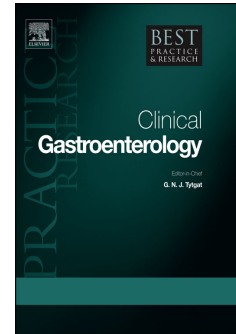


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# Hepatitis B virus infection and the immune response: The big questions

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## Practice Points

- Clinical events during the natural history of chronic hepatitis B virus (HBV) are intricately linked to the host immune system, but the details of host-virus interactions remain insufficiently understood
- HBV is sensed by the immune system, but the ensuing antiviral responses are not sufficient to prevent chronicity in most newborns and some adults

## Research Agenda

- To understand the basic mechanisms of HBV clearance, persistence and long lasting HBV cure by using the most advanced and detailed biomedical techniques, down to the single cell level.
- To gather more information on the intrahepatic, rather than the peripheral, immune populations, as their phenotype and function are very different
- To focus on longitudinal assessment of immune parameters, thereby excluding effects of cohort variation in HBV research

36 **Abstract**

37 Clinical events and the host immune response during hepatitis B virus (HBV) infection are  
38 intricately linked. Despite decades of research, important questions concerning the  
39 immunopathogenesis of chronic HBV infection remain unanswered. For example, it is  
40 unclear which immune parameters facilitate persistence, and if HBV can be completely  
41 cleared from the human liver. Recent technological breakthroughs now allow researchers to  
42 address these seemingly basic, but essential questions surrounding HBV immunity. It will be  
43 important to better define the molecular underpinnings of immune cell function and  
44 dysfunction during chronic disease and in controlled infection, with particular focus on the  
45 liver, as little information is available on the intrahepatic compartment. In the near future, it  
46 may be possible to solve some of the controversy surrounding the immune responses to  
47 HBV, and establish the features of both the innate and adaptive arms of the immune system  
48 required to achieve sustained control of HBV infection.

## 49 **HBV and the immune response: The big questions**

50 The natural history of HBV infection is exceptionally complex, and, not surprisingly, so are  
51 the host immune responses. For almost every clinical or virological change during the course  
52 of HBV infection, there are proven or suspected correlates in host immunity, some of which  
53 might represent the underlying cause of the observed alterations in disease, while others will  
54 be consequences of the change, for example in viral load. Recent advances in experimental  
55 and analytical capabilities now allow researchers to address seemingly basic but still  
56 unsolved questions surrounding HBV immunity. We have formulated several questions  
57 regarding host-virus interactions whose answers we deem most relevant for a better  
58 understanding of HBV immunopathogenesis and for the development of novel therapeutic  
59 strategies (Figure 1). After addressing these issues, we will highlight some of the progress  
60 that has already been made in the field of chronic HBV.

61 First is the question of what determines the different outcome in acute HBV infection,  
62 as the virus is spontaneously controlled in some subjects, while becoming chronic in others.  
63 Here we have to consider two completely distinct scenarios: adult HBV infection typically  
64 leads to HBV control, whereas natal infection results in chronicity in almost all cases, with  
65 declining but still very significant chronicity rates if infection occurs before the age of 5.  
66 However, not much is known of the immunological processes, resulting in these striking  
67 differences. We know much more about acute and chronic infection “after the fact”, i.e. in  
68 adult patients who have established chronic infection or successful HBV control. Studies  
69 during the early phase of infection, when the outcome is not yet determined, are much more  
70 difficult to undertake. The best opportunity for further insights will be studies in young  
71 children who present with early infection. Immunological studies in this population are  
72 feasible, but until recently had to be very limited in scope, in part because only small  
73 amounts of blood can be drawn for analysis. New technologies now enable analysis of low  
74 volume samples with high resolution and without the requirement of research facilities close  
75 to the clinic.

