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Reassessing Immune Control of Hepatitis A Virus

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Abstract

There is renewed interest in hepatitis A virus (HAV) pathogenesis and immunity after 2–3 decades of limited progress. From a public health perspective, the average age at infection has increased in developing countries, resulting in more severe hepatitis that is poorly understood mechanistically. More fundamentally, there is interest in comparing immunity to HAV and hepatitis C virus (HCV): small, positive-strand RNA viruses with very different infection outcomes. Here, we review evidence that circulating HAV virions are cloaked in membranes, with consequences for induction of innate immunity and antibody-mediated neutralization. We also consider the contribution of CD4+ helper versus CD8+ cytotoxic T cells to antiviral immunity and liver injury, and present a model of non-cytotoxic immune control of HAV infection.

Introduction

Hepatitis A virus (HAV) is a positive-strand RNA virus classified in the family *Picornaviridae*. A prominent cause of fecal-orally transmitted acute viral hepatitis (Figure 1) and prevalent where sanitation is poor, it was first visualized in 1973 by immune electron microscopy in the feces of human volunteers [1]. Only a single HAV serotype exists, and it has never been shown to establish longterm persistent infections. Interest in HAV peaked in the late 1980s, but then declined with introduction of successful formalin-inactivated vaccines and the discovery of hepatitis C virus (HCV). HCV, also a positive-strand RNA virus (family *Flaviviridae*), has a striking capacity to establish persistence and strong

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association with chronic hepatitis, progressive hepatic fibrosis and liver cancer – clinical outcomes never linked to HAV. However, recent years have marked a resurgence of interest in HAV, sparked in part by the desire to understand these different infection outcomes. Many potential explanations have been advanced [2], but the mechanisms underlying HCV persistence remain elusive. Contrasts in the host response to HAV and HCV that are now emerging may provide important clues to this mystery. Recent studies have brought to light several unexpected aspects of the innate and adaptive immune response to HAV, and revealed paradigm-breaking features of HAV structure and the HAV lifecycle. Here, we review these recent developments and outline the questions they pose for future investigations.

Structure of the infectious HAV particle

While the organization of the HAV genome resembles other picornaviruses (Figure 2), recent crystallographic studies show the capsid to be intermediate in structure between that of ‘primitive’ insect dicistroviruses and mammalian picornaviruses such as poliovirus [3]. Although it was recognized that the HAV structure must differ from other picornaviruses given its impressive physical stability and a distinct morphogenesis pathway [4,5], the degree of difference comes as a surprise and indicates that HAV diverged from other picornaviruses eons ago. The capsid is also generally devoid of the surface topology that provides binding sites for cellular receptors on other picornaviruses [3], raising questions as to how HAV enters cells.

Even more surprising is the discovery that HAV is released from infected hepatocytes cloaked in host membranes and thereby hidden from neutralizing antibodies [6]. These membrane-wrapped virions (‘eHAV’) (Figure 2) are infectious and possess key attributes of conventional enveloped viruses, including loss of infectivity upon extraction with organic solvents. The membrane cloaking the virus is not decorated with virally-encoded glycoproteins, however, providing an important distinction and leading us to consider these eHAV virions to be “quasi-enveloped” [7]. While the largest of the 4 capsid proteins, VP1, is 274 amino acid residues in length in ‘naked’, non-enveloped HAV virions, it has an 8 kDa carboxyterminal extension (pX, also known as 2A) and is approximately 71 residues longer in eHAV (VP1-pX) [6]. pX is unrelated to any other known protein. It plays a critical role in capsid assembly and likely eHAV envelopment, but is cleaved from the capsid upon loss of the membrane [5,6].

The biogenesis of quasi-enveloped eHAV particles is dependent upon ALIX and VPS4B [6], components of the cellular endosomal sorting complex required for transport (ESCRT) commonly involved in budding of conventional enveloped viruses [8]. ALIX appears to bind tandem YPX_3L ‘late domains’ in VP2 [6]. Although confirmatory ultrastructural data are lacking, this likely promotes the budding of assembled capsids into multivesicular bodies (MVBs), leading to eHAV envelopment and a release mechanism resembling exosome biogenesis [9]. Since the VP2 late domains are buried beneath the surface of the naked capsid in the X-ray structure [3], the capsid appears to undergo significant conformational rearrangement upon membrane dissolution and loss of pX.

