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# Interaction between macrophages, hepatocytes and hepatitis B virus: from reprogramming of macrophages phenotypes towards establishment and maintenance of the infection

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Suzanne Faure-Dupuy. Interaction between macrophages, hepatocytes and hepatitis B virus: from reprogramming of macrophages phenotypes towards establishment and maintenance of the infection. Virology. Université de Lyon, 2018. English. NNT: 2018LYSE1073 . tel-01812772

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N° d'ordre NNT : 2018LYSE1073

## **THESE de DOCTORAT DE L'UNIVERSITE DE LYON**

opérée au sein de  
**l'Université Claude Bernard Lyon 1**

**Ecole Doctorale** N° accréditation  
**Biologie Moléculaire Intégrative et Cellulaire (BMIC)**

**Spécialité de doctorat** : Virology and Immunology  
**Discipline** : Infectiologie

Soutenue publiquement le 20/04/2018, par :  
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# **Interaction between macrophages, hepatocytes and hepatitis B virus: From reprogramming of macrophages phenotypes towards establishment and maintenance of the infection**

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“All we have to decide is what to do with the time that is given us.”

**Gandalf the Grey**

**J.R.R Tolkien**



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## Abstract

### **Interaction between macrophages, hepatocytes and hepatitis B virus: From reprogramming of macrophages phenotypes towards establishment and maintenance of the infection**

Hepatitis B virus (HBV) chronically infects over 250 million people worldwide. Several treatments can be used to prevent the formation of *de novo* particles. However, they do not allow the total eradication of the infection. Therefore, it is necessary to develop new therapeutic strategies, including immune-therapeutic ones, which would be more likely to lead to an immunological control of HBV infections. We have recently shown that IL-1 $\beta$  is the most effective antiviral cytokine against the replication of HBV *in vitro*. In the liver, IL-1 $\beta$  is mainly produced by resident macrophages (also called Kupffer cells) or infiltrating cells (inflammatory monocytes differentiated into macrophages). Recent studies have shown that HBV is able to partially inhibit the induction of innate immune responses. Hence, it was necessary to determine if HBV was also able to block the production of IL-1 $\beta$  by the different types of macrophages.

The objective of this thesis was to study the effect of HBV on macrophage phenotypes and the impact of those modifications on the establishment of HBV infection in hepatocytes.

Blood monocytes and liver macrophages were purified, respectively, from peripheral blood or hepatic resections, and were exposed to HBV during their differentiation and/or activation for monocytes, or only during activation for liver macrophages which are already in a differentiated state. HBV was able to partially inhibit the secretion of IL-6 and IL-1 $\beta$  by pro-inflammatory macrophages. Moreover, HBV was able to inhibit IL-1 $\beta$  secretion by liver macrophages stimulated by different ligands and, conditioned medium of pro-inflammatory macrophages could inhibit the establishment of infection in hepatocytes. This effect was reverted when macrophages were exposed to HBV, concomitantly with a lower production of IL-6 and IL-1 $\beta$ .

In summary, HBV is able to modify macrophage phenotypes to favour the establishment and persistence of HBV infection. The full understanding of the mechanistic basis of how HBV phenotypically modifies macrophages will be a first step towards the development of new therapeutic strategies.

**Key words:** Macrophages; pro-inflammatory; IL-1 $\beta$ ; reprogramming; antiviral.



## Résumé

### **Interaction entre les macrophages, les hépatocytes et le virus de l'hépatite B : de la reprogrammation du phénotype des macrophages vers l'établissement et la persistance de l'infection**

Le virus de l'hépatite B (HBV) infecte chroniquement plus de 250 millions de personnes. Des traitements existent permettant de contrôler la production de particules infectieuses. Cependant, aucun des traitements actuels ne permet d'éradiquer complètement l'infection. Il est donc nécessaire de développer de nouvelles stratégies thérapeutiques, incluant des approches immunothérapeutiques pour permettre un meilleur contrôle immunologique des infections HBV. Dans une étude récente menée au sein du laboratoire, il a été montré que l'IL-1 $\beta$  est la cytokine ayant le plus fort effet antiviral contre la réplication d'HBV dans les hépatocytes. Dans le foie, la cytokine IL-1 $\beta$  est principalement produite par les macrophages résidents (les cellules de Kupffer) ou infiltrants (monocytes inflammatoires différenciés en macrophages). De nombreuses études récentes ont montrés qu'HBV était capable de bloquer partiellement l'induction des réponses immunitaires innées. Il est donc important de déterminer si HBV est capable d'empêcher la production d'IL-1 $\beta$  par les différents types de macrophages.

L'objectif de cette thèse était d'étudier l'effet du virus sur le phénotype des macrophages et les implications de ces modifications phénotypiques sur l'établissement de l'infection dans les hépatocytes.

Des monocytes du sang ou des macrophages du foie ont été purifiés, respectivement, du sang périphérique ou de résections hépatiques, et ont été exposés au virus pendant leur différenciation et/ou leur activation pour les monocytes, ou seulement pendant leur activation pour les macrophages hépatiques déjà différenciés. Il a été démontré que le virus de l'hépatite B est capable d'inhiber la sécrétion d'IL-6 et d'IL-1 $\beta$  par les macrophages pro-inflammatoires. De plus, HBV est capable d'inhiber la sécrétion d'IL-1 $\beta$  par les macrophages hépatiques stimulés par différents ligands. Finalement, les surnageants de macrophages pro-inflammatoires sont capables de bloquer l'établissement de l'infection, ce qui n'est pas le cas quand les macrophages ont été exposés au virus.

Il apparait donc qu'HBV est capable de modifier le phénotype des macrophages pour favoriser l'établissement et la persistance de l'infection. La compréhension des mécanismes de subversion du phénotype des macrophages par le virus de l'hépatite B serait un premier pas vers le développement de nouvelles stratégies thérapeutiques.

**Mot clés :** Macrophages ; pro-inflammatoire ; IL-1 $\beta$  ; reprogrammation; antiviral.

# Résumé substantiel

## **Interaction entre les macrophages, les hépatocytes et le virus de l'hépatite B : de la reprogrammation du phénotype des macrophages vers l'établissement et la persistance de l'infection**

Travaux de thèse réalisés par Suzanne Faure-Dupuy au sein du Centre de Recherche en Cancérologie de Lyon (CRCL), UMR INSERM 1052, CNRS 5286, Centre Léon Bérard, dirigé par Alain Puisieux

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### **Introduction :**

Le virus de l'hépatite B (HBV) infecte chroniquement plus de 250 millions de personnes dans le monde et reste un problème majeur de santé publique. La chronicité de l'infection par HBV est associée, avec le temps, à l'apparition d'une fibrose et d'une cirrhose pouvant conduire au développement d'un carcinome hépatocellulaire. Des traitements existent pour la prise en charge des patients chroniquement infectés et permettent de contrôler la charge virale sérique. Cependant, aucun des traitements actuels ne permet d'éliminer le minichromosome viral (ADNccc), c'est-à-dire la matrice nucléaire des ARN viraux, au sein des hépatocytes. Ces traitements doivent ainsi être administrés à vie, car une interruption est quasi universellement associée à une réactivation virale. Il est donc nécessaire de trouver de nouvelles cibles thérapeutiques permettant l'éradication totale de l'infection ou bien *a minima* un contrôle immunologique durable.

Parmi les nouvelles molécules en cours de développement contre HBV sont retrouvés des agonistes de récepteurs de l'immunité innée, les « patterns recognition receptors » (PRR). Ces agonistes se fixent sur des récepteurs spécifiques et permettent d'induire la sécrétion de cytokine pro-inflammatoire et/ou d'interférons. Notamment, un ligand du « toll like receptor » 7 (TLR7 ; le GS-9620 développé par Gilead Sciences) a montré des résultats très prometteurs *in vitro* et *in vivo* chez la marmotte et est récemment passé en étude clinique de phase II. D'autres ligands de PRR sont également étudiés en phase préclinique. Avec le développement de ces agonistes dans la lutte contre HBV, il apparaît nécessaire de connaître l'expression des récepteurs correspondants dans les cellules résidentes du foie, ainsi que la capacité de ces cellules à répondre à un stimulus donné.

De plus, dans une étude récente menée au sein du laboratoire, il a été montré que la cytokine interleukine-1-beta (IL-1 $\beta$ ) avait un fort effet antiviral contre HBV, avec une IC<sub>50</sub> (concentration inhibitrice 50%) largement inférieure à l'interféron-alpha (IFN- $\alpha$ ), couramment utilisé dans le traitement des hépatites B chroniques. Au sein du foie, l'IL-1 $\beta$  est principalement produite par les macrophages résidents (les cellules de Kupffer) ou infiltrants (monocytes inflammatoires différenciés en macrophages). HBV n'induit que peu ou pas de réponses immunes. De nombreuses études récentes ont montrés qu'HBV était capable de bloquer partiellement l'induction d'une réponse immunitaire. Il est donc maintenant important de déterminer si HBV est capable d'empêcher la production et/ou la sécrétion d'IL-1 $\beta$  par les différents types de macrophages retrouvés dans le foie. Il sera aussi important de contrôler l'impact des sécrétions des macrophages sur l'établissement de l'infection dans les hépatocytes, et si l'effet sur l'infection est annulé par l'exposition préalable des macrophages au virus.

**Objectifs du projet de recherche :**

Cette thèse a eu comme objectif : 1) l'étude de l'expression des senseurs de l'immunité dans les cellules résidentes du foie et les modèles de lignées cellulaires correspondantes ; 2) l'étude de l'effet du virus sur le phénotype des macrophages et l'implication de ces modifications phénotypiques sur l'établissement de l'infection dans les hépatocytes.

**Matériel et méthodes :**

Pour la première étude, les différentes cellules résidentes du foie (les hépatocytes, les cellules de Kupffer, les cellules sinusoidales endothéliales et les cellules hépatiques étoilées) ont été purifiées à partir de résections hépatiques. L'expression basale des senseurs au niveau ARN et protéique a été déterminée ainsi que la capacité de ces différents types cellulaires à répondre (sécrétion d'IL-6 et d'IP-10) à différents ligands de récepteurs de l'immunité. L'accès à des résections hépatiques étant limité, l'expression des senseurs et les réponses aux stimuli ont été comparées à celles observés dans les lignées cellulaires disponibles pour établir le modèle le plus relevant.

Pour la deuxième étude, des monocytes du sang ou des macrophages du foie ont été purifiés, respectivement, du sang périphérique et à partir de résections hépatiques, et ont été exposés au virus pendant leur différenciation et/ou leur activation pour les monocytes, ou seulement pendant leur activation pour les macrophages hépatiques déjà différenciés. Les sécrétions de

différentes cytokines ont été déterminées en absence ou en présence du virus ainsi que l'impact de ces sécrétions sur l'établissement de l'infection dans les hépatocytes.

**Résultats :**

La plupart des adaptateurs des voies de l'immunité sont exprimés dans toutes les cellules du foie. Cependant, bien que seulement une partie des récepteurs analysés soient détectés à l'état basal sur les cellules du foie, une réponse au stimulus de chaque récepteurs « toll-like » est détectée sur au moins l'une des cellules hépatiques.

De plus, le virus de l'hépatite B est capable de partiellement interférer avec la différenciation des monocytes du sang en macrophage pro-inflammatoire, sécréteur d'IL-1 $\beta$ . En effet, une baisse de la sécrétion de l'IL-6 et de l'IL-1 $\beta$  a pu être observée quand le virus est présent pendant la différenciation des cellules. En adéquation avec ces résultats, HBV est capable d'inhiber la sécrétion de l'IL-1 $\beta$  par les macrophages hépatiques stimulés par des ligands des inflammasomes canoniques (AIM2) ou non-canoniques (NLRP3). De plus, l'IL-1 $\beta$  recombinant ou les surnageants de macrophages pro-inflammatoires sont capables de bloquer l'établissement de l'infection dans les hépatocytes. Ce phénotype peut être inversé par les modifications de sécrétion induite par le virus sur les macrophages.

**Conclusions et perspectives :**

Pour la première fois, nous faisons état, certes de manière non exhaustive, mais avec des cellules primaires, de l'expression des senseurs de l'immunité innée dans les différentes cellules résidentes du foie. Cela pourra permettre de mieux comprendre les réponses immunitaires induites au niveau hépatiques lors de l'utilisation d'agonistes de ces récepteurs. Nous avons également montré que le virus est capable d'interférer avec la différenciation des macrophages et/ou leur activation pour inhiber la sécrétion de l'IL-1 $\beta$ . Cette cytokine a un fort effet antiviral à la fois sur l'établissement et la persistance de l'infection. Il apparaît donc que HBV, par des mécanismes encore non déterminés, est capable de modifier le phénotype des macrophages, dans lesquels ils ne se répliquent pas, pour favoriser l'établissement et la persistance de l'infection. La compréhension des mécanismes de subversion du phénotype des macrophages par le virus de l'hépatite B serait un premier vers le développement de nouvelles cibles thérapeutiques. En effet, il est important de développer de molécules thérapeutiques n'agissant pas directement sur le cycle viral et les cellules infectées mais sur la réactivation d'un système immunitaire fonctionnel.



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## Abbreviations

<b>3TC:</b> Lamivudine	<b>HBsAg:</b> Hepatitis B surface Antigens / surface proteins
<b>aa:</b> amino acids	<b>HBSP:</b> Hepatitis B Splicing Protein
<b>ADF:</b> Adefovir dipivoxil	<b>HBV:</b> Hepatitis B virus
<b>ALT:</b> Alanine aminotransferase	<b>HBeAg:</b> Hepatitis B e Antigen
<b>APC:</b> antigen presenting cell	<b>HBsAg:</b> Hepatitis B S Antigen
<b>BCP:</b> Basal Core Promoter	<b>HBxAg:</b> Hepatitis B x Antigen / X protein
<b>cccDNA:</b> covalently closed circular DNA	<b>HCC:</b> Hepatocellular Carcinoma
<b>CCL:</b> C-C motif chemokine	<b>HCV:</b> Hepatitis C virus
<b>CXCL:</b> C-X-C motif chemokine	<b>HDV:</b> Hepatitis D virus
<b>CCR:</b> C-C motif chemokine receptor	<b>HIV:</b> Human Immunodeficient Virus
<b>CXCR:</b> C-X-C motif chemokine receptor	<b>HSC:</b> Hepatic Stellate Cell
<b>CD:</b> Cluster of Differentiation	<b>HSPG:</b> Heparan Sulfate Proteoglycans
<b>CDS:</b> Cytosolic DNA Sensors	<b>HTA:</b> Host-Targeting-Agents
<b>cGAS:</b> cyclic GMP-AMP Synthase	<b>IFN:</b> Interferon
<b>CHB:</b> Chronic Hepatitis B	<b>IL:</b> Interleukin
<b>CTD:</b> C-Terminal “protamine” Domain of HBcAg	<b>ILC:</b> Innate Lymphoid Cell
<b>DAA:</b> Direct-Acting-Agent	<b>iMATES:</b> Intrahepatic Myeloid-cell Aggregates
<b>DAMP:</b> Danger Associated Molecular Pattern	<b>IRF:</b> Interferon Regulatory Factor
<b>DC:</b> Dendritic Cell	<b>ISG:</b> Interferon Stimulated Gene
<b>DDX3:</b> Dead box helicase 3	<b>ISRE:</b> Interferon Stimulated Response Element
<b>dHepaRG:</b> differentiated HepaRG	<b>JAK:</b> Janus Kinase
<b>DNA:</b> Deoxyribose Nucleic Acid	<b>JNK:</b> c-Jun N-terminal Kinases
<b>dsDNA:</b> double stranded DNA	<b>KC:</b> Kupffer Cells
<b>Enh:</b> Enhancer	<b>LPS:</b> Lipopolysaccharide
<b>ER:</b> Endoplasmic Reticulum	<b>LSEC:</b> Liver Sinusoidal Endothelial Cell
<b>ERK:</b> Extracellular signal-Regulated Kinases	<b>LT:</b> T Lymphocytes
<b>ETV:</b> Entecavir	<b>MΦ:</b> Macrophage
<b>EZH2:</b> Enhancer of Zeste Homolog 2	<b>M1:</b> type 1 macrophage / pro-inflammatory macrophage
<b>HAP:</b> Heteroarylhydroypyrimidine	<b>M2:</b> type 2 macrophage / anti-inflammatory macrophage
<b>HBcAg:</b> Hepatitis B core Antigen / capsid protein	

**MAIT:** Mucosal-Associated Invariant T-cell  
**MAVS:** Mitochondrial Antiviral-Signalling protein  
**MDA5:** Melanoma Differentiation-Associated protein 5  
**mDC:** myeloid Dendritic Cell  
**MDSC:** Myeloid-Derived Suppressor Cell  
**MF:** Myofibroblasts  
**MMP:** Matrix Metalloproteinase  
**MyD88:** Myeloid Differentiation primary response 88  
**NA:** Nucleoside Analogs  
**NAP:** Nucleic Acid Polymer  
**NFκB:** Nuclear Factor-kappa B  
**NK:** Natural Killer  
**NKT:** Natural Killer T  
**NLR:** NOD-Like Receptors  
**NLRC4:** NLR family CARD domain-containing protein 4  
**NLRP:** NACHT, LRR and PYD domains-containing protein  
**NLS:** Nuclear Localization Signal  
**NTCP:** Sodium Taurocholate Cotransporting Polypeptide  
**NTD:** N-Terminal Domain of HBcAg  
**ORF:** Open Reading Frame  
**PAMP:** Pathogen Associated Molecular Pattern  
**PBMC:** Peripheral Blood Mononuclear Cell  
**PD-1:** Programmed Death receptor 1  
**PD-L1:** Programmed Death Ligand 1  
**pDC:** plasmacytoid Dendritic Cell

**PF-rcDNA:** Protein Free rcDNA  
**pgRNA:** pregenomic RNA  
**PHH:** Primary Human Hepatocyte  
**Pol:** viral Polymerase  
**PRR:** Pathogen Recognition Receptors  
**rcDNA:** relaxed circular DNA  
**RLR:** RIG-Like Receptors  
**RNA:** Ribonucleic Acid  
**RT:** Reverse Transcriptase  
**ssDNA:** single stranded DNA  
**STAT:** Signal Transducer and Activator of Transcription  
**STING:** Stimulator of Interferon Genes  
**SVP:** subviral non-infectious particles  
**SVR:** Sustained Virologic Response  
**TAK1:** Transforming growth factor β-Activated Kinase 1  
**TAM:** Tumor Associated Macrophage  
**TBK1:** TANK Binding Kinase 1  
**TDF:** Tenofovir Disoproxil Fumarate  
**TIRAP:** Toll-Interleukin 1 Receptor domain containing Adaptor Protein  
**TLR:** Toll-Like Receptors  
**TNFα:** Tumor Necrosis Factor alpha  
**TP:** Terminal Protein  
**TRAIL:** Tumor-necrosis-factor Related Apoptosis Inducing Ligand  
**TRIF:** TIR-domain-containing adapter-inducing interferon-β  
**WHV:** Woodchuck Hepatitis B Virus

# Chapter I: Background

Part of this introduction has been adapted from two reviews I wrote in collaboration with Dr. David Durantel and Dr. Julie Lucifora, which are presented in appendices 1.2 and 1.3.

## 1. Immunity: Basic knowledge and liver specificity

### 1.1. Innate and adaptive immunity: basic insights

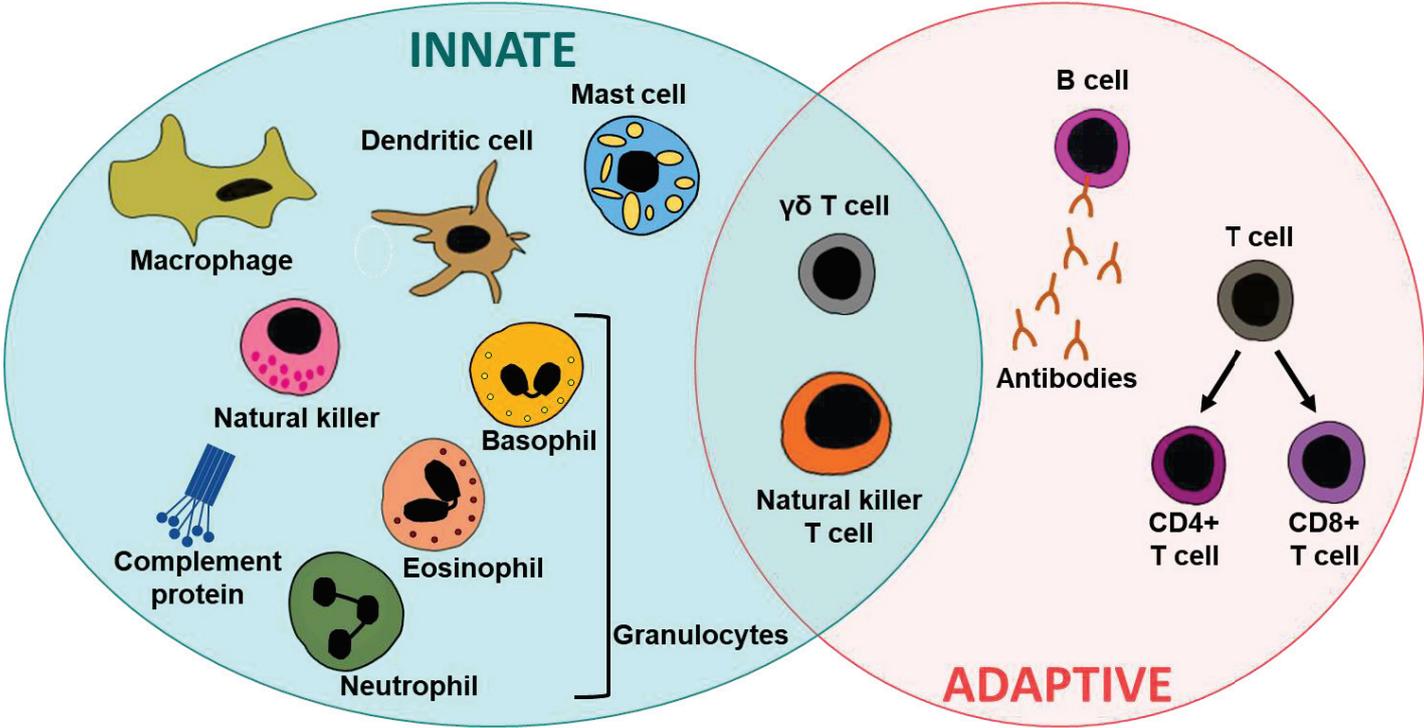
To protect the body against external pathogens (microbes, parasites, viruses...) and/or against aberrant self-components (cancerous cells, apoptotic cells ...), a functional immune response is mandatory in order to maintain self-integrity. Immune responses can be divided into two phases of responses: innate and adaptive immunities (**Table 1**). This introduction will only provide a highly simplified presentation of the overall immune system and will concentrate on the innate immune system.

**Table I. Major features of Innate and Adaptive immunity**

	<b>Innate</b>	<b>Adaptive</b>
<b>Receptors</b>	Primitive and broad	Highly specific (T and B cell receptors)
<b>Kinetics</b>	Fast (hours-days)	Slow (days-weeks)
<b>Regulation</b>	+/-	++++
<b>Amplification</b>	No (insignificant)	Yes
<b>Self discrimination</b>	-	++++
<b>Duration</b>	Short (days)	Long (months/years)
<b>Memory</b>	-	++++

The **Innate immunity** is the first line of defence against pathogens (Baron et al., 2000). It invokes a fast response that is able, within hours or days, to recognize a large range of aberrant immunological and non-self features. The induction of innate immunity is poorly regulated, as false responses are better than no responses, whereas its termination is very well controlled to prevent chronic inflammation and autoimmune diseases. Indeed, innate immunity is a global response, which does not discriminate “self” from pathogens. After a few days, the response will be terminated without any “memory” of it having happened. A large number of

Figure 1. Major components of innate and adaptive immunities (adapted from Dranoff et al., 2004).

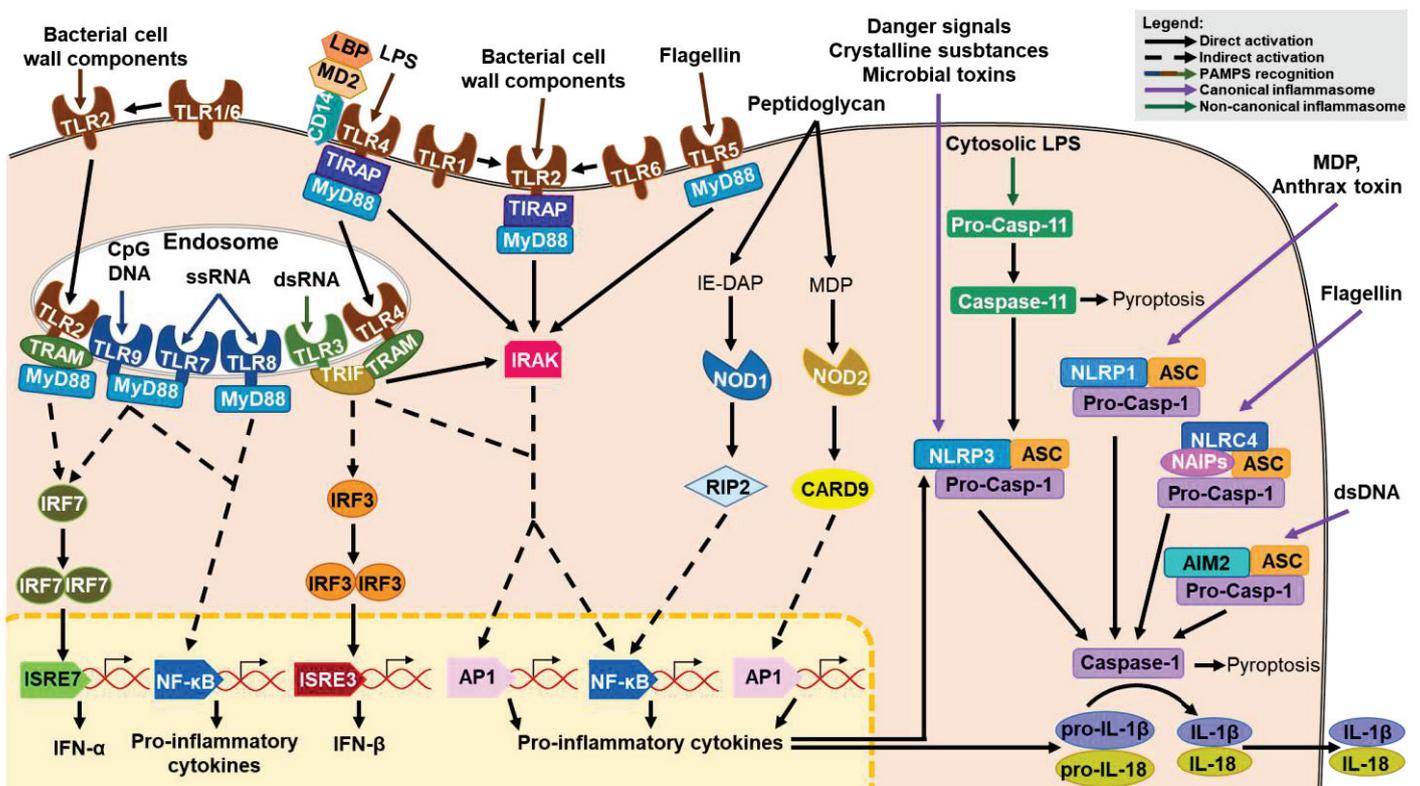


cellular and molecular mediators are implicated in the launching of an innate immune response (**Figure 1**):

- **Mastocytes:** Inflammatory cells of 10 to 15  $\mu\text{m}$  diameter with multi-lobed nucleus activated by inflammatory reaction, implicated in mediator/effector secretion (lipids, amines, cytokines...).
- **Dendritic cells (DC):** Cells of 25 to 30  $\mu\text{m}$  diameter, found in an immature state in tissues but that migrate to second lymphoid organs upon activation and maturation. They are activated by pathogen recognition. Immature DC play a key role in antigen captures and lymphocyte inactivation, whereas mature DC activate and recruit naive lymphocytes to the site of infection.
- **Macrophages (M $\Phi$ ):** Terminal differentiated monocytes, M $\Phi$  are from 25 to 50  $\mu\text{m}$  in diameter and are a heterogeneous population according to their organ localization. The description of M $\Phi$  will be further developed in the section 1.4.1 on “macrophages principal features and functions”.
- **Natural killer cells (NK):** Lymphocytes of 15 to 20  $\mu\text{m}$  in diameter located in the blood, second lymphoid organs or infected tissues; NK are implicated in the elimination of tumours or infected cells through the degranulation of granzyme and perforin.
- **Basophils:** Very similar to mastocytes (10 to 15  $\mu\text{m}$  with multi-lobed nucleus), they are also able to secrete mediators/effectors (lipids, amines, cytokines...) and play a key role in inflammation.
- **Eosinophils:** Cell of 12 to 17  $\mu\text{m}$  in diameter with bi- or tri-lobed nucleus, eosinophils are implicated in anti-parasitic cytotoxicity as well as the secretion of mediators implicated in allergic responses.
- **Neutrophils:** Cell of 9 to 16  $\mu\text{m}$  in diameter with a three to five lobed nucleus, they are implicated in phagocytosis of pathogens and in the release of inflammatory mediators.

The **adaptive immunity** is the second line of defence, activated by cells involved in the innate immune response, called antigen-presenting cells (APC). This response is highly specific for a given peptide, which arises from antigen processing, and is presented by MHC molecules and recognized by B or T cell receptors. Due to two important features of adaptive immunity, the necessity of antigen presentation by innate immune cells and the necessity of adaptive cells amplification/differentiation, this response is slow (days to weeks). The adaptive response is

**Figure 2. Toll-like receptors, NOD-like receptors and inflammasome signalling pathways (adapted from Invitrogen).** Representation of TLR, NLR and inflammasomes, their activating molecules, their adaptors and their simplified signalling pathways.



highly regulated and able to discriminate “self” from pathogens. After elimination and resolution of the problem, a memory of the response persists. T or B lymphocytes are the major effectors of adaptive immunity ([Figure 1](#)). The narrow cell type involved is compensated by the billions of different receptors expressed on lymphocyte populations due to genetic recombination, called V(D)J recombination. Characteristics of adaptive cells are the following:

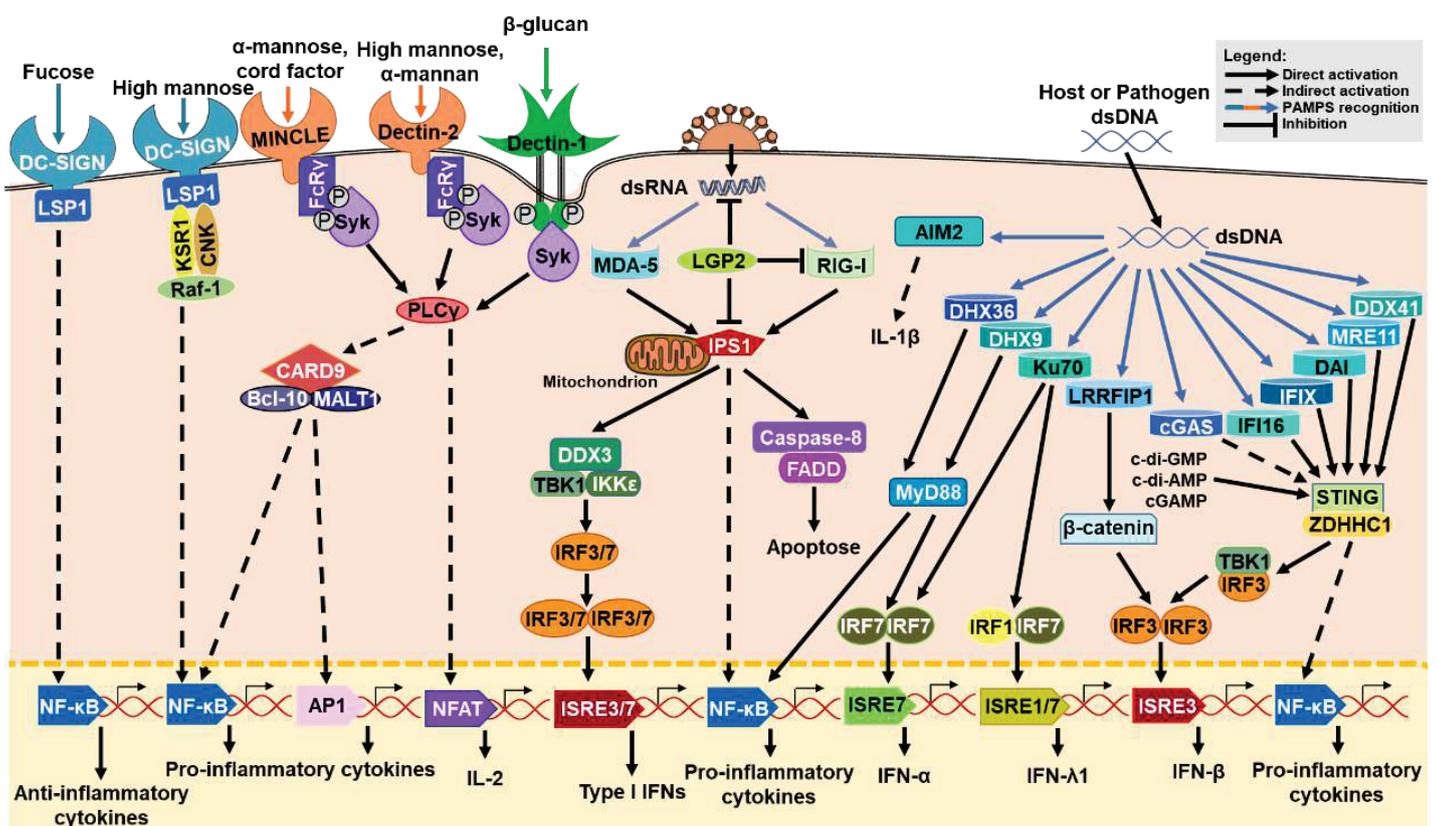
- **B lymphocytes:** heterogenic population that develops in the bone marrow (B cell). Upon activation, they can differentiate into plasmocytes that are the cells producing antibodies
- **T lymphocytes:** heterogenic population that develops in the thymus (T cell). Two major types of T cells exist: CD4<sup>+</sup> or CD8<sup>+</sup> cells. The CD4<sup>+</sup> cells produce different cytokines depending on their differentiation (Th1, Th2, Th17, pro-inflammatory cytokine secretors, or Treg, anti-inflammatory cytokine secretors). The CD8<sup>+</sup> cells are cytotoxic lymphocytes, which, upon activation, can secrete perforin, granzyme, and granulysin to kill infected or cancerous cells.

## 1.2. Innate immune sensors

The early and non-specific detection of pathogens generally occurs, at subcellular/molecular levels, via the recognition of Pathogen-Associated Molecular Patterns (PAMP) by innate immunity sensors, also called **Pathogen Recognition Receptors** (PRR). Amongst PRR, there are toll-like (TLR), Retinoic acid-Inducible Gene I (RIG)-like (RLR), nucleotide-binding oligomerization domain-containing protein (NOD)-like (NLR), C-type Lectin (CLR), and DNA-sensing (CDS) receptors, which are differentially or ubiquitously expressed on various types of epithelial/endothelial cells, as well as professional and non-professional immune cells (Pandey et al., 2014). Their localization and activation is different between PRRs ([Table II](#)).

Upon interaction between a PRR and its cognate PAMP, various downstream signalling pathways are activated and sequentially involve: (1) adaptor/co-adaptor molecules (e.g., myeloid differentiation primary response gene 88 (MyD88), TIR-domain-containing adapter-inducing interferon- $\beta$  (TRIF), etc.); (2) kinases (e.g., TANK binding kinase 1 (TBK1), transforming growth factor  $\beta$ -activated kinase 1 (TAK1), etc.) and (3) transcription factors (e.g., interferon regulatory transcription factors (IRFs), nuclear factor kappa-B (NF $\kappa$ B), c-fos/c-jun) (Pandey et

**Figure 3. C-type lectin receptors, RIG-like receptors and cytosolic DNA sensors signalling pathways (adapted from Invitrogen).** CLR, RLR and CDS, their activating molecules, their adaptors and their simplified signalling pathways are detailed.



al., 2014) (**Figures 2 and 3**). This leads to the expression of effector genes, including interferon-stimulated-genes (ISG) and NF- $\kappa$ B-inducible or pro-inflammatory genes. Primary effectors include the various interferons (IFN; IFNs- $\alpha$  (13 different alleles), IFN- $\beta$ , IFNs- $\lambda$  (four alleles), and IFN- $\gamma$ ), as well as pro-inflammatory cytokines/chemokines (e.g., interleukin (IL)-6, tumour necrosis factor (TNF- $\alpha$ )) which collectively have direct or indirect anti-microbial actions, and/or are involved in the recruitment of innate and adaptive immune cells to the infected or damaged site.

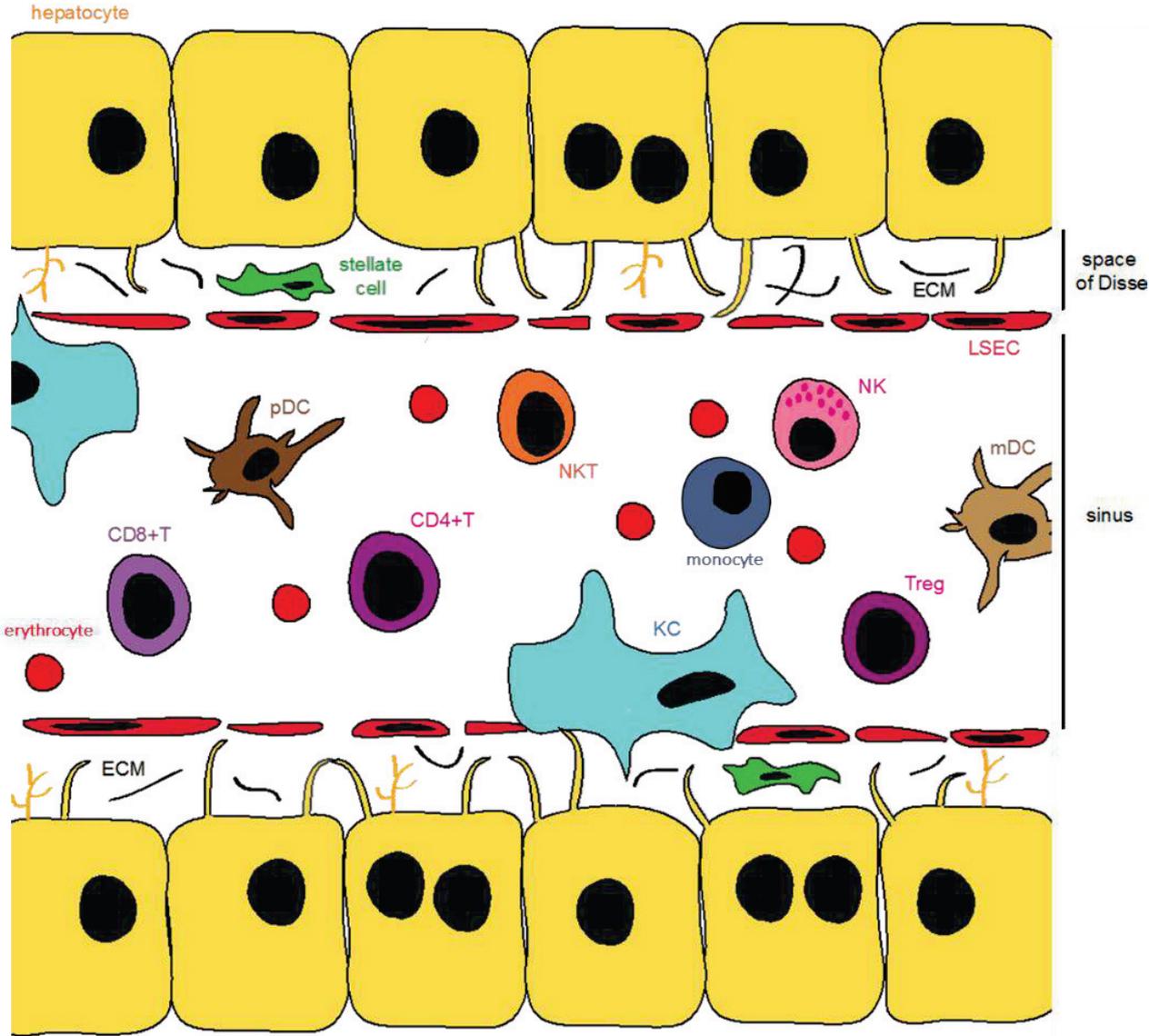
**Table II. Cellular localization and activation signal of TLR, NLR, CLR, RLR and CDS**

	Localisation	Activation signal
<b>TLR</b>	membranes (cellular or endosomal)	extracellular and endosomal pathogens
<b>NLR</b>	cytosol	cytosolic pathogens or danger molecules
<b>CLR</b>	cellular membrane	carbohydrates
<b>RLR</b>	cytosol	cytosolic dsDNA
<b>CDS</b>	cytosol	cytosolic dsDNA

Other innate detection systems rely on canonical or non-canonical **inflammasomes** (Broz and Dixit, 2016; Crowley et al., 2017). Four different inflammasomes exist: NACHT, LRR and PYD domains-containing protein 1 and 3 (NLRP1 and NLRP3), NLR family CARD domain-containing protein 4 (NLRC4), and absent in melanoma 2 (AIM2), which all share a common adaptors ASC. They are induced by PAMP or Danger-Associated Molecular Patterns (DAMP) and lead to the activation of the Caspase-1, which in turn cleaves precursor molecules (e.g., pro-IL-1 $\beta$  and pro-IL-18) to liberate active IL-1 $\beta$  and IL-18 in the infected or damaged microenvironment (**Figure 2**).

Finally, some receptors can regulate the inflammation. This is the case of **Programmed Death receptor 1** (PD-1) and its ligands (PD-L1). PD-L1, which is expressed by resident cells or cancerous cells, can bind to its receptor PD-1 that is expressed on T lymphocytes, leading to a down-regulation of TCR-mediated IL-12 secretion and subsequently to the down-regulation of T cell proliferation (Arasanz et al., 2017; Dal Bello et al., 2017). PD-1/PD-L1 are factors involved in immune tolerance, and play a key role in cancer development and escape from the immune system (Bertucci et al., 2016).

**Figure 4. Liver organization.** Schematic representation of the liver organization with the parenchymal cells (i.e. the hepatocytes), the liver resident non-parenchymal cells (i.e. the stellate cells, the liver endothelial cells (LSEC), and the Kupffer cells (KC)), and the flowing-through blood cells (i.e. plasmacytoid and myeloid dendritic cells (pDC and mDC), CD4 and CD8 T lymphocytes (CD8+T and CD4+T), regulatory lymphocytes (Treg), natural killer (NK and NKT), monocytes, and erythrocytes).

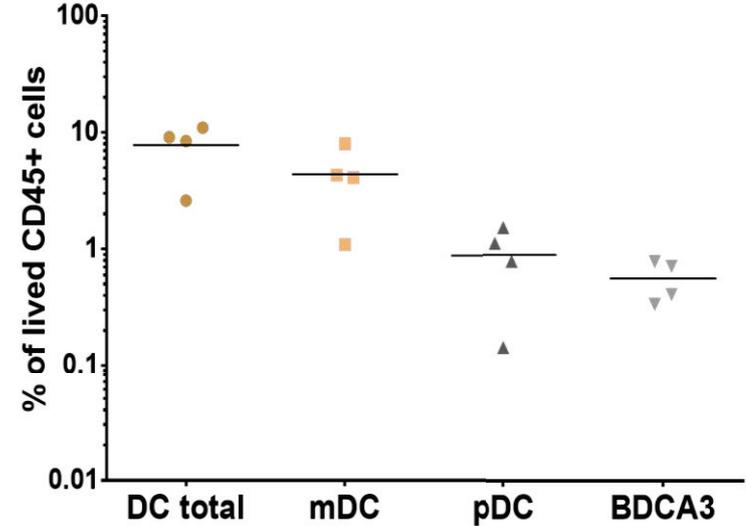
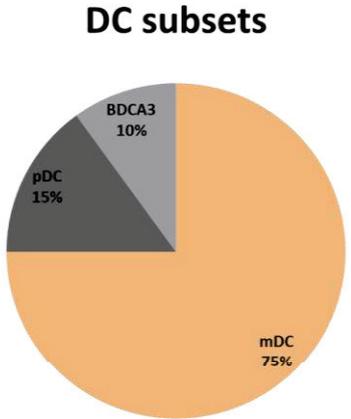
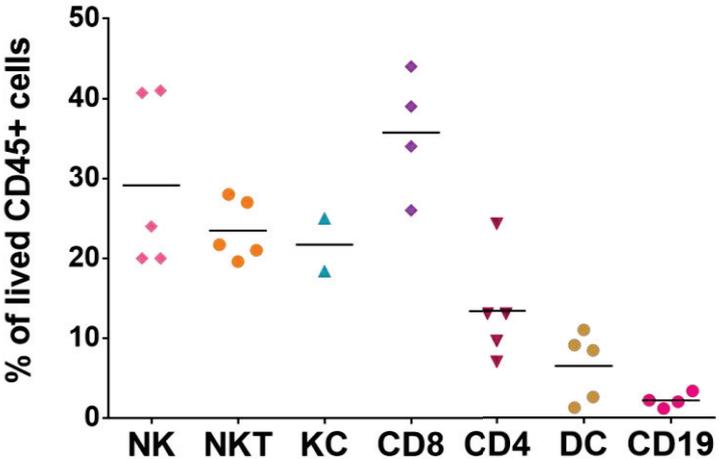
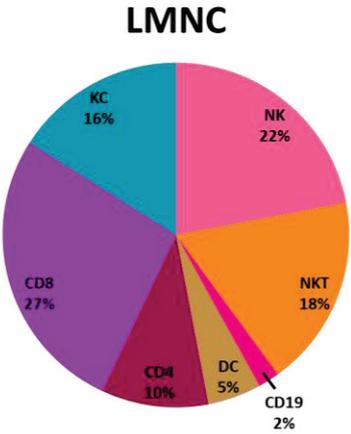


### 1.3. Liver immunity

The liver is often considered as a **secondary lymphoid organ** due to the amount of blood flowing-through, infiltrating, and the resident immune cells it contains (Crispe, 2009, 2011). Notably, innate immune cells, as well as non-parenchymal/non-professional cells endowed with innate functions (e.g., liver sinusoidal endothelial cells (LSEC), hepatic stellate cells (HSC)) (Protzer et al., 2012), are particularly enriched in this solid organ (**Figure 4**). By order of decreasing abundance, innate cells of (**Figure 5**): (1) lymphoid origin (natural killer and natural killer T-cells (NK/NKT) but also innate lymphoid cells (ILC), and mucosal-associated invariant T-cell (MAIT)); (2) myeloid origin (e.g., Kupffer cells (KCs), myeloid-derived suppressive cells (MDSC), monocytes, neutrophils); and (3) various types of dendritic cells (DCs; mDC-BDCA1<sup>+</sup>, mDC-BDCA3<sup>+</sup>, plasmacytoid DCs; pDCs), can be found in a healthy liver. These cells, as well as the cytokinic/chemokinic effectors that they produce, can be beneficially involved in the early containment of pathogen infection and the orchestration of pathogen-specific adaptive responses (Crispe, 2009; Protzer et al., 2012). However, they can also be involved in immune-driven pathogenesis, if a return to homeostasis is impaired by a pathogen or a recurrent exposure to xenobiotics/alcohol (Guidotti and Chisari, 2006; Heymann and Tacke, 2016; Knolle and Thimme, 2014). With respect to the latter, sterile or pathogen-driven chronic inflammation, as well as non-specific and uncontrolled cytotoxic activities, can be associated with the development of steatohepatitis, fibrosis, cirrhosis, and hepatocellular carcinoma (Guidotti and Chisari, 2006; Heymann and Tacke, 2016; Knolle and Thimme, 2014).

Many liver innate immune cells are involved in the so-called tolerogenic microenvironment, which prevails and protects this organ against permanent exposure to gut-derived microbial degradation products or a low concentration of living bacteria (Crispe, 2014). Incidentally, the liver “hosts” many chronic infections, including type B and C viral hepatitis, as well as parasitic infections (Protzer et al., 2012). Despite this intrinsic immune tolerance, the liver is well equipped to mount a potent antimicrobial response, and successful pathogens have also evolved strategies to either passively or actively evade innate and adaptive immune responses in order to persist.

Figure 5. Percentage of liver infiltrating immune cells (Adapted from Gruffaz, Testoni, Isorce, Luangsay et al., in preparation).



### 1.3.1. Hepatocytes

**Hepatocytes** represent 80% of the liver mass. Their primary function is to ensure the homeostasis of the liver through different metabolic functions such as drug detoxification, urea elimination, cholesterol synthesis or storage of glucose as glycogen. However, these cells are also able to participate in liver immune responses. In normal/non-transformed hepatocytes, it has recently been shown that most PRR are expressed and functional (Crispe, 2016; Luangsay et al., 2015a; Vegna et al., 2016), with the potential exception of the cyclic GMP-AMP Synthase/Stimulator of Interferon Gene (cGAS/STING) axis (Thomsen et al., 2016), and the lack of inflammasome activities (Zannetti et al., 2016). Moreover, several studies have shown that hepatocytes, which do not express co-stimulatory factors CD80 and CD86, are able to activate and induce proliferation of specific naive CD8<sup>+</sup> T cells in the absence of exogenously added cytokines, a property only shared by professional APC (Bertolino et al., 1998; Herkel et al., 2003). T cells activated by hepatocytes have lost their cytolytic function after 3 days of co-culture, which correlates with T cell death, suggesting that despite efficient activation, proliferation and transient CTL functions, T cells activated by hepatocytes do not survive. In summary, hepatocytes can participate in the activation of an innate immune response and can activate T lymphocytes in the liver.