76 Immune responses of chronically infected adults have been performed for decades,  
77 but, as we will describe in this review, often have conflicting results. These variations are  
78 partly caused by methodological differences, but the variation among patient characteristics  
79 also has significant impact [1]. In addition to establishing the range and variation of immune  
80 cell parameters during frequent subtypes of chronic infection, we also need to know if, and to  
81 what degree, antiviral therapy is able to reconstitute those parts of the HBV immune  
82 response previously insufficient for functional control. At least based on the different antibody  
83 patterns observed in treated patients, it seems obvious that restoration of immunity is not  
84 uniform, and certainly in most subjects not sufficient for viral control in the absence of

85 therapy. But what exactly is restored, whether immune restoration is just a consequence of  
86 diminished viral replication that is lost in the absence of antiviral treatment, or whether  
87 restored immunity is independent or even the cause of viral suppression, needs to be  
88 evaluated in much more detail. In this context it is also important to define whether circulating  
89 viral proteins, such as HBs antigen (HBsAg), are indeed the key immunosuppressive agents  
90 that *in vitro* studies have suggested[2].

91 Another important question is what level of HBV control can actually be achieved  
92 through the host immune response. It is clear that even after HBsAg clearance from the  
93 blood, covalently closed circular DNA (cccDNA) can remain in hepatocytes indeterminately,  
94 evident from the well-documented cases of HBV reactivation in anti-HBsAg positive subjects  
95 undergoing immune-ablative therapies. Whether this is the dominant or even the only  
96 scenario in “resolved” HBV infection, or whether some patients are indeed able to completely  
97 clear HBV DNA based on an even more effective immune response, will require larger  
98 studies analyzing liver tissue for cccDNA in these populations.

99 Finally, greater insights need to be generated into what actually happens in the liver  
100 as the site of infection, since both the composition of immune populations as well as their  
101 functional and phenotypic profiles are different from what is observed in the blood. New  
102 technologies now allow analysis of rare immune populations in the liver, down to cells on the  
103 single cell level. Similarly, we should utilize the improved tools for the integrative analysis of  
104 cellular processes and immune functions in order to understand the immune response to  
105 HBV more holistically. Many components of the immune response, both innate and adaptive,  
106 have to act in concert in different scenarios of viral control and viral persistence. Below we  
107 will broadly summarize the current knowledge of both innate and adaptive arms of the  
108 immune responses to HBV infection. While the studies described below do not yet provide  
109 definitive answers, they are the foundation for future investigations into the issues raised  
110 above.

111

112

## 113 **Innate immune responses to HBV**

114 HBV is transmitted upon contact with blood or body fluids of an infected person. A minute  
115 amount of HBV virions in the bloodstream is sufficient for infection of hepatocytes [3]. The  
116 HBV virion enters the hepatocyte via the sodium taurocholate co-transporting polypeptide  
117 (NTCP), a bile receptor located on the basolateral membrane, contributing to its specificity to  
118 human or chimpanzee hepatocytes [4]. After the envelope protein mediates fusion of the viral  
119 and endosomal membranes, the capsid enters the cytoplasm and the viral DNA is released  
120 into the nucleus through nuclear pores. Upon import into the nucleus, HBV can integrate into  
121 the host genome or be present as non-integrated covalently closed circular DNA (cccDNA).  
122 The cccDNA molecule will serve as a template for replication leading to infection of more  
123 hepatocytes, and can persist even after HBsAg loss [5]. As circulating blood passes the liver,  
124 HBV can easily spread to other hepatocytes.

125

## 126 **The detection of HBV by infected hepatocytes**

127 The sensing of HBV and triggering of intracellular antiviral mechanisms can occur on the cell  
128 membrane by Toll-like receptors (TLR), in the endosome by TLR7 or TLR9, and in the  
129 cytoplasm by sensors, such as intracellular retinoic acid inducible gene I (RIG-I) and  
130 melanoma differentiation gene 5 (MDA5) upon ligation with viral proteins or nucleic acids.  
131 Although some controversy exists, it has been reported that TLR2, MDA-5 and RIG-I are  
132 involved in sensing of HBV. For the cytoplasmic sensor RIG-I, it was demonstrated that HBV  
133 pre-genomic RNA triggered its activation, resulting in the release of interferon (IFN)- $\lambda$  but not  
134 type-I IFN by HBV infected primary human hepatocytes and hepatoma cell lines[6]. Release  
135 of IFN by hepatocytes and possibly other cells induces expression of hundreds of IFN-  
136 stimulated genes (ISGs) with potent antiviral activity. However, the HBV-induced IFN  
137 responses are weak [7, 8], which is reflected by the usual lack of clinical symptoms during  
138 the acute HBV infection. Also, early data from animal models showed that HBV does not  
139 induce the release of type-I IFN [9, 10]. The absence of symptoms and the modest IFN  
140 induction by HBV led to adaptation of the term 'stealth virus'. However, IFN responses and  
141 ISG induction are present, albeit marginal compared to other chronic viruses [7, 8]. The use  
142 of cccDNA as a transcriptional template in the nucleus likely contributes to HBV's capacity to  
143 limit detection in hepatocytes. Adding to this, viral proteins, like HBV polymerase and HBx  
144 protein, directly inhibit the cellular machinery that detects replication intermediates. It is  
145 currently unknown which pattern recognition receptor or signaling pathway is essential for  
146 early viral control *in vivo*, and perhaps more relevant to the majority of patients, to HBV  
147 persistence in humans.