Only quasi-enveloped eHAV is detected in serum and plasma during acute infection, whereas non-enveloped virions are shed in feces [6]. While not well understood, these naked virions are probably produced in the liver and secreted in bile. They may be released from hepatocytes as eHAV, but converted to naked virions in the proximal biliary canaliculus where local bile salt concentrations could be sufficient to dissolve the membrane [10]. However, available data do not exclude an intestinal source [11]. The non-enveloped virion is remarkably stable to heat, low pH, and drying, facilitating viral transmission [3,4]. This dual lifestyle, quasi-enveloped and cloaked from neutralizing antibodies within the host while devoid of membranes and stable in the environment, provides unique opportunities for spread within and between hosts.

Cellular entry of eHAV occurs via a chloroquine-sensitive endocytic pathway distinct from entry of non-enveloped virions [6]. Entry of both virion types is dependent upon the phosphatidylserine receptor, T cell immunoglobulin and mucin domain-*1* (TIM-1, also referred to as HAVCR1), but little else is known about this. Important questions that remain unresolved is how the eHAV membrane alters cellular tropism, and whether a distinct receptor is involved in eHAV entry.

Innate and cell-intrinsic immune responses

Type 1 interferon (IFN-α/β) is both a first line of defense against viruses and important in optimal priming of subsequent adaptive cellular immunity. HAV evokes a minimal intrahepatic type I IFN response in chimpanzees, far less quantitatively than that observed in acute HCV infections (Figure 3) [12]. Despite this, intrahepatic viral RNA is 100- to 1000-fold more abundant in acute HAV versus HCV infection. There are several possible explanations for these differences. Both viruses express proteases that cleave MAVS and TRIF, key adaptor proteins in RIG-I-like receptor (RLR) and Toll-like receptor 3 (TLR3) signaling, respectively. This represents an interesting example of convergent evolution, as the responsible HAV proteases, 3ABC and 3CD [13,14], are structurally and phylogenetically unrelated to the HCV protease, NS3/4A [15]. However, the mature HAV protease, $3C^{pro}$, also cleaves NEMO, a bridging adaptor required for NF-κB activation and IFN-β expression [16]. The targeting of NEMO by HAV may provide an additional level of disruption in interferon signaling beyond that imposed by HCV, possibly contributing to less interferon-stimulated gene (ISG) expression in hepatitis A.

Differences may also exist in the plasmacytoid dendritic cell (pDC) response to these infections. pDCs are activated and produce IFN through a TLR7 pathway when placed in co-culture with HCV-infected cells [17]. Although they do not sense some picornaviruses unless the virus is complexed with antibodies [18,19], they do produce substantial amounts of IFN-α when co-cultured with HAV-infected cells [20]. pDCs preferentially take up quasi-enveloped eHAV virions, which stimulate IFN production in the absence of genome replication. pDCs sense HCV RNA carried as cargo from infected cells by exosomes [21], a mechanistically similar process since eHAV resemble exosomes and may share a similar biogenesis. A key difference between HAV and HCV, however, may be in how pDCs are recruited to the liver. In chimpanzees, numerous pDCs are present within the liver by the end of the first week of HAV infection (Figure 1) [20]. For unknown reasons, they disappear

and cannot be detected at the peak of virus replication and acute inflammation 2–3 weeks later. Less is known about temporal aspects of the pDC response in HCV infection, but pDCs appear to be abundant in chronically infected livers where ISG expression is often strong [22].

HCV may also replicate less efficiently than HAV, resulting in lower expression of HCV proteins and therefore less efficient antagonism of IFN signaling. HCV is exquisitely and uniquely sensitive to oxidative membrane damage, whereas HAV is not [23]. Because HCV infection induces oxidative stress, an auto-regulatory circuit unique to HCV may ensure that replication is maintained at low levels within the liver.

Adaptive Immunity and Control of HAV Infection.