*In vitro*, several cell types are currently used to study hepatocyte function, and they can be divided into 2 types of cell:

- **Transformed cells:** hepatocytes isolated from cancerous resections. Such cell lines (HuH, HuH 7.5, and HepG2) are a useful model to study hepatocyte functions and/or liver specific infection (Aden et al., 1979; Nakabayashi et al., 1984; Quinkert et al., 2005). However, due to transformation/cancerisation processes, these cell lines do not have a functional intrinsic immunity (Luangsay et al., 2015a).
- **Non-transformed cells:** The HepaRG cell line is a human hepatic progenitor cell line. HepaRG can be differentiated into hepatocytes (dHepaRG) by a 4 weeks differentiation process (Gripon et al., 2002). dHepaRG have a functional immune system and, so, are useful for the study of immune responses in hepatocytes (Luangsay et al., 2015a). However, the efficacy of infection by HBV of dHepaRG is only approximatively of 10%, while 100% of hepatocytes in HBV infected patients' biopsies can be stained positive for HBV proteins.

Nowadays, the more relevant model, *in vitro*, are primary human hepatocytes (PHH) which can be isolated from liver resections by a collagenase treatment (Lecluyse and Alexandre, 2010). PHH have a functional immune system. Their infection rate with HBV is higher than dHepaRG (90% on average), and thus is closer to what is found in patients. However, access to liver resections is limited, highlighting the need for a good surrogate model. The closest available model to study immune responses *in vitro* in hepatocytes is thus at present the HepaRG cell line (Luangsay et al., 2015a).

### 1.3.2. Liver endothelial sinusoidal cells (LSEC)

LSECs compose the **sinusoidal wall** of sinusoids and feature inter and intracellular fenestrations of 100 to 150 nm, allowing the diffusion of molecules and macromolecular complexes (**Figure 4**). LSECs are the key actors of liver angiogenesis, liver regeneration, and, by extension, liver tumorigenesis and tumour vascularization. They are also implicated in liver clearance through their scavenger-dependent endocytic capacities. LSECs express a large range of adhesion molecules and secrete numerous chemokines, and are, altogether, involved in immune cells' activation and recruitment. They are capable of presenting circulating antigen to CD4<sup>+</sup> and CD8<sup>+</sup> T cells, making LSECs major actors of hepatic immune-surveillance (Knolle and Limmer, 2003). These cells also play a key role upon danger signals leading to fibrosis since, they will undergo cytoskeletal remodelling, leading to a loss of fenestration upon shear stress (Eggert and Greten, 2017; Sørensen et al., 2015).

For the *in vitro* study of LSECs, 2 types of cell are available: the TRP3 (a LSEC cell line) and primary LSEC isolated from liver resections. **TRP3** is a cell line of LSEC isolated from a 65 year-old female with a history of hepatic arteriovenous malformations related to hereditary haemorrhagic telangiectasia. TRP3 display similar phenotypes to primary LSEC (Parent et al., 2014), making this cell line a good surrogate model. **Primary LSEC** are isolated from liver resection by a positive selection on the CD146, as described in article 5.

### 1.3.3. Hepatic stellate cells (HSC)

HSCs are localized in the liver sinusoids, in the space of Disse, between LSECs and hepatocytes, with which they are in close contact through their cytoplasmic extensions (**Figure 4**). HSC are

equivalent to the **liver fibroblast**, specialized in the storage and metabolism of lipids such as vitamin A (retinol) and triglycerides. They are the producers of extra cellular matrix (ECM) in the space of Disse, allowing cohesion, communication and differentiation of the different cells. These cells normally represent 5 to 8% of the total number of the cells in the liver, however upon chronic inflammation, HSC undergo transformation to become myofibroblasts (MFs), the activated state of HSC (Eggert and Greten, 2017; Silva et al., 2017; Tsuchida and Friedman, 2017). Once activated, these cells proliferate and start secreting numerous components of the extracellular matrix creating a scarred tissue (Moran-Salvador and Mann, 2017). During an uncontrolled inflammation/scarring process, the over-production of extracellular matrix induces fibrosis, which can end up in cirrhosis and lead through time to the development of HCC (Eggert and Greten, 2017; Moran-Salvador and Mann, 2017; Silva et al., 2017; Zoubek et al., 2017). It has also been suggested that HSCs could play a role of APC (Crispe, 2009).

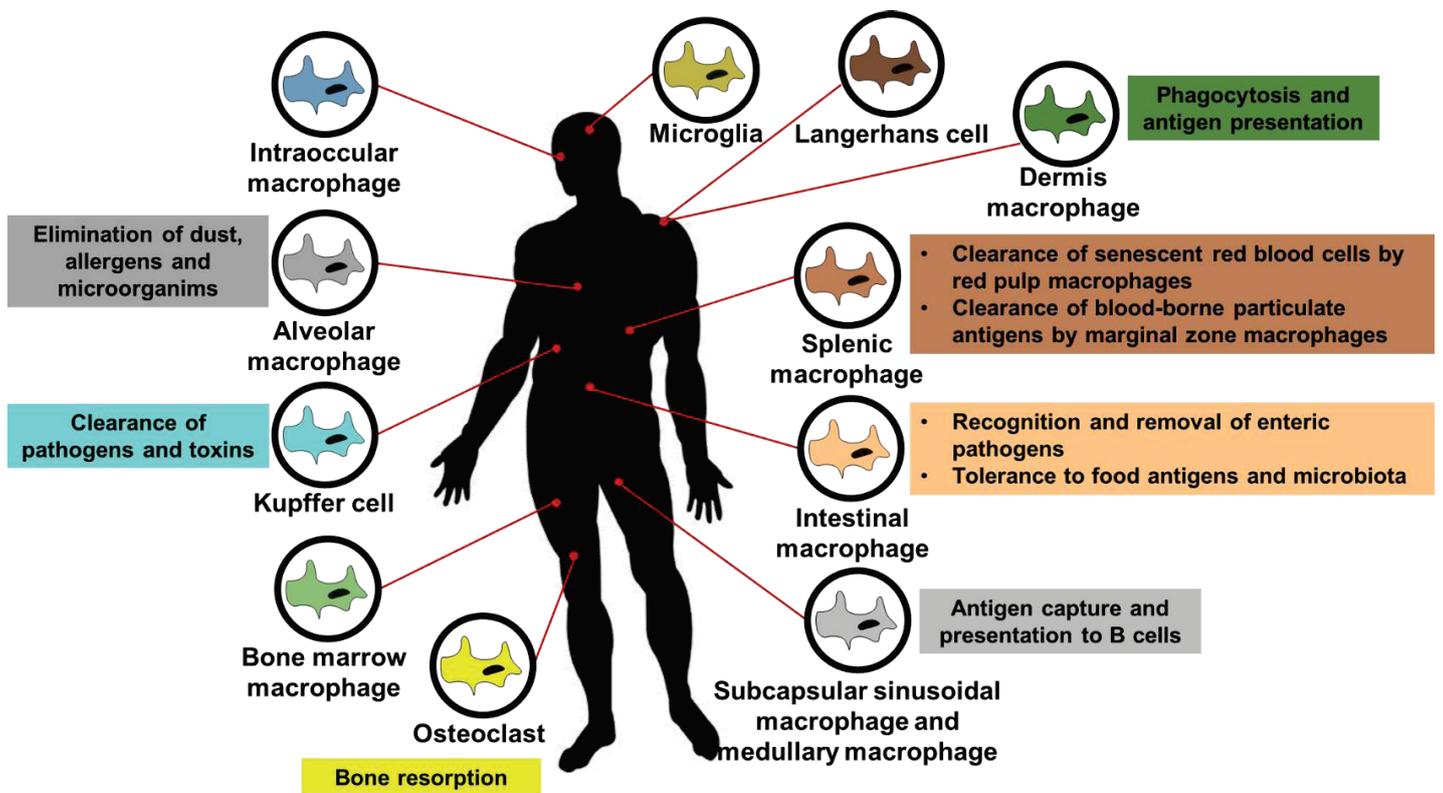
Primary HSC can be purified from liver resection, as described in article 5. However, due to limited access to liver resections, two cell lines have been created a decade ago (Xu et al., 2005): **LX-1** and **LX-2**. Both cell lines have allowed the study of liver disease as they both retain HSCs features. However, LX-2 presents two main advantages over LX-1: their viability in serum free media and high transfectability.

#### 1.3.4. Kupffer cells (KC)

KC, the **liver resident macrophages**, represent 80% of the total macrophage count within the body (Krenkel and Tacke, 2017). As macrophages, they form the first line of defence against pathogens and are specialized in their recognition (Seki et al., 2000). They are localized within blood vessels (i.e., in sinusoids) and through cytoplasmic extensions can pass the sinusoidal fenestrations to interact with hepatocytes (**Figure 4**). Specific features of Kupffer cells will be discussed in the next-section.

Primary KC can be purified from liver resection, as described in the article 5. However, as access to liver resections is limited, macrophage cells lines can be a useful tool for the study of KC *in vitro*. One cell line commonly used for the study of macrophages is **THP1**, which is a monocyte line that can be differentiated into macrophages *in vitro*. However, as liver resident macrophages and blood monocytes have different origins and features (see macrophages

Figure 6. Tissue resident macrophages (adapted from Singh et al., 2014).



principal features and functions), we have created a cell line from primary KC, to obtain an immortalized Kupffer cell line (**IKC**).

## 1.4. Macrophages

### 1.4.1. Principal features and functions

Macrophages (M $\Phi$ ) were first described, by **Ilya Metchnikoff** at the end of the 19<sup>th</sup> century, as evolutionary conserved phagocytes that have evolved for more than 500 million years (Gordon, 2016). Over the last 2 centuries, a lot has been learnt about M $\Phi$  embryonic origins, phenotypes of different subpopulations, and their various functions.

In solid organs, M $\Phi$  can have two origins. **Resident macrophages** comes from the yolk sac whereas **infiltrating macrophages**, which are recruited upon pathogen contamination and/or injury, are derived from the differentiation of peripheral blood circulating monocytes, themselves primarily coming from the bone marrow (Ginhoux and Guilliams, 2016). Depending on the tissue, it is not always possible to distinguish resident M $\Phi$  from infiltrating ones. For example, in the human liver, no specific marker has been identified to differentiate these two types of M $\Phi$ .

**Tissue resident M $\Phi$**  represent the first line of defence against pathogens such as bacteria and viruses, and are therefore a major component of the innate immune response to infections (**Figure 6**). In addition to their physiologic phagocytic capacity, which allows the elimination of physiologically aging, or infected, or cancerous cells, M $\Phi$  can also largely contribute to adaptive immunity by processing and presenting antigens after pathogen engulfment and their recognition through Pattern Recognition Receptors (PRR) (Hume, 2015). Upon activation, M $\Phi$  can secrete a large spectrum of pro-inflammatory cytokines involved in different activities such as lymphocyte polarization (IL-1, IL-12, IFN- $\gamma$ ...) or neutrophil recruitment (IL-8, TNF $\alpha$ ...), and also anti-inflammatory cytokines involved in inflammation regulation (IL-10 and TGF $\beta$ ), as well as chemokines involved in leukocyte recruitment to the sites of injury (CXCL8...) (Martins-Green et al., 2013) and others factors, such as VEGF implicated in angiogenesis (Pollard, 2008).

### 1.4.2. Pro- and anti-inflammatory phenotypes

MΦ can have a large variety of phenotypes that can be classified by specific marker expression and/or secreted cytokines (Hume, 2015; Murray et al., 2014). Several classifications have been proposed over the years, but a consensus is yet to be found. Nevertheless, they can be divided into two major phenotypes: pro- and anti-inflammatory. Of note, the pro-/anti-inflammatory dichotomy is a simplified view since each MΦ expresses a set of genes that on its own could define a new type of MΦ (Hume, 2015).

**Pro-inflammatory MΦ**, called M1, have been defined as mediators of the defence against bacterial and viral pathogens. They are implicated in inflammation, tumour resistance and killing of intracellular pathogens (**Figure 7**) (Mantovani et al., 2004). M1 are commonly described as STAT1<sup>+</sup>, IRF5<sup>+</sup>, and iNOS<sup>+</sup>, and can secrete a large panel of pro-inflammatory cytokines (IL-6, IL-1 family, IL-12, IL-23, TNFα...), chemokines (CXCL9, CXCL10, CXCL11, CCL3, CCL5...), and inflammatory mediators (i.e. reactive oxygen species or ROS) (Mantovani et al., 2004). M1 MΦ have a “broken Krebs cycle” leading to the accumulation of citrate and subsequently NO production, as well as the accumulation of succinate, HIF1α and IL-1β secretion (O’Neill, 2015). M1 MΦ are reported to be able to activate tumour-killing mechanisms and to amplify Th1 responses, inducing a positive feedback in the anti-tumour response (Biswas and Mantovani, 2010). Indeed, M1 macrophages can present antigens to induce an adaptive immune response but can also reactivate lymphocytes locally, at the injury/infection site. Although pro-inflammatory MΦ are mainly implicated in the elimination of noxious signals or microorganism invaders, M1-like macrophage-derived cytokine production have also been identified as key factors in several autoimmune and chronic inflammatory diseases, including Crohn’s disease, multiple sclerosis, and autoimmune hepatitis (Murphy et al., 2003; Smith et al., 2009).

**Anti-inflammatory MΦ**, commonly called M2, are mainly implicated in the resolution of inflammation and wound healing, but also, in some circumstances and by mechanisms not fully understood, in the defence against parasites (**Figure 7**) (Chávez-Galán et al., 2015). M2 are commonly described as STAT6<sup>+</sup>, IRF4<sup>+</sup>, and CD163<sup>+</sup>, and can secrete anti-inflammatory cytokines (IL-10, TGFβ), chemokines (CCL1, CCL17, CCL24...) and growth/angiogenic factors (PDGF, VEGF, EGF). In contrast to M1, M2 MΦ have a “functional Krebs cycle” allowing production of ATP and glycosylation of M2-associated receptors (O’Neill, 2015). M2

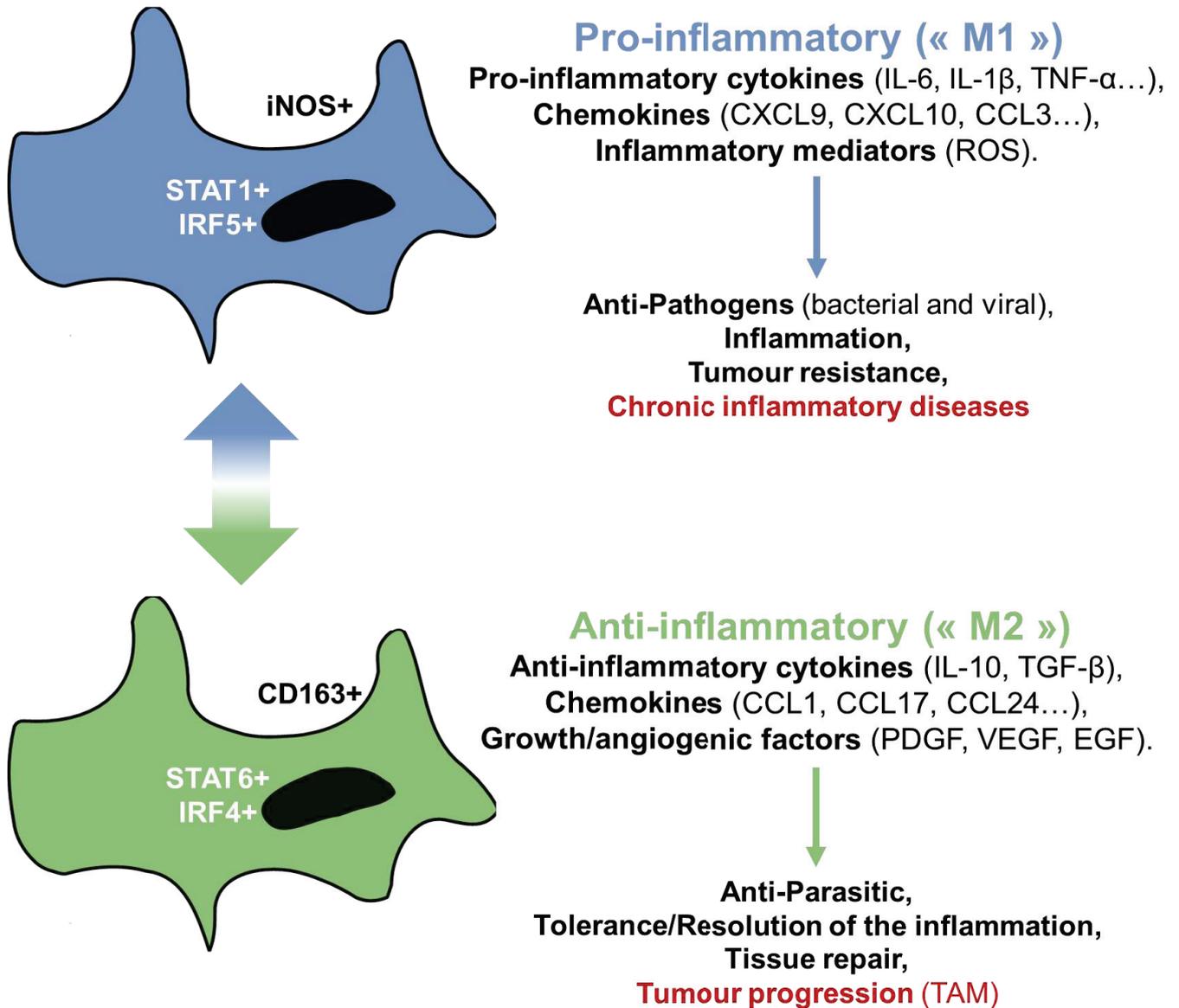
macrophages are mandatory for the resolution of inflammation, and tissue repair after an injury and/or cytotoxic activity (Mantovani et al., 2004). However, M2 are also associated with tumour progression through the secretion of negative immuno-modulators, and are called, in the tumour micro-environment, TAM for “tumour associated macrophages” (Tang et al., 2013). TAM have been associated to the progression of numerous cancers and as such are potential new target for therapeutic purposes (Murray and Wynn, 2011; Ries et al., 2014; Tang et al., 2013).

### 1.4.3. Kupffer cells specificity

**Kupffer cells** (KC), the liver resident M $\Phi$ , was first described by Fahimi in 1970 as peroxidase positive cells (Fahimi, 1970). KC are the largest M $\Phi$  population in the human body as they represent 80% of the total human M $\Phi$  count under normal physiological conditions (Crispe, 2009; Seki et al., 2000). KC are localized in liver blood vessels, seeded on liver sinusoidal endothelial cells and their fenestration, where they can phagocytose debris, aging blood cells or pathogens. After phagocytosis, pathogens can be processed into phagolysosomes and antigens can be presented to lymphocytes to activate or recall a local immune response (Crispe, 2009; Liaskou et al., 2012). As resident M $\Phi$ , KC express all common M $\Phi$  markers (i.e. CD68, CD14...) (Ginhoux and Guilliams, 2016) and have more precisely been defined as CD14<sup>+</sup>, HLA-DE<sup>+</sup>, HLA-ABC<sup>+</sup>, CD86<sup>+</sup>, DC-SIGN<sup>+</sup> with low expression of CD1b, CD40 and CD83 (Tu et al., 2008). As previously mentioned, in humans, no specific marker has been identified to differentiate KC from infiltrating macrophages, whereas in the mouse (i.e. Clec4F) (Scott et al., 2016) and rat (i.e. ED-1 and ED-2) (Zeng et al., 2013) markers have been described. In addition, KC have a long life-span (more than a year) and are self-renewing cells with a slow replicating activity. Thus the liver M $\Phi$  pool can be renewed, unlike monocyte derived macrophages which have a limited life-span (Naito et al., 1997). Interestingly, a recent study by Scott and colleagues described that in mice monocyte derived M $\Phi$  can, with time, acquire KC phenotype (including self-renewal capacity) after depletion of the resident M $\Phi$  in a model of KC expressing the diphtheria toxin receptor (Scott et al., 2016).

KC play a critical role in **liver tolerance**. Indeed, the liver is at the crossroads between the systemic and enteric circulation, with 80% of the blood reaching the liver coming from the

Figure 7. Pro- and anti-inflammatory phenotype of macrophages.

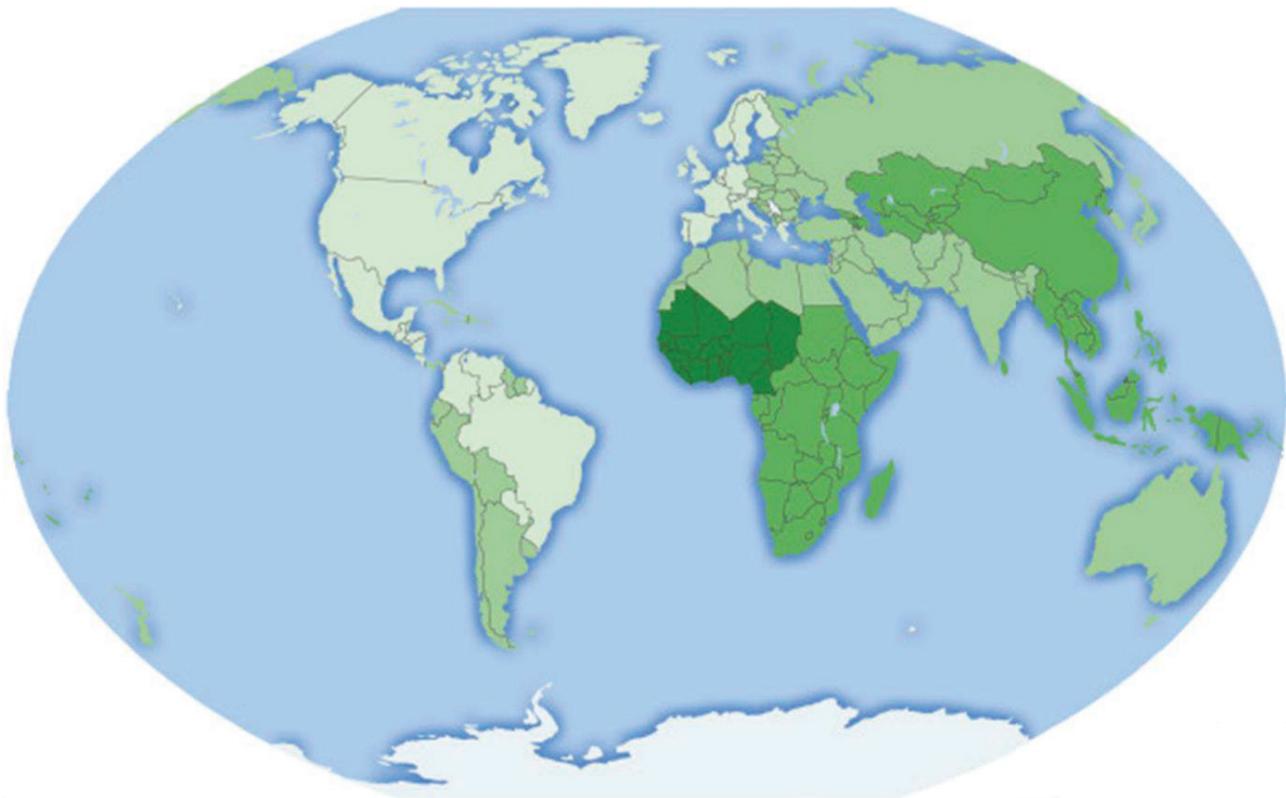


hepatic portal vein. Thus, liver cells are constantly exposed to pathogens. Indeed, lipopolysaccharide (LPS) endotoxin is found in measurable amounts (few nano grams) in the enteric circulation (Prytz et al., 1976). This phenomenon should theoretically lead to a constant inflammation within the liver but none is observed in healthy individuals. The liver is indeed described as an immune-tolerant organ (Crispe, 2009). This tolerance has been defined as a systemic or “oral” tolerance of enteric pathogens, which depends on the normal connection between the liver and gut (Yang et al., 1994). Yet, the full role of KC in this tolerance phenomenon still need further investigation, especially by assessing KC phenotype/state in healthy liver. Indeed, the anti- or pro-inflammatory phenotype of KC at steady state is yet to be properly determined. Depending on their localization in the liver, MΦ may have different phenotypes. It could be hypothesised that near the enteric circulation, where the exposition to bacteria is constant, KC will be more likely to be M2 MΦ, the MΦ implicated in tolerance, through the secretion of immune-regulatory mediators (Ju and Pohl, 2005). In contrast, near the systemic circulation, where the presence of pathogens is a sign of infection, KC would be more likely to be in a pre-pro-inflammatory state in order to quickly eliminate any blood infections. Moreover, other factors could play a role in the fate of KC phenotype. The oxygen gradient in the liver is linked to the expression of hypoxia regulated genes (HIF family), among which is HIF1 $\alpha$ , a factor linked to the accumulation of IL-1 $\beta$  in M1 MΦ (Kietzmann, 2017).

As previously described, even in this tolerant environment, KC are able to respond to the **detection of specific pathogens** (Knolle et al., 1995a; Seki et al., 2000; Tu et al., 2008). KC express a large spectrum of pattern recognition receptor (PRR), among which, TLR1/2, TLR2/6, TLR3, TLR4, TLR8 and RIGI/MDA5 (Sun et al., 2016), leading, after stimulation, to the secretion of a large spectrum of immune factors such as ROS, cytokines (IL-6, IL-10, IL-12, TNF $\alpha$ ...), and chemokines (CCL3, CCL5...) among others (Knolle et al., 1995b; Sica et al., 2014; Tacke, 2017; Tu et al., 2008). KC have also been described to be functional for several inflammasomes (AIM2, NLRP3), which, upon stimulation, induce the production, maturation and secretion of IL-1 $\beta$  and IL-18 (Yu et al., 2017; Zannetti et al., 2016). Chemokines and cytokines secretion will further recruit and activate monocytes from the circulating blood that will be locally differentiated and further reinforce the MΦ population and inflammation (Tacke, 2017). Thus in order to chronically infect the liver, pathogens (such as hepatitis viruses) must have evolved strategies to escape or prevent recognition by the immune system and liver inflammation.

**Figure 8. Seroprevalence of hepatitis B virus infection worldwide (adapted from CDC, 2016).**

Prevalence of hepatitis B is defined as the percentage of people infected with HBV categorized in four groups: low (<2%), low-intermediate (2-4.9%), high-intermediate (5-7.9%) and high (>8%).

**Prevalence of Hepatitis B:**

- High: > 8%
- High intermediate: 5% - 7.9%
- Low intermediate: 2% - 4.9%
- Low: < 2%
- No data

In summary, the liver is both an immune-tolerant (Crispe et al., 2006) and immune-competent organ (Crispe, 2009), in which Kupffer cells play a central role in preventing the circulation in the blood of pathogens and aberrant cells.

## 2. Hepatitis B

### 2.1. Generalities

#### 2.1.1. Epidemiology

**Hepatitis B virus** (HBV) chronically infects 257 million people worldwide (WHO, 2017). The persistence of viral replication is associated with a high morbidity and mortality, due to major complications (see natural history). Indeed, it is estimated that acute and chronic HBV infections are responsible for around 800,000 deaths per year worldwide (i.e., ranked 15<sup>th</sup> in the Global Burden of Disease Study) (Locarnini et al., 2015). Seroprevalence of HBV, as measured by HBsAg carriage, is globally of 3.61% (Chang and Nguyen, 2017). On this criteria, endemicity is categorized as low (<2%), low-intermediate (2-4.9%), high-intermediate (5-7.9%) and high (>8%), with the highest levels of 8.63% being found in Africa (**Figure 8**) (Chang and Nguyen, 2017).

HBV is divided into at least **8 genotypes** (A-H), based on a nucleotide sequence diversity of more than 7.5%. Major genotypes vary from one region to the other (**Figure 9**). Two additional Asian genotypes (I and J) have been proposed but have not been approved yet by the international committee on the taxonomy of viruses. Genotypes can be further divided into sub-genotypes (a diversity greater than 4%) and recombinants have also been described (Dény and Zoulim, 2010; Locarnini et al., 2013). Correlations between genotypes, clinical progression and treatment response have been established, although it is not possible to attribute to each genotype a predictive score for the severity of the associated disease. On the one hand, genotype C has been associated with an increased mutation frequency in the core protein promoters and subsequent resistance to IFN therapies (Kao et al., 2000a). Genotype C has also been associated with more pronounced liver diseases compared to genotype B which has been associated to a higher HCC development rate in the Taiwanese population

Figure 9. Genotype prevalence of hepatitis B virus infection worldwide (adapted from Osiowy, Coffin and Andonov, 2016). Major genotypes found in different regions of the world.



(Kao et al., 2000b, 2002). On the other hand, genotype D has been particularly associated with mutations in the PreCore region that abolish the secretion of HBeAg (Dény and Zoulim, 2010; Knipe and Howley, 2013).

### 2.1.2. Transmission pathways

HBV can either be transmitted vertically and horizontally (**Figure 10**) (Dény and Zoulim, 2010):

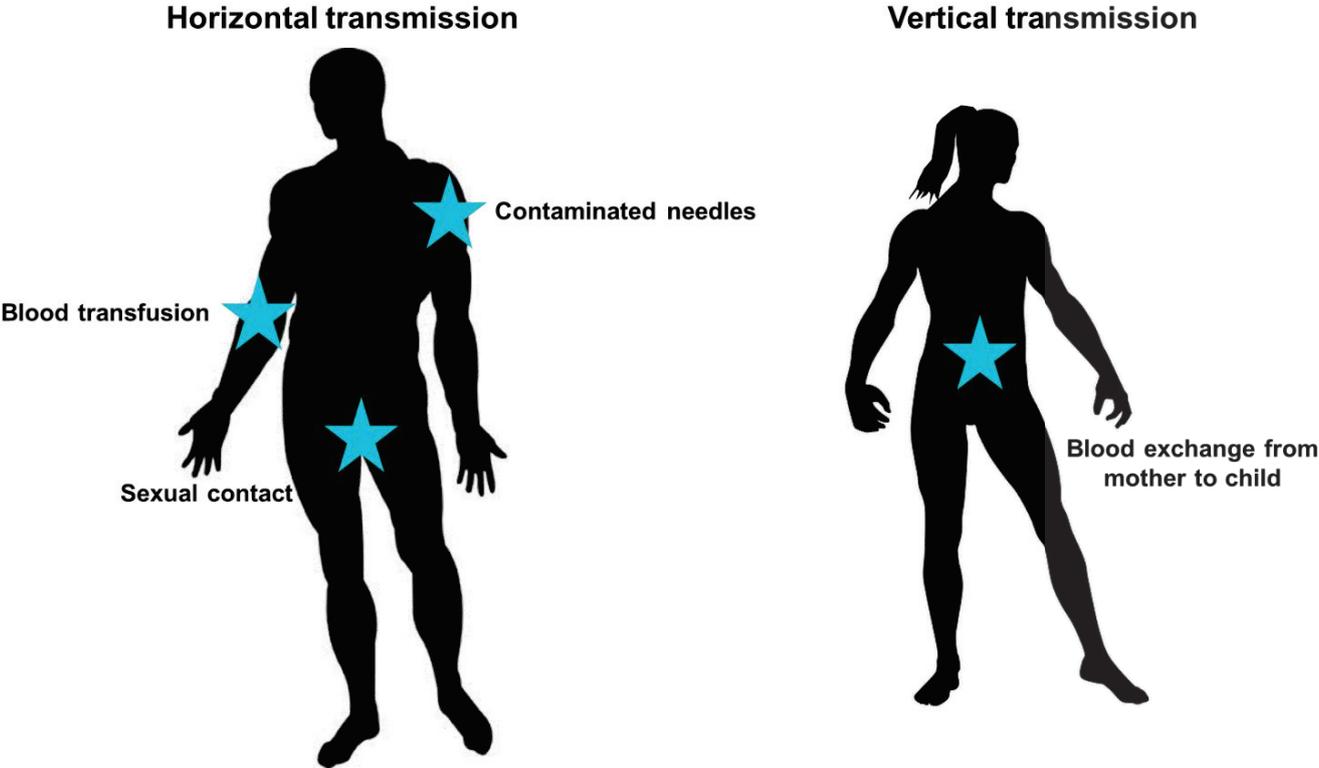
- **Vertical transmission**, from mother to infant, is frequent in highly endemic zones and occurs during the peripartum or the initial months after birth. No significant placental transmission has been demonstrated. Acute HBV infection in the third semester, high viral load and HBeAg positivity are associated with the highest risk of transmission;
- **Horizontal transmission** occurs, mainly, by parenteral exposure to infected blood (blood transfusion, contaminated needles) or unprotected sexual contact with exchange of body fluids (Kidd-Ljunggren et al., 2006).

## 2.2. HBV biology

### 2.2.1. Viral classification

Hepatitis B virus is a partially dsDNA virus. Thus, in the Baltimore classification, HBV belong to group VII, namely dsDNA viruses which replicate through an RNA intermediate. HBV is classified in the *Hepadnaviridae* family with other hepatotropic DNA viruses. Those viruses have been regrouped together by four common features: hepatotropism, genetic organisation, viral morphology and replication mechanisms (a retro-transcription step in their viral life cycle). Hepadnaviridae are divided into two genera: the genus orthohepadnavirus, includes HBV along with other mammalian viruses (as woodchuck hepatitis virus (WHV), ground squirrel hepatitis virus, woolly monkey hepatitis virus and bat hepatitis virus), the genus avihepadnavirus includes avian virus, as the duck hepatitis B virus (DHBV) and heron HBV (Schaefer, 2007).

**Figure 10. Hepatitis B virus transmission pathways.** HBV can either be transmitted horizontally by blood or fluid exchanges or vertically from mother to infant.



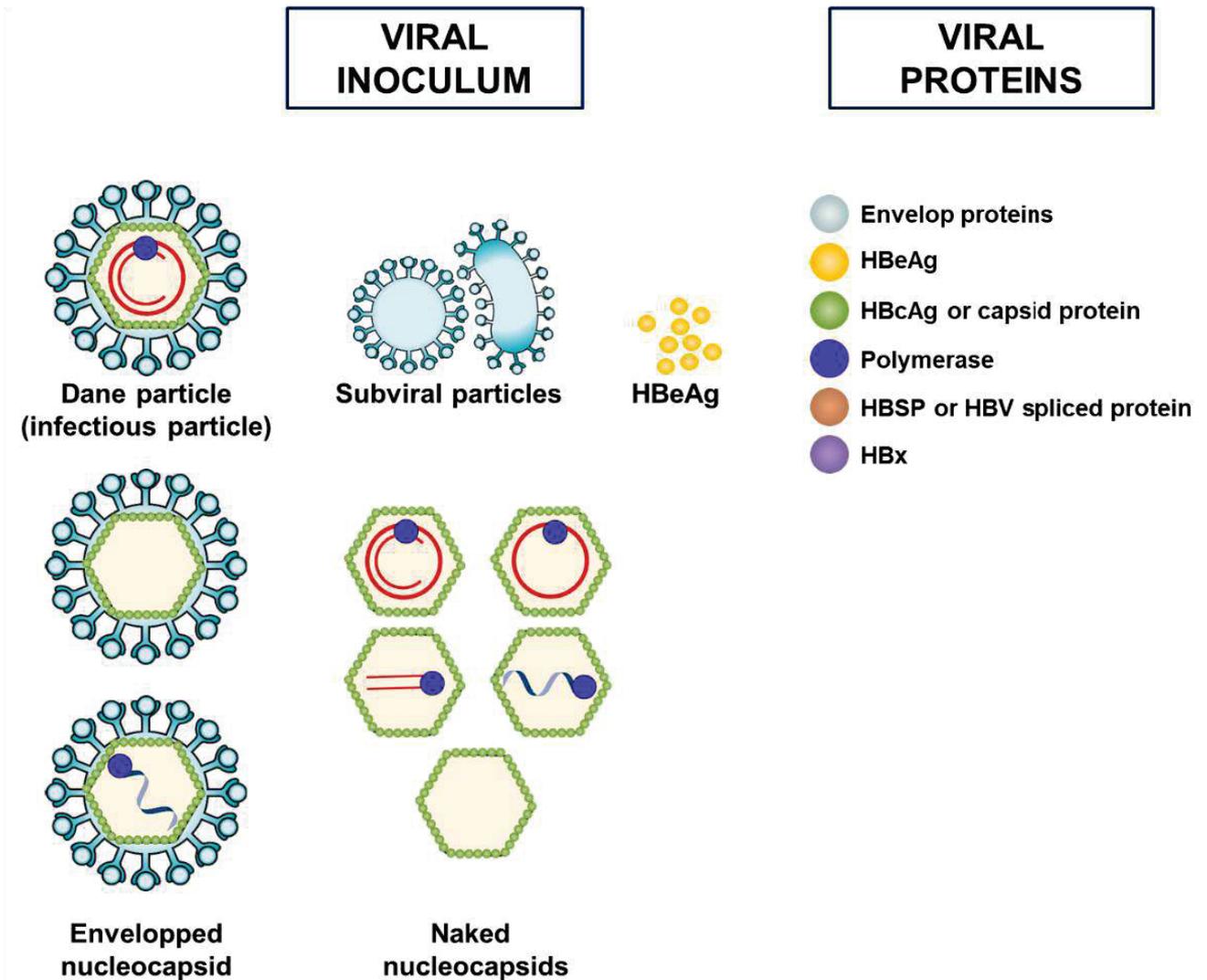
## 2.2.2. Viral structure

### 2.2.2.1. Virions and subviral particles

An HBV inoculum is composed of a secreted dimeric antigen, HBeAg (see viral proteins), and several major viral particles (**Figure 11**):

- **Complete virions**, also called **Dane particles** named after their discoverer, are the infectious form of the virus. In the serum of patients, viral titer can go up to  $10^{10}$  genome-copies/ml. The Dane particle is a 42 nm sphere with an inner icosahedral symmetry (Dane et al., 1970; Hruska et al., 1977). Virions are composed, from the surface to the interior, of: a lipoprotein membrane containing the three forms of viral surface proteins (S-, M- and L-HBsAg in a ratio of 4:1:1), an icosahedral nucleocapsid composed of 120 dimers of HBc proteins, and one single copy of the HBV genome, called relaxed circular DNA (rcDNA), covalently linked to the viral polymerase (Knipe and Howley, 2013).
- **Subviral non-infectious particles (SVP)** containing only envelop proteins. SVP are of, approximatively, 22 nm in diameter and circulate in the serum of patients in 1 000 to 10 000 fold excess compared to virions (Urban et al., 2014). Two types of SVP exist: spheres with an octahedral symmetry and filaments, which are asymmetrical with variable length and width. Filament possess the same HBsAg ratio as Dane particles. However, there is no L-HBsAg in spheres which have a ratio of 4:1 of S-HBsAg:M-HBsAg. The role of SVP in HBV infection is still a matter of debate. They may favour the infection through the saturation of humoral responses by their large excess and, so, seem to play a key role in immune subversion.

**Figure 11. Viral components secreted released during apoptosis by HBV-infected hepatocytes and their protein composition.** The viral inoculum is composed of the Dane particles (the infectious form of the virus), two subviral particles form (spheres and filaments) and secreted HBeAg. Upon apoptosis of hepatocytes, naked nucleocapsid can be released. In addition to Dane particles, other forms of enveloped nucleocapsid have been described in patients' serum. The different viral proteins are presented in the right panel.



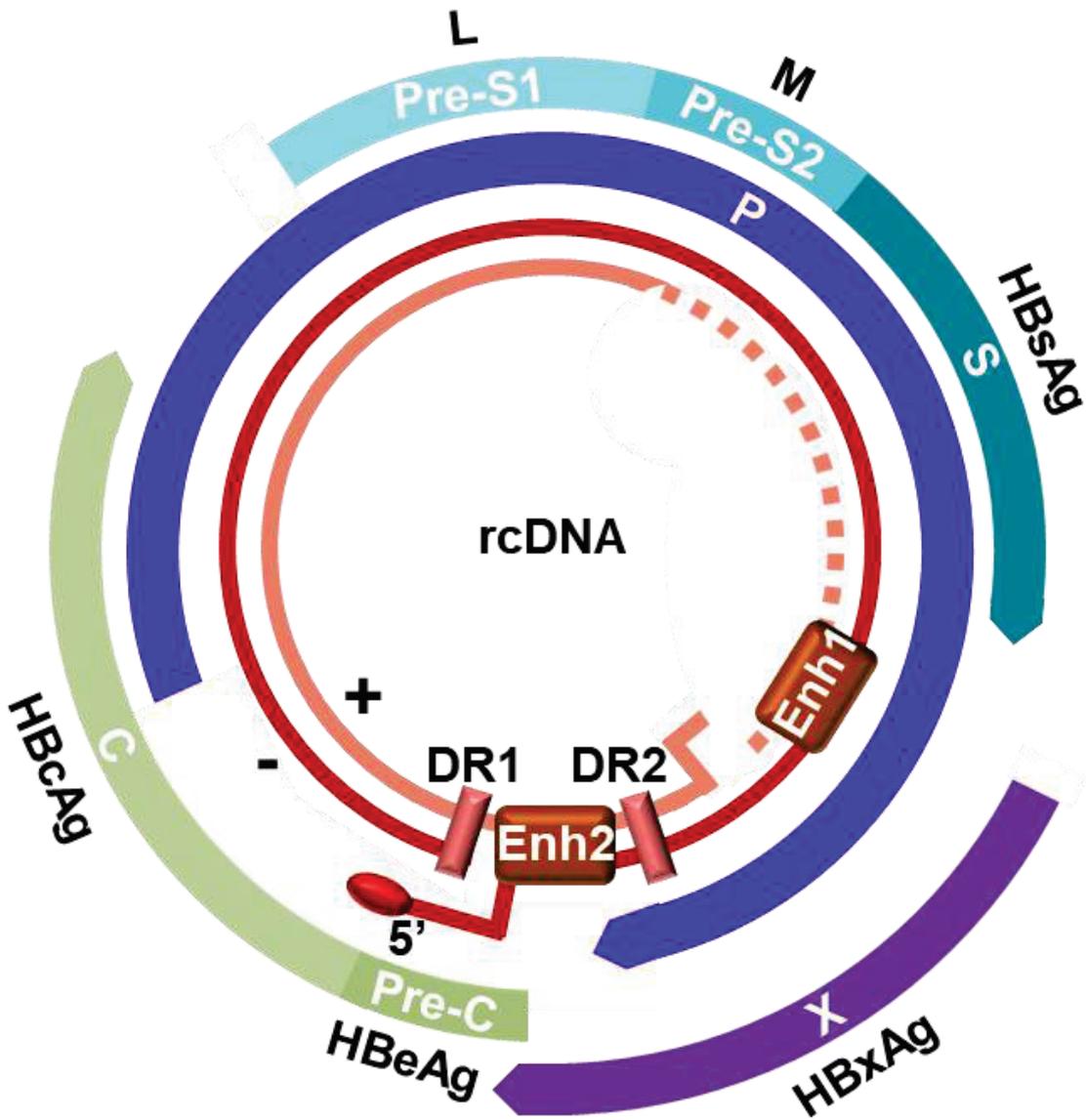
In addition to these HBsAg containing particles, identified from patients' sera, two other sub-viral forms have been described:

- **Non-enveloped nucleocapsids**, found to be secreted *in vitro* by HBV replicating hepatoma cell lines and suggested to play a role in virus spreading (Cooper and Shaul, 2006; Watanabe et al., 2007). They have also been shown to be released upon hepatocytes apoptosis (Arzberger et al., 2010). These nucleocapsids may contain viral DNA, but also all the intermediates between RNA encapsidation to final DNA retro-transcription (see viral life cycle). Their existence and role *in vivo* is still to be demonstrated;
- **Enveloped empty capsids** have been described in patients' sera, but their relevance and function is still to be determined (Luckenbaugh et al., 2015).

### 2.2.3. Viral DNAs

The HBV genome is found in secreted viral particles and in mature capsid in the cytoplasm of infected hepatocytes. The viral genome is a partially double-stranded and relaxed-circular DNA (rcDNA) of 3.2 kb. The complete strand of the genome, of negative polarity, is the template for transcription and is covalently linked to the viral polymerase at its 5' extremity. The incomplete stand of the genome, of positive polarity, contains a gap and has a 3' extremity of variable length (Summers et al., 1975). The positive strand is associated with a RNA oligomer, which derives from the pre-genomic RNA (pgRNA) and serves as the template for the completion of the negative strand. The circularity of the HBV genome is ensured by a 200 nucleotides long region in the positive strand, which overlaps the 5' and 3' extremity of the negative strand. The overlapping region contains the direct repeat region (DR) 1 and 2, mandatory for viral replication (Knipe and Howley, 2013). The genome contains 4 open-reading-frames (ORF), 4 promoters, 2 enhancer elements and 1 single polyadenylation signal that is used to terminate all RNAs (Figure 12). All nucleotides are implicated in the transcription of, at least, one viral mRNA (due to overlapping ORFs, each nucleotide may be in the transcript of several proteins) (Nassal, 2015) and transcription regulation regions overlap with coding information.

**Figure 12. HBV genome organization (adapted from Liang et al., 2009).** Organization of HBV genome includes key regulatory elements and open reading frames.



HBV ORFs are all oriented in the same direction and led to the production of the 7 viral proteins:

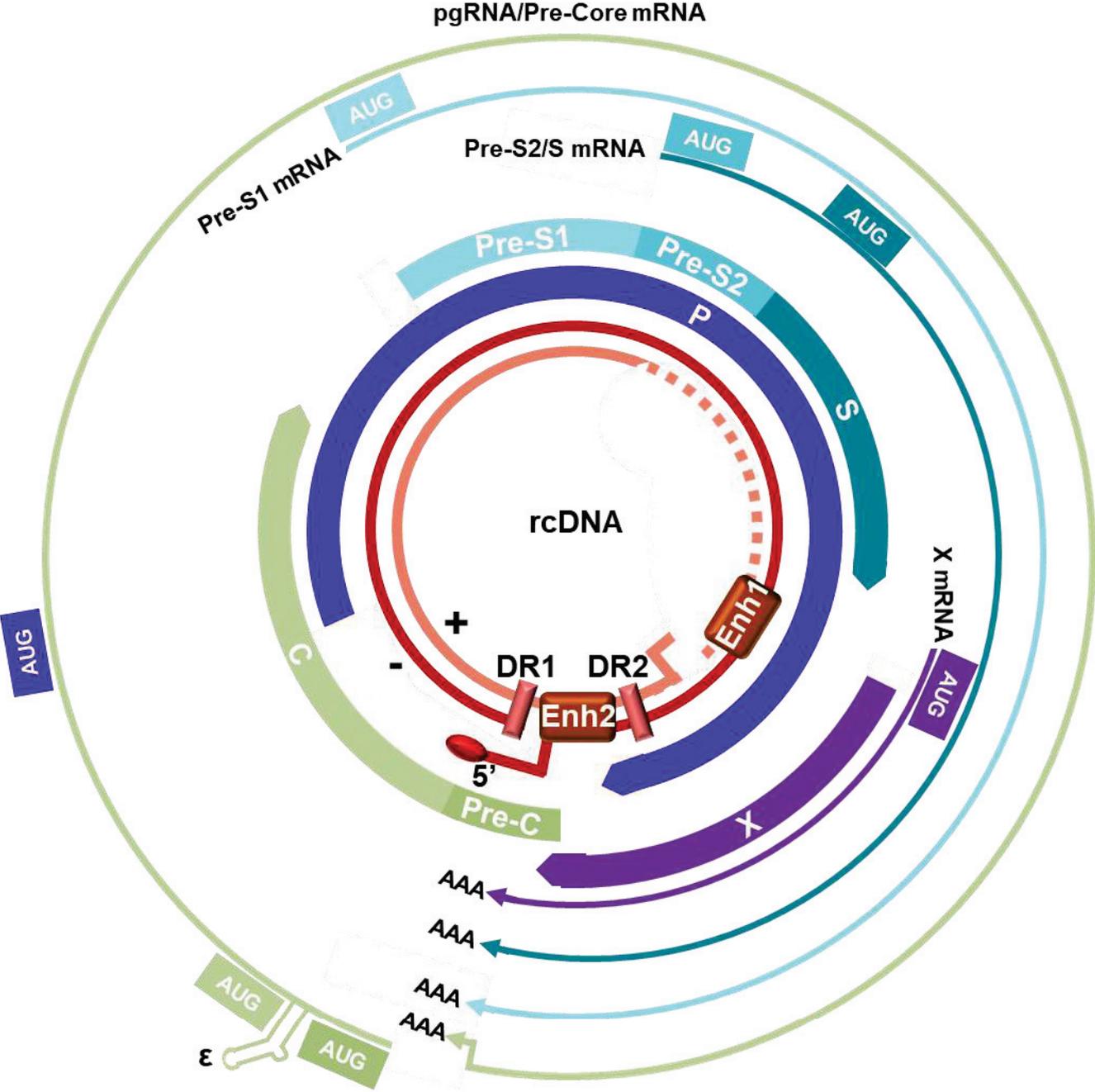
- **Polymerase ORF** is the largest ORF, representing 80% of HBV viral genome and codes for the viral polymerase.
- **Pre-S1/Pre-S2/ S ORF** is within the Polymerase ORF and contains 3 in-phase start codons which code for the 3 forms of HBV envelop protein (S-, M-, and L-HBsAg, for small, medium, and large HBsAg).
- **Pre-Core/Core ORF** contains 2 in-phase initiation codons and codes for secreted HBeAg and HBcAg (capsid protein).
- **X ORF** codes for the X protein.

