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# Hepatitis B Surface Antigen Seroclearance: Immune Mechanisms, Clinical Impact, Importance for Drug Development

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Tarik Asselah has acted as a speaker and investigator for Janssen, Gilead, Roche, and Merck.

Nathalie Boyer has acted as a speaker and investigator for Janssen, Gilead, Roche and Merck. Issam Tout, Dimitri Loureiro, Abdellah Mansouri and Vassili Soumelis declare no competing interests.

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### **Author contributions**

TA designed and supervised the manuscript. IT and TA prepared the manuscript. All the authors contributed to the drafting of the review, the critical revision of the manuscript and the final approval of the version.

**Keywords:** Chronic HBV infection, HBsAg seroclearance, Immune system, Interferon, Nucleos(t)ide analogues HBV therapy

### **Lay Summary**

HBsAg loss is a rare event in the natural history of CHB and is associated with a lower risk of HCC. HBsAg contributes to the deregulation of the innate and adaptive immunity during CHB. Future therapeutic strategies targeting HBsAg are crucial to restore the anti-HBV response and achieve a functional cure of CHB infection.

### **Abbreviations**

aa, amino acid ; ALT, Alanine aminotransferase; anti-HBsAg, hepatitis B surface antigen antibodies; AST, Aspartate aminotransferase; AIM2, Absent in melanoma 2; atMBC, Atypic Memory B cells; cccDNA, covalently closed circular DNA; CHB, chronic hepatitis B; CTL, Cytotoxic T cell; DC, Dendritic cells; ETV, entecavir; FDA, Food and Drug Administration; FNA, Fine needle aspiration; HBcAg, Hepatitis B core antigen; HBcrAg, Hepatitis B core-related antigen; HBeAg, Hepatitis B e antigen; HBsAg, Hepatitis B surface antigen; HBV, hepatitis B virus; HBV; SVPs, Hepatitis B virus sub-viral particles; HCC, hepatocellular

carcinoma; hNTCP, human sodium taurocholate cotransporting polypeptide; IFN- $\alpha$ , interferon alpha; IL, Interleukin; IRF7, Interferon regulatory factor 7; JNK, c-Jun N-terminal protein kinase; KC, Kupffer cells; LAG-3, Lymphocyte-activation gene 3; L-HBsAg, Large Hepatitis B surface antigen; LSEC, Liver sinusoidal endothelial cells; MAIT, mucosal associated invariant T cells; MDSC, Myeloid-derived suppressor cells; M-HBsAg, Medium Hepatitis B surface antigen; mRNA, messenger RNA; MVP, Major vault protein; MyD88, Myeloid differentiation primary response 88; NA, nucleoside analogue; NAP, Nucleic Acid Polymer; NK cells, Natural Killer cells; ORF, open reading frames; PD-1, Programmed cell death protein 1; PEG-IFN, pegylated interferon; pgRNA, pregenomic RNA; PRR, Pattern recognition receptor; rcDNA, relaxed circular DNA; S-HBsAg, Small Hepatitis B surface antigen; siRNA, small interfering RNA; TAF, tenofovir alafenamide; T-bet, T-box protein expressed in T cells; TDF, tenofovir disoproxil fumarate; TFH, T Follicular Helper cell; TFV, tenofovir; Tim-3, T-cell immunoglobulin and mucin-domain containing-3; TLR, toll-like receptor; TNF- $\alpha$ , Tumor necrosis factor alpha; Treg, Regulatory T cells.

## **Abstract**

Hepatitis B surface antigen (HBsAg) seroclearance occurs rarely in the natural history of chronic hepatitis B (CHB) infection and is associated with a reduced risk of hepatocellular carcinoma (HCC). Many factors are associated with HBsAg seroconversion, including immune, and viral factors. However, the immune mechanisms associated with HBsAg seroclearance are still difficult to elucidate.

The aim of the treatment of hepatitis B virus (HBV) infection is the seroclearance of HBsAg. Unfortunately, this goal is rarely reached with current approved treatments. Understanding HBsAg loss mechanisms appears important to achieve an HBV cure drug development. While studies from HBV animal models are giving insights on the potential

immune mechanisms and interactions occurring between the immune system and HBsAg, they do not recapitulate all features of CHB in human and are subject to variability due to their complexity. In this article, we review recent studies on these immune factors, their influence on CHB progression, and HBsAg seroconversion. These data provide new insights in the development of immune therapeutic approaches to partially restore the anti-HBV immune response. Targeting HBsAg will ideally relieve the immunosuppressive effects on the immune system and help to restore anti-HBV immune responses.

## **Key Points**

- HBsAg seroclearance is a rare event in the natural history of CHB.
- HBsAg seroclearance is associated with a reduced risk of HCC.
- HBsAg contributes to the deregulation of both innate and adaptive immune cells.
- The decrease of HBsAg can potentially restore anti-HBV immune responses.
- Combination of antivirals and immune therapy is crucial for drug development.

1

## **2 Introduction**

3 Hepatitis B virus (HBV) infection represents a global health problem with approximately 257  
4 million people chronically infected[1]. HBV chronic infection is the major contributor to the  
5 development of cirrhosis, hepatocellular carcinoma (HCC) and liver-related death  
6 worldwide[2–5]. Currently, two approved therapeutic strategies are available, Pegylated  
7 interferon (PEG-IFN) or nucleos(t)ide analogues (NAs), which suppress HBV replication and  
8 slow disease progression. However, these treatments do not generally achieve a cure[3–5].

1 The ultimate endpoint of chronic HBV treatment is sustained HBsAg loss with or without  
2 seroconversion to hepatitis B surface antibody (anti-HBs). Seroclearance and conversion of  
3 HBsAg represents of the most important outcomes for chronic hepatitis B trials because it  
4 represents immunity to HBV and indicates a better prognosis[6]. However, due to the  
5 persistence of intrahepatic, covalently closed circular HBV DNA (cccDNA)[7], HBsAg  
6 seroclearance and conversion to anti-HBsAg in chronic infection is a rare event. It can occur  
7 spontaneously, but the reported rates have been variable since it is affected by a myriad of  
8 patient characteristics such as age, cirrhosis, HBeAg status, HBV DNA level, and serum  
9 HBsAg level[8,9]. Prior cohort studies, from Southeast Asia, showed that the annual HBsAg  
10 seroclearance rate can range from a low of 0.12% to a high of 2.38%[8,10]. The host immune  
11 response to HBV is closely related to the natural course of HBV infection. CHB has been  
12 associated with the exhaustion of T and B cell responses[11,12], HBsAg being a major  
13 contributor to the immunopathogenesis of CHB. This review focuses on the immune factors  
14 affected by HBsAg and the immunological changes associated with HBsAg seroclearance  
15 which could be potential targets for immune therapy for CHB.

16

## 17 **Molecular virology and structure of HBsAg**

18 HBV is a small hepatotropic enveloped DNA virus belonging to *Hepadnaviridae*[13]. HBV  
19 virions have an icosahedral nucleocapsid formed by HBV core proteins (HBcAg) containing  
20 the partly double-stranded DNA in relaxed circular conformation (rcDNA). The nucleocapsid  
21 is surrounded by the viral envelop composed of three types of HBsAg: L-(large), M-(middle)  
22 and S-(small) HBsAg[13]. HBsAg plays a crucial role in the attachment to the hepatocyte via  
23 the high affinity interaction between the 75 aa on the N-terminus of L-HBsAg (PreS1 domain)  
24 with the 157 to 165 residues on the HBV receptor, the human sodium taurocholate co-  
25 transporting polypeptide receptor (hNTCP, SLC10A1) that allows hepatocytes infection[14–

