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Kupffer cells: friend or foe during hepatitis B infection?

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2
3 17 **Abbreviations:**
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5 18 HBV: hepatitis B virus
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8 19 CHB: chronic hepatitis B
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10 20 cccDNA: covalently closed circular DNA
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13 21 M Φ : macrophage
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16 22 PRR: pattern recognition receptor
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19 23 ROS: reactive oxygen species
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22 24 KC: Kupffer cells
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25 25 LPS: lipopolysaccharide
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28 26 HIF: hypoxia induced factor
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30 27 HBeAg: hepatitis B e antigen
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33 28 HBsAg: hepatitis B surface antigen
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36 29 NK: natural killer
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39 30 gMDSC: granulocyte myeloid derived suppressor cell
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42 31 rcDNA: relaxed circular DNA
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45 32 NF- κ B: nuclear factor-kappa B
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48 33 HBc: hepatitis B capsid
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50 34 FASL: FAS ligand
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53 35 IFN: interferon
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56 36 PBMC: peripheral blood mononuclear cell
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3 37 PD1: programmed cell death 1
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5 38 PD-L1: programmed cell death-ligand 1
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8 39 DHBV: duck hepatitis B virus
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11 40 NPC: non-parenchymal cell
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14 41 MMP9: matrix metalloprotease 9
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17 42 IFNAR1: interferon alpha receptor 1
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19 43 MAPK: mitogen activated protein kinase
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22 44 HNF: hepatocyte nuclear factor
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25 45 PPAR β/δ : peroxisome proliferator-activated receptors
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28 46 HMGB1: high-mobility group box 1
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3 48 **Abstract**
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5 49 The Hepatitis B virus chronically infects the liver of 250 million people worldwide.
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7 50 Over the past decades, major advances have been made in the understanding of
8
9 51 Hepatitis B virus life cycle in hepatocytes. Beside these parenchymal cells, the liver
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11 52 also contains resident and infiltrating cells involved in immune responses to
12
13 53 pathogens and much less is known about their interplay with Hepatitis B virus. In this
14
15 54 review, we summarized and discussed the current knowledge of the role of Kupffer
16
17 55 cells, the liver resident macrophages, in HBV infection.
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24 57 **Word count:** 86
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27 58 **Key words:** Hepatitis B virus; Kupffer cells; innate immunity inhibition; tolerance
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- Hepatitis B virus can be recognized by macrophages in specific conditions (i.e. hepatocytes cell death).
 - Hepatitis B virus inhibits pro-inflammatory responses in macrophages that are described to have an anti-viral effect.
 - Hepatitis B virus favours anti-inflammatory responses macrophages and liver tolerance.
 - Kupffer cells participate in hepatitis B virus associated pathogenesis through activation of hepatic stellate cells and through inhibition of T cell responses by anti-inflammatory secretions.
 - Understanding the mechanisms by which hepatitis B virus inhibits macrophages responses could be the first step towards development of new therapies for chronically infected patients.

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60 **Introduction**

61 Hepatitis B virus (HBV) is a hepatotropic virus that chronically infects around 250
62 million people worldwide (WHO, 2017). Upon exposure to the virus, 90-95% of
63 immune-competent adults will clear the infection, whereas 5-10% will develop a
64 chronic infection. In these chronically HBV-infected (CHB) individuals, the risk of
65 developing, in the long-term, cirrhosis and hepatocellular carcinoma is greatly
66 increased¹. Despite an efficient prophylactic vaccine that protects from infection and
67 treatments that induce a virosuppression (i.e., reduction of viral load in blood), a
68 complete cure of CHB individuals is not achievable yet, due to the persistence of an
69 episomal viral DNA template, called cccDNA (i.e., covalently-closed-circular DNA),
70 inside the nucleus of infected cells². The development of new treatments is therefore
71 still necessary. One major cause of HBV chronicity comes from a non-optimal
72 immune response against the virus and infected cells during the acute infection
73 phase and after progression to a persistent infection^{3,4}. Why and how the virus
74 interacts with and impairs immune cell functions still remains to be fully understood.
75 In this review, we focus on the interaction between the virus and one specific type of
76 immune cells: the macrophages.

77

78 **Macrophages phenotypes and functions**

79 Macrophages (MΦ) were first described, by Ilya Metchnikoff at the end of the 19th
80 century, as evolutionary conserved phagocytes that have evolved for more than 500
81 million years⁵. Over the last 2 centuries, a lot has been learnt about MΦ embryonic
82 origins, phenotypes of different subpopulations, and their various functions.

1
2
3 83 In solid organs, M Φ can have two origins. Resident macrophages comes from the
4
5 84 yolk sac whereas infiltrating macrophages, which are recruited upon pathogen
6
7 85 contamination and/or injury, are derived from the differentiation of peripheral blood
8
9 86 circulating monocytes, themselves primarily coming from the bone marrow⁶.
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11 87 Depending on the tissue, it is not always possible to distinguish resident M Φ from
12
13 88 infiltrating ones. For example, in the human liver, no specific marker has been
14
15 89 identified to differentiate these two types of M Φ .

16
17
18 90 Tissue resident M Φ represent the first line of defence against pathogens such as
19
20 91 bacteria and viruses, and are therefore an important component of the innate
21
22 92 immune response to infections. In addition to their physiologic phagocytic capacity,
23
24 93 which allows the elimination of physiologically aging, or infected, or cancerous cells,
25
26 94 M Φ can importantly also contribute to adaptive immunity by processing and
27
28 95 presenting antigens after pathogen engulfment and their recognition through Pattern
29
30 96 Recognition Receptors (PRR)⁷. Upon activation, M Φ can secrete a large spectrum of
31
32 97 pro-inflammatory cytokines involved in different activities such as lymphocyte
33
34 98 polarization (IL-1, IL-12, IFN- γ ...) or neutrophil recruitment (IL-8, TNF α ...), and also
35
36 99 anti-inflammatory cytokines involved in inflammation regulation (IL-10 and TGF β), as
37
38 100 well as chemokines involved in leukocyte recruitment to the sites of injury
39
40 101 (CXCL8...)⁸ and others factors, such as VEGF implicated in angiogenesis⁹.

