IC50 shift in parasite killing in response to primaquine in donors with differential enzymatic activity (Gural et al, unpublished data, 2018).

In a recent study, metabolic clearance functions of a collection of monoculture and coculture platforms were interrogated across a diverse set of enzyme markers, including 5 major cytochrome P-450 enzymes. Specifically, this study focused on SACC and MPCC cultures at 1-week postseeding and found higher activity in the MPCCs across all enzymes monitored, with most pronounced difference being in the CYP2D6 enzyme. With respect to their use in long-term studies, the MPCC platform has been demonstrated to maintain hepatic-specific function for more than 4 weeks, whereas function in SACCs has not been studied beyond 3 weeks, to date. Finally, although the MPCC system uses a single source of primary hepatocytes per assay, SACCs pool cells from multiple human hepatocyte donors, thus preventing interrogation of donor-specific properties, such as CYP2D6 activity, which is important within the context of required drug bioactivation, as described previously.

Stem Cell–Derived 2-Dimensional Culture Systems

The platforms described in the previous section depend on access to sources of adult, primary human hepatocytes. Human embryonic stem cells and induced pluripotent stem cells offer a renewable and alternative source of these essential cells. Numerous studies have differentiated human embryonic stem cells and induced pluripotent stem cells to yield hepatocyte-like cells (HLCs) that exhibit phenotypic and functional traits resembling those of fetal liver cells.[44,45] These HLCs offer a wide array of traceable genetic backgrounds, and the potential to be generated in a patient-specific manner, enabling investigation of host genetics within the context of host-pathogen interactions, and may also be used in target identification for drug development. Notably, use of these cells necessitates proactive genetic characterization to screen for any aberrations that might occur during the proliferation and differentiation processes.[46]

Several groups have demonstrated that induced pluripotent stem cells–derived HLCs (iHLCs) express HCV and HBV entry receptors, and support productive viral infection with HCV or HBV particles,[41,47–49] Viral load in iHLCs is higher than in hepatoma cell lines,[47] and antiviral innate immune response is readily detectable on HCV or HBV infection, with increased expression of various type I interferon-stimulated genes similar to that observed in primary human hepatocytes in MPCCs.[39,41] Furthermore, infected iHLCs can be used for drug screening purposes because they respond to anti-HCV and -HBV drugs.[39,41,47]

During the differentiation process, cultures of pluripotent stem cells that achieve the definitive endoderm stage are not yet permissive to HCV infection, whereas more mature hepatic progenitor cells support the entire viral life cycle. Interestingly, Wu et al[48] characterized this transition from resistance to susceptibility by showing that cells become permissive with induction of liver-specific microRNA-122 and other host factors including epidermal growth factor receptor and EphA2, and a down-regulation of some interferon-stimulated genes. iHLC infection of *P falciparum* and *P vivax* revealed that permissiveness of iHLCs began at the hepatoblast stage. More importantly, it was shown that in this system, iHLCs could be chemically matured to enable primaquine bioactivation, opening the door to potential antimalarial drug testing applications.[50] Together, these studies demonstrate that the use of patient-derived iHLCs may enable personalized *in vitro* culture models for hepatotropic infections in the future.

3-Dimensional Cell Culture Systems

Although 2D systems are easy to create and enable straightforward microscopic monitoring of infection, culture conditions in these systems lack the complexity and architecture of the liver microenvironment, which thus prohibit interrogation of the functional role of the extracellular matrix, and/or the impact of different cell types on liver infections. In light of these limitations, several 3D-engineered liver models have been created to recapitulate certain aspects of liver function that cannot be monitored in 2D. Early attempts to model HCV infection in 3D included the use of a 3D radial-flow bioreactor to grow a human hepatoma cell line.[51] The bioreactor is a vertically extended cylindrical matrix with porous bead microcarriers, providing sufficient oxygen and nutrient supply to cells. Huh7 cells grown in the bioreactor maintain a polarized state, and can support productive HCV infection for months.[51]

Similarly, hollow fiber systems[52] and 3D rotating wall vessels[53] are used to model HCV infection in 3D. These systems, however, require a large quantity of cells, and are challenging to scale up. Alternative 3D cultures of hepatoma cell lines using polyethylene glycol–based hydrogels,[54] thermoreversible gelatin polymers,[55–57] alginate,[58] galactosylated cellulosic sponges,[59] Matrigel,[60,61] and collagen[62] have been developed and shown to be permissive to HCV or HBV infections. In these systems, cell lines were shown to maintain more differentiated states, exhibit better hepatic function,[58] and differential gene expression profiles,[55,56,60,62] as compared with their 2D counterparts. Moreover, several groups demonstrated that 3D liver spheroids could be manufactured at specific sizes and easily scaled up.

