Hepatitis C is a leading etiology of liver cancer and cause for liver transplantation. Although new therapies have improved the rates of sustained response, a large proportion of patients (~50%) fail to respond to antiviral treatment, thus remaining at risk for disease progression. While chimpanzees have been used to study HCV biology and treatments, their cost is quite high and their use is strictly regulated; indeed, the NIH no longer supports the breeding of chimpanzees for study. The development of HCV therapies has been hindered by the relative paucity of small animal models to study HCV pathogenesis. This review presents the strengths of the human liver transplant, highlighting the advances derived from this model, including insights into viral kinetics and quasispecies, viral receptor binding and entry, innate and adaptive immunity. Moreover, consideration is made of current and emerging antiviral therapeutic approaches based on translational research results.

HCV is a major health problem

As described in many excellent articles in this Journal, liver disease related to HCV is the single leading indication for liver transplantation throughout the world, and its significance as a clinical problem cannot be overstated. In this review, we will highlight what has been learned in the past decade about liver transplantation as a model to study HCV pathogenesis, including important insights into the roles of viral kinetics and quasispecies, hepatitis C receptor binding and viral entry, innate and adaptive immunity, and how these insights might be applied to novel preventative and therapeutic approaches.

HCV Animal Models: Challenges to studying HCV

One factor limiting the development of HCV therapies is the paucity of animal models for HCV infection that simulates human infection. While chimpanzees have been used to study HCV biology and treatments, their cost is quite high and their use is strictly regulated; indeed, the NIH no longer supports the breeding of chimpanzees for study. The SCID/Alb-uPA mouse has emerged as the current “gold standard” of small animal models of HCV infection. After transplant with human hepatocytes in the first few weeks of life, the subacute liver failure induced by the transgene leads to a strong proliferative stimulus to hepatocytes. The native murine hepatocytes are inhibited from responding, leaving the human hepatocytes (protected from xenograft rejection by the SCID status) to proliferate and achieve repopulation of levels up to 90% of the liver. The mice can then be infected.
with HCV of defined origin [e.g., H77, JFH-1] or from clinical serum samples, maintaining high-level infection titers for many months. Improvements in methods of hepatocyte generation, adjuvant immune interventions and improved breeding strategies have markedly reduced earlier limitations to the number of mice that can be produced, rendering larger studies more practical than with other in vivo models of HCV infection, but there are only a few laboratories in the world that can generate and maintain these mice. Moreover, the SCID status of these mice precludes immunologic analyses unless cells are added back (e.g., adoptive immunotherapy).

Advantages of human liver transplantation as a model system

The human liver transplantation model provides a unique opportunity and research framework to examine HCV pathogenesis for a number of reasons (Table 1). Liver explants are enriched with HCV-specific T lymphocytes.

HCV infections that follow a chronic course are usually marked by low frequencies of antigen (Ag)-specific T cells targeting few epitopes. Most studies of the intrahepatic compartment to date in humans and chimps have relied on non-specific expansion to yield sufficient number of cells for analysis. Because the whole organ is removed at the time of liver transplantation, it is possible to characterize intrahepatic cells directly ex vivo without in vitro expansion. As shown in Figure 1, the liver is enriched for HCV-specific cytotoxic CD8+ T cells (CTLs). Intrahepatic lymphocytes typically demonstrate distinct phenotypic profiles associated with exhaustion, including up-regulation of PD-1 and down-regulation of CD127. Understanding the molecules associated with T cell exhaustion within the hepatic compartment provides insights and rationale for novel therapeutic targets. For example, blockade of the PD-1/PD-L (ligand) pathway restores the functional competence of HCV-specific CTLs. A number of studies are ongoing to target this pathway either by blocking interactions between the receptor and its ligand(s) or by down regulating PD-1.

Accelerated natural history allows definition of distinct disease outcomes within a relatively short period of time

In the immunocompetent setting, chronic HCV infection is a very slowly progressive disease, making prospective evaluation of its natural history very problematic. Consequently, long periods of time are required to document any clear-cut evidence of progressive liver injury. In contrast, the proportion of HCV-positive liver transplant recipients who develop cirrhosis at 5 years ranges from 21–35%. Accordingly, the median and mean rates of fibrosis development (which are non-linear) are significantly higher than that observed pre-transplantation (p <.0001). As a result, annual protocol liver biopsies are recommended in HCV-positive liver allograft recipients. The telescoped natural history of HCV has allowed identification of specific factors associated with disease progression; donor age and use of T cell depletion for treatment of rejection (e.g., OKT3) have consistently been associated with more rapid disease progression. Interestingly, prospective analyses of non-transplant patients has shown that age at index biopsy (rather than duration of infection) is an important predictor of progression, suggesting that HCV becomes more fibrogenic with advancing age. Conversely, as shown in the immunoglobulin anti-D cohort, young age appears to be protective. The importance of the T cell response in mediating HCV clearance has been underscored in a number of studies (see below as well).
**Prospective tracking of patients: serum, PBMC, and liver biopsies**

One of the main limitations of studies involving patients with HCV is the cross-sectional or retrospective nature of most analyses. An inherent advantage to the transplant model is that the time of infection of the allograft is known and patients are prospectively followed as part of their clinical care, allowing collection of blood and serial analysis of liver tissue. Because acute infection of the allograft invariably occurs, there is an opportunity to study the innate and adaptive immune responses triggered in the early stages of infection. Moreover, in order to study the role of HCV quasispecies in disease progression usually requires some perturbation in the immune response, either by the immune system in the acute setting or under treatment-induced pressure. Thus, whereas the immunocompetent setting is associated with stable viral replication that does not vary to a significant degree over months to years, liver transplantation is characterized by a marked increase (~16–20 fold) in circulating viral titers, allowing both viral kinetics and viral sequence evolution analyses.