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45 102 M Φ can have a large variety of phenotypes that can be classified by specific marker
46
47 103 expression and/or secreted cytokines^{7,10}. Several classifications have been proposed
48
49 104 over the years, but a consensus is yet to be found. Nevertheless, they can be divided
50
51 105 into two major phenotypes: pro- and anti-inflammatory. Of note, the pro-/anti-
52
53 106 inflammatory dichotomy is a simplified view since each M Φ expresses a set of genes
54
55 107 that on its own could define a new type of M Φ ⁷.

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3 108 Pro-inflammatory MΦ, called M1, have been defined as mediators of the defence
4
5 109 against bacterial and viral pathogens. They are implicated in inflammation, tumour
6
7 110 resistance and killing of intracellular pathogens¹¹. M1 are commonly described as
8
9 111 STAT1⁺, IRF5⁺, and iNOS⁺, and can secrete a large panel of pro-inflammatory
10
11 112 cytokines (IL-6, IL-1 family, IL-12, IL-23, TNFα...), chemokines (CXCL9, CXCL10,
12
13 113 CXCL11, CCL3, CCL5...), and inflammatory mediators (i.e. reactive oxygen species
14
15 114 or ROS)¹¹. M1 MΦ have a “broken Krebs cycle” leading to the accumulation of citrate
16
17 115 and subsequently NO production, as well as the accumulation of succinate, HIF1α
18
19 116 and IL-1β secretion¹². M1 MΦ are reported to be able to activate tumour-killing
20
21 117 mechanisms and to amplify Th1 responses, inducing a positive feedback in the anti-
22
23 118 tumour response¹³. Indeed, M1 macrophages can present antigens to induce an
24
25 119 adaptive immune response but can also reactivate lymphocytes locally, at the
26
27 120 injury/infection site. Although pro-inflammatory MΦ are mainly implicated in the
28
29 121 elimination of noxious signals or microorganism invaders, M1-like macrophage-
30
31 122 derived cytokine production have also been identified as key factors in several
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33 123 autoimmune and chronic inflammatory diseases, including Crohn’s disease, multiple
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35 124 sclerosis, and autoimmune hepatitis^{14,15}.

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40 125 Anti-inflammatory MΦ, commonly called M2, are mainly implicated in the resolution of
41
42 126 inflammation and wound healing, but also, in some circumstances and by
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44 127 mechanisms not fully understood, in the defence against parasites¹⁶. M2 are
45
46 128 commonly described as STAT6⁺, IRF4⁺, and CD163⁺, and can secrete anti-
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48 129 inflammatory cytokines (IL-10, TGFβ), chemokines (CCL1, CCL17, CCL24...) and
49
50 130 growth/angiogenic factors (PDGF, VEGF, EGF). In contrast to M1, M2 MΦ have a
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52 131 “functional Krebs cycle” allowing production of ATP and glycosylation of M2-
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54 132 associated receptors¹². M2 macrophages are mandatory for the resolution of
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3 133 inflammation, and tissue repair after an injury and/or cytotoxic activity¹¹. However, M2
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5 134 are also associated with tumour progression through the secretion of negative
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7 135 immuno-modulators, and are called, in the tumour micro-environment, TAM for
8
9 136 “tumour associated macrophages”¹⁷. TAM have been associated to the progression
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11 137 of numerous cancers and as such are potential new target for therapeutic purposes
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13 138 ^{17–19}.

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19 140 **Kupffer cells: the liver macrophages**

21
22 141 Kupffer cells (KC), the liver resident M Φ , was first described by Fahimi in 1970 as
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24 142 peroxidase positive cells²⁰. KC are the largest M Φ population in the human body as
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26 143 they represent 80% of the total human M Φ count under normal physiological
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28 144 conditions^{21,22}. KC are localized in liver blood vessels, seeded on liver sinusoidal
29
30 145 endothelial cells and their fenestration, where they can phagocytose debris, aging
31
32 146 blood cells or pathogens. After phagocytosis, pathogens can be processed into
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34 147 phagolysosomes and antigens can be presented to lymphocytes to activate or recall
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36 148 a local immune response^{21,23}. As resident M Φ , KC express all common M Φ markers
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38 149 (i.e. CD68, CD14...)⁶ and have more precisely been defined as CD14⁺, HLA-DE⁺,
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40 150 HLA-ABC⁺, CD86⁺, DC-SIGN⁺ with low expression of CD1b, CD40 and CD83²⁴. As
41
42 151 previously mentioned, in humans, no specific marker has been identified to
43
44 152 differentiate KC from infiltrating macrophages, whereas in the mouse (i.e. Clec4F)²⁵
45
46 153 and rat (i.e. ED-1 and ED-2)²⁶ markers have been described. In addition, KC have a
47
48 154 long life-span (more than a year) and are self-renewing cells with a slow replicating
49
50 155 activity. Thus the liver M Φ pool can be renewed, unlike monocyte derived
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52 156 macrophages which have a limited life-span²⁷. Interestingly, a recent study by Scott
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54 157 and colleagues described that in mice monocyte derived M Φ can, with time, acquire