148 As the human liver is the site of HBV replication and contains high viral protein  
149 concentrations, the most appropriate approach for addressing basic questions concerning  
150 HBV detection is to evaluate liver material. Unfortunately, such studies are rare. Lebosse et  
151 al. [11] analyzed RNA extracted from liver biopsies of chronic HBV patients and showed low  
152 intrahepatic IFN $\alpha$  expression relative to healthy controls, which was unaffected by viral  
153 replication. Previously, we analyzed liver biopsies from patients in specific clinical phases of  
154 chronic HBV. By comparing the transcriptome of liver and blood samples from patients in  
155 distinct clinical phases of HBV we found that, ISG are transcribed even in patients presumed  
156 to be 'immune tolerant' to HBV [12]. Transcription of catalytic polypeptide-like 3B, a protein  
157 related to cccDNA degradation [13], was increased in the immune tolerant phase, which  
158 suggests that the capacity to limit the establishment of high amounts of cccDNA may be  
159 different between phases, but not absent in any phase. These two studies using human liver  
160 tissue raise significant doubt about the labels 'stealth virus' and 'immune tolerant', as HBV is  
161 indeed sensed by the host immune system and antiviral responses are initiated, even as they  
162 are not sufficient to halt viral replication and spread in persons with chronic infection.  
163 Whether these differences of innate intrahepatic responses are caused by chronic HBV  
164 replication, or reflect immune characteristics that select patients for persistent infection,  
165 remains to be clarified using longitudinally acquired samples of human liver.

166

### 167 **The interaction of antigen presenting cells and HBV**

168 Intrahepatic leukocyte populations lining the sinusoidal lumen of the portal branches as well  
169 as hepatocytes are constantly in contact with huge amounts of bacterial antigen derived from  
170 the gut. Tolerance during exposure to high antigen loads is essential for host survival, and  
171 the relative tolerogenic milieu of the liver is well described[14]. Illustrative of the complex  
172 balance of the liver immune system is the fact that, despite its tolerant nature, HBV is cleared  
173 after transmission in the vast majority of infected individuals. For the development of  
174 treatment strategies, it is important to evaluate the leukocyte populations involved in this  
175 process. Liver residing antigen presenting cells, like Kupffer cells and dendritic cells (DC),  
176 could potentially modulate host immune responses to a phenotype enabling chronic viral  
177 infection, but limited information exists on the interaction between these cells and HBV  
178 (Figure 2). DC are crucial for their ability to efficiently present antigen to naïve CD4 T cells, or  
179 to CD8 T cells via cross-priming. At present, the impact of persistent HBV infection on the  
180 DC compartment is not fully clear. *In vitro* studies have demonstrated that the presence of  
181 viral antigen may limit DC functionality[15]. Both plasmacytoid and myeloid DC can present  
182 antigens to T cells, and can, depending on the cytokine milieu, skew T cell effector function  
183 towards tolerance or activation. Plasmacytoid DC are specialized in the production of high  
184 levels of type I and type III IFN, thereby potentially initiating innate immune responses to

185 HBV after recognition of viral nucleic acids via TLR7 or TLR9. However, based on *ex vivo*  
186 assays, DC function during chronic HBV patients may be hampered. The frequency of both  
187 myeloid DC and plasmacytoid DC is not changed in the majority of studies with patient  
188 material, but others have reported various functional differences compared to healthy  
189 controls [16-19]. Possibly via downregulation of TLR7 or TLR9, plasmacytoid DC seem to be  
190 less capable to produce IFN $\alpha$ , especially when DC are derived from patients with  
191 considerable liver inflammation [17, 20].