Another example where the recreation of a 3D liver-like microenvironment allows the study of early hepatic infection is the migration of *E hystolytica* through human liver sinusoidal endothelial cells and Huh7 cell layers.[62,63] The layers of human cells are separated by a collagen scaffold, enabling imaging of cell barrier crossing and 3D migration of the parasites toward hepatocytes, which recapitulates

initial invasion of the liver and eventually leads to parenchyma destruction and abscess formation.

To further mimic shear stress, blood flow, and the extracellular environment within a tissue, several liver-on-a-chip models have recently been created[64–67] and hold great potential for modeling liver-specific pathogens. As an added benefit, different cell types within the liver or from other organs can be assembled on the same chip, making it possible to study the effects of different cell types on the progress of infection. Alternatively, decellularized pig and human livers have been repopulated with human cells to create 3D structures with physiologically relevant architectures.[68,69] Although not many groups have directly used these more complex models to study hepatotropic infections, we believe that these techniques can potentially offer novel insights into host-pathogen interactions. Selection of the most appropriate model system is guided by the combination of the specific question under interrogation, and the desired method for tracking infection responses under those circumstances.

Humanized Mouse Models

In particular infection studies, it is necessary to consider organ-organ interactions, not to mention more complex 3D architecture than what has been achieved thus far *in vitro*; however, conventional mouse models cannot be used for studying pathogens that have strict hepatotropism. To overcome this barrier, mouse models with "humanized" livers, or that bear ectopic human liver structures, have been developed. The strategy used entails induction of liver injury with physical, chemical, or surgical insults, followed by transplantation or implantation of primary human hepatocytes. One added advantage of these systems over other 3D liver models is the possibility of incorporating human immune cells in the mice, which may have an impact on pathogen development, clearance, and drug responses.

The first model used to monitor HCV and *P falciparum*,[70,71] infections *in vivo* was the Alb-uPA/SCID chimeric mouse where human hepatocytes were transplanted into urokinase-type plasminogen activator-transgenic SCID mice, which undergo liver failure. Subsequently, FNRG/FRG (human hepatocytes transplanted into Fah[-/-], Rag2[-/-], and Il2rγ[-/-] mice with or without a NOD background), and TK-NOG (human hepatocytes transplanted into herpes simplex virus type-1 thymidine kinase mice) chimeric mouse models were validated for HCV,[72,73] HBV,[72,73] *P falciparum*,[74] *P vivax*,[75] and *Plasmodium ovale*[76] infections. Interestingly, Calattini et al[72] showed that HCV particles produced in the FRG model have different biophysical properties and receptor usage compared with cell culture–derived virions, suggesting fundamental differences between *in vitro* and *in vivo* pathogenesis of HCV infection.

Humanized mouse models can also be used for drug screening purposes, although they are better served as a bridge between *in vitro* models and human trials, because their maintenance and cost are bottlenecks. Mailly et al[77] demonstrated in Alb-uPA/SCID mice that antibodies targeting HCV entry factor Claudin-1 can act as antiviral therapy, as shown by inhibition of HCV entry, cell-cell transmission, and virus-induced signaling, leading to a clearance of persistent HCV infection. The FRG mouse model was used to test a canonical drug for *P vivax*, primaquine, in early treatment mode (causal prophylaxis), and reported clearance of hypnozoites.[75]

Another caveat of xenotransplantation mice models is the difficulty to create and maintain them.[78,79] To overcome this issue, Dorner et al[80] created a genetically humanized mouse expressing human factors. Transgenic mice expressing human entry receptors (CD81 and occludin) crossed with an immune-deficient inbred mouse supported HCV infection in murine hepatocytes. As a more affordable alternative to the available humanized mouse models, Ng et al[81] created microporous polyethylene glycol cryogels implanted into mice as ectopic livers, without the need for injuring the mouse liver, offering the added advantage that it can be rapidly generated for immediate use. More recently, human tissue seeds composed of primary human hepatocytes, human endothelial cells, and fibroblasts in degradable hydrogels were implanted into FNRG mice where the host-derived regenerative stimuli favored organized expansion of the grafts. The resulting graft contained bile duct–like structures and a vascular network, supported by the presence of red blood cells in the seeds.[82] The potential ability to connect these ectopic grafts to the vascular network of the animal raises the prospect of studying local and systemic effects of hepatotropic pathogens in the future.