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3 158 KC phenotype (including self-renewal capacity) after depletion of the resident M Φ in
4
5 159 a model of KC expressing the diphtheria toxin receptor²⁵.
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8 160 KC play a critical role in liver tolerance. Indeed, the liver is at the crossroad between
9
10 161 systemic and enteric circulation, with 80% of the blood reaching the liver coming from
11
12 162 the hepatic portal vein. Thus, liver cells are constantly exposed to pathogens. Indeed,
13
14 163 lipopolysaccharide (LPS) endotoxin is found in measurable amounts (few nano
15
16 164 grams) in the enteric circulation²⁸. This phenomenon should theoretically lead to a
17
18 165 constant inflammation within the liver but none is observed in healthy individuals. The
19
20 166 liver is indeed described as an immune-tolerant organ²¹. This tolerance has been
21
22 167 defined as a systemic or “oral” tolerance of enteric pathogens, which depends on the
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24 168 normal connection between the liver and gut²⁹. Yet, the full role of KC in this
25
26 169 tolerance phenomenon still need further investigation, especially by assessing KC
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28 170 phenotype/state in healthy liver. Indeed, the anti- or pro-inflammatory phenotype of
29
30 171 KC at steady state is yet to be properly determined. Depending on their localization in
31
32 172 the liver, M Φ may have different phenotypes. It could be hypothesised that near the
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34 173 enteric circulation, where the exposition to bacteria is constant, KC will be more likely
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36 174 to be M2 M Φ , the M Φ implicated in tolerance, through the secretion of immune-
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38 175 regulatory mediators³⁰. In contrast, near the systemic circulation, where the presence
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40 176 of pathogens is a sign of infection, KC would be more likely to be in a pre-pro-
41
42 177 inflammatory state in order to quickly eliminate any blood infections. Moreover, other
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44 178 factors could play a role in the fate of KC phenotype. The oxygen gradient in the liver
45
46 179 is linked to the expression of hypoxia regulated genes (HIF family), among which is
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48 180 HIF1 α , a factor linked to the accumulation of IL-1 β in M1 M Φ ³¹.
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53 181 As previously described, even in this tolerant environment, KC are able to respond to
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55 182 the detection of specific pathogens^{22,24,32}. KC express a large spectrum of PRR,
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3 183 among which, TLR1/2, TLR2/6, TLR3, TLR4, TLR8 and RIGI/MDA5^{33,34}, leading,
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5 184 after stimulation, to the secretion of a large spectrum of immune factors such as
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7 185 ROS, cytokines (IL-6, IL-10, IL-12, TNF α ...), and chemokines (CCL3, CCL5...)
8
9 186 among others^{24,35-37}. KC have also been described to be functional for several
10
11 187 inflammasomes (AIM2, NLRP3), which, upon stimulation, induce the production,
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13 188 maturation and secretion of IL-1 β and IL-18^{38,39}. Chemokines and cytokines secretion
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15 189 will further recruit and activate monocytes from the circulating blood that will locally
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17 190 differentiated and further reinforce the M Φ population and inflammation³⁶. Thus in
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19 191 order to chronically infect the liver, pathogens (such as hepatitis viruses) must have
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21 192 evolved strategies to escape or prevent recognition by the immune system and liver
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23 193 inflammation.

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27 194 In summary, the liver is both an immune-tolerant⁴⁰ and immune-competent organ²¹,
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29 195 in which Kupffer cells play a central role in preventing the circulation in the blood of
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31 196 pathogens and aberrant cells.

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35 36 37 198 **Interaction between KC and HBV**

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40 199 The study of HBV infection often focuses on the hepatocytes in which the virus
41
42 200 replicates. Viral antigens are defined as secreted viral components which can be
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44 201 protein (hepatitis B e antigen; HBeAg), subviral particles (hepatitis B surface antigen
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46 202 embed in lipid membrane; HBsAg), and virions containing an icosahedral capsid
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48 203 enveloped by surface protein (empty virions, containing no viral genome or Dane
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50 204 particles, the infectious particles). However, the effect of the production of infectious
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52 205 particles and subviral particles in the liver and in the systemic circulation is still largely
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54 206 unknown. To note, several studies have shown that HBV replicative intermediate can
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3 207 be find in peripheral blood mononuclear cells⁴¹⁻⁴³, yet, HBV is not able to replicate
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5 208 inside KC (i.e. no secretion of HBeAg and HBsAg, and no detection of HBV RNA)
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7 209 (our unpublished data). It has been described that HBV infection can modify the
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9 210 functions of several immune cells such as natural killer (NK) cells, T cells, and
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11 211 granulocytic myeloid derived suppressive cells (gMDSC)⁴⁴. We will now focus on the
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13 212 interaction of HBV and macrophages from four different angles: i) Can macrophages
14
15 213 recognize HBV? ii) Can HBV modify the phenotype and function of MΦ? iii) Do MΦ
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17 214 play a role in the establishment or persistence of the infection? iv) Do MΦ play a role
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19 215 in the development of pathologies associated with HBV infection?
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217 **Can liver macrophages recognize hepatitis B virus?**

218 Liver MΦ are equipped to recognize pathogens and mount an efficient pro-
219 inflammatory response if needed to prevent, control and delay infections that could
220 become chronic, as described above. A study in Pekin duck revealed that minutes
221 after inoculation HBV was taken up by KC, highlighting their interaction *in vivo*⁴⁵. As a
222 DNA virus with RNA intermediates, HBV could theoretically be recognized by several
223 PRR expressed by KC such as TLR3 or RIGI/MDA5 as well as some cytosolic DNA
224 sensors³⁴. However, the recognition of HBV nucleic acids may be impaired by (i) the
225 physical separation of encapsidated rcDNA (relaxed circular DNA, a partially double
226 stranded DNA) from cytosolic DNA sensors, and (ii) the resemblance between HBV
227 RNAs and host mRNAs as viral RNAs are transcribed by the host polymerase.
228 Indeed, several studies have shown that in animal models, primary exposition to HBV
229 elicits little to no immune responses^{3,4}. Even though the authors did not specifically
230 analyse the responses in macrophages, the lack of immune responses in liver
231 biopsies may be also interpreted as an absence of response in macrophages.