HAV-specific humoral and cellular immune responses typically appear 4–5 weeks after infection with the onset of hepatitis (Figure 1). Increased numbers of plasmablasts secreting IgM with a variety of specificities are present at this point in time [24], but this rapidly transitions to a neutralizing IgG response that provides life-long protection from hepatitis A [25]. Passive transfer of anti-HAV antibodies or vaccination up to two weeks after exposure to the virus can prevent liver disease [26], indicating that antibodies also have the potential to modulate the course of an established infection. Neutralizing antibodies recognize a small number of closely-positioned epitopes in the highly conserved VP1, VP3 [27] and possibly VP2 [3] capsid proteins.

Non-enveloped HAV are readily neutralized when pre-treated with antibodies before inoculation onto cultured cells [28], and thus it has been assumed that immunization or immune globulins protect against disease by neutralizing circulating virus. However, quasi-enveloped eHAV virions (the only virion type found in blood) are completely resistant to neutralization in classical infectious focus-reduction assays since the membrane effectively cloaks the capsid [6]. Despite this, replication is inhibited when anti-capsid antibodies are added to cells several hours after eHAV infection [6]. Neutralization probably occurs within late endosomes or lysosomes, where the membrane is likely to be removed and the capsid exposed during entry of the virus. The kinetics of such post-endocytic neutralization suggest that eHAV entry is relatively slow, requiring 4–6 hours for dissolution of the membrane. In contrast, antibodies have no effect when added even immediately after infection of cells with non-enveloped HAV [6].

HAV-specific cytotoxic CD8+ T cell responses were first described in the blood [29] and liver [30] of jaundiced patients with acute hepatitis A 25 years ago. Since the icteric phase of infection typically coincides with a sharp decline in viremia, this CD8+ T cell response was correlated kinetically with control of virus replication (Figure 1). The detection of cytotoxic CD8+ T cells during this acute phase of the infection was also consistent with liver injury being immune mediated, since robust virus replication occurs during the preceding 2–3 week prodromal period without liver disease. However, a series of recent studies have provided fresh insight into cellular immune responses using newer methods for more precise measurement of T cell frequency and function. Epitopes presented by defined class I epitopes were mapped in human subjects infected during a recent hepatitis A outbreak [31].

Whereas CD8+ T cells targeting these epitopes were successfully expanded from blood of patients with acute HAV infection, most circulating CD8+ T cells were present at frequencies too low for direct visualization with class I tetramers. Effector functions were not assessed. A survey of CD4+ helper and CD8+ cytotoxic T cell activity was also undertaken in two chimpanzees with relatively mild transient hepatitis 3–4 weeks after experimental challenge [32]. In that study, CD8+ T cells were visualized in blood with class I tetramers, but they targeted few epitopes and did not gain effector functions until after viremia and hepatitis had substantially declined (Figure 1). On the other hand, multi-functional HAV-specific CD4+ T cells targeting over 30 discrete class II epitopes appeared in blood at much higher frequency well before CD8+ T cells were detected. Control of viremia was more closely linked to expansion of functional CD4+ T cells than CD8+ T cells [32]. The frequency of HAV-specific CD4+ T cells declined very slowly in blood after termination of viremia and fecal shedding of virus. Slow contraction of CD4+ T cells paralleled gradual clearance of HAV RNA from the liver over 8–9 months [12,32]. While the continued presence of intrahepatic HAV RNA for such a long period of time was unexpected, it is consistent with a role for residual viral antigen in prolonging CD4+ but not CD8+ T cell contraction as described recently in lymphocytic choriomeningitis virus (LCMV)-infected mice [33].

While the detection of CD8+ T cells in patients with acute hepatitis A provided an early conceptual framework explaining both acute liver injury and immune control of HAV (Figure 4, left panel), liver injury can range from inapparent to severe (even fatal) in acute HAV infection. This suggests that cytotoxic CD8+ T cell activity is likely to be a variable feature in hepatitis A. More recent studies of patients infected during a recent nationwide epidemic of hepatitis A in South Korea established an inverse correlation between the frequency and function of regulatory T cells (Treg) in blood and the severity of liver injury as reflected by increases in in serum alanine aminotransferase (ALT) [34]. The frequency of circulating HAV-specific CD8+ T cells did not correlate with Treg activity [34], raising questions about the identity of effector cells that mediate immunopathology. Little is known about the role of NK and NKT cells in this infection. Moreover, the potential for HAV to modulate NKT cell cytotoxic activity [35] as well as Treg function [36] through direct interaction of the viral capsid with the TIM-1 receptor highlights the complexity of host virus-interactions in this infection.

