Commentary

Intrahepatic T Cells in Hepatitis B: Viral Control versus Liver Cell Injury

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Clearance of the hepatitis B virus (HBV), a noncytopathic double-stranded DNA virus, requires the coordinated response of innate and adaptive, humoral and cellular immune systems. In more than 90% of immunocompetent adults who become infected, this immune response is quite vigorous, resulting in acute, self-limited hepatitis with rapid reduction of viral load and long-lasting, protective humoral and cellular immunity. However, in 5% of HBV-infected immunocompetent adults, and most cases of vertical transmission of HBV, persistent infection and chronic necroinflammatory liver disease evolve which may eventually lead to liver cirrhosis and hepatocellular carcinoma.

In HBV infection, the cellular immune response is thought to contribute to both viral clearance and liver cell injury. These two opposing functions have even been attributed to the same cell: upon cognate recognition of viral peptides on MHC class I molecules of HBV-infected cells, $CD8^+$ T cells acquire the capacity to either cure HBV-infected cells via noncytopathic, cytokine-mediated inhibition of HBV replication or to destroy them via perforin-, Fas ligand (FasL)-, and TNF-α–mediated death pathways. Both effector functions have been observed during resolution of acute hepatitis B (1).

Individuals with acute, self-limited HBV infection characteristically mount a vigorous, polyclonal, and multispecific Th and CTL response to epitopes within the HBV envelope (HBe), nucleocapsid, and polymerase proteins that is readily detectable in the peripheral blood. This response coincides with the maximum elevation of serum alanine aminotransferase (ALT) levels (2) and precedes clearance of HBe and surface (HBs) antigens and development of neutralizing antibodies (3). In contrast, the HBV-specific immune response is weak or undetectable in the blood of chronically infected patients, although individual, HBV-specific T cell clones have been isolated and expanded from liver biopsies (4, 5). Since HBV is considered a noncytopathic virus and the degree of the intrahepatic inflamma-

tory leukocytic infiltrate is regarded as the histologic hallmark of the severity of chronic hepatitis B, it has been postulated that the HBV-specific immune response is too weak to eliminate HBV from all infected hepatocytes, but sufficiently strong to continuously destroy HBV-infected hepatocytes and to induce chronic inflammatory liver in persistently infected individuals. The reason for this inefficient, yet harmful nature of the cellular immune response in chronic hepatitis B is currently not known. Is the absolute number of intrahepatic and circulating HBV-specific T cells too low to clear HBV early in the infection? Are HBV-specific T cells anergized by high viral load? Do intrahepatic HBV-specific T cells exert different effector functions in chronically infected patients than in those who control HBV replication? Do intrahepatic T cells recognize different, e.g., subdominant, HBV epitopes in chronically infected patients? Or are functional HBV-specific T cells antagonized by emerging HBV mutants?

Finding answers to these questions has been hampered by the fact that HBV does not grow in tissue culture and that the chimpanzee is the only animal that can be infected with the virus. HBV-specific T cells have been isolated from liver biopsies of chronically infected patients, but phenotypic and functional characterization generally required extensive in vitro expansion (4, 5). This technique unavoidably introduced a selection bias for T cells that could be expanded in tissue culture, and thus, allowed neither correct quantitation of HBV-specific T cells within the intrahepatic infiltrate nor direct ex vivo assessment of T cell function.

HBV-specific CTLs and Control of HBV Replication

In this issue, Maini et al. (6) use three HBV peptide HLA-A2 tetramers to compare the number and function of circulating and intrahepatic HBV-specific T cells in two well-defined groups of persistently infected, $HBsAg^+$ patients with chronic hepatitis B: a group of $HBeAg^-$ patients with low levels of viremia (<2 pg/ml serum), normal serum ALT levels, and no histologic evidence of intrahepatic inflammation, and a second group of $HBeAg^+$ patients with high levels of viremia (>800 pg/ml serum), elevated serum ALT levels, and histologic evidence of chronic hepatitis (6).