Transcription of the 4 ORF is regulated by 4 promoters, 2 enhancers and a cis-acting negative regulatory element. Regulatory elements are functional in the nucleus after rcDNA to cccDNA (circular covalently closed DNA) conversion (Moolla et al., 2002). Further details of transcription regulation will be addressed in the cccDNA paragraph.

**cccDNA** is a form of HBV DNA that persist within infected hepatocytes. cccDNA existence has been reported in other cells (Lu et al., 2009) but the proper demonstration of its presence and functionality has only been established in hepatocytes. cccDNA is an episomal, plasmid-like, replicative intermediate DNA form that is only found in the nucleus of infected cells. This chromatin-like structure, also called viral minichromosome, is associated to histones and other host and viral proteins (Levrero et al., 2009) and is the only template for transcription of viral RNAs. The cccDNA forms a stable pool in the liver of infected patients (mean copy number/cells=1.8 [0.008-54] in HBeAg positive patients and 0.09 [0.01-15] in HBeAg negative patients) (Volz et al., 2007). This minichromosome can persist in quiescent cells without any effect on its viability. However, its half-life and persistence in mitosis is controversial (Knipe and Howley, 2013). Eradication of cccDNA is a rare event but a partial clearance can be observed in acute infections. Three mechanisms for HBV clearance have been proposed:

- **Non-cytolytic elimination** by action of cytokines directly on infected cells without hepatocytes' death or regeneration;
- **Cytolytic elimination** through hepatocytes' death;

**Figure 13.** The different viral transcript (adapted from Liang et al., 2009). Schematic representation of HBV RNA transcript with initiation codons and shared polyA tail.



- **Absence of neo-formation/dilution** that can happen when patients are treated with nucleoside analogues (NA) inhibiting Dane particle formation and recycling (see Treatments). Then, the cccDNA pool will be eliminated through a combination of hepatocytes' death and mitotic loss (Allweiss et al., 2017; Levrero et al., 2009; Nassal, 2015).

#### 2.2.4. RNA transcripts

HBV replication occurs, as characteristic of all hepadnaviruses, through RNA intermediates. RNA-polymerase II carries out transcription, using the cccDNA as a template. Five transcripts (2 genomic and 3 sub-genomic, which all possess a 5' cap and a shared polyA tail) can be identified in infected cells (**Figure 13**):

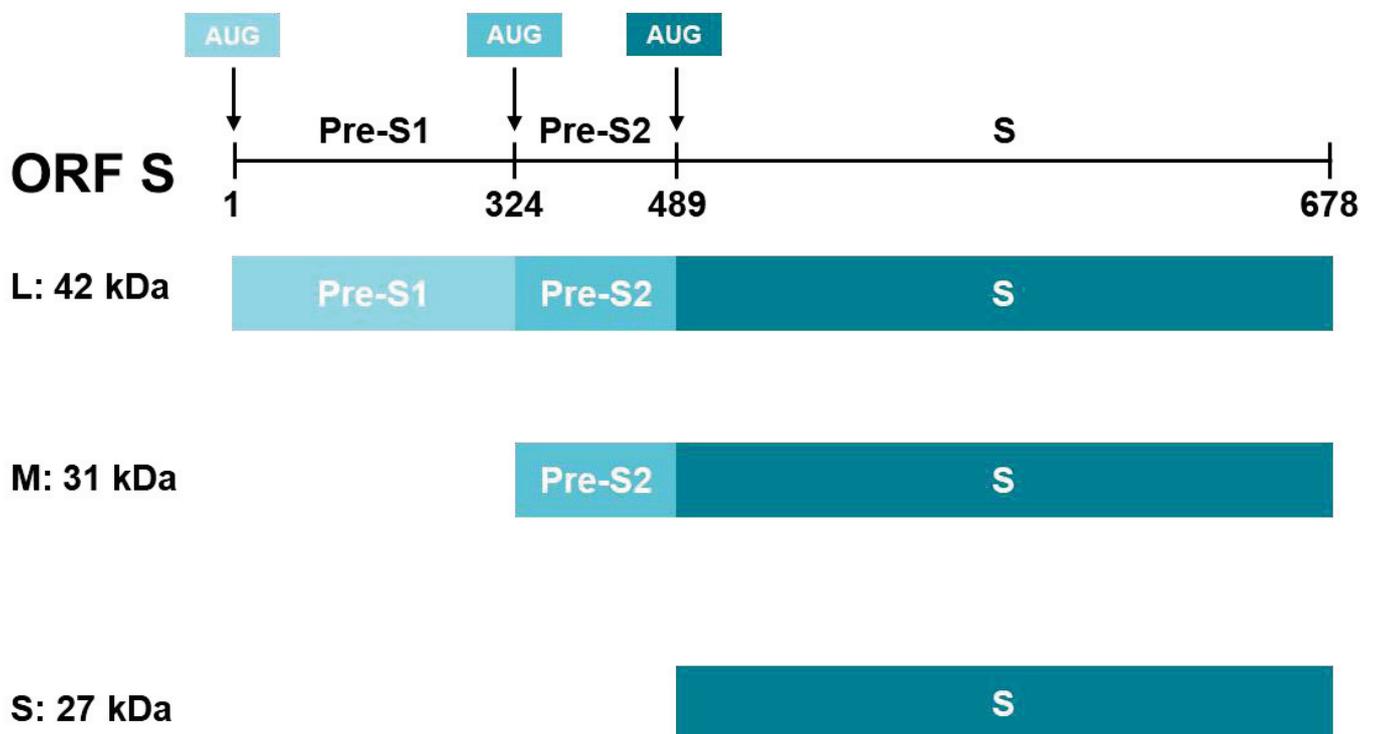
- **Pregenomic RNA** (3.5 kb) is the template for the translation of the viral polymerase and core proteins and is the template for *de novo* synthesis of HBV DNA genome through reverse transcription;
- **Pre-Core mRNA** (3.5 kb; few bases longer than pgRNA) is the template for translation of the pre-core protein and, ultimately, for the secreted HBeAg;
- **Pre-S1 mRNA** (2.4 kb) is the template for the translation of L-HBsAg;
- **Pre-S2/S mRNA** (2.1 kb) is the template for the translation of M- and S-HBsAg;
- **X mRNA** (0.7 kb) is the template for the translation of the X protein (Moolla et al., 2002).

To note, the X transcript is only occasionally detected in infected tissues (Knipe and Howley, 2013).

#### 2.2.5. Viral proteins

The HBV genome codes for **7 viral proteins**: HBV polymerase, Core protein (HBcAg), Pre-core (later cleaved into HBeAg), large, medium and small envelope proteins (L-, M-, S-HBsAg) and the X protein (Seeger and Mason, 2015). An eighth viral protein have been found in liver biopsies of HBV infected patients, the hepatitis B spliced protein (HSBP) which can be produced through alternative splicing of pgRNA (Soussan et al., 2003).

**Figure 14. The three forms of HBV envelop proteins (S, M, and L).** Translation is initiated at different in-frame positions with the L form containing the 108 aa Pre-S1 domain and the 55 aa Pre-S2 domain, the M-HBsAg containing the Pre-S2 and the shortest S form just the S protein. The three forms share the same C-terminus.

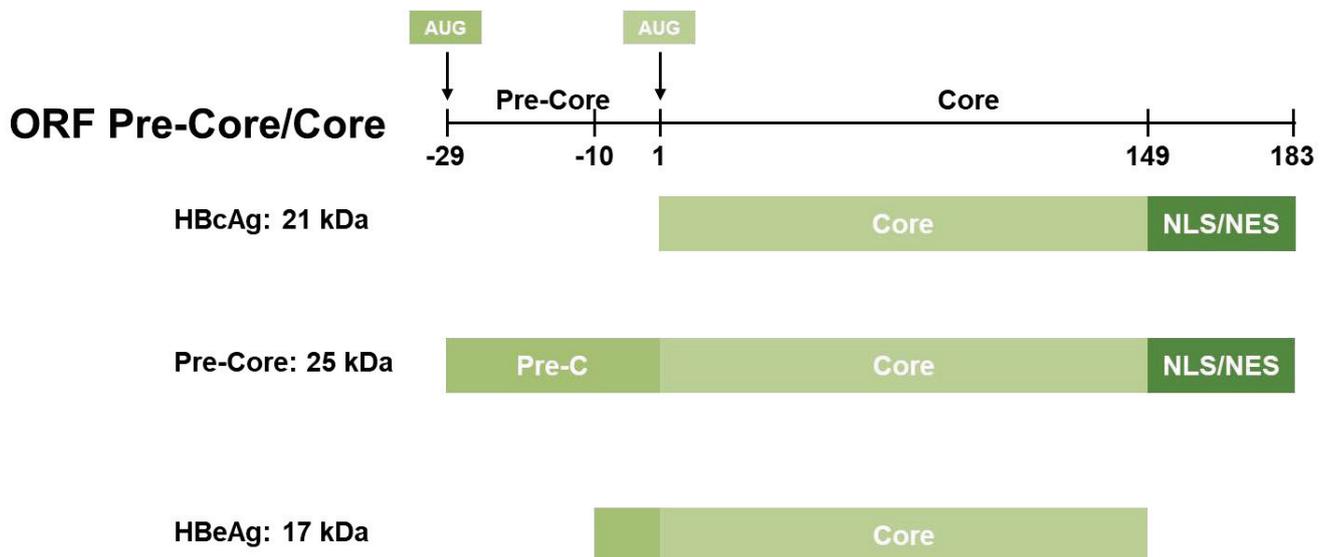


### 2.2.5.1. Surface proteins (HBsAg): small, medium and large

The HBV genome codes for **three envelope proteins**, small (S), medium (M) and large (L), starting with three different ATG but ending at the same terminal stop codon (**Figure 13**). The S protein is a 226 amino-acid long polypeptide and is the most abundant form found in infected cells (Bruss and Ganem, 1991). The M protein is larger by 55 residues (Pre-S2 region) and the L protein contains 108 to 119 more residues (depending on the strain) than M protein (Bruss and Ganem, 1991) (**Figure 14**). Surface proteins are synthesized in the endoplasmic reticulum (ER) and assemble in hetero- or homo-dimeric complexes through cysteine residues contained in the S domain (Wounderlich and Bruss, 1996), to form the envelope. The L protein is essential for both capsid envelopment during virion morphogenesis as well as viral entry via the binding to the sodium-taurocholate cotransporting polypeptide (NTCP) HBV receptor, which is expressed at the surface of hepatocytes (Schulze et al., 2010). L and S proteins are also required for virion secretion, whereas M is not (Bruss and Ganem, 1991). In addition, surface proteins with SVPs are secreted in large excess (on average 1,000 to 10,000 times more than Dane particle) in the blood stream of infected patients as spheres and filaments in the absence of capsid or viral genome (Ganem and Prince, 2004). Each subtype of particles has a different proportion of HBsAg proteins. Spheres (around 20 nm in diameter) contain mostly S and some M proteins, whereas filaments (22 nm large for variable length) contains also L proteins (**Figure 11**) (Gilbert et al., 2005; Heermann et al., 1984; Patient et al., 2009). Variation of SVPs morphology (sphere VS filament) could be dependent on the S/L ratio and the quantity of L incorporated during morphogenesis. However, a too high quantity of L protein in subviral particles, inhibits their secretion and promote their retention within the cytoplasm (Persing et al., 1986).

SVPs are known to play a key role in immunity control, as discussed later in this introduction, and in the decoy for the recognition Dane particles by the adaptive immune cells (Bruss, 2007; Patient et al., 2009). Indeed, antibodies against surface proteins cannot overcome the quantity of SVPs in the blood stream (up to 1 mg/ml) allowing Dane particles to circulate and re-infect other hepatocytes (Bruss, 2007; Patient et al., 2009). All the roles of HBsAg have not been investigated yet, nor if the proportion of the three proteins is modified during the course of infection and what the impact is on the virus interaction with the host.

**Figure 15. Hepatitis B virus pre-core and core proteins.** HBcAg, Pre-Core and HBeAg all share the Core domain whereas only HBcAg and Pre-Core proteins contains the NLS/NES sequence. The NLS sequence as well as a part of the Pre-Core domain is cleaved from Pre-core protein to form the secreted HBeAg.



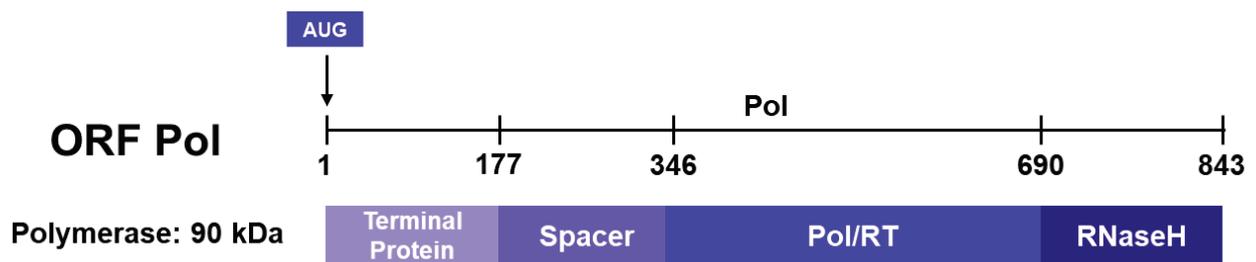
### 2.2.5.2. Capsid protein (HBcAg)

The capsid protein, **HBcAg**, is 183 aa long and has a molecular weight of 21 kDa. HBcAg is the structural unit of the viral nucleocapsid. HBcAg contains 2 domains (**Figure 15**): the N-terminal domain (NTD) implicated in dimerization/assembly of the protein and recruitment to building sites, as well as the C-terminal “protamine” domain (CTD) involved in nucleic acid integration, pgRNA encapsidation and *in fine* DNA replication. The level of CTD phosphorylation regulates capsid maturation. Indeed, DNA synthesis is induced after core dephosphorylation and conformational reorganisation of CTD. This change exposes binding sites for interaction with the envelop protein, which leads to the assembly of mature virions (Mabit and Schaller, 2000; Perlman et al., 2005). CTD has a nuclear localization sequence (NLS) that mediates nuclear transport of the entering and part of the newly formed capsid (also called recycling) (Yeh et al., 1990). HBcAg is highly immunogenic. Although, HBcAg is only detected in the liver of infected patients, anti-HBcAg antibodies are a good marker of current and past infection.

### 2.2.5.3. Pre-Core protein and HBeAg

PreCore mRNA translation gives rise to a precursor protein of 25 kDa, that contains the same domains (NTD and CTD) as the core protein extended by a 29 aa at the N-terminus (**Figure 15**) (Yeh et al., 1990). The Pre-Core protein is transported to the ER where it is processed by sequential cleavage to the form that is secreted, the 17 kDa **HBeAg** (Standring et al., 1988). Although, the amino acid primary sequence is identical between HBcAg and HBeAg, they possess *in fine* different antigenic properties (Salfeld et al., 1989). Patient’s serum HBeAg levels are used as a surrogate marker of active replication even if HBeAg is not directly involved *sensus stricto* in HBV replication as shown by a normal replication of the HBeAg negative strain *in vitro* (Parekh et al., 2003) (see viral life cycle). The seroconversion of HBeAg to anti-HBeAg is a marker of the end of active replication and the beginning of the clinical evolution towards an inactive carrier state. But this process can takes years. The clinical loss of HBeAg is the result of the selection of mutants, bearing either Pre-Core stop mutations or BCP (i.e. basal core promoter) mutations, which abrogate or greatly lower its synthesis. These patients albeit having no circulation of HBeAg may have continuous disease activity (Volz et al., 2007).

**Figure 16. Domains of hepatitis B virus polymerase.** The four functional domains are represented from N- to C-terminus: TP (terminal protein), RT (reverse transcriptase), and RNase-H.



#### 2.2.5.4. Polymerase (Pol)

**HBV polymerase** (230 aa; 90 kDa) is the only enzyme coded by the HBV genome. The viral polymerase is the target of current direct-acting antiviral (DAA) drugs (see treatment). Sequence and functional analyses of the polymerase have revealed 3 sub-domains (**Figure 16**): the terminal protein (TP), the reverse transcriptase (RT), and RNase-H domains. A spacer sequence of 56 aa, with no clear function, serves as a linker between TP and RT domains (Nassal, 2008).

The **TP domain** is essential for the interaction with the “epsilon”  $\epsilon$  packaging signal of pgRNA which confers template specificity to the HBV polymerase. A covalent bond links the polymerase to the minus-strand of HBV DNA (Nassal, 2008).

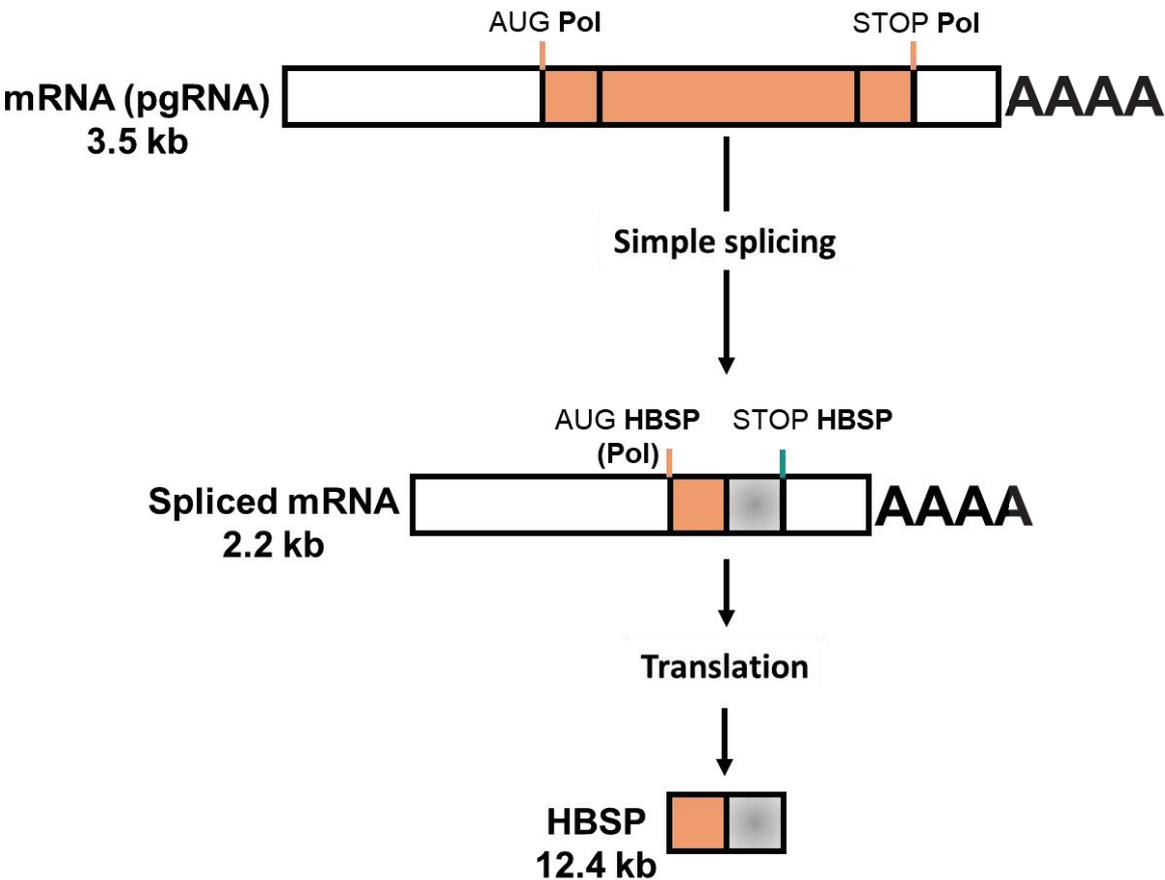
The **RT domain** has 2 enzymatic functions: the reverse transcription of pgRNA into the minus-strand of HBV DNA and DNA-dependant DNA synthesis of HBV positive-strand from the minus strand. However, the RT has no 3'-5' exonuclease activity, which makes HBV polymerase error-prone and leads to high HBV genome variability (Okamoto et al., 1987).

The **RNase-H** domain is responsible for pgRNA degradation after synthesis of the minus-strand as well as generation of short RNA primers required for DNA positive-strand synthesis (Wei and Peterson, 1996). RNase-H inhibitors have been shown to block HBV replication and could therefore be a potential target for therapeutic options (Tavis and Lomonosova, 2015).

#### 2.2.5.5. X protein (HBxAg)

The **X protein** is the smallest protein coded by the HBV genome (154 aa; 17 kDa). X is implicated in HBV replication *in vivo* and is also a trans-activator of a number of cellular promoters. The precise perimeter of X activity is still elusive and varies depending on the study models used (Bouchard and Schneider, 2004). This is the reason why it will not be discussed *in extenso* here. For sure, the X protein plays a role in the establishment and maintenance of HBV replication, *in vitro* (Lucifora et al., 2011) and *in vivo*, as shown in the woodchuck model (Zoulim et al., 1994). Furthermore, X has a role in epigenetic activation of cccDNA

**Figure 17. Formation of Hepatitis B Spliced Protein, HBSP (adapted from Assrir et al., 2010).** Schematic representation of pgRNA splicing to form the spliced mRNA of 2.2 kb later translated to form HBSP. pgRNA: pre-genomic RNA; Pol: polymerase ORF.

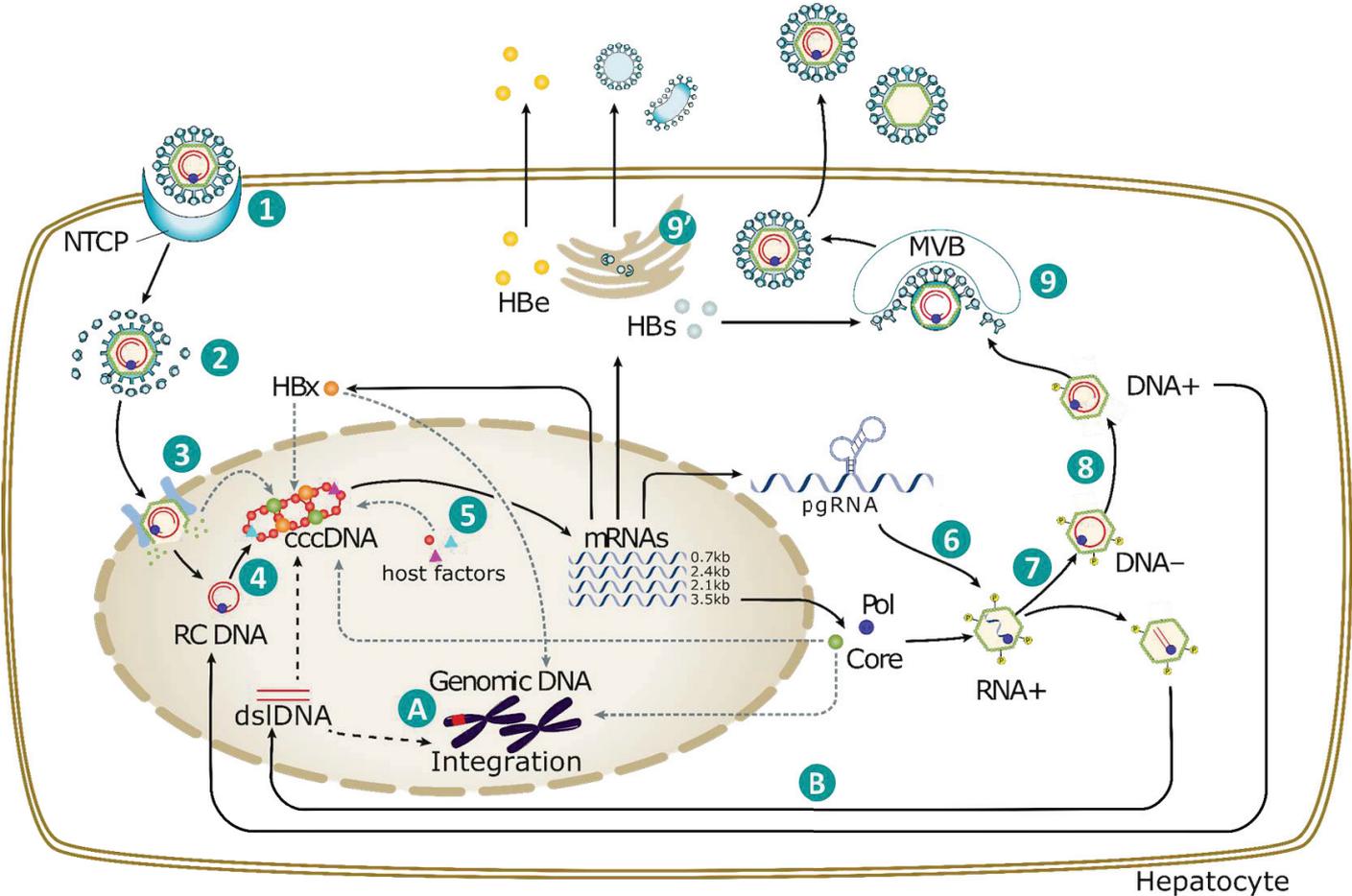


(Belloni et al., 2009). Regulation of nucleocapsid phosphorylation status by X protein has also been postulated to be essential for HBV replication (Melegari et al., 2005). Moreover, X could be a trans-activator for all HBV promoters (Moolla et al., 2002) particularly from extrachromosomal DNA templates, and not from integrated DNA (van Breugel et al., 2012). Even though X protein does not directly bind DNA, it can interact with transcription factors and regulate expression of host genes (Lara-Pezzi et al., 1998). Interaction of X with histone modification enzymes plays a key role in epigenetic regulation of gene expression (Tian et al., 2013). The X protein is also implicated in cell cycle regulation, apoptosis, the DNA damage response and in the modulation of innate immune response (Wei et al., 2010a, 2010b). Through its interaction with all these different signalling pathways, HBx has been proposed to be involved in HBV-associated HCC (Fallot et al., 2012). Finally, more recently, a study has shown that X protein is implicated in the degradation of the SMC5/6 complex (a host restriction factor of cccDNA), through an DDB1/E3 ubiquitin ligase dependant mechanism (Decorsière et al., 2016).

#### 2.2.5.6. Hepatitis B splicing-regulated protein (HBSP)

The **Hepatitis B Spliced protein** (HBSP) was discovered in HBV chronically infected patient's biopsies. HBSP is produced from a 2.2 kb spliced form of the pgRNA, called SP1, which is later translated into a 12 kDa protein of 93 aa (**Figure 17**) (Soussan et al., 2000). Translation of this protein starts from the polymerase AUG codon, and, therefore, the first 46 aa of HBSP and viral polymerase are identical. The following 47 aa are a new sequence generated by the new spliced RNA sequence. The biological functions of this protein are still being studied. However, HBSP seems to play a role in the pathogenicity and/or persistence of HBV infection (Soussan et al., 2003). Indeed, HBSP can reduce liver inflammation during chronic HBV infection and through this may promote infection by down-regulation of immune responses (Pol et al., 2015). Recently, HBSP expression in mice liver was shown to limit liver damage during induced fibrosis (Duriez et al., 2017a). This protective effect was associated with a decrease in the recruitment of inflammatory monocytes/macrophages, due to an inhibition of C-C motif chemokine ligand 2 (CCL2) expression in hepatocytes.

**Figure 18. HBV viral life cycle.** Detailed explanations are given in the text for the indicated numbers.



## 2.2.6. Viral life cycle

### 2.2.6.1. Viral Entry

L-HBsAg is mandatory for viral entry into hepatocytes. Moreover, the antigenic loop of S-HBsAg also seems to play a role, as shown by a lack of infectivity when there is a modification of this antigen loop (Julithe et al., 2014; Le Duff et al., 2009). The envelope of HBV virions interacts with heparan sulfate proteoglycans (HSPG) in an electrostatic manner, a process that is necessary but not sufficient for the permissive entry of virions (Leistner et al., 2008; Schulze et al., 2007). Recently, the HBV receptor, **NTCP**, has been discovered (**Figure 18.1**). NTCP is located at the basolateral membrane of hepatocytes and play a role in enterohepatic circulation of bile salts (Yan et al., 2012). A peptide of 75 aa at the N-terminal sequence of Pre-S1 binds to NTCP to allow entry (Urban et al., 2014). Further internalisation processes remains largely understood. A recent study has highlighted the implication of glypican 5 as a co-entry factor of HBV (Verrier et al., 2016), and other factors are currently being investigated.

### 2.2.6.2. Nuclear import and cccDNA formation

After entry, the nucleocapsid is released in the cytoplasm by a mechanism which is not fully deciphered mechanistically and is transported to the nucleus by the microtubular system (**Figure 18.2**). The nuclear pore is then crossed thanks to the interaction of the core NLS sequences with importin  $\alpha$  and  $\beta$  (**Figure 18.3**). How the capsid disassembles at the nuclear pore remain to be elucidated. A model has been proposed where the capsid would disintegrate within the nuclear pore and release the rcDNA inside the nucleus. Of note, only hypophosphorylated capsids would disintegrate. So only mature capsids containing rcDNA and not immature capsids containing pgRNA will be able to disassemble (see pgRNA transcription, encapsidation and retro-transcription) (Kann et al., 2007). The cccDNA is synthesized from the rcDNA (**Figure 18.4**), either incoming (**Figure 18.1**) or recycling virions (**Figure 18.B**). The conversion from **rcDNA to cccDNA** requires several steps (Levero et al., 2009; Nassal, 2015):

- The removal of HBV polymerase from the 5' minus-strand of the rcDNA. The existence of a protein free rcDNA (PF-rcDNA) has been suggested *in vitro* (Levero et al., 2009);

- The RNA primers covalently linked to the 5' end of the positive-strand of rcDNA as well as one copy of the terminal redundancy of the minus-strand are then removed;
- The positive-strand is completed to form a full-length dsDNA. Both ends of the two strands are ligated together. It is hypothesised that host enzymes are hijacked for this process however they remain to be identified.

**Viral DNA integration** is not mandatory for viral replication as it is for retroviruses. However, integration of the viral DNA can happen in patients in 10 to 100 % of hepatocytes during HCC (**Figure 18.A**). The integration have been proposed to play a role in the development of HCC but a positive correlation has not been established yet (Seeger and Mason, 2015). A model has been proposed in which HBV ds-linear-DNA-containing particles in the inoculum might facilitate the integration of HBV DNA into host genome, through homologous recombination at the sites of double strand breaks in the host DNA (Tu et al., 2017).

### 2.2.6.3. pgRNA transcription, encapsidation and retro-transcription

Viral RNAs are transcribed from cccDNA by the DNA-dependent RNA polymerase II, and all have a 5' cap and a common poly-A tail. The transcripts are then recognized and translated as cellular RNAs by ER associated ribosomes (**Figure 18.5**).

The viral polymerase binds the  $\epsilon$  loop of the 5' end of the pgRNA, from which it has been translated. Viral polymerase and pgRNA are co-packaged into nucleocapsid by interaction of pgRNA with the CTD sequence of HBc (**Figure 18.6**) (Bartenschlager et al., 1990; Nassal, 2015). The induction of conformational changes, by the interaction with HBc favour the initiation of the reverse transcription. Firstly, pgRNA is reversed transcribed into the minus-strand of the rcDNA. The RNA template is then digested with the exception of the 5' end, which serves as the primer for positive-strand synthesis (**Figure 18.7**). The negative strand is the template for positive strand synthesis (**Figure 18.8**). The reverse transcription is closely linked to the capsid maturation. Indeed, pgRNA-containing capsids are hyperphosphorylated (immature capsid) while reverse transcription is associated with a progressive dephosphorylation (mature capsid). Phosphorylation status changes have two consequences on the virion:

- Conformational changes allowing the interaction with HBsAg and secretion;

- Immature, phosphorylated, capsids are stable and do not disintegrate upon interaction with the nuclear pore (Bruss, 2007; Kann et al., 2007).

#### 2.2.6.4. Assembly and secretion

HBsAg proteins can have 2 different fates: **envelopment of nucleocapsid** (L-HBsAg is mandatory) or **subviral particles formation**. The secretion pathways differ between virions and subviral particles:

- Virions will be assembled and secreted through multi vesicular bodies (MVB) (**Figure 18.9**)
- Subviral particles will be assembled and secreted through ER-Golgi intermediate compartment and following Golgi secretory pathway (**Figure 18.9'**) (Huovila et al., 1992; Watanabe et al., 2007).

#### 2.2.6.5. Transcriptional regulation of cccDNA

HBV genome contains several regulatory sequences (4 promoters and 2 enhancers) (**Figure 12**).

**Enhancer I** (Enh I) is 270 bp long and is located between Pre-S1/Pre-S2/S and X ORFs. Enh I is the major element of transcription regulation. It particularly induces the activation of Core and X and more moderately of Pre-S and S. It is the binding site for ubiquitously transcription factors (such as AP1 and NF- $\kappa$ B) and liver specific transcription factors (such as HNF1, HNF3, and HNF4) (Moolla et al., 2002). By sequence analysis, an ISRE (interferon stimulated response element), was found in Enh I, which could explain the mechanisms of action of interferon on the regulation of HBV transcription. This response is mediated by STAT1 and STAT2 binding on Enh I (Belloni et al., 2012; Tur-Kaspa et al., 1990).

**Enhancer II** (Enh II) is 105 bp long and is located upstream of the core promoter. It modestly induces Pre-S/S and X and to a lesser extent Core. HNF3, HNF4, and SP1 are able to bind to the Enh II (Moolla et al., 2002).

CccDNA transcription is also and mainly regulated by **epigenetic modifications** of attached DNA molecules and/or attached histones. Due to its chromatinized form, the cccDNA displays

a characteristic “beads-on-a-string” arrangement on electronic microscopy and has a nucleosome organisation (Bock et al., 2001; Newbold et al., 1995). On cccDNA, the histone predominant species are H3 and H2B, and to a lower extent H4, H2A, and H1. HBc can also be part of the nucleosomes. Numerous cellular transcription factors and enzymes (CREB, STAT1/2, EZH2...) have been shown to bind to cccDNA in hepatoma cell lines (Belloni et al., 2009; Pollicino et al., 2006). The amino terminal tails of 4 core histones of nucleosomes can undergo translational modification (lysine acetylation, lysine and arginine methylation, serine phosphorylation, ubiquitination and sumoylation). Histone acetylation allows the initiation of the transcription. The decrease of acetylation in H3 and H4 N-terminal ends leads to the recruitment of histone methyltransferase, DNA methylation, and following gene repression. Transcription activity of the cccDNA varies during the infection course and can be modulated by immune factors and X binding (Belloni et al., 2009; Levrero et al., 2009). The methylation status of the 6 CpG islands on HBV DNA contributes to the regulation of gene expression and transcriptional repression (Zhang et al., 2013). Major epigenetic modifications of cccDNA and their effect on the gene expression are listed in [Table III](#).

Mark (DNA or histones)	Site	Function
<b>DNA methylation</b>		
Methylated cytosine (meC)	CpG islands	Repression
<b>Histone Post-Translational modifications</b>		
Acetylated lysine (K Ac)	H3 (9, 14, 18, 59) H4 (5, 8, 13, 16) H2A, H2B	Activation
Phosphorylated serine/threonine (S/T)	H3 (3, 10, 28) H1A, H2B	Activation
Methylated arginine (R Me)	H3 (17, 23) H4 (3)	Activation
Methylated lysine (K Me)	H3 (4, 36, 79) H3 (9, 27), H4 (20)	Activation Repression
Ubiquitylated lysine (K Ub)	H2B (120) H2A (119)	Activation Repression
Sumoylated lysine (K Su)	H2B (6/7) H2A (126)	Repression

**Table III. cccDNA epigenetic modifications (adapted from Levrero, 2009).** Type, site and functions of known chromatin marks. The numbers between brackets represent the amino acid residues involved in the given modifications.

### 2.2.6.6. Models for HBV replication study

The complete HBV replication cycle has been described to happen only in differentiated hepatocytes and in a few species. Viral genome delivery to host cells is not sufficient to allowed a HBV replicative cycle, therefore other factors (host and viral) are essential for replication. Host and liver specific transcription factors have been shown to be involved in RNA transcription but others roles earlier in the HBV life cycle (before cccDNA formation) are not excluded (Seeger and Mason, 2015). Moreover, in a mouse model expressing the human sodium-taurocholate cotransporting polypeptide (hNTCP, HBV entry receptor), HBV cannot replicate and, although it replicates in a transgenic mice model, cccDNA is not detected (Guidotti et al., 1995; Yan et al., 2013). Whether such a restriction is due to a lack of specific factors or the presence of inhibitory elements remains to be determined.

Due to the narrow range of susceptible and permissive hosts to HBV infection, a limited number of models to study HBV life cycle are available both for *in vitro* and *in vivo* studies:

- ***In vitro***, most of the studies on the late stages (after viral DNA delivery to the nucleus) of HBV life cycle were performed in a model of transfection of HBV DNA in hepatoma cell lines (not an infectious model due to lack of viral receptor). Up to recently, complete HBV life cycle could only be studied in primary human hepatocytes (PHH) or in dHepaRG cell line (Gripon et al., 2002; Zeisel et al., 2015). However, new models to study the complete HBV life cycle have been recently characterized. They consist of hepatoma cells, which express the viral receptor (e.g. HepG2-hNTCP or Huh7-hNTCP; see viral entry) (Li and Urban, 2016). Of note, as the cell lines used are hepatoma ones, innate immune pathways are impaired, making this model not suitable for immune studies
- ***In vivo***, three types of models have been implemented:
  - **Natural infection:** Surrogate models, based on host-adapted viruses belonging to the same family of viruses have been often used to get insights on HBV biology. Among these models are the Pekin Duck infected by DHBV or the Woodchuck infected by WHB. The best available model to allow infection with the human virus is the chimpanzee model. However, the use of those animals is highly restricted (Dandri and Petersen, 2017).

- **Transgenic mice:** to study HBV pathogenesis, several mice models expressing viral components or replicating HBV to a certain degree, have been created. However, a lot of bias might come from studying these models which, nevertheless, provide good insights into HBV pathogenesis (Iannacone and Guidotti, 2015).
- **Infectable mice:** Firstly, liver humanized mice models present the advantage of using human hepatocytes in their 3D environment and natural infection (Kremsdorf and Strick-Marchand, 2017). However, even though new models allowed the study of immune responses in double humanized models, humanized mice are difficult to handle and often die before the end of experiments. Secondly, AAV-HBV infected mice are easy to handle and allow the study of all HBV viral steps with the exception of viral entry. Indeed, cccDNA can be formed and detected in this model (Lucifora et al., 2017). This mouse model presents a functional immune system.
- **Infectable macaques:** Macaques cannot be naturally infected by HBV as they do not possess a NTCP sufficiently similar to the human one. However, a recent study has shown that expressing the hNTCP, through an AAV-NTCP, enables the macaques to be infected by the human HBV. Macaques may be less easy to handle than mice, but they have the advantage of bearing an immune response closer to what is observed in the human (Burwitz et al., 2017). Altogether, the macaque is a promising model for the *in vivo* study of HBV infection course (Protzer, 2017).

## 3. Interaction between hepatitis B virus and innate immunity

### 3.1. Natural history of HBV disease

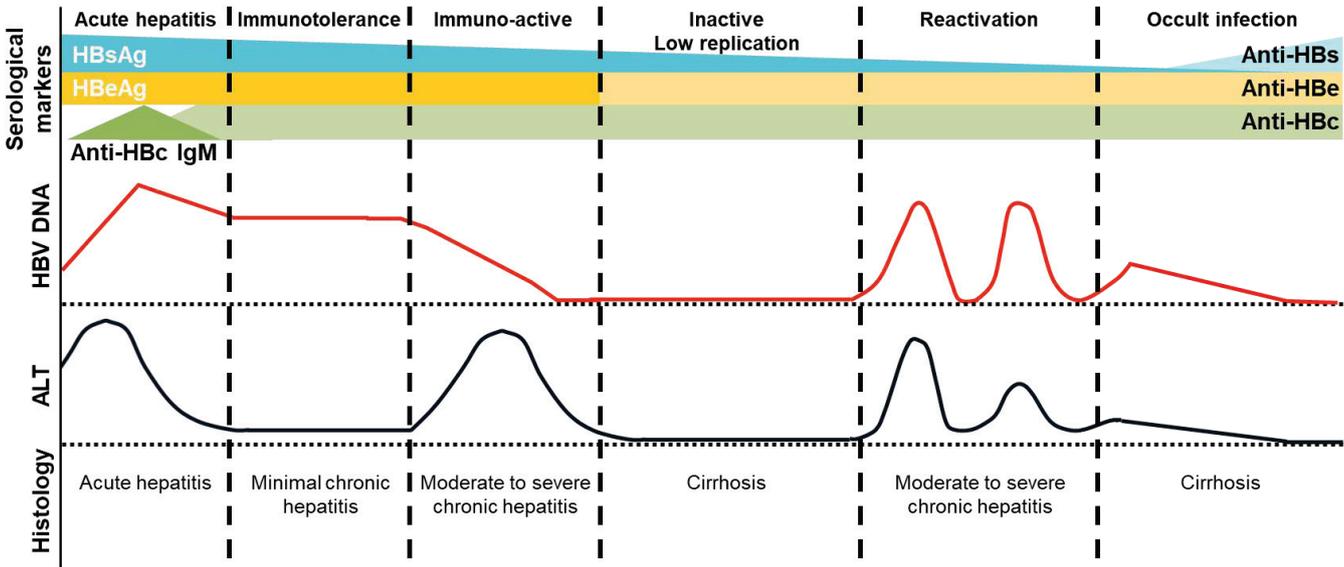
After exposure to HBV, the incubation period ranges from 1 to 6 months, before an acute hepatitis can be evidenced.

**Acute hepatitis B** (lasting 1-2 weeks) is asymptomatic in 60-80% of cases. Fulminant hepatitis (acute liver failure) occurs in less than 1% of cases. After acute infection, infected infants present a risk of 90% of developing a chronic infection whereas adults present a risk of 5% to progress to chronicity (Lai and Yuen, 2007).

**Chronic hepatitis B** (CHB) is defined by the persistence of HBsAg for more than 6 months. Chronic infection can be divided in 5 phases of variable duration (**Figure 19**) (Locarnini et al., 2015):

- **“Immune tolerant”** phase is characterized by high viral replication (HBeAg positivity and high viral load) in the absence of liver damage (normal alanine aminotransferase (ALT) levels, minimal histological activity). This concept of immune tolerance has been challenged through evidences suggesting that some immune mediated liver damage could occur during this phase (Bertoletti and Kennedy, 2015; Bertoletti et al., 2017). Moreover, it was recently shown that viral integration and hepatocyte clonal expansion could also occur during this phase, thus suggesting that molecular events which are later associated with the development of HCC could occur during this early phase of infection (Mason et al., 2016). Pathology of this phase is difficult to study since in the absence of liver damage, liver biopsies are rarely performed;
- **“Immune active”** phase is associated with the occurrence of an anti-HBV immune response. This phase is characterized by a drop in viral load and an increase in ALT levels. During this phase HBe seroconversion can occur;

**Figure 19. Natural course history of hepatitis B infection (adapted from Deny and Zoulim, 2010).** Evolution of serologic, hepatic and viral markers throughout the course of the disease.



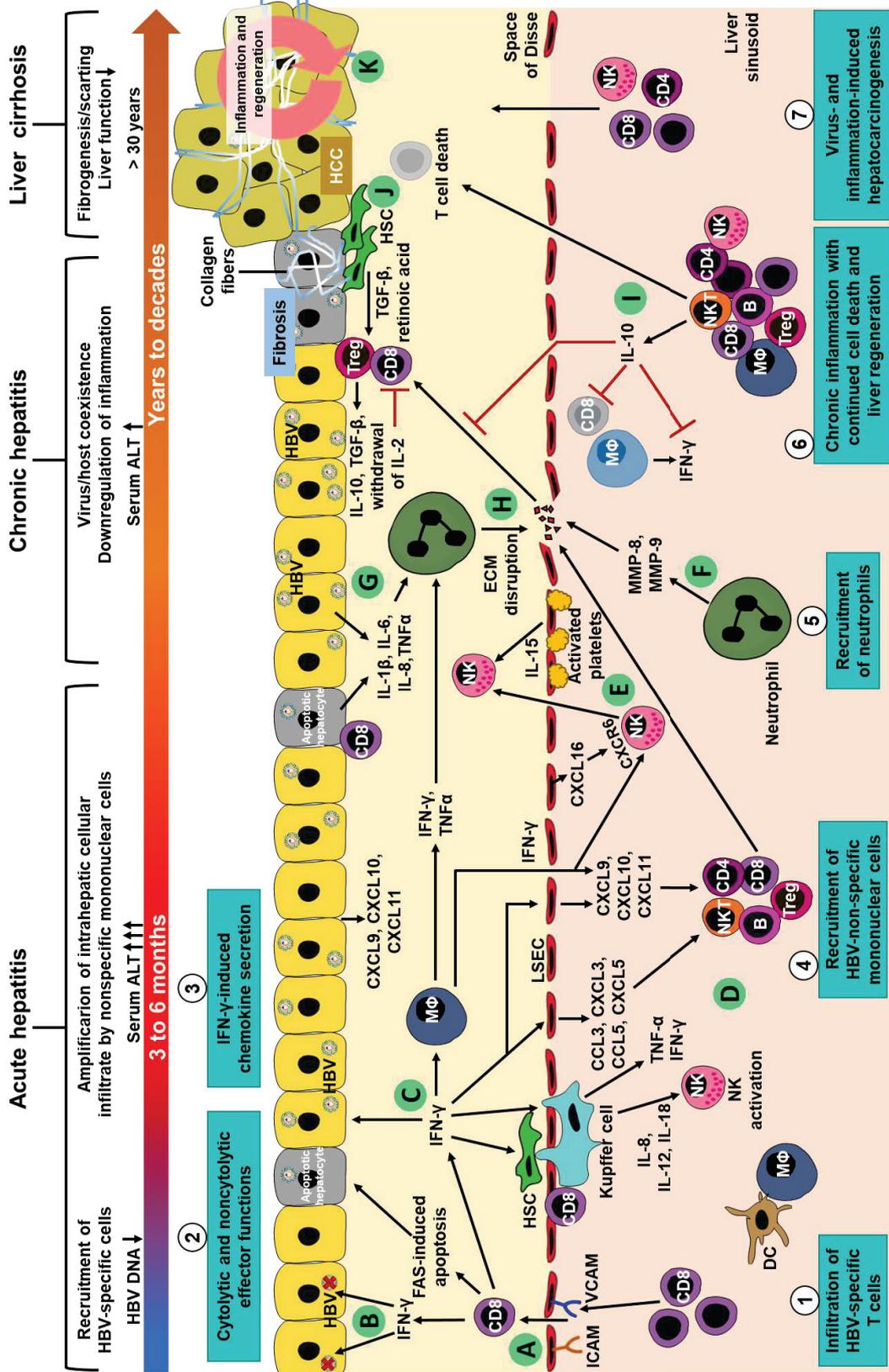
- **“Inactive carrier”** phase is characterized by a low viral replication following HBeAg to HBe antibody seroconversion. Viral load is barely detectable or undetectable (below 2 000 UI/mL), and serum aminotransferases levels are normal. HBsAg loss and seroconversion to anti-HBs antibody may occur spontaneously in 1-3% of cases. Nevertheless, viral infection is not eliminated and disease progression and its complications may still occur;
- **“Reactivation”** phase is characterized by fluctuating serum HBV DNA and aminotransferase level increases and, active hepatitis. HBeAg remains undetectable, due to a selection of pre-core and basal core promoters mutants;
- **“Occult infection”** phase is characterised by the presence of HBV DNA within the liver (with or without detection in the serum) in the absence detectable of HBsAg (Kwak and Kim, 2014). Occult HBV is a result of a low HBV replicative activity, that may be associated with cccDNA epigenetic silencing (Levrero et al., 2009).

Untreated CHB patients will progress to develop fibrosis, cirrhosis and HCC, at variable rates, depending on disease activity. In the inactive carrier phase, progression is slow (less than 1% per year) whereas progression from the immune reactive and HBeAg negative hepatitis phase to cirrhosis can be as high as 2-10% per year. Globally, in CHB patients a cumulative risk of cirrhosis development ranges from 8 to 20%. Cirrhotic patients present an increased risk of liver decompensation, HCC and death (Locarnini et al., 2015). In cirrhotic patients, the main risks factors for progression towards HCC are HBeAg positivity (3.6 times higher incidence than HBeAg negative patients) and HBV DNA levels. Overall, the outcome of CHB is determined by both viral (HBV DNA levels, genotype, and particular mutation patterns) and host factors (age, gender, genetic background, and immune status) (Liang, 2009).

### 3.2. HBV immune induced pathogenesis

The association between HBV infection and HCC risk is well established (Blumberg et al., 1975). In comparison to healthy controls, HBV infected patients were estimated to have a 233 risk increase to develop HCC (Beasley et al., 1981), leading to the classification of HBV as an

**Figure 20. Pathogenesis of HBV-related liver disease (adapted from Rehermann 2013).**  
Simplified schematic presentation of key factors that contribute to the pathogenesis of acute and chronic liver disease (see text for full details).