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3 232 In the KC, the situation may be different as although they do not support viral
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5 233 replication (i.e. no neo formation of HBV RNAs), viral components could be present
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7 234 after phagocytosis and viral epitopes could be recognised by immune receptors.
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9 235 Indeed, after purification of different subsets of human liver cells, Hösel *et al.* showed
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11 236 that non-parenchymal cells (i.e., all liver cells except hepatocytes) and especially KC
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13 237 were activated after a 3h of exposure to HBV. They observed a transient activation of
14
15 238 nuclear factor kappa B (NF- κ B) and the secretion of pro-inflammatory cytokines (IL-6,
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17 239 IL-8, TNF- α , and IL-1 β) without any induction of an interferon response⁴⁶ (Figure 1,
18
19 240 path#1). This results were recently confirmed by Cheng and colleagues who showed
20
21 241 that in monocyte derived macrophages exposed to high quantities of HBV, a
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23 242 transient (between 0.5 to 6h) increase of IL-6, IL-1 β , and TNF- α mRNAs was
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25 243 observed⁴⁷ (Figure 1, path#1).
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29 244 Unexpectedly, TLR2, a PRR implicated in the recognition of bacterial components,
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31 245 has been described to recognize HBV capsid/core protein (HBc)⁴⁸. Cooper and
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33 246 colleagues found that, in a monocyte cell line, THP1, a full-length HBc was bound to
34
35 247 monocyte receptors, leading to their differentiation into M Φ . Moreover, this binding
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37 248 strongly induced the production of the pro-inflammatory cytokines TNF- α , IL-6, and
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39 249 IL-12p40⁴⁸ (Figure 1, path#2). These inductions were dependent of NF- κ B, ERK-1/2,
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41 250 and p38 MAPK activation. The authors demonstrated the necessity of binding
42
43 251 between the arginine-rich C terminus of HBc and membrane heparin sulphate for a
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45 252 recognition by monocytes⁴⁸. However, the relevance of this model is to be questioned
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47 253 as capsid proteins are normally enveloped in the virion when circulating outside of
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49 254 the hepatocytes, which should prevent recognition by TLR2 ligands, and M Φ
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51 255 activation. Nevertheless, in the case of cell death, it may be possible that naked
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53 256 capsid could be released out of the hepatocytes. For example, a study by Arzberger
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3 257 *et al*, showed that apoptosis of HBV infected hepatocytes led to the release of naked
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5 258 nucleocapsid⁴⁹ which could theoretically induce TLR2-mediated M Φ activation
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7 259 (Figure 1, path#2'). In addition, a study performed with samples from HBV-positive
8
9 260 patients showed that KC stained positive for HBsAg and were more activated than
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11 261 those from control livers⁵⁰. Internalization of HBsAg was also observed *in vitro* using
12
13 262 purified KC or monocyte-derived M Φ and led to a strong induction of pro-
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15 263 inflammatory cytokines such as IL-6, TNF- α , and IL-15 as well as the anti-
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17 264 inflammatory cytokine IL-10, the chemokines CCL4 and CXCL8⁵⁰ (Figure 1, path#3)
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19 265 and the subsequent activation of IFN- γ production by NK⁵⁰ (Figure 1, path#4).
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21 266 Moreover, a study in HBsAg-transgenic mice showed that KC secretes IL-12 upon
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23 267 TLR9 ligand inducing liver injury caused by NK⁵¹. In this model, an increase of Fas
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25 268 ligand (FasL) and Fas proteins were observed respectively on NK and PHH, leading
26
27 269 to an induction of a cytotoxic response against PHH⁵¹ (Figure 1, path#2').

30
31 270 Some studies have shown that HBV could also induce an interferon-dependent
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33 271 immune response, which should have a direct antiviral effect on HBV infection in
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35 272 hepatocytes, indeed Peg-IFN α is currently used to treat HBV infection⁵². Using a
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37 273 transgenic mouse model allowing high levels of HBV replication, Real *et al.* recently
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39 274 showed that HBV replication lead to a TLR3 dependent interferon response in non-
40
41 275 parenchymal liver cells⁵³ (Figure 1, path#5). A significant up-regulation of IFN- β 1 and
42
43 276 subsequently ISG15 and LFIT1 mRNAs was observed in mouse model in which the
44
45 277 small HBsAg is not produced (Figure 1, path#5'). Of note, HBsAg has previously
46
47 278 been described to attenuate immune responses facilitating chronic infection (see
48
49 279 section on HBV modification of KC phenotype)^{38,54,55}.

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53 280 In summary (Figure 1), contradictory results regarding the potential activation or lack
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55 281 of activation of an immune response in M Φ by HBV have been reported. These
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3 282 discrepancies might come from the differences in model (chimpanzees, woodchuck,
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5 283 transgenic mice...) and virus (genotypes, purification, naked nucleocapsid...) used in
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7 284 different experimental approaches. The lack of responses in HBV-infected animal
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9 285 models^{3,4}, compared to results demonstrating that macrophages seems to be
10
11 286 equipped to recognize HBV^{46-48,50,51,53}, raises the possibility of an active HBV-driven
12
13 287 evasion mechanism which could contribute to the establishment and persistence of
14
15 288 infections *in vivo*.
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22 290 **Can HBV modify macrophage phenotype and function?**

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24 291 Several studies have described inhibitory mechanisms used by HBV to block the
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26 292 induction of a potential pro-inflammatory/anti-viral response, including inflammasome
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28 293 inhibition, immune receptor down-regulation, pro-inflammatory secretion inhibition,
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30 294 increase of anti-inflammatory secretion, and negative immune checkpoint induction.
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33 295 First, HBV has been shown to interfere with inflammasome activation. Zannetti *et al.*
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35 296 determined that KC production of IL-1 β by AIM2 inflammasome engagement was
36
37 297 blocked by HBV, through an HBsAg-dependent mechanism³⁸ (Figure 2, path#1). This
38
39 298 inhibition seemed to be specific to the AIM2 inflammasome, as HBV was not capable
40
41 299 of blocking IL-1 β production driven by other inflammasomes, including NLRP3³⁸.
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43