1 16]. HBV entry is followed by the nucleocapsid translocation into the nucleus of infected  
2 cells. Then, rcDNA is remodeled by host factors into cccDNA minichromosome and serves as  
3 a template for the transcription of all HBV viral transcripts including HBsAg's[17].  
4 Interestingly, cccDNA is not the only source of HBsAg production as HBV integration in the  
5 host genome can lead to cancer development and HBsAg secretion[18]. Thus, it is possible to  
6 block HBV transcription (cccDNA) without affecting HBsAg quantification. HBsAg proteins  
7 are encoded from two viral transcripts, PreS1 and PreS2/S mRNA (2,4 and 2,1 kb), which are  
8 transcripts from one of the four overlapping open reading frames (ORFs) in the HBV  
9 genome[19]. L- and M-HBsAg proteins are produced respectively from PreS1 and PreS2/S  
10 mRNA. The presence of a second weak start codon on the PreS2/S mRNA leads to S-HBsAg  
11 translation[20]. HBsAg proteins differ in their N-terminus but share a common S domain with  
12 four putative transmembrane domains on their C-terminus. S-HBsAg is the smallest HBsAg  
13 with 227 aa and contains only the S domain. M-HBsAg contains on their N-terminus an  
14 additional domain, the PreS2 domain, with an extension of 55 aa. L-HBsAg proteins have the  
15 two PreS2 and PreS1 domains at their N-terminus. Additionally, the N-terminus of M-HBsAg  
16 ends with an acetylate group and L-HBsAg with an myristate group at glycine 2[21,22]. Fig. 1  
17 shows the different viral transcripts as well as the 3 HBsAg proteins structure. Myristoylated  
18 L-HBsAg at N-terminus are important for NTCP receptor attachment by increasing receptor  
19 binding[23,24]. After their translation, HBsAg proteins accumulate in the endoplasmic  
20 reticulum (ER) where they form agglomerates via covalent disulfide bridges with different  
21 cysteines in their S domain[25]. Surface proteins are required for the capsid envelopment  
22 except M-HBsAg which is not essential for virions morphogenesis and secretion[26,27]. L-  
23 and M-HBsAg can act as transcriptional activators and are able to activate some  
24 promoters[28]. S and PreS1 domains interact with HBcAg leading to virions secretion[29].  
25 The excessive production of HBsAg leads to the production of non-infectious spherical and

1 filamentous sub-viral particles (SVPs) and HBV infectious particles. Fig. 2 shows the various  
2 HBsAg roles within the HBV life cycle.

### 3 **HBsAg detection and quantification**

4 Circulating HBsAg levels may reflect cccDNA transcription and act as an additional marker  
5 of on-treatment efficacy[30]. The availability of standardized commercial assays has renewed  
6 interest in quantitative serum HBsAg as a biomarker to stratify the risk of disease progression,  
7 relapse, and predict treatment response[31]. Two assays are currently used in HBV  
8 diagnostic to detect epitopes in the “a” determinant region of HBsAg, a highly  
9 conformational, hydrophilic domain from positions 124 to 147[32]. The most used is the  
10 Architect HBsAg QT (Abbott Diagnostics) assay, an automatized chemiluminescent  
11 microparticle immunoassay based on a calibration curve standardized by the WHO[33]. It is  
12 able to measure, in two steps, HBsAg concentration from 0,05 to 250,0 IU/mL with a  
13 sensitivity of 99,8% and a specificity of 95%. The other HBsAg quantification assay is an  
14 automated Roche Diagnostics Elecsys® HBsAg II screening assay who is able to quantify  
15 HBsAg concentration from 0,05 to 52 000 UI/mL with a high specificity (>99,8%)[34].  
16 Recently, serum HBV pre-genomic (pg)RNA (henceforth referred to as HBV RNA) has been  
17 considered as a new biomarker for cccDNA[35], especially in virally suppressed patients with  
18 low detectable HBV DNA under NAs therapy. HBsAg quantitative detection methods have  
19 been widely used in antiviral efficacy prediction. However, many factors may render difficult  
20 the use of serum HBsAg as a surrogate for cccDNA transcription activity with long-term NAs  
21 treatment, such as HBV DNA integration [13] and long-term NAs treatment related HBsAg  
22 retention[36]. Unlike HBsAg, HBV RNA is derived only from cccDNA, and its quantification  
23 is not affected by viral antigens or antibody complexes and therefore, it may reflect more  
24 accurately cccDNA transcriptional activity. A study of 291 treatment-naïve CHB patients  
25 showed the « superiority » of serum HBV RNA in differentiating the ‘HBeAg-negative

1 reactive' phase compared to HBV DNA and HBsAg as serum HBV RNA levels increased if  
2 there is reactivation to active hepatitis[37]. Numerous studies showed a strong to moderate  
3 correlation between HBsAg and serum HBV RNA, except for HBeAg-negative patients,  
4 where the correlation was weak [37,38].  
5 Finally, serum hepatitis B core-related antigen (HBcrAg) is a surrogate marker of both  
6 intrahepatic cccDNA and its transcriptional activity[39].

7

### 8 **HBsAg seroclearance: prevalence and clinical significance**

9 Spontaneous HBsAg seroclearance, defined as the loss of serum HBsAg on two occasions at  
10 least 6 months apart and remaining absent up to the last visit[40], is a rare event in the natural  
11 history of CHB infection. Interestingly, HBsAg seroclearance, in perinatally acquired HBV  
12 infection, is rare in comparison with immune competent adults who acquire infection later in  
13 life. HBsAg seroclearance is rarely observed when CHB is established, probably due to the  
14 induction of tolerance. In a systematic review and pooled meta-analysis, the annual incidence  
15 of HBsAg seroclearance was 1–2% worldwide[41]. Similarly, another meta-analysis found  
16 low rate of HBsAg seroclearance in untreated and treated patients (pooled annual rate  
17 approximately 1%)[42]. The natural course of chronic HBV infection is described by five  
18 distinct phases[3–5]. First, the HBeAg-positive chronic HBV infection, consisting of high  
19 HBV DNA levels, HBeAg positivity, and normal Alanine Aminotransferase (ALT) levels.  
20 The second phase is the HBeAg-positive chronic hepatitis B, characterized by high levels of  
21 HBV DNA and ALT and moderate to severe liver necroinflammation. Most patients can  
22 achieve HBeAg seroconversion and enter the third phase, HBeAg-negative chronic HBV  
23 infection, with positive anti-HBe, undetectable/low (<2,000 IU/ml) HBV DNA levels and  
24 normal ALT levels [2]. Other patients can progress to the fourth phase, HBeAg-negative CHB  
25 (reactivation), characterized by moderate to high levels of serum HBV DNA and ALT. The

1 fifth phase, the HBsAg-negative phase is characterized by serum negative HBsAg with or  
2 without anti-HBs. Of note, spontaneous HBsAg loss occurred rarely[40,43] and mainly in  
3 inactive carriers[43–45] usually more than 10 years after they have entered inactive  
4 phase[46].

5 Interestingly, HBsAg seroclearance was associated with a lower baseline HBV DNA level  
6 (6.61 log<sub>10</sub> IU/mL) vs not having HBsAg seroclearance (7.71 log<sub>10</sub> IU/mL) and with a lower  
7 level of HBsAg at baseline (2.74 log<sub>10</sub> IU/mL) vs not having HBsAg seroclearance (3.90  
8 log<sub>10</sub> IU/mL). HBsAg seroclearance was not associated with gender, HBV genotype or  
9 treatment history. Heterogeneity was substantial across the studies. HBsAg seroclearance is  
10 associated with a reduced risk of HCC compared with patients who are HBsAg-persistent  
11 carriers[47]. In a population-based prospective study, 1-2% (8/652) of participants with  
12 HBsAg seroclearance developed HCC over a 9-year surveillance program[47]. The  
13 cumulative HCC incidence rate was significantly lower in patients with HBsAg seroclearance  
14 than in HBsAg-persistent carriers but was slightly higher than in HBV-uninfected controls. In  
15 another study, among 20,263 CHB patients, those with NA-induced HBsAg seroclearance  
16 with complete viral suppression had a lower risk of HCC (but not hepatic events) than those  
17 only achieving complete viral suppression with long-term NA treatment[48].

18

## 19 **Immune cells and HBsAg**

20 In CHB, liver necroinflammation which is the driver for fibrosis, is affected by a dynamic  
21 imbalance of HBV, liver cells, and the host's immune system. Although many useful  
22 immunological insights into HBV pathogenesis have been made by studying peripheral blood,  
23 a large proportion of relevant responses are enriched in the liver as tissue-resident immune  
24 subsets play vital roles in front-line immunosurveillance in the liver and other organs. Liver  
25 biopsies and Fine needle aspiration (FNA) allowed the identification of tissue and liver

1 resident immune cells, not seen in the peripheral blood, including HBV-specific PD-1<sup>hi</sup> T  
2 cells and natural killer (NK) cells, together with PD-L1-expressing hepatocytes. A  
3 preferential accumulation of PD-1<sup>hi</sup> Tbet<sup>hi</sup> atMBC in the liver was shown compared to the  
4 peripheral blood[12]. The liver is also enriched with several innate-like populations such as  
5 mucosal associated invariant T (MAIT) cells and LSEC.  
6 HBV particles can inhibit innate immune responses in hepatocytes leading to a decreased  
7 expression of antiviral cytokines[49]. HBsAg is involved in immune evasion processes which  
8 are presented in table 1. Many immune cells contribute to the immunopathogenesis of HBV  
9 infection, such as NK cells, cytotoxic T lymphocytes, dendritic cells (DCs), memory and  
10 plasma B cells, and myeloid-derived suppressor cells (MDSCs) among others[50–56].  
11 However, the immune mechanisms underlying HBsAg loss have not been studied in detail.  
12 Understanding the cellular basis of these immune interactions may help in improved strategies  
13 for viral clearance. We will discuss the role of innate and adaptive immune cells involved in  
14 CHB as well as their interactions and correlations with HBsAg (Table 1).