The major disadvantages of the liver transplant model are related to the large number of variables (e.g., ischemic injury, immunosuppression, degree of HLA mismatch, donor genetic factors) preclude simple interpretation. Moreover, acute infection of the allograft occurs in a patient who is not immunologically naïve to HCV, and therefore extrapolation to the acute infection setting is not perfect. Nonetheless, as outlined below, the liver transplant model has provided us with important insights into the roles of viral kinetics and quasispecies, hepatitis C receptor binding and viral entry, innate and adaptive immunity.

**Implications of viral kinetics for de novo allograft infection**

Viral kinetics refers to the serial quantification of viral amount in serum of infected patients. This has been used for multiple purposes, such as measurement of disease progression, response to antiviral therapy and prognosis following liver transplantation. The serum viral load at the time of measurement likely reflects a complex interaction between viral production by infected cells and clearance by the host immune system. After liver transplantation, the relative contribution of each of these factors likely differs depending on what point in time the patient’s serum is sampled. Though often used to evaluate degree of liver infection, measurement of the amount of virus in the serum may not be as relevant as the amount of virus in the liver. Intrahepatic and membrane associated virus, rather than freely circulating virus, likely causes liver injury; therefore, liver viral load may better reflect magnitude of infection than serum viral load. As patients differ in rates of viral clearance, it seems reasonable to suppose that liver and serum derived viral loads may differ, although this is not clearly defined. Whereas Terrault et al. found that serum and liver viral loads differed widely (ratio of liver to serum viral load ranged from 17 to 286), Sreekumar et al. demonstrated that serum and liver viral loads correlated well ($r=0.77–0.93$, $p<0.01$), though intrahepatic levels were always higher (on average 79-fold). Both groups compared serum and liver viral loads after liver transplant using the same technique, and these differences may have resulted from the narrow dynamic range of detection for their assays. These early generation branched DNA assays discriminated a 3-log range of concentrations compared with current PCR techniques which routinely can quantify virus over 6-log viral concentration. How well serum and liver amounts correlate using current PCR methodology normalized to known standards in immunocompromised transplant patients remains unknown.

During the liver transplant operation and early post-transplant, there are likely important differences in serum and liver viral loads. García-Retortillo et al. showed that the allograft acts as an absorption column for virus. Serum viral load rapidly decreases with reperfusion (Figure 2) of the allograft, presumably as the liver removes virus from the circulation and intrahepatic viral amount increases (intrahepatic viral load was not determined in this study).
The rate of viral removal or efficiency of absorption during reperfusion was variable between patients, suggesting that some livers are more capable of binding virus. Those livers with significant ischemia-reperfusion (I-R) injury appeared less capable of associating with virus, as viral half-life was prolonged compared with clearance for livers without I-R injury. This may be due to cell surface receptor injury and impaired hepatocyte interaction with HCV. Though I-R injury may diminish viral uptake by the liver, its overall effect may be to hasten recurrence due to other mechanisms (e.g., impaired regenerative capacity of liver). This is suggested by data demonstrating that increasing warm ischemia time contributes to increased rates of viral recurrence and that preservation injury has a more pronounced impact on outcomes for HCV-infected patients than for HCV-uninfected patients. By demonstrating that early after transplant allografts differ in the number of infected cells (immunohistochemical staining for the viral antigen), prior studies support the notion that allografts differ in their ability to bind virus. Characterization of the factors contributing to the rate of viral clearance by the liver might allow pre-transplant identification of allografts more susceptible to infection and avoid the use of organs likely to have higher affinity for HCV.

Viral-specific factors also contribute to the variability in viral absorption by the liver. It has been shown that only a portion of the virus in a recipient’s bloodstream infects the liver, and the fraction of virus capable of infection differs between patients. A larger infectious fraction might result in more rapid viral clearance, but this remains unknown. Furthermore, greater inoculum size should predict worse outcomes. With primary HCV infection, inoculum size predicts likelihood of seroconversion and severity of subsequent infection in vivo and amount of virus internalized within cells in vitro. By analogy, the level of viremia at the time of allograft reperfusion may predict worse outcomes following liver transplant. This remains unclear as early studies measuring pretransplant viral load quantified viral amount prior to initiation of the transplant operation, not immediately prior to reperfusion. Therefore, these results are likely confounded by blood loss and ongoing resuscitation during this intervening period. No recent studies are available to clarify this potential discrepancy. Furthermore, secondary sites of viral infection (e.g. lymph nodes) may also contribute to variability in amount of virus available to infect the liver and net rate of viral clearance. Both inoculum size and the proportion that is infectious should contribute to how much virus infects the liver, and this may more accurately predict outcomes than currently measured predictors.

Serum viral load reaches a nadir 8–24 hours after reperfusion, likely representing saturation of cell surface receptors for HCV in the allograft. The subsequent increase in viral load should represent established infection and production of new virus by the infected allograft. During the first 7 days following transplant, viral kinetics appear highly variable between individuals and may be related to an attenuated immunologic response of the recipient. In this regard, Garcia-Retortillo et al. observed that 5 of the 6 patients with a second-phase decline in HCV viral level did not receive corticosteroids, whereas only 1 of the 13 patients who received corticosteroids as part of their immunosuppressive regimen showed a second-phase decline in viral level. Fukumoto et al. similarly demonstrated that for patients with steroid induction, serum viral load increases early (typically by postoperative day 2). These observations are consistent with studies demonstrating that viremia increases with corticosteroid administration for both immunocompetent patients with chronic hepatitis and immunosuppressed liver transplant patients treated for rejection. This may represent a steroid-specific effect rather than diminished immunocompetence, as cyclosporine A administration in chronically HCV infected patients does not increase viremia (though there is controversy as to whether cyclosporine has antiviral action). It is likely that the steroid effect impairs immunologic clearance rather than stimulate viral
production by the allograft, as steroid administration does not appear to alter rates of viral replication in livers after transplantation\textsuperscript{54}.