44 300 However, Yu *et al.* recently showed that HBV could also inhibit NLRP3 induced IL-1 β
45
46 301 secretion by KC and that this inhibition was HBeAg dependent³⁹ (Figure 2, path#2).
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48 302 This difference in results may come from differences in the HBV inocula used and
49
50 303 especially the quantity of HBeAg in the viral preparations. Although mechanisms
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52 304 involved may differ, both studies described an inhibition of IL-1 β secretion by KC
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54 305 after exposure to HBV components. HBV has likely evolved several strategies to
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3 306 block the production of IL-1 β , which has been recently described as the most efficient
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5 307 cytokine, amongst several tested (including IFNs) against the replication of HBV *in*
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7 308 *vitro*⁵⁶.
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10 309 In addition, HBV has been shown to prevent pro-inflammatory cytokine secretion by
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12 310 influencing PRR expression in M Φ . A down-regulation of TLR2 and TLR3 was found
13
14 311 in peripheral blood mononuclear cells (PBMC) and KC of chronically HBV-infected
15
16 312 patients compared to healthy controls^{57,58} (Figure 2, path#3). As TLR2 and TLR3
17
18 313 have been shown to, respectively, recognize HBc protein⁴⁸ and HBV genome
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20 314 replication in non-parenchymal cells⁵³, a down-regulation of their expression in
21
22 315 hepatocytes, KC and PBMCs may contribute to the lack of an efficient immune
23
24 316 response against HBV⁵³. However, another study showed a higher secretion of IFN- α
25
26 317 and - β , as well as IL-6 and IL-10 upon TLR3 stimulation in PBMC from HBV patients
27
28 318 compared to healthy controls, contradicting the results of a down-regulation of TLR3
29
30 319 in monocytes⁵⁵. Moreover, in HBV patients, TLR2 expression on KC and peripheral
31
32 320 monocytes was reduced in HBeAg-positive chronic HBV patients (i.e. immune-
33
34 321 tolerant/immune active patients) compared to controls individuals, whereas it was
35
36 322 significantly increased in HBeAg-negative chronic HBV patients (e.g., in inactive HBV
37
38 323 carrier) compared to controls⁵⁸. This modification of expression was specific to TLR2,
39
40 324 as TLR4 expression did not differ between groups. It appears that the effect of HBV
41
42 325 on TLR expression may be differentially regulated during the different phases of HBV
43
44 326 chronic infection that would partially explain the difference of immune responses
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46 327 observed during those phases.
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51 328 HBV may also directly target pro-inflammatory cytokines secretion. For example, in
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53 329 CHB patients, HBsAg induced an inhibition of IL-12 secretion by
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55 330 monocytes/macrophages after TLR2 stimulation through the interference of HBV with
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3 331 the JNK/MAPK pathway⁵⁹ (Figure 2, path#4). To further decipher what happens in
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5 332 the liver, the team of Pr Schlaak purified murine KC and exposed them to some TLR-
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7 333 ligands in the presence or absence of HBsAg, HBeAg or HBV virions. Upon
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9 334 exposition to any of these viral components, expression of interferon sensitive genes
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11 335 and pro-inflammatory cytokines was inhibited in KC⁵⁵ (Figure 2, path#5). In addition,
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13 336 Wu *et al.* have shown that stimulating KC with TLR3 or TLR4 ligands induced the
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15 337 production of IFN β and the inhibition of viral replication in hepatocytes in mice⁶⁰.
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17 338 However, this antiviral effect was lost when KC were pre-incubated with HBV⁵⁴
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19 339 (Figure 2, path#5). Among the mediators inhibiting HBV infection, IFN- α and IFN- γ
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21 340 were identified and shown to directly inhibit HBV replication in patients⁶¹. Moreover, a
22
23 341 recent study showed that HBV virions hijack enzymes involved in lipid metabolism to
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25 342 infect hepatocytes⁶². It could be hypothesised that the same pathways could be also
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27 343 hijack by HBV in M Φ to enter or pass-through these cells to be delivered to
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29 344 hepatocytes.
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34 345 Aiming at the same goal, others studies have shown that in addition to preventing
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36 346 pro-inflammatory responses, HBV also increases anti-inflammatory responses. In
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38 347 purified rat KC exposed to different quantities of HBV virions, no morphological
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40 348 changes were observed on KC, but, compared to non-infected animals, TGF- β
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42 349 secretion was increased from 5.38 at day-3 to 7.75 fold at day-7 post-exposure⁶³
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44 350 (Figure 2, path#6). In this study, no modification in the levels of pro-inflammatory
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46 351 cytokines IL-1, IL-6 and TNF- α was observed compared to non-HBV-exposed cells.
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48 352 However, the authors neither indicated the secretion level of those cytokines in both
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50 353 controls and HBV exposed KC nor did they examine if the pro-inflammatory cytokines
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52 354 could be secreted by KC after TLR stimulation and modified by incubation with
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54 355 HBV⁶³. In addition, Jiang *et al.* demonstrated that in non-parenchymal liver cells
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3 356 exposed to HBV, the expression of anti-inflammatory cytokines (IL-10 and TGF β)
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5 357 was enhanced⁵⁵ (Figure 2, path#6). This phenotype was associated with an inhibition
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7 358 of NF- κ B, IRF-3 and MAPKs pathways in these cells. Specific T-cell activation by KC
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9 359 was also impaired when cells were exposed to HBsAg, but this phenotype could be
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11 360 reverted by a treatment with IL-10 antibodies, suggesting that the effect of HBV on
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13 361 KC impairment of T cell response was mediated by this cytokine⁵⁵ (Figure 2,
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15 362 path#6'). Furthermore, in an established HBV carrier mouse model, Li *et al.* showed
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17 363 that TLR2 deficiency improved HBV elimination, thus indicating that TLR2 could
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19 364 modulate HBV persistence⁶⁴. Liver M Φ were shown to have an increased TLR2
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21 365 expression in HBV-carrier mice and to produce more IL-10 upon TLR2 activation by
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23 366 an HBcAg stimulation leading to a poor induction of CD8+ T cells^{64,65} (Figure 2,
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25 367 path#7). Indeed, IL-10 deficiency or anti-IL-10R treatment resulted in a functional
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27 368 CD8+ T cell response and HBV elimination in this mice model^{64,65}. To get further
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29 369 insights into the context of a primary infection, Dunn *et al.* analysed a cohort of 21
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31 370 patients with acute HBV, including 8 patients analysed before the peak of viremia,
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33 371 when the infection is still being established⁶⁶. At the peak of viremia, during the
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35 372 inhibition of lymphocyte activation, a peak of IL-10 was observed in the serum.
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37 373 Moreover, in chronically infected HBV patients an increase of CD68⁺ CD86⁺ M Φ was
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39 374 observed, promoting a specific Th2 response, thus promoting of phagocytic-
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41 375 independent inflammation⁶⁷. Lastly, Nebbia *et al.* described that in CHB patients
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43 376 there is an up-regulation of Galectin-9 on KC as well as an up-regulation of HBV
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45 377 specific Tim3⁺ CD8 T cell⁶⁸ (Figure 2, path#8). This Tim-3/Galectin-9 pathway up-
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47 378 regulation could be the cause of T cell exhaustion during chronic infection⁶⁸.
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53 379 Finally, Programmed Death-1 (PD-1) and its ligands (PD-L1), proteins implicated in
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55 380 tolerance⁶⁹, have been described to be essential to balance antiviral immunity and
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3 381 inflammation in acute HBV patients⁷⁰. A study carried-out with 32 chronic HBV
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5 382 patients, showed an up-regulation of PD-1 and PD-L1 in liver biopsies compared to
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7 383 healthy controls⁷¹ (Figure 2, path#9). The same results were found in a chronic
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9 384 carrier mouse model⁷². PD-1/PD-L1 up-regulation correlated with hepatic
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11 385 inflammation and increased alanine transaminase levels in patients⁷¹ and with
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13 386 impaired cytotoxic T lymphocytes response in mice⁷². However, PD-L1 expression
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15 387 was lower in liver residential antigen presenting cells, including KC⁷¹, during the
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17 388 inactive carrier phase. Moreover, in mice, PD-L1 blockade or KC depletion led to an
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19 389 inhibition of HBV viral parameters, as well as an increase of IFN- γ ⁺ CD8⁺ cells⁷².
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21 390 Another study further showed that PD-L1 expression was up-regulated in CD14+
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23 391 circulating cells during chronic HBV infection, liver cirrhosis and hepatocellular
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25 392 carcinoma⁷³. PD-1/PD-L1 interaction may therefore play an important role in
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27 393 balancing liver damage during chronic HBV infection⁷¹.
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31 394 In summary (Figure 2), HBV seems to block pro-inflammatory responses in KC and
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33 395 peripheral monocytes/macrophages, correlating with a poor elimination of the virus.
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35 396 On the other hand, HBV strongly activates anti-inflammatory cytokines secretion (IL-
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37 397 10 and TGF- β) as well as inhibitory checkpoint factors (e.g., PD-1/PD-L1). Overall, it
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39 398 seems that HBV concomitantly inhibits a pro-inflammatory/anti-viral M1-like
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41 399 phenotype, and activates an anti-inflammatory/pro-tolerogenic M2-like phenotype.
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48 401 **Do macrophages promote the establishment or persistence of the infection?**