Conclusion: Towards a more flexible model of HAV immunity and pathogenesis

The new findings summarized above demand a rethinking of the relationships between innate and adaptive immune responses and acute liver injury, as well as immune elimination of HAV. A new model might place less emphasis on cytotoxic elimination of HAV-infected hepatocytes by CD8+ T cells, particularly if the delay in acquisition of effector function observed in chimpanzees [32] is recapitulated in future studies of human subjects (Figure 4, right panel). A defect in early antiviral effector function by CD8+ T cells is probably not caused by an absence of help because CD4+ T cells in the HAV-infected chimpanzees produced IL-2, IL-21, and IFN-γ [32]. Interference with class I antigen processing and

presentation has been described for other picornaviruses [37], but the relevance of this to generation of CD8+ T cell immunity in HAV infection remains to be determined. Weak type I IFN responses in the liver [12] might also dampen CD8+ T cell immunity as this cytokine delivers an important differentiation signal in some virus infections. As discussed above, more needs to be learned about the fate of pDCs in the acutely infected liver in order to better understand the paucity of type I IFN responses and how it might limit development of CD8+ T cell responses.

The concept that CD4+ T cells provide direct control of virus infections through production of antiviral cytokines is gaining favor [38]. Non-cytotoxic control of virus replication by CD4+ T cells could be a general mechanism for terminating HAV infection regardless of CD8+ T cell activity. CD4+ T cells could also have a protective role in the post-convalescent phase of infection, when HAV RNA genomes are gradually lost from liver. Clinical relapse associated with apparent recrudescent infection after the initial resolution of symptoms of hepatitis A [39], and prolonged presence of HAV RNA in serum of some adults [40], suggests the existence of a non-cytotoxic mechanism of immune surveillance that is effective in most infections. CD4+ T cells, that contract gradually after apparent resolution of infection [32], could serve this function. However, the persistence of viral RNA in the liver for months after the cessation of fecal shedding [12] remains to be explained. One interesting possibility is that virions may remain complexed to neutralizing antibodies within endolysosomes.

Finally, despite its major fecal-oral route of transmission, the role of the gut as a site for HAV replication and perhaps as a regulator of immune responses to the virus has received insufficient attention [41]. Early studies in owl monkeys suggest HAV may replicate in the lower gastrointestinal tract [11], but this has never been confirmed in humans. Local inflammatory signals elicited by even limited replication of HAV in the gut could substantially influence the nature of immunity and disease severity in liver [41].

A better understanding of HAV pathogenesis and immunity could provide general insight into mechanisms of immune evasion and control of other viruses that infect the liver, including HCV. A focus on human subjects who are infected as a result of sporadic and epidemic spread of the virus provides one path forward. However, there is a need for a renewed effort to better characterize non-human primate models of hepatitis A and perhaps even the adaptation of HAV to replication in rodents to facilitate access to tissue and experimental manipulation of immune responses.

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the potential for non-cytotoxic control of HAV infection and perhaps immune surveillance in liver where HAV genomes decay slowly over several months following resolution of viremia. **

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HIGHLIGHTS

- Circulating virions are cloaked in membranes and resistant to neutralization
- Acute HAV infection induces a meager intrahepatic type I interferon response
- CD4+T cells appear earlier and acquire effector function before CD8+ T cells
- Immune control of HAV may be predominantly non-cytotoxic and cytokine driven