Once infection of the allograft is established, serum viral level may more accurately reflect intrahepatic amount of virus, and this may explain how early serum viral load can predict subsequent outcomes. In the first month posttransplant, serum viral load increases to as much as 20 times pre-transplant levels\textsuperscript{45}, possibly because the allograft is better able to support viral replication than the now removed, cirrhotic liver\textsuperscript{55}. How high the viral load rebounds early (<30 days) can predict severity of disease recurrence\textsuperscript{56}; however, data are mixed regarding the predictive power of serum viral load beyond the first month of transplant\textsuperscript{31, 48, 57–59}. Therefore, severity of initial infection likely plays a significant role in subsequent outcomes, and later viral load patterns become more complex to interpret when multiple factors (i.e. immunosuppression, robustness of host immune system, viral fitness for propagation and allograft support of replication) likely determine viral replication.

**What is the differential contribution of the allograft in supporting viral replication?**

Certain allografts may serve as more efficient hosts for viral replication than others. Negro et al. demonstrated that rates of viral replication in allografts (as determined by anti-genomic strand-specific RT-PCR) appear to differ between patients and seem unrelated to immunosupression\textsuperscript{54}. This study was limited by its semi-quantitative nature and lack of consistent, defined protocol biopsy time-points. Other than this study, rates of viral replication in the liver have not been well studied after transplantation, likely due to the challenges of anti-genomic, or negative strand-specific, RT-PCR. The ability of the positive-strand to self-prime the negative-strand replicative intermediate has necessitated the use of less sensitive thermostable reverse transcriptases, and therefore sensitive but poorly quantitative Southern blot to visualize product\textsuperscript{39}. With the development of strand-specific real-time RT-PCR allowing for more reliable and discriminatory quantification of replication\textsuperscript{60, 61}, future studies evaluating differences in viral replication between allografts is warranted.

It has been shown that Huh 7.5 cells, which have a single point mutation in the dsRNA sensor retinoic acid-inducible gene-I (RIG-I), have impairment of innate antiviral defenses within the hepatocyte and are more permissive to viral replication, yielding viral titers that are ~50-fold higher and more efficient spread of the infection\textsuperscript{62}. The possibility that differential interferon signaling, including the phosphorylation and nuclear translocation of IFN regulatory factor-3 (IRF-3) may play an important role early after transplantation is an important hypothesis that has yet to be tested. It was first suggested 10 years ago\textsuperscript{63, 64} that cytokine gene polymorphisms within the allograft might mediate the inflammatory and antiviral microenvironment early post-transplantation, but whether there is coupling between innate immunity within the hepatocyte, e.g., IFN production, and subsequent expansion of innate and adaptive lymphocytes remains unexplored (see below). If associations between genetic markers and HCV-related allograft injury are confirmed, these results would have important implications for identification of optimal donors (particularly in the living donor setting where there would be time to screen for these markers) and identification of high risk patients earlier, allowing institution of antiviral therapy either pre-emptively or with the first signs of histologic recurrence.

**Role of HCV Viral Quasispecies in De Novo Allograft Infection**

Due to the genetic variability of the virus, the viral envelope proteins that mediate attachment to liver cells mutate rapidly and frequently, resulting in multiple “quasispecies” within an individual\textsuperscript{65}. The second viral envelope protein (E2) is thought to be the primary
interface with host cells.\textsuperscript{66, 67} Within this region of the viral envelope is a highly variable region (HVR1) consisting of 27 amino acids\textsuperscript{68–71} thought to derive its variability from a combination of immunologic pressure\textsuperscript{71} and the high error rate of the RNA dependent RNA polymerase the virus uses for replication. These different amino acid sequences within each individual each represent a quasispecies variant. The role of quasispecies evolution with established infection is controversial, and most studies have focused on quasispecies diversity as a surrogate for immunologic pressure (i.e., more pressure = more diversity; less pressure = less diversity). While Sullivan et al. found that higher levels of diversity correlated with less severe recurrence\textsuperscript{72} (presumably because the immune system mounted a stronger humoral response to the virus), Pessoa et al. showed that immunosuppressed transplant patients had greater quasispecies diversity than immunocompetent non-transplant patients\textsuperscript{73}. In fact, the quasispecies nature of established infection with all RNA viruses has been called into question\textsuperscript{74}.

Regardless of the state of flux for viral mutation and immunologic pressure, certain HVR1 proteins sequences appear to be selected out by the liver as it is infected with the virus. A complex population of quasispecies generated in the liver and secondary sites of infection such as lymph nodes\textsuperscript{47} is available to bind and infect the liver. Hughes et al. demonstrated that only a portion of this infectious inoculum present in patient serum prior reperfusion of allografts went on to infect the liver, and that this quasispecies selection began immediately upon reperfusion\textsuperscript{39, 40}. There was a sudden shift in the relative frequency of certain quasispecies variants over others, prior to any detectable viral replication in the liver. The authors obtained serum samples prior to reperfusion of liver allografts, and liver biopsies after reperfusion but prior to completion of the operation as well as 5–10 days after transplant. By sequencing PCR product, they then determined which quasispecies from the infecting serum were also present in liver samples.

As demonstrated for one representative patient (Figure 3), the HVR1 quasispecies recovered from the preperfusion serum were quite diverse, and not all were infectious. Of the 14 variants identified, only three were recovered from the post-perfusion liver, and only one of the variants selected out by the liver went on to infect the allograft at week one. Though usually considered an immunologic decoy, HVR1 has been shown to have properties that could allow it to interface with the host cells. Despite its high rate of nucleotide variability, the chemical-physical properties and conformation of this exposed region on the viral envelope are highly conserved, with basic residues consistent with a role in protein receptor and glycosaminoglycan binding\textsuperscript{75, 76}. Selection for HVR1 variants have also been observed \textit{in vitro}\textsuperscript{77–79} and in chimpanzees\textsuperscript{80}, and antibodies directed against HVR1 have prevented infection\textsuperscript{67, 81–84}. Therefore, HVR1 must play some role in viral attachment to cells.

