

Review

HBV pathogenesis in animal models: Recent advances on the role of platelets[☆]

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Hepatitis B virus (HBV) causes acute and chronic necroinflammatory liver diseases and hepatocellular carcinoma (HCC). HBV replicates noncytopathically in the hepatocyte, and most of the liver injury associated with this infection reflects the immune response. While the innate immune response may not contribute significantly to the pathogenesis of liver disease or viral clearance, the adaptive immune response, particularly the cytotoxic T lymphocyte (CTL) response, contributes to both. Recent observations also reveal that antigen-nonspecific inflammatory cells enhance CTL-induced liver pathology and, more surprisingly, that platelets facilitate the intrahepatic accumulation of CTLs, suggesting that the host response to HBV infection is a highly complex but coordinated process. The notion that platelets contribute to liver disease and viral clearance by promoting the recruitment of virus-specific CTLs into the liver is a new concept in viral pathogenesis, which may prove useful to implement treatments of chronic HBV infection in man.

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1. Introduction

HBV is an enveloped, noncytopathic and hepatotropic DNA virus that causes a liver disease of variable duration and severity [1]. Over 95% of acutely infected adults completely and spontaneously recover from the infection, while most neonatally transmitted infections become persistent [2]. Chronic HBV infection often progresses to the development of life-threatening complications such as cirrhosis and hepatocellular carcinoma (HCC) [3]. On a worldwide basis, over 350 million people are chronically infected by HBV and about 1 million of them die each year from the complications of chronic infection [2]. As many of these patients do not have a sustained response to currently available therapies (nucleoside analogues and/or interferon) [2], it is very

important to improve our understanding of HBV pathogenesis if we are to develop better treatments.

The experimental approaches examining HBV pathogenesis have been difficult because the host range of HBV is limited to man and chimpanzees, and because *in vitro* systems for the propagation of HBV do not exist. Studies of HBV pathogenesis using models of HBV-related hepadnavirus infections in the woodchuck, ground squirrel and Pekin duck have also been difficult because the immune systems of these outbred species have not been characterized. To overcome these limitations, we developed transgenic mice that express the viral genes individually [4–7], and also mice that express all of the viral gene products and replicate HBV at high levels in the primary hepatocyte *in vivo* [8].

The availability of these small animal models with a well-defined immune system and the experimental use of HBV-infected chimpanzees have helped to elucidate in recent years many immunological mechanisms involved in HBV pathogenesis [1]. In the course of these studies several other previously unknown aspects of viral pathogenesis have been discovered, and they repre-

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sent the main subject of this review. In particular, we will examine current evidence pertaining to the pathogenetic and antiviral role of innate and adaptive immune responses, with particular emphasis on the cellular and molecular mechanisms responsible for liver damage and the role that platelets play in these processes.

2. Innate immune responses

As viruses infect target cells, the host rapidly triggers early innate defense mechanisms in order to contain viral spread. These mechanisms comprise the induction of apoptosis by the virus [9], the production of antiviral cytokines such as IFN- α/β by the infected cells [10], and the activation of effector functions of cellular components of the innate immune system (such as NK and NKT cells) [11].

There is currently no evidence that HBV can trigger apoptosis. During the early phase of HBV infection in chimpanzees (i.e., before virus-specific T cells enter the liver) there is no histological or biochemical evidence of hepatocellular injury [12,13]. In addition, HBV is able to replicate at high levels in the liver of both patients and transgenic mice noncytopathically, when cellular immune responses are pharmacologically suppressed, anergic or deleted [1,8]. The evidence that HBV induces the production of IFN- α/β by the infected cells is also lacking. Indeed, global gene expression profiling performed on liver RNA samples from HBV-infected chimpanzees and transgenic mice indicates that no IFN- α/β or IFN- α/β -responsive genes are induced in the organ before the entry of adaptive immune responses [14]. Although activation of NK cells, NKT cells or engagement of Toll-like receptors has been shown to inhibit viral replication in HBV transgenic mice [15–17], there is still no evidence that these cells or pathways of the innate immune system play a role in disease pathogenesis or viral clearance during the initial phase of HBV infection. Indeed, activated NK cells and NKT cells are an abundant source of IFN- γ [18,19], but neither IFN- γ nor IFN- γ -inducible genes are detected in the liver of chimpanzees as HBV spreads throughout the organ noncytopathically [12,13]. Collectively, these results indicate that early innate defense mechanisms may not significantly contribute to the pathogenesis of liver injury or to viral clearance, and that HBV remains quite undetected until the adaptive immune response enters the liver.