**oncogenic virus.** Worldwide 50% of HCC are due to previous or on-going infection by HBV (El-Serag, 2012).

Development of HCC is a long and multifactorial process, in which, both immune response (associated with hepatocytes death and regeneration) and viral components are implicated. Currently known viral factors of HCC development includes: i) the transactivator role of HBx, and its involvement on the epigenetic regulation of cell transcription; ii) the induction of oxidative stress in the infected cells, associated with the accumulation of envelope proteins (PreS2 mutations, that lead to the synthesis of a truncated M-HBsAg); and iii) the random integration of HBV DNA in the host genome, that was shown to happen in transcriptionally active regions, in the proximity of cells' genes (El-Serag, 2012; Fallot et al., 2012). In the majority of patients, HCC develops after decades of infection and is associated with liver fibrosis, revealing the importance of **liver inflammation and regeneration** in the pathogenic process. Indeed, during acute hepatitis, HBV-specific T cells will infiltrate the liver and released cytolytic (FAS-induced apoptosis) and non-cytolytic (IFN- $\gamma$ ) effectors leading to HBV DNA decreased within the liver (**Figure 20.A**) (Musch et al., 1998). IFN- $\gamma$ , has a direct anti-viral effect on hepatitis B replication within hepatocytes (**Figure 20.B**) (Brunelle et al., 2010), but also induced the activation of several liver resident cells (HSC, LSEC and KC) and already infiltrating cells (macrophages) leading to the secretion of numerous chemokines (CCL3, CXCL3, CCL5, ...), cytokines (TNF- $\alpha$ , IL-8, IL12, ...) and IFN- $\gamma$  (**Figure 20.C**). Chemokines further induce the recruitment and activation of other circulating mononuclear cells (NK, macrophages, T CD4<sup>+</sup>, T CD8<sup>+</sup>, Treg, and B lymphocytes...), a response which may not be specific to HBV (**Figure 20.D**) (Rehermann, 2013). This infiltration and immune response is associated with serum ALT increased. At this point, activated platelets are found in liver fenestration, leading to the activation of NK (**Figure 20.E**). Liver inflammation is further reinforced by neutrophils recruitment within the liver, which, through matrix metalloproteinase (MMP) 8 and 9 secretion, induced damage in the LSEC wall (**Figure 20.F**) (Sitia et al., 2004). Neutrophils are activated by pro-inflammatory secretion (IL-1 $\beta$ , IL-6, IL-8, and TNF- $\alpha$ ) in the liver micro-environment (**Figure 20.G**). Activated neutrophils disrupt the extracellular matrix, allowing further infiltration of immune cells (**Figure 20.H**). During chronic hepatitis, inflammation continues the death of hepatocytes and liver regeneration associated with IL-10 secretion and immune control (**Figure 20.I**). This process can last from years to decades, and is associated with the development of fibrosis through HSC activation (**Figure**

**20.J)** (Tsuchida and Friedman, 2017). With time, fibrogenesis and scarring will lead to the down-regulation of liver function and to the development of HCC, associated with T cell death (**Figure 20.K**).

Most of these mechanisms have been learnt from transgenic mice that express HBsAg or replicate complete HBV genome in their hepatocytes and are intravenously injected with HBs-specific CD8 T cells (Rehermann, 2013). However, it has been shown, in chronic HBV infected patients, that nonspecific infiltrating mononuclear cell but not the HBV-specific T cell count was correlated with liver pathogenicity (Maini et al., 2000). So, **recruitment of HBV-nonspecific mononuclear cells** seems to be a key factor to HBV pathogenesis. To note, nucleoside analogues that completely suppress viral load but do not modify the amount of intrahepatic cccDNA, do not abrogate the risk of HCC development.

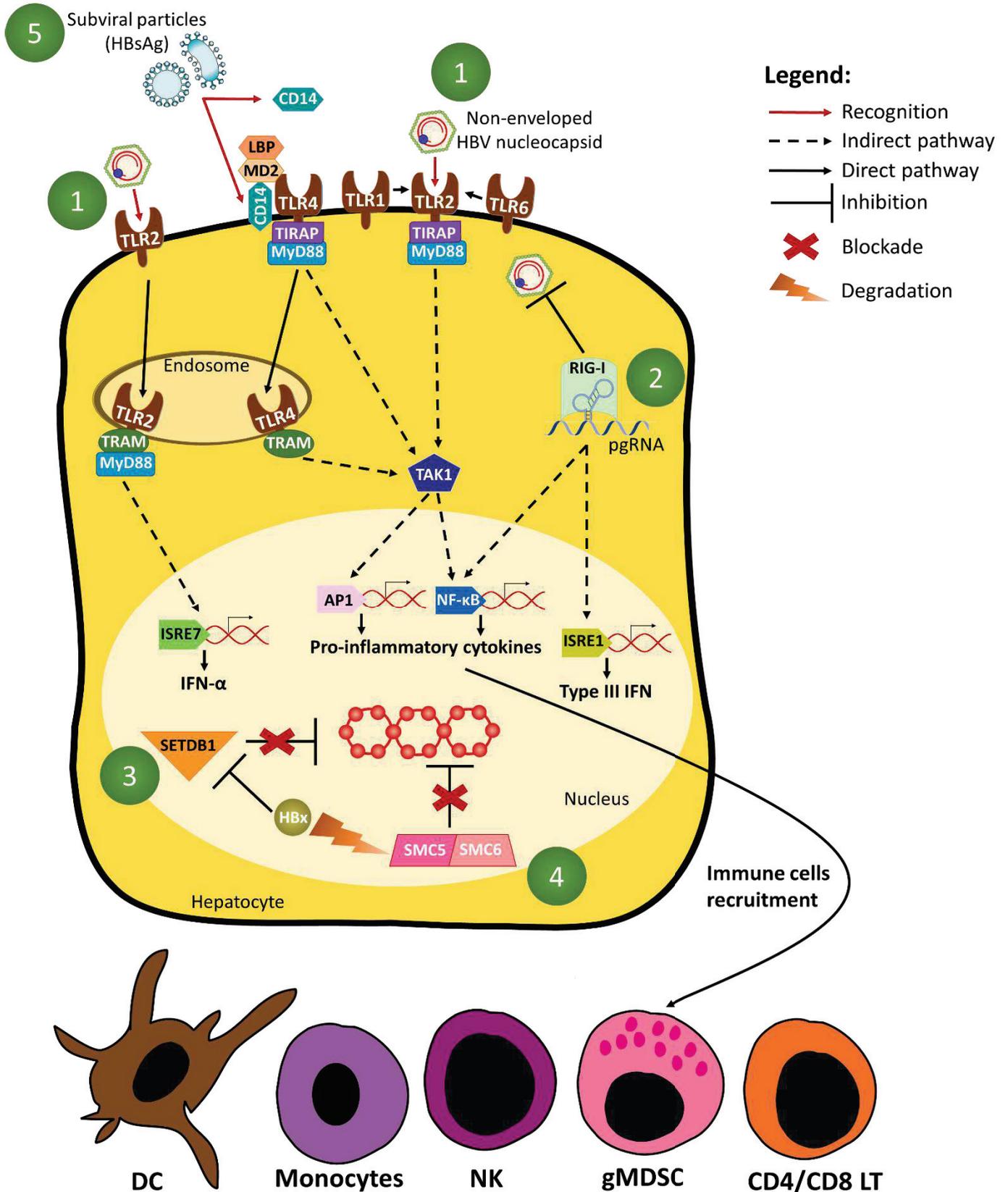
### 3.3. HBV Recognition by Innate Sensors

Whether HBV is genuinely detected by PRR upon very short exposures (i.e., contact between viral particles/incoming materials and PRR), or after the amplification of its genome and expression of its proteins during its replicative life-cycle in hepatocytes, is a matter of debate. One difficulty comes from the fact that *in vitro* models used to study HBV replication are suboptimal. Indeed, to obtain a strong and productive infection in primary human hepatocytes, 100 to 1000 virus-genome-equivalents/cell of recombinant HBV virions are needed (Gripon et al., 2002), whereas, as a basis for comparison, a multiplicity of infection of <0.1 colony-forming-unit/cell is only needed to infect hepatoma cells with HCV. Moreover, for an unknown reason, HBV is not capable of spreading/diffusing into a monolayer of hepatocytes *in vitro*, thus indicating that there are impairments in virion egress or/and re-entry, which prevents the spreading, and consequently, the possibility of an increased exposure to PRR. Another problem relies on the fact that the “quality” of HBV inoculum used for *in vitro* experiments needs to be optimal to generate genuine results. Most researchers use HBV produced by HepG2.2.15 (Sells et al., 1987) or HepAD38 cells (Ladner et al., 1997a), in order to efficiently infect PHH (Gripon et al., 1988), dHepaRG (Gripon et al., 2002), or NTCP-expressing hepatoma cells (Li and Urban, 2016). Various levels of non-enveloped nucleocapsids may contaminate these inocula (supplementary material in (Luangsay et al., 2015b)) and be at the

origin of artefactual results. Moreover, an HBcAg synthesized in bacteria may be contaminated with lipopolysaccharide (LPS)-like ligands, thus further amplifying the problem (Vanlandschoot et al., 2007). Nevertheless, it has been suggested that the HBV nucleocapsid is a ligand for TLR2 (Cooper et al., 2005) and that TLR2-engagement by cognate ligand leads to the strong production of pro-inflammatory cytokines by hepatocytes and/or other TLR2-positive cells (**Figure 21.1**) (Cooper et al., 2005; Huang et al., 2015; Luangsay et al., 2015b, 2015a; Yoneda et al., 2016). However, the existence of this non-enveloped HBcAg ligand *in vivo* is still a matter of debate. The use of HBV virions purified from a patient would guaranty the relevance of experiments; however, so far, there is no clear demonstration that they can be used to reproducibly and efficiently infect hepatocytes *ex vivo/in vitro*. In contrast to other viral entities, HBV is a **poor inducer of innate immunity** because of the intrinsic characteristics of its life cycle (Knipe and Howley, 2013). The viral rcDNA, as well as single-stranded DNA (ssDNA) or linear double-stranded DNA (dsDNA; intermediates/side products of reverse transcription), are likely recognized as damaged, non-methylated, and non-self DNAs. In contrast, the other viral nucleic acids, including cccDNA and various viral RNAs, are “host-like” molecules, and are therefore non-differentiable from “self” (**Figure 21**). Being synthesized within nucleocapsids, rcDNA is protected from a potential recognition by DNA sensors. One possible HBV PAMP could be the ternary complex formed by the pgRNA, HBc and polymerase proteins (Knipe and Howley, 2013). In this respect, RIG-I was proposed as a sensor of the “Epsilon ( $\epsilon$ ) stem-loop” within pgRNA, leading to both a measurable production of type-III IFN in PHH, and a “sequestration of pgRNA” associated with an effector-independent antiviral decrease in rcDNA synthesis (i.e., intrinsic-restriction-like mechanism) (**Figure 21.2**) (Sato et al., 2015).

If cccDNA ends up being a minichromosome-like structure, likely indistinguishable from self-DNA, it appears that its initial establishment from rcDNA, as well as its early transcription after the onset of infection, are modulated by host proteins, which could be defined as “**intrinsic restriction factors**” (**Figure 21.3**). Hence, it was shown that both the histone-methyltransferase SETDB1 (Rivière et al., 2015) and the complex SMC5/6 (Decorsière et al., 2016) are capable of impairing cccDNA transcription early-on after the onset of infection, and that HBx is needed to either counteract the action of the former (Rivière et al., 2015) or induce the

**Figure 21. Hepatitis B virus (HBV) detection by innate immune sensors and regulation by host factors.** Details explanations are present in the text according to the indicated numbers.



degradation of the latter in a DDB1-Cul4-E3-ubiquitin-ligase-dependent manner (**Figure 21.4**) (Decorsière et al., 2016).

Besides the potential detection of the different HBV nucleic acids, it has also been reported that subviral particles could interact with CD14, a co-receptor of TLR4 in myeloid cells, via their phospholipid moiety (Vanlandschoot et al., 2002); this could lead to the internalization of HBsAg into these cells and their activation (Gehring et al., 2013). In fact, the soluble fraction of CD14, bound to HBsAg, could be responsible for this activation (**Figure 21.5**) (van Montfoort et al., 2016). The fact that the lipid moiety of HBsAg subviral particles is instrumental for this activation represents an interesting finding. Indeed, the lipidomic of viral particles and its evolution during the natural course of infection in a human is completely unknown; potential changes in lipid composition, with the incorporation of “toxic lipid” (e.g., peroxydated lipid), could be associated with changes in the immune characteristics of HBV subviral particles (**Figure 21**).

To summarize, the molecular determinants of a potential recognition of HBV PAMPs by PRRs are still poorly defined and further studies are necessary. This understanding is instrumental in the context of the development of PRR agonists, which could be used in immune-therapeutic combinations.

### 3.4. Mechanisms of HBV-Driven Inhibition of Innate-Signalling Pathways

For HBV, it has been clearly shown that adaptive responses are needed for an efficient and persistent control of infection (Bertoletti and Ferrari, 2016; Park and Rehermann, 2014). However, the role of innate immunity has often been overlooked, as HBV infection is usually diagnosed several weeks after the onset of infection, when the virus has already escaped from early immune responses and viremia is high (Bertoletti and Ferrari, 2012; Maini and Gehring, 2016). For this reason, the role of innate immune cells and their effectors, in HBV persistence and associated-pathogenesis, has yet to be actively investigated.

Previous studies have shown that an acute exposure to/infection by HBV, *in vitro*, in an animal model and in humans, does not lead to a strong activation of IFN and pro-inflammatory responses. The first demonstration came from a microarray study performed with the biopsies of experimentally-infected chimpanzees, showing that, in comparison with HCV, the exponential increase in HBV viremia was not associated with a significant/measurable gene expression change in their liver, including innate immunity genes (Wieland et al., 2004a). In agreement with this analysis, no (or a weak) production of IFNs and cytokines/chemokines was found in the serum of acutely HBV-infected patients (i.e., patients who consulted before clearing acute infection or progressing to chronic stage), as compared to what could be seen with similar patients infected by HIV or HCV (Stacey et al., 2009a). A production of the immunosuppressive cytokine IL-10 was, however, evidenced during the viral load increase and was associated with a transient inhibition of NK functions (**Figure 22.1**) (Dunn et al., 2009). This increased production of IL-10 correlates with virus replication, and was also detected during virus-induced flares occurring in long-term chronically infected patients (Das et al., 2012). This points toward a virus-mediated manipulation of cytokines/chemokines production in favour of **immune suppression** (**Figure 22**).

When studied *ex vivo/in vitro*, the acute exposure of hepatocytes or other liver cell types to HBV is also not globally associated with a strong induction on IFNs and pro-inflammatory cytokines/chemokines. Differentiated HepaRG and PHH infected by “qualitatively relevant” recombinant HBV inoculum did not secrete a measurable amount of IL-6 or IFN- $\beta$ , despite a very weak and transient activation of gene expression (Luangsay et al., 2015b). Similarly, KCs exposed to the virus only transiently released a very weak amount of IL-6 (Hösel et al., 2009a) or IL-1 $\beta$  (Zannetti et al., 2016), suggesting that HBV could be detected/sensed, but could also rapidly impair nascent responses. This potential lack of measurable events could either be due to: (1) the viral genotype used, as an HBV genotype-C led to a measurable secretion of IFN- $\lambda$  by infected hepatocytes (in contrast to genotype D used in other studies (Sato et al., 2015)); or (2) to the cell cultivation conditions, as micro-patterned PHH on fibroblast feeder cells exposed to HBV showed a significant induction of type-III IFN gene expression (**Figure 22**) (Shlomai et al., 2014).

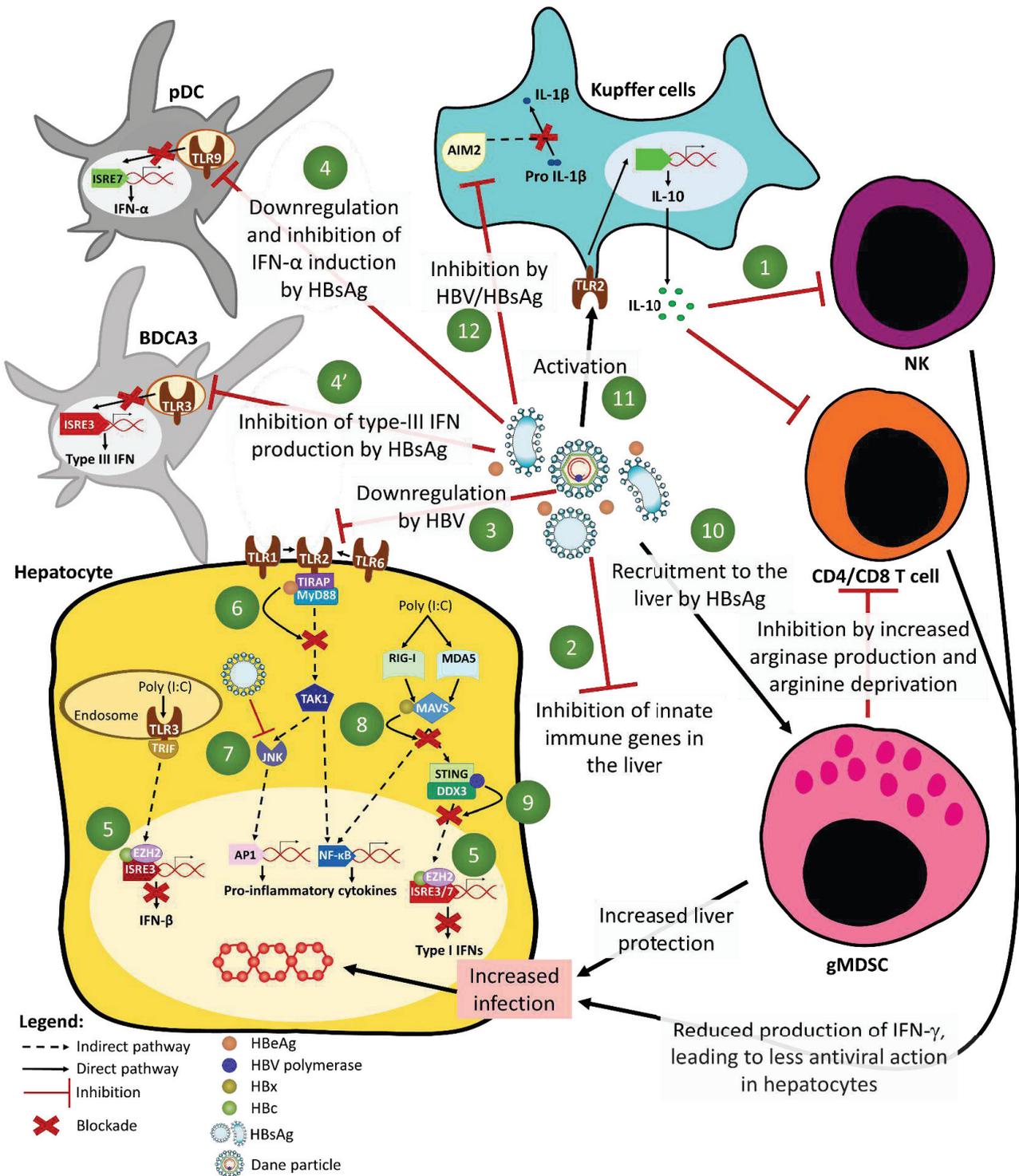
The **weak response** or lack of IFN and/or pro-inflammatory responses, could also be due to viro-implemented active mechanisms. In this respect, the recently performed analyses in

Chronic Hepatitis B (CHB) patient biopsies have shown that many genes of innate immunity were down-regulated when compared to control patients, and the magnitude of this inhibition was dependent upon the HBeAg status and serum levels of HBsAg (**Figure 22.2**) (Lebossé et al., 2017). Even if these results were only obtained at the RNA level, they confirm previous studies that had shown a down regulation, at the protein level, of innate sensors such as TLR2 (**Figure 22.3**) (Visvanathan et al., 2007) or TLR9 (Vincent et al., 2011). These results are in sharp contrast with what has been observed in the woodchuck model, in which an increase of the expression of innate immunity genes was observed (Fletcher et al., 2012). Moreover, in mice with humanized liver, which are immune-deficient, HBV infection led to a very weak increased expression of some innate immune genes, but not a significant decrease (Giersch et al., 2015). This emphasizes that animal models may not always be predictive of what happens in humans (**Figure 22**).

In contrast to HDV (Alfaiate et al., 2016; Giersch et al., 2015), HBV infections seem to be associated with a lack of induction or even a decreased expression of ISGs or other pro-inflammatory genes, thus pointing toward mechanisms of **active inhibition**. The first demonstration that HBV could inhibit PRR signalling pathways came from a study, which showed that both HBeAg and HBsAg could antagonize the antiviral action of PRR agonists in murine parenchymal and non-parenchymal cells (Wu et al., 2009a). Despite its interest, the results of this study were challenged due to the murine nature of the cell types used, as well as because of the apparent lack of specificity of the observed inhibitory phenotype (i.e., all viral forms led to the inhibitory phenotype) and the lack of insights on the underlying mechanism of action.

More recently, we have shown that HBV could be sensed by hepatocytes, leading to an early (i.e., between 2h and 8h post infection) and moderate elevation of the expression of some innate immunity genes (Luangsay et al., 2015b). However, we observed that this response was transient, and that incoming HBV virions were capable, in the absence of neo-synthesized viral proteins, of blocking the induction of IFN genes triggered in trans by cognate ligands of TLR3, RIGI, or Melanoma Differentiation-Associated protein 5 (MDA5). We then found that the core/capsid/HBc protein was responsible for this inhibitory phenotype through the recruitment of a histone-methyl-transferase, i.e., Enhancer of Zeste Homologue2 (EZH2), on targeted gene promoters, which was in turn capable of establishing repressive epigenetic

**Figure 22. HBV modulation of innate immune sensors.** Details explanations are present in the text according to the indicated numbers.



marks to prevent their inducibility (**Figure 22.5**) (Gruffaz et al., 2013). If the capacity of HBc to bind to synthetic dsDNA, the host genome, or cccDNA was already known (Bock et al., 2001; Fernández et al., 2003; Guo et al., 2011), its ability to recruit epigenetic modifying enzymes and modulate gene expression is a novel discovery, and “upgraded” this otherwise structural protein to a potential innate immunity regulator in infected hepatocytes.

Other viral proteins have been suggested to interfere with innate signalling pathways in hepatocytes (Yi et al., 2015). The two which are secreted, HBsAg and HBeAg, are thought to be involved in many immune evasion processes. As previously discussed, they are capable of impairing the activation of all TLR pathways (Jiang et al., 2014a; Wu et al., 2009a). However, regarding their potential role in the inhibition of intrahepatic signalling pathways, experimental data are less clear. It has been proposed that HBe or/and a related intracellular form called p22, could bind to the co-adaptor of Myd88, TIRAP (also called MAL2), and therefore interfere with the TLR2 signalling pathway (**Figure 22.6**) (Lang et al., 2011). More surprisingly, it has also been suggested that HBs could inhibit this pathway by a mechanism involving the inhibition of the c-Jun N-terminal protein Kinase (JNK), which in turn, would prevent IL-12 production (**Figure 22.7**) (Wang et al., 2013). The HBx protein has also been endowed with many biological activities, including the regulation of innate pathways. Hence, it has been proposed that HBx could inhibit the dsRNA-mediated IFN response by either competitively interacting with host-factors of these pathways (e.g., Mitochondrial Anti-Viral-Signalling protein (MAVS), TRIF, IRF3) and/or inducing their degradation by proteasome (**Figure 22.8**) (Hong et al., 2015; Jiang and Tang, 2010; Kumar et al., 2011; Wei et al., 2010a). However, it is worth noting that the over-expression of this protein has often been associated with many irrelevant activities (Slagle et al., 2015). Finally, the HBV Pol has also been described to inhibit the dsRNA-mediated IFN response by interfering with STING and Dead box protein 3 (DDX3) functions (**Figure 22.9**) (Liu et al., 2015; Wang and Ryu, 2010).

To conclude, there is a lot of published data on HBV-driven inhibition of innate-signalling pathways, however there are still many caveats in demonstrations and an overall lack of knowledge of underlying mechanisms. It seems appropriate to indicate that if the HBV-driven strategies to inhibit immune responses are genuine, they are only adapted to what HBV itself would be able to trigger as a weak inducer of innate responses; indeed, it seems that HBV-mediated inhibitory phenotypes are not very potent and are likely not capable of fully

counteracting a more aggressive induction of innate responses, like that mediated, for instance, by Hepatitis delta virus (HDV) (Alfaiate et al., 2016).

### 3.5. Modulation of Innate Cell Number/Frequency by HBV

One way for HBV to initially and persistently escape innate immune cell antiviral functions would be to modulate their numbers, ideally within the infected liver. There are no robust and convincing data showing that the number of CD14<sup>+</sup> monocytes, resident KCs, pDC (BDCA2<sup>+</sup> cells), mDC-BDCA3<sup>+</sup>, and NK/NKT cells within the infected liver, or in the peripheral blood, is affected by HBV throughout the natural history of infection (van der Aa et al., 2016; Boltjes et al., 2014; Gehring et al., 2013; de Groen et al., 2017; Martinet et al., 2012a; Tjwa et al., 2016).

Therefore, HBV infections may instead be associated with the **impairment of functions** rather than the number/frequency of cells. There is at least one convincingly described exception to this, which is the number of myeloid-derived suppressive cells in CHB patients (Pallett et al., 2015a). Indeed, it was recently reported that the number of granulocytic subsets of MDSC is increased in the low-inflammatory (immune tolerant) phase of the HBV natural history. Their number is inversely proportional to the level of liver inflammation, thus suggesting that these cells contribute to the protection of the liver, while likely favouring HBV replication. Although the mechanism has not been completely elucidated, HBV is likely to induce their recruitment/expansion in the liver to its own benefit. In the infected organ, MDSC inhibit HBV-specific CD4<sup>+</sup> and CD8<sup>+</sup> T-cell responses via metabolic means. Indeed, MDSC secrete a very high amount of arginase in the liver microenvironment, which in turn, decreases the amount of available arginine, an amino acid crucial for lymphocyte physiology and growth. This leads to their starving and functional inhibition. HBsAg could trigger their recruitment/expansion by acting through the ERK/Interleukin-6/Signal Transducer and Activator of Transcription 3 (ERK/IL-6/STAT3) signalling pathway (**Figure 22.10**) (Fang et al., 2015).

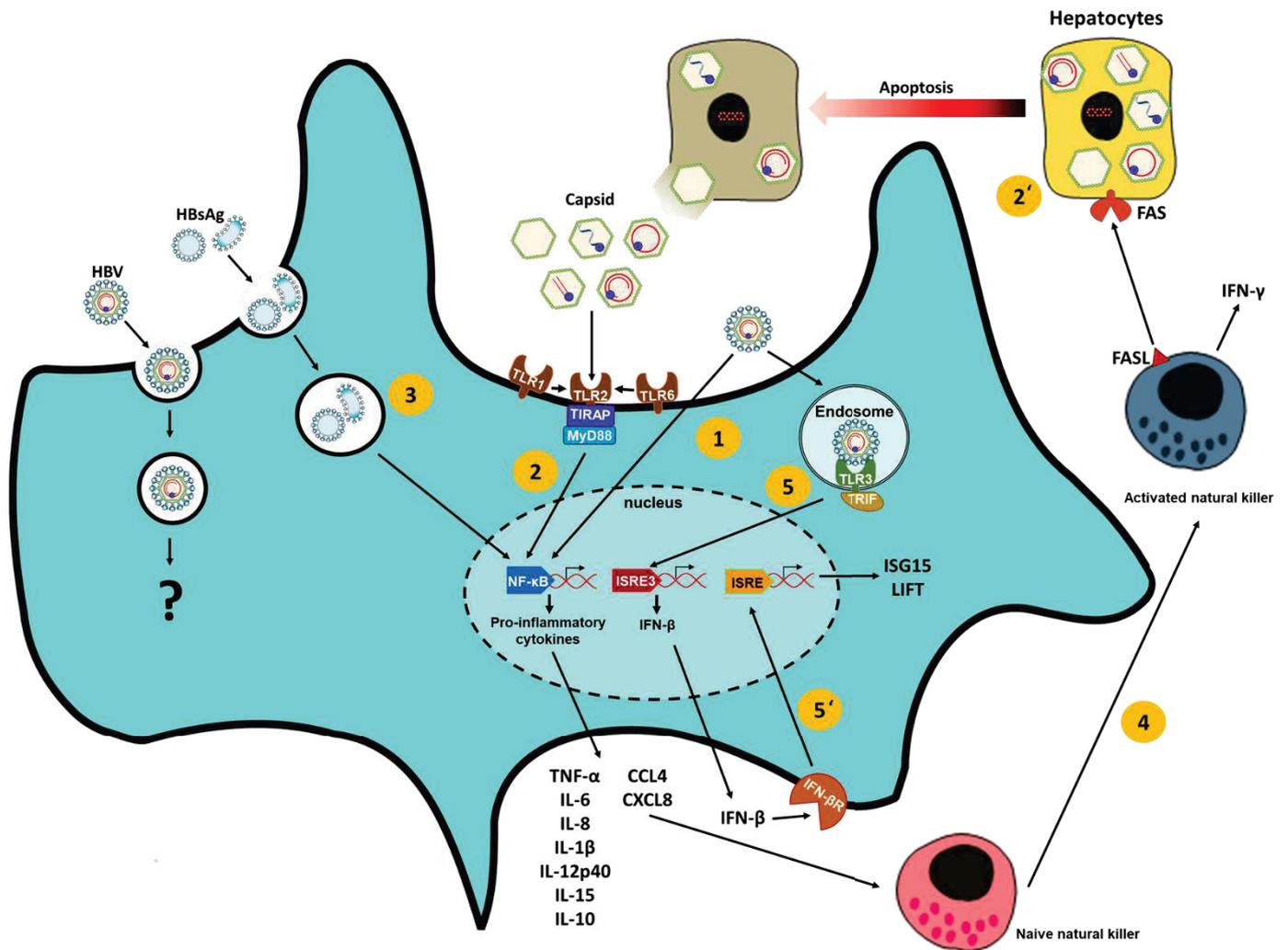
### 3.6. Modulation of Dendritic and NK/NKT Cells Functions by HBV

More than affecting their numbers, HBV seems to impair the functions of many innate immune cells. An investigation on HBV-driven inhibitory phenotypes and underlying mechanisms is not easy in the absence of a good animal model and little access to human liver samples. Moreover, studying liver phenotypes is more relevant than studying peripheral blood ones, as intimate contact/interaction between infected hepatocytes and immune cells can only take place within the liver. Unfortunately, most of the studies performed so far have been with blood-derived cells, thus limiting their meaningfulness and feeding the debate around the “systemic perturbation” of innate functions by HBV.

pDCs are a unique DC subset specialized in IFN- $\alpha$  production, representing 10% of total DC in the liver (**Figure 5**), and are essential antiviral innate cells (Swiecki and Colonna, 2015). Their number in CHB patients seems to be unchanged in both the blood and liver compartments (Martinet et al., 2012a; Woltman et al., 2011). However, it was shown that blood- and liver-derived pDC from CHB patients in an *ex vivo* study were less capable of producing IFN- $\alpha$  upon TLR9 challenges (Martinet et al., 2012a; Vincent et al., 2011; Woltman et al., 2011), less capable of cross-talking and activating the cytolytic activity of naive NK cells (Martinet et al., 2012a), and more prone to inducing the generation of regulatory T-cells (Hong and Gong, 2008). HBsAg was suggested to be the main driver of these modulations, recapitulating these phenotypes *ex vivo* (Xu et al., 2009). It may act by interacting with the regulatory receptor BDCA2 and triggering the downstream inhibitory SYK/MEK-ERK1/2 pathway (Gilliet et al., 2008), or by inducing the down-expression of TLR9 itself by an unknown mechanism (**Figure 22.4**) (Vincent et al., 2011).

BDCA3<sup>+</sup>/Clec9A<sup>+</sup> myeloid DCs (van der Aa et al., 2015), which are responsible for IFN- $\lambda$  production through the activation of TLR3 in particular and representing 10% of total DC in the liver (**Figure 5**), were also recently shown to be functionally impaired in the blood and liver compartment of CHB patients (van der Aa et al., 2016). HBsAg would also be involved in the inhibition of these cells, as the exposition of naive cells to this antigen recapitulates the inhibitory phenotype. Thus, HBV would be capable of reducing the production of type-I and III

**Figure 23. Detection of HBV by macrophages.** Mechanisms schematically presented in the figure are described in the main text.



IFNs, which are two the main classes of antiviral cytokines, by targeting both subsets of DC and therefore inhibiting relevant NK cell activation (**Figure 22.4'**).

NK cells are particularly enriched in the liver and are a major source of IFN- $\gamma$ , a cytokine with direct anti-HBV activity (Isorce et al., 2016; Xia et al., 2016) and immune-stimulatory properties. They likely play a major role in the resolution of acute HBV infection (Dunn et al., 2009; Fiscaro et al., 2009; Guy et al., 2008; Webster et al., 2000) and, not surprisingly, their impaired functionality in CHB patients, in particular regarding their reduced capacity to produce IFN- $\gamma$ , has been shown (Oliviero et al., 2009; Peppia et al., 2010). IL-10 and TGF- $\beta$  present in an HBV-infected liver would be responsible for this inhibition (**Figure 22.1**) (Peppia et al., 2010). Regarding their cytotoxic activities in CHB, data are more in favour of an exacerbation of NK-mediated liver damage (Okazaki et al., 2012; Zhang et al., 2011). Interestingly, these features of preserved NK cell cytotoxicity and impaired IFN- $\gamma$  production are found in both chronic HBV and HCV infections (Rehermann, 2015), thus pointing toward convergent mechanisms in chronic hepatitis. This complexity of the HBV-driven modulation of NK functions has significant implications with respect to therapeutic manipulation.

### 3.7. Interaction between Kupffer cells and HBV

#### 3.7.1. Can macrophages recognized hepatitis B virus?

Liver M $\Phi$  are equipped to recognize pathogens and mount an efficient pro-inflammatory response if needed to prevent, control and delay infections that could become chronic, as described above. A study in the Pekin duck revealed that minutes after inoculation HBV was taken up by KC, highlighting their interaction *in vivo* (Tohidi-Esfahani et al., 2010). As a **DNA virus with RNA intermediates**, HBV could theoretically be recognized by several PRR expressed by KC such as TLR3 or RIGI/MDA5 as well as some cytosolic DNA sensors (**Figure 23**). However, the recognition of HBV nucleic acids may be impaired by (i) the physical separation of encapsidated rcDNA (relaxed circular DNA, a partially double stranded DNA) from cytosolic DNA sensors, and (ii) the resemblance between HBV RNAs and host mRNAs as viral RNAs are transcribed by the host polymerase. Indeed, several studies have shown that in animal models, primary exposition to HBV elicits little to no immune responses (Fletcher et

al., 2012; Wieland et al., 2004a). Even though the authors did not specifically analyse the responses in macrophages, the lack of immune responses in liver biopsies may be also interpreted as an absence of response in macrophages.

In the KC the situation may be different as although they do not support viral replication (i.e. no neo formation of HBV RNAs), viral components could be present after phagocytosis and **viral epitopes recognised** by immune receptors. Indeed, after purification of different subsets of human liver cells, Hösel *et al.* showed that non-parenchymal cells (i.e., all liver cells except hepatocytes) and especially KC were activated after a 3h of exposure to HBV. They observed a transient activation of nuclear factor kappa B (NF- $\kappa$ B) and the secretion of pro-inflammatory cytokines (IL-6, IL-8, TNF- $\alpha$ , and IL-1 $\beta$ ) without any induction of an interferon response (Hösel et al., 2009b) (**Figure 23.1**). These results were recently confirmed by Cheng and colleagues who showed that in monocyte derived macrophages exposed to high quantities of HBV, a transient (between 0.5 to 6h) increase of IL-6, IL-1 $\beta$ , and TNF- $\alpha$  mRNAs was observed (Cheng et al., 2017) (**Figure 23.1**).

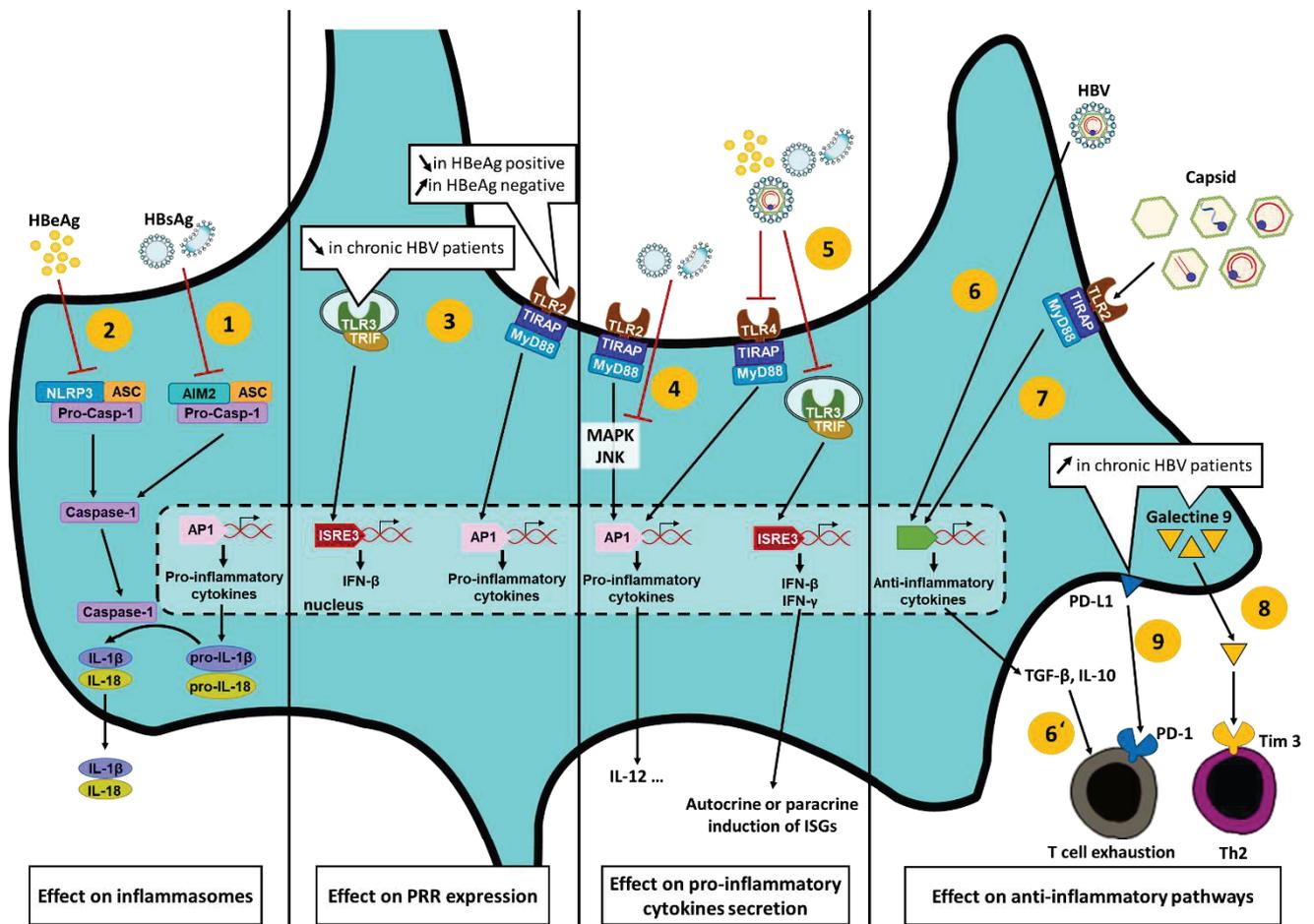
Unexpectedly, **TLR2**, a PRR implicated in the recognition of bacterial components, has been described to recognize HBV capsid/core protein (HBc) (Cooper et al., 2005). Cooper and colleagues found that, in a monocyte cell line, THP1, a full-length HBc was bound to monocyte receptors, leading to their differentiation into M $\Phi$ . Moreover, this binding strongly induced the production of the pro-inflammatory cytokines TNF- $\alpha$ , IL-6, and IL-12p40 (Cooper et al., 2005) (**Figure 23.2**). These inductions were dependent of NF- $\kappa$ B, ERK-1/2, and p38 MAPK activation. The authors demonstrated the necessity of binding between the arginine-rich C terminus of HBc and membrane heparin sulphate for a recognition by monocytes (Cooper et al., 2005). However, the relevance of this model is to be questioned as capsid proteins are normally enveloped in the virion when circulating outside of hepatocytes, which should prevent recognition by TLR2 ligands, and M $\Phi$  activation. Nevertheless, in the case of cell death, it may be possible that naked capsid could be released from the hepatocytes. For example, a study by Arzberger *et al.*, showed that apoptosis of HBV infected hepatocytes led to the release of naked nucleocapsid (Arzberger et al., 2010) which could theoretically induce TLR2-mediated M $\Phi$  activation (**Figure 23.2'**). In addition, a study performed with samples from HBV-positive patients showed that KC stained positive for HBsAg and were more activated than those from control livers (Boltjes et al., 2015). Internalization of HBsAg was also

observed *in vitro* using purified KC or monocyte-derived M $\Phi$  and led to a strong induction of pro-inflammatory cytokines such as IL-6, TNF- $\alpha$ , and IL-15 as well as the anti-inflammatory cytokine IL-10, the chemokines CCL4 and CXCL8 (Boltjes et al., 2015) (**Figure 23.3**) and the subsequent activation of IFN- $\gamma$  production by natural killer (NK) (Boltjes et al., 2015) (**Figure 23.4**). Moreover, a study in HBsAg-transgenic mice showed that KC secretes IL-12 upon TLR9 ligand exposure inducing liver injury caused by NK (Hou et al., 2016). In this model, an increase of Fas ligand (FasL) and Fas proteins were observed respectively on NK and PHH, leading to an induction of a cytotoxic response against PHH (Hou et al., 2016) (**Figure 23.2'**).

Some studies have shown that HBV could also induce an **interferon-dependent immune response**, which should have a direct antiviral effect on HBV infection in hepatocytes, indeed Peg-IFN $\alpha$  is currently used to treat HBV infection (Durantel and Zoulim, 2016). Using a transgenic mouse model allowing high levels of HBV replication, Real *et al.* recently showed that HBV replication lead to a TLR3 dependent interferon response in non-parenchymal liver cells (Real et al., 2016) (**Figure 23.5**). A significant up-regulation of IFN- $\beta$ 1 and subsequently ISG15 and LFIT1 mRNAs was observed in a mouse model in which the small HBsAg is not produced (**Figure 23.5'**). Of note, HBsAg has previously been described to attenuate immune responses facilitating chronic infection (see section on HBV modification of KC phenotype) (Jiang et al., 2014a; Wu et al., 2009a; Zannetti et al., 2016).

In summary (**Figure 23**), contradictory results regarding the potential activation or lack of activation of an immune response in M $\Phi$  by HBV have been reported. These discrepancies might come from the differences in model (chimpanzees, woodchuck, transgenic mice...) and virus (genotypes, purification, naked nucleocapsid...) used in different experimental approaches. The lack of responses in HBV-infected animal models (Fletcher et al., 2012; Wieland et al., 2004a), compared to results demonstrating that macrophages seems to be equipped to recognize HBV (Boltjes et al., 2015; Cheng et al., 2017; Cooper et al., 2005; Hösel et al., 2009b; Hou et al., 2016; Real et al., 2016), raises the possibility of an active HBV-driven evasion mechanism which could contribute to the establishment and persistence of infections *in vivo*.

**Figure 24. Modification of macrophages phenotype by HBV.** Mechanisms schematically presented in the figure are described in the main text.



### 3.7.2. Can HBV modify macrophage phenotype and function?

Several studies have described inhibitory mechanisms used by HBV to block the induction of a potential pro-inflammatory/anti-viral response, including inflammasome inhibition, immune receptor down-regulation, pro-inflammatory secretion inhibition, increase of anti-inflammatory secretion, and negative immune checkpoint induction.

First, HBV has been shown to **interfere with inflammasome activation**. Zannetti *et al.* determined that KC production of IL-1 $\beta$  by AIM2 inflammasome engagement was blocked by HBV, through an HBsAg-dependent mechanism (Zannetti *et al.*, 2016) (**Figure 24.1**). This inhibition seemed to be specific to the AIM2 inflammasome, as HBV was not capable of blocking IL-1 $\beta$  production driven by other inflammasomes, including NLRP3 (Zannetti *et al.*, 2016). However, Yu *et al.* recently showed that HBV could also inhibit NLRP3 induced IL-1 $\beta$  secretion by KC and that this inhibition was HBeAg dependent (Yu *et al.*, 2017) (**Figure 24.2**). This difference in results may come from differences in the HBV inocula used and especially the quantity of HBeAg in the viral preparations. Although mechanisms involved may differ, both studies described an inhibition of IL-1 $\beta$  secretion by KC after exposure to HBV components. HBV has likely evolved several strategies to block the production of IL-1 $\beta$ , which has been recently described as the most efficient cytokine, amongst several tested (including IFNs) against the replication of HBV *in vitro* (Isorce *et al.*, 2016).

In addition, HBV has been shown to **prevent pro-inflammatory cytokine secretion** by influencing PRR expression in M $\Phi$ . A down-regulation of TLR2 and TLR3 was found in peripheral blood mononuclear cells (PBMC) and KC of chronically HBV-infected patients compared to healthy controls (Huang *et al.*, 2013b; Visvanathan *et al.*, 2007) (**Figure 24.3**). As TLR2 and TLR3 have been shown to, respectively, recognize HBc protein (Cooper *et al.*, 2005) and HBV genome replication in non-parenchymal cells (Real *et al.*, 2016), a down-regulation of their expression in hepatocytes, KC and PBMCs may contribute to the lack of an efficient immune response against HBV (Real *et al.*, 2016). However, another study showed a higher secretion of IFN- $\alpha$  and - $\beta$ , as well as IL-6 and IL-10 upon TLR3 stimulation in PBMC from HBV patients compared to healthy controls, contradicting the results of a down-regulation of TLR3 in monocytes (Jiang *et al.*, 2014a). Moreover, in HBV patients, TLR2 expression on KC and peripheral monocytes was reduced in HBeAg-positive chronic HBV patients (i.e. immune-

tolerant/immune active patients) compared to controls individuals, whereas it was significantly increased in HBeAg-negative chronic HBV patients (e.g., in inactive HBV carrier) compared to controls (Visvanathan et al., 2007). This modification of expression was specific to TLR2, as TLR4 expression did not differ between groups. It appears that the effect of HBV on TLR expression may be differentially regulated during the different phases of HBV chronic infection that would partially explain the difference of immune responses observed during those phases.

HBV may also directly target the secretion of **pro-inflammatory cytokines**. For example, in CHB patients, HBsAg induced an inhibition of IL-12 secretion by monocytes/macrophages after TLR2 stimulation through the interference of HBV with the JNK/MAPK pathway (Wang et al., 2013) (**Figure 24.4**). To further decipher what happens in the liver, the team of Pr Schlaak purified murine KC and exposed them to some TLR-ligands in the presence or absence of HBsAg, HBeAg or HBV virions. Upon exposition to any of these viral components, expression of interferon sensitive genes and pro-inflammatory cytokines was inhibited in KC (Jiang et al., 2014a) (**Figure 24.5**). In addition, Wu *et al.* have shown that stimulating KC with TLR3 or TLR4 ligands induced the production of IFN $\beta$  and the inhibition of viral replication in hepatocytes in mice (Wu et al., 2007). However, this antiviral effect was lost when KC were pre-incubated with HBV (Wu et al., 2009a) (**Figure 24.5**). Among the mediators inhibiting HBV infection, IFN- $\alpha$  and IFN- $\gamma$  were identified and shown to directly inhibit HBV replication in patients (Isorce et al., 2015). Moreover, a recent study showed that HBV virions hijack enzymes involved in lipid metabolism to infect hepatocytes (Esser et al., 2018). It could be hypothesised that the same pathways could be also be hijacked by HBV in M $\Phi$  to enter or pass-through these cells to be delivered to hepatocytes.

Aiming at the same goal, other studies have shown that in addition to preventing pro-inflammatory responses, HBV also **increases anti-inflammatory responses**. In purified rat KC exposed to different quantities of HBV virions, no morphological changes were observed on KC, but, compared to non-infected animals, TGF- $\beta$  secretion was increased from 5.38 at day-3 to 7.75 fold at day-7 post-exposure (Li et al., 2012) (**Figure 24.6**). In this study, no modification in the levels of pro-inflammatory cytokines IL-1, IL-6 and TNF- $\alpha$  was observed compared to non-HBV-exposed cells. However, the authors neither indicated the secretion level of those cytokines in both controls and HBV exposed KC nor did they examine if the pro-

inflammatory cytokines could be secreted by KC after TLR stimulation and modified by incubation with HBV (Li et al., 2012). In addition, Jiang *et al.* demonstrated that in non-parenchymal liver cells exposed to HBV, the expression of anti-inflammatory cytokines (IL-10 and TGF $\beta$ ) was enhanced (Jiang et al., 2014a) (**Figure 24.6**). This phenotype was associated with an inhibition of NF- $\kappa$ B, IRF-3 and MAPKs pathways in these cells. Specific T-cell activation by KC was also impaired when cells were exposed to HBsAg, but this phenotype could be reverted by a treatment with IL-10 antibodies, suggesting that the effect of HBV on KC impairment of T cell response was mediated by this cytokine (Jiang et al., 2014a) (**Figure 24.6'**). Furthermore, in an established HBV carrier mouse model, Li *et al.* showed that TLR2 deficiency improved HBV elimination, thus indicating that TLR2 could modulate HBV persistence (Li et al., 2015). Liver M $\Phi$  were shown to have an increased TLR2 expression in HBV-carrier mice and to produce more IL-10 upon TLR2 activation by an HBcAg stimulation leading to a poor induction of CD8<sup>+</sup> T cells (Li et al., 2015; Xu et al., 2014) (**Figure 24.7**). Indeed, IL-10 deficiency or anti-IL-10R treatment resulted in a functional CD8<sup>+</sup> T cell response and HBV elimination in this mice model (Li et al., 2015; Xu et al., 2014). To get further insights into the context of a primary infection, Dunn *et al.* analysed a cohort of 21 patients with acute HBV, including 8 patients analysed before the peak of viremia, when the infection is still being established (Dunn et al., 2009). At the peak of viremia, during the inhibition of lymphocyte activation, a peak of IL-10 was observed in the serum. Moreover, in chronically infected HBV patients an increase of CD68<sup>+</sup> CD86<sup>+</sup> M $\Phi$  was observed, promoting a specific Th2 response, thus promoting of phagocytic-independent inflammation (Said et al., 2016). Lastly, Nebbia *et al.* described that in CHB patients there is an up-regulation of Galectin-9 on KC as well as an up-regulation of HBV specific Tim3<sup>+</sup> CD8 T cell (Nebbia et al., 2012) (**Figure 24.8**). This Tim-3/Galectin-9 pathway up-regulation could be the cause of T cell exhaustion during chronic infection (Nebbia et al., 2012).