Using direct, ex vivo analysis of intrahepatic T cells with HBV epitope–specific HLA-A2 tetramers, a technique that

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does not require in vitro T cell expansion, Maini et al. (6) identified CD8$^+$ T cells, specific for the same HBV epitopes that were recognized by patients with self-limited hepatitis B (2), in the blood and livers of chronically infected patients who were able to control both viral load and liver injury. In the liver, CD8$^+$ T cells that recognized an HBV nucleocapsid epitope constituted up to 9% of all intrahepatic CD8$^+$ T cells and expressed the activation marker DR$^+$ (6). In the blood, these cells displayed the phenotype of antigen-experienced resting cells and were able to mount rapid IFN-γ, cytotoxic, and proliferative responses. Since none of the patients displayed neutralizing antibodies against HBsAg, the authors suggest that these intrahepatic HBV-specific CD8$^+$ T cells contributed to control of HBV replication and, importantly, that they were able to do so without inducing hepatic immunopathology. Thus, the results that Maini et al. obtained in persistently infected, HBeAg$^-$ patients with low viral load (6), and additional studies by other investigators (7, 8) are leading to a new appreciation of the function of HBV-specific CD8$^+$ T cells favoring a protective rather than a pathogenic role in HBV infection. In addition, several new and puzzling questions arise with regard to the intrahepatic infiltrate in HBeAg$^+$ patients with high viral load and elevated liver enzymes. These will be discussed below.

The report by Maini et al. (6) suggests that chronic hepatitis B is not characterized by complete absence of HBV-specific T cells—instead, there appears to be a balance between HBV-specific T cells and small amounts of replicating virus that can be maintained without induction of liver injury. Notably, similar findings have also been reported in patients who were studied decades after clinical recovery from acute and chronic HBV, even after serological clearance of all viral antigens, long-lasting neutralizing antibody responses, and immunity to reinfection (9–11). The strength of the HBV-specific CTL response after clinical recovery correlated with persistence of very small amounts of HBV DNA in the serum and PBMCs (10). Since the CTL response was specific for HBV nonstructural proteins that required synthesis by infected cells, these observations imply continuous priming of new CTLs by minute traces of transcriptionally active virus. HBV-specific antibodies and T cells, in turn, may prevent viral spread and reinfection and therefore control the remaining virus. Indeed, HBV reactivation or transmission has been reported if immunological control became impaired, e.g., during immunosuppression (12) and after transplantation of organs from recovered (anti-HBs$^+$, anti-HBcIgM$^-$) donors into immunosuppressed recipients (13). Conceivably, therefore, virus-specific T cells may persist not only in the peripheral blood, but also in the liver, as recently reported for intrahepatic hepatitis C virus (HCV)-specific CD8$^+$ T cells after recovery from acute, self-limited HCV infection (14).

How is this control mediated and why is it not effective in patients with high viral load? This is one of the key questions that arise from this study (6). Unfortunately, the number of tetramer$^+$ cells that were isolated from a given liver biopsy was too small to compare the function of HBV-specific, intrahepatic T cells in persistently infected patients who did or did not control viral load (6). Although Maini et al. (6) did not exclude cytotoxic effector functions of HBV-specific intrahepatic CD8$^+$ T cells, the sparse scattering of these T cells within liver lobules among a large number of hepatocytes suggests a more efficient mechanism that does not require "one to one" contact between effector and target cell. Specifically, IFN-α/β, IFN-γ, and TNF-α have been shown to downregulate HBV replication and gene expression noncytopathically in infected cells (8). HBV nucleocapsid particles, replicative viral intermediates, and the transcriptional template of the virus, the episomal covalently closed circular (ccc) HBV DNA, are all susceptible to these cytokine-mediated effects (7). This mechanism plays an important role in the early phase of acute HBV infection because downregulation of HBV replication precedes massive infiltration of CD8$^+$ T cells and manifestation of liver disease (7). Even a small number of HBV-specific, intrahepatic T cells may lead to sufficiently high intrahepatic cytokine concentrations to inhibit HBV replication. McClary et al. reported in a recent study that constitutive expression of IFN-γ contributed to a threefold downregulation of HBV replication in the liver of HBV transgenic mice compared with IFN-γ receptor and IFN-α/β receptor knockout mice (15). Moreover, IFN-γ, produced by HBsAg-specific, adoptively transferred CTLs, was sufficient to inhibit HBV replication in the livers of HBV transgenic mice that were either unable to respond to TNF-α or IFN-α/β (TNF-α receptor or IFN-α/β receptor knockout mice) or unable to produce IFN-γ (IFN-γ knockout mice), respectively (15). Thus, it is possible that curative, IFN-γ–mediated, rather than immunopathogenic, cytolytic effects of HBV-specific T cells prevail, if the intrahepatic infiltrate is small and HBV-specific CD8$^+$ T cells constitute most of it. In addition, IFN-γ secretion by intrahepatic CTLs may contribute to optimal in vivo processing of HBV antigens (16), since it was recently shown that an immunodominant HBV core CTL epitope was only generated by IFN-γ–induced immunoproteasomes, not by constitutive proteasomes (17).