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50 402 As the first line of defence against pathogens, KC should counteract infection of
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52 403 hepatocytes by HBV. A study performed in ducks infected with DHBV (duck hepatitis
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54 404 B virus) showed that endotoxin stimulation could inhibit DHBV replication in primary
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3 405 duck hepatocytes (i.e., accumulation of viral proteins and amplification of the nuclear
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5 406 extrachromosomal DHBV DNA templates)⁷⁴. The authors further observed that this
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7 407 inhibition was due to non-parenchymal cells (NPC)-mediated cytokine secretion, and
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9 408 more probably to KC, which contaminates isolated hepatocytes. Nevertheless, HBV
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11 409 is able to chronically infect the liver, and counteract/escape these immune
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13 410 responses. The role of KC in the establishment and persistence of the infection
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15 411 remains unclear.

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18 412 Interestingly, in a mouse model the depletion of KC prior to HBV infection prevented
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20 413 the establishment of a chronic infection⁶⁵ (Figure 3, path#1). This effect was
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22 414 correlated with the secretion of IL-10 by KC, which impaired the humoral response
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24 415 against HBV infected hepatocytes⁶⁵. Moreover, Chen *et al.* have shown that the
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26 416 matrix metalloproteinase 9 (MMP-9) is up-regulated in PBMCs of chronic hepatitis B
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28 417 patients and can be activated by HBV in PBMCs and macrophages *in vitro*⁷⁵. This
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30 418 up-regulation may favour the infection as the authors further showed that MMP-9
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32 419 enhanced HBV replication in hepatocytes through binding to IFNAR1 (IFN α receptor
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34 420 1), inducing its ubiquitinylation and degradation⁷⁵ (Figure 3, path#2). IFNAR1
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36 421 degradation prevents the binding of type I IFN and the associated antiviral effects.