Finally, **Programmed Death-1 (PD-1) and its ligands (PD-L1)**, proteins implicated in tolerance (Wang et al., 2016), have been described to be essential to balance antiviral immunity and inflammation in acute HBV patients (Kassel et al., 2009). A study carried-out with 32 chronic HBV patients, showed an up-regulation of PD-1 and PD-L1 in liver biopsies compared to healthy controls (Xie et al., 2009) (**Figure 24.9**). The same results were found in a chronic carrier mouse model (Tian et al., 2016). PD-1/PD-L1 up-regulation correlated with hepatic

inflammation and increased alanine transaminase levels in patients (Xie et al., 2009) and with impaired cytotoxic T lymphocytes response in mice (Tian et al., 2016). However, PD-L1 expression was lower in liver residential antigen presenting cells, including KC (Xie et al., 2009), during the inactive carrier phase. Moreover, in mice, PD-L1 blockade or KC depletion led to an inhibition of HBV viral parameters, as well as an increase of IFN- $\gamma$ <sup>+</sup> CD8<sup>+</sup> cells (Tian et al., 2016). Another study further showed that PD-L1 expression was up-regulated in CD14<sup>+</sup> circulating cells during chronic HBV infection, liver cirrhosis and hepatocellular carcinoma (Huang et al., 2017). PD-1/PD-L1 interaction may therefore play a significant role in balancing liver damage during chronic HBV infection (Xie et al., 2009).

In summary (**Figure 24**), HBV seems to block pro-inflammatory responses in KC and peripheral monocytes/macrophages, correlating with a poor elimination of the virus. On the other hand, HBV strongly activates anti-inflammatory cytokines secretion (IL-10 and TGF- $\beta$ ) as well as inhibitory checkpoint factors (e.g., PD-1/PD-L1). Overall, it seems that HBV concomitantly inhibits a pro-inflammatory/anti-viral M1-like phenotype, and activates an anti-inflammatory/pro-tolerogenic M2-like phenotype.

### 3.7.3. Do macrophages promote the establishment or persistence of the infection?

As the first line of defence against pathogens, KC should counteract infection of hepatocytes by HBV. A study performed in ducks infected with DHBV (duck hepatitis B virus) showed that endotoxin stimulation could inhibit DHBV replication in primary duck hepatocytes (i.e., accumulation of viral proteins and amplification of the nuclear extrachromosomal DHBV DNA templates) (Klöcker et al., 2000). The authors further observed that this inhibition was due to NPC-mediated cytokine secretion, and more probably to KC, which contaminates isolated hepatocytes. Nevertheless, HBV is able to chronically infect the liver, and counteract/escape these immune responses. The role of KC in the establishment and persistence of the infection remains unclear.

Interestingly, in a mouse model the depletion of KC prior to HBV infection prevented the establishment of a chronic infection (Xu et al., 2014) (**Figure 25.1**). This effect was correlated with the secretion of IL-10 by KC, which impaired the humoral response against HBV infected

hepatocytes (Xu et al., 2014). Moreover, Chen *et al.* have shown that the matrix metalloproteinase 9 (MMP-9) is up-regulated in PBMCs of chronic hepatitis B patients and can be activated by HBV in PBMCs and macrophages *in vitro* (Chen et al., 2017). This up-regulation may **favour the infection** as the authors further showed that MMP-9 enhanced HBV replication in hepatocytes through binding to IFNAR1 (IFN $\alpha$  receptor 1), inducing its ubiquitinylation and degradation (Chen et al., 2017) (**Figure 25.2**). IFNAR1 degradation prevents the binding of type I IFN and the associated antiviral effects.

In contrast, some studies have shown that KC may play a role in HBV elimination. Hösel *et al.* showed that KC exposed to HBV secreted more IL-6, inducing the activation of the mitogen-activated protein kinases (MAPK) in PHH, leading to the inhibition of hepatocytes nuclear factor (HNF) 1 $\alpha$  and 4 $\alpha$ , two transcriptional factors essential for HBV replication (Hösel et al., 2009b) (**Figure 25.3**). Another study conducted with HepG2 cells, showed that a treatment with TGF- $\beta$ 1 (cytokine secreted by KC exposed to HBV (Li et al., 2012)) could suppress HBV replication (Hong et al., 2012). TGF- $\beta$ 1 specifically reduced HBV core promoter activity, and, subsequently, viral pre-genomic RNA, core protein, and HBV replication, through repression of HNF 4 $\alpha$  expression (Hong et al., 2012) (**Figure 25.4**).

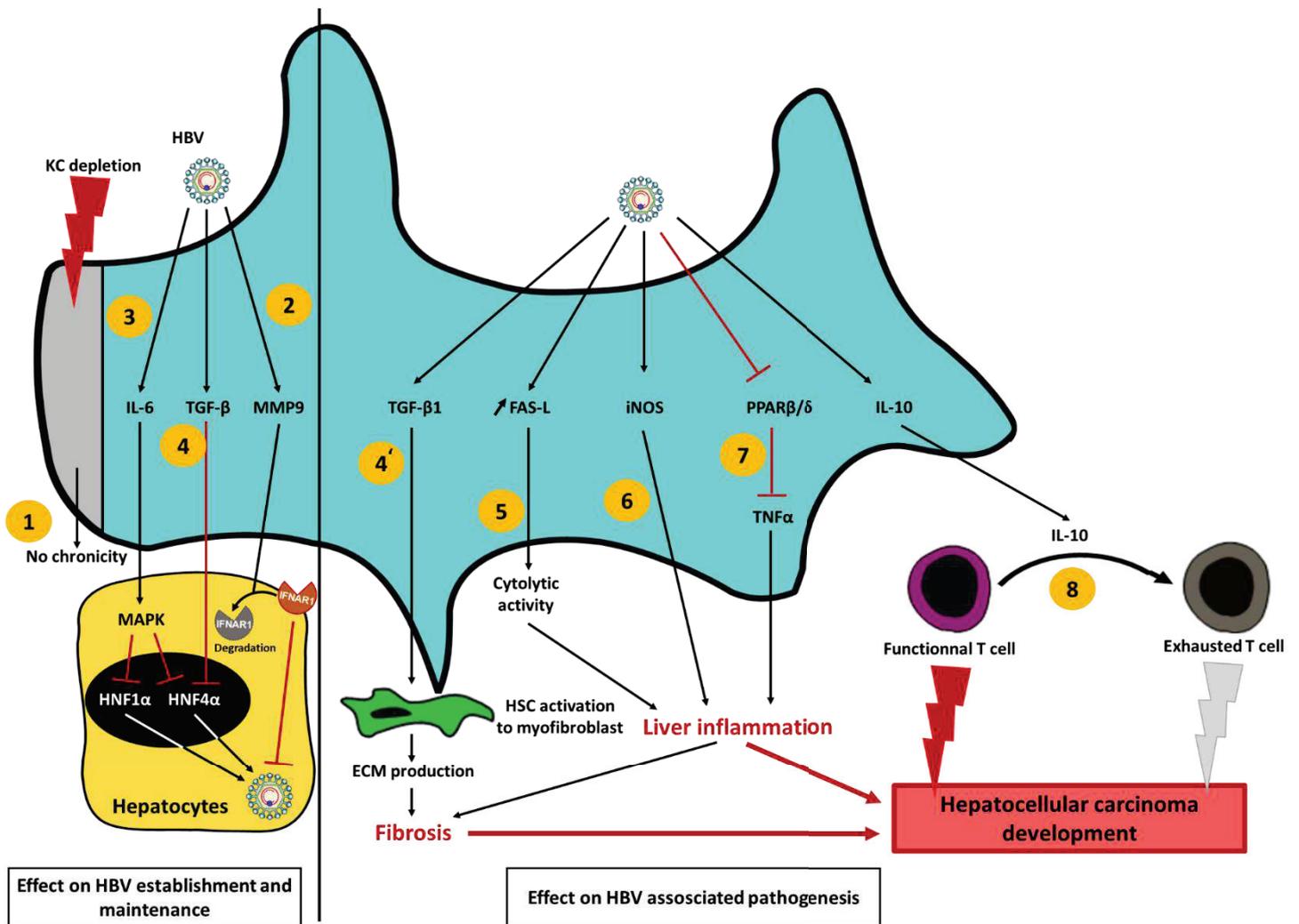
In summary (**Figure 25**), it is still unclear if KC plays a role in the establishment and persistence of the infection and further studies are required. The discrepancies between results may be due to the phenotype (pro- or anti-inflammatory) of KC during primary infection and could be one of the key factors involved in the infection outcome.

#### 3.7.4. Do macrophages play a role in the development of pathologies associated to HBV infection?

KC have been described to play a role in several liver associated pathologies including aberrant responses to treatment (Ju and Pohl, 2005; Seki et al., 2000) and may be associated with HBV-associated liver pathologies including fibrosis, cirrhosis and the progression to hepatocellular carcinoma (Levrero and Zucman-Rossi, 2016).

As described above, Li *et al.* have shown an **increase of TGF- $\beta$ 1** secretion by KC in the presence of HBV (Li et al., 2012). TGF- $\beta$ 1 can directly promote fibrosis by inducing the differentiation of

**Figure 25. Effect of KC in HBV establishment and pathogenesis.** Mechanisms schematically presented in the figure are described in the main text.



hepatic stellate cells into myo-fibroblasts, subsequently stimulating the production of extracellular matrix components (Wynn and Barron, 2010) (**Figure 25.4'**).

In addition to liver fibrosis, KC plays an important role in **liver inflammation/tolerance and injury associated to uncontrolled inflammation**. Different populations of macrophages have recently been described in the liver of chronically infected animal models and patients. A study showed that end-stage HBV liver disease is associated with an accumulation of CD14<sup>+</sup>HLA-DR<sup>hi</sup>CD206<sup>+</sup> macrophages (Tan-Garcia et al., 2017). These cells expressed spontaneously pro-inflammatory markers (TNF- $\alpha$ , GM-CSF, Caspase 1...) and had a low expression of anti-inflammatory markers (arginine 2, PPARG...) with the exception of IL-10 which was increased (Tan-Garcia et al., 2017). This population has been described as resistant to LPS tolerization. Another population of CD205<sup>+</sup> macrophages has been described in the liver of HBV transgenic mice and chronic hepatitis B patients (Yong et al., 2017). This population presented a higher expression of activation markers, pro-inflammatory cytokines, chemokines/chemokine receptors, and phagocytosis related genes (Yong et al., 2017). Moreover, in patients with high ALT levels, a large decrease of CD8<sup>+</sup> T lymphocytes and increase of Fas-L positive cells, especially KC, were shown (Tang et al., 2003). This observation led the authors to the hypothesis that during chronic infection, liver injury could not be the result of CD8<sup>+</sup> T cell response but an increase of Fas-L expression on KC and, through an increased cytolytic activity (Tang et al., 2003) (**Figure 25.5**). Another study performed in liver biopsies from 74 chronically infected patients revealed that iNOS intrahepatic expression was significantly higher in patients with chronic HBV (Wu et al., 2008). In these samples, Kupffer cells stained positive for iNOS, a marker of pro-inflammatory M $\Phi$ , the level of which correlated with ALT levels, and with the grading of liver inflammation and staging of liver fibrosis (Wu et al., 2008) (**Figure 25.6**). Moreover, as previously described, proliferator-activated receptor- $\beta/\delta$  (PPAR $\beta/\delta$ ) activation was shown to inhibit steatosis and inflammation, well-known cancer risks factors (Nagasawa et al., 2006). In a model of HBV transgenic mice, activation of PPAR $\beta/\delta$  reduced steatosis and tumour multiplicity (Balandaram et al., 2016). PPAR $\beta/\delta$  activation was also correlated with reduced *Tnfa* mRNA levels, as well as, reduced ALT level but increased apoptotic signalling (Balandaram et al., 2016) (**Figure 25.7**). Ligand activation of PPAR $\beta/\delta$  inhibited LPS signalling and though *Tnfa* expression, which could be prevented in a model of KC non-expressing-PPAR $\beta/\delta$  (Balandaram et al., 2016). This data suggested a role of KC in liver

inflammation during chronic HBV infection through TNF- $\alpha$  secretion. Finally, tumour progression in chronic HBV infection could be related to the increased of IL-10 secretion described by several teams (Dunn et al., 2009; Li et al., 2015; Xu et al., 2014). Indeed, a tolerogenic environment in the liver could lead to a non-efficient lymphocyte response and consequently, to cancer progression (Trehanpati and Vyas, 2017) (**Figure 25.8**). However, a study by Sitia *et al.* showed that in a chronic HBV carrier mouse model, KC, through scavenging receptors, removed apoptotic PHH, blocking the release of high-mobility group box 1 (HMGB1) and neutrophil infiltration in the liver (Sitia et al., 2011). The authors' hypothesized that this may prevent liver inflammation and damage during chronic infection.

Taken together these data show that HBV may modify KC phenotype in favour of the development of liver-associated pathologies such as fibrosis, particularly through TGF- $\beta$ 1 secretion, and, ultimately, HCC, through aberrant expression of factors implicated in carcinogenesis. Moreover, KC could play an important role in liver inflammation during chronic HBV and prevent a functional humoral response by largely increasing IL-10 secretion by macrophages.

## 4. Treatments

### 4.1. Current available treatments

#### 4.1.1. Vaccination

The first generation of HBV vaccines was obtained by purification of spheres from patient's blood with CHB and was commercialized in 1981 (Lavanchy, 2004). Half a decade later, recombinants vaccines against HBV were created. These second generations of vaccines have an efficacy of 95% against the establishment of HBV infection after immunization with a protection of more than 10 years. After the implementation of an universal immunisation program, in Taiwan, HBsAg prevalence went from 9.8% in 1988 to 1.3% in 1994 in children of less than 15 years old (Chen et al., 1996). Moreover, HBV vaccine is the first to have proven its efficacy in cancer prevention. Another study in the Taiwanese population has shown a

decrease of the annual incidence of HCC in children from 6 to 14 years old during the first vaccination campaign (Chen et al., 1997).

#### 4.1.2. Antiviral drugs

Nowadays, there is no specific therapy for acute hepatitis B treatment, other than current CHB treatments. Seven drugs are available for the treatment of CHB patients: 2 different formulation of IFN- $\alpha$  (conventional or PEGylated) and 6 nucleos(t)ide polymerase inhibitors (lamivudine, telbivudine, adefovir dipivoxil, tenofovir disoproxil fumarate, entecavir, and TAF) each with different efficacies (**Table IV**).

**IFN- $\alpha$**  effects on HBV infection have been known for a long time (Greenberg et al., 1976). It has been accepted for the treatment of CHB patients in the nineties and it is recommended for HBeAg positive patients with low viral load and persistent liver necro-inflammatory activity (high ALT). The mechanisms behind IFN- $\alpha$  effect on HBV are not fully understood yet, even though several mechanisms have been suggested. Among others, several effect of IFN- $\alpha$  on viral replication inhibition have been described (non-exhaustive list):

- Blockade of RNA-containing core particle formation (Wieland et al., 2000);
- Acceleration of decay of replication-competent core particles (Xu et al., 2010);
- Degradation of pgRNA (Li et al., 2010);
- MxA mediated inhibition of nuclear export of HBV mRNA (Gordien et al., 2001);
- Inhibition of HBV transcriptional activity, associated with repression of Enh 1 activity (Rang et al., 1999; Tur-Kaspa et al., 1990; Uprichard et al., 2003);
- Epigenetic regulation of cccDNA transcriptional activity (Belloni et al., 2012; Liu et al., 2013);
- Non-hepatotoxic degradation of cccDNA (Lucifora et al., 2014).

**Table IV. Outcome of commonly used HBV therapies (adapted from Zoulim& Durantel, 2015).**

Patients	Entecavir	Tenofovir	PEG-IFN $\alpha$ -2a
HBeAg positive	n=354	n=176	n=271
HBV DNA undetectable	67%	76%	25%
HBeAg seroconversion	21%	21%	27%
ALT normalization	68%	68%	39%
HBsAg loss	2%	3,20%	2,90%
HBeAg negative	n=325	n=250	n=177
HBV DNA undetectable	90%	93%	63%
ALT normalization	78%	76%	38%
HBsAg loss	0,30%	0%	0,60%

IFN- $\alpha$  treatment has been shown to induce activation of NK and NKT in patients (Micco et al., 2013), as well as the specific induction of CD8<sup>+</sup> T cell (Chen et al., 2010). However, another study has shown that IFN- $\alpha$  treatment in HBeAg negative patients reduced strikingly the number of CD8 T cell (Micco et al., 2013). Authors showed an increase in the total number of NK as well as an increase of activation markers/receptors (i.e. NKp46...), IFN $\gamma$ , and TRAIL. Thus, IFN- $\alpha$  efficacy may be limited due to its depleting effect on CD8<sup>+</sup> T cells.

**DAAs** currently used for CHB patients target the inhibition of HBV polymerase by mimicking natural nucleosides and by incorporating in newly synthesized HBV DNA or RNAs leading to chain termination (Koumbi, 2015). This treatment lead to a very slow (up to decades) decrease in amounts of cccDNA (Werle-Lapostolle et al., 2004). Those molecules have been classified into 3 groups:

- **L-nucleoside** such as Lamivudine (3TC) and telbivudine;
- **Acyclicphosphonate**, including adefovir dipivoxil (ADF) and tenefovir disoproxil fumarate (TDF);
- **Cyclopentanes**, represented by entecavir (ETV).

However, a long life treatment is mandatory to prevent HBV reactivation as interruption of treatment leads to relapses (return of detectable HBV DNA quantity in serum of patients with clinical exacerbation).

### 4.1.3. Investigated drugs in development

Even with an effective vaccines to prevent HBV infection, and several treatment options to inhibit viral replication, one major challenge persists: total viral eradication and the rate of HBsAg seroconversion (called “functional cure”) remains very low in treated patients (Zeisel et al., 2015).

Nowadays, the main focus of HBV drug development research is on the inhibition of cccDNA by i) preventing its formation and ii) promoting its degradation or silencing. The current drugs in development are ([Table V](#)):

- **Entry inhibition:** Myrcludex<sup>®</sup> B can block interaction between HBV envelop and hNTCP, with an IC50 of 80 pM *in vitro* (Schulze et al., 2010). This molecule inhibits the establishment and spreading of HBV particles (Lütgehetmann et al., 2012; Volz et al., 2013). Myrcludex<sup>®</sup> B is currently in phase II trial. Preliminary results show a decrease of HBV viremia at week 24 after initiation of treatment (Bogomolov et al., 2014).  
**cccDNA destabilization:** Some molecules have been identified in pre-clinical studies to reduce the cccDNA pool. First, disubstituted sulforamide compounds inhibit the formation of cccDNA *in vitro* (Cai et al., 2012). Moreover, some enzymes and cytokines have been shown to induce non-cytolytic cccDNA degradation, such as zinc-finger nucleases and the CRISP/CAS9 system, and agonists of the lymphotoxin  $\beta$  receptor (Lucifora et al., 2014; Weber et al., 2014).
- **Transcriptional repression:** cccDNA is subject to epigenetic regulation. It is tempting to use compounds targeting chromatin-modifying enzymes to inhibit cccDNA transcriptional activity. Strategies can include inhibition of histones hypo-acetylation and induction of DNA, and specific histones methylation (Levrero et al., 2009).
- **Encapsidation inhibition:** As discussed in the Hepatitis B chapter, HBV nucleocapsid is essential for viral replication. Indeed, in addition to being mandatory for virion secretion, encapsidation of pgRNA is essential for retro-transcription to rcDNA. Capsids modulators have been explored for more than a decade and 2 classes of molecule have been identified: Heteroaryldihydropyrimidines (HAP), which when used at low doses accelerate the assembly of capsid and formation of aberrant particles, whereas at higher doses HAP increase capsid degradation (Bourne et al., 2008); Phenylpropenamide derivatives, which target pgRNA encapsidation by promoting

**Table V. Current, in development, and possible therapies to treat hepatitis B chronic infection.**

Treatment	Cellular target	Stage of development	Effect	Problems encountered	
<b>Currently used or in development strategies</b>	Peg-IFN- $\alpha$	Currently used	10% of HBsAg loss	Induced NK/NKT killing of HBV specific T cell by overexpression of TRAIL death receptor + Little to no effect on cccDNA amount	
	NUC	Currently used	Loss of HBV DNA production and restoration of HBV specific T cell function	No effect on HBsAg secretion + Little to no effect on cccDNA amount	
	Peg-IFN- $\alpha$ + NUC	-	Long term treatment lead to HBs loss in more cases than IFN $\alpha$ alone	Same as Peg-IFN- $\alpha$ alone	
	Peg-IFN- $\lambda$	Phase II (discontinued)	Same results as Peg-IFN- $\alpha$	Same as Peg-IFN- $\alpha$ alone	
	TLR7 ligands	Liver cells expressing TLR7	Reduction of viral parameters in WHB model	Doses used in human maybe too low to be efficient	
	PD1 blockade	T cell	Prevent T cell inhibition by tumour cells	May need an additional stimulation to activate T cell response	
	Core Assembly modulator	Infected hepatocytes	Reduction of capsid formation and virion excretion	Little to no effect on cccDNA amount	
	Pre-S1 peptide	Infected hepatocytes	Blockade of HBV entry	Little to no effect on cccDNA amount in already infected hepatocytes	
	<b>Feasible therapies</b>	<b>Treatment</b>	<b>Cellular target</b>	<b>Expected effect</b>	<b>Possible problems</b>
		Anti-TRAIL monoclonal antibody	NK/NKT cells	Restoration of a specific HBV T cell response	Uncontrolled immune response
Cytokines (IFN- $\beta$ , IFN- $\gamma$ , IL-6, TNF- $\alpha$ , IL-1 $\beta$ )		Liver cells expressing this cytokines receptors	Greater inhibitory effect and HBsAg loss rate than Peg-IFN- $\alpha$	Cytokine storm	
LT- $\beta$ R agonist		Liver cells expressing LT- $\beta$ R	cccDNA loss	Uncontrolled immune response	
IL-1 $\beta$ restoration		Macrophages	IL-1 $\beta$ production and inhibition of infection	Liver inflammation	
TLR2 agonist		Liver cells expressing TLR2	Activation of IRF and NF- $\kappa$ B pathway and pro-inflammatory cytokine production	Cytokine storm	
NUC + TLR2 agonists		Infected hepatocytes + Liver cells expressing TLR2	Inhibition of viral proteins that could block the response + Activation of NF- $\kappa$ B pathway and pro-inflammatory cytokine production	Cytokine storm	
TLR9		pDC	Induction of endogenous IFN- $\alpha$ production	Cytokine storm	
NUC + TLR7 agonists		Infected hepatocytes + Liver cells expressing TLR7	Inhibition of viral proteins that could block the response + Activation of pro-inflammatory cytokine production	Cytokine storm	

capsid formation without pgRNA inside (no effect on total HBcAg amounts but this treatment affects cccDNA amounts).

- **Viral assembly inhibition:** A new class of molecules has recently been developed: the nucleic acid polymers (NAP). NAPs inhibit HBsAg secretion and viral particle formation, in pre-clinical evaluations (Noordeen et al., 2013a, 2013b). *In vitro*, NAPs were shown to have an dose-dependent inhibition of viral entry whereas they did not elicit any anti-viral effect after infection (Guillot et al., 2017).

## 4.2. Innate Immunity modulation: Therapeutic Insights

### 4.2.1. Can We Improve the Efficacy and Use of IFN- $\alpha$ - Based Therapy?

The past and current clinical use of recombinant IFN- $\alpha$  as an anti-HCV and anti-HBV drug is the best proof of concept that innate immune effectors can be therapeutically valuable, despite their intrinsic low safety profile. The treatment of CHB patients with Peg-IFN- $\alpha$  leads to less than 10% of HBsAg loss (i.e., in a setting of trials; even less in real life), which is currently recognized as a good marker of a functional cure (Konerman and Lok, 2016). The reasons why some patients benefit from such a treatment are unclear. Besides virologic and biochemical reasons (i.e., pre-therapeutic viral load, viral genotype, level of pre-therapeutic inflammation), the genetics of the host is likely involved. It would be great to identify biomarkers associated with sustained virologic response (SVR) off-treatment to prevent the unnecessary exposure of CHB patients to Peg-IFN- $\alpha$ , which is responsible for multiple side effects and is not well-tolerated (**Table V**) (Konerman and Lok, 2016).

Virus-related mechanisms of **resistance to IFN- $\alpha$**  at subcellular levels are currently unknown. It could be that some viral proteins impair the Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway, as shown *in vitro* or in humanized liver mouse models (Christen et al., 2007; Lütgehetmann et al., 2011). Recently, it was shown that IFN- $\alpha$  could induce, by epigenetic remodelling, the silencing of cccDNA (i.e., inhibition of transcription (Belloni et al., 2012)), which may account for the antiviral effect of the drug in humans, and even in some circumstances, to its degradation via an APOBEC3A-dependent mechanism (Lucifora et al., 2014).

If IFN- $\alpha$  administration is meant to boost immunity and help **break tolerance to the virus**, it has nevertheless been shown that a strong exposure to IFN- $\alpha$  in CHB patients was associated with a NK/NKT-mediated increased killing of HBV-specific T-cells over-expressing the TNF-Related Apoptosis-Inducing Ligand (TRAIL) death receptor (Micco et al., 2013; Penna et al., 2012). As the use of nucleoside analogs (NA) in CHB was associated with a restoration of T-cell functions (Boni et al., 2012), there was a rationale for combining Peg-IFN- $\alpha$  and a potent second generation NA (Thimme and Dandri, 2013).

The results of a large open-label active-controlled study aiming at determining the benefit of a combination between Peg-IFN- $\alpha$  and Tenofovir, indeed showed that the long-term combination of these two drugs increases the HBsAg loss rate (Marcellin et al., 2016), thus encouraging further clinical studies in this respect. In particular, it would be interesting to have biomarkers to identify patients that would benefit the most from IFN-based therapies. In the future, it would be interesting to block the TRAIL-dependent killing of HBV-specific T-cells by monoclonal antibodies, as for other check-point inhibition strategies, to further improve the Peg-IFN- $\alpha$ /NA combination by correcting NK/NKT altered functions (Maini and Gehring, 2016).

#### 4.2.2. Is There a Place for Other Innate Immune Stimulators?

Beside IFN- $\alpha$ , the use of the less toxic **IFN- $\lambda$**  was also proposed to combat viral hepatitis. Indeed, the pattern of expression of the IFN- $\lambda$  receptor was more restricted to epithelial cells and fewer immune cells, and this IFN has been shown to induce less side effects, as compared to IFN- $\alpha$ . In the clinical trial, it was recently shown that Peg-IFN- $\lambda$  is as efficient as, but not superior to, Peg-IFN- $\alpha$  for treatment (Chan et al., 2016). If further development for CHB is unlikely, the clinical evaluation of Peg-IFN- $\lambda$  is pursued in the context of co-infection with the hepatitis delta virus.

Recently, a large body of evidence has shown that many **IFNs/cytokines**, including IFN- $\alpha$ , IFN- $\gamma$ , IFN- $\beta$ , IFN- $\lambda$ , IL-6, TNF- $\alpha$ , and IL-1 $\beta$  were capable of inhibiting HBV replication in hepatocytes, in the absence of immune cells (**Table V**) (Isorce et al., 2015, 2016; Lucifora et al., 2014; Xia et al., 2016). These innate effectors are capable of inhibiting the transcription of cccDNA (thought to be through distinct molecular mechanisms; unpublished data), and some

of them are also capable of inducing the **degradation of cccDNA *in vitro***, as initially shown with LT- $\beta$ R agonists, which were particularly potent in this respect (Lucifora et al., 2014). If the development of LT- $\beta$ R agonists (i.e., monoclonal antibodies and genetically engineered agonists) is going to be pursued, the use of injectable recombinant pro-inflammatory cytokines in humans, other than IFNs, is unlikely. A sound approach to take advantage of the direct antiviral characteristics of these molecules would be to allow their endogenous, local, and timely controlled *de novo* synthesis in CHB patients. For this, further fundamental studies are needed to determine, in particular, how HBV is capable of blocking their production and/or antiviral effect *in vivo*. In this respect, research aiming at a better understanding of the mechanism of the blockade of IL-1 $\beta$  production (IL-1 $\beta$  being the most active cytokines tested *in vitro* (Isorce et al., 2016)), by liver macrophages/monocytes is essential.

#### 4.2.3. Development of PRR Agonists for Combinational Therapeutic Approaches

Currently, no PRR agonists have been approved for the treatment of a chronic infection (**Table V**). Some PRR agonists are currently developed as adjuvant for prophylactic or therapeutic vaccinations in the field of infectiology and oncology. In the HBV field, the antiviral properties of TLR agonists have been described more than ten years ago in HBV-transgenic mice (Isogawa et al., 2005). More recently, we reported another comparative screen of the *in vitro* activity of TLR and RLR agonists (Lucifora et al., 2015). In this latest screen, TLR2 and TLR3 ligands were shown to be of particular interest, as compared to TLR7 or TLR9 agonists for instance, as they could not only stimulate immune cells, but also have a direct effect in infected hepatocytes, through the activation of IRFs and/or NF $\kappa$ B pathways. The **TLR2 ligand** Pam3CSK4 was found to be extremely potent (activity in the nM range) when used in primary hepatocytes (Lucifora et al., 2015), as compared to its reported efficacy in hepatoma cell models (Zhang et al., 2012). Interestingly, it was shown in the woodchuck model that a treatment with NA leading to a decrease in Woodchuck hepatitis virus (WHV) viremia and was associated with a re-expression of TLR2 in the liver. Hence, a combination between the TLR2 agonist and NA, or other Direct-Acting-Agents (DAAs) developed against HBV, could be a way forward to circumvent the HBV-induced inhibition of TLR2 expression observed in CHB patients (Durantel and Zoulim, 2012; Visvanathan et al., 2007). An obstacle to the therapeutic development of TLR2 agonist

is the fear of a “cytokine storm” that could occur upon systemic administration. The vectorization of the TLR2 agonist into nanoparticles, which could be specifically delivered to the liver, could help circumvent this problem and would be useful to study.

**TLR9 agonists** have a theoretical interest as they are the only ones capable of inducing the formation of iMATEs in a mouse liver, which are a tertiary immune structure composed of myeloid cells providing a niche for the proliferation and maturation of T-cells (Huang et al., 2013a). Interestingly, the HBV genome contains CpG motifs that have been shown to be either inhibit or activate TLR9 (Vincent et al., 2011), thus suggesting that HBV could naturally modulate the TLR9 pathway. In this respect, it was also shown that TLR9 expression in the Peripheral Blood Mononuclear Cell (PBMC) of infected patients was reduced; the demonstration of this is pending in liver-derived mononuclear cells. This would have consequences on the use of the TLR9 agonist in therapy. However, TLR9 ligands have been successfully encapsulated into nanoparticles and have shown an improved efficacy in boosting prophylactic HBV immunization (Lv et al., 2014). This could be helpful to improve the efficacy of TLR9 agonists *in vivo*, as TLR9 agonists have so far been shown to be less active as compared to those of TLR2 or TLR7 in the woodchuck model (Meng et al., 2016). Other PRR agonists, including those working through cGAS and STING (Dansako et al., 2016; Guo et al., 2015; He et al., 2016), or those working through RIG-I/NOD2 (Korolowicz et al., 2016), are being investigated for their potential anti-HBV properties.

The current clinical development of **TLR7 agonists** for CHB is more opportunistic than based on a demonstrated superiority of action on the virus. Indeed, amongst all PRR agonists, TLR7 ligands, which are small heterocyclic molecules, are the only ones to be deliverable orally, which is a tremendous advantage over other ligands. The best-characterized and advanced TLR7-L agonist is GS-9620. This agonist has demonstrated, in mono-therapy, an extremely potent anti-HBV activity in infected chimpanzees and woodchucks, with a very strong, yet unexpected, effect on the cccDNA level in the latter model (Lanford et al., 2013; Menne et al., 2015). These results have prompted phase-1b/2 clinical evaluations and official results regarding virologic aspects are awaited. One critical point is related to the doses that have been selected for administration; it is possible that they will end-up suboptimal, as safety was the main concern of these trials. Mechanistically, GS-9620 works by inducing the production of IFN- $\alpha$ , mainly by pDC, or related mucosal cells, in the upper gut (Gane et al., 2015; Lawitz

et al., 2015). An interesting path forward will be to test this molecule in association with either NA or other DAA (e.g., core assembly inhibitors) in current development, as well as with therapeutic vaccines. Indeed, PRR agonists are mainly meant to have adjacent activity in the context of combination therapy. In this respect, the excellent results obtained in monotherapy in animal models are more indicative than predictive of what will happen in humans.

#### 4.2.4. Manipulation of the Numbers or Biological Activity of Innate Immune Cells with Impaired Functions in CHB Patients to Help Restore HBV Immune Control

Therapeutic concepts, based on processes such a manipulation, are more complex to implement, as we have to keep in mind that immune responses in CHB patients are a subtle equilibrium between the lack of antiviral activity and excessive pro-inflammatory or unspecific killing activities, which could worsen immune-pathogenesis.

Beside inspecific innate immune activation via immune-stimulators (IFN- $\alpha$  or PRR agonist that was discussed above), one could envisage to specifically restore beneficial or inhibit detrimental **innate immune cell functions** (Table V). Myeloid cells endowed with M2-like functions, similar to those of TAMs, are likely to be involved in the persistence of HBV replication. In this respect, MDSC were clearly shown to be associated with a dampening of HBV-specific and bystander T-cell responses in CHB. The depletion (or re-differentiation into antiviral myeloid cells) of such cells or an inhibition of their functions could help to restore the immune function, particularly in low-inflammatory/immune tolerant patients with high viremia, no ALT elevation and, yet, a relevant number of HBV-specific T-cells (as compared to a later stage of the disease). This concept was exemplified by the use of anti-CSF-R1 antibodies to inhibit TAM function in cancer biology (Ries et al., 2014) and a similar strategy could be implemented by targeting a specific marker of MDSC. On the biochemical side, Tadalafil, a FDA-approved small molecule of the PDE5 inhibitor family, was shown to inhibit the suppressive functions of MDSCs in cancer patients, notably by decreasing arginase expression (Tobin et al., 2017). As for other check-point inhibitors (Ipilimumab, Nivolumab, Pembrolizumab), the repositioning of these drugs to chronic infections is only a matter of time and carefully implemented clinical trials.

If the stimulation of immune cells capable of locally and temporally producing either IFN- $\alpha$  (via pDC), IFNs- $\lambda$  (via BDCA3<sup>+</sup> cells) or IL-1 $\beta$  (via myeloid cells), which have direct anti-HBV properties (Isorce et al., 2015), which is a sound approach theoretically, and could be achieved for instance by PRR agonists, a cell-therapy based on pDC or BDCA3<sup>+</sup> DC loaded with HBV-derived peptides could also be an interesting approach. Indeed, these cells could both produce cytokines and elicit T-cell functions via their APC properties. A proof-of-concept study was recently reported in the context of HBV, by Martinet and colleagues (Martinet et al., 2012b).

## Chapter II: Research project

### 1. Hypothesis and objectives

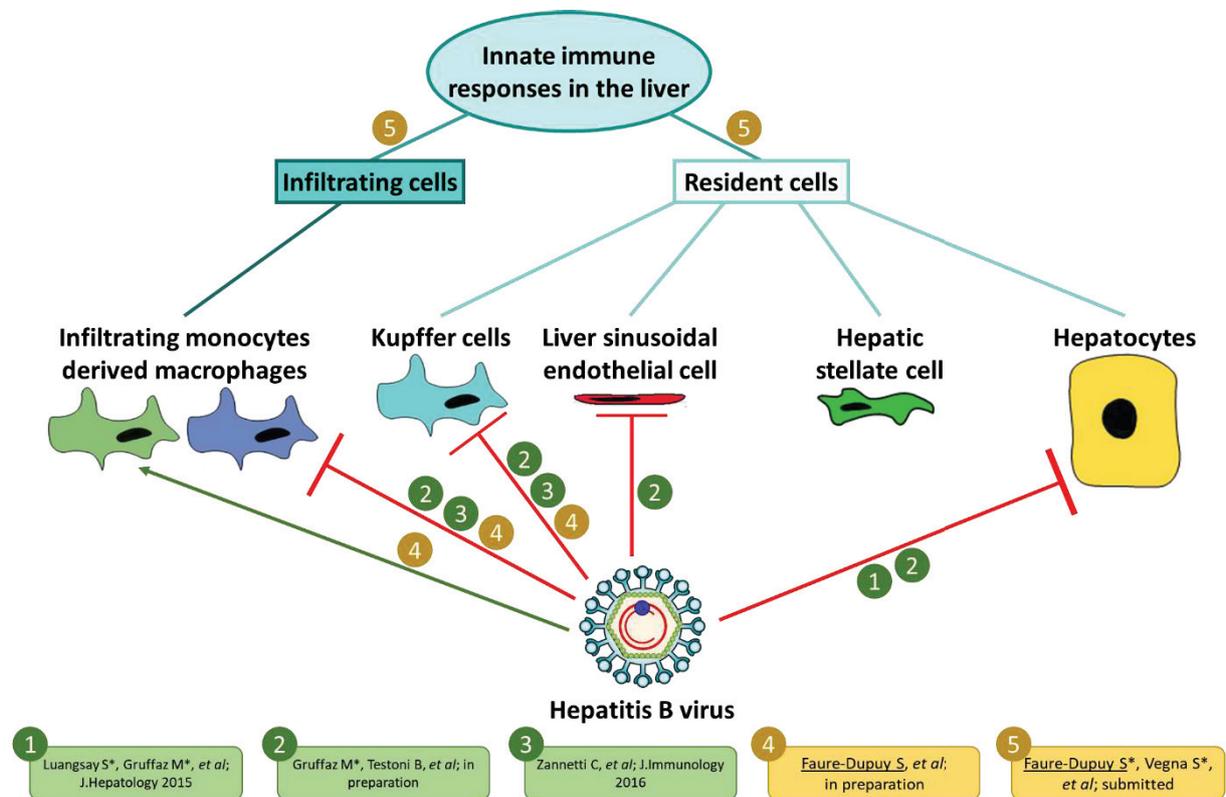
As mentioned in the previous section, despite the existence of an effective prophylactic vaccine, HBV infection remains a major global health issue with more than 250 million infected patients having an increased risk of hepatic complications. Indeed, current available treatments, such as interferon and nucleoside analogues, do not eradicate completely the infection due to the persistence of cccDNA in the nucleus of infected hepatocytes and many infected patients are resistant to such therapies.

Several studies have shown that during the primary HBV infection, no activation of the host's immune response is detected (Fletcher et al., 2012; Suslov et al., 2018; Wieland et al., 2004a). This observation had led to the hypothesis that HBV is a stealth virus (i.e. a virus invisible to the immune system). In contrast, other studies have shown that HBV can be recognized by the innate immune system (Boltjes et al., 2014; Zhang et al., 2012) but can block the induction of this immune response (Christen et al., 2007; Yu et al., 2017) leading to the persistence of the infection.

Thus, the effect of HBV on innate immune responses remains unclear. The debate is further amplified by the models used for the study of the innate immune responses during HBV infection which could be suboptimal. Indeed, *in vitro*, the use of transformed cell lines, in which the innate immune responses is impaired (Luangsay et al., 2015a), could mask HBV's effect on immune responses. Moreover, due to the difficulties associated with working on an animal model which can be infected with the human HBV (i.e. chimpanzees), the majority of the *in vivo* studies on HBV have been performed in animals infected with other hepadnaviruses (WHB, DHBV) or in transgenic mice. Both types of models have different advantages but neither supports a "normal" human HBV infection. In addition, the difference in the inoculum used for the different studies (i.e. quantity of SVPs/HBeAg or the presence of endotoxins or the viral genotype) might have different impacts on the innate immune responses.

The objectives of our team are to study the interaction of HBV with innate immunity and the intrinsic responses of liver cells using relevant models.

I have participated in five studies during my PhD that are presented in this manuscript. The interaction between the different studies is presented in **Figure 26**.



**Figure 26. Summary of the thesis objectives and paper I was involved in.** In yellow, papers where I have been the principal investigator; in green, papers that I was involved in to different extents. \*: authors contributed equally.

The aim of the first study, by Luangsay, Gruffaz, *et al* (published in 2015), was to analyse if HBV infection in hepatocytes could modulate the induction of innate immune responses by PRR agonists.

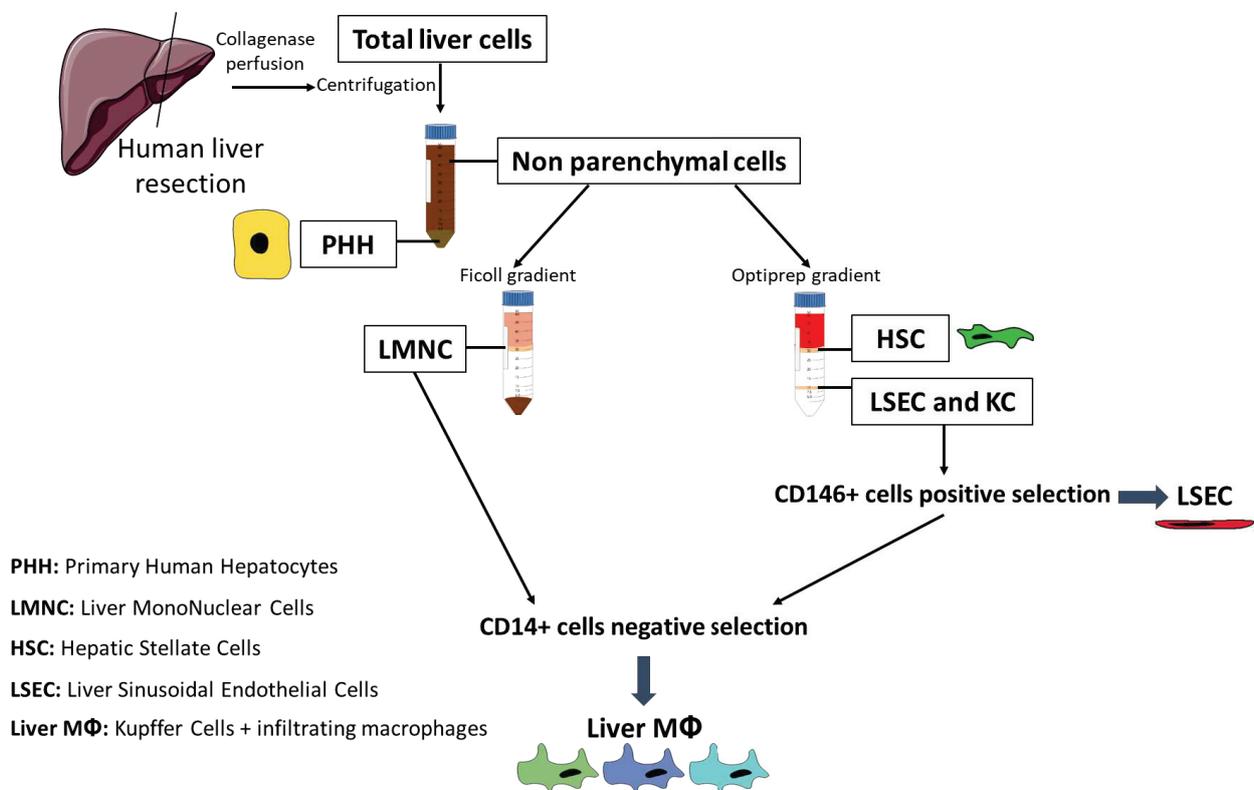
The aim of the second study, by Gruffaz, Testoni, *et al* (in preparation), was to analyse if HBV could modulate the innate immune responses at early times points after the infection (i.e. minutes to hours), and which viral proteins were responsible for this effect.

The aim of the third study, by Zanetti *et al* (published in 2016), was to analyse the functionality of different inflammasomes in hepatocytes and Kupffer cells and the modulation by HBV of the induction of these inflammasomes.

The aim of the fourth study, by Faure-Dupuy *et al* (in preparation), was to analyse the effect of HBV on infiltrating and resident macrophage phenotypes and their activation and how the modulation of this phenotype influenced the establishment of the infection in primary human hepatocytes.

Finally, the aim of the fifth study, by Faure-Dupuy, Vegna, *et al* (submitted), was to perform a non-exhaustive analysis of the expression of PRR, and proteins involved in their signalling pathways in liver resident cells. This work was performed in order to have a better view of which cells could respond to which PRR agonists and, thus, which agonist could be used to develop new experimental strategies to treat HBV chronic infection.

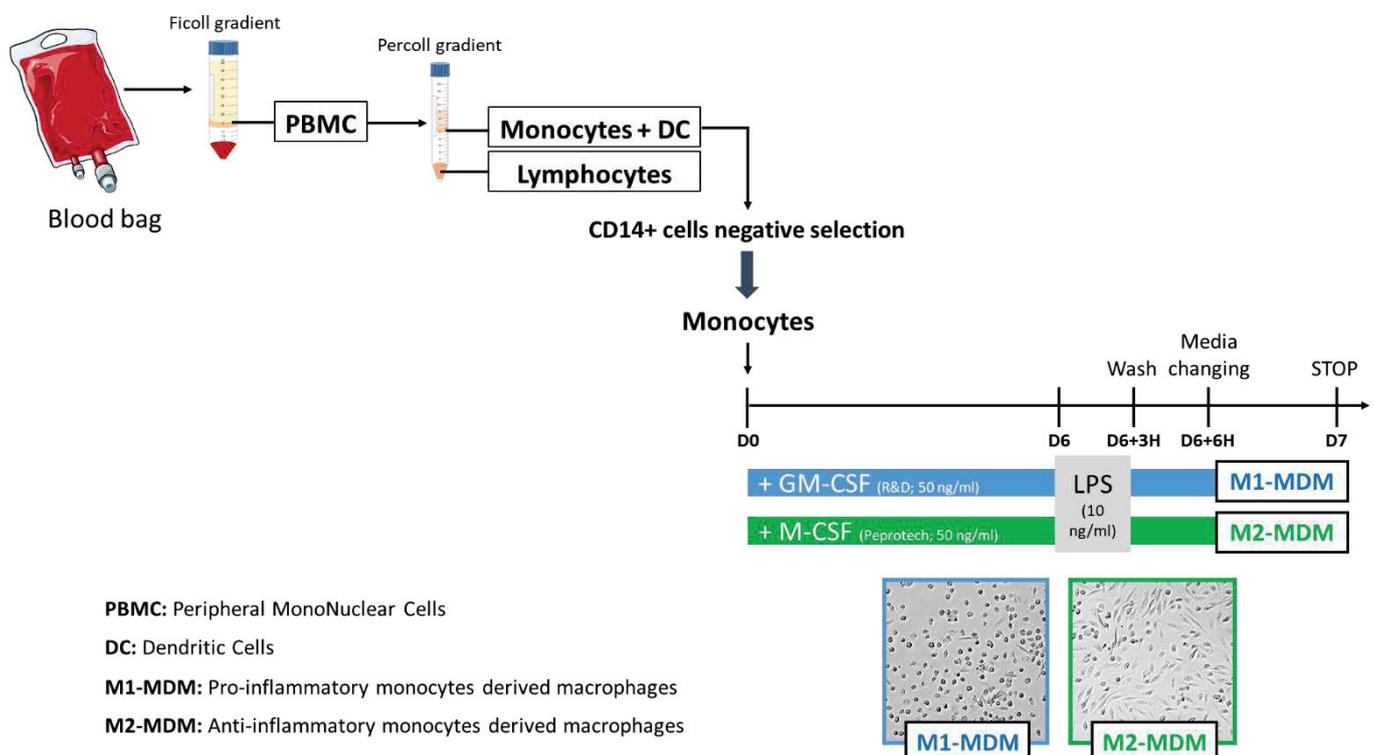
## 2. Experimental strategies



**Figure 27. Purification method of the different types liver resident cells.**

For the studies on hepatocytes, we used two types of *in vitro* models: primary human hepatocytes (PHH) which are isolated from liver resections (Figure 27) and the HepaRG cell line which have been previously shown to have a profile of expression of TLRs and RLRs closer to that seen in primary hepatocytes than that in transformed cell lines (Luangsay et al., 2015a). Hepatocytes were exposed to different ligands of PRR after the infection to study the impact of HBV on the activation of innate immune responses or they were exposed to cytokines (recombinant or produced by macrophages) before and during the infection process to determine the impact of factors secreted by macrophages on the establishment of HBV infection.

To study the effect of HBV on macrophages' phenotype and function, we used two types of *in vitro* models: total liver macrophages (i.e. liver resident M $\Phi$  containing Kupffer cells and infiltrating macrophages) purified from liver resections (Figure 27) and blood monocytes purified from peripheral blood and differentiated *in vitro* into either M1- or M2-MDM (Figure 28). M $\Phi$  were exposed to HBV during differentiation and/or activation and were stimulated with different ligands of TLRs to assess the effect of HBV on their phenotypes and secretions.



**Figure 28. Method of purification of monocytes from peripheral blood and differentiation into M1- or M2-MDM.**

Finally, to assess the expression of different PRR in the liver resident cells, PHH, hepatic stellate cells, liver M $\Phi$ , and liver sinusoidal endothelial cells were purified from liver resections (**Figure 27**). The different cell types were either analysed without any further treatment (assessment of basal expression levels of the PRR and their signalling pathway) or stimulated with PRR ligands to assess their ability to respond to the different stimulated pathways.