The results of the Hughes et al. study\textsuperscript{39} may partially explain the variable rates of viral clearance by allografts during reperfusion reported by Garcia-Retortillo et al\textsuperscript{85}. In addition to the patient from Figure 3, six other patients demonstrated variable degrees of quasispecies selectivity, with different infectious fraction size (number of variants in the reperfusion liver/number of variants in the pretransplant serum) between patients. It seems likely that a patient with large infectious fraction would have a faster rate of viral clearance during reperfusion and greater magnitude of infection than one with a smaller infectious fraction. It is also possible that persistence of a predominant variant from pretransplant serum to postperfusion liver would result in higher rates of clearance and greater magnitude of infection. This may explain the results reported by Gretch et al\textsuperscript{85}, who found that persistence of a predominant serum variant from pre-transplant serum to post-transplant serum was associated with recurrent HCV disease, whereas failure of predominant variants to persist posttransplant was associated with no early recurrence. For the predominant variant to persist and lead to recurrent disease, it may represent the most infectious variant leading to a
greater infectious fraction and magnitude of infection, although the relationship between quasispecies selection and viral kinetics has not been assessed and therefore that conclusion cannot be made. When evaluated overall, the post-perfusion liver selected out a population of variants more closely related to one another than those in the infecting serum (Figure 4). This selection for a closely related population of quasispecies (out of a diverse population of potentially infectious quasispecies) by the liver suggests that there is an as yet unidentified common property to infectious HVR1 sequences that could be targeted to decrease the magnitude of initial allograft infection.

After infection is established in the allograft and viral replication and mutation proceeds, quasispecies continue to evolve, and likely do so independently from the role HVR1 has in cell attachment. A variant that represents a small proportion of the virus that infected the liver may have a survival advantage over other variants. This variant may go on to represent a majority of quasispecies within an individual as infection progresses, as small fitness differences may contribute to more efficient replication and propagation. The rapid increase noted in HCV viral loads after transplantation proves the high capacity of HCV to adapt to a new environment. In particular, viral escape from a dominant immune response early after liver transplantation could play a central role in viral persistence by enhancing viral survival when it is most susceptible to immune selection (i.e., during massive infection of the allograft). Transplantation of an HCV-infected liver into an HCV-positive recipient represents a model of superinfection, and Vargas and colleagues demonstrated that superinfection of the liver recipient by the donor’s strain was associated with significantly milder disease than when the recipient strain became (or remained) dominant. In addition, genotype 1 or 1b consistently predominated over non-1 or non-1b genotypes in recipients of infected grafts, suggesting replicative differences among viral strains.

Despite the co-existence of virus-specific immune responses, HCV is able to persist for a virtually indefinite period of time in a tug-of-war with the host as a complex of heterogeneous and dynamic genomes. Most HCV quasispecies analyses in liver transplant patients have focused on HVR1, located at the N-terminus of the E2/NS1 region, and the results have been conflicting. As mentioned previously, Gretch et al. showed that successful propagation of pre-transplant major quasispecies was associated with a more severe form of HCV disease recurrence, a finding that was subsequently confirmed by Doughty and colleagues. In contrast, Pessoa et al. found that, in the subset of patients with fibrosing cholestatic hepatitis, divergence of quasispecies was enhanced, resulting in emergence of many new variants. However, differences in quasispecies are not in themselves definitive evidence for the existence of immune selection. The assumption that RNA viruses are in mutation-selection equilibrium has recently been called into question, i.e., the state of flux in mutants previously ascribed to immune pressure may depend more on the relative fitness of viral subpopulations. In this model, lower viral loads in patients with epitopic sequence variation may simply reflect compromised replicative activity of the variant. These considerations are particularly relevant in the OLT setting where HCV may have a direct viral cytopathic effect and where no protective role for virus-specific antibody responses has ever been established. Moreover, studies anti-HCV immunoglobulins prepared from a pool of highly immune human plasma have failed to prevent graft infection in HCV-infected liver transplant recipients.

**Allograft Cell-Surface Receptors for HCV**

CD81, expressed on the hepatocyte cell surface, likely contributes to HCV infection of allografts after transplantation. This widely expressed 25-kD protein in the tetraspanin superfamily is involved in cell adhesion and signal transduction. The protein spans the cell membrane four times and forms two extracellular loops that are exposed on the cell surface.
Whereas the intracellular and transmembrane segments of the protein are highly conserved across multiple species, the extracellular loops are diverse and conserved only for humans and chimpanzees (species permissive for HCV infection)\(^{93, 94}\). The major extracellular loop has been shown to bind the second envelope protein of HCV, and antibodies the prevent infection in vivo (chimpanzees) neutralize binding of HCV to CD81\(^{93}\).

E2 can bind CD81 independent of any other cell surface receptors, and several areas (including HVR1) within E2 have variable impact on binding affinity\(^{94}\). The role of HVR1 within E2 is controversial. Conflicting studies have demonstrated that HVR1 does not appear to alter the conformation of E2 or the ability of E2 to bind CD81\(^{95, 96}\) and that HVR1 is a key determinant of E2 binding of CD81\(^{97}\).

The amount of CD81 on the cell surface determines the amount of E2 that can bind the cell\(^{94}\) and cells may need a certain threshold level of the receptor for infection\(^{98}\). By analogy, allografts that remove virus from the circulation during reperfusion at a faster rate than other allografts\(^{33}\) may have a greater density of CD81. In the only study evaluating CD81 expression in liver transplants, the authors demonstrated that allografts differ in CD81 density at the time of transplantation\(^{39}\). Figure 5 demonstrates anti-CD81 staining in an allograft from one representative patient at the time of transplant and up to 4 months following transplant. Though allografts appeared to express high levels of CD81 by one month, they differed in the amount at the time of reperfusion. Whether or not these differences resulted in variable magnitude of infection remains unknown, but it is reasonable to hypothesize that some allografts are more susceptible to infection than others based on the amount of CD81 expressed on the cell surface.