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40 422 In contrast, some studies have shown that KC may play a role in HBV elimination.
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42 423 Hösel *et al.* showed that KC exposed to HBV secreted more IL-6, inducing the
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44 424 activation of the mitogen-activated protein kinases (MAPK) in PHH, leading to the
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46 425 inhibition of hepatocytes nuclear factor (HNF) 1 α and 4 α , two transcriptional factors
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48 426 essential for HBV replication⁴⁶ (Figure 3, path#3). Another study conducted with
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50 427 HepG2 cells, showed that a treatment with TGF- β 1 (cytokine secreted by KC
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52 428 exposed to HBV⁶³) could suppress HBV replication⁷⁶. TGF- β 1 specifically reduced
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3 429 HBV core promoter activity, and, subsequently, viral pre-genomic RNA, core protein,
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5 430 and HBV replication, through repression of HNF 4 α expression⁷⁶ (Figure 3, path#4).
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8 431 In summary (Figure 3), it is still unclear if KC plays a role in the establishment and
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10 432 persistence of the infection and further studies are required. The discrepancies
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12 433 between results may be due to the phenotype (pro- or anti-inflammatory) of KC
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14 434 during primary infection and could be one of the key factors involved in the infection
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16 435 outcome.
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22 437 **Do macrophages play a role in the development of pathologies associated with**
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24 438 **HBV infection?**
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27 439 KC have been described to play a role in several liver associated pathologies
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29 440 including aberrant responses^{22,30} and may be associated with of HBV-associated liver
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31 441 pathologies including fibrosis, cirrhosis and the progression to hepatocellular
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33 442 carcinoma⁷⁷.
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36 443 As described above, Li *et al.* have shown an increase of TGF- β 1 secretion by KC in
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38 444 the presence of HBV⁶³. TGF- β 1 can directly promote fibrosis by inducing the
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40 445 differentiation of hepatic stellate cells into myo-fibroblasts, subsequently stimulating
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42 446 the production of extracellular matrix components⁷⁸ (Figure 3, path#4').
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45 447 In addition to liver fibrosis, KC plays an important role in liver inflammation/tolerance
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47 448 and injury associated to uncontrolled inflammation. Different populations of
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49 449 macrophages have recently been described in the liver of chronically infected animal
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51 450 models and patients. A study showed that end-stage HBV liver disease is associated
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53 451 with an accumulation of CD14⁺HLA-DR^{hi}CD206⁺ macrophages⁷⁹. These cells
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55 452 expressed spontaneously pro-inflammatory markers (TNF- α , GM-CSF, Caspase 1...)
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3 453 and had a low expression of anti-inflammatory markers (arginine 2, PPARG...) with
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5 454 the exception of IL-10 which was increased⁷⁹. This population has been described as
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7 455 resistant to LPS tolerization. Another population of CD205⁺ macrophages has been
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9 456 described in the liver of HBV transgenic mice and chronic hepatitis B patients⁸⁰. This
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11 457 population presented a higher expression of activation markers, pro-inflammatory
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13 458 cytokines, chemokines/chemokine receptors, and phagocytosis related genes⁸⁰.
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15 459 Moreover, in patients with high ALT levels, a large decrease of CD8⁺ T lymphocytes
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17 460 and increase of Fas-L positive cells, especially KC, were shown⁸¹. This observation
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19 461 led the authors to the hypothesis that during chronic infection, liver injury could not be
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21 462 the result of CD8⁺ T cell response but an increase of Fas-L expression on KC and,
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23 463 through an increased cytolytic activity⁸¹ (Figure 3, path#5). Another study performed
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25 464 in liver biopsies from 74 chronically infected patients revealed that iNOS intrahepatic
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27 465 expression was significantly higher in patients with chronic HBV⁸². In these samples,
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29 466 Kupffer cells stained positive for iNOS, a marker of pro-inflammatory MΦ, which level
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31 467 correlated with ALT level, and with the grading of liver inflammation and staging of
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33 468 liver fibrosis⁸² (Figure 3, path#6). Moreover, as previously described, peroxisome
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35 469 proliferator-activated receptor-β/δ (PPARβ/δ) activation was shown to inhibit
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37 470 steatosis and inflammation, well-known cancer risks factors⁸³. In a model of HBV
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39 471 transgenic mice, activation of PPARβ/δ reduced steatosis and tumour multiplicity⁸⁴.
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41 472 PPARβ/δ activation was also correlated with reduced *Tnfa* mRNA levels, as well as,
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43 473 reduced ALT level but increased apoptotic signalling⁸⁴ (Figure 3, path#7). Ligand
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45 474 activation of PPARβ/δ inhibited LPS signalling and though *Tnfa* expression, which
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47 475 could be prevented in a model of KC non-expressing-PPARβ/δ⁸⁴. This data
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49 476 suggested a role of KC in liver inflammation during chronic HBV infection through
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51 477 TNF-α secretion. Finally, tumour progression in chronic HBV infection could be
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3 478 related to the increased of IL-10 secretion described by several teams⁶⁴⁻⁶⁶. Indeed, a
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5 479 tolerogenic environment in the liver could lead to a non-efficient lymphocyte response
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7 480 and consequently, to cancer progression⁸⁵ (Figure 3, path#8). However, a study by
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9 481 Sitia *et al.* showed that in a chronic HBV carrier mouse model, KC, through
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11 482 scavenging receptors, removed apoptotic PHH, blocking the release of high-mobility
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13 483 group box 1 (HMGB1) and neutrophil infiltration in the liver⁸⁶. The authors
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15 484 hypothesized that this may prevent liver inflammation and damage during chronic
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17 485 infection.

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20 486 Taken together these data show that HBV may modify KC phenotype in favour of the
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22 487 development of liver-associated pathologies such as fibrosis, particularly through
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24 488 TGF- β 1 secretion, and, ultimately, HCC, through aberrant expression of factors
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26 489 implicated in carcinogenesis. Moreover, KC could play an important role in liver
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28 490 inflammation during chronic HBV and prevent a functional humoral response by
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30 491 largely increasing IL-10 secretion by macrophages.

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35 36 37 493 **Conclusion**

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40 494 Contradictory data has been published with respect to the lack or activation of
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42 495 immune responses in M Φ during HBV infection. The absence of responses seen in
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44 496 HBV-infected animal model^{3,4}, despite the fact that liver macrophages are capable of
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46 497 recognising HBV^{46-48,50,51,53}, raises the possibility of an active HBV-driven immune
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48 498 evasion mechanism, which could contribute to the establishment and persistence of
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50 499 infections *in vivo*. Several studies have concluded that HBV does modulate M Φ
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52 500 phenotypes by: i) inhibiting pro-inflammatory responses in KC and peripheral
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54 501 monocytes/macrophages, ii) promoting anti-inflammatory cytokines secretion (IL-10

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3 502 and TGF- β), and/or iii) increasing inhibitory checkpoint factors expression (e.g., PD-
4 503 1/PD-L1). Taken together, HBV would concomitantly inhibit the pro-inflammatory/anti-
5 504 viral M1-like phenotype, and promote an anti-inflammatory/pro-tolerogenic M2-like
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7 505 differentiation. This phenotype switch may initially favour viral replication but in the
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9 506 long term may also promote the pathogenesis of liver disease, such as fibrosis,
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11 507 cirrhosis and, ultimately, HCC as collateral damage.
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16 508 A specific modulation of the function of macrophages to restore more favourable T-
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18 509 cell-mediated immune responses against HBV should be tested within the
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20 510 development of complex immune therapeutic strategies to defeat HBV⁸⁷. Therefore a
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22 511 substantial effort should be made to better understand the complex interplays
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24 512 between liver macrophages and HBV infections in order to determine whether a
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26 513 therapeutic intervention aiming at reversing the phenotype switch induced by HBV
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28 514 could favour the inhibition of HBV infection (i.e. by direct mechanisms for example
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30 515 through IL-1 β secretion⁵⁶, or by indirect mechanisms through the activation of a
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32 functional adaptive response) and prevent disease progression.
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25 715 **Figure Legends**

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28 716 **Figure 1: Detection of HBV by macrophages.** Detailed mechanisms are given in
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30 717 the text.

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33 718 **Figure 2: Modification of macrophages phenotype by HBV.** Detailed mechanisms
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35 719 are given in the text.

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38 720 **Figure 3: Effect of KC in HBV establishment and pathogenesis.** Detailed
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40 721 mechanisms are given in the text.