15 (Table 1 references : [57,58] , [59] , [60,61] , [51,52,62,63] , [56] , [51] , [64,65] , [66] , [67])

## 16 **Innate Immune cells**

### 17 18 **Kupffer cells**

19 Kupffer cells (KCs) are the resident macrophages of the liver[68], accounting for  
20 approximately 20% of liver parenchyma cells. Their most important function is the removal  
21 of toxins from the circulating blood, but KCs can also effectively remove viruses, bacteria,  
22 and other pathogens mostly via TNF- $\alpha$ , interleukin-1 (IL)-1, IL-6, oxygen free radicals and  
23 the inflammasome. HBV and HBsAg can abrogate AIM2 inflammasome responses by  
24 deregulating IRF7 expression and binding on the AIM2 promoter in human KCs[57].  
25 Furthermore, KCs directly interacted with HBsAg *in vivo* and *in vitro*[58] which induced the  
26 production of pro-inflammatory cytokines, such as TNF- $\alpha$ , IL-6, and CXCL8.

1 HBsAg-induced cytokine production by KCs and monocyte-derived macrophages and  
2 subsequent NK cell activation may be an early event in viral containment and may support the  
3 induction of HBV-specific immunity upon HBV infection.

#### 4 **Dendritic cells**

5 DCs are crucial immune sentinels which orchestrate antiviral immunity. They can detect  
6 viruses and their components through multiple pattern recognition receptors (PRR), to  
7 subsequently produce large amount of antiviral cytokines especially type I and type III IFNs,  
8 and to cooperate with other immune effectors as well as their role in uptaking antigens,  
9 performing cross-presentation and priming virus-specific cytotoxic T cells[69]. Myeloid or  
10 conventional dendritic cells (cDCs) that express TLR3, TLR4 and TLR8 can be distinguished  
11 from plasmacytoid dendritic cells (pDCs) which express mainly TLR7 and TLR9[70–72]. In  
12 CHB infection, functional perturbations in DCs have been described in numerous  
13 studies[51,60–63,73,74]. Moreover, two recent studies showed that HBsAg can affect the  
14 maturation of DCs[75] and that HBV subverts DCs in both blood and liver[76]. A study on  
15 PBMCs of patients undergoing HBsAg seroclearance showed increased dendritic cell  
16 frequencies with enhanced expression of TLRs as well as increased CD8+ T cells and plasma  
17 B cells frequencies suggesting that DC may play a crucial role in the mechanism of HBsAg  
18 seroclearance[77].

#### 19 **NK cells**

20 NK cells are important immune lymphocytes in the liver, accounting for approximately one-  
21 third of intrahepatic lymphocytes[78]. NK cell receptors have activating or inhibitory  
22 properties upon engagement by molecules on the surface of target cells. The balance, between  
23 these signals, controls immediate effector functions: cytotoxicity and IFN- $\gamma$  secretion. They  
24 have an important role in the defense against intracellular pathogens and tumors[79] and they  
25 play a crucial antiviral role during HBV infection[80–82]. During CHB, NK cells have been

1 shown to express an inhibitory phenotype with blunt functional responses[83] and to mediate  
2 virus-specific CD8<sup>+</sup> T-cell depletion through a death receptor pathway[84]. Furthermore,  
3 HBV can generate suppressive monocytes, which initiate regulatory NK cells differentiation  
4 resulting in T cell inhibition[85]. Changes in the NK cell phenotype may predict efficient  
5 immune reconstitution before anti-HBsAg seroconversion. Furthermore, an induction of the  
6 proliferation and expansion of CD56 bright NK cell numbers in peripheral blood and an  
7 increase in cytotoxicity function and IFN- $\gamma$  expression were associated with decreased serum  
8 HBsAg levels[86–88]. Increased NK cell function is associated with active hepatitis and  
9 HBsAg seroclearance following structured NA cessation[89]. However, the role of NK cells  
10 in the process of HBsAg loss is still unknown.

## 11 **Adaptive Immune cells**

### 12 **Cytotoxic CD8<sup>+</sup> T cells**

13 HBV-specific CD8<sup>+</sup> T-cell response, have been suggested to play an important role in viral  
14 clearance. In contrast to CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cell responses have been studied more widely.  
15 In acute hepatitis B (AHB) patients, the presence of strong and multiple CTL response in  
16 peripheral blood reinforced the concept[90,91] while being barely detectable in the peripheral  
17 blood of CHB patients[92]. T cell exhaustion has been documented in the literature: CTLs are  
18 unable to mediate complete eradication of the virus, and they subsequently recruit HBV-  
19 nonspecific inflammatory cells, including bystander T cells, NK cells, and neutrophils, that  
20 inevitably cause the immunopathology of CHB[93]. The low clearance rate of HBsAg is  
21 possibly related to a weak CTL response to HBsAg. CD8<sup>+</sup> T cell exhaustion in chronic HBV  
22 infection mirrors that described in other chronic viral infections in mice and humans, with the  
23 sustained expression of inhibitory receptors, such as PD-1, TIM-3 and 2B4, reduced  
24 proliferative capacity and poor effector functions such as reduced IFN- $\gamma$  and IL-2  
25 secretion[64,94]. Inhibition of indoleamine 2,3-dioxygenase activity enhances the HBsAg-

1 specific Tc1 immune response and induction of CTLs after immunization with HBsAg,  $\alpha$ -  
2 GalCer, IL-2, and IL-12b, which are vital cytokines for inducing the antigen-specific Tc1  
3 response[95,96]. A genome-wide expression profiling of exhausted HBV-specific CD8<sup>+</sup> T  
4 cells from CHB patients revealed an extensively downregulated gene-expression program  
5 when compared to functionally competent CD8<sup>+</sup> T cells from patients who spontaneously  
6 resolved infection[97]. Among the various dysregulated processes, mitochondrial function  
7 seemed to be extensively defective, and its restoration by mitochondria-targeted antioxidants  
8 elicited functional T cell reconstitution. These results may lead to novel strategies for  
9 improving HBsAg clearance.

10

#### 11 **Th CD4<sup>+</sup> cells**

12 Virus-specific CD4<sup>+</sup> T cells are key regulators of both efficient B cell/antibody and CD8<sup>+</sup> T  
13 cell responses [98,99]. However, in CHB infection, T cell responses are described as hardly  
14 detectable and display a functionally exhausted phenotype[65,100]. T cells of patients with  
15 subsequent HBsAg loss, following cessation of NA treatment, had a less exhausted and more  
16 activated phenotype, compared to patients with retained HBsAg[101]. T cells expressed low  
17 levels of PD-1 and KLRG1. Furthermore, PD-1<sup>+</sup> CD8<sup>+</sup> T cells positively correlated with  
18 HBsAg levels at baseline. In HBV-infected mice and blood from CHB patients, a T follicular  
19 helper (TFH)-cell response to HBsAg is required for HBV clearance, and blocking Treg-cell  
20 activity restored the ability of TFH cells to clear HBV infection[102]. Circulating PD-1<sup>hi</sup>  
21 CXCR5<sup>+</sup> CD4<sup>+</sup> T cells were associated with decreased HBsAg levels in CHB patients  
22 receiving PEG-IFN- $\alpha$  therapy[103]. Indeed, HBV-specific IFN- $\gamma$  producing CD4<sup>+</sup> T cells are  
23 associated with viral clearance in CHB patients and their frequency was positively correlated  
24 with the decrease of HBsAg[104]. Finally, in a cohort of 209 CHB patients, there was a  
25 strong negative correlation between CD4<sup>+</sup> T cells IL-4<sup>+</sup> and qHBsAg levels[105].