192 Kupffer cells are liver resident macrophages, and make up 15-20 % of the  
193 intrahepatic leukocytes [21]. We have previously shown that Kupffer cells can interact with  
194 HBsAg *in vivo*, which led to increased pro-inflammatory cytokine production compared to  
195 healthy controls[22]. However, HBV proteins like HBeAg may interfere with Kupffer activation  
196 by downregulation of TLR expression as shown *in vitro*[23]. In line with this finding, patient-  
197 derived peripheral monocytes were also found to have lower TLR2 expression compared to  
198 healthy controls[24]. Kupffer cells can play different roles in the presence of HBV antigens,  
199 as was illustrated in a rat model, where HBV causes Kupffer cells to produce more of the  
200 pro-fibrogenic and tolerogenic cytokine TGF- $\beta$ 1, as opposed to the pro-inflammatory  
201 cytokines IL-6, IL-1 and TNF[25]. To prevent continuous activation and excessive  
202 intrahepatic inflammation, Kupffer cells become refractory to subsequent endotoxin  
203 challenge, which may contribute to the tolerogenic liver environment. Furthermore, Kupffer  
204 cells can interact and influence other immune cells, either directly via cell-cell contact or  
205 indirectly via the activity of cytokines. Indirect activation of natural killer (NK) cells can take  
206 place via Kupffer cell-derived IL-12 and IL-18[22, 26].

207

### 208 **NK cell function and phenotype in chronic HBV**

209 NK cells can recognize and lyse infected cells, and may therefore be an important effector  
210 cell that plays a role during persistence of HBV. Indeed, the frequency and function of NK  
211 cells in peripheral blood of chronic patients has been studied extensively, and it seems clear  
212 that the balance of NK cell stimulatory and inhibitory signals significantly impacts their  
213 activity, which in turn correlates with patient outcomes during the natural history of chronic  
214 HBV. One of the major effector functions of NK cells, the production of the antiviral and anti-  
215 fibrotic cytokine IFN $\gamma$ , is reported to be unaffected during chronic HBV by some studies [27,  
216 28] but reduced in others[25, 29, 30]. IFN $\gamma$  release by NK cells mediates the non-cytolytic  
217 clearance of HBV by virus-specific CD8 T cells[31, 32]. Another beneficial effect may be that  
218 IFN $\gamma$  induces the production of the antiviral APOBEC proteins A3A and A3B directly affecting  
219 the integrity of cccDNA [33]. In a transgenic mouse model, the potential of IFN $\gamma$  was  
220 dramatically demonstrated by injection of the NKT cell activating agent alpha-  
221 galactosylceramide, which stopped HBV from replicating, and led to infiltration of NK cells



222 into the liver[34]. In humans, however, alpha-galactosylceramide affected HBV DNA levels in  
223 only a subset of human chronic HBV patients, and the treatment was not well tolerated [35].  
224 During chronic HBV infection, the cytotoxic capacity of NK cells seems to remain intact[36],  
225 which together with the impaired cytokine production as described by some studies,  
226 suggests the existence of a functional dichotomy of NK cells in chronic HBV. However, given  
227 the conflicting reports in the literature, it is obvious that more detailed and more controlled  
228 studies need to be performed. A recent study performed by our group compared NK cell  
229 phenotype and function in blood among HBV patients in different clinical phases.  
230 Interestingly, despite big differences in viral load and ALT levels between patients, the NK  
231 cell compartment demonstrated only subtle differences between the patients cohorts[37].  
232 Concerning their activation, a combination of IL-12, IL-15 and IL-18 secreted by other  
233 intrahepatic immune cell populations, including activated Kupffer cells, seems to be the most  
234 likely mechanism. Direct contact with virally infected cells or HBV DNA in the blood may be a  
235 less effective stimulus for NK cells, based on work by Bonoroni et al., who describe no  
236 correlation between viral load and peripheral NK activation [38], which is in line with our  
237 previous study[37]. Also supporting this finding is the observation that tenofovir-induced viral  
238 load reduction did not significantly alter intrahepatic NK cell activation as demonstrated using  
239 the analysis of fine-needle aspirate liver biopsies, even after six years of viral  
240 suppression[39]. The capacity of intrahepatic NK cells to inhibit fibrogenesis through IFN $\gamma$   
241 production [40], or by killing stellate cells that are driving collagen syntheses in the liver [41],  
242 may also be relevant for clinical outcomes. However, in contrast to the above described  
243 beneficial effects of NK cells, their activation may alternatively facilitate HBV persistence, as  
244 was recently illustrated by the finding that HBV-specific CD4 T cells expressing death ligands  
245 (not seen in healthy controls) can be targets for NK cell mediated killing [42]. In a similar  
246 fashion, NK cells have been shown to kill HBV-specific CD8 T cells.