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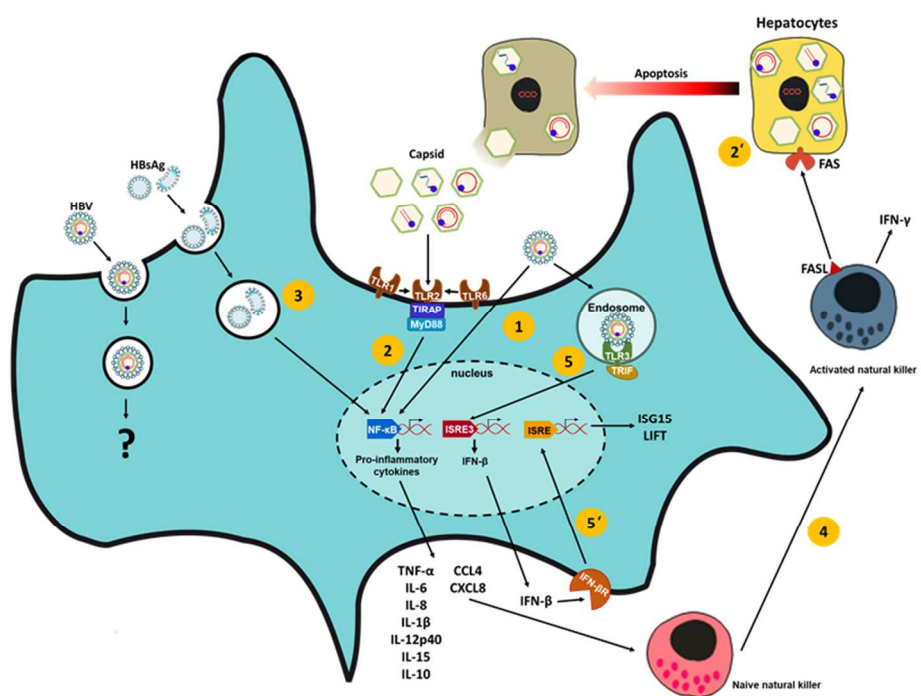


Figure 1: Detection of HBV by macrophages. Detailed mechanisms are given in the text.

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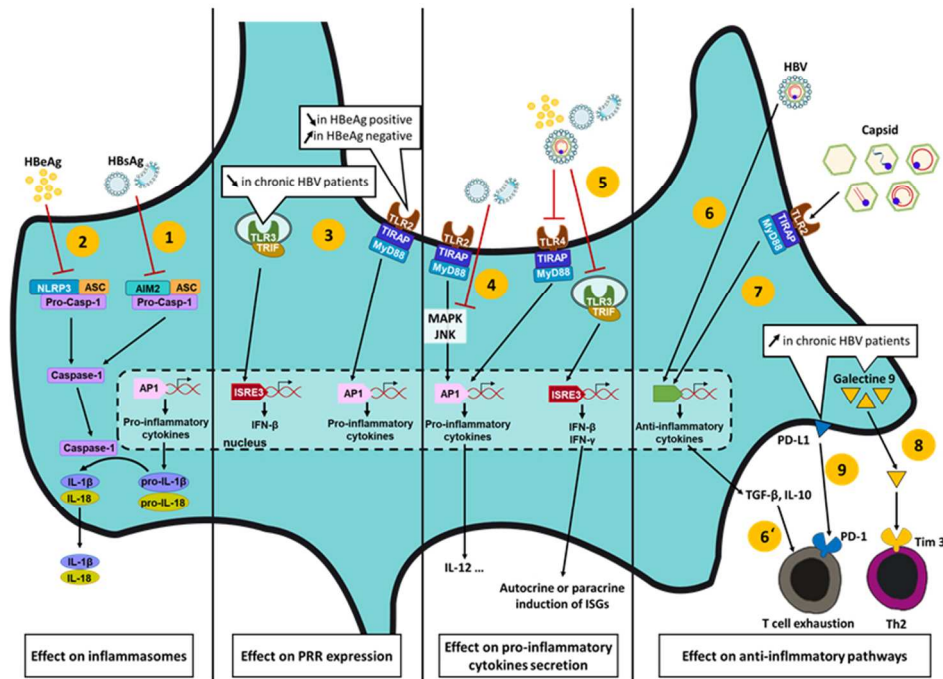
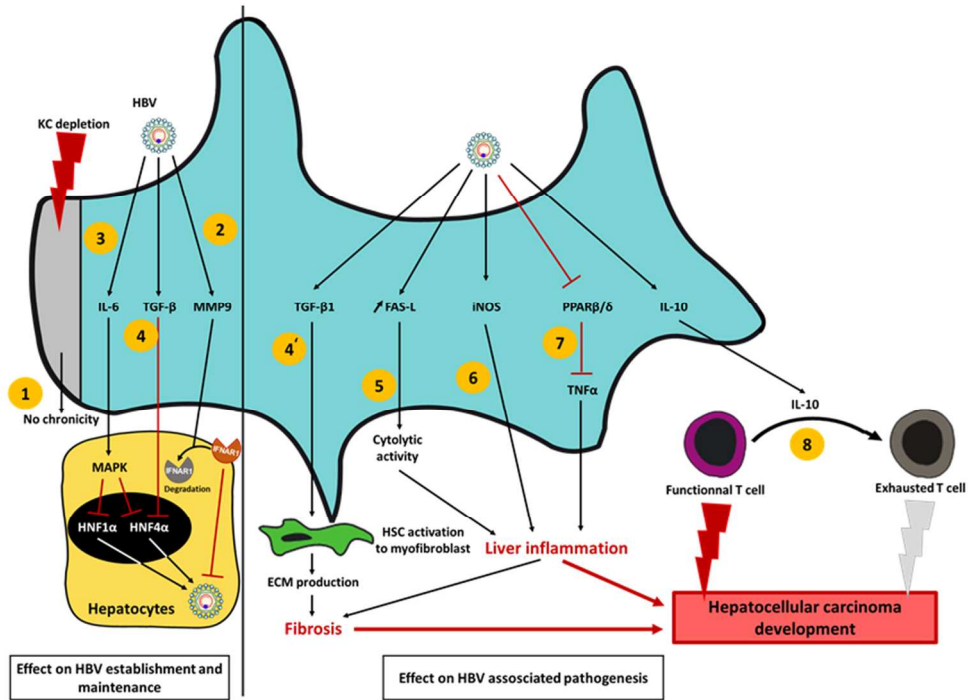


Figure 2: Modification of macrophages phenotype by HBV. Detailed mechanisms are given in the text.

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