Decreasing the ability of CD81 to interact with HCV may alter allograft infection. Ischemia-reperfusion injury (as previously mentioned) may alter allograft affinity for HCV\(^{33}\) and quasispecies selectivity\(^{40}\), possibly through damage to CD81. Furthermore, antibodies directed against CD81 have been shown to prevent infection in an in vivo SCID mouse/human hepatocyte model\(^{99}\). These results need to be further validated, as it remains unclear what role CD81 plays in allograft infection.

CD81 likely acts in concert with other cell-surface receptors to capture and internalize the virus. In the Hughes et al. study of quasispecies selection by allografts\(^{39}\), the authors demonstrated that only a fraction of virus captured by allografts went on to infect the allograft. This suggests that multiple receptors bind virus, but not all receptors internalize virus. CD81 by itself does not appear to result in infection, as tamarins (species of monkey) that express CD81 which binds E2 with high affinity\(^{100}\) and mice expressing human CD81\(^{101}\) are both refractory to HCV infection. Despite having a high binding efficiency similar to that of the human immunodeficiency virus (HIV) gp120-CD4 interaction, only 30% of CD81 internalizes after binding (compared with 50–80% internalization of CD4)\(^{102}\). CD81 many act as an intermediary between attachment and internalization of the virus, as anti-CD81 antibody prevents internalization of pseudoviral particles bound to hepatocytes\(^{103}\).

The low-density lipoprotein (LDL) receptor (LDLr) may act alone or in concert with CD81 to bind and internalize the virus. HCV has been found to associate with circulating LDL in the serum\(^{104–107}\), bind LDLr on hepatocytes, transfer into cells, and then begin replicating\(^{108–110}\). Agnello et al. demonstrated that the amount of intracellular virus correlated with LDLr density and that anti-LDLr antibodies markedly diminished infection\(^{108}\). The fact that anti-LDLr antibodies incompletely prevented infection and that fibroblasts deficient in LDLr were still infected with the virus argues that more than one cell surface receptor is involved with infection. It has also been demonstrated that the first
envelope protein (E1)\textsuperscript{111–113}, in addition to E2\textsuperscript{113} which binds CD81, likely interacts with LDL and leads to internalization of the LDL-HCV complex. Furthermore, CD81 may be a necessary co-receptor for LDLr mediated internalization of the HCV-LDL complex\textsuperscript{113}.

The recent discovery of other candidate receptors for the virus, human scavenger receptor type B-class I (SR-BI)\textsuperscript{114} and Claudin-1\textsuperscript{115}, adds to the evidence that HCV infects cells through a complex interplay between other cell-surface receptors, viral envelope proteins (including HVR1) and lipoproteins. SR-BI has been found to bind E2 in an HVR1 dependent manner\textsuperscript{114, 116} requiring co-expression of CD81 for viral infection\textsuperscript{116, 117}. SR-BI may enhance viral attachment and entry via binding HDL\textsuperscript{116}, but it remains unclear how E2/HVR1 interacts with HDL. Evidence suggests that E2 binds SR-BI directly and then is internalized through cholesterol transfer from HDL\textsuperscript{118–120}. Anti-SR-BI antibodies prevent HCV infection in the presence of HDL\textsuperscript{121}, but unlike for LDL and LDLr, HDL does not appear to associate with and carry HCV into cells\textsuperscript{119}. Moreover, the recent demonstration that human occludin is an essential HCV cell entry factor whose silencing decreases HCV infectivity opens up a novel target for therapy\textsuperscript{122}. Future studies assessing the efficacy and safety of antibody cocktails to block the specific binding and internalization of HCV in the early stages of reperfusion are warranted.

**Innate Immune Lymphocyte Responses**

Innate immune lymphocytes are believed to play important roles in immediate response to viral infections by production of IFN-γ and/or the recognition of viral-infected cells\textsuperscript{123}. Natural killer NK cells mediate the lysis of virus-infected cells via natural cytotoxicity and antibody-dependent cellular cytotoxicity and are controlled by positive and negative cytolytic signals. Accordingly, injection drug users who remain human immunodeficiency virus-1 uninfected despite many years of high-risk exposure demonstrate significantly augmented NK cell lytic activities and cytokine secretion when compared to human immunodeficiency virus-1 infected injection drug users\textsuperscript{124}. The liver has a uniquely specialized immune system enriched for NK and natural T (NT) cells\textsuperscript{123, 125}. Studies\textsuperscript{126, 127} have demonstrated that binding of the HCV envelope protein, HCV-E2, to CD81 directly blocks NK cell functional activation, proliferation, cytokine production, and cytotoxic granule release, instead of giving a co-stimulatory signal as with T cells. CD81-mediated inhibition of NK cells was seen for activated and resting NK cells and in NK cells from healthy uninfected donors as well as patients with chronic HCV infection, suggesting that the inhibitory effect is a general function of NK cell-CD81 ligation and could occur at all stages of infection\textsuperscript{128}. Additional work has shown that NK cells are depleted and phenotypically altered in chronic HCV infection\textsuperscript{129–131}.