247

## 248 **Adaptive immune responses during chronic HBV**

249 Adaptive immune responses by virus-specific CD4 and CD8 T cells, B cells and antibodies  
250 are indispensable for HBV control[43]. These responses develop relatively late in HBV  
251 infection compared to other viral infections: at ten to twelve weeks post exposure[44]. This  
252 late emergence of adaptive immunity is thought to be a consequence of the stealthy nature  
253 of early HBV infection, with low viral replication and minimal to no activation of innate  
254 immunity. This delayed response is not per se a factor in HBV chronicity, as it is also  
255 observed in the vast majority of adult patients who go on to control the virus spontaneously.  
256 While we have learned many important details about how adaptive immune responses  
257 emerge and evolve from the early phase of infection throughout viral resolution or chronic

258 viremia[43], much remains to be determined in order to fully appreciate the exact  
259 mechanisms driving successful and failing adaptive immune responses targeting HBV. This  
260 is especially true as our appreciation of the complexity and diversity of different players of the  
261 adaptive immune response, mostly defined in animal models, has grown faster than our  
262 ability to translate such integrative concepts into human infection[45].

263

#### 264 **Detection of HBV-specific CD4 T cells**

265 Virus-specific CD4 T lymphocytes are key regulators of both efficient B cell/antibody and  
266 CD8 T cell responses and thus are thought to be essential to the control of most viral  
267 infections [46]. Unfortunately, virus-specific CD4 T cells are also notoriously difficult to study,  
268 as they are rather low in frequency and complex in their phenotypes and function. Overall,  
269 our understanding of the HBV-specific CD4 response remains incomplete. The seminal  
270 chimpanzee studies defining the importance of CD4 T cells by analyzing the impact of  
271 CD4 T cell depletion on the course of infection are not straightforward in their findings, as  
272 depletion of CD4 T cells before infection leads to chronic HBV infection, while depletion just  
273 before the rise of viremia and liver enzymes did not alter the natural course of infection[46,  
274 47]. These findings support a critical role of CD4 T cells in the control of HBV infection, but  
275 also raise questions about the exact role of T cell help and its mode of action. Generally,  
276 HBV-specific CD4 T cells appear in the blood seven to ten weeks after infection, in parallel  
277 with the emergence of HBV-specific CD8 T cells and antibodies[44, 48]. The CD4 T cells  
278 mostly target epitopes in HBV core, though minor responses against surface, polymerase  
279 and x protein have also been described[49-52]. In patients with acute or controlled HBV  
280 infection, CD4 responses are more broadly directed and more vigorous, compared to  
281 patients with established chronic viremia. Functionally, HBV-specific CD4 T cells have been  
282 shown to predominantly secrete Th1 type cytokines[53], though overall studies assessing the  
283 phenotype and function of the cells directly *ex vivo* are very few and rather limited. In this  
284 context it is important to remember that most of these studies were performed in the era  
285 when standard proliferation and ELISpot assays were the only means to assessing virus-  
286 specific CD4 T cell responses, and as we have learned from other infections such as with  
287 HCV, these functional assays might miss significant parts of the response[54]. In addition,  
288 HBV-specific CD4 T cells at the site of infection might be quite different than those analyzed  
289 in the blood. Indeed, after *in vitro* expansion from liver biopsies obtained in patients with  
290 chronic infection, intrahepatic CD4 T cells have shown a distinct functional profile compared  
291 to those derived from blood, with the secretion of IL-4 and IL-5 in addition to Th1 type  
292 cytokines[55]. A reassessment of HBV-specific CD4 T cell responses using current  
293 methodologies, i.e. HLA class II tetramers, for direct *ex vivo* phenotyping and flow based cell  
294 sorting, followed by omics analyses, seems imperative in order to obtain a more detailed

295 understanding of this central component of the adaptive immune response. These methods  
296 enable analysis of single cells, and thus might allow *ex vivo* studies even in infection at  
297 young age, for which we currently have no significant data about the CD4 T cell response.