3. Adaptive immune responses

Virus-specific CD4⁺ T helper cells and CD8⁺ CTLs participate in tissue damage and viral clearance either by killing infected cells or by producing soluble factors

such as cytokines and chemokines that contribute to the inflammatory process and/or inhibit viral replication [20]. T-cell derived cytokines and chemokines also promote the shaping of antiviral antibody responses that take part in viral clearance, mainly by blocking virus entry into susceptible cells and by removing infectious virions from the circulation.

As mentioned earlier, we lack cell culture systems capable of being productively infected by HBV. Therefore, the kinetics and function of neutralizing Abs in the resolution or prevention of HBV is still poorly understood. Evidence that Abs with neutralizing activity emerge following a self-limited HBV infection is supported by the observation that chimpanzees that resolved a previous infection are completely protected from rechallenge [21]. The appearance of neutralizing Abs, however, is thought to occur relatively late after HBV exposure and, thus, it is unlikely to contribute to the early phase of viral clearance during acute infection [21].

It is also unlikely that CD4⁺ T helper cells play an important pathogenetic role in HBV infection, despite the fact that they have been shown to have cytolytic activity in other infection systems [22]. Indeed, recent observations in an acutely HBV-infected chimpanzee that was depleted of CD4⁺ T cells at the peak of infection indicated that the liver disease in this animal was comparable to that detected in immunologically unmanipulated controls [13]. Thus, CD4⁺ T helper cells may contribute to the control of HBV infection mainly by facilitating the induction and maintenance of virus-specific CTLs, as has been suggested for hepatitis C virus (HCV) [23]. In keeping with this, relatively vigorous HBV-specific T helper responses are always associated with quantitatively and qualitatively significant CTL responses in humans and chimpanzees that resolve HBV infection [21].

In contrast to CD4⁺ T helper cells that are primed within secondary lymphoid organs by antigen presenting cells (APCs) that have internalized soluble viral antigens, priming of CTLs requires the processing of viral proteins that are either endogenously produced within or phagocytosed by professional APCs [24,25]. For viruses like HBV that do not considerably infect professional APCs, tissue-derived dendritic cells that have internalized apoptotic virus-infected cells and debris are expected to migrate to the regional lymph nodes to permit T cell priming to occur [24,25]. The recognition of antigen by naïve CD8⁺ CTL precursors circulating through secondary lymphoid organs initiates the development of effector CTLs, which clonally expand and leave the lymph nodes, due to the altered expression of surface molecules [26]. Indeed, developing effector CTLs modify the expression of selectin ligands such as P-selectin glycoprotein ligand (PSGL)-1 [27], as well as distinct chemokine receptors and integrins, which direct their

recruitment to nonlymphoid vascular beds, like those of the liver [28]. It is also noteworthy that activated platelets express abundant P-selectin (see below) and functional PSGL-1 is expressed at particularly high levels in effector T cells polarized in the direction of type 1 [29,30], like the murine HBV-specific CTL lines and clones we have used over the years in many studies involving HBV transgenic mice.