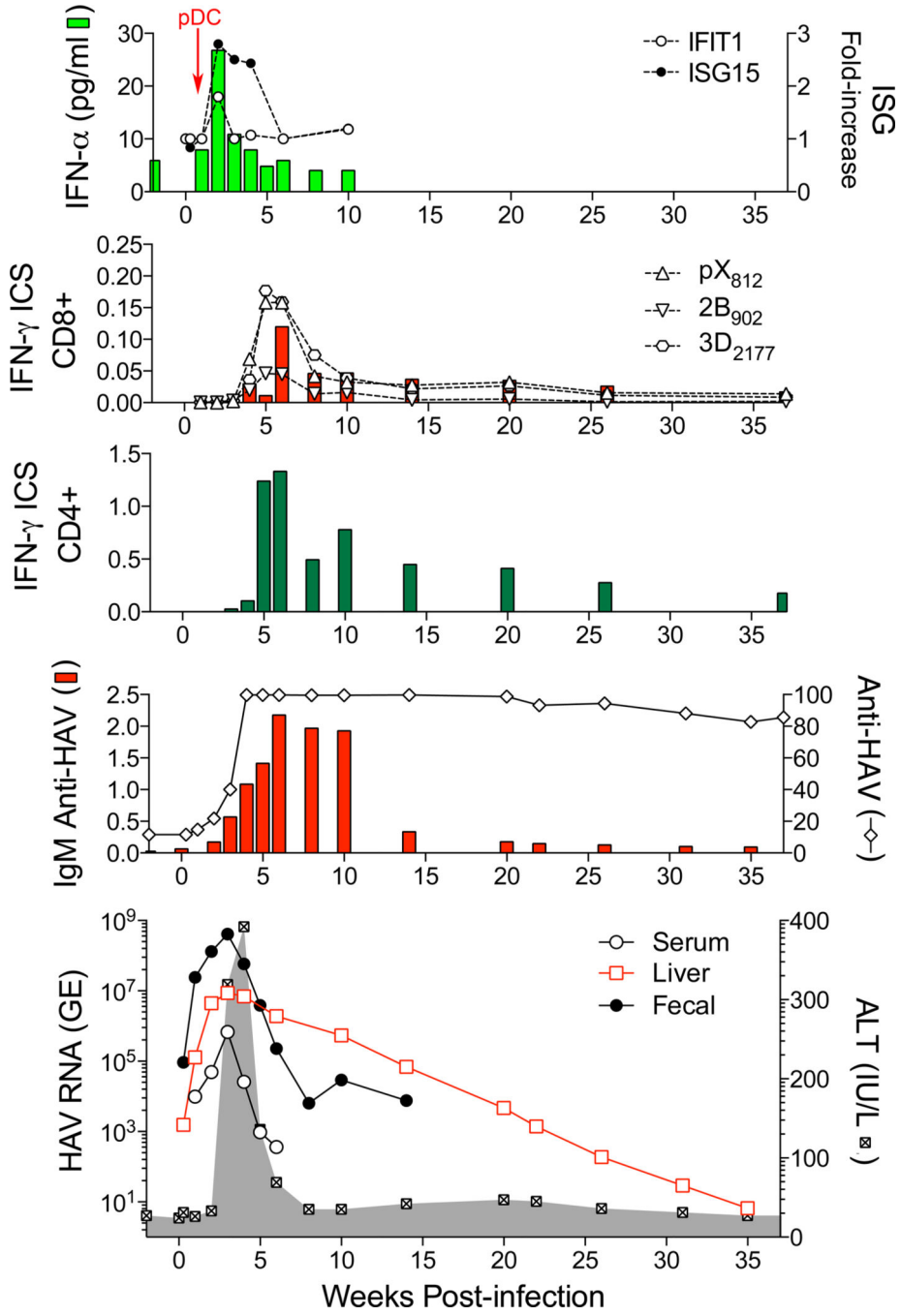

Figure 1.

Virologic and immunologic events during acute HAV infection in a chimpanzee inoculated intravenously with wild-type HAV [12,20,32]. Working from the bottom up, the lowest panel shows the presence of viral RNA (GE, genome equivalents) in serum (GE/ml), feces (GE/gm), and liver tissue (GE/μg total RNA) in relationship to serum alanine aminotransferase (ALT) activity shown in the shaded zone [12]. The prolonged persistence of intrahepatic HAV RNA is surprising. The panel immediately above shows total anti-HAV antibody (% blocking in a competitive ELISA assay) and IgM anti-HAV (ELISA O.D.)