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**B cells**

B cells are major contributors in the humoral immune response and have dual roles: being professional antigen-presenting cells (APC), they recognize antigens and prime T cells. Also, they differentiate into memory cells and antibody-producing plasma cells which are responsible for the production of antibodies. B cells play a vital role during HBV infection by secreting anti-HBsAg antibodies, a sign of the resolution of the infection. Conversely, HBsAg could disrupt the mechanisms of innate and adaptive immunity cell responses and result in suppression of immune responses against HBV[66]. However, while the quantity and function of HBV-specific T cells have been clearly defined in CHB patients[97,106], less attention has been paid to the role of neutralizing anti-HBsAg and detailed characterization of the anti-HBV-specific B cell response is lacking. In addition, B cell-depleting drugs, such as the anti-CD20 antibody rituximab, induce HBV reactivation in HBV carriers and even in patients with “resolved” HBV in whom nuclear HBV cccDNA persists as a viral reservoir for decades[107]. B cells from CHB patients showed a reduced proliferative capacity and were incapable of producing anti-HBsAg upon stimulation. This functional defect reverted after HBsAg loss[54,108]. Recently, two studies were able to characterize HBsAg-specific B cells. While present in CHB context as the same frequencies as in resolved HBV infection, they were unable to mature into anti-HBsAg-secreting cells and thus, present functional alterations that resemble atMBC with low expression of CD21 and CD27 and high expression of PD-1 and T-bet[12,55]. The function of these HBsAg-specific B cells can be partially restored in vitro by PD-1 blockade[55]. These results determine B cell dysfunction rather than antibody depletion as the main reason for the lack of anti-HBsAg and thus present B cells as targets for novel antiviral strategies.

## 1 **Regulatory cells**

2 Regulatory T cells (Treg) are a specialized subpopulation of T cells that act to suppress T cell  
3 proliferation and cytokine production thereby maintaining homeostasis. HBsAg can enhance  
4 Treg cell activity and mDC costimulatory molecules and can suppress monocyte activation  
5 and pDC function[109]. On the other hand, mMDSCs are strong inhibitors of the T cell  
6 response[110]. A recent study found that HBsAg levels were positively correlated with  
7 mMDSC frequency in CHB patients and that HBsAg can maintain HBV persistence by  
8 increasing the differentiation of monocytes into mMDSCs[67]. The polarization of MDSCs  
9 by HBsAg can restrain the activation of T cells in CHB infection[67]. Despite these data,  
10 several key questions remain unanswered. The ideal would be to determine immune  
11 parameters associated with persistence, clearance and recurrence of HBV and to study the  
12 function and phenotype of both peripheral and intrahepatic lymphocyte populations as well as  
13 hepatocytes, which may aid in the rational design of immunotherapeutic strategies.

14

## 15 **Cytokines and HBsAg**

16 Cytokines act as key coordinators of the inflammatory responses involved in HBV  
17 pathogenesis. IL-6 is produced by a variety of cells and is involved in many biological roles,  
18 including induction of cell differentiation, generation of B immunoglobulin, promotion of T  
19 cell proliferation among others. A recent study showed that IL-6 polymorphisms 572G/C and  
20 -597G/A are significantly associated with CHB risk[111]. Furthermore, Bouezzedine et al.  
21 show that IL-6 can strongly inhibit HBsAg secretion which was confirmed by observation of  
22 a severe reduction in cccDNA after IL-6 treatment[112]. IL-12 have numerous roles:  
23 promoting the differentiation of Th1 cells, enhancing NK cell cytotoxicity and activation of  
24 IFN $\gamma$  pathways and rescuing the antiviral function of exhausted HBV-specific T cells[113].

1 Direct interaction of HBsAg with human monocytes and macrophages regulates the  
2 production of IL-12[67].

### 3 **TLRs and HBsAg**

4 The early and non-specific detection of pathogens generally occurs, at subcellular/molecular  
5 levels, via the recognition of Pathogen-Associated Molecular Patterns (PAMP) by innate  
6 immunity sensors, also called Pathogen Recognition Receptors (PRR) expressed in various  
7 types of epithelial/endothelial cells, as well as professional and non-professional immune  
8 cells[114]. Among PRRs, TLRs belong to a conserved family of transmembrane glycoprotein  
9 receptors capable of sensing a wide variety of PAMPs and DAMPs. Viral proteins have been  
10 suggested to interfere with innate signaling pathways in hepatocytes and in immune cells. In  
11 fact, HBsAg is capable of impairing the activation of all TLR pathways[115]. HBsAg  
12 downregulates TLR9 in pDCs leading to the inhibition of IFN $\alpha$  production[74,116]. In B  
13 cells, HBsAg has been shown to downregulate TLR9 expression and function leading to  
14 deficient TLR9-mediated B cell responses[66]. Moreover, HBsAg can selectively inhibit  
15 TLR2 ligand-induced IL-12 production in monocytes/macrophages by interfering with JNK  
16 activation[59]. These data may suggest that strategies targeting HBsAg production or  
17 secretion may lead to the restoration of an efficient immune response in CHB patients and  
18 therefore combination therapy should be considered. Fig. 3 represents the proposed immune  
19 mechanisms of HBsAg seroclearance following decreased HBsAg levels and the potential  
20 restoration of anti-HBV immune response.

### 21 **Insights for HBsAg seroclearance in animal models**

22 Animal models are important tools to explore mechanisms of HBV immunopathogenesis and  
23 evaluation of new therapies. Some important insights in assessing the efficacy of new  
24 therapies include GS-9620, a TLR7 agonist, that was tested in HBV chronically infected  
25 chimpanzees[117]. A 100-fold decline in viral load as well as a dramatic drop in the number

1 of HBsAg positive cells was observed during therapy. A phase II study with GS-9620, in  
2 virally suppressed HBV patients on NA showed evidence of immune activation but no  
3 significant decline in HBsAg[118]. Another study, with ARC-520, an RNAi antiviral  
4 targeting HBV transcripts including HBsAg, in combination with NAs, showed a profound  
5 reduction in HBsAg, with a maximum reduction of more than 2 logs[18]. Additionally, a  
6 novel long-acting modified IFN $\alpha$  (PASylated-IFN $\alpha$ ) induced anti-HBsAg seroconversion in  
7 HBV-transgenic mice after three weeks[119]. Recently, GLP-26, a capsid assembly  
8 modulator, was tested in a humanized mouse model in combination with entecavir (ETV) and  
9 induced decrease in viral loads and viral antigens that was sustained for up to 12 weeks after  
10 treatment cessation[120].

11

## 12 **HBsAg seroclearance and current therapies**

13 The goal of treatment is to improve survival by preventing the risk of end-stage liver disease,  
14 HCC and to improve quality of life. However, it is difficult to demonstrate survival  
15 improvement and surrogate markers are needed. HBsAg seroclearance is a surrogate marker  
16 of survival and therefore is the ideal endpoint for treatment. Current treatments include PEG-  
17 IFN, which have been shown to exert dual actions, including an immunomodulatory effect  
18 and minimal direct antiviral activity against HBV or NAs such as lamivudine, telbivudine,  
19 adefovir dipivoxil, ETV, tenofovir disoproxil fumarate (TDF), and tenofovir alafenamide  
20 (TAF), which directly target the reverse transcription functions of polymerase and thus  
21 suppress HBV replication effectively, used as monotherapy, both of which offer limited  
22 efficacy in achieving HBsAg loss[121]. PEG-IFN have the advantage of inducing sustained  
23 response after a define course of treatment (usually 48 weeks), however, with a low rate of  
24 response and a poor tolerability. NAs require life-long administration because they don't  
25 eliminate the persistent cccDNA within the infected hepatocytes. Among NUCs, ETV, TDF

1 and TAF are preferred as first choice therapy because the rare risk of resistance and due to  
2 their high antiviral potency. A significantly greater proportion of patients receiving TDF plus  
3 PEG-IFN for 48 weeks had HBsAg loss than those receiving TDF or PEG-IFN alone, and this  
4 combination can be suitable in a subset of patients [122]. However, the observed rate of  
5 HBsAg loss in the study was lower than that assumed in the study design, reducing the power  
6 for comparison between groups. Finally, prolonged follow-up of patients who had not  
7 restarted TDF treatment would be required to determine the long-term benefits of response  
8 and durability of outcome. Further studies are required to identify the optimal combination  
9 therapy regimen that would allow more patients to achieve and sustain HBsAg loss.