Effector CD8⁺ CTLs are thought to play a fundamental role in the pathogenesis of liver disease and viral clearance during HBV infection, and this is supported by the following data. First, the beginning of liver injury kinetically corresponds with the influx of virus-specific CTLs into the liver of chimpanzees infected by HBV, and depletion of these cells at the peak of viremia delays the onset of biochemical, histological and clinical evidence of viral hepatitis as well as viral clearance [12,13]. Second, the association between the magnitude of virus-specific CTL responses, liver disease severity and viral clearance has been reported not only in infected chimpanzees but also in many studies of patients infected with HBV. Indeed, patients with acute viral hepatitis, who successfully clear HBV, mount a relatively vigorous multispecific polyclonal CTL response to several HBV-encoded antigens that is usually associated with a relatively severe degree of hepatocyte damage [21,31]. In contrast, liver cell injury is rather limited in chronically infected patients in whom the CTL response is extremely weak and narrowly focused [21,31]. Third, the adoptive transfer of HBV-specific CTL lines and clones into immunologically tolerant HBV transgenic mice triggers a necroinflammatory liver disease that shares the same histologic features of acute viral hepatitis in man and results in the inhibition of HBV replication [32].

The latter studies in mice have also taught us that the antiviral potential of virus-specific CTLs is largely mediated by noncytolytic mechanisms involving the local production of IFN- γ by these cells early after antigen recognition [32]. Indeed, it has been reported that IFN- γ (mostly *via* its capacity to induce nitric oxide in the liver [33]) prevents the assembly of replication-competent HBV RNA-containing capsids in the hepatocyte [34] in a proteasome- [35] and kinase-dependent [36] manner. During this process, the viral nucleocapsids disappear from the cytoplasm of the hepatocytes [37] and the viral RNAs are destabilized by a SSB/La-dependent mechanism in the nucleus [38–40], yet the hepatocytes remain perfectly healthy. The notion that IFN- γ produced by activated CTLs plays a direct role in viral clearance is corroborated by studies in chimpanzees acutely infected with HBV. It was shown in those animals that most of the viral DNA disappeared from the liver before the peak of liver disease, concomitant with the initial intrahepatic appearance of IFN- γ [12]. Moreover, neither intrahepatic IFN- γ

induction nor viral clearance occurred in HBV-infected chimpanzees that were depleted of CTLs at the peak of infection [13].

Interestingly, recent work in the HBV transgenic mouse model indicates that, upon entry into the liver, the capacity of virus-specific effector CTLs to secrete IFN- γ rapidly subsides, and this phenotype is maintained until HBV antigens are cleared from the liver [41]. The results suggest that sustained antigen stimulation (as it occurs during chronic infection) is responsible for the lack of IFN- γ production by CTLs. Even more interesting is the observation that this process is kinetically followed by the intrahepatic expansion of IFN- γ -nonproducing CTLs with increased cytotoxic capabilities [41], suggesting that the antiviral (i.e., production of IFN- γ), but not pathogenetic (i.e., killing of hepatocytes), potential of intrahepatic CTLs is likely to be impaired in chronically infected patients. According to this scenario, chronic HBV infection may be characterized by a numerically deficient CTL response that contributes more to liver damage than to viral clearance.

Based on the aforementioned results, it is apparent that HBV replicates noncytopathically in the hepatocyte, and that most of the liver damage associated with this infection reflects the immune response. It is also evident that the innate immune response does not contribute significantly to the pathogenesis of liver disease or viral clearance, while the adaptive immune response, especially the virus-specific CTL response, contributes to both. Although hepatocellular injury is initiated and mediated by the CTLs, however, it is becoming increasingly clear that antigen-nonspecific inflammatory cells exacerbate CTL-induced immunopathology. Results pertaining to this subject are summarized below.

4. The pathogenetic role of antigen-nonspecific inflammatory cells

As mentioned above, we produced HBV-replicating transgenic mice that show no signs of liver disease [8] until the adoptive transfer of virus-specific CTLs, whereupon they develop a necroinflammatory liver disease that is histologically similar to acute viral hepatitis in man [32]. As CTLs reach the liver parenchyma, the first step in the disease process is antigen recognition by these cells, which rapidly induces hepatocellular apoptosis [42]. The initial apoptotic process, however, involves a relatively small number of hepatocytes. As time progresses, many host-derived, antigen-nonspecific inflammatory cells are recruited into the liver, thereby contributing to the formation of necroinflammatory foci scattered throughout the liver parenchyma, in which apoptotic hepatocytes and virus-specific CTLs are outnumbered by host-derived mononuclear and polymorphonuclear inflammatory cells [43].