Readout Modalities of Infection

An important determinant when choosing engineered systems for studying hepatotropic pathogens is the readout modality used to assess infection properties. Although 2D systems generally offer a variety of simple and scalable readouts, 3D systems and animal models require more complex and laborious methods to monitor infection processes. Readouts can be terminal, such as polymerase chain reaction (PCR) or cell fixation followed by immunohistochemistry, or real-time, such as genetic modification of the pathogen of interest for live imaging, or detection of secreted molecules. Next we briefly describe the assays typically used for the main hepatic-amplified pathogens (HBV, HCV, and *Plasmodium*) in the different systems. The selection of each assay and model system may vary based on the experimental question at hand.

PCR-based assays and fixed sample imaging are both commonly used terminal assays for quantification of total pathogen burden. PCR is highly sensitive, but cannot determine the number or type of cells infected, and so is suboptimal for questions regarding the heterogeneity of host cell susceptibility. HBV infection is classically analyzed by PCR quantification of viral DNA, cccDNA, and pgRNA, which are indicative of active viral replication in infected cells. Similarly, HCV and *Plasmodium* infections can be monitored by detection of viral RNA or *Plasmodium* 18S rRNA, respectively.[50,74,75] In contrast, imaging of fixed

samples provides structural information about the pathogen and quantification of disease load. For example, the distribution of viruses and parasites inside hepatocytes and across the tissue system can be visualized by immunostaining of pathogen-specific antibodies.[10,38,50,74,75] As an alternative method, Ramanan et al[83] demonstrated that single-molecule fluorescence in situ hybridization can be used to detect viral RNA levels in fixed samples by labeling both strands of HCV viral RNA with high specificity and sensitivity. This single cell phenotypic analysis enables studies of subpopulation infection patterns and kinetics. Together, these methods offer a wealth of information about the pathogen's infection level, and are compatible with the 2D and 3D models described previously, although in the case of the latter these methods are more laborious because they require sectioning of the liver constructs or humanized livers.

Live imaging offers the advantage of dynamic monitoring of infection in real-time. Because of the tissue scattering effect, live fluorescence imaging is feasible in 2D systems, but becomes more challenging in 3D or animal models. Fluorescent protein-based reporter lines have been created for HCV and *P falciparum* by inserting the reporter gene into the pathogen's genome, allowing visualization in live cells.[84,85] One observed caveat for the genetically modified HCV virus was attenuated RNA replication compared with wild-type virus.[86] To overcome this bottleneck, a cell-based reporter system has been developed to distinguish between HCV infected and uninfected cells. In this system, protease activity of the HCV nonstructural protein 3-4A in infected cells causes a mitochondrial-anchored fluorescence reporter to be shuttled into the nucleus, allowing monitoring of viral replication in real time.[87] For pathogens like *P vivax* where no transgenic lines exist, live imaging of parasite development has been challenging, thus many questions remain regarding the processes involved in reactivation of hypnozoites, development of schizonts, and release of merosomes from the liver. Although 2D systems are amenable to live fluorescent imaging, bioluminescent live imaging is a better option for monitoring HCV[86] and *P falciparum*[88] infections in 3D and animal models.

An alternative readout modality that enables kinetic monitoring of the infection process without the need to perturb infected cells is detection of released molecules or pathogen progeny into the environment. On completing the viral replication cycle, for example, HBV- and HCV-infected cells release newly synthesized viral particles, which can be quantified without the need for terminating the experiment. For example, during HBV infection, surface and envelope antigens can be detected by enzyme-linked immunosorbent assay in culture supernatants or animal sera. For *Plasmodium* parasites, propagation of the parasite beyond the liver stage offers a unique readout modality.[89,90] Quantification of infected blood can serve as a proxy for final liver stage burden. Parasites that have egressed out of hepatocytes go on to infect red blood cells, if accessible to them. In the case of humanized mouse models, FNRG mice must be injected with human erythrocytes immediately before parasite egress from the liver, whereas the TK-NOG model can sustain >80% human erythrocyte chimerism for up to 5 weeks after a single engraftment.[76] To date, the only model where the entire *P falciparum* life cycle could be maintained without the need for exogenous human erythrocyte injection is the DRAG model,[91] where HLA class II molecules are expressed in NRG mice to favor human hematopoietic stem cell engraftment. *In vitro* overlay of MPCCs with erythrocytes or reticulocytes also results in blood stage infection for both *P falciparum*[38] and *P vivax* (Gural et al, unpublished data, 2018).