In a liver transplantation study comprised of four patient groups [patients with mild HCV recurrence (n = 9), severe HCV recurrence (n = 10), patients with non-HCV-related liver failure (n = 10), and normal healthy subjects (n = 10)], we found that higher levels of CD56\textsuperscript{+} lymphocytes are protective, i.e., associated with milder HCV recurrence (Figure 6)\textsuperscript{132}. Moreover, HCV is associated with impaired lymphokine-activated killing and natural cytotoxicity and higher expression of the inhibitory receptor NKG2A relative to HCV-negative controls with liver disease. Taken together, these data showing decreased CD16 expression, decreased circulating frequency of CD8\textsuperscript{+}CD56\textsuperscript{+} lymphocytes, and increased FasL on CD56\textsuperscript{+} lymphocytes depict a model of global dysfunction in HCV infection with impaired antibody-dependent cellular cytotoxicity and natural cytotoxicity as well as enhanced apoptosis. A recent prospective study evaluated the population dynamics of innate immune cells for 1 year, variations in the frequencies of circulating NK, NT and γδ T cells occurring within the first week after surgery, generally returning to baseline values one
Importantly, HCV RNA levels were statistically associated with the proportion of circulating NK cells. The authors propose that these observations may be due to de novo graft infection resulting in an acute inflammatory process and homing of innate and adaptive immune cells to the liver in order to exert their effector function.

The activity of NK cells is controlled through a complex quantitative and qualitative balance of cell surface activating and inhibitory receptors that react with MHC Class I and Class I like molecules. The killer cell immunoglobulin-like receptors (KIR) represent a diverse family of activatory and inhibitory receptors interacting with self-MHC class I ligands. Recently, Espadas de Arias et al. explored the role of KIR genotypes and their HLA ligands in HCV disease recurrence and progression in the setting of liver transplantation in a well-defined cohort of 151 donor-recipient pairs. The main findings of this study were: mismatching of KIR-HLA-C ligands between donor-recipient pairs were associated with recurrent hepatitis; the presence of KIR2DL3 in the recipient correlated with fibrosis progression; furthermore, in the presence of KIR2DL3, mismatching of KIR-HLA-C ligands favored progression of recurrent hepatitis to fibrosis. Although these results indicating a genetic NK component contributing to allograft injury need to be confirmed independently, they might have important implications in terms of frequency of protocol biopsies and threshold for consideration of antiviral therapy.

Adaptive Immune Responses

A failure to specifically mount an efficient immune response to HCV antigens, either because of selective defects in the host immune system, the effect of immunosuppressive drugs, or because high viral titers affect the normal function of the immune cells might account for why the majority of HCV-infected transplant recipients develop allograft hepatitis. An early study by our laboratory demonstrated that approximately 40% of patients with minimal or self-limited recurrent HCV demonstrated proliferative responses to HCV antigens whereas none of the patients with severe recurrence did so. Subsequent data derived from our laboratory and others indicate that use of more sensitive assays (e.g., ELISPOT and intracellular cytokine staining) indeed do demonstrate viral-specific T cell responses in a higher proportion of patients with end-stage disease and following transplantation than initially appreciated using more conventional assays. CD8+ T cells are the primary effector lymphocytes for provision of protective immunity against intracellular pathogen infection of parenchymal cells. Moreover, we provided evidence that T-cell responses emerge after liver transplantation, in particular HCV-specific CTLs, correlating with improved virologic response and clinical outcomes (see Table II).

As examples, Figure 7A and Figure 7B show two HCV-positive recipients who were HLA A2+ recipients of HLA A2+ donor livers, allowing for detailed comparison of HCV-specific CTL responses using HLA A2-restricted tetramers. The first patient had high frequencies of multispecific CD4+ T-cell responses specific to HCV NS3, NS4, and NS5 antigens on the day of transplantation that decreased in the ensuing months. Levels of tetramer responses to HCV-specific peptides were essentially negative at the first 3 time points, but became detectable after initiation of therapy. The CTLs that peripherally reconstituted after transplantation were terminally differentiated (CD28lowCCR7low) memory cells with effector function, and clonotypically identical to CTLs present in the explant liver the day of transplantation. Taken together, these data support a model where recipient-derived CTLs repopulate the allograft post-transplant, and these efflux into the peripheral blood after viral load is brought under control.

In contrast, the second patient failed to respond to antiviral therapy and consequently died 16 months after OLT. Recent work in the acute infection (non-transplant) model has shown...
that priming of CTLs in the absence of vigorous HCV-specific CD4 help is associated with ultimate demise of these cells and lack of viral control. By analogy, as shown in Figure 7B, CD4+ T-cell enzyme-linked immunospot (ELISPOT) responses were lacking at all but one time; NS3 1073-specific CD8+ T-cell frequencies declined progressively, despite the initiation of antiviral therapy, from 0.58% the day of transplantation to 0.02% by the 10th postoperative month. We excluded the presence of viral escape mutations as a potential cause for the changes in tetramer-specific frequencies by direct sequencing of the epitope coding region at various times, in accordance with prior work showing no association with HCV sequence variation within CTL epitopes and severity of recurrence.

The fact that liver transplantation is performed with no regard to specific matching of donor-recipient HLA alleles may result as a barrier to the development of protective (i.e., antiviral) cell-mediated immunity directed against infected cells within the allograft. A study from our group has shown the generation of new HCV-specific T cells that are restricted by donor HLA alleles, yet derived from the recipient’s original T-cell pool (Figure 8). For the purposes of this study, we selected HLA A2-negative recipients of HLA A2+ grafts. As shown in Figure 8, recipient HCV-specific CTL clones cultured with HLA A2+ lymphoid cell lines that had been pulsed with cognate peptide (NS31406–1415, KLVALGINAV) but not irrelevant HCV core peptide elicited a strong immune response by interferon-γ ELISPOT. Moreover, when these recipient CTLs were cultured with recipient-derived (syngeneic) lymphoid cell lines and cognate peptide, there was no appreciable immune response. Thus, these results indicate that these HCV-specific CTLs circulate within the recipient and display functional antiviral activity (i.e., secrete interferon-γ and show cytolysis) only when they encounter allograft-derived HLA molecules and viral peptide. These results underscore the plasticity of the TCR, as well as the need to assess both allograft- and recipient-restricted CTLs to comprehensively understand the nature of protective immunity to HCV after liver transplantation.