The recruitment process of antigen-nonspecific mononuclear cells is a chemokine-dependent event, since blocking the chemokines CXCL9 and CXCL10 reduces the trafficking of these cells into the liver without affecting the homing capacity of virus-specific CTLs and polymorphonuclear neutrophils (PMNs) [44]. The association of reduced liver disease with reduced recruitment of antigen-nonspecific mononuclear cells implies that these cells can amplify the liver damage initiated by the CTLs. Similar mechanisms may contribute to the pathogenesis of viral hepatitis in man, where, as in our system, the number of HBV-specific T cells detected in the liver is outnumbered by recruited nonvirus-specific T cells and other mononuclear inflammatory cells [45].

Further studies also demonstrated that the severity of CTL-induced liver disease in this model is ameliorated by the depletion of Gr-1⁺ cells (Gr-1 is an antigen highly expressed by PMNs), which, secondarily, abolishes the intrahepatic recruitment of all antigen-nonspecific Gr-1⁻ mononuclear cells (NK and NKT cells, T and B lymphocytes, monocytes and, macrophages, dendritic cells) despite the strong induction of chemokine gene expression [46]. Those results suggest that in addition to chemokine expression, other CTL-induced functions are necessary for mononuclear cell recruitment to occur. These functions likely include the production of matrix-degrading metalloproteinases (such as MMP-8 and MMP-9) by PMNs, since these enzymes are rapidly activated into the liver after CTL transfer, and their functional inhibition reduces the intrahepatic recruitment of antigen-nonspecific mononuclear cells and much of the attendant liver disease [47]. The results are compatible with the hypothesis that PMNs are the first cell type to be recruited into the liver following antigen recognition by the CTLs. According to this, the production of MMPs by PMNs may cleave components of the extra-

cellular matrix and, thus, facilitate the trafficking of mononuclear cells into the liver parenchyma in response to their own chemoattractants (i.e., CXCL9 and CXCL10).

Recent data also indicate that hepatocellular damage itself may be involved in the initial CTL-induced recruitment of PMNs in our model. Studies by others have shown that high-mobility group box 1 (HMGB1) protein, an abundant nuclear protein acting as an architectural chromatin-binding factor, can be passively released by necrotic or damaged cells and chemoattract PMNs [48,49]. Following transfer of virus-specific CTLs into HBV transgenic mice, HMGB1 translocates from the nucleus to the cytoplasm of injured hepatocytes, and this phenotype likely leads to the release/secretion of HMGB1 into the extracellular space [50]. Treatment of CTL-injected HBV transgenic mice with HMGB1 inhibitors decreases the intrahepatic recruitment of PMNs and, secondarily, the homing of antigen-nonspecific mononuclear cells that amplify the CTL-induced liver damage [50]. A cartoon summarizing the results we described thus far in this section is provided in Fig. 1.

It is also noteworthy that HMGB1 inhibitors did not impair the homing capacity of HBV-specific CTLs [50], similar to what occurred when we blocked CXCL9 and CXCL10, we depleted PMNs or we inhibited the function of PMN-dependent MMPs [44,46,47]. These results indicate that antigen-specific CTLs can enter the liver parenchyma and recognize antigen independently of HMGB1, CXCL9, CXCL10 or PMNs, suggesting that other processes may mediate their intrahepatic recruitment.