Importantly, however, the same mechanisms that contribute to viral clearance in acute hepatitis B may also prevent complete recovery of chronically infected patients. Partial downregulation of HBV replication and antigen expression without complete viral clearance may allow infected cells to escape from immune recognition and may result in low level viral persistence in the absence of liver injury. This scenario may indeed reflect the apparent balance between low viral load, absence of liver injury, and resting, antigen-experienced T cells in the peripheral blood of persistently infected patients that Maini et al. described (6).

Function of HBV-specific T Cells—Influenced by Viral Load?

How does the intrahepatic cellular infiltrate of persistently infected patients with low viral load differ from that of patients with high viral load? As expected and typical for a severe, chronic active form of hepatitis, patients with high

viral load displayed a more extensive intrahepatic inflammatory infiltrate, extending from the portal areas into the liver parenchyma (6). Surprisingly, however, Maini et al. (6) now demonstrate that the absolute number of HBV-specific intrahepatic T cells that bind to the HB core (HBc)18–27 peptide HLA tetramer did not differ between patients with high and those with low viral load. Instead, HBc18–27-specific T cells were more diluted among liver infiltrates in highly viremic patients, indicating that the recruitment of these other cells may have contributed to liver injury. While quantitative differences of T cell populations that are specific for other HBV epitopes cannot be fully excluded, Maini et al. (6) raise the important question of whether the quality rather than the quantity of HBc18–27 specific T cell response differed between both patient groups.

Because of the limited number of lymphocytes that can be isolated from a liver biopsy, functional aspects of the HBV-specific immune response were assessed using HBV-specific T cells derived from the peripheral blood (6). This provided valuable and interesting, but not complete, information because circulating and intrahepatic HBV-specific T cells displayed different phenotypes with regard to their activation status, and thus may have differed in their effector functions such as cytotoxicity and IFN-γ production as well (6). HBV-specific CD8+ T cells, isolated from the blood of patients with low viral load and normal ALT values, displayed a resting phenotype, but mounted rapid and vigorous proliferative, IFN-γ, and cytotoxic responses upon reexposure to antigen in vitro. In contrast, the number of circulating HBV-specific T cells derived from the blood of patients with high viral load and elevated serum ALT levels was lower, and these cells displayed a poor expansion potential in vitro. Avoiding the bias introduced by prolonged cell culture, these results now confirm earlier analyses that depended on in vitro expansion of cells (11, 18). Since it has recently been suggested that individual effector functions may exhibit different sensitivity to stimulation as well as to functional impairment (19), it will be important to study additional effector functions of circulating and intrahepatic HBV-specific T cells in patients with high viral load with direct ex vivo techniques.

Impaired functions of virus-specific CD8+ T cells have also been reported in persistent infections with the lymphocytic choriomeningitis virus (LCMV) (20) and the neurotropic JHM strain of mouse hepatitis virus (JHMV), respectively (21). In acute viral infections, functional impairment of virus-specific CD8+ T cells has been observed when viral load of HBV (2) or LCMV (22) were high. Interestingly, impaired functional T cell responsiveness may be reversible, and the proliferative and cytotoxic function of HBV-specific T cells recovered after the acute phase of HBV when their frequency decreased and HBeAg was cleared (2). Similarly, a small percentage of chronically infected patients experience a spontaneous decrease of HBV DNA levels and clearance of HBeAg every year, a process that may be followed by clearance of HBsAg, development of neutralizing anti-HBs antibodies, and complete recovery