Future Therapeutic Approaches

In conclusion, the past 10 years of research have yielded important insights into mechanisms that govern viral replication and mediate HCV-related allograft injury. These results may help identify patients more likely to develop severe HCV recurrence and therefore benefit from current antiviral therapy, as well as provide a rationale for the future use of novel therapeutic approaches. Although a number of promising drugs that specifically inhibit the HCV cell cycle have been withdrawn at early stages of clinical development, it is expected that newer agents will become available for the treatment of chronic HCV in the near future. When used as monotherapy, current inhibitors show a low barrier to genetic resistance, a potential problem for antivirals given the high rate and error-prone nature of HCV replication. In theory, achieving steady-state levels for peginterferon and ribavirin may reduce the selection and expansion of resistant strains related to STAT-C (specifically targeted antiviral therapy for patients with chronic HCV). This challenge is likely greater in liver transplant recipients because of higher viral levels and because a higher proportion of patients require interferon/ribavirin dose reduction (and even cessation). So far, no data are available on the pharmacokinetics and pharmacodynamics of these STAT-C in the liver transplant setting.

A number of approaches and their challenges merit further consideration. Although trials with hepatitis C immunoglobulin have been disappointing, it is hoped that more comprehensive antibody discovery approaches will provide neutralizing protection in the post-transplant setting as demonstrated in the human liver–chimeric mouse model. Antibodies that block molecules involved in viral binding and entry (e.g., antibodies directed against CD81) will likely need to be of very high affinity (affinity constant ≥10−9) to...
overcome the high affinity binding of E2 to CD81. Perfusion of livers with molecules designed to block viral binding \textit{ex vivo} under cold conditions will likely be a challenge as E1 and E2 binding to cells at 4°C is 7- and 12-fold less than at 32°C. Removal of virus during the anhepatic phase of transplantation possibly in conjunction with portal or systemic bypass may provide an innovative approach to reduction in the viral inoculum. This will likely pose a challenge, as current technology (e.g., double filtration plasmapheresis) does not allow for the high flow rates necessary for the bypass circuit and unfortunately removes critical serum elements such as fibrinogen. Infusion of activated innate lymphocytes (e.g., NK cells) derived from the donor might limit HCV replication within the allograft early post-transplant. Adoptive transfer of lymphocytes with high avidity T cell receptors specifically targeting HCV (personal communication, Michael Nishimura) might represent a future approach without eliciting alloimmunity.

\textbf{Acknowledgments}

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\textbf{Literature Cited}


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*Liver Transpl.* Author manuscript; available in PMC 2010 November 1.
Figure 1.
The liver is enriched for antigen-specific cytotoxic T lymphocytes (CTLs). A) Flow cytometric dot plot gated on CD3<sup>+</sup>CD8<sup>+</sup> T cells showing HCV-specific CTLs A2–1073 (R4) enriched within the hepatic compartment. Expression of PD-1 on Ag-specific (71.74%) cells relative to bulk CTLs (6.02%) is shown in the histogram. CTLs shown in R5 may include cells reactive against other regions of HCV. B) Total HCV-specific PD-1 expressed as a percentage of pentamer<sup>+</sup> CD8<sup>+</sup> T cells as the (mean fluorescence intensity) MFI. Chronic HCV infection is associated with a greater percentage of PD-1<sup>+</sup> HCV-specific CTLs in the periphery (n = 17 patients, 45 pentamers responses), as well as in the liver (n = 9 patients, 29 pentamers). The data are shown for seven patients (21 pentamers) with resolved infection. The intensity of PD-1 staining is also higher on the cells of chronically infected patients, showing the most concentrated PD-1 expression in the liver<sup>14</sup>.
Figure 2.
Schematic of Perioperative Viral Kinetics (Adapted from Garcia-Retortillo et al.\textsuperscript{33}). Serum viral load remains fairly stable during hepatectomy as the liver continues to release virus into the circulation. During the anhepatic phase, viral load begins to decrease, as the virus producing liver has been removed. Rate of viral decrease during this stage is related to blood loss and dilution from resuscitation. With reperfusion of the allograft, virus is rapidly removed from the circulation by the previously uninfected allograft. It appears that allografts are variable in their ability to bind and remove virus, as rates of viral clearance differ markedly. Viral load reaches a nadir (likely due to saturation of cell-surface receptors for the virus) and then starts to increase with established infection and viral replication.
Figure 3.
Quasispecies selective nature of allograft infection. As demonstrated in a single, representative patient, only a portion of HVR1 quasispecies binds and subsequently infects the liver. New quasispecies are rapidly generated with allograft infection and intrahepatic viral replication (taken with permission from Hughes et al.39). First sequence represents the consensus sequence. Each letter of sequence represents the amino acid at the given position. Hyphens represent conserved amino acids. Number next to each sequence represents the number of times that sequence was identified in the given sample.
Figure 4.
HVR1 quasispecies selected out by the liver are more closely related than the pool of quasispecies in the pretransplant serum inoculum. With generation of new quasispecies, diversity increases (taken with permission from Hughes et al.\(^{39}\)). MAAD=maximal amino acid diversity. Grey lines represent individual patients. Black line represents mean values.
Figure 5.
CD81 expression (brown staining) following liver transplantation: (a) explant liver; allograft (b) 2 hours after reperfusion, (c) 3 days, (d) 1 week, and (e) 2 months posttransplant (taken with permission from Hughes et al.39).
Figure 6.
Levels of CD56⁺ lymphocytes in chronic HCV patients and HCV-negative liver disease patients prior to liver transplantation as well as normal healthy controls. Multi-parameter flow cytometric analysis was used to estimate the levels of CD56 lymphocytes, NK (CD56⁺CD3⁻) and NT (CD56⁺CD3⁺) cells in chronic HCV infection prior to liver transplantation (A). Total CD56⁺ lymphocyte levels are significantly decreased in all chronic HCV patients compared to normal uninfected control subjects. This reflects a deficiency in both NK and NT cells. A decrease in total CD56⁺ cells was also observed for control non-HCV chronic liver disease patients due to a significant reduction in NT but not NK cells. Chronic HCV patients were stratified into two groups depending on severity of disease recurrence post liver transplantation. Of interest, all CD56⁺ lymphocyte populations were decreased in the patient group with subsequent severe outcome compared to those who had mild recurrence of HCV liver disease. Compared to non-HCV liver disease, this reduction was significant for total CD56⁺ and NK (but not NT) cells only for the severe group (B). *p < 0.05. (From Rosen et al.¹³²).
Figure 7.
Longitudinal analyses of HCV-specific in two patients with severe cholestatic HCV who received antiviral therapy. Both patients were HLA A2+ recipients of HLA A2+ donor livers. A) Reconstitution of HCV-specific cellular immunity in a patient with severe cholestatic HCV recurrence who responded to antiviral therapy (HCV RNA expressed as $10^6$ copies/mL). (Top) IFN-γ ELISPOT (enzyme-linked immunospot) responses to HCV recombinant proteins, viral load and serum bilirubin. (Middle) CD8+ T-cell responses to NS3 1073 tetramer. (Bottom) Amino acid sequence of NS3 1073–1081 epitope at 4 time points (HCV genotype 1a prototype sequence: CINGVCWTV). B) HCV-specific immune responses in a patient with severe cholestatic HCV recurrence who failed to respond to antiviral therapy (HCV RNA expressed as $10^6$ copies/mL). (Top) IFN-γ ELISPOT responses to HCV recombinant proteins, viral load, and serum bilirubin. (Middle) CD8+ T-cell responses to NS3 1073 tetramer. (Bottom) Amino acid sequence of NS3 1073–1081 epitope at 4 time points (HCV genotype 1a prototype sequence: CINGVCWTV); amino acid substitution (V for I at position 2 was detected but remained stable over time). (Taken from Weston et al.\textsuperscript{137}). (Editor: provided are original color figures, can use the figures as they appear in Hepatology manuscript).
Recipient-derived T cells that recognize HCV peptides in the context of donor HLA molecules (HLA A2). Taken together, these results suggest that T cells circulate in liver transplant recipients and are not activated until they encounter donor alleles containing HCV peptides.