298

### 299 **Detection of HBV-specific CD8 T cells**

300 In contrast to CD4 T cells, CD8 T cell responses have been studied much more widely and in  
301 greater detail, including direct *ex vivo* analyses[56]. They are essential for HBV control, as  
302 their depletion invariably led to chronicity in chimpanzees[46]. HBV-specific CD8 T cells  
303 become readily detectable six to eight weeks after infection in adults, and their appearance  
304 coincides with a decrease in viral load that is observed even before the onset of liver  
305 injury[44], supporting the contribution of non-cytolytic elimination of intracellular virus to viral  
306 control. CD8 lymphocytes targeting all HBV proteins have been identified[43] and shown to  
307 have cytolytic as well as non-cytolytic effector functions[57]. Non-cytolytic effector functions  
308 could be especially relevant, as hepatocytes have been shown to be quite resilient to cell-  
309 mediated killing *in vitro*[58]. While the exact immunological determinants of CD8 mediated  
310 HBV control are difficult to define with certainty in humans, the development of a sustained,  
311 broad and polyclonal CD8 T cell response that is highly functional is seen as *conditio sine*  
312 *qua non* for HBV control. To what degree such a CD8 response is dependent on other arms  
313 of the immune response, be it CD4 T cell help or a preceding innate response, is not fully  
314 clear.

315

316 Importantly, there is also a good amount of literature on intrahepatic CD8 T cells targeting  
317 HBV, mostly from chronic infection. Detection of HBV-specific CD8 T cells by tetramer  
318 staining of cultured intrahepatic lymphocytes revealed that the frequencies of virus specific  
319 CD8 T cells were inversely correlated with the degree of liver injury[59], supporting the  
320 hypothesis that specific responses might be not only important for disease resolution, but  
321 also for protection from progressive liver disease. Another pioneering study using fine needle  
322 aspirate biopsies to analyze intrahepatic lymphocytes during acute infection provided  
323 longitudinal characterization of HBV-specific CD8 T cells directly *ex vivo* without *in vivo*  
324 expansion. HBV-specific CD8 T cells were highly enriched in the liver during the acute phase  
325 of infection and remained detectable after HBsAg seroconversion and full clinical  
326 recovery[60]. A similar approach was also employed in chronically infected patients;  
327 revealing that virus specific CD8 T cells were most readily detected during the inactive carrier  
328 phase[61]. Altogether, current evidence illustrates differences in phenotype and function  
329 between peripheral and intrahepatic lymphocytes, and during different stages of HBV  
330 infection. Since one can now analyze cells from liver biopsies or fine needle aspirates with

331 much more powerful analytic tools, this opportunity should be used to deepen our  
332 understanding of the CD8 T cell response in acute and chronic infection, and during therapy.