[12]. The next two panels show frequencies of HAV-specific CD4+ and CD8+ T cells among peripheral blood mononuclear cells, as determined in an IFN-γ intracellular staining (ICS) assay [32]. CD8+ cells were also quantified on the basis of staining with tetramers targeting epitopes in pX, 2B, and $3D^{pol}$ (see Figure 2). Note the difference in scale between CD4+ and CD8+ T cell frequencies. The top panel shows type I IFN responses to HAV infection as reflected in minimal and only early serum IFN-α levels detectable by cytokine ELISA, and minimal increases in intrahepatic expression of IFN-stimulated genes: IFIT1 and ISG15 [12]. pDCs were detected in liver tissue only at 1 week after viral challenge (arrow) [20]. (*The authors gratefully acknowledge the essential involvement of Dr. Robert Lanford in these comprehensive chimpanzee studies.*)

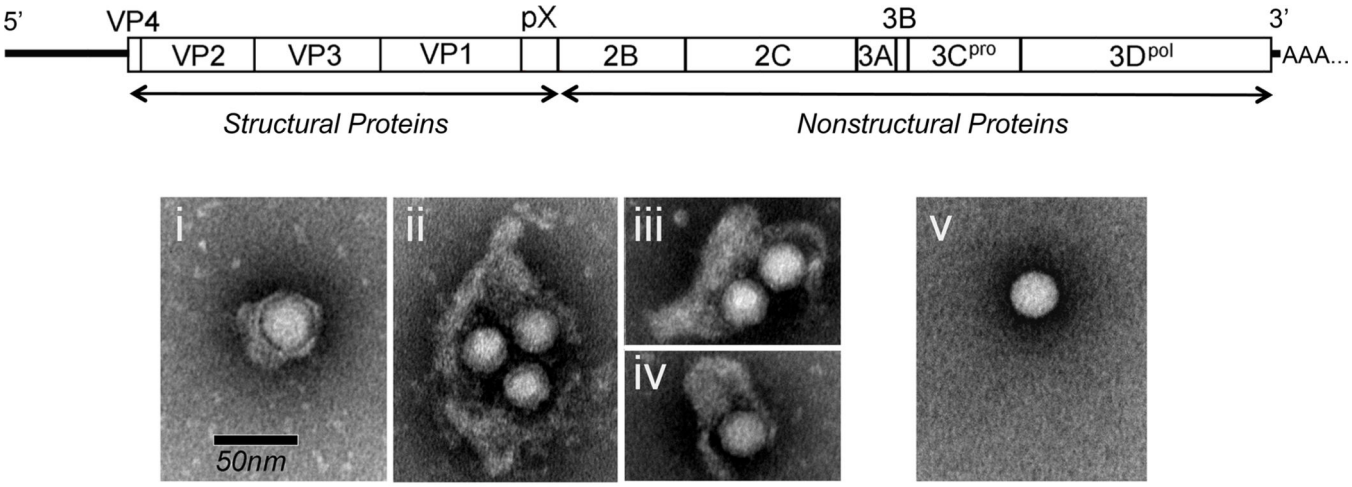

Figure 2.

Schematic showing organization of the 7.5 kb single-stranded, positive-sense RNA genome of HAV. The 2227 amino acid residue polyprotein is comprised of both structural and nonstructural proteins, and is flanked by 5. and 3. untranslated RNA segments containing regulatory elements. Below are shown electron microscopic images of gradient purified quasi-enveloped eHAV (panels i–iv) and naked, non-enveloped HAV (panel v) released from infected hepatoma cell cultures. (*Reproduced with permission from Feng et al. Nature 2013, 496:367–371*).