As mentioned in our Summary, recent observations pertaining to the role of platelets in the trafficking of CTLs within the liver provided new insight regarding this issue. Interestingly, this work (which will be described at length in the following section) was preced-

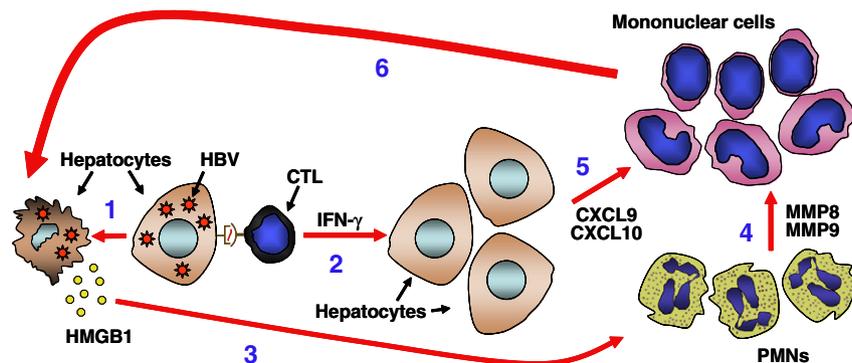


Fig. 1. Pathogenesis of liver disease and viral clearance in HBV transgenic mice. Following antigen recognition, HBV-specific CTLs (CTL) get activated and kill a small number of hepatocytes (1). Activated CTLs also secrete IFN- γ (2), which inhibits viral replication noncytopathically. Damaged or necrotic hepatocytes release HMGB1 (3), which attracts antigen-nonspecific polymorphonuclear cells (PMNs) into the liver. Production of MMPs by these cells (4) remodels the extracellular matrix and facilitates the intrahepatic migration of antigen-nonspecific mononuclear cells (i.e., NK cells, T and B cells and monocytes). The migration of these mononuclear cells also requires CXCL9 and CXCL10 (5), two chemokines produced by parenchymal and nonparenchymal cells of the liver in response to IFN- γ . Once they reach the liver parenchyma, antigen-nonspecific mononuclear cells amplify the liver disease initiated by the CTLs (6).

ed by the observation that the intrahepatic production of nitric oxide (a potent inhibitor of platelet activation [51]) is responsible for limiting CTL recruitment and liver disease severity in our HBV transgenic mouse system as well as in normal inbred mice infected with hepatotropic viruses [33].

5. The role of platelets in HBV pathogenesis

It is long known that by their capacity to adhere to areas of vascular injury, platelets become activated, aggregate and contribute to the formation of vessel-repairing clots [52,53]. Besides being cellular effectors of hemostasis, platelets are rapidly deployed to sites of injury or infection and potentially modulate inflammatory processes by interacting with leukocytes and by secreting cytokines, chemokines and other inflammatory mediators [52,53].

Using two different models of acute viral hepatitis, HBV transgenic mice as recipients of HBV-specific CTLs and normal inbred mice acutely infected with adenovirus, we recently showed that platelets are detectable within CTL-containing intrahepatic necroinflammatory foci, alongside apoptotic hepatocytes and inflammatory cells (including virus-specific CTLs) [54]. Importantly, platelet depletion greatly ameliorates the severity of liver disease [54]. The profound reduction in liver disease severity observed in platelet-depleted animals is associated with a nearly proportional reduction in the intrahepatic accumulation of virus-specific effector CTLs, both of which are restored upon reconstitution with normal platelets, but not upon reconstitution with platelets treated with prostaglandin (PG) E₁, (a known inhibitor of platelet activation) [54]. Although reduced in number, CTLs recovered from the liver of animals whose platelets are either absent or incapable of becoming activated are intact in their capacity to kill target cells, produce cytokines and chemokines and recruit antigen-nonspecific inflammatory cells into the infected organ [54].

The mechanisms through which platelets facilitate the intrahepatic accumulation of CTLs are poorly understood, and efforts to elucidate these processes are currently pursued. *In vitro* findings suggest that, under the low shear rate flow conditions likely to occur in the venous circulation of the liver, virus-specific effector CTLs tightly interact with activated platelet and, again, this process is inhibited when platelets are treated with PGE₁ [54]. Thus, the results of *in vivo* and *in vitro* studies are in agreement with the hypothesis that an initial inflammatory response within the liver may result in changes of the vessel wall that promote platelet activation and activation-dependent events resulting in interaction with CTLs. This interaction may eventually facilitate CTLs to egress from the bloodstream, enter the liver parenchyma and perform pathogenetic and/or

antiviral functions. A cartoon summarizing this concept is provided in Fig. 2.