10

#### 11 **HBsAg seroclearance during IFN therapy**

12 A 48 weeks treatment with PEG-IFN allows the potential for immune control of HBV  
13 infection, when compared to NUCs, with higher rates of HBeAg seroconversion and the  
14 possibility of viral suppression after stopping treatment, with loss of HBsAg in a proportion  
15 of patients who maintain undetectable HBV DNA. After PEG-IFN, sustained off-therapy  
16 virological response (SVR) is defined as serum HBV DNA levels < 2,000 IU/ml after the end  
17 of therapy. In HBeAg-negative CHB patients, a phase III trial evaluating PEG-IFN  
18 monotherapy has showed SVR rates of 44% at 6 months and 28% at 3 years after the end of  
19 therapy[123]. In HBeAg-negative patients with genotype D or E, PEG-IFN was less effective  
20 with a SVR around 20%. The rate of HBsAg loss progressively increased after PEG-IFN- $\alpha$   
21 discontinuation, from 3% at month 6 to 9% at year 3 to 12% at year 5 in the registrational  
22 trial. Overall, among sustained responders, approximately 30% clear HBsAg in the long-term.  
23 HBsAg loss rates increase after the end of PEG-IFN $\alpha$  therapy in initially HBeAg-positive  
24 CHB patients with SVR [124]. Of the patients with an initial HBeAg seroclearance, 30%

1 experienced HBsAg seroclearance after 3 years of follow-up. The sustainability of HBsAg  
2 loss and seroconversion after PEG-IFN $\alpha$  have been documented[122].  
3 HBsAg loss was evaluated in patients receiving the combination of TDF and PEG-IFN  $\alpha$ -2a  
4 for a finite duration in a randomized trial, and compared to TDF monotherapy and PEG-IFN  
5 monotherapy[122]. Seven hundred and forty CHB patients were randomly assigned to receive  
6 TDF plus PEG-IFN for 48 weeks, TDF plus PEG-IFN for 16 weeks followed by TDF for 32  
7 weeks, TDF for 120 weeks, or PEG-IFN for 48 weeks. At week 72, 9% of subjects in group  
8 receiving TDF plus PEG-IFN for 48 weeks combination had HBsAg loss compared with less  
9 than 3% in other groups.

10

### 11 **HBsAg seroclearance during NAs therapy**

12 ETV and TDF are potent HBV inhibitors with a high barrier to resistance and should be used  
13 as first-line monotherapies [125]. More than 95% of persons treated with the highly potent  
14 TDF and ETV achieve virological undetectability. NAs are administered orally, tolerability is  
15 favorable and efficacy is strong[126]. In HBeAg positive mainly Caucasian patients, HBsAg  
16 loss was around 10% after 5 years of TDF and was more likely to occur in Caucasian patients  
17 [127]. No HBsAg loss was observed after 2 years of TDF or ETV. Table 2 resumes all  
18 reported spontaneous and after treatment HBsAg seroclearance rates (table 2).

19 (Table 2 references: [8,41] , [128–130] , [127,131,132] , [133] , [123,134] , [135,136] , [137] ,  
20 [122]).

21

### 22 **HBsAg decrease as a predictor of treatment response**

23 To identify responders in the early phase of PEG-IFN $\alpha$ -2a therapy, decrease in serum  
24 quantitative HBsAg is a validated on-treatment marker predicting sustained off-treatment  
25 response[138]. In a proof of concept study of 48 CHB HBeAg-negative patients receiving

1 PEG-IFN $\alpha$ -2a, a decrease of 0,5 and 1 log<sub>10</sub> IU/mL of serum HBsAg levels at weeks 12 and  
2 24 of therapy had a 90% negative predicting value and a 89% positive predicting value for  
3 week 12 and 97% negative predicting value and a 92% positive predicting value for week 24  
4 sustained response, respectively[139]. This was the first study to suggest early kinetics (week  
5 12) of HBsAg to differentiate sustained responders from non-responders to PEG-IFN.

6

### 7 **The role of HBsAg in NAs cessation**

8 Interestingly, Chen et al. evaluated in a retrospective study the role of HBsAg quantification  
9 in predicting HBsAg loss and HBV relapse[140]. In HBeAg $\square$ positive and negative patients  
10 reports that EOT HBsAg levels < 300 IU/ml, 300–1000 IU/ml and >1000 UI/ml in  
11 HBeAg $\square$ positive patients were associated with sustained HBeAg loss in 55.6%, 7.7% and  
12 3.3% respectively. EOT HBsAg cut $\square$ offs <120 IU/ml, 120–1000 IU/ml and > 1000 IU/ml in  
13 HBeAg $\square$ negative patients were associated with HBsAg loss in 79.2%, 14.3% and 0%,  
14 respectively, and HBsAg cut $\square$ offs < 200 IU/ml, 200–1000 IU/ml and > 1000 IU/ml were  
15 associated with an SVR in 93%, 11.1% and 15.4% respectively. Similar results were reported  
16 in patients whose treatment was discontinued; an EOT HBsAg level <100 IU/ml was  
17 associated with high SVR, while > 1000 IU/ml was associated with a 1 year post $\square$ treatment  
18 relapse in 70%[141]. A systemic review to summarize the role of HBsAg in NAs cessation  
19 among Asian CHB patients showed that HBsAg loss ranged 21.1-58-8% in pts with HBsAg <  
20 100 IU/ml, vs 3.3-7.4% in pts with HBsAg >100 IU/ml[142].

21

### 22 **HBsAg seroclearance and novel therapies**

23 Several strategies, including antivirals targeting various stages of the HBV life cycle (HBV  
24 entry, viral replication, cccDNA production, and viral protein expression), as well as

1 immunotherapeutic agents, are being explored in experimental models or have reached  
2 clinical testing, which may have the potential to complement PEG-IFN and NA based  
3 therapy[17,143–145]. Strategies inhibiting viral gene expression either through cccDNA  
4 transcription or viral mRNA translation can decrease serum HBsAg levels. Nucleic acid-  
5 based polymers (NAPs) are a new class of broad-spectrum antiviral compounds which act  
6 against HBV infection by blocking the release of the surface antigen protein (HBsAg) from  
7 infected hepatocytes[146]. This pharmacological activity blocks replenishment of HBsAg in  
8 the circulation, allowing host mediated clearance and has important clinical significance as it  
9 may potentiate the ability of immunotherapies (resumed in Fig. 4) to restore functional control  
10 of HBV infection. Removal of HBsAg would enhance the effect of PEG-IFN $\alpha$ -2a and can  
11 lead to the creation of a favorable immunological activation, appearance of free anti-HBsAg  
12 antibodies and clearance of HBV virions in the blood. Among HBsAg-targeting antiviral  
13 strategies (Fig. 5), NAPs are being investigated in preclinical evaluations and in several  
14 clinical trials that have evaluated the activity of REP 2139, REP 2055 and REP 2165 in  
15 monotherapy and in combination with immunotherapy[146–149]. 9 of 12 patients showed  
16 substantial reduction of HBsAg and seroconversion to anti-HBsAg in response to REP 2139-  
17 Ca (NCT02646189)[147]. Recently were reported results of an open label, randomized,  
18 controlled, phase 2 study involving 40 CHB patients, treated with two different NAPs, REP  
19 2139-Mg or REP 2165-Mg in association with PEG-IFN and TDF. No severe adverse events  
20 were reported. Efficacy was impressive with around half of the patients achieving HBsAg  
21 seroconversion[149]. These interesting results need to be confirmed in larger studies.  
22 Furthermore, the potential for cytotoxicity resulting from intracellular retention of HBsAg  
23 should be investigated and the problem of NAPs accumulating in the liver should be  
24 addressed.

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**Conclusion**

HBsAg seroclearance is a rare event in the natural history of HBV and it is associated with reduced risk of HCC. Current antiviral therapies using PEG-IFN and NAs can suppress HBV replication and improve the prognosis of CHB, but they fail to clear HBsAg to achieve a functional cure. Several guidelines proposed HBsAg seroclearance as a crucial surrogate marker of thorough HBV clearance. HBsAg may contribute to the impairment of innate and adaptive immunity and the exhaustion of T cell and B cell responses. Therefore, a reduced serum HBsAg level could facilitate the recovery of the host's immune system. Numerous immune cells have been shown to interact with HBsAg contributing to the immunopathogenesis of CHB but the immune mechanisms underlying HBsAg seroclearance are still unclear. A better understanding of the interaction between HBsAg and these immune factors could contribute to the development of effective immune therapies.