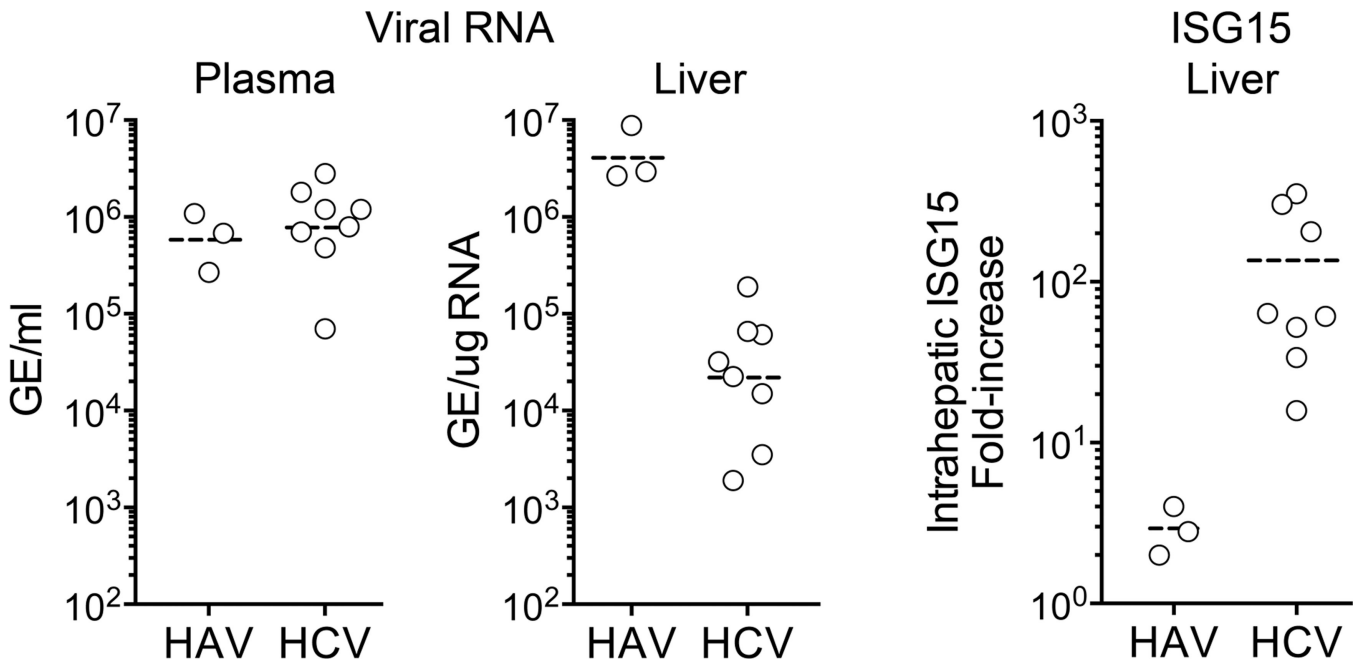

Figure 3.

Comparison of maximum intrahepatic and serum viral RNA abundance and interferon-stimulated gene (ISG15) expression in acute, resolving HAV (n = 3) and HCV (n = 8) infections in experimentally infected chimpanzees. Differences in intrahepatic genome copy numbers (p=0.01) and ISG expression (p=0.01) were significant by two-sided Mann-Whitney test. Adapted from Lanford et al. [12].