Platelet activation is a complex process that includes cytoskeletal assembly and shape changes, secretion of agonists such as thromboxane A₂ (TXA₂) and ADP that promote further activation and aggregation, and functional expression of molecules such as P-selectin, GPIIb/IIIa or PSGL-1 [52,53,55] that can mediate leukocyte interaction. Pertinent to this, it is noteworthy that platelet P-selectin has been shown to interact with PSGL-1 on leukocytes (including T cells) and promote their rolling along the endothelium of lymph nodes [56]. Upon interaction with platelets (*via* platelet P-selectin), leukocytes are also thought to roll on the endothelium of cutaneous post-capillary venules thanks to platelet expression of PSGL-1 and GPIIb/IIIa [57]. While platelet PSGL-1 may facilitate this process by directly binding endothelial P-selectin, platelet GPIIb/IIIa may do so by binding fibrinogen, which secondarily interacts with endothelial ICAM-1 [57]. Along these lines, intravital microscopy studies in mesenteric venules have recently suggested that, after directly supporting an initial rolling of leukocytes in a P-selectin-dependent manner, platelets (again through P-selectin) stimulate endothelial cells to become activated, express P-selectin themselves, and further sustain leukocyte rolling [58]. Following ischemia-reperfusion liver injury, the rolling of polymorphonuclear neutrophils (PMNs) on the endothelium of post-sinusoidal venules has also been shown to require P-selectin [59].

The aforementioned results suggest that molecules such as P-selectin, PSGL-1 and/or GPIIb/IIIa could be

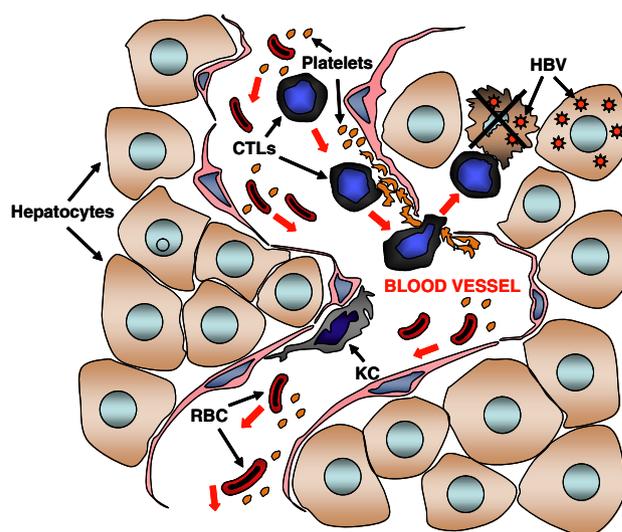


Fig. 2. Platelets facilitate the accumulation of CTLs in the infected liver. Inflammation-induced changes of the vessel wall may promote platelet adhesion and activation, which in turn favor the exit of virus-specific CTLs from the bloodstream and their accumulation within the liver parenchyma where HBV is replicating. RBC, red blood cells; KC, Kupffer cells.

involved in mediating platelet/CTL interactions within the inflamed liver. Through the use of intravital microscopy studies, platelets, virus-specific CTLs, HBV transgenic mice, adenovirus-infected normal inbred mice and mice genetically deficient for molecules such as P-selectin, PSGL-1 and GPIIbIIIa, we plan to address this hypothesis in the future. Defining the molecular mechanisms whereby platelets interact with CTLs and mediate liver disease and/or viral clearance may shed new light on previously unknown and important aspects of the pathogenesis of HBV and possibly other viruses (i.e., HCV) which infect the liver and whose clearance depends on CTLs [23,60].