The presence of cccDNA and integrated DNA on one hand, and the particular liver microenvironment and immune deregulations induced by chronic infection on another hand, are key challenges for antiviral approaches[144]. Overall, the safety and efficacy of the newly developed strategies, whether antivirals or immune based, need to be tested, and their ability to remove HBsAg needs to be investigated. Strategies focusing on reducing HBsAg by siRNA, NAPs or antibody-mediated neutralization will be crucial to help restoring immune responses. Developing effective combination therapies targeting HBsAg may further induce the appearance of an anti-HBV specific immune response and lead to a functional cure for CHB.

1

## 2 **Figure legends**

### 3 **Fig. 1. Schematic model of Dane particle and HBsAg proteins.**

4 HBV genome is housed in a capsid structure formed by core (HBcAg) proteins surrounded by  
5 three different HBsAg, L-, M- and S-HBsAg. All HBsAg share the S domain which contains  
6 four putative transmembrane domains. M-HBsAg contains an additional PreS2 domain and L-  
7 HBsAg have both PreS1 and PreS2 domains. The NTCP binding domain is present in the  
8 PreS1 domain and is important for HBV infection. The “a” determinant is immunogenic in its  
9 S domain which is important for antibody neutralization.

10

### 11 **Fig. 2. HBsAg lifecycle and main effects in infected hepatocytes.**

12 HBsAg is produced from both cccDNA transcripts and HBV integration in the host genome.  
13 M- and L-HBsAg can act as transcriptional activators for host genes. HBsAg proteins are  
14 accumulated in ER and can induce the activation of cellular stress pathways. HBsAg plays a  
15 crucial role in HBV encapsidation and virions secretion. Secreted HBsAg can subvert the  
16 antiviral immune response.

17

### 18 **Fig. 3. Proposed immune mechanisms for HBsAg seroclearance.**

19 Decreased HBsAg levels could facilitate the recovery of the host’s immune system. mDCs  
20 regain their antigen presenting capacities to activate T cells, as well as TNF $\alpha$  secretion. pDCs  
21 restore TNF $\alpha$  and IFN $\gamma$  secretion and activate NK cells. Restoration of NK cell effector  
22 functions: cytotoxicity and IFN $\gamma$  secretion, activation of T cells. Recovery from T cell  
23 exhaustion: restored proliferation, increase in HBsAg-specific CTLs, direct cytolytic activity  
24 on infected hepatocytes and IFNs secretion. Suppression of excessive functions of Treg and  
25 MDSCs. KCs induce cytokines production, have restored inflammasome functions and

1 activate NK cells. Restoration of functional HBsAg-specific plasmocytes secreting  
2 neutralizing anti-HBsAg. Upward green arrows signify a restoration of function and/or  
3 frequency while downward red arrows signify a decrease in function and/or frequency.

4

5 **Fig. 4. Developed immune-based approaches to clear HBV.**

6 (1) Therapeutic vaccination could restore dysfunctional T and B cell responses during CHB.  
7 (2) Stimulation of innate immunity by (i) TLRs and RIG-I agonists leading to the activation  
8 of hepatocytes, intrahepatic dendritic, NK and MAIT cells. (ii) TCR-like antibodies allow  
9 direct recognition of HBV infected hepatocytes. (iii) Cytokines inhibit HBV replication. (3)  
10 Antibody-mediated neutralization could prevent HBV infection of hepatocytes and reduce  
11 HBsAg circulating levels. (4) Anti-HBV T cell boosting by (i) T cell engineering. (ii) and  
12 (iii) Checkpoint blockade, modulation of regulatory cells and metabolic modulation. (5)  
13 Functional maturation of dysfunctional HBsAg-specific B cells by boosting Th cells or anti-  
14 PD1 therapy.

15

16 **Fig. 5. HBsAg-targeting antiviral approaches.**

17 Schematic representation of HBsAg-targeting strategies. HBV life cycle is shown, and  
18 different approaches are developed to reduce high levels of HBsAg in CHB patients. (1)  
19 Inhibition of HBsAg release by nucleic acid polymers (NAPs). (2) Silencing and eliminating  
20 cccDNA. Targets against cccDNA include antiviral cytokines (PEG-IFN), blockade of  
21 rcDNA and epigenetic regulation to undergo its degradation. Technologies such as  
22 CRISPR/Cas9 are being utilized to eliminate cccDNA along with the use of histone  
23 deacetylase (HDAC) inhibitors. (3) Suppression of HBsAg expression by HBV-targeting  
24 siRNA.