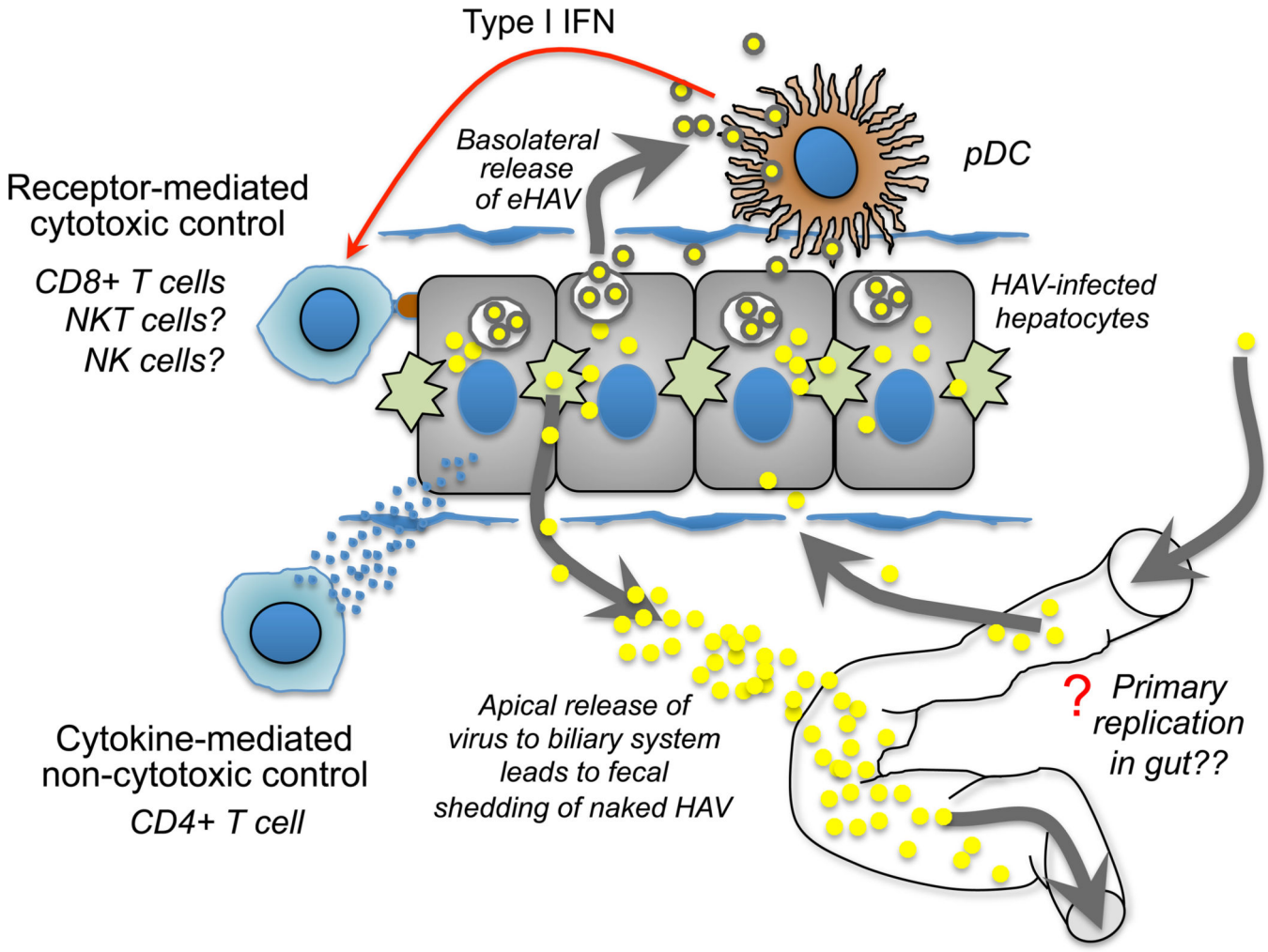

Figure 4.

Proposed cellular interactions in the liver during acute hepatitis A. HAV infection of the liver is thought to be non-cytopathic, resulting in release of quasi-enveloped virions from the basolateral plasma membrane of hepatocytes into the circulation and apical release of virus into the biliary system resulting in fecal shedding of naked HAV virions [6]. Contact between infected hepatocytes and plasmacytoid dendritic cells (pDC) results in transfer of eHAV to pDCs and signaling for production of type I IFN [20], a cytokine important to development of adaptive cellular immune responses. Cytotoxic cells, including virus-specific CD8+ T cells, NK cells, and NKT cells, have been implicated in acute hepatocellular injury during HAV infection. Liver damage can range from mild and inapparent, to severe and fatal, and may be regulated in part by the strength of the innate immune response, including type I IFN production by pDC. We propose that non-cytotoxic control of HAV replication is a central feature of infection and immune control of the virus regardless of disease severity. CD4+T helper cells are a potential source of antiviral cytokines because they appear in blood and acquire effector function earlier than CD8+ T cells [32], as shown in Figure 1. A primary site of virus replication within the gut after per-

oral infection with the virus, or secondary to shedding of virus from the liver, remains speculative but could influence infection and immunity in the liver.