### 3. First study

#### **Early inhibition of hepatocytes innate responses by hepatitis B virus**

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**Published in 2015 in the Journal of Hepatology.**



## Early inhibition of hepatocyte innate responses by hepatitis B virus

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**Background & Aims:** The outcome of hepatitis B virus (HBV) infection may be influenced by early interactions between the virus and hepatocyte innate immune responses. To date, the study of such interactions during the very early step of infection has not been adequately investigated.

**Methods:** We used the HepaRG cell line, as well as primary human hepatocytes to analyze, within 24 h of exposure to HBV, either delivered by a physiologic route or baculovirus vector (Bac-HBV), the early modulation of the expression of selected antiviral/pro-inflammatory cytokines and interferon stimulated genes. Experiments were also performed in the presence or absence of innate receptor agonists to investigate early HBV-induced blockade of innate responses.

**Results:** We show that hepatocytes themselves could detect HBV, and express innate genes when exposed to either HBV virions or Bac-HBV. Whereas Bac-HBV triggered a strong antiviral cytokine secretion followed by the clearance of replicative intermediates, a physiologic HBV exposure led to an abortive response. The early inhibition of innate response by HBV was mainly evidenced on Toll-like receptor 3 and RIG-I/MDA5 signaling pathways upon engagement with exogenous agonist, leading to a decreased expression of several pro-inflammatory and antiviral cytokine genes. Finally, we demonstrate that this early inhibition of

dsRNA-mediated response is due to factor(s) present in the HBV inoculum, but not being HBsAg or HBeAg themselves, and does not require *de novo* viral protein synthesis and replication. **Conclusions:** Our data provide strong evidence that HBV viral particles themselves can readily inhibit host innate immune responses upon virion/cell interactions, and may explain, at least partially, the “stealthy” character of HBV.

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### Introduction

Viral hepatitis represents a major health problem worldwide, with hundreds of millions of chronic carriers who have a high risk of developing liver cirrhosis and hepatocellular carcinoma [1]. To establish and maintain persistent replication, hepatitis B virus (HBV) has evolved multiple strategies to evade the host innate and adaptive immune responses [2]. To restore immune control of the virus, virus-mediated inhibitory mechanisms could be defeated/unlocked in an interventional therapeutic perspective. A better knowledge of the underlying molecular mechanisms responsible for virus-induced blockade of immune responses is crucial before envisaging such strategies to improve the success of current antiviral treatments [3].

Most viruses are detected early after infection by both immune and/or infected cells via pathogen recognition receptors (PRR), including Toll-like receptors (TLR) and RIG-I like receptors (RLR) and NOD-like receptors (NLR) [4]. It is still unclear if HBV is recognized by the innate immune system and/or if the virus can actively suppress or avoid early antiviral responses that drive the control of HBV infection [2,5,6]. The few data obtained in acutely infected humans, chimpanzees and woodchucks have shown that during the natural course of HBV infection, the activation of innate responses is predominantly weak or absent [7–10]. In particular, a seminal work performed in chimpanzees, has shown that HBV does not induce a strong modulation of gene expression in the liver of an infected animal as compared to hepatitis C virus (HCV) [10]. Following this work HBV was qualified as a “stealth virus” [11] as opposed to HCV. A stealth virus can be a virus that

**Keywords:** Hepatitis B virus; Hepatocytes; Early host/virus interaction; IFN response; dsRNA-mediated innate immunity.

Received 24 November 2014; received in revised form 22 June 2015; accepted 13 July 2015; available online 26 July 2015

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**Abbreviations:** Bac, baculovirus; DMSO, dimethyl sulfoxide; GFP, green fluorescent protein; HBV, hepatitis B virus; HCV, hepatitis C virus; IFN, interferon; ISG, interferon stimulated genes; KC, Kupffer cells; LSEC, liver sinusoidal endothelial cells; MDA, melanoma differentiation-associated gene; MOI, multiplicity of infection; NLR, NOD-like receptor; PEG, polyethylene glycol; PHH, primary human hepatocytes; PRR, pathogen recognition receptor; RIG, retinoic-acid-inducible protein; RLR, RIG-like receptor; SV, Sendai virus; TLR, toll-like receptor; VGE, virus genome equivalent.



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does not induce measurable innate responses because of a lack of detection by PRR, or a virus that is able to actively inhibit nascent innate responses. In this respect, some other data suggest that, during chronic infection, HBV could negatively regulate host immune responses by interfering with TLR expression and signaling pathways [12–14], or by inhibiting interferon (IFN) response [15–19]. Underlying molecular mechanisms could involve several distinct HBV proteins as recently reviewed [2,5,6].

However to date, there are no very early kinetic interaction study between HBV and hepatocytes that have been performed to determine if the virus could; i) be initially detected by host cells; ii) modulate host immune gene expression; and then iii) inhibit innate responses. Only one study, performed in primary human hepatocyte (PHH) cultures showed that HBV could induce the transient production of interleukin (IL)-6, thus suggesting that the virus could be initially “sensed”, at least by liver macrophages (i.e. present in PHH cultures), and lead to the production of pro-inflammatory cytokines. However, HBV could, 24 hour (h) after the onset of infection, inhibit the production of IL-6 by a yet unknown mechanism [20].

To better characterize the early interplay between HBV and hepatocyte innate immunity in terms of recognition and evasion, we used a non-transformed human hepatocyte cell line, differentiated HepaRG (dHepaRG), which is permissive for persistent HBV infection and is devoid of contamination by immune cells [21–23]. We showed that hepatocytes themselves could “sense” HBV and initiate an antiviral response, when delivered to cells by baculoviral transduction, as previously observed [24], but also during a more physiologic infection with HBV virions. While an efficient antiviral response was observed against HBV replication, when launched by baculovirus, this response was abortive in the context of a physiological HBV infection. In this case, the suppression of innate responses was exerted by viral component(s) already present within the inoculum not requiring *de novo* viral synthesis. This active suppression of pathogen-sensing pathways in the very early phase of infection, which prevented the establishment of a competent innate immunity, correlated with the development of a persistent infection *in vitro*.

### Materials and methods

#### HBV and Sendai viral inocula

HBV inoculum was either concentrated from filtered HepG2.2.15 (wild-type virus) or K6 (HBx negative virus) [25] supernatants by polyethylene glycol (PEG) precipitation as previously described [22], or partially purified by heparin chromatography [26], then concentrated using centrifugal filters devices (Amicon Ultracel 100K, Millipore). A mock “HBV-negative” inoculum (mock control) was generated by depletion of Dane particles, HBsAg and HBeAg using centrifugal filters devices (Amicon Ultracel 10K, Millipore). After DNA extraction (QIAamp Ultra-sens Virus kit, Qiagen), HBV inoculum was titrated by qPCR with forward 5'-GCTGACGCAACCCCACT-3' and reverse 5'-AGGAGTCCGAGATATGG-3' probes using a standard curve from a quantified HBV encoded plasmid. All preparations were tested for the absence of endotoxin (Lonza Verviers, Belgium). Sendai virus (SV; Cantell strain; titer: 4000 HAU/ml) was obtained from Charles River Laboratories (Bois des Oncins, France) and used according to recommendations.

#### HBV virion and viral protein purification

Dane particles were purified from the PEG precipitated HBV inoculum by sequential ultracentrifugation through a cushion of sucrose first, then on a sucrose density gradients at 35,000 rpm for 16 h at 4 °C in a Beckman SW41Ti Rotor. Collected fractions were tested for sucrose density, HBV DNA (qPCR), HBcAg (Western blot with anti-HBc (Dako)), HBeAg and HBsAg (ELISA). The overall purity of preparation was investigated by SDS-PAGE and SYPRO-Ruby Protein

Gel Staining (Life Technologies). The concentrations of HBsAg and HBeAg were measured by commercial immunoassay kits, according to the manufacturer's protocols (Autobio Diagnostics Co., China). One NCU, i.e. unit used in “HBeAg detection and relative quantification ELISA” from Autobio, is equivalent to 13.33 ng.

#### Human hepatocyte culture and HBV infection

The human liver progenitor HepaRG cells were cultured and infected as previously described [21,22]. PHHs were prepared from surgical liver resections as previously described [27]. They were infected similarly to differentiated HepaRG.

#### Baculovirus vectors and cell transduction

Two baculoviruses were used in this study: a 1.1x genome-length HBV recombinant baculovirus vector (Bac-HBV) and a control baculovirus expressing GFP instead of HBV pgRNA. Baculoviral transduction of mammalian cells was performed as previously described [24].

#### Cell stimulation

Cells (10<sup>6</sup> per well) were stimulated with TLR agonists (Invivogen) and harvested after 6 h (except for RIG-I/MDA5 after 24 h) for the analysis of IL-6 protein production by ELISA: TLR1/2 (pam3CSK4, 0.8 µg/ml), TLR3 (poly(I:C), 10 µg/ml), TLR4 (LPS 0.4 µg/ml), TLR5 (flagellin, 0.1 µg/ml), TLR6 (FSL-1, 0.1 µg/ml), TLR7/8 (ssRNA, 10 µg/ml), RIG-I/MDA5 (transfection of poly(I:C) with the reagent Iyovec, 0.2 µg/ml). For the cytokine gene expression analysis following poly(I:C) stimulation, cells were harvested after 3 h of stimulation.

#### Nucleic acid purification, RT-qPCR, and qPCR

Total RNA or DNA were respectively purified with the Nucleospin RNA II or MasterPure™ DNA Purification kits according to manufacturer's instructions (Macherey Nagel or Epicentre). cDNA was obtained after reverse transcription using the SuperScript® III Reverse Transcriptase (Life technologies) and real time quantitative qPCR (the sequence of primer pairs are listed in Table 1) was performed using the EXPRESS SYBR® GreenER™ qPCR Supermix Universal (Life technologies), and run on the MyiQ Biorad machine. Relative mRNA expression was analyzed with q-base software (Biogazelle, Belgium) using the comparative cycle threshold (2<sup>-ΔΔCt</sup>) method with two housekeeping genes (*RPLP0* and *β-actin*) previously tested for their stability in the HepaRG cells and the PHH, and normalized to the control conditions (-1). The relative HBV mRNA level was quantified using the same primer pairs used for the HBV PCR quantification.

#### Analysis of cytokine production

At selected time points, cell culture supernatants were harvested and tested for the secretion of IL-6, IFN-λ1/3 (R&D system), IFN-α, and IFN-β (PBL Interferon Source) according to the manufacturer's instructions.

#### UV inactivation

HBV and mock inocula were irradiated or not at room temperature on a UV Transilluminator (Appligene) delivering 3.3 mw/cm<sup>2</sup> for 30, 60, or 90 min. The efficacy of HBV replication with UV-inactivated inocula was analyzed after exposure to HepaRG cells by RT-qPCR and secretions of HBeAg and HBsAg were analyzed by ELISA (see Supplementary Fig. 3A).

**Table 1. Sequences of human primer pairs used for RT-qPCR.**

Gene	Forward	Reverse
IFN-α	gtgaggaaacttccaagaataac	tctcatgattctgctgacaa
IFN-β	gcgcattgaccatgatgaga	gagatcttcagttcggaggaac
IL-29	gfggtgctgggacattgg	ctcctggtgacagagatttg
IL-6	accctgacccaaccacaaat	agcgcgacagaatgagatgatt
IL-1-β	aatctgacctgctgctgggtt	tggttaattttgggctcacactct
ISG56	agccaaacatgctctacagac	cttcaaccactgittcaatgc
OAS1	agggtgtaaaagggtgctcc	acaacaggtcagcagctcagat
b-ACTIN	tggcattgcccagcagatgc	tctctggaggtggacagcga
RPLP0	caccattgaatctgagtgatgt	tgaccagcccaagagagaag

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### Statistical analysis

Statistical analysis was performed using the Dixon and the non-parametric Mann-Whitney tests using the GraphPad Prism software. For all tests, a  $p$  value  $\leq 0.05$  (\*),  $\leq 0.005$  (\*\*), or  $\leq 0.0005$  (\*\*\*) was considered as significant.

### Results

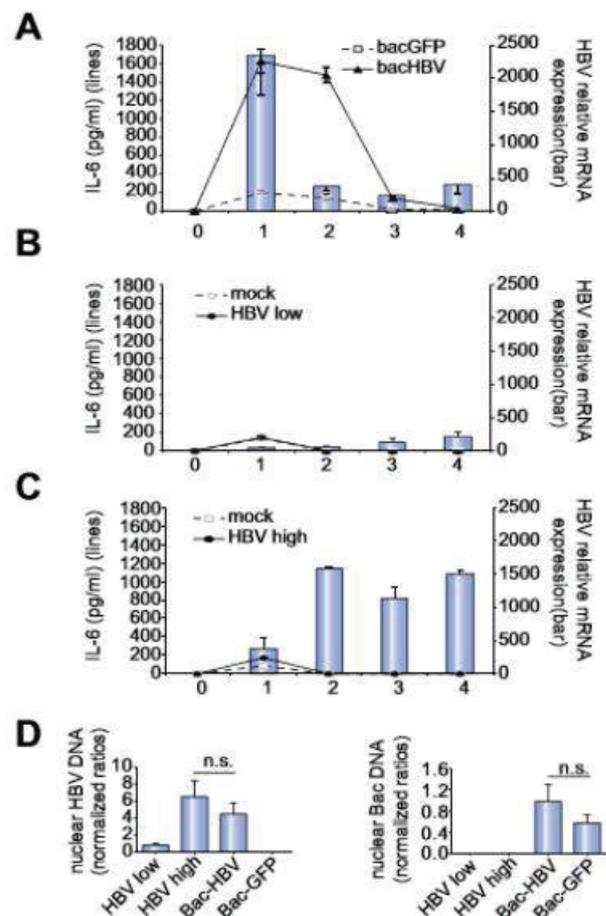
#### *IL-6 production during vector-mediated or physiologic HBV infection of dHepaRG cells*

To start examining the modulation of hepatocyte innate immunity during persistent or resolving HBV infection *in vitro*, dHepaRG cells were either infected with recombinant HBV or transduced with Bac-HBV, a recombinant baculovirus carrying 1.1 HBV genome unit, which is able to launch synchronized and strong intracellular HBV replication [24,28]. The former infection model leads to a persistent infection which can last for months in dHepaRG cells [22], whereas the latter leads to a strong initial replication peaking at 24 h post-transduction (p.t.) followed by a non-cytopathic, IFN-driven, elimination of HBV replicative intermediates (referred as “clearance of viral replication” hereafter) [24].

In transduced-dHepaRG cells, the rapid and transient synthesis of HBV RNA correlated with a strong secretion of *IL-6* ( $\approx 1500$  pg/ml), which peaked at 24 h p.t., remained high until day 3 p.t., before returning to baseline at day 6 p.t. (Fig. 1A). The elimination of replication intermediates in these cells was very fast, likely due to the antiviral effect of secreted IFNs [24] and other inflammatory cytokines, including *IL-6* which has been recently shown to exhibit direct antiviral activity [20]. dHepaRG transduced with an identical amount (i.e. 100 pfu/cell) of control baculovirus (Bac-GFP) did not lead to significant release of *IL-6*, thus showing that *IL-6* secretion was the consequence of the synthesis of HBV replicative intermediates. In sharp contrast, cells infected with either a low (Fig. 1B) or high (Fig. 1C) dose of HBV virions showed only a weak (<200 pg/ml) and transient secretion of *IL-6* (detection only at 24 h post-infection (p.i.); undetectable after), and was not associated with an inhibition of HBV replication, as intracellular HBV RNA started to increase at day 3 or day 6 according to initial multiplicity of infection (m.o.i). It is worth noting that both transduction with Bac-HBV (or its control Bac-GFP) and a high m.o.i with HBV led to non-significantly different amounts of initially nuclear-delivered baculoviral and/or HBV DNA (Fig. 1D), and HBV RNA in cells (Fig. 1A and C). These results supported the relevance of the comparison between baculovirus-mediated and physiologic HBV infection, in terms of correlation between initial response and outcome of infection. However in the case of viral transduction, the maximal accumulation of HBV RNA occurred shortly (i.e. before 24 h p.t.) after the onset of infection and was associated with a strong production of *IL-6*, whereas in the case of natural infection with a high m.o.i, HBV RNA started to accumulate only at day 3 after a lag phase. These results suggest that a “physiological” HBV infection does not induce a significant innate response in dHepaRG cells, as measured here by *IL-6* secretion, and inevitably leads to persistence, whereas during an experimental, baculovirus-mediated infection, a strong and rapid production of HBV replication intermediates induces a strong innate response, leading to the elimination of replicative intermediates *in vitro*.

#### *Detailed analysis of the modulation of innate gene expression during vector-mediated or physiologic HBV infection of dHepaRG cells*

One main advantage of *in vitro* HBV infectious models, over biopsy-based approach in chimpanzees [10], is that the hepatocyte innate responses to HBV can be monitored by sensitive RT-qPCR to detect very early and subtle variations in the expression of host innate genes in a tight time course-dependent manner. To this end, we demonstrated that a productive HBV infection, measured by HBeAg and HBsAg quantification, was obtained several days post-infection in PHH and dHepaRG cells with an initial inoculation time as short as 2 h (Supplementary Fig. 1A–D). We confirmed that infection is more efficient in PHH than in dHepaRG (Supplementary Fig. 1A–B vs. C–D), and that the yield of infection is dependent upon the initial m.o.i (i.e. amount of virus genome equivalent (vge)/cell) (Supplementary Fig. 1E–F).



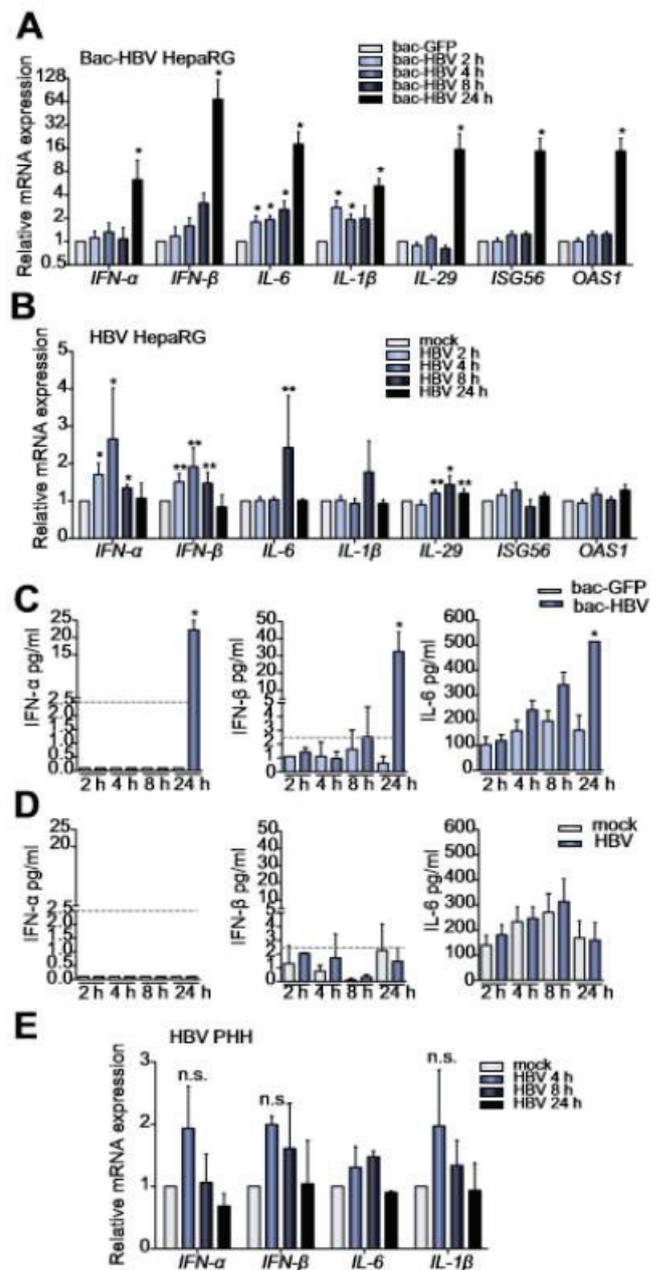
**Fig. 1. Kinetics of HBV replication and *IL-6* secretion in transduced or infected dHepaRG cells.** dHepaRG cells were either transduced with Bac-HBV (A), or infected with a low dose (100 vge/cell) (B), or high dose (1000 vge/cell) (C) of HBV. Cell supernatants were harvested at the indicated time points to measure *IL-6* protein secretion (left axis) and compared to the control media (Bac-GFP or mock-HBV) (A, B, C). The relative HBV mRNA expression in these cells was quantified in parallel by RT-qPCR (right axis). Results are represented as the mean  $\pm$  SEM of two independent experiments (A, B, C). Baculoviral and/or HBV DNA were also quantified in nuclear extract at 24 h post-transduction or infection by qPCR using primers specific to baculovirus (i.e. IE-1 gene) backbone or HBV (D).

Rather than going for pan-genomic analysis, we have chosen to focus on the expression of some antiviral/pro-inflammatory cytokines (*IFN- $\alpha$* , *IFN- $\beta$* , *IL-29*, *IL-6*, *IL-1 $\beta$* ) and two prototypic interferon stimulated genes (ISGs; *ISG56* and *OAS1*). Following Bac-HBV transduction, the expression of all antiviral/pro-inflammatory cytokine genes was significantly upregulated, *IFN- $\beta$*  being the most induced transcript (Fig. 2A). Corroborating this increased gene expression at RNA level, a measurable secretion of *IFN- $\alpha$*  (20 pg/ml), *IFN- $\beta$*  (30 pg/ml), and *IL-6* (500 pg/ml) was observed at 24 h p.t. in the supernatant of Bac-HBV-transduced cells (compared to the Bac-GFP control) (Fig. 2B). Again in sharp contrast, following a natural HBV infection, a weak but significant gene induction of type I *IFN*, *IL-29* and *IL-6* expression was observed between 4 h to 8 h p.i., which rapidly returned to baseline within 24 h p.i. No induction of ISG (*ISG56* and *OAS1*) expression was detected in HBV infected cells (Fig. 2C). As a result of this weak increase of gene expression, there was no detectable secretion of cytokines in the supernatant of HBV infected cells (Fig. 2D). In PHHs, as for HepaRG cells, a weak and transient innate response was also observed upon HBV infection (Fig. 2E), despite a higher infectivity rate (Supplementary Fig. 1C, D), and was similarly associated with the establishment of a persistent infection.

Therefore, a strong intracellular HBV replication, experimentally launched by Bac-HBV transduction, could be easily sensed by innate receptors and induced potent antiviral responses, which in turn led to the elimination of replicative intermediates. In contrast, during physiological HBV infection, a modest and transient response, with no detectable production of cytokines in cell supernatant, that can be qualified as abortive response, was observed and associated with the persistence of replication *in vitro*. Since such responses could be measured at least at the level of gene expression, it does indicate that HBV was "sensed" by cells, but the virus seemed to rapidly disarm this nascent response. The next step was to determine whether HBV is able to inhibit the hepatocyte innate responses experimentally engaged by "exogenous" ligands of PRRs.

#### HBV actively represses dsRNA-mediated innate responses in hepatocytes shortly after the onset of infection

To investigate whether HBV could rapidly repress hepatocyte innate responses, dHepaRG cells were infected with HBV at low or high multiplicity for 24 h, then stimulated by various PRR agonists known to induce the production of *IL-6* in these cells (unpublished data and [29]), to measure the induction of the innate response. While stimulation of TLR5 or TLR6 receptors led to a stronger secretion of *IL-6* in HBV infected cells in comparison to controls (production of *IL-6* in control mock-infected cells represented 100%), stimulations of TLR2, TLR4 or TLR7/8 led to similar *IL-6* protein secretion. Interestingly, stimulation of dsRNA sensors (TLR3 and RIG-I/MDA5) with either poly(I:C) or transfected poly(I:C), led to significantly less *IL-6* protein secretion by HBV infected cells (Fig. 3A). Moreover, analysis of the induction of selected host innate gene expression in poly(I:C)-stimulated cells also showed a strong inhibition reaching 60-70% at the highest m.o.i for *IL-6* gene expression, but also for type I and type III IFNs (*IFN- $\beta$* , *IL-29*) and *OAS1* (i.e. used as a prototypic ISG) in HBV infected hepatocytes (Fig. 3B). Although this HBV-mediated



**Fig. 2. Kinetics of the relative mRNA expression of cytokine and ISG after either Bac-HBV-transduction or HBV infection in human hepatocytes.** Bac-HBV-transduced cells (A and B) and HBV infected dHepaRG cells (C and D) or HBV infected primary human hepatocytes (PHH) (E) were harvested at the indicated time points (2, 4, 8, or 24 h), and RNA extracted and subjected to RT-qPCR. The fold induction of the relative mRNA expression level of the cytokines (A, C, and E) was normalized to housekeeping genes and compared to either Bac-GFP or mock controls. The supernatants from these cells were also harvested at the indicated time point to measure the secreted proteins and compared to the control media (Bac-GFP or mock) (B and D). Results are given as a mean  $\pm$  SEM of at least three independent experiments ( $n = 3$  to 7 when required to shown significance) and differences were considered as statistically significant to the control conditions (Bac-GFP or mock) when the  $p$  value was  $<0.05$  (\*) or  $<0.005$  (\*\*). The results for the relative mRNA expression for PHH are given as a mean  $\pm$  SEM of two independent experiments (E).

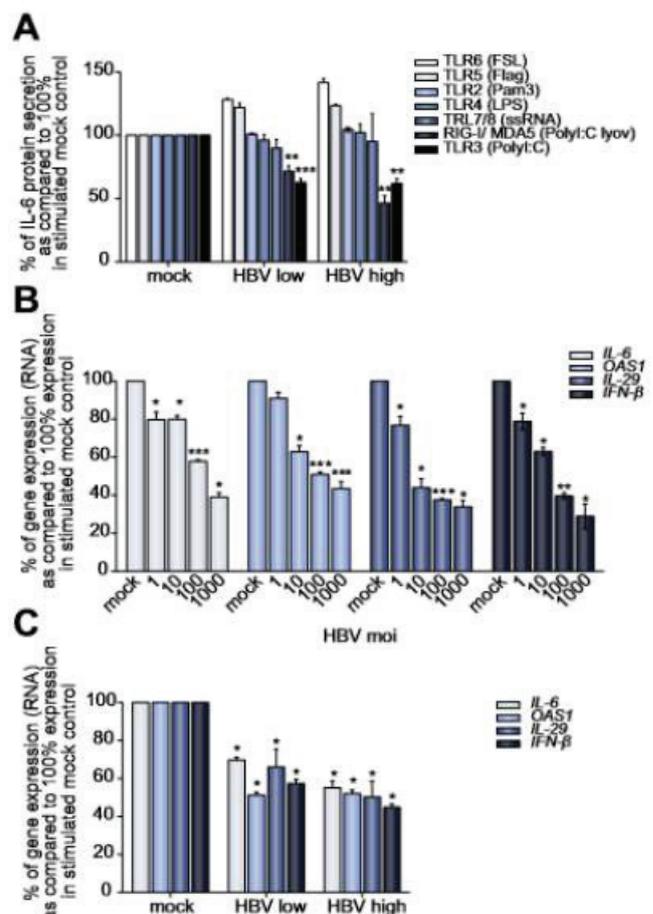
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inhibition of TLR3, RIG-I/MDA5 pathways by poly(I:C) seemed to be dependent on the amount of virus used to initially inoculate dHepaRG cells, it seemed that a very low quantity of HBV virus (i.e. 1 vge/cell) was sufficient to significantly repress the expression of *IFN- $\beta$* , *IL-29*, and *IL-6* in infected hepatocytes (Fig. 3B). Similar observations were also made in HBV infected PHHs at both RNA (*IFN- $\beta$* , *IL-29*, *IL-6*, *OAS1*) and, more importantly, at level of secreted proteins (*IL-6*, *IFN- $\lambda$* , and *IFN- $\beta$* ) following poly(I:C) stimulation (Fig. 4A, B). The latter suggests that an inhibition observed at RNA level translates into a stronger inhibitory phenotype at the level of secreted cytokines, thus strengthening the relevance of the results. To functionally confirm this inhibition, we used two types of assays. First, we could observe a significant inhibition of *IFN- $\beta$*  gene 24 h following SV super-infection in dHepaRG cells previously infected with HBV, compared to cells not infected with HBV but super-infected by SV in a similar manner (Fig. 5A, B). Second we extended Bac-HBV mediated intracellular replication in super-infection experiments (Supplementary Fig. 2); indeed we had previously shown that HBV intracellular replication launched by Bac-HBV delivery was abortive due to a strong IFN response [30]. Pre-infection of cells with HBV was capable to block IFN response and therefore extend Bac-HBV-mediated replication. Both assays suggested that HBV could suppress a strong induction of the IFN response, in a context of super-infection. Finally, the repressive effect of HBV on the dsRNA-mediated innate response observed after only 24 h of inoculation with HBV was maintained after 12 days of infection when HBV infection is well established, which suggests that HBV is capable to induce a long lasting inhibition of the dsRNA-mediated innate response in infected cells (Fig. 3C).

*The very early inhibition of the dsRNA-mediated innate response in hepatocytes does not require de novo viral protein synthesis, but is initiated by viral proteins associated with HBV virions*

So far the inhibition of the dsRNA-mediated innate response in hepatocytes was measured after 24 h of inoculation with the virus. To determine whether this inhibition could be set even earlier, we shortened the inoculation time down to 2 h. Cells were inoculated with HBV for either 2, 4, 8, or 24 h, then stimulated for 3 additional hours with poly(I:C) after removal of the viral inoculum. In this setting, the HBV-mediated inhibition of *IFN- $\beta$*  and *IL-29* gene expression and to a lesser extent of *OAS1* and *IL-6* gene expression seemed to occur very rapidly after the virus addition; in fact, as early as 2 h after virus inoculation. Interestingly, the inhibition of expression of *IFN- $\beta$*  and *IL-29* upon poly(I:C) stimulation reached its maximum after only 4 h of viral exposure (Fig. 6A), which suggests that no viral proteins were required for this very early inhibition.

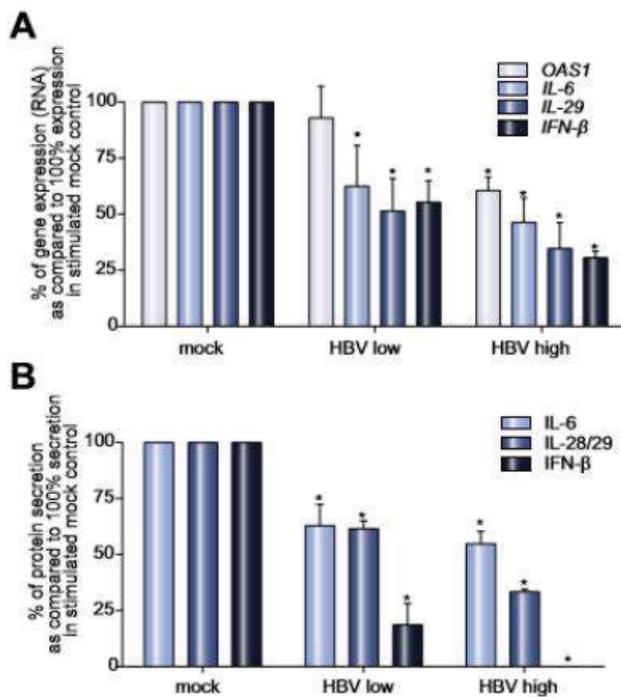
To demonstrate that no viral replication (i.e. no *de novo* production of replication intermediates and viral proteins) was needed, a UV-inactivated HBV inoculum, deficient in triggering a productive infection (Fig. 6B) was used. We stimulated non-UV and UV-inactivated HBV inoculated cells (at 24 h post-exposure) with poly(I:C), to measure the impact on induction of dsRNA-mediated innate gene expression (Fig. 6A). In both non-UV and UV-HBV exposed cells showed a comparable strong inhibition of the induction of innate gene expression (*IFN- $\beta$* , *IL-29*, *IL-6*, and *OAS1*), demonstrating that a HBV factor (or a host-factor associated with HBV virions) present in the inoculum was sufficient to mediate a prompt and strong repression of the



**Fig. 3. Cytokine expression following the stimulation of PRR in HBV infected dHepaRG cells.** The induction of the innate response was first evaluated through *IL-6* secretion in the supernatant of cells infected with HBV (low = 100 vge/cell or high = 1000 vge/cell) for 24 h, then stimulated with indicated PRR agonists for 6 h (A). The induction of the relative innate gene expression level in poly(I:C) 3 h-stimulated cells was analyzed by RT-qPCR in dHepaRG cells infected for 24 h with increasing amount of virus (1, 10, 100, or 1000 vge/cell) (B), or after 12 days of infection (100–1000 vge/cell) (C). The mRNA expression level for each gene was normalized to housekeeping genes and relatively compared to the control mock-infected cells. The percentage of activation of each gene was then compared to the stimulated control mock-infected cells (~100%). Results are given as a mean  $\pm$  SEM of at least three independent experiments ( $n = 3$  to 15) and differences were considered as statistically significant to the control condition when the  $p$  value was  $<0.05$  (\*),  $<0.005$  (\*\*), or  $<0.0005$  (\*\*\*)

TLR3 and RIG-I/MDA5 signaling pathways. Similarly UV-inactivated HBV, as well as HBx negative HBV could block Sendai-mediated IFN responses (Fig. 5C).

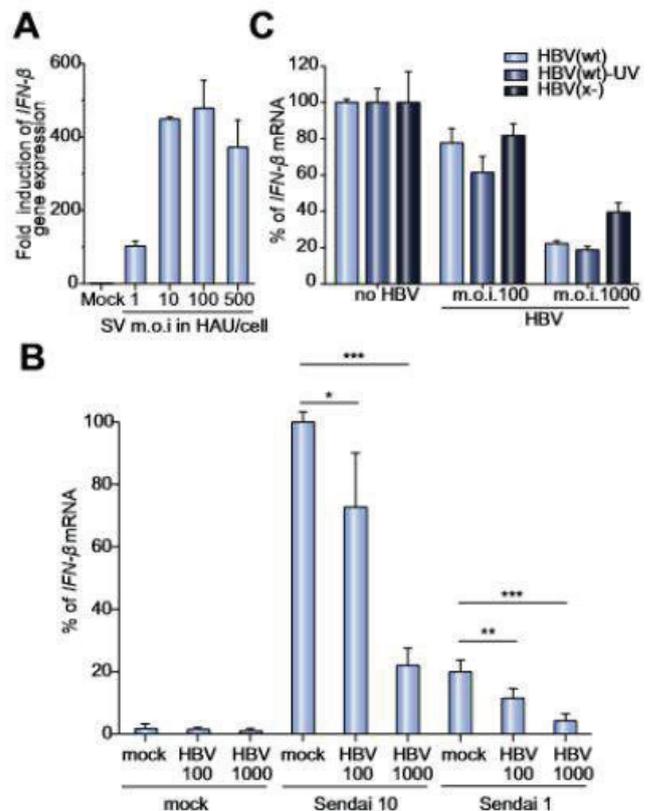
To identify which viral determinants could be responsible for the suppressive effect on the innate response, we purified each viral component of the HBV inoculum (i.e. infectious viral Dane particles, subviral particles (HBsAg), and HBeAg) (Supplementary Fig. 4A). Differentiated HepaRG cells were exposed with either a full inoculum free of non-enveloped capsids (called HBV) (Supplementary Fig. 4B), or to the various purified viral components including infectious Dane particles purified by a double cushion/gradient methodology to insure purity (Supplementary Fig. 4C), subviral particles (SVPs; i.e. HBsAg), and HBeAg for 24 h. The concentration of infectious particles, SVPs and HBeAg



**Fig. 4. Cytokine expression and secretion in HBV-infected PHH following TLR3 stimulation.** PHH were infected for 24 h with HBV (low = 100 vge/cell, high = 1000 vge/cell) and stimulated with poly(I:C) for 3 h. The induction of the innate response was first evaluated through the induction of the relative gene expression level of several innate genes (*IL-6*, *IL-29*, *IFN-β*, *OAS1*) (A), and protein secretion of *IL-6*, *IFN-λ*, and *IFN-β* by ELISA. (B) The mRNA expression level for each gene was normalized to housekeeping genes and relatively compared to the control mock-infected cells. The percentage of activation of each gene was then compared to the stimulated control mock-infected cells (=100%). Results are given as a mean ± SEM of at least three independent experiments (n = 3 to 6) and differences were considered as statistically significant to the control condition when p value was ≤0.05 (\*).

was harmonized to that of full inoculum as follow: HBsAg at 5 ng/10<sup>6</sup> cells, HBeAg at 4.4 NCU/10<sup>6</sup> cells, and HBV DNA at 100 vge/cell. After 24 h of exposure to HBV or various viral components, the impact on *IL-6*, *IFN-β*, *IL-29*, and *OAS1* gene expression following poly(I:C) stimulation was evaluated. While *IL-6* expression was significantly inhibited by each viral component, a strong inhibition of type I/III IFNs and *OAS1* gene expression was only observed with the HBV full inoculum and purified infectious Dane particles (Fig. 6B). To further exclude the implication of HBeAg and HBsAg in this phenotype, we also used recombinant antigens produced in *Pichia Pastoris* (HBeAg and HBsAg from commercial source) or produced in HepaRG cells (Supplementary Fig. 5) and performed similar experiments to find that neither HBeAg nor HBsAg, yet used at a 10x higher equivalent m.o.i as compared to full HBV inoculum or purified Dane particles, were capable to inhibit the induction of *IFN-β* expression after poly(I:C) stimulation (Fig. 6C). To further demonstrate that the very early inhibitory phenotype was associated with the entry of infectious particles themselves, we treated cells with Myrcludex<sup>®</sup>, a peptide capable of blocking, in a specific manner, HBV entry into cells [31]. Indeed, we found that a concentration of as low as 100 nM of Myrcludex<sup>®</sup> could prevent viral entry and replication (Supplementary Fig. 3B), and revert HBV-mediated inhibition of *IFN-β* gene induction (Fig. 7). This

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**Fig. 5. HBV can inhibit the innate response triggered by a super-infection with Sendai virus in HepaRG cells.** (A) dHepaRG cells were infected at different multiplicity of infection (m.o.i.) (between 1 and 500 HAU/cell) of Sendai virus (SV), and expression of *IFN-β* gene analyzed by RT-qPCR 24 h post-infection. (B) dHepaRG cells were either mock or infected for 24 h with HBV at two different doses (100 or 1000 vge/cell), and then mock or super-infected with SV at a m.o.i. of 1 or 10 HAU/cell (Sendai 10 or 1). The induction of the innate response was evaluated through the induction of the relative gene expression level of *IFN-β* gene. The condition mock-infection followed by super-infection with SV at an m.o.i. of 10 was set as the 100% of induction. (C) dHepaRG cells were either mock or infected for 24 h with either wild-type HBV, UV-inactivated HBV, or HBx negative HBV at two different doses (100 or 1000 vge/cell), and then super-infected with SV (10 HAU/cell). The induction of the innate response was evaluated through the induction of the relative gene expression level of *IFN-β* gene. Results are given as a mean ± SEM of at least two independent experiments (n = 2–3), and differences were considered as statistically significant to the control condition when p value was ≤0.05 (\*) or ≤0.005 (\*\*).

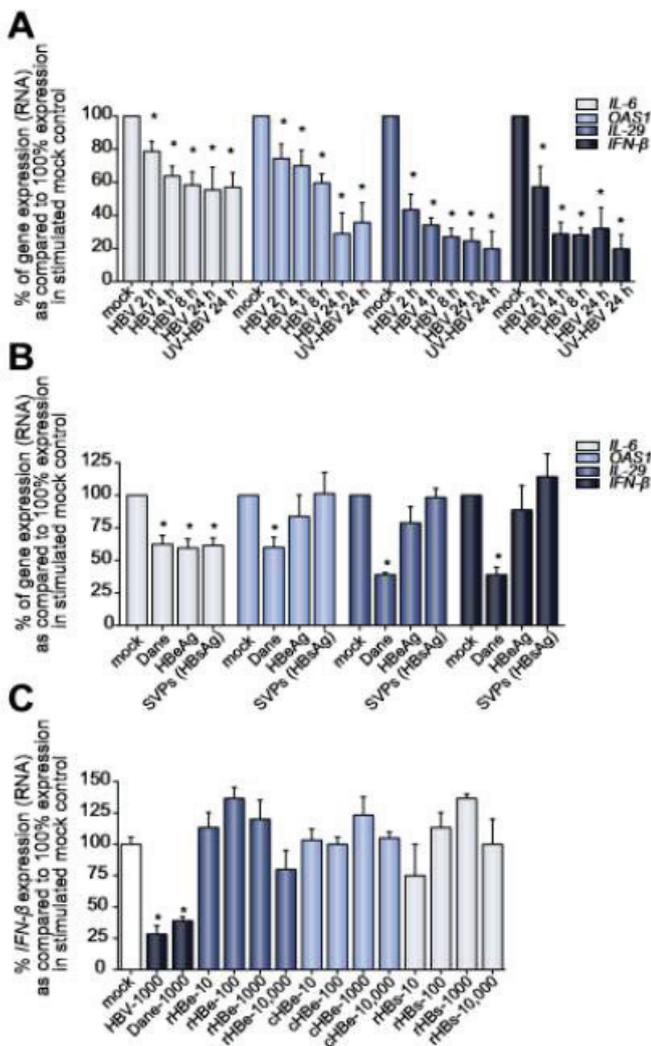
latter data further demonstrates that a UV-resistant viral component within HBV infectious virions, is responsible for the early repression of IFN responses.

## Discussion

In this study, we used two relevant hepatocyte culture models, based on “immunocompetent” PHH and HepaRG cells, the latter being also devoid of non-parenchymal or liver resident immune cells, to decipher how HBV early interactions with hepatocytes may lead to the establishment of a persistent infection or, on the contrary, to the elimination of HBV replicative intermediates.

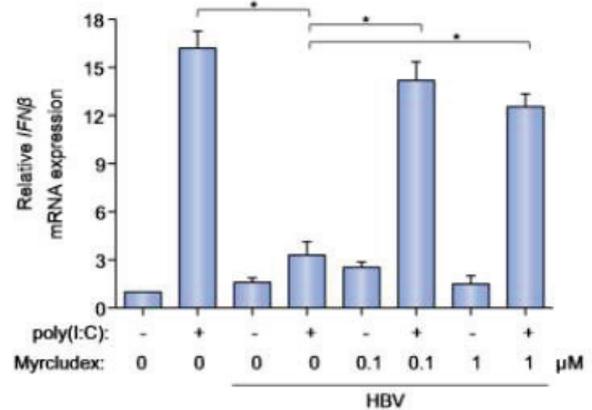
Collectively, our results showed that HBV could rapidly interfere with the hepatocyte antiviral responses mediated by the host

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**Fig. 6. Inhibition of dsRNA-induced gene expression by HBV is a very early event and is caused by HBV proteins associated with virions.** (A) dHepaRG cells were mock or infected at a m.o.i. of 100 vge/cell with wild-type HBV for 2, 4 or 8 h, or wild-type or UV-inactivated HBV for 24 h, and then stimulated by poly(I:C) for 3 h. RT-qPCR were performed to analyze the induction of indicated genes. Results are given as a mean  $\pm$  SEM of three independent experiments and differences were considered as statistically significant to the control condition when  $p$  value was  $\leq 0.05$  (\*). (B) dHepaRG cells were exposed for 24 h to a purified infectious virions (Dane) (equivalent of 100 vge/cell), HBeAg (4.4 NCU/10<sup>6</sup> cells; equivalent of 100 vge/cell) or SVPs (HBeAg) (5 ng/10<sup>6</sup> cells; equivalent of 100 vge/cell). Cells were then stimulated by poly(I:C) for 3 h, and RT-qPCR performed as in panel A. (C) dHepaRG cells were exposed for 24 h to increasing amount of HBeAg (rHBe), or HBeAg produced in yeast (rHBe) or in HepaRG cells (chHBe), then stimulated by poly(I:C) for 3 h, and RT-qPCR performed to monitor *IFN-β* expression. For all panels, the mRNA expression level for each gene was normalized to housekeeping genes and relatively compared to the control mock-infected cells. The percentage of activation of each gene was then compared to the stimulated control mock-infected cells (~100%). Results are given as a mean  $\pm$  SEM of at least three independent experiments ( $n = 3$  to 6) and differences were considered as statistically significant to the control condition when  $p$  value was  $\leq 0.05$  (\*).

viral recognition system, in particular by innate receptors detecting dsRNA pathogen-associated molecular patterns. When HBV strongly replicates following Bac-HBV transduction, a strong antiviral response that could not be counteracted by the virus and led to the elimination of replicative intermediates, was



**Fig. 7. Entry inhibitor treatment reverts the inhibitory phenotype.** dHepaRG were mock or HBV infected (100 vge/cell) in absence or presence of the indicated amount of Myrcludex (entry inhibitor) for 24 h, then stimulated (or not) with poly(I:C) for 3 h. The expression of *IFN-β* gene was analyzed by RT-qPCR. Results are given as a mean  $\pm$  SEM of three independent experiments and differences were considered as statistically significant to the control condition when  $p$  value was  $\leq 0.05$  (\*).

observed. However, in the context of a more physiologic HBV infection with a recombinant virus, HBV induced only a transient and modest increase of IFN and pro-inflammatory gene expression, which was associated *in fine* to a persistent infection. We provide evidence that HBV actively impair dsRNA-mediated recognition, including heterologous SV in the context of super-infection, during the very early phase of infection. Because inhibition of the hepatocyte innate response already occurred within the first 24 h of infection and was maintained until at least 12 days after infection, which is considered as a “persistent” infection in cell culture [22], our results may in part explain why HBV was previously considered as a stealth virus [10]. Furthermore, we showed that HBV components and/or host factors associated with HBV present in the viral inoculum were necessary and sufficient to suppress the innate response driven by the dsRNA sensors. Following this very early inhibition, neo-synthesis of viral proteins in infected cells beginning a few hrs post-infection, and thereafter, may be responsible for amplification of the inhibitory phenotype and its maintenance over time.

By studying the modulation of IFN and pro-inflammatory gene expression a few hours after exposure to the virus, we have confirmed that HBV can be initially sensed by hepatocyte PRRs. The fact that HBV could be sensed *in vitro*, i.e. in primary hepatocyte cultures, was previously shown by Hösel and coworkers [20]. Our detailed kinetic studies enabled to detect an early, weak, transient, yet reproducible, upregulation of the expression of downstream genes, which were not reported previously due to the late monitoring after the onset of infection, when the virus has already established inhibitory strategies.

While it is not yet established which sensors are involved in the detection of HBV virions and intracellular intermediates of replication, our data clearly demonstrate the particular ability of the virus to efficiently and promptly inhibit, within 2 h of viral exposure and in the absence of *de novo* viral protein synthesis, the TLR3 and RIG-I/MDA5-mediated innate response. While HBeAg and SVPs containing HBeAg alone seemed sufficient to downregulate the *IL-6* response at a comparable level to infectious Dane particles and the whole HBV inoculum, neither HBeAg nor HBeAg could repress the type I/III IFNs and ISGs response

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when cells were exposed to a complete HBV inoculum, which contained the same viral protein concentration. Only viral components of the infectious Dane particles (i.e. HBcAg, viral polymerase, HBV genome, or host-associated proteins) [32] seemed to exert a specific inhibition on IFN response. Further studies will be needed to determine which of these viral or host components composing the Dane particle could rapidly impact on the IFN response and by which mechanisms. Recently, Wu and coworkers [14] showed, in primary murine hepatocytes, a significant suppression of NF- $\kappa$ B activity and to a lesser extent of IFN- $\beta$  secretion in TLR3 stimulated primary murine hepatocytes, LSEC and Kupffer cells exposed to human HBV or secreted HBV antigens (e.g. HBeAg, HBsAg) that had been produced by an immortalized hepatocyte cell line derived from primary mouse hepatocyte cultures (HBV-met cells) [33]. However, in this setting, the consequences on HBV infection could not be studied, as murine hepatocytes are not susceptible to HBV. In addition, the authors observed the inhibition of the *IL-6* response in the HBV-met cells following TLR4, TLR7, and TLR9 stimulation, which implies that human HBV could suppress the murine hepatocyte innate responses [14]. In our model of human hepatocyte infection by HBV, none of these sensors was modulated by HBV, at least early after the onset of the infection. The mechanism described here may be hepatocyte specific, while HBV may adopt other suppressive mechanisms on immune cells as described elsewhere in human monocyte and dendritic cell populations [2,34–39].

Our results shed light on the early interplay between HBV and hepatocytes and demonstrate a suppressive effect of HBV on hepatocyte innate responses (i.e. dsRNA-mediated) that occurs before the onset of viral replication (i.e. no viral protein neo-synthesis) and therefore may contribute to the establishment of a persistent infection. This inhibitory process was timely and quantitatively adjusted to HBV, which is, due to the nature of its genome and life cycle, a weak inducer of innate responses, but noteworthy strong enough to counteract strong inflammation induced by the SV or enable extension of HBV intracellular replication mediated by a Bac-HBV virus, which is otherwise abortive due to a strong IFN response [30]. Further studies are now required to decipher the inhibitory mechanisms mediated by the different components of HBV during the very early phase of infection in the liver microenvironment. The understanding of the host-virus interactions and the mechanisms that underlie the regulation of innate responses of parenchymal and non-parenchymal liver cells is an essential step for the development of future treatment intervention targeting specifically the HBV-induced repression of innate responses in the infected liver.

### Conflict of interest

SL, MG, JL, NI, BT, MM, SFD, MAG, RP, and MR have nothing to declare. DD, and FZ received a research grant from Hoffmann-La-Roche to perform experiments. HJ is employee of Hoffmann-La-Roche.