(23). A previously published prospective study supports the hypothesis that activation of HBV-specific T cell responses in the peripheral blood precedes spontaneous clearance of HBeAg in chronically infected patients (24) and suggests that latent, immune-mediated clearance mechanisms can become spontaneously activated in some individuals and may eventually contribute to clearance of HBsAg and complete recovery. Since Maini et al. (6) did not have the opportunity to prospectively study these patients, we cannot be sure that circulating HBV-specific CD8+ T cell responses increased before the decrease in viral load and clearance of HBeAg, or whether they appeared afterwards. This raises the possibility that the CTL response may have been secondary to the reduction in viral load rather than causing it. A recent publication supports this hypothesis (25). Treatment of HBV-infected patients with the antiviral agent lamivudine induced a reduction of viral load that was followed by recovery of functional, HBV-specific CD4+ T cell responses in the peripheral blood (25). Thus, the current study (6) also raises a cautionary note suggesting that therapeutic enhancement of HBV-specific T cell responses may not be sufficient to induce viral clearance and resolution of liver disease and instead, should be accompanied or preceded by attempts to reduce viral load.

Immunopathogenesis of HBV

Since the absolute number of tetramer+, HBc18–27-specific T cells did not differ between patients with and without liver injury (6), Maini et al. suggest that the remaining intrahepatic T cells that were not stained with this tetramer might be responsible for liver injury. Importantly, these unstained T cells were more frequently found in the liver of patients with elevated ALT levels than in those with normal ALT levels. Since this non-HBc18–27-specific cellular infiltrate may either consist of HBV-specific T cells that recognize different HBV epitopes or of HBV-nonspecific bystander cells, several questions arise that are not answered in this study (6): Is liver injury predominantly due to bystander effects of antigen-nonspecific cells? Is it due to direct interaction between liver-infiltrating cells and hepatocytes, or is it the consequence of the high turnover of liver-infiltrating CD8+ T cells that presumably do not leave the liver alive (26)?

During acute, self-limited HBV, maximum ALT levels coincide with intrahepatic infiltration of CD8+ T cells, suggesting that these cells mediate acute necroinflammatory liver disease (7). Proof of this hypothesis was derived from a series of experiments performed with transgenic mice that expressed either HBsAg or all HBV proteins in their hepatocytes and replicated the virus. Intravenous injection of HBsAg-specific CTLs induced an acute, self-limited necroinflammatory liver disease (27), and antigen recognition by the transferred CTLs was identified as the earliest detectable pathological event, followed by apoptosis of individual hepatocytes. Both FasL and perforin pathways had to be activated simultaneously by the same CTLs in order to kill the hepatocyte (28). Importantly, however, most of the liver injury was not mediated by HBV-specific CTLs, but

by antigen-nonspecific inflammatory cells that were recruited into the liver 4–12 h later (29). These cells surrounded HBV-specific CTLs and formed necroinflammatory foci with hepatocellular necrosis (29). Because of the limited life span of the transferred CTLs that initiated this cascade, the induced hepatitis was generally transient and nonfatal in these mice. If the intrahepatic inflammatory infiltrate initiated by HBV-specific T cells persisted, as observed in transgenic mouse models of chronic hepatitis B, chronic necroinflammatory liver disease (30, 31) and ultimately hepatocellular carcinoma (30) was induced.

Recruitment and Fate of Intrahepatic T Cells

Why do HBV-nonspecific cells migrate into the liver? And, provided that the majority of tetramer⁻ cells were indeed HBV nonspecific in the study by Maini et al. (6), why were they predominantly attracted into the livers of persistently infected, HBeAg⁺ patients with high viral load, and less frequently into those of persistently infected, HBeAg⁻ patients with low viral load? These questions lead again to the critical point of the study, to the function of HBV-specific intrahepatic T cells. If the absolute number of HBV-specific intrahepatic T cells was comparable in both patient groups, is it possible that functional differences extend not only to their antiviral effects as suggested by Maini et al. (6), but also to their ability to recruit antigen-nonspecific T cells? Or are the size and composition of the intrahepatic inflammatory infiltrate influenced by viral load and intrahepatic expression and secretion of HBeAg?