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Fig1

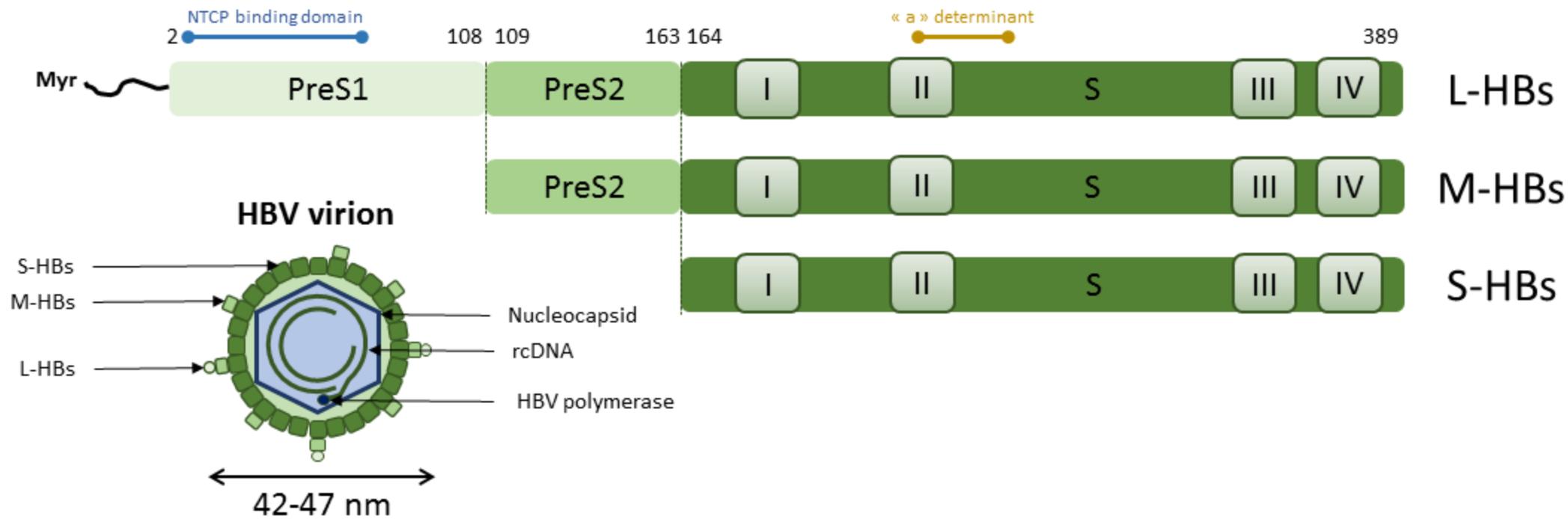


Fig. 2

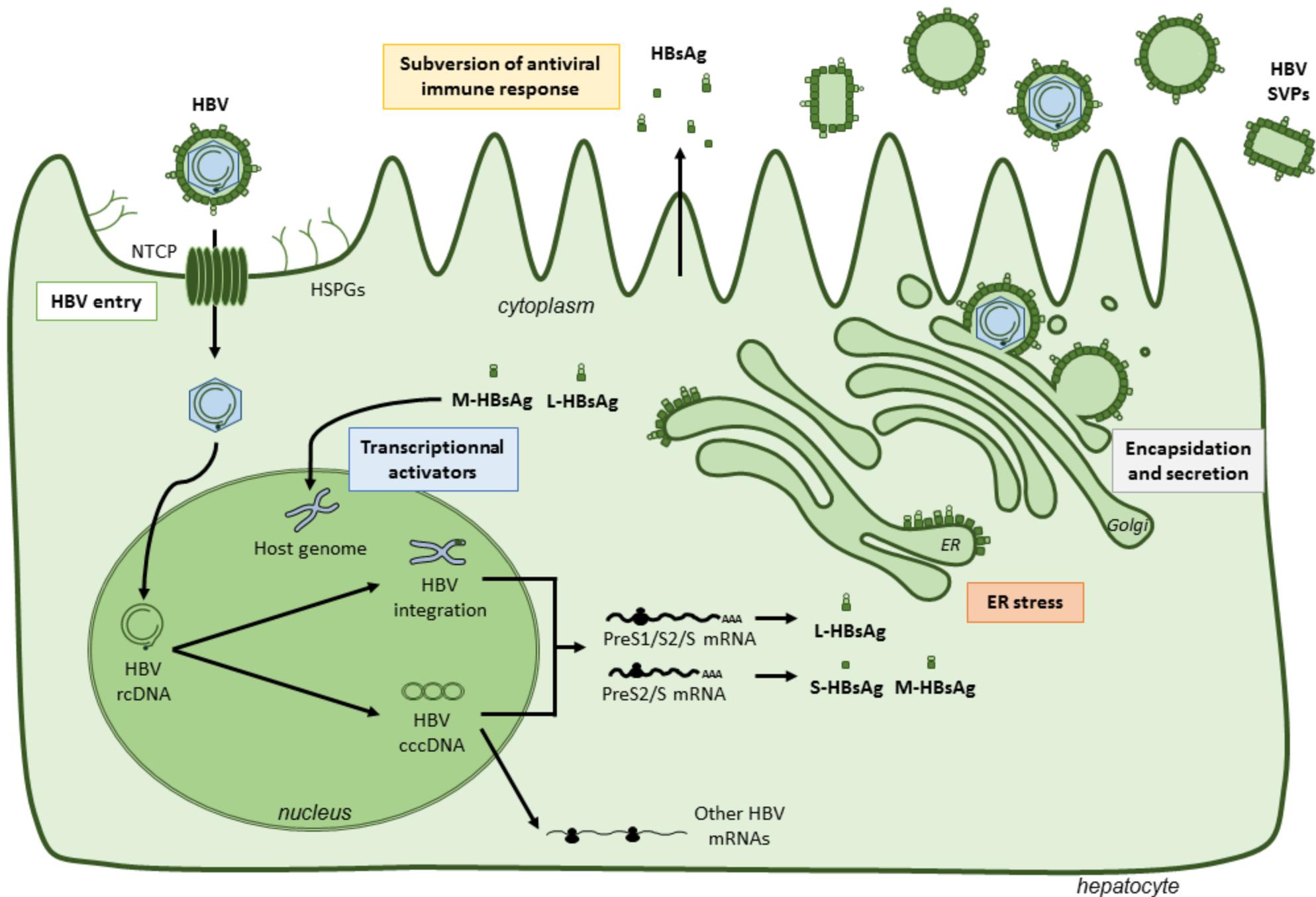


Fig. 3

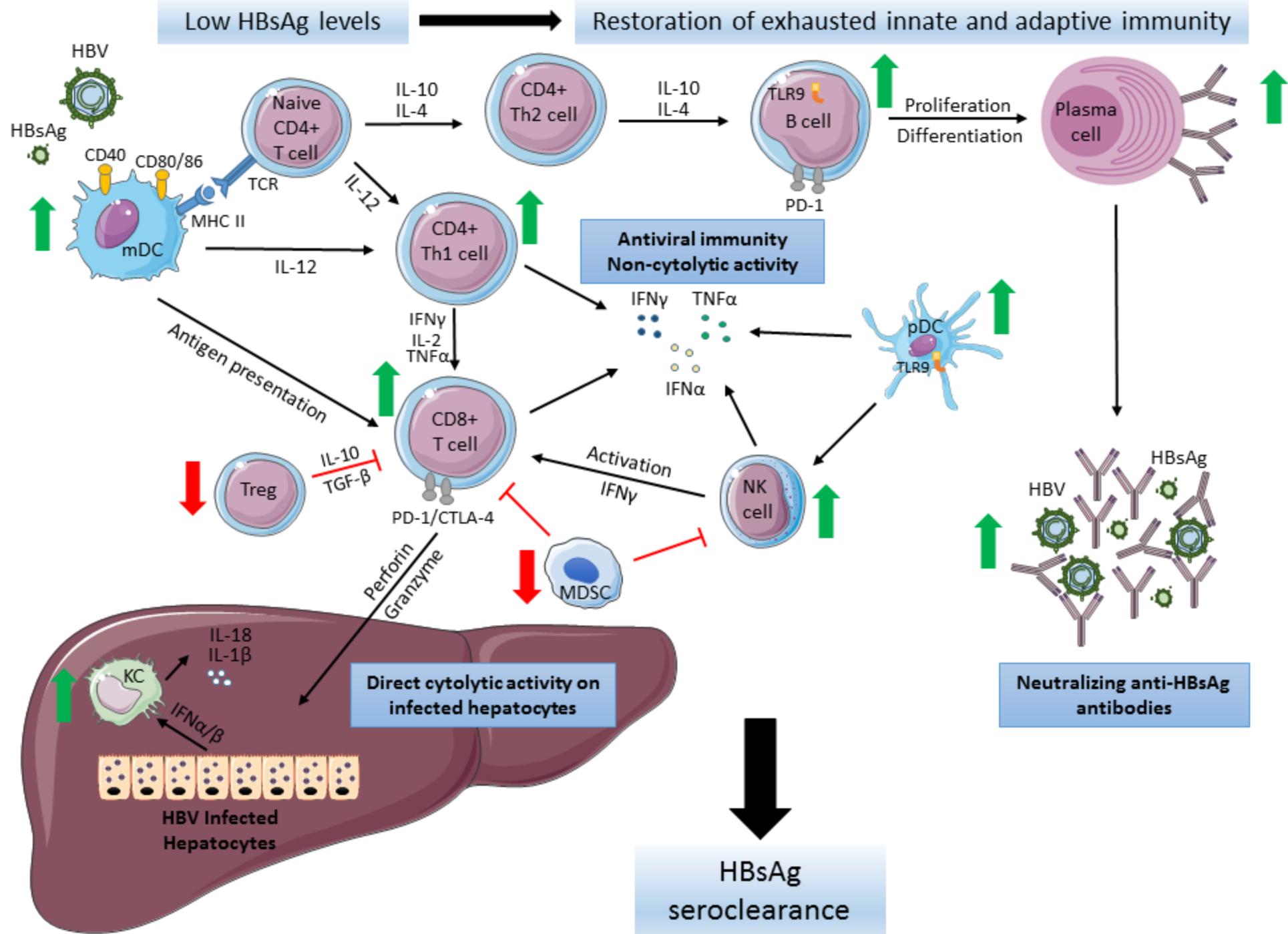


Fig. 4

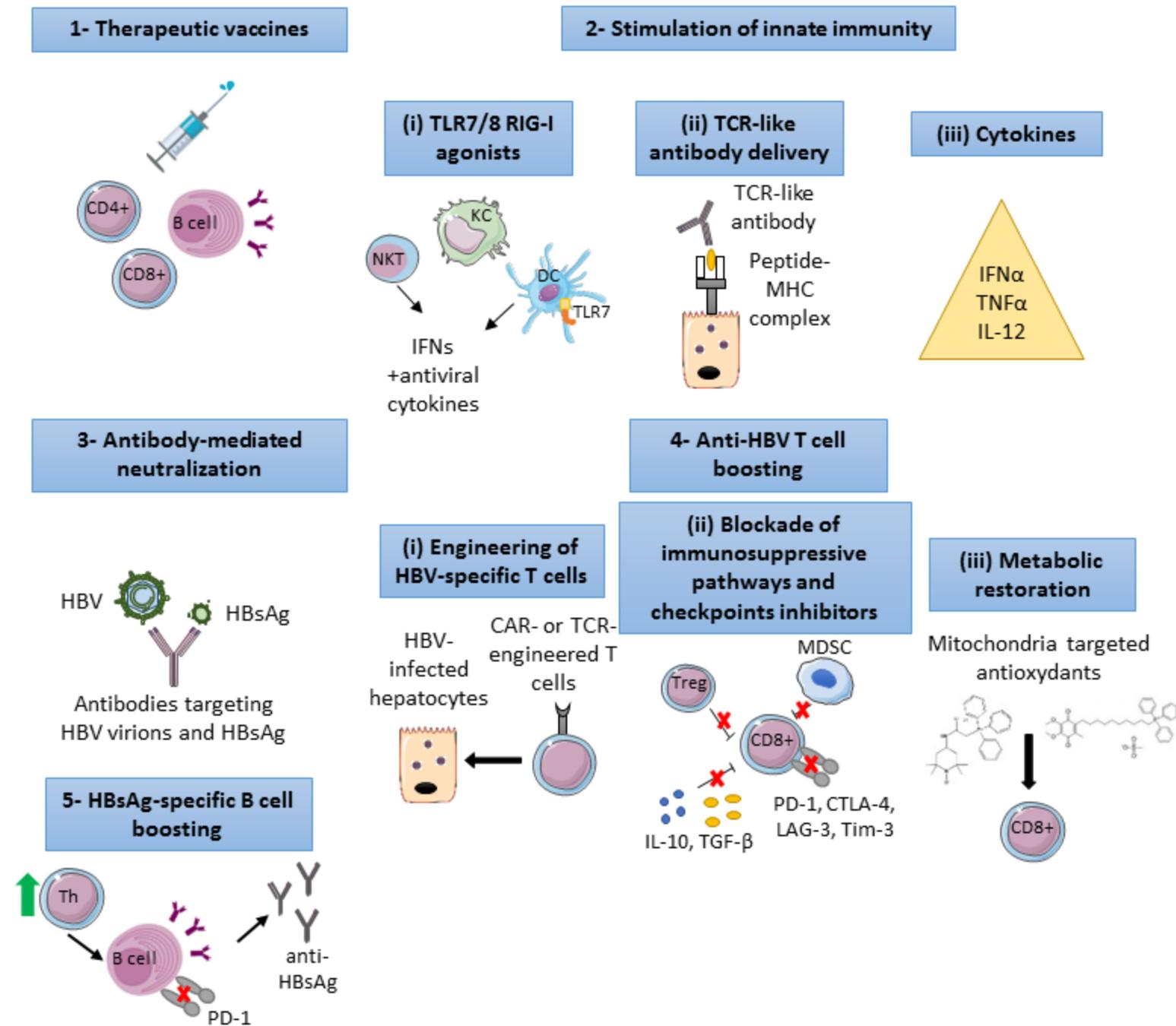
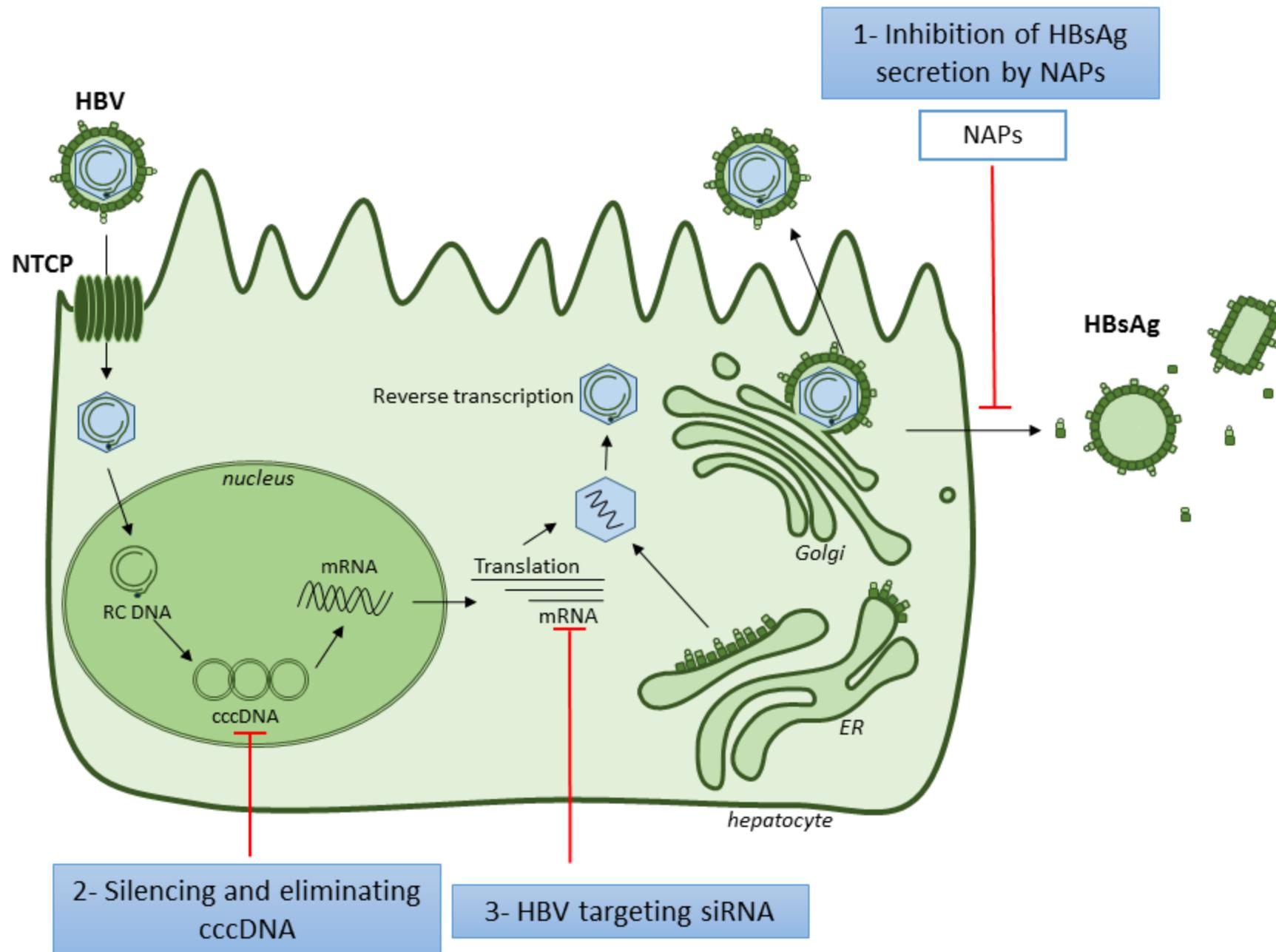


Fig. 5



**Table 1. Hepatitis B surface antigen (HBsAg) effects on immune cells**

Immune cell type	HBsAg effects	Reference(s)
Kupffer cell	Directly interacts with KCs in vivo and in vitro ↓ the AIM2-inflammasome and ↓ the production of IL-1 $\beta$	57,58
Monocyte/ Macrophage	↓ TLR2 and c-Jun N-terminal protein kinase (JNK), thus ↓ production of IL-12	59
mDC	↓ frequency and function	60,61
pDC	↓ TLR9-mediated activation and IFN- $\alpha$ production	51,52,62,63
PBMC	↓ the interaction of MVP with MyD88 in infected cells, thus ↓ type I IFN responses	56
NK cell	↓ cytolytic activity	51
T cell	Exhaustion of CD4+ and CD8+ T cells by ↑ of PD-1 and Lag-3 expression	64,65
B cell	↓ TLR9 expression and B cell related functions	66
Monocyte/ MDSC	↑ the differentiation and ↑ the expansion of monocytes into MDSCs	67