333

### 334 **Chronic HBV and functional T cell impairment**

335 Once patients are chronically infected, especially in those patients exposed in early  
336 childhood, there is usually an extended phase that has been described as immune tolerant,  
337 with high levels of viremia but no or very little liver disease. It has been postulated that during  
338 this phase T cell responses are mostly absent and/or non-functional. Recent data indicate  
339 that this is not the case, and instead T cells are readily detectable in these patients and  
340 seem, if anything, more and not less functional than those in later, so called immune active,  
341 stages of disease[12]. This “immune tolerant” phase of HBV might hold more surprises and  
342 certainly warrants further investigation. During later stages of chronic infection, the repertoire  
343 of detectable HBV-specific T cells is limited and their frequencies are rather low, at least in  
344 the blood, and especially so in patients with high viral loads[62]. Preserved HBV-specific  
345 CD8 T cells become gradually more functionally impaired, or exhausted, which is thought to  
346 be driven by persistent antigen exposure. CD8 T cell exhaustion in chronic HBV infection  
347 mirrors that described in other chronic viral infections in mice and humans, with the sustained  
348 expression of inhibitory receptors, such as PD-1, TIM-3 and 2B4, reduced proliferative  
349 capacity and poor effector functions such as reduced IFN $\gamma$  and IL-2 secretion [63-65]. The  
350 exhausted state is also associated with distinct expression patterns of transcription factors,  
351 compared to functional effector or memory T cells, most notably low expression of T-bet[66]  
352 The cells seem also increasingly susceptible to apoptosis through upregulation of Bim and  
353 TRAIL-R2[67, 68], two key regulators of cell death. Whether the functional exhaustion of  
354 HBV-specific CD8 T cells is the sole or dominant contributor to CD8 T cell failure, or whether  
355 the virus can also escape through the generation of viral escape sequence variants, like in  
356 HIV and HCV infection, is currently an open question. HBV as a DNA virus is much more  
357 genetically stable compared to HIV and HCV, and early studies supported the idea that HBV  
358 displayed little variability that could be linked to immune pressure[69]. However, a recent  
359 study revealed viral variation compatible with escape mutations in both the core and  
360 envelope sequence and to a lesser extent in the polymerase sequence of HBV[70, 71].  
361 Future studies in this area should further define the mechanisms of T cell exhaustion and  
362 whether exhausted T cells in chronic HBV infection could potentially be reinvigorated through  
363 immunotherapeutic interventions. In addition, we need to define the contribution of viral  
364 variants to the failure of HBV adaptive immunity.

365

### 366 **Functional T-cell restoration under treatment**

367 If T cell exhaustion is principally maintained by persistent antigen exposure, long-term  
368 therapy with successful control of viremia, should, to some degree, be capable of restoring T  
369 cell function. This would be especially relevant for immunotherapeutic approaches that most  
370 likely will be applied in the context of antiviral therapy. This has been studied in some detail,  
371 though the complexity of antiviral treatments, but also of the course of chronic HBV infection  
372 itself, makes interpretation of the data challenging [72-75]. Boni et al compared nucleos(t)ide  
373 treated chronic HBV infected patients to those with untreated or resolved infection. PBMC  
374 were analyzed either directly *ex vivo* with class I dextramers or for their proliferative capacity  
375 and cytokine production after *in vitro* expansion. *Ex vivo* analysis of virus-specific T cells  
376 suggested continued impairment even after long-term treatment, though after *in vitro*  
377 expansion some functional properties were partially restored, indicating some improvement  
378 of the T cell populations[73]. A similar study design has also been performed in IFN $\alpha$  treated  
379 chronic HBV patients and failed to detect improved T cell function, at least in terms of  
380 cytokine production[74]. Whether the modest T cell recovery is due to the remaining high  
381 levels of HBsAg in serum, despite the control of viral replication, is an important and  
382 controversial question. While it is widely assumed that surface antigen has a directly  
383 negative effect on T cell function, this hypothesis is mostly based on *in vitro* studies using  
384 high doses of recombinant proteins. One wonders how this effect mediated by circulating  
385 proteins could be specific to HBV-specific immune cells as there is no experimental evidence  
386 that adaptive immune responses to other pathogens are similarly impaired as those targeting  
387 HBV. Clinically chronic HBV patients are also not showing signs of significant immune  
388 impairment. Further studies analyzing the impact of circulating HBV antigens on HBV  
389 immune responses *in vivo* are needed, together with a more detailed and comprehensive  
390 assessment of the integrated adaptive immune response in well-defined longitudinal cohorts  
391 undergoing structured antiviral treatment and treatment interruptions.

392

### 393 **Regulatory T cells**

394 Regulatory T (Treg) cells are T cells that can regulate the local immune response via cell-cell  
395 contact or via secretion of cytokines, such as TGF- $\beta$  or IL-10. Treg cells play a central role in  
396 immunological tolerance to self- and foreign antigens by suppressing activation, proliferation  
397 and effector functions of a wide range of lymphocyte subsets[76]. The best-known are  
398 CD25+FoxP3+CD4 natural Treg cells that directly inhibit other T cells. In addition, an  
399 increasing number of other regulatory T cell types have been described, including CD8 T  
400 cells with inhibitory functions. Most studies in HBV have focused on natural CD4 Treg cells in  
401 the peripheral blood, where these cells usually constitute between 3 and 10% of the total  
402 CD4 T cell population. Confusingly, while some studies have found increased intrahepatic  
403 and peripheral Treg cell levels in chronic HBV compared to healthy individuals and self-