In addition, the kinetics of T cell recruitment into the liver and T cell turnover in the liver have to be considered. Low blood flow and branched structure of the intrahepatic vascular bed and mobility of the Kupffer cells facilitate recruitment of T cells from the peripheral blood (32) by expression of chemokines and adhesion molecules (26). Since certain adhesion molecules are constitutively expressed in the noninflamed liver and upregulated during infection, activated CD8⁺ T cells are continuously trapped in the liver regardless of the antigen specificity (26). Accordingly, a high percentage of influenza-specific T cells has been detected in the liver of influenza virus–infected mice (33). What is the fate of these T cells? Mehal et al. recently described that activated, CD8⁺ but not CD4⁺ T cells undergo apoptosis within 18 h of entry into the liver (26). Indeed, the liver has been described as a graveyard for activated T cells (33) and is thought to contribute to homeostasis of the T cell repertoire after pathogen-induced expansion of antigen-specific T cell clones.

Thus, even an equal number of HBV-specific T cells that Maini et al. (6) describe in liver biopsies of HBV-infected patients with high and low viral load may still be compatible with different rates of T cell recruitment and turnover. After antigen recognition and exertion of their specific effector function, virus-specific effector CTLs are prone to undergo activation-induced cell death. This cascade of events may occur rapidly in the presence of high concentrations of HBV antigens and the absence of sufficient costimulation. Notably, with regard to chronic HCV

infection, Nuti et al. estimated a loss of 2×10^8 T cells in the liver, which equals 0.1% of the total body lymphocytes, per day (32). Thus, an accelerated T cell turnover in the liver would be compatible with the lower frequencies of circulating antigen-specific T cells that Maini et al. (6) described in patients with high viral load. If, on the contrary, the concentration of intrahepatic viral antigens and the degree of inflammation are low, HBV-specific T cells may enter the liver less frequently and succumb to a slower death due to cytokine starvation. While this notion is strictly speculative at present, analysis of the kinetics of T cell induction and turnover may represent an important area of research, especially if the liver is the primary organ of viral replication.

Further Questions

The study and findings reported by Maini et al. (6) will certainly stimulate efforts to study both quantitative and qualitative aspects of the intrahepatic, HBV-specific cellular immune responses with techniques that allow direct, ex vivo analysis of antigen-specific T cells. In addition, they may draw attention to the composition, effector function, migration pattern, and fate of the intrahepatic cellular infiltrate in chronic HBV. Several interesting questions are raised and remain to be addressed.

The first set of questions addresses the functional state of intrahepatic, HBV-specific T cells in chronically infected patients with high viral load and evidence of liver injury. Why are these intrahepatic HBV-specific T cells not able to exert sufficient antiviral control? Note that they are found in the same quantity as in the liver of patients who control viral load. Are they inhibited or are their effects outnumbered by supposedly HBV-nonspecific T cells? Do HBV-nonspecific bystander cells shift the cytokine balance in an unfavorable way? Or are HBV-specific T cells anergized, as recently reported in a prospective study of patients with chronically evolving, acute HBV (34)? If the number of intrahepatic T cells is the same in both patient groups, it should be possible, although technically difficult, to compare their phenotype and function with direct, ex vivo techniques.

Related questions address the phenotype, specificity, and function of the intrahepatic cellular infiltrate that did not react with the HBV tetramers used in this study: Are these lymphocytes HBV-nonspecific bystander cells or are they targeted against other, potentially subdominant HBV epitopes? If they are HBV-nonspecific bystander cells, why do they become activated during HBV infection? Do they express specific chemokine receptor, such as CC chemokine receptor 5 (CCR5), that attracts them to the liver? What is the fate and life span of HBV-specific and -nonspecific T cells once they reach the liver? Can migration and/or function of these liver-infiltrating HBV-nonspecific T cells be inhibited?

Finally, the last group of questions addresses the function and role of specialized antigen-presenting cells that are characteristic for the liver. Both Kupffer cells and sinusoidal endothelial cells have been shown to internalize and

present soluble antigen and thus, may exert immune regulatory functions. Do they exert stimulatory (35) or tolerogenic (36) effects on intrahepatic lymphocytes? Do they initiate recruitment of antigen-nonspecific cells? Which stimuli are required to induce production of antiviral cytokines in intrahepatic macrophages, which are considered potent producers of TNF-α and IFN-α/β and play an important role in downregulation of HBV replication? Is their function impaired in chronic HBV?

Answers to these questions may contribute to our future understanding of the immunopathogenesis of chronic HBV and the tight balance between liver injury and viral control. Since both liver injury and viral control can be mediated by the immune system of the infected host, more information is needed before antigen-specific immunotherapy of chronic HBV infection should be attempted.

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