AIM2, Absent in melanoma 2; HBsAg, Hepatitis B surface antigen; IFN- $\alpha$ , interferon  $\alpha$ ; IL-12, interleukin 12; KC, Kupffer cell; mDC, myeloid dendritic cell; LAG-3, Lymphocyte-activation gene 3; MDSC, Myeloid derived suppressor cell; MVP, major vault protein; Myd88, Myeloid differentiation primary response 88; NK cell, natural killer cell; PBMC, peripheral blood mononuclear cell; PD-1, Programmed cell death protein 1; pDC, plasmacytoid dendritic cell; TLR2, Toll-like receptor 2; TLR9, Toll-like receptor 9.

**Table 2. Spontaneous and after treatment HBsAg seroclearance**

<b>Mode</b>	<b>Treatment</b>	<b>Therapy duration (weeks)</b>	<b>Follow-up duration (weeks)</b>	<b>Number of participants (n)</b>	<b>HBsAg seroclearance (%)</b>	<b>Reference(s)</b>
Spontaneous	N/A	N/A	260 - 520	1965 - 42588	4,03 - 8,1	8,41
Under current treatment (monotherapy)	ETV	52 - 260	52 - 260	146 - 709	1,4 - 5,1	128 - 130
	TDF	48 - 260	48 - 260	266 - 585	3 - 11	127,131,132
	TAF	96	96	576	1	133
	PEG-IFN	48 - 52	48 - 52	136 - 177	4 - 7	123,134
Combination therapy	ETV+TDF	92 - 96	96	57 - 197	1,7 - 3,6	135,136
	PEG-IFN+ETV	48	48	85	1,17	137
	PEG-IFN+TDF	48	72	186	9,1	122

ETV, Entecavir; HBsAg, Hepatitis B surface antigen; N/A, Not applicable; PEG-IFN, Pegylated interferon; TAF, Tenofovir alafenamide; TDF, Tenofovir disoproxil fumarate.