Conclusions and Future Work

Because liver-targeting pathogens are diverse, often with narrow tropism, models that recapitulate authentic host-pathogen interactions are critical for the development of liver-acting therapeutic interventions. With the landscape of available models, and the evolution of new platforms that continue to be developed to support infection by a diverse repertoire of hepatotropic pathogens, it remains essential that researchers match their selected model and readout pairing to the specific question at hand.

The model systems and readout modalities described in this review form a list of combinations that can be used to interrogate different aspects of pathogen development. For example, 2D cultures are easier to setup and offer the added benefit of portability, especially for such pathogens as *P vivax*, which lack laboratory-adapted strains and thus require on-site experimentation in endemic areas. 2D systems are also more amenable to high throughput drug screening than their 3D counterparts. The biologic complexity that human hepatoma cell lines lack can be overcome by use of primary human hepatocytes, which more closely resemble a functional human liver. Furthermore, donor variability of human hepatocytes can be used as a tool for interrogating donor-specific biology, such as host factors that determine invasion and development, and pathogen response to bioactivation-requiring drugs. Stem cell–derived systems offer a renewable alternative and provide the benefit of fine-tuning specific aspects of host factors. However, it should be noted that these cultures require significant culture expertise, and may not represent a fully differentiated adult hepatocyte phenotype. In comparison with 2D models, 3D models enable the study of more complex microenvironments on pathogen development, and can be adapted for use in perfused organ-organ "on chip" models, which may be of particular value for hepatropic pathogens that impact multiple tissues (eg, malaria, LASV, EBOV). Finally, humanized mouse models offer a multiorgan system to study aspects of pathogen development and clearance mechanisms that require a more complex microenvironment. During the course of liver infection, the invading pathogen encounters several liver-resident and/or recruited immune cells. However, except for few humanized animals that incorporate both human hepatocytes and immune cells,[92] most of the models described here lack this aspect of the liver environment, although several are amenable to the addition

of immune cells. In one example, Kupffer cells were successfully added to the MPCC platform, but this configuration has yet to be probed in the context of infection.[93] Addition of other key innate effector cells, such as complement, neutrophils, and natural killer cells, and antibodies could enable the study of immunologic responses against human pathogens *in vitro*.

Novel readout modalities that can monitor live cultures would be a valuable addition to the existing repertoire and further unleash the power of engineered liver platforms. Efforts should focus on improving genetic engineering tools to modify the genomes of HBV and *P vivax*, for which no reporter lines are available. For HBV, reporter viruses have not been successfully created because of the compact nature of the viral genome. For *P vivax*, lack of a continuous blood culture makes genetic modification of the parasite impossible. Furthermore, discovery of new secreted biomarkers could aid in kinetic tracking of infection. An example could be the adaptation of a protease detection system, such as those already developed for cancer[94–97] and HCV[87] monitoring. This would be especially useful within the context of *Plasmodium* infections, because no secreted biomarkers have been described to date. It is known that malaria parasites encode a serine protease, which can be found in the serum of malaria-infected individuals and is hypothesized to play a role in parasite egress out of infected hepatocytes.[98] Such an approach could allow live tracking of not only laboratory strains but also clinical isolates of both *P falciparum* and *P vivax*. Lastly, with the advent of high-throughput sequencing techniques and single-cell analysis modalities, such as laser capture microdissection,[70,99] Drop-seq,[100] and Seq-well,[101] one can expect a more thorough understanding of the biology of both the pathogen and its host with single-cell resolution.

In recent years, exciting clinical progress has been made in the study of hepatotropic pathogens, notably with the cure of HCV,[102,103] and licensing of the first malaria vaccine, RTS,S. Nevertheless, infectious liver diseases continue to prevail and pose a great global health burden, particularly given that the access to medical interventions is not universal. Although tissue engineering has contributed greatly to the current available knowledge of hepatotropic pathogens, we envision a continued effort to develop better liver models. This goal is of importance not only for the major hepatotropic pathogens discussed in this review but also for pathogens that have exclusively been studied in human hepatoma cell lines, such as EBOV or LASV. These hemorrhagic fever viruses are prime examples of zoonotic viruses that accidentally infect humans, causing severe disease and high mortality, yet they remain understudied. The recent EBOV outbreak led to growing public concern regarding the devastating risks such pathogens pose to human health, and demand on the scientific community to develop better prevention and treatment options. Building on the knowledge of HCV, HBV, and *Plasmodium*, we anticipate that adaptation of the described engineered models may significantly advance the understanding of the pathogenesis of these new global threats.

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Conflicts of interest
The following author discloses the following: Sangeeta N. Bhatia is a cofounder of Ascendance, which commercially manufactures and distributes micropatterned co-cultures. The remaining authors disclose no conflicts.