In addition to P-selectin, PSGL-1 and GPIIbIIIa, activated platelets express CD154 (or CD40L), cytokines such as interleukin (IL)-1- β and chemokines such as CCL5 (i.e., RANTES), all of which have the potential to induce endothelial cells to become activated and facilitate leukocyte rolling through the expression of integrins and intercellular (ICAMs) or vascular (VCAM-1) adhesion molecules [52,53]. Although it has been reported that platelet CD154 induces (at least under nonflow conditions *in vitro*) endothelial expression of adhesion molecules [61], its involvement in leukocyte rolling has been denied by intravital microscopic studies within mesenteric venules [58]. Notably, both IL-1- β and CCL5 are rapidly and strongly expressed in the liver of CTL-injected HBV transgenic mice [44,62]. Passive neutralization of IL-1- β in these animals, however, affects neither the pathogenetic nor the antiviral potential of CTLs [62], indicating that this cytokine is likely not involved in our system. As per CCL5, it is worth mentioning that its secretion and/or deposition on the microvasculature occurs in a platelet P-selectin-dependent manner [63].

While the identification of the molecules involved in platelet/CTL interaction remains to be determined, the aforementioned data suggest that pharmacologic intervention targeting the pro-activating functions of platelet agonists may result in the inhibition of CTL recruitment into the liver and the amelioration of CTL-induced liver disease. Indeed, preliminary experiments indicate that the combined administration of aspirin and clopidogrel drastically inhibited both events in our mouse models of CTL-mediated acute viral hepatitis without causing bleeding side effects (Iannacone et al., unpublished data). Aspirin, the most widely used inhibitor of platelet function, affects TXA₂ production by irreversibly inactivating cyclooxygenase-1 (COX-1), thus, blocking a feedback activation mechanism [64]. Clopidogrel is a prodrug that needs to be converted into an active metabolite by the liver and irreversibly inactivates the ADP-receptor P2Y₁₂, which is required for stable platelet aggregation [65,66]. Of note, aspirin and clopidogrel not only inhibit platelet activation and aggregation in an additive manner [64], but also reduce P-selectin

expression, GPIIbIIIa activation and the formation of platelet–leukocyte aggregates [67–69].

The notion that anti-platelet treatment diminishes the severity of CTL-induced liver disease has therapeutic potential for the treatment of chronic HBV infection in man. Indeed, chronic HBV infection is characterized by an inefficient CTL response that is unable to completely eradicate the virus from the liver, often resulting in continuous cycles of low-level liver cell destruction. Persistence of these events for long periods of time is a major cause for the development of life-threatening complications, including HCC. As such, the CTL response in chronically infected patients may cause more harm than good, and, therefore, it is of some interest determining whether continuous administration of aspirin/clopidogrel may reduce its detrimental impact. For this reason, we plan to define in the future whether this aspirin/clopidogrel treatment may prevent the onset or decrease the incidence of HCC in a HBV transgenic mouse model of CTL-mediated chronic liver injury [70,71]. We believe that findings emerging from these studies may set an important proof of principle for a pharmacologically based anti-platelet treatment that would control unfavorable immunopathology without causing generalized immune suppression. This knowledge may also pave the road for the design of new and more specific platelet inhibitors (like small molecules inhibiting platelet/CTL interactions).

6. Concluding remarks

Our comprehension of the pathogenesis of HBV infection has significantly advanced in recent years, particularly because of the experimental use of chimpanzees and transgenic mice. It is becoming increasingly apparent that HBV replicates noncytopathically within the hepatocyte, and that the adaptive immune response, mainly the virus-specific CTL response, plays a crucial role in both liver disease and viral clearance. Recent studies also indicate that antigen-nonspecific inflammatory cells enhance CTL-induced immunopathology in the liver, and that platelets are required for virus-specific CTLs to accumulate within the liver and perform pathogenetic and/or antiviral effector functions. Future work intended to address the molecular basis of platelet/CTL interactions will not only expand our current knowledge of the host-virus relations that determine the pathogenesis of infection, but they may also guide us to the discovery of new approaches for the treatment of chronic HBV infection and its life-threatening complications.

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