404 limited HBV infection, others did not, similar to results in HCV infection[77-82]. This is  
405 complicated by different methods to define the Treg populations, which have evolved over  
406 time from simple staining of CD25 antigen to increasingly more complex and specific  
407 combinations of phenotypic markers, including FoxP3, PD-1 and CD127. Given their local  
408 mode of action, the presence and functionality of intrahepatic Treg cells should be most  
409 consequential, but almost all data is from the blood. It remains to be seen whether their main  
410 role is enabling chronic infection or rather protection from active liver disease in the context  
411 of long-term viremia.

412

### 413 **B cells and antibodies**

414 HBV-specific antibodies are clearly able to provide sterilizing immunity after vaccination.  
415 Clinically, different antibody profiles are important for the diagnosis and characterization of  
416 acute and chronic HBV infection. Much less is known about the relative contribution of B cells  
417 and HBV antibodies to viral control once infection has been established. B cells have been  
418 reported to display an activated phenotype and seem functionally intact, even at later stages  
419 of infection. We also recently demonstrated that blood gene signatures indicative of B cell  
420 responses were highly active during the immune active phase in chronic HBV patients, using  
421 a systems biology approach [12]. HBV antibodies target the surface, polymerase, core and x  
422 proteins of HBV, and appear ten to twelve weeks after infection. Detection of antibodies  
423 against HBsAg is the clinical correlate of protective immunity, but HBs antibodies likely  
424 contribute to control of viremia even in chronic infection where they are not detected by the  
425 standard antibody assays as they form immune complexes that prevent viral attachment and  
426 entry. In contrast, the core antibody (anti-HBc) is detectable in all stages of infection and  
427 considered not to mediate viral control, though the passive immunization of anti HBc/HBe  
428 does seem to prolong the incubation period in chimpanzees[83]. Overall a better  
429 characterization of how both B cells and antibody responses contribute to viral control during  
430 acute and chronic infection is urgently needed, as most likely a concerted effort by T cells  
431 and antibodies will offer the highest likelihood of effective HBV control. In this context it  
432 should also be noted that treatment with immunomodulatory drugs can lead to reactivation of  
433 controlled HBV infection[84]. The classic example is rituximab, a monoclonal antibody  
434 targeting CD20-expressing B cells, thus eliminating B cells and suppressing antibody  
435 production. It is not clear, however, whether the effect on antibodies is the sole or main  
436 cause for HBV reactivation, as rituximab treatment also impacts CD4 T cells and potentially  
437 indirectly also CD8 T cell memory[84, 85]. A detailed characterization of virus-specific  
438 immune responses during treatment with such agents should reveal important insights into  
439 immunological changes that might lead to diminished HBV control.

440

441 **Summary**

442 Despite numerous immunological studies performed in HBV infected patients several key  
443 questions remain unanswered. For decades, the lack of HBV models facilitating replication of  
444 human strains hampered scientific progress, and even patient data can be misleading due to  
445 the huge variation among study cohorts of patients with chronic infection. Through careful  
446 patient selection and the use of modern biomedical techniques, like genomics and  
447 proteomics, researchers can now tackle basic questions regarding HBV  
448 immunopathogenesis. The aim should be to determine immune parameters associated with  
449 persistence, clearance and recurrence of HBV. Also, the mechanisms of recognition of viral  
450 antigens in chronic infections by hepatocytes in vivo remain unclear, with a particular need  
451 for ex vivo assays. Potential antiviral effector cells like Kupffer cells, natural killer cells and  
452 dendritic cell populations may be less functional during chronic infection, possibly leading to  
453 infrequent and exhausted HBV-specific T cells in adults. By the use of modern techniques,  
454 the function and phenotype of both peripheral and intrahepatic lymphocyte populations as  
455 well as hepatocytes can be determined, which may aid in the rational design of  
456 immunotherapeutic strategies.

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468 **Figure Legends**

469

470 Figure 1: Important questions concerning the clinical events and immunopathogenesis of  
471 chronic HBV infection remain unanswered.

472

473 Figure 2: The persistence of HBV infections is determined by the complex interactions of  
474 multiple leukocytes.

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