### Authors' contributions

- Study concept and design: SL, MG, DD, and FZ
- Acquisition of data: SL, MG, JL, NI, BT, MM, SFD, SM, MAG, and DD
- Analysis and interpretation of data: SL, MG, HJ, DD, and FZ
- Drafting of the manuscript: SL, DD, and FZ

- Critical revision of the manuscript for important intellectual content: HJ, DD, and FZ
- Statistical analysis: SL and BT
- Technical, or material support: MR, RP, and MM

### Acknowledgments

The authors would like to thank Lydie Lefrançois and Judith Fresquet for the isolation of primary human hepatocytes, as well as the staff from Pr Michel Rivoire's surgery room for providing liver resection samples. We are grateful to William Mason (Fox Chase Cancer Center, Philadelphia, USA) for the critical reading of the manuscript.

This work was supported by grants from ANRS (French national agency for research on AIDS and viral hepatitis), FINOVI (Foundation for innovation in infectiology), FRM (Foundation for medical research; DEQ20110421327), Hoffmann-La-Roche (pRED, Basel, Switzerland) and by INSERM core grants. This work was also supported by the DEVweCAN LABEX (ANR-10-LABX-0061) of the "Université de Lyon", within the program "Investissements d'Avenir" (ANR-11-IDEX-0007) operated by the French National Research Agency (ANR).

### Supplementary data

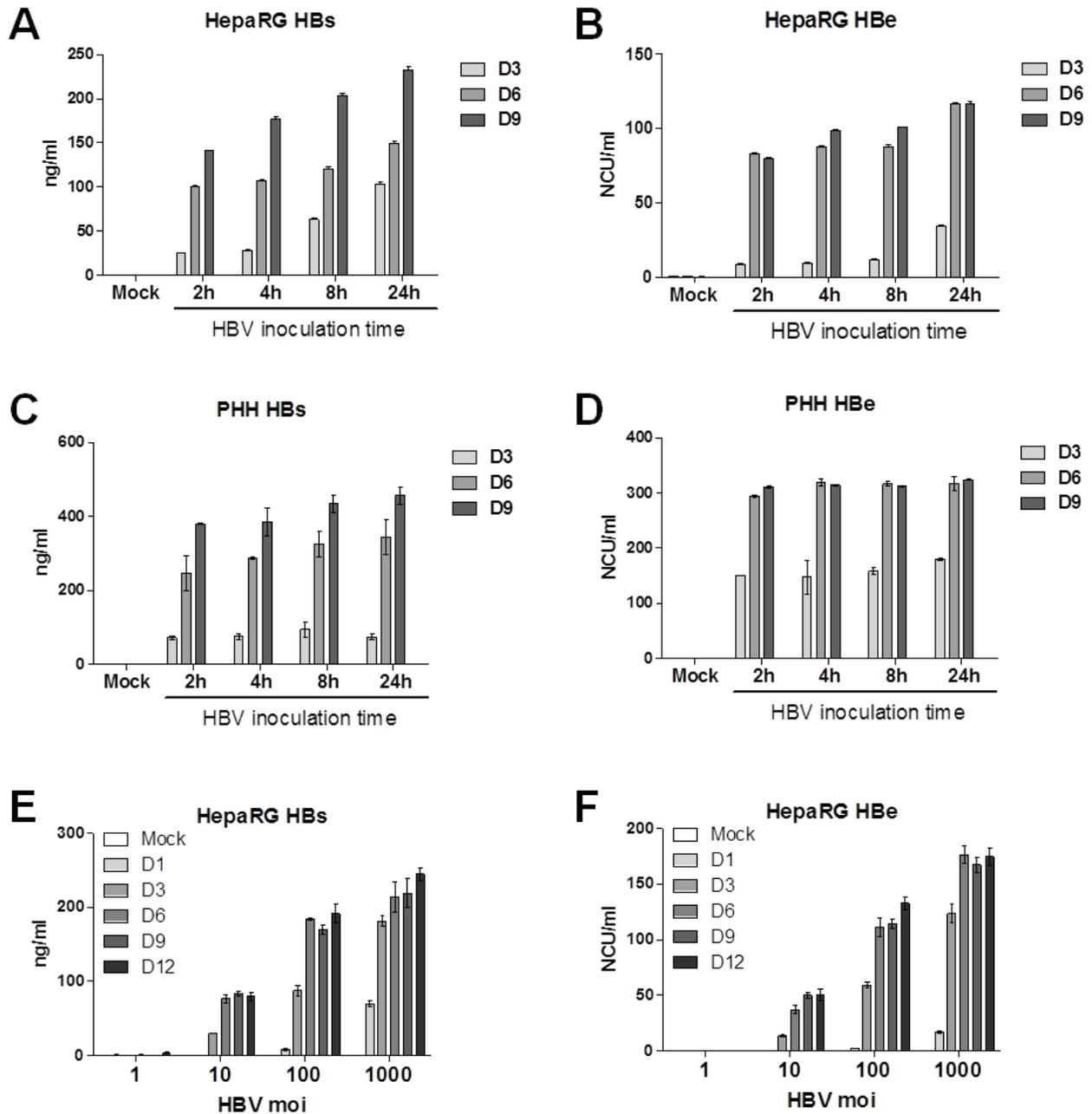
Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.jhep.2015.07.014>.

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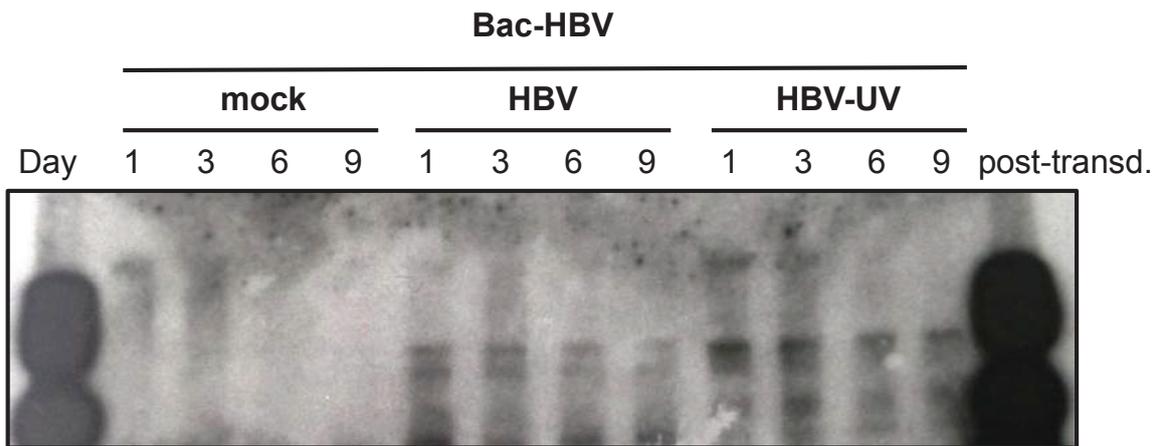
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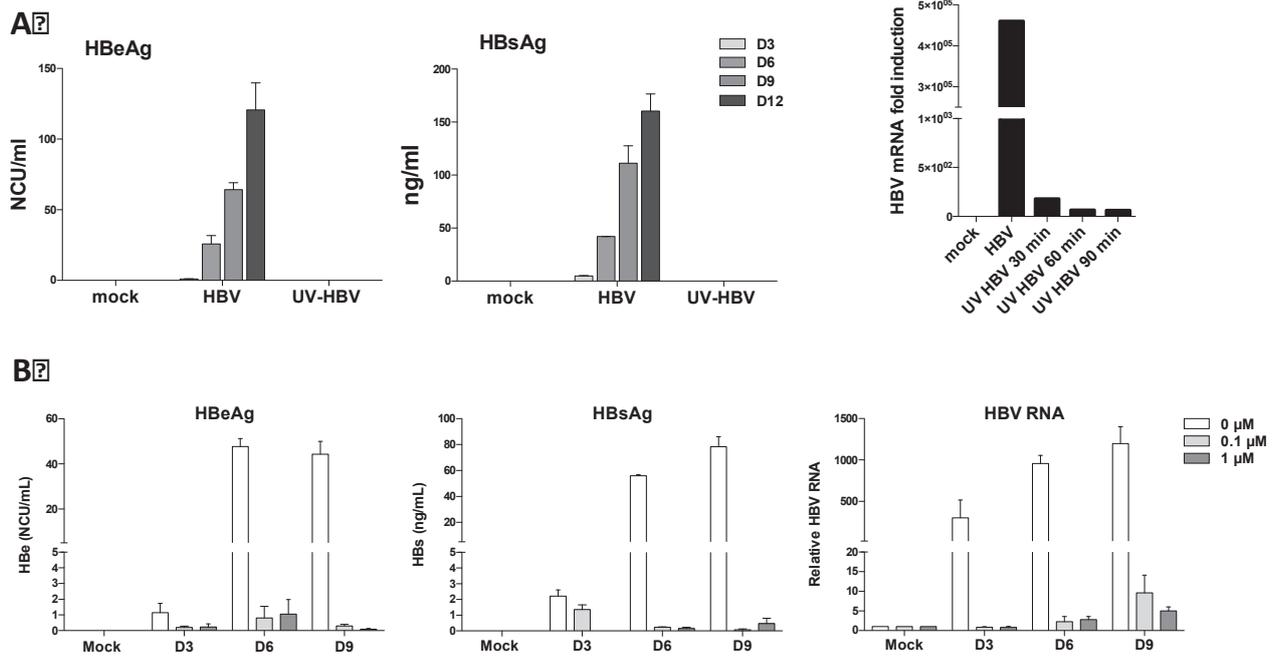
**Supplementary Figure 1. Kinetics of HBV replication in dHepaRG cells and PHH.**

Cells were infected with HBV (100 vge/cell) for 2, 4, 8 and 24 hr (A-D), or infected with increasing amount of virus (1-10-100-1000 vge/ml) for 24 hr (E- F). The secretion of HBsAg and HBeAg was measured by ELISA at day-3, 6 and 9 post-infection in dHepaRG cells (A-B, E-F), and PHH (C-D). Results are given as a mean  $\pm$  SD of one representative experiment.



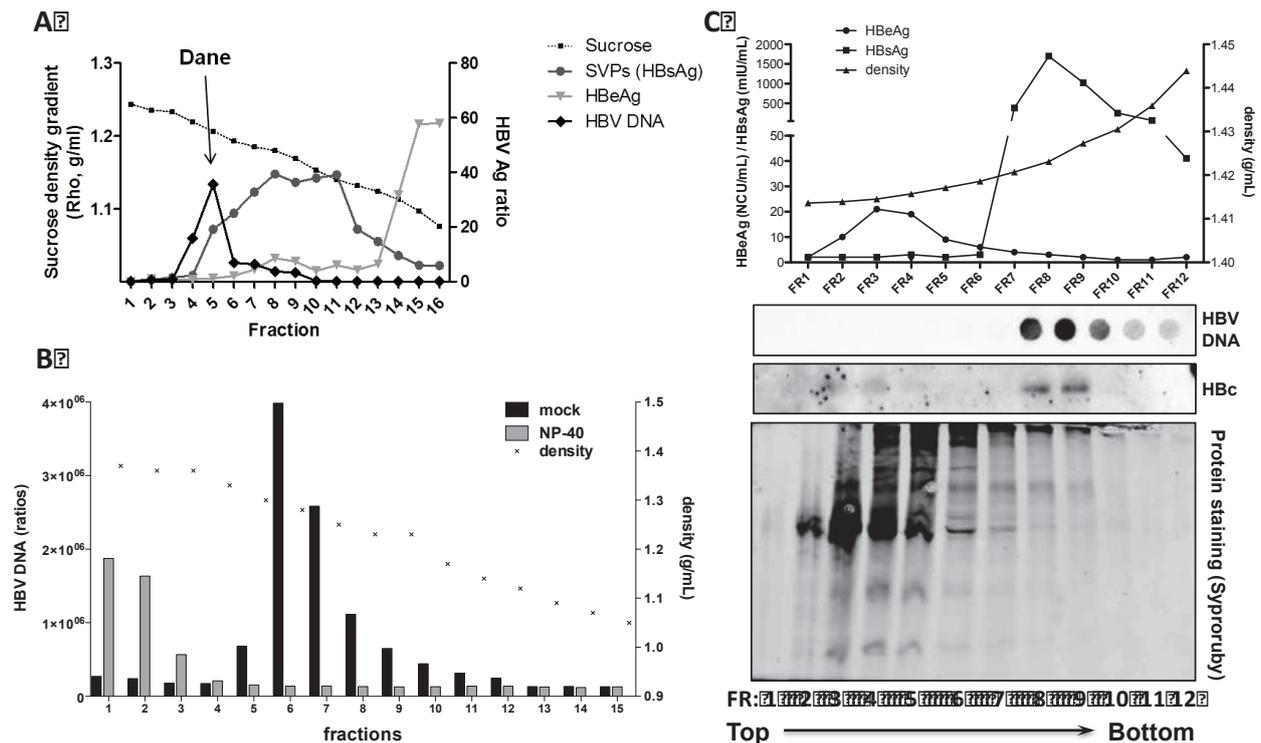
**Supplementary Figure 2. Kinetics of Bac-HBV-mediated intracellular replication in mock or HBV-pre-exposed dHepaRG.**

dHepaRG cells were either mock, HBV, or UV-inactivated HBV infected (100 vge/cell) for 24 hr, then super-transduced with Bac-HBV at a m.o.i. of 25 pfu/mL. A m.o.i. of 25 was used here instead of 100 as used in other part of the work, to prevent cell toxicity. Total DNA was then extracted at indicated days post-transduction, run into a 1% agarose gel in 1x Tris/Borate/EDTA buffer, transferred into positively charged nylon membrane, and subjected to Southern blot analysis using a P<sup>32</sup> radioactive probe against HBV.



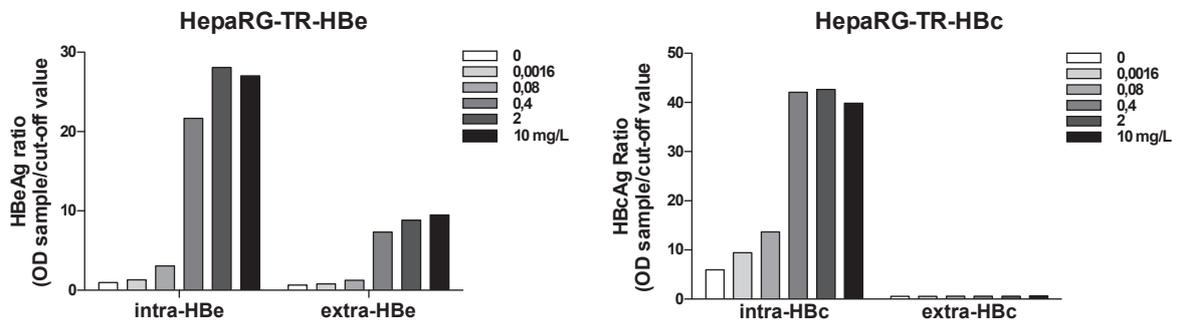
**Supplementary Figure 3. Kinetics of HBV replication after infection with UV-inactivated HBV or after inhibition with entry inhibitor myrcludex.**

(A) HBV/ mock inoculum was irradiated or not for 30, 60 and 90 min and dHepaRG cells were incubated for 24h with either HBV or UV-inactivated HBV (UV-HBV) inocula (A). The mRNA level of HBV was measured by RT-qPCR 12 days post-infection to follow the effect of the UV-inactivation on the virus replication and the secretion of HBeAg and HBsAg was monitored from day-3 to day-12 post-infection in the HBV or UV-HBV infected cells and results were given as a mean  $\pm$  SD of one representative experiment. (B) dHepaRG were mock-or HBV-infected (100 vge/cell) in absence or presence of the indicated amount of Myrcludex (entry inhibitor) for 24 hr. At the end of the inoculation time cells were extensively washed with cold PBS, and HBV replication monitored over-time by ELISA (HBe and HBsAg detection) and RTqPCR (HBV RNA detection).



**Supplementary Figure 4. Purification of viral components and verification of the purity of viral inocula by sucrose gradient ultracentrifugation.**

(A) HBV infectious particles (Dane particles), subviral particles SVPs (HBsAg) and HBeAg were purified through a sucrose cushion, then a sucrose gradient as described in the experimental procedure section. The purity of each fraction was tested by qPCR (for the Dane particles) and by ELISA for HBsAg and HBeAg (left axis), and the density determined by refractometry (right axis). (B) PEG precipitated HBV inocula were either mock or NP40-treated, then loaded onto a sucrose gradient (20-60%), and subjected to ultracentrifugation at 100,000g for 16 hr at 4°C. HBV DNA was detected in each fraction by qPCR (left axis) and the density determined by refractometry (right axis). (C) Typical HBV inoculum used for experiments was passed through a 5.6-56% iodixanol gradient to separate viral components. Twelve fractions were collected then analyzed by WB to detect the core protein, by dot blot to detect viral DNA, by ELISA to detect HBs and HBe antigens, and by SDS-PAGE/Syproruby stain to check purity of Dane containing fractions.



### Supplementary Figure 5. Expression of HBeAg in HepaRG-TR-HBe cell line.

A recombinant HepaRG cell line was engineered using two lentiviruses to transfer genes encoding respectively tetracycline repressor (TR) and preCore gene (HBe). The cell line was named HepaRG-TR-HBe. Another cell line was created with the core gene, named HepaRG-TR-HBc, and used as control. Both cell lines were used to monitor the expression of respective proteins upon tetracycline induction. Increasing doses of tetracycline (in indicated mg/L) were added to cells for a period of 48 hr. Then, both cell supernatants and intracellular protein extracts were assayed in ELISA to detect HBe and HBc. HBe protein can be secreted, whereas HBc could not.



## 4. Second study

### **Virion-associated Hepatitis B core (HBc) protein is an early negative regulator of hepatocyte Interferon (IFN) response**

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**In preparation.**

## Summary

Hepatitis B virus (HBV), as many other viruses, has evolved different strategies to evade immune responses to establish and maintain viral persistence. In particular, HBV does not seem to induce any measurable IFN and pro-inflammatory responses during acute infection. The absence of innate immune response could be due to an impaired detection of HBV by the host innate sensors or could result from an active evasion via the prompt establishment of inhibitory mechanisms.

In a previous work, we showed that HBV virion could trigger a very early inhibition of the hepatocyte-mediated IFN response immediately after infection. Here, we demonstrate that HBV core protein (HBc) is the main viral component involved in this inhibitory process. After HBV entry and migration of nucleocapsids to the nuclear pore, HBc enters the nucleoplasm, where, after binding to target promoters, it impairs the activation of specific innate immunity gene expression upon dsRNA sensors engagement. The recruitment of EZH2 histone methyltransferase is responsible for the establishment of repressive epigenetic histone marks (H3K27) at specific ISG-related HBc-bound-promoters, affecting IFN gene response immediately after the onset of infection. This inhibitory mechanism is maintained during persistent infection thanks to the recycling of nucleocapsids to the nucleus. Interestingly, we also found that this inhibition is at work in non-parenchymal cells (Kupffer and liver endothelial sinusoidal cells) into which HBV does penetrate and deliver HBc to the nucleus compartment.

HBV has evolved a strategy to impair IFN response immediately after the onset of infection by using the incoming virion structural protein HBc to regulate gene expression by epigenetic mean.

## Introduction

Around 250 million individuals are chronically infected with hepatotropic viruses (WHO), which represent, if left untreated, a high risk of developing liver cirrhosis and hepatocellular carcinoma (Arzumanyan et al., 2013; El-Serag, 2012). Hepatitis B virus (HBV), a DNA virus belonging to the *Hepadnaviridae* family, is highly endemic in some area of the globe where parenteral transmission was until recently possible. The natural history of HBV infection is quite complex with distinct physiopathology and therapeutic stages. The natural course of HBV infection consists of four phases: i) the previously called “immune-tolerance” phase (IT), now referred as “low-inflammatory” phase, during which the virus replicates at very high levels without immune control and apparent liver damage, ii) a chronic-active-hepatitis (CAH) phase, iii) an inactive-carrier phase, and iv) potential reactivation episodes (Busch and Thimme, 2015; Fattovich et al., 2008; McMahon, 2010).

To establish and maintain a chronic infection, HBV has evolved various strategies to evade the host innate and adaptive immune responses (Ait-Goughoulte et al., 2010; Bertoletti and Ferrari, 2012; Dandri and Locarnini, 2012; Ferrari, 2015; Tan et al., 2015). Several HBV proteins, including those expressed within the infected hepatocytes (e.g. HBx, HBV polymerase) or the HBV antigens released in the bloodstream (i.e. HBsAg and HBeAg), have been shown or proposed to bear immune-modulatory functions within infected hepatocytes or the liver micro-environment, with subsequently a lack of immune cell cross-talk and antigen presentation, as well as a general HBV-specific T cell exhaustion (Ait-Goughoulte et al., 2010; Bertoletti and Ferrari, 2012; Dandri and Locarnini, 2012; Ferrari, 2015; Tan et al., 2015). To restore/favour an immune control of the virus, virus-mediated inhibitory mechanisms would have to be defeated/unlocked in an interventional therapeutic perspective. To this end, a better knowledge of the underlying molecular mechanisms responsible for the blockade of host immune responses is crucial before envisaging such strategies.

The interferon response is a central component of host antiviral strategies (de Weerd and Nguyen, 2012; Randall and Goodbourn, 2008; Wang and Fish, 2012). This response, which involves the production of a wide range of IFNs (type-I, II and III), may occur within the infected cells or in the microenvironment by the activation of professional innate immune cells (i.e. IPC; IFN producing cells such as pDC or mDC2-BDCA3+). This response is crucial to hold the early spreading of viruses, to reduce the intensity of their replication, to finely tune cellular

innate response and to finally enable the orchestration of subsequent adaptive response. In turn, viruses, in particular those that progress to chronicity, have evolved many strategies to evade this IFN response to their own benefit (Bowie and Unterholzner, 2008; Heim, 2013; Manel and Littman, 2011; Randall and Goodbourn, 2008). In this respect, HBV is a peculiar virus, as, in contrast to HIV or HCV, it does not seem to induce a measurable systemic production of IFN neither in the early (Dunn et al., 2009; Stacey et al., 2009) nor in the late phase of human infection (Rehermann and Bertoletti, 2015). If the results obtained with human samples are subjected to caution, because of the difficulty to get robust and homogeneous cohort of patients during the acute phase of infection, the experiments performed in self-resolving HBV-infected chimpanzees have suggested that innate immunity genes were not up-regulated in their liver when HBV viremia was exponentially rising (i.e. few weeks post infection) (Wieland et al., 2004). Since then, HBV was considered as a “stealth” virus, which does not induce IFN response, likely due to a lack of detection or activation, by innate sensors (pattern recognition receptors; PRRs) (Wieland et al., 2005). Corroborating this, a close look at the viral life cycle indeed suggests that viral PAMPs (i.e. pathogen associated molecular patterns), in particular viral nucleic acids, are not highly exposed to PRRs (Seeger et al., 2015). However, a stealth virus can also be a virus that is able to actively inhibit nascent responses.

Substantiating this hypothesis, our recent work, performed *in vitro* in infected primary human hepatocytes (PHH) or closely related in HepaRG cells {Luangsay, 2015 #96}, has shown that HBV could be weakly detected by PRRs in hepatocytes; however, HBV was capable to actively and rapidly counteract nascent innate response. The inhibitory mechanism involved a viral component present in HBV virions, but was neither due to HBsAg nor HBeAg {Luangsay, 2015 #95}. The core protein (HBc) and HBV polymerase, with a stoichiometry of 240 unit to 1 in favour of HBc (Seeger et al., 2015), were potentially the two viral proteins composing the infectious virions that could mediate this inhibitory effect. In this respect, it was shown that over-expression of the viral polymerase could both inhibit RIGI/MDA5 and STING-mediated IFN- $\beta$  induction by either interfering with DDX3, a regulating component of the IRF3 kinase complex (Wang and Ryu, 2010) or the K63-linked ubiquitination of STING {Liu, 2015 #98}. Other studies have also suggested, using a reporter gene and a non-physiologic expression system, that HBc could inhibit the expression of IFN- $\beta$  and MxA by directly interfering with

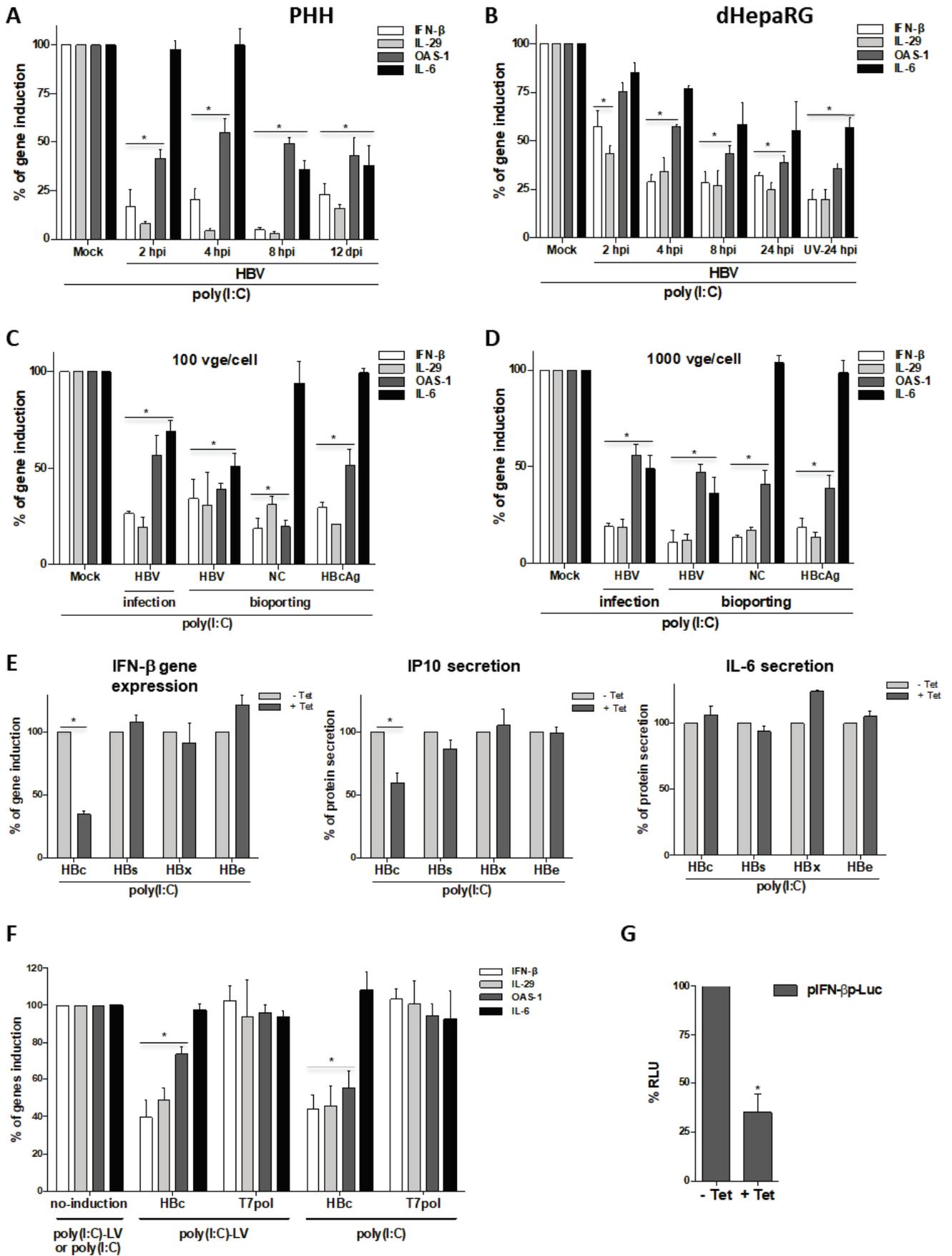
their transcription (Fernandez et al., 2003; Rosmorduc et al., 1999; Whitten et al., 1991). However, detailed mechanisms about this inhibition remained until now largely unknown.

The aim of this work was to identify the HBV-virion associated factor responsible for the early inhibition of IFN response we evidenced in a previous work {Luangsay, 2015 #95}, and to get more insights on the molecular underlying inhibitory mechanism. In this study, HBc was identified as the major viral determinant responsible for this inhibition. This is the first demonstration that a viral structural protein of HBV virions is able to modulate the host innate immune response by epigenetic regulation of type-I IFN gene expression, shortly after viral entry/penetration.

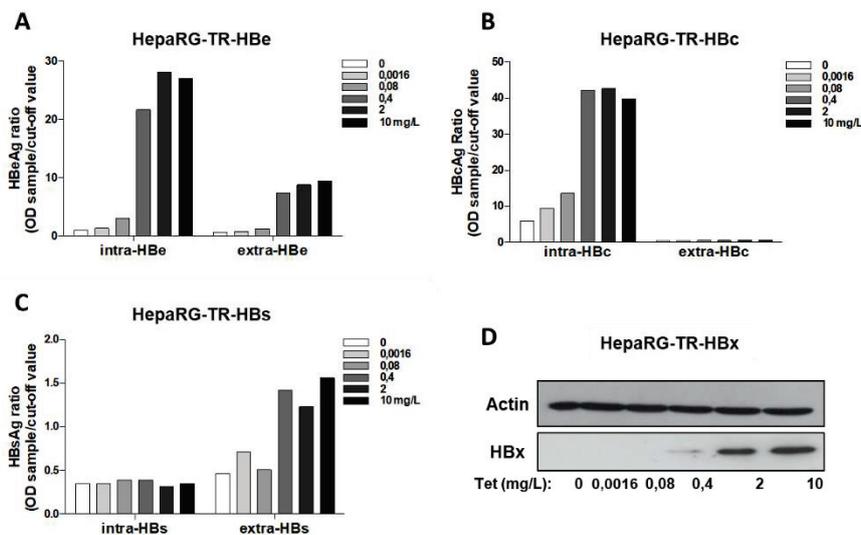
## Results

### ***Virion-associated HBc is the viral determinant involved in the early inhibition of hepatocyte IFN response***

We recently reported that HBV could actively and promptly, i.e. within minutes/hours after the onset of infection, and in the absence of *de novo* viral synthesis, inhibit PRR agonist-mediated IFN response in both differentiated HepaRG (dHepaRG) cells and primary human hepatocytes (Luangsay et al., 2015b). We extended these results by very early kinetic studies (Figures 1A and 1B). Again, a strong and early inhibition of a dsRNA-mediated IFN response (i.e. prototypic type-I (*IFN- $\beta$* ), type-III (*IL-29*) IFN, as well as *OAS-1* as a prototypic ISG) in HBV-infected PHH stimulated with poly(I:C) was observed as early as 2h post-HBV exposure, whereas a delayed inhibition, starting at 8h p.i., was observed for *IL-6* (i.e. prototypic pro-inflammatory gene). Interestingly, we showed that a HBV virion/inoculum-associated factor was responsible for this very early inhibition, as the inhibitory phenotype was still maintained with UV-inactivated virus in HBV-infected dHepaRG (Figures 1B). Since HBsAg and HBeAg, the two secreted viral antigens present in the HBV inoculum, were previously ruled out as early negative regulator of IFN response (Luangsay et al., 2015b), we reasoned that HBc, the major protein composing viral nucleocapsid (240 units/capsid) within infectious particles, could be potentially responsible for this inhibition.

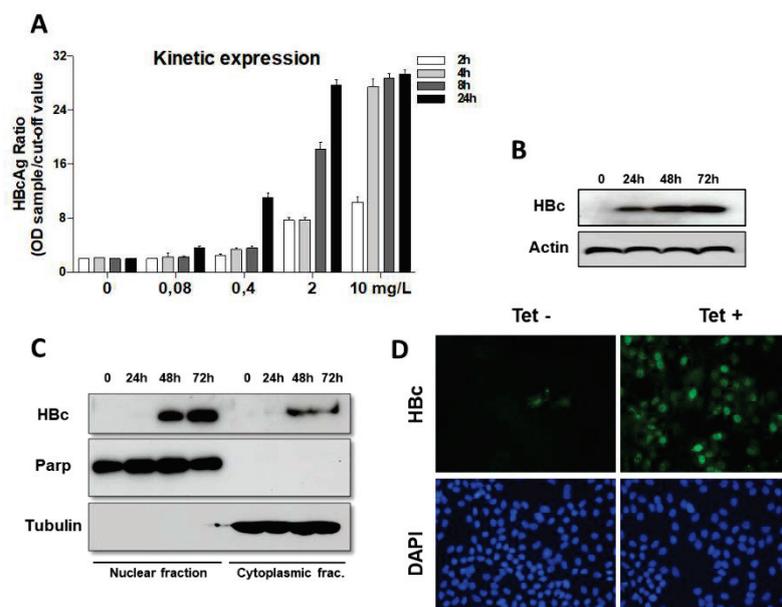


**Figure 1. HBc protein is the viral determinant involved in the early inhibition of dsRNA-mediated IFN responses in hepatocytes.** (A) PHH or (B) differentiated HepaRG were exposed/infected with 1000 vge/cell of wild type or UV-inactivated HBV for the indicated time, then stimulated with poly(I:C)-LMW (10 µg/mL) for 4h. After total RNA purification, the expression of innate immunity genes (*IFN-β*, *IL-29*, *OAS-1* and *IL-6*) was analysed by RT-qPCR and compared to the mock control normalized to 100%. Proliferative HepaRG cells were either mock- or lipo-transfected for 4 hours with (C) low or (D) high amounts of complete HBV inoculum, nucleocapsids or recombinant HBcAg (100 or 1000 vge/cell and HBcAg at 50 or 500 pg /cell) prior to poly(I:C) stimulation (4h). Expression of indicated genes was analysed by RT-qPCR and compared to the mock control normalized to 100%. (E) dHepaRG expressing in a tetracycline dependent manner (Tet at 10 µg/mL; 48h of expression of the corresponding protein) either HBc , HBs, HBx, or HBe were stimulated with poly(I:C) for 4h. Then *IFN-β* gene expression as well as IL-6 or IP-10 secretion were monitored by RT-qPCR or ELISA. (F) Differentiated HepaRG-TR-HBc and HepaRG-TR-T7 cells were treated or not for 24h with tetracycline (Tet, 10 µg/mL) prior to stimulation with poly (I:C) for 4h and gene expression analysed by RT-qPCR and compared to the non-tet-treated cells normalized to 100%. (G) HepaRG-TR-HBc cells were transfected with the pIFN-βp-Luc plasmid, induced or not with tetracycline for 48h, then stimulated with poly(I:C) for 4h. Luciferase activity was analysed on cell lysate with a Luminoskan Ascent. All results are represented as the mean ± SEM of at least 3 independent experiments.

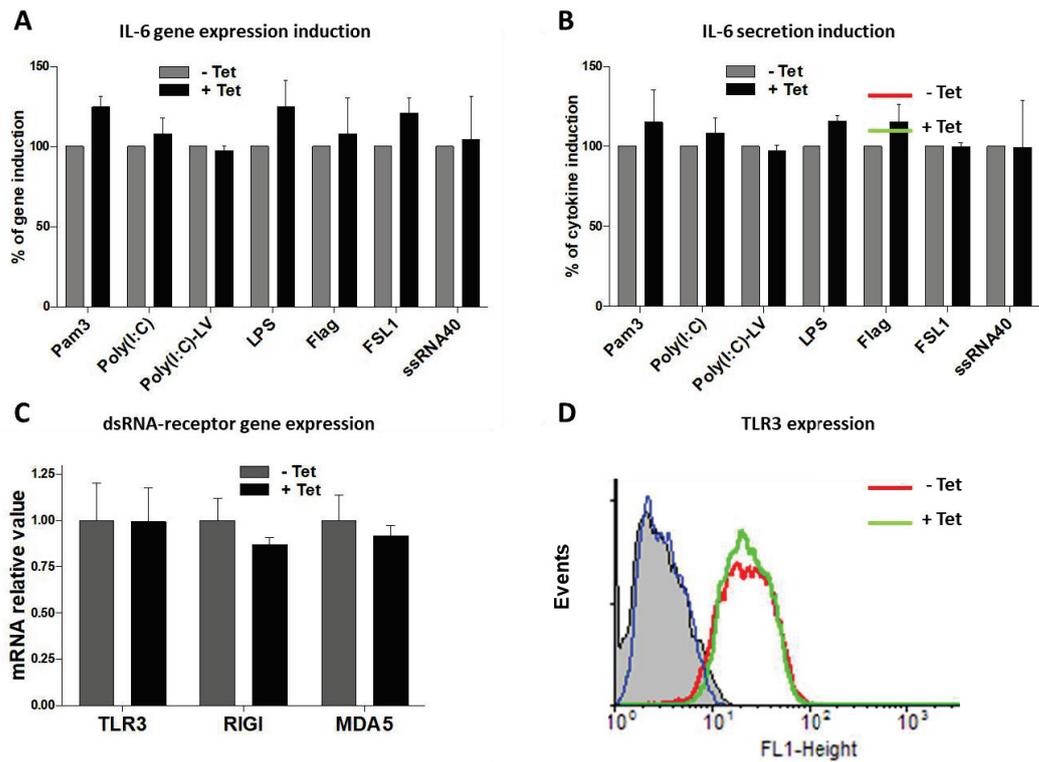


**Suppl. Fig. 1. Analysis of HBV protein expression in various cell lines.** (A, B, and C) Intra- and extracellular expression of HBeAg, HBcAg, and HBsAg quantified by ELISA in indicated cell lines at 24h post-induction with indicated doses of tetracycline. (D) Intracellular expression of the HBx protein by the HepaRG-TR-HBx cell line at 24h post-induction with indicated doses of tetracycline analysed by western blot with anti-V5 (detection of C-terminally tagged HBx) and anti-actin antibodies.

To test this hypothesis, we exposed dHepaRG cells to standardized amounts (100 or 1000 vge/cell) of HBV complete inoculum (i.e. containing HBV virions and subviral particles (SVPs; HBsAg and HBeAg)), non-enveloped nucleocapsids only (NCs, gradient-purified), or recombinant HBc (rechHBc). To allow their cell penetration, NCs and rechHBc, were delivered by lipo-transfection as previously reported (Rabe et al., 2006). Twenty four hours later, cells were stimulated by poly (I:C) for an additional 4h after washing the excess of viral inoculum, nucleocapsids or core proteins. Transfection of dHepaRG cells with complete HBV inoculum, as for that obtained with a conventional infection, could also lead to a significant inhibition of poly (I:C)-mediated induction of the expression of *IFN- $\beta$* , *IL-29*, *OAS-1*, and *IL-6* genes (Figures 1C and 1D). Importantly, similar results were obtained with both transfected NCs and rechHBc, with the exception that induction of *IL-6* gene expression was not affected by these two forms of HBV core protein. This suggests that HBc *per se* is responsible for the specific inhibition of the IFN response, whereas a different type of inhibition of NF- $\kappa$ B-mediated pro-inflammatory response by other HBV proteins could be at play (Luangsay et al., 2015b).



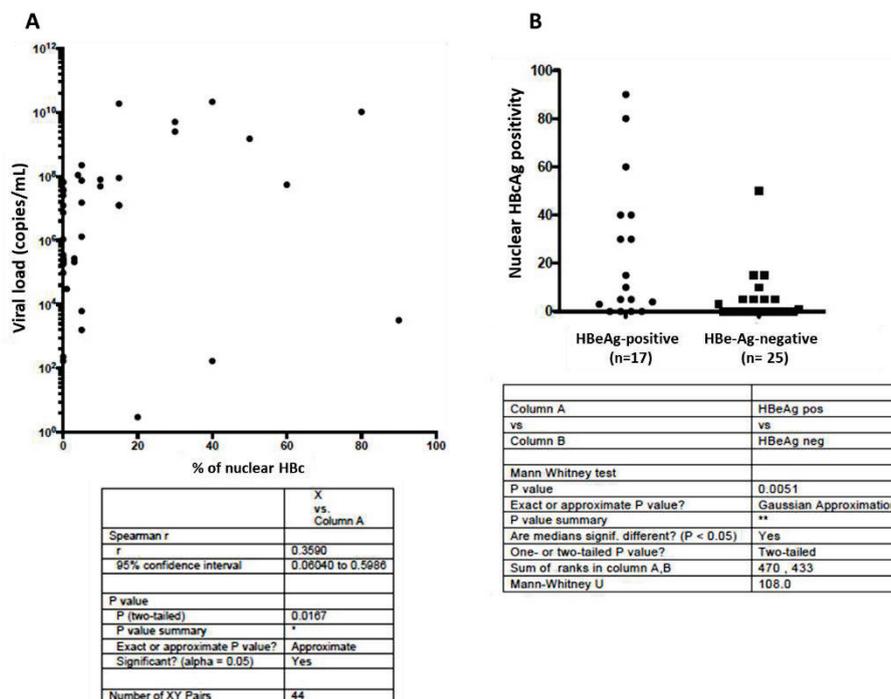
**Suppl. Fig. 2. Core expression and subcellular localization in the inducible HepaRG-TR-HBc cell line.** (A) Kinetic expression of HBcAg analysed by ELISA in differentiated HepaRG-TR-HBc cells, following treatment with several dose of tetracycline (0.08, 0.4, 2 and 10 µg/ml) for 2h, 4h, 8h and 24h. (B) Kinetic of HBc expression after induction with 10 µg/mL of tetracycline for the indicated time analysed by WB. (C) Kinetic and subcellular localization of HBc in tet-induced HepaRG-TR-HBc cells after cytoplasm and nucleoplasm separation analysed by WB. (D) Immunofluorescence analysis of HBc expression (with anti-HBc C1 Abcam) in tet-induced HepaRG-TR-HBc cells at 24h post induction. Cell nucleus were stained with Hoechst reagent.



**Suppl. Fig. 3. Core expression and subcellular localization in the inducible HepaRG-TR-HBc cell line.** (A) IL-6 cytokine gene expression, and (E) protein secretion were analysed by RT-qPCR and ELISA respectively in dHepaRG-TR-HBc cells treated or not for 24h with prior stimulation with several TLRs ligands (TLR2, Pam3CSK4; TLR3, Poly(I:C); RIGI/MDA5, Lyovec-Poly(I:C); TLR4, LPS; TLR5, Flagellin; TLR6, FSL1; TLR7/8, ssRNA40) for 4 hours and compared to the non-tetracycline stimulated HepaRG-TR-HBc cell line normalized to 100%. (C) Expression of *TLR3*, *RIGI*, and *MDA5* genes analysed by RT-qPCR on total RNA extracted from dHepaRG-TR-HBc cells induced or not with tetracycline (10 µg/mL) for 24h. (D) FACS analysis of TLR3 expression dHepaRG-TR-HBc cells induced or not with tetracycline (10 µg/mL) for 24h.

To further demonstrate the prominent role of HBc in the early inhibition of hepatocyte IFN response, we engineered an inducible HepaRG cell line expressing HBV core protein under the control of tetracycline (i.e.). Similar cell lines were also generated for HBs, HBe and HBx expression as well as a control cell line expressing the T7 polymerase protein (Figure S1). In HepaRG-TR-HBc cells, HBc was detected as early as 2 hours post-induction, was not secreted, reached a plateau of expression by 48-72 h post-induction, and was mainly localized within the nucleus of cells (Figure S2). Following HBc, HBs, HBe, or HBx mock- or tet-induction, cells were stimulated for 4h with TLR3-L to measure *IFN-β* gene expression, as well as IP10 and IL-6 secretion. Among the four inducible cell lines, only that expressing HBc showed a reduced induction of *IFN-β* gene expression and IP10 secretion following stimulation, thus confirming

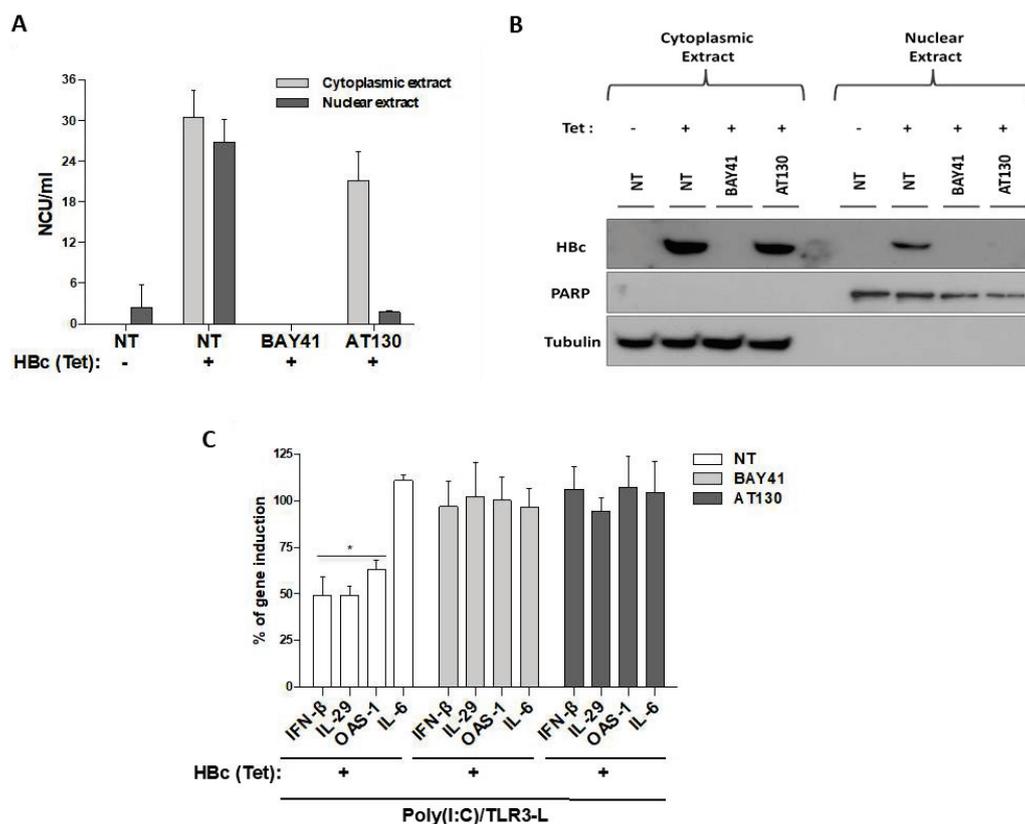
that HBe is the main contributor to this inhibition (Figures 1E). Similar results were also obtained with other prototypic genes of the IFN response, and with a RIGI/MDA5 ligand (poly(I:C)-LMW-Lyovec) (Figure 1F), or using an *IFN- $\beta$*  luciferase reporter construct transfected in HBe-expressing cells (Figure 1G). These results corroborated with those obtained in dHepaRG cells exposed to purified HBV nucleocapsids or recHBe (Figures 1C and 1D). Similarly, in this cell line, IL-6 expression or secretion was not affected by HBe and interestingly none of the other viral protein tested (HBs, HBx, HBe) could modulate its secretion as well (Figures 1E and 1F). The fact that, in our experimental conditions, HBe did not seem to regulate HBV-mediated NF- $\kappa$ B-dependent pro-inflammatory pathways was further confirmed by using other TLR agonists (TLR1/2, TLR3, RIGI/MDA5, TLR4, TLR5, TLR6, and TLR8 agonists) involved in NF- $\kappa$ B dependent IL-6 induction (RNA and protein levels) (Figures S3A and S3B). Importantly, the inhibition of the poly(I:C)-mediated induction of *IFN* and *ISG* genes, observed in HBe-expressing cells, was not due to a modification of expression of dsRNA sensors in the time frame of the experiment (Figures S3C and S3D). Altogether, these results suggest that HBe is an early inhibitor of the IFN response in hepatocytes exposed to HBV.



**Suppl. Fig. 4. HBe immunohistochemistry in biopsies of HBeAg positive and HBe-Ag negative patients.** A total of 42 biopsies from HBe-Ag positive (n= 17) and HBe-Ag negative (n=25) were labeled with an anti-HBe antibody and revealed by immunoperoxidase staining. The percentage of cells with nuclear staining was established and plotted in function of viremia (A) or HBeAg status (B). Statistics were done with the Mann-Whitney test.

### ***Nuclear localization of Hbc is required to efficiently impair dsRNA-mediated IFN response***

It has been previously proposed that Hbc could be mainly localized in the nucleus of hepatocytes in patients with high viremia (i.e. IT/low inflammatory phase or HBeAg+ patients in CAH phase), whereas a cytosolic or mixed subcellular localization was observed in patients well-advanced in CAH phase (HBeAg negative patients). This suggested that the nuclear localization of Hbc could be associated with a high viremia and a poor/lack of immune response (Akiba et al., 1987; Chevallier-Queyron and Chemin, 2011; Chu and Liaw, 1987). By performing ISH staining on liver biopsies from a cohort of 45 patients in CAH (18 HBeAg-positive and 27 HBeAg-negative patients), correlations between the viral load and percentage of hepatocytes positive for HbcAg in the nucleus (Figure S4A), and between HBeAg positivity and percentage of hepatocytes positive for HbcAg in the nucleus (Figure S4B) were found. Our results confirmed that high viremia seems to correlate with a higher localization of HbcAg within the nucleus of infected hepatocytes.



**Figure 2: Capsid assembly inhibitor treatment reverts Hbc-mediated inhibitory effect.** (A and B) Differentiated HepaRG-TR-Hbc cells were treated or not for 72h with Bayer41-4109 (1  $\mu$ M) or AT130 (3  $\mu$ M) prior stimulation with tetracycline (10  $\mu$ g/mL) for 24h. Nuclear and cytoplasmic protein extract from dHepaRG-TR-Hbc cells

stimulated with tetracycline (10 µg/ml) for 24h were extracted and HBcAg were evaluated by ELISA (A) and western blot (B) using homemade anti-HBc antibody, anti-PARP (nucleus marker), and anti-tubulin (cytoplasmic marker) antibodies. (C) Differentiated HepaRG-TR-HBc cells were treated or not for 72h with Bayer41 (1 µM) or AT130 (3 µM) prior to be stimulated with tetracycline for 24h (2µg/ml) and then treated with Poly (I:C) ligand for 4h. Expression of indicated genes was analyzed by RT-qPCR and compared to the non-tetracycline stimulated HepaRG-TR-HBc cells normalized to 100%. Results in panels A and C are represented as the mean ± SEM of 3 independent experiments.