Top) ELISPOT assay was performed with 1,000 T cells, 20,000 LCLs expressing A2 allele (top row), A3 allele (middle row), or syngeneic (recipient-derived) LCLs (bottom row) cocultured with no peptide, cognate peptide (NS31406–1415, KLVALGINAV) or irrelevant HCV core peptide (core35–44, YLLPRRGPRL). (Taken from Rosen et al. 143).

Bottom) Demonstration that these HCV-specific clones do not react to allo-HLA alone. Lymphoid cell lines (LCLs) expressing all potential HLA class I alleles from donor and recipient were used as antigen presenting cells. Briefly, no peptide or cognate peptide (KLVALGINAV) was added to 3 X 10^6 LCLs and 1.5 X 10^6 clones were co-cultured for a total of 6 hrs (Monensin 2μM added after 2 hrs). Cells were rinsed twice with PBS + 1% BSA and stained for 30–40 minutes with tetramer; cells were rinsed twice, fixed in 200μL of 4% paraformaldehyde for at least 15 minutes then permeabilized with 2 mL 1X PermWash (Pharmingen) for half an hour at 4°C. Permeabilized cells were stained with monoclonal antibodies to IFNγ. Stained cells were washed twice in 2mL PBS + 1% BSA and fixed in 300μL 2% paraformaldehyde. Acquisition was performed within 24 hours of staining; all flow cytometry data were collected using a FACS-Calibur flow cytometer (BD Biosciences) and analyzed with CellQuest software (BD Biosciences). A) no peptide, LCL HLA A2A3B14B44; B) cognate peptide and LCL HLA A2A3B14B44; C) cognate peptide and LCL HLA A3A24B62B18; D) cognate peptide and LCL HLA A30A33B13B14; E) cognate peptide and LCL HLA A1A3B7B50; F) cognate peptide and LCL HLA A3A30B7B13 (recipient derived).
### Table I
Advantages and Disadvantages of the Human Liver Transplant Model

<table>
<thead>
<tr>
<th>Advantages</th>
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<tbody>
<tr>
<td>• Explant liver contains high number of HCV-specific T cells</td>
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<td>• Post transplant natural history is accelerated</td>
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<td>• Time of infection is known, allowing viral kinetics and host immune studies</td>
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<td>• Sequential serum specimens and biopsies typically available</td>
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<table>
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<tr>
<th>Disadvantages</th>
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<tr>
<td>• Immunosuppression</td>
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<td>• HLA mismatch (and its effect on antigen recognition)</td>
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<tr>
<td>• Extrapolation to acute infection confounded by the fact recipient is not naive to HCV infection</td>
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Evidence for protective and adverse immunological mechanisms in HCV recurrence (with references)

<table>
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<tr>
<th>Protective</th>
<th>Adverse</th>
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<tr>
<td>• HCV-specific CD4+ T cells early post infection (136,137)</td>
<td>• Decreased CD56+ lymphocytes pre-transplant associated with severe recurrence (132)</td>
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<tr>
<td>• HCV-specific CD8+ T cells in setting of antiviral therapy (137,138)</td>
<td>• T cell depletion treatments (23, 52)</td>
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<tr>
<td>• Pre-transplant Level of CD56+ lymphocytes (132)</td>
<td>• mismatching of KIR-HLA-C ligands between donor-recipient pairs and the presence of KIR2DL3 in the recipient (135)</td>
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<td>• Cytokine gene polymorphisms (63,64)</td>
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<td>• Impaired innate interferon signaling within hepatocytes (unknown)</td>
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<td>• Allo-restricted T cell responses (unknown, see references 125, 143)</td>
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<td></td>
<td>• Relative allograft expression of CD81 and other HCV receptors with immune properties (unknown, see reference 39)</td>
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