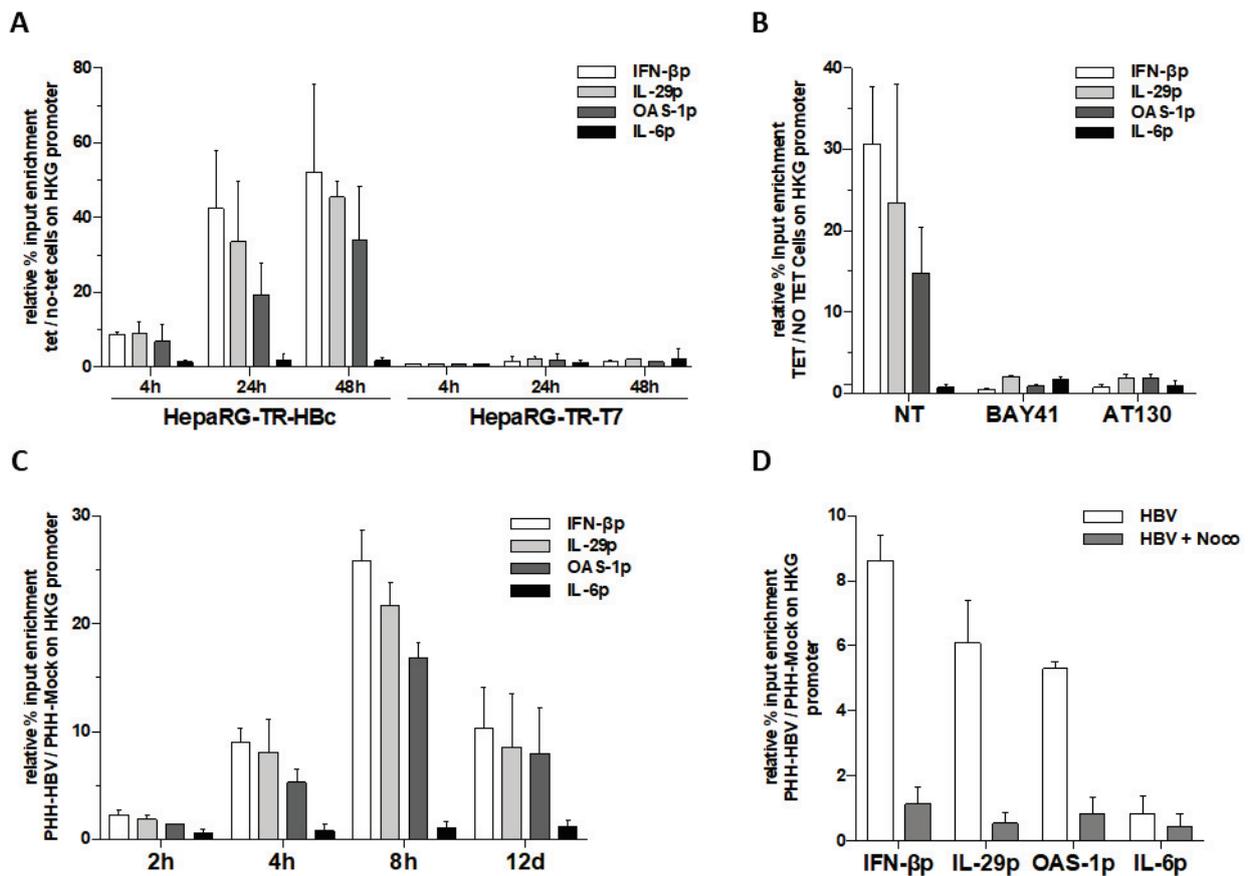
To further investigate whether the nuclear localization of HBc could significantly impact on the hepatocyte innate immune responses *in vitro*, HepaRG-TR-HBc cells were treated, prior to tetracycline induction, with two different capsid assembly inhibitors, an heteroaryldihydropyrimidine (i.e. BAY41-4109) or a phenylpropenamide derivative (i.e. AT-130), which both impair nucleocapsid assembly (Deres et al., 2003; Feld et al., 2007). These two treatments, which could prevent both HBc trafficking and translocation into the nucleus (Figure 2A and 2B), reverted the phenotype of HBc-mediated inhibition of expression of *IFN-β*, *IL-29*, *OAS-1* genes (Figure 2C). These results suggest a correlation between the nuclear localization of HBc and the inhibition of IFN and ISG gene expressions upon engagement of TLR3 or RIGI/MDA5 signaling pathway.

### ***Upon HBV infection, HBc can rapidly interact with hepatocyte innate gene promoters to control IFN response***

In conditions of overexpression of HBc in hepatoma cells, it has been suggested that HBc could interact with host promoters to regulate gene expression. For instance, *IFN-β* and *MX-A* promoters were shown to be repressed by HBc (Rosmorduc et al., 1999; Whitten et al., 1991), whereas HBc was shown to directly bind to ISRE-containing DNA in EMSA experiments (Fernandez et al., 2003). More recently, using liver biopsies from HBV-infected patients and chromatin immunoprecipitation (ChIP) microarrays, it was also shown that HBc could bind *in vivo* to a broad spectrum of CpG rich human gene promoters and potentially modulate their expression, even though functional analyses to validate the results were only performed *in vitro* with few genes (Guo et al., 2012).

To determine whether HBc could interact with our studied gene promoters, we performed ChIP-qPCR experiments on tet-induced dHepaRG-TR-HBc cells using an anti-HBc Ab (and

control Ab) and primers specific for *IFN- $\beta$* , *IL-29*, *OAS-1* and *IL-6* promoters, or control gene promoters (i.e. *HKG*, *GAPDH*, or *DHFR*). HbC was found to interact with *IFN- $\beta$* , *IL-29* and *OAS-1* promoters, but not with *IL-6* one, as early as 4 hours post-induction (Figure 3A). Interestingly, the blockade of the nuclear localization of HbC by capsid assembly inhibitor treatments that prevent HbC entry into the nucleus resulted in a marked decrease of the interaction between HbC and gene promoters in HbC-expressing cells (Figure 3B).



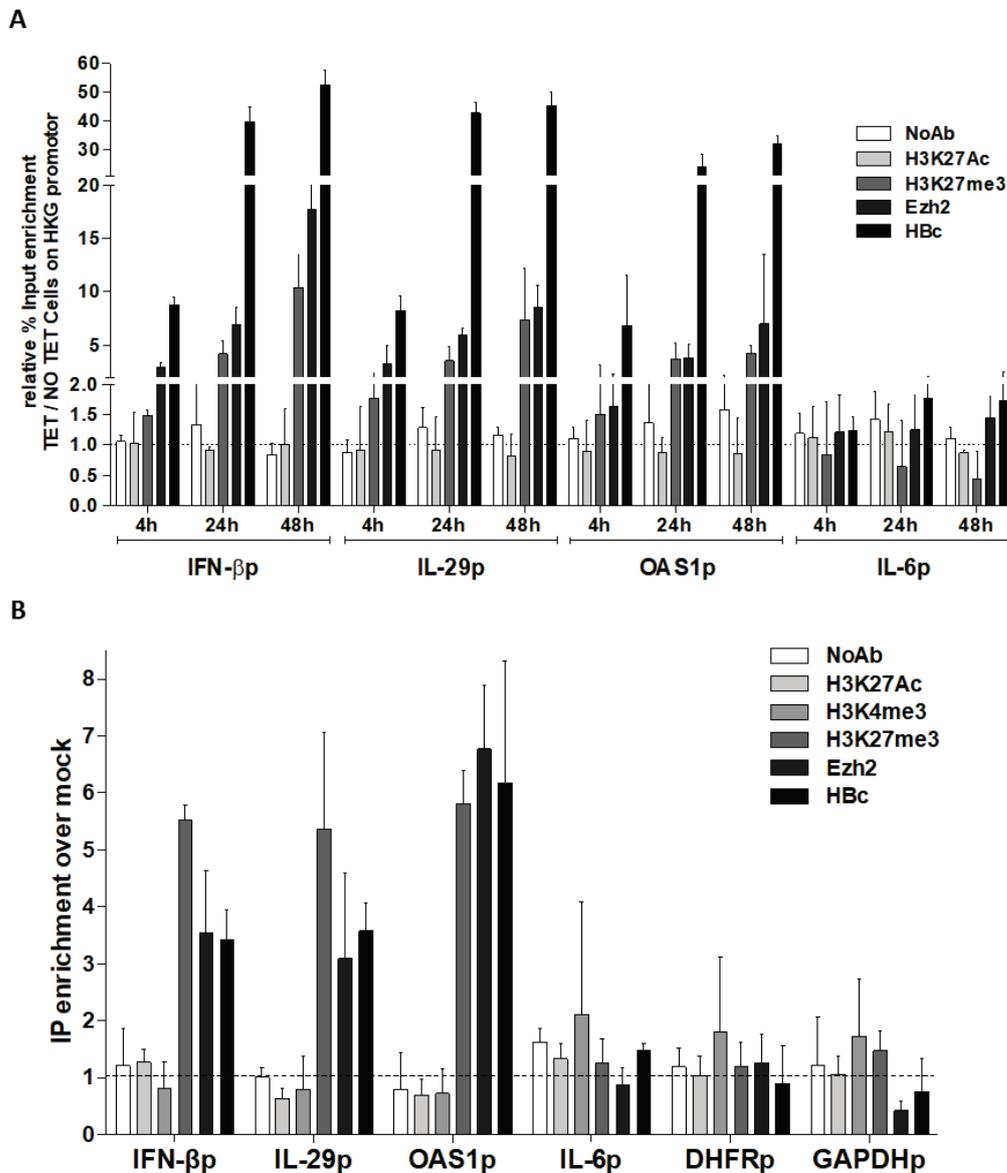
**Figure 3: HbC protein interacts rapidly with innate gene promoters to inhibit dsRNA-mediated IFN response in hepatocytes.** (A) Differentiated HepaRG-TR-HbC and HepaRG-TR-T7 cells were treated for 4h, 24h and 48h with tetracycline (10  $\mu$ g/mL), or (B) dHepaRG-TR-HbC cells were treated or not for 72h with Bayer41-4109 (1  $\mu$ M) or AT130 (3  $\mu$ M) prior to be stimulated with tetracycline (10  $\mu$ g/mL) for 24h, and ChIP-qPCR was performed with homemade anti-HbC (or isotopic antibodies) and specific primers for *IFN- $\beta$* , *IL-29*, *OAS-1* and *IL-6* promoters. The percentage of input enrichment was normalized on the non-tetracycline stimulated HepaRG-TR-HbC cells and on HKG promoter. (C) PHH were infected with a complete HBV inoculum for 2h, 4h 8h, or 12 days and ChIP-qPCR performed as in (A and B). (D) PHH were infected with a complete HBV inoculum for 8h in the presence or absence or nocodazole, and ChIP-qPCR was performed as in (A and B). All results are represented as the mean  $\pm$  SEM of 3 independent experiments.

ChIP analysis was also performed in HBV-infected PHH. The binding of HBc to *IFN- $\beta$* , *IL-29* and *OAS-1* promoters was evidenced, whereas no binding to *IL6* one at any of the time points was observed (Figure 3C). These results are consistent with the observation that *IFN- $\beta$* , *IL-29* and *OAS-1* gene expressions were inhibited by HBc, while the expression of *IL6* was not (Figure 1A). Of note, the binding of HBc was long lasting, as at day-12 pi, HBc could be still “ChIPed” on promoters. Importantly, no binding of HBc on *IL-6* promoter was again observed, thus confirming that HBc did not regulate *IL-6* transcription in our experimental conditions. We previously showed a correlation between HBc nuclear localization and gene expression modulation. To support this observation, we used short-term and non-cytotoxic treatment with nocodazole, which inhibits trafficking and translocation of HBc to nucleus (Rabe et al., 2006), and in turn prevents the binding of HBc to target promoters evidenced by ChIP-qPCR experiment (Figure 3D). Altogether, our results suggest that in the very early phase of infection, HBc from “incoming” virions could, after trafficking through the cytosol and translocation into the nucleus, negatively modulate the IFN response by binding to the promoters of some innate genes and, thereby, interfering with their expression.

***HBc induces the recruitment on target genes of EZH2, a histone methyl-transferase involved in the establishment of repressive epigenetic marks***

Several viruses were shown to manipulate the epigenetic state of the host genome notably by modifying the histone methylation status (Berger, 2007; Ferrari et al., 2012; Fonseca et al., 2012; Niller et al., 2009). We postulated that HBc-mediated repression of innate genes could be due to its binding to targeted promoters and further epigenetic modulations. To demonstrate this, we performed ChIP studies on HBc-expressing cells using antibodies directed either against hyper-methylated or acetylated histone H3 or against EZH2, a methyl-transferase involved in histone H3 methylation at position K27 (Czermin et al., 2002; Wu et al., 2011). Results showed significant enrichment of the repressive H3K27me3 mark on *IFN- $\beta$* , *IL-29* and *OAS-1* promoters, as early as 4h post-HBc expression (Figure 4A). Mirroring the pattern of HBc expression after tet-induction and defining a dose response, the enrichment of this repressive mark further increased at 24h and 48h post-induction, while an active mark (H3K27Ac) remained low throughout the kinetic. Confirming this result, we also found EZH2,

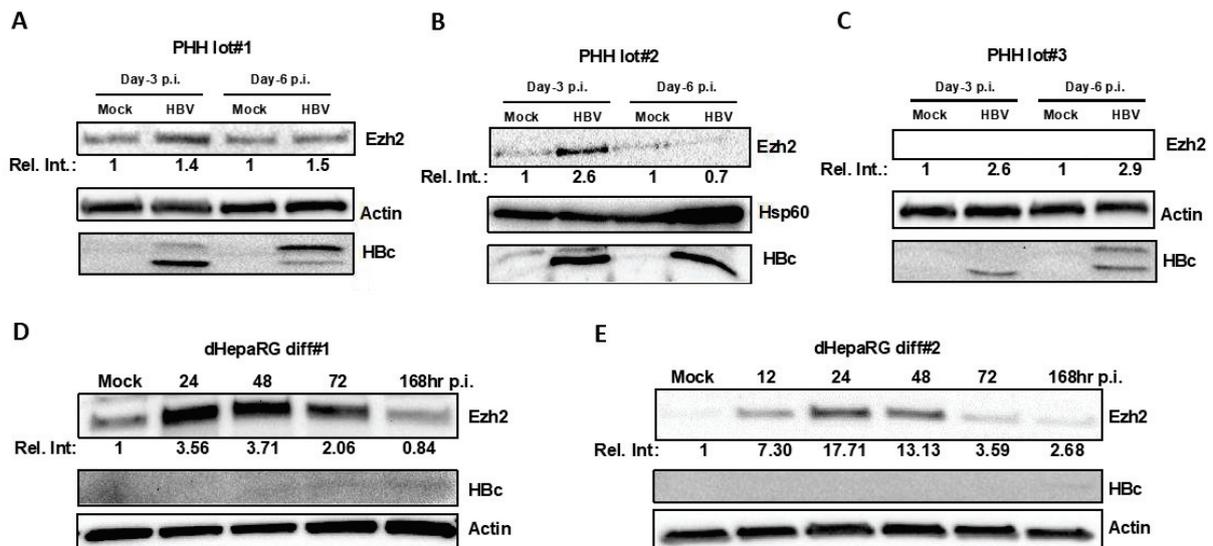
which contributes to the establishment of the H3K27me3 modification, on HBC-regulated promoters (Figure 4A).



**Figure 4: HBC affects host gene epigenetic status to inhibit IFN response in hepatocytes.** (A) Differentiated HepaRG-TR-HBC cells were treated for 4h, 24h, or 48h with tetracycline (10  $\mu\text{g}/\text{mL}$ ), or (B) PHH were infected by 100 vge/cell of HBV for 24h, then histone modifications, recruitment of the histone methyl-transferase EZH2, and binding of HBC to *IFN- $\beta$* , *IL-29*, *OAS-1*, and *IL-6* promoters were assessed by ChIP-qPCR, using either none, control, anti-H3K27Ac, anti-H3K27me3, anti-EZH2, or anti-HBC antibodies. The percentage of input enrichment was normalized either to the non-tetracycline stimulated HepaRG cell lines (A) or to mock infected PHH (B) and on H3G promoter. All results are represented as the mean  $\pm$  SEM of 3 independent experiments.

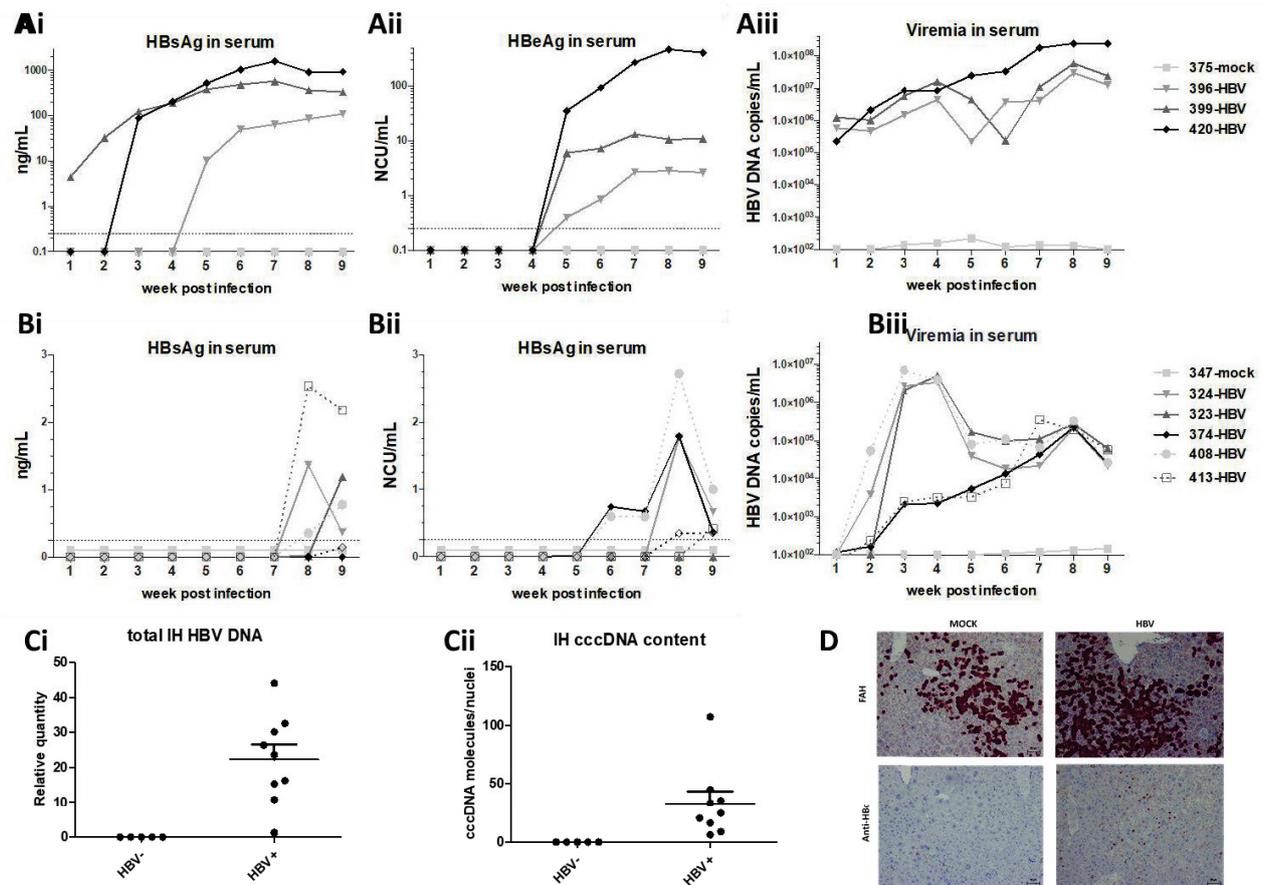
ChIP experiments were also performed on HBV-infected PHH at 24h post-infection and showed a concomitant occupation of *IFN- $\beta$* , *IL-29*, and *OAS-1* promoters by HBC, EZH2, and K27me3 histone H3, while no active marks (H3K27Ac and H3K4me3) were found (Figure 4B).

This result is consistent with the fact that the binding of HBc to specific innate gene promoters could result in a direct or indirect recruitment of EZH2, which in turn contributed to the establishment of epigenetic repressive marks by tri-methylation of H3 at position K27. Therefore, these results strongly suggest that the inhibition of IFN response is due to a very early epigenetic reprogramming of innate immunity genes caused by the active transport into the nucleus and binding of incoming HBc to their promoters.



**Figure 5: HBV induces EZH2 expression very early after the onset of infection.** Three independent batches of PHH (A to C) and two different lots of differentiated HepaRG (D and E) were infected with 500 vge/cell of HBV and protein harvested at indicated times. Cell protein extracts were subjected to western blotting using anti-EZH2, anti-HBc and anti-Actin or anti-HSP60 antibodies.

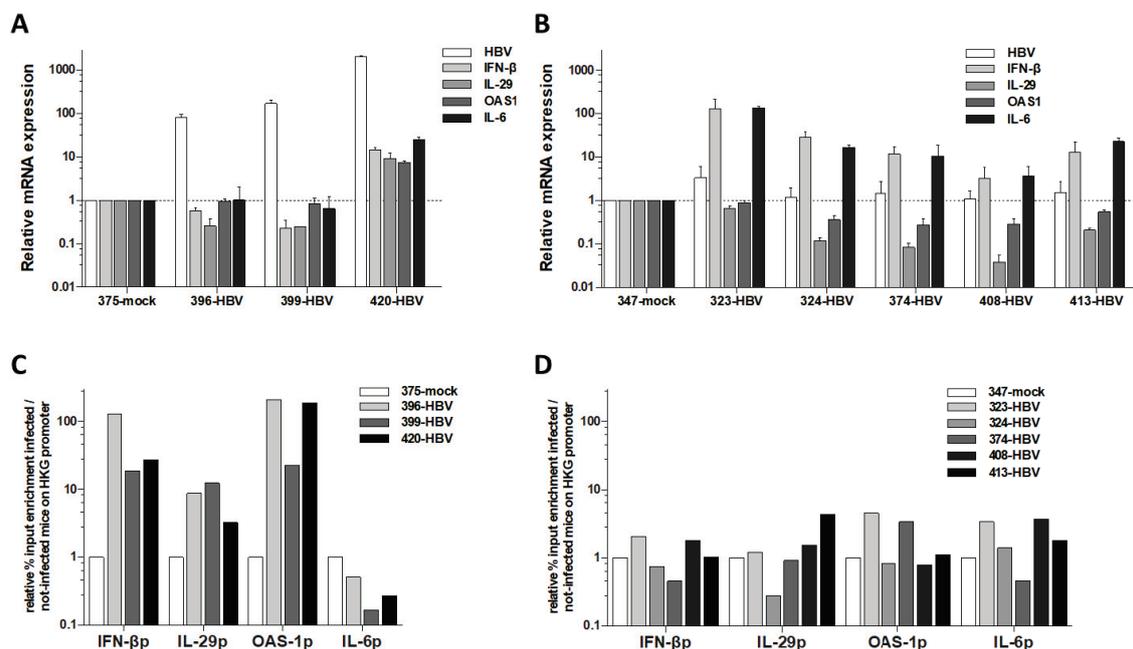
In order to determine whether HBc could be directly responsible for the recruitment of EZH2 on target promoters, we attempted to co-immunoprecipitate HBc and EZH2 using specific antibodies, but failed to detect interaction between these proteins in HepaRG-TR-HBc cell lines, as well as in HBV-infected PHH. We also analyzed the effect of HBV infection on EZH2 expression. In both HBV-infected PHH (Figure 5A-5C) and dHepaRG (Figures 5D and 5E), we found an increased expression of EZH2, as compared to kinetically-paired and non-infected hepatocyte. It is worth noting that the weak/lack of HBc detection in HepaRG cells is due to very low level of HBV replication in this model as compared to PHH.

**Long-term *in vivo* maintenance of the HBc-mediated inhibition of IFN response**

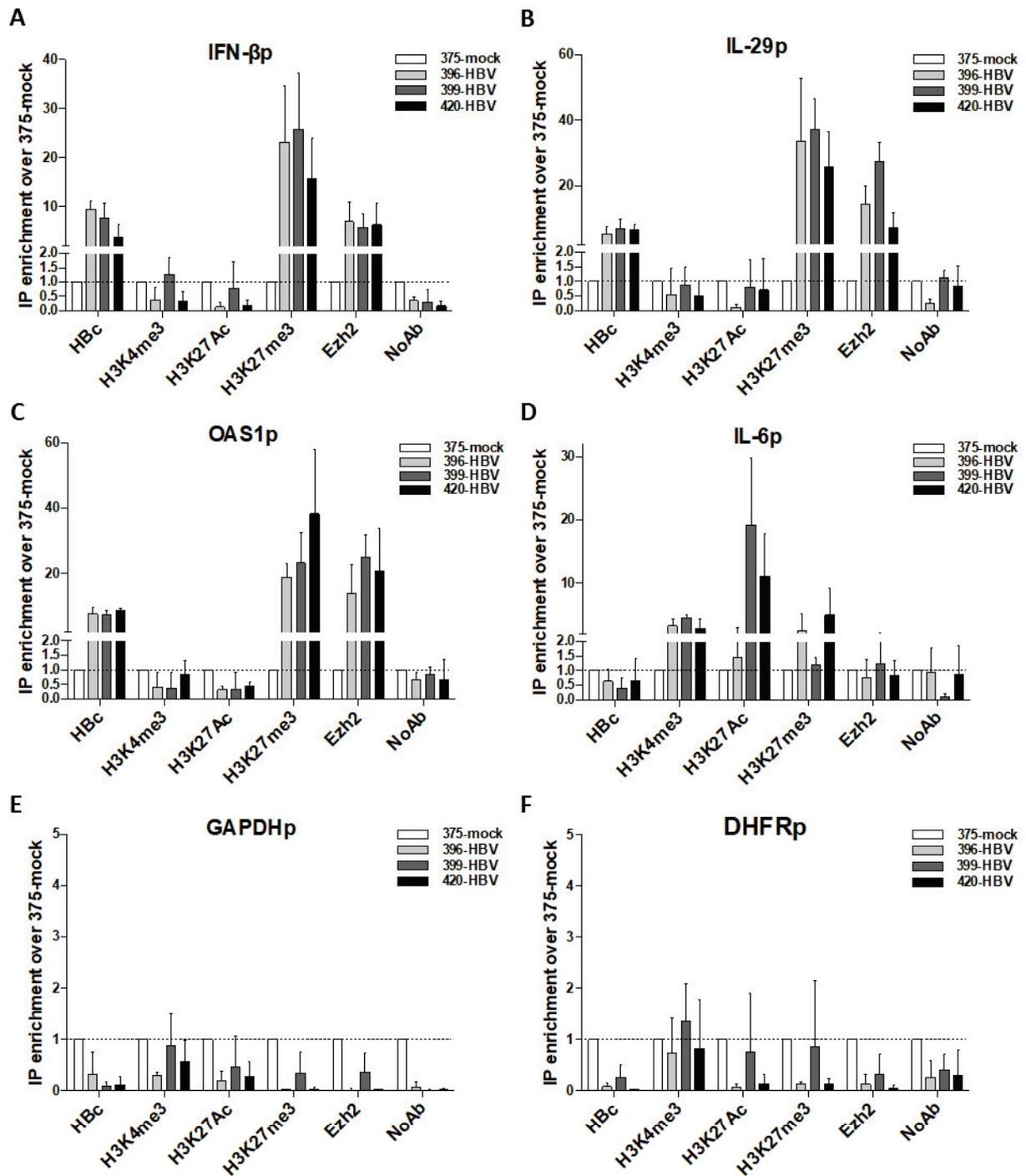
**Suppl. Fig. 5. Viremia, antigenemia, and intra-liver virologic parameters in HBV-infected FRG mice.** (A and B) Viremia and antigenemia (HBeAg and HBsAg) was monitored every week for 9 weeks after HBV ( $5.10e8$  vge/mouse for panels A;  $5.10e7$  vge/mouse for panels B) infection by qPCR and ELISA. (C) Intra-liver total HBV DNA and cccDNA levels were obtained by qPCR and cccDNA specific qPCR {Werle, 2004 #88}. (D) Immunohistostaining was performed on paraffin embedded mouse liver samples with anti-HBc (Dako) and human-specific anti-FAH (Cliniscience) antibodies. Representative picture of immune-staining in mock- and HBV-infected mice are shown.

The next step was to determine whether HBc could play the same role *in vivo*. We first investigated the sub-cellular localization of HBc in human hepatocytes engrafted in liver-humanized FRG mice (Bissig et al., 2010), which were chronically infected with HBV. In mice showing persistent infection and high levels of HBV DNA and viral proteins in the serum and liver (Figure S5A and S5C, i.e. mice 396, 399, and 420 versus mock infected 375), we assessed for the cellular distribution of HBc in hepatocytes and found it mainly in the nucleus (Figure S5D, lower right panel). These results were consistent with the data obtained with HBc-

expressing HepaRG cells (Figure S2) and in liver biopsies of chronically HBV infected patients during the immune-tolerance phase (Akiba et al., 1987; Chevallier-Queyron and Chemin, 2011; Chu and Liaw, 1987). Then, ChIP experiments were performed in the liver of these chronically HBV-infected mice to measure Hbc binding to innate gene promoters, as well as the association of EZH2 and related epigenetic marks (Figure 6). Results showed that these highly HBV replicative mice featured concomitant similar enrichment to IFN-related gene promoters for Hbc, EZH2 and the repressive H3K27me3 mark, whereas active marks (H3K27Ac and H3K4me3) were not changed. In addition, in these mice the epigenetic profile was associated with a low expression of innate genes (Figure S6A and S6C). Moreover, on the *IL-6* promoter, neither Hbc, nor EZH2 and the associated repressive mark H3K27me3 were found enriched, whereas positive marks (H3K27Ac and H3K4me3) were found with a low level of enrichment, thus confirming the absence of Hbc effect on *IL-6* promoter. Interestingly, in mice with low viremia and antigenemia (Mice 323, 324, 374, 408 and 413; Figure S5B), Hbc was not found associated to *IFN- $\beta$* , *IL-29*, and *OAS-1* promoters (Figure S6D), and *IFN- $\beta$*  and *IL-6* genes were found significantly up-regulated (Figure S6B), which nicely correlated with the low replicative status.



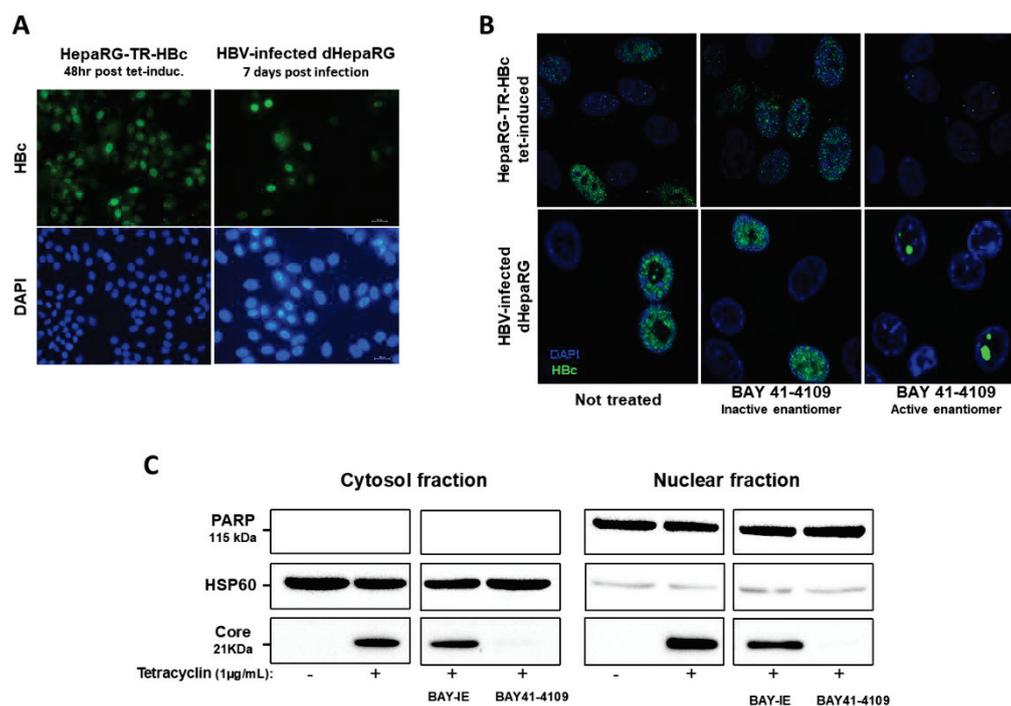
**Suppl. Fig. 6. Innate immune gene expression and ChIP analysis with mouse liver samples.** Expression of indicated innate immune genes and level of HBV RNA in high (A) and low (B) viremia mice as measured by RT-qPCR. Results of ChIP-qPCR experiments done with anti-Hbc and primers specific to indicated promoters in high (C) and low (D) viremia mice.



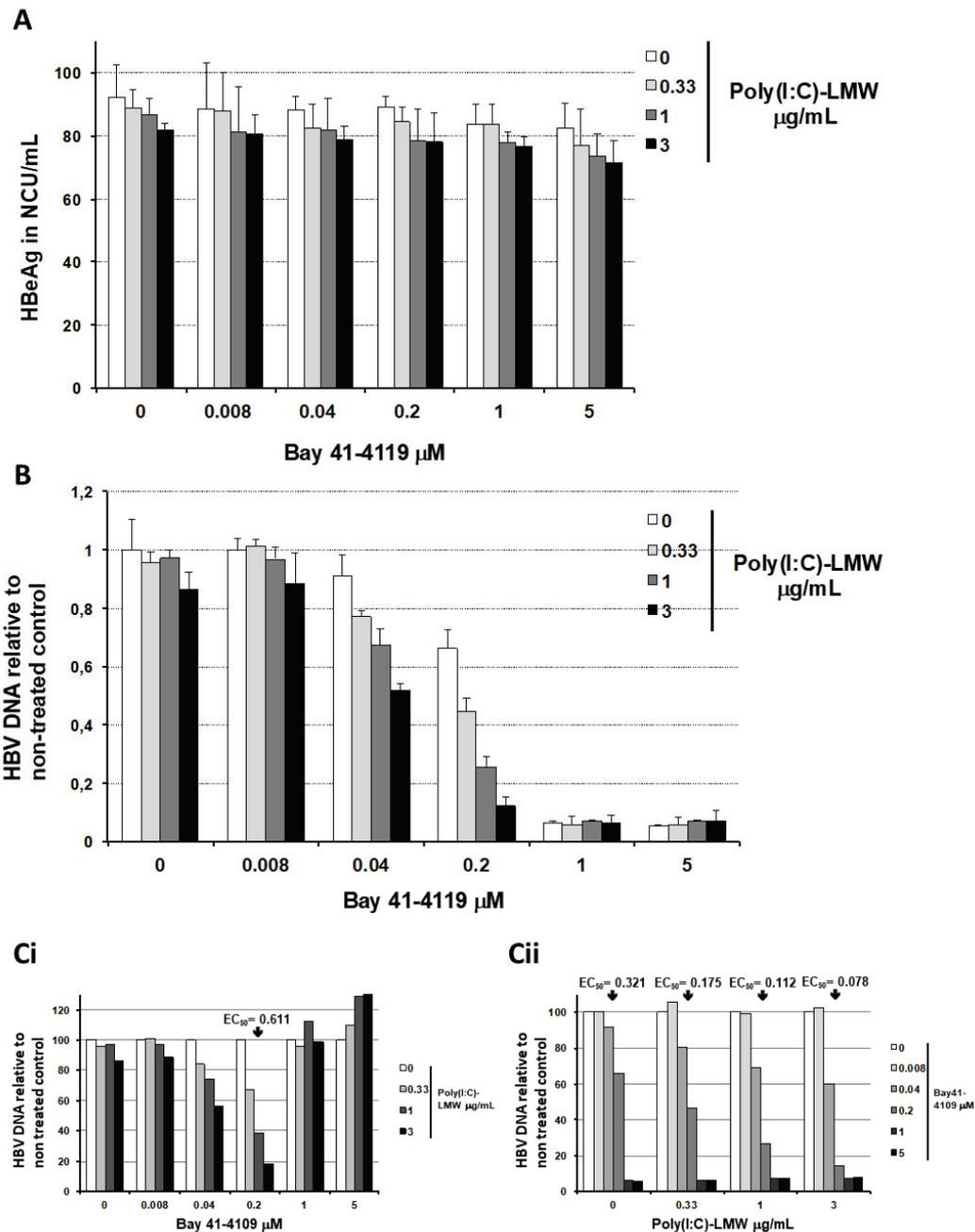
**Figure 6: Long-term inhibition of hepatocyte innate response by HBV core protein.** Persistently HBV-infected liver-humanized FRG mice were sacrificed 8 weeks post-infection and livers store at  $-80^{\circ}\text{C}$  after snap freezing in liquid nitrogen. ChIP-qPCR was performed using none, control, antiH3K4me3, anti-H3K27Ac, anti-H3K27me3, anti-EZH2, or anti-HBc antibodies and specific primers for *IFN-β*, *IL-29*, *OAS-1*, *IL-6*, *GAPDH* and *DHFR* promoters. The percentage of input enrichment was normalized to the non-infected mice. All results are represented as the mean  $\pm$  SEM of 3 independent experiments performed with 3 independent extraction of the same liver samples.

### ***Capsid assembly inhibitors increase the anti-HBV effect of an IFN-response inducer***

As Hbc interfere with the dsRNA-mediated induction of IFN response, we wondered whether administration of capsid assembly inhibitors, interfering with Hbc localization in infected hepatocyte prior to dsRNA receptor stimulation, could potentiate IFN-mediated antiviral effect. To this end, we used the active enantiomer of BAY41-4109 (Deres et al., 2003), which is capable to block trafficking of Hbc to nucleus in our models (Figure S7) and has an efficient concentration 50% ( $EC_{50}$ ) of around 320 nM (see panel Cii of Figure 7). Persistently HBV-infected dHepaRG cells were treated twice (at day-7 and 10 p.i.) with various concentrations (between 0 to 5  $\mu$ M) of the active enantiomer of BAY41-4109, then co-treated once (day-13) with the same concentrations of BAY41-4109 and suboptimal antiviral concentrations (0, 0.33, 1, and 3  $\mu$ g/mL; see Figure S8 for the data on antiviral effect) of a TLR3 ligand (i.e. poly(I:C)-LMH). While the effect of combined treatments on HBeAg secretion was minor (Figure 7A), the inhibition of the intracellular HBV DNA accumulation by poly(I:C)-LMW was potentiated by increasing amounts of the capsid assembly inhibitor (Figure 7B and Ci); and vice versa, the inhibition of the intracellular HBV DNA accumulation by BAY41-4109 was also potentiated by increasing amount of poly(I:C)-LMW, with an  $EC_{50}$  for the former going from 0.321 nM to 0.078 nM (Figure 7Cii). The removal of Hbc from the nucleus by specific inhibitors could therefore represent an interesting strategy to restore IFN responsiveness of infected hepatocytes.



**Suppl. Fig. 7. Effect of capsid assembly inhibitors on HBeAg trafficking and subcellular localization.** (A) Immunofluorescence analysis with anti-HBeAg (C1; Abcam) in tetracycline-induced HepaRG-TR-HBeAg and HBV-infected dHepaRG cells. (B) Immunofluorescence analysis, as in panel A, on cell treated or not with inactive and active Bay41-41-09 [Deres, 2003 #66]. (C) Western blot analysis with anti-HBeAg, anti-HSP60, and anti-PARP antibodies using cell lysates from mock-or tetracycline-induced HepaRG-TR-HBeAg, which have been fractionated into cytosol and nuclear fraction.



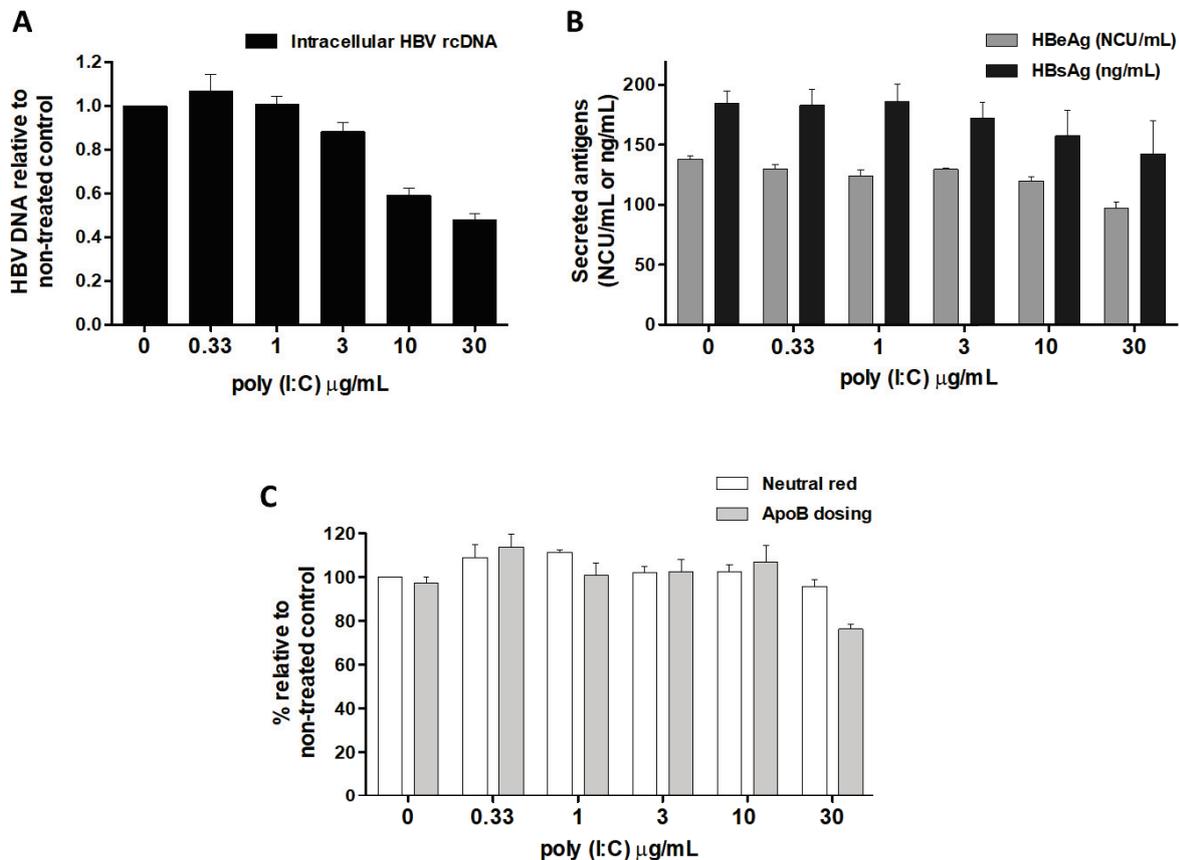
**Figure 7: HBeAg inhibitor treatment potentiates the anti-HBV effect of TLR3-L.** Differentiated HepaRG cells were infected with HBV (100 vge/cell) and left untreated for 7 days. Infected cells were treated twice (at days 7 and 10 p.i.) with various concentration of an HBeAg inhibitor (BAY41-4109) as indicated, then co-treated (day 13) for 72 h with various concentrations of BAY41-4109 and poly (I:C). Secretion of HBeAg was monitored in the

supernatant of cells at the end of treatment (day 16) (A), and intracellular HBV DNA accumulation by qPCR (B). All results are represented as the mean  $\pm$  SEM of 3 independent experiments. On panels (Ci and Cii), the same data as in (B) were re-analyzed in order to standardized in a reciprocal manner to 100% for the no drug condition of a given drug while the other is varying in concentration. This could enable the visualization of EC<sub>50</sub> that were calculated using the “polynomial equation of individual curves” for each experiment. The precise EC<sub>50</sub> is given at the top of the graph.

### ***HBc can also inhibit dsRNA-mediated IFN response in non-parenchymal cells exposed to HBV***

So far we have shown that HBc is capable to inhibit IFN response in HBV-infected hepatocytes (i.e. liver parenchymal cells). But other cells can be exposed to the virus in the liver, including liver-resident macrophages (i.e. Kupffer cells) and liver endothelial sinusoidal cells (LSEC), the two main scavenger cell types that are also involved in foreign antigen presentation and have innate immune functions in the liver (Crispe, 2011; Knolle, 2016). Moreover, both cell types could also contribute to the active delivery of HBV (or related virus) to hepatocytes by transcytosis mechanisms (Breiner et al., 2001b) (Esser et al., unpublished data), thus suggesting that HBV could penetrate into and deliver HBc to the nucleus of these cells, without replicating in them. To check whether HBV and HBc could also inhibit dsRNA-mediated IFN response in these cell types, we used purified KC from human liver resections, as previously reported (Zannetti et al., 2016), and an immortalized LSEC cell line we previously generated (called iLSEC here) (Parent et al., 2014), exposed them for 24h to 1000 vge/cell of HBV or 500 pg/cell (i.e. equivalent of 1000 vge/cell) of recombinant HBc, then stimulated them with poly(I:C) for 4h. An immunostaining with an anti-HBc at 24h post exposition to HBV (1000 vge/cell) is shown to evidence viral penetration into iLSEC cells (Figure 8A); auto fluorescence of KC did not allow to generate such data in this cell type. A significant reduction of the induction of *IFN- $\beta$* , *OAS-1*, and *IL-6* was observed in both cell types with HBV, whereas HBc was only able to inhibit induction of *IFN- $\beta$*  and *OAS-1* (Figure 8B and C), which is consistent with results obtained in hepatocytes (Figure 1). ChIP experiments were also performed on HBV-exposed iLSEC and KC to measure HBc binding to innate gene promoters. As previously

shown in infected hepatocytes or in the liver of infected mice, Hbc was found on *IFN- $\beta$*  and *OAS-1* promoters, and not on *IL-6* one in both cell types (Figure 8D).

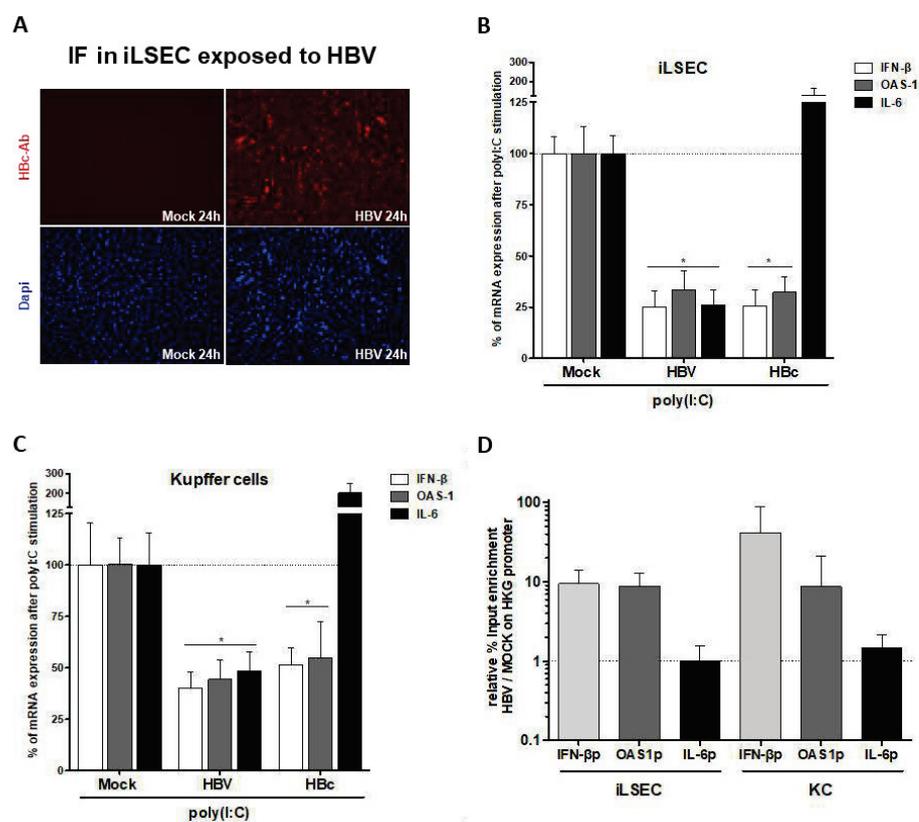


**Suppl. Fig. 8. Antiviral effect of TLR3-L on HBV replication in infected dHepaRG.** Persistently HBV-infected dHepaRG (day-7 p.i.) were treated for 3 days with indicated concentrations of poly (I:C)-LMW. (A) Intracellular HBV DNA was monitored by qPCR on total DNA. (B) HBV secreted antigens were quantified by ELISA in the supernatant of treated cells. (C) Toxicity was evaluated by neutral red staining on cell and dosing of secretion of apolipoprotein B in supernatant with ALerCHEK ELISA.

## Discussion

Neither acute human (Dunn et al., 2009; Stacey et al., 2009) or chimpanzee (Wieland et al., 2004) HBV infections, nor chronic HBV infections (Tan et al., 2010) are accompanied by the production of IFN in the bloodstream of patients and the induction of innate immunity gene expression in the liver. This is in sharp contrast with chronic HCV or HIV infections, in which some interferon-stimulated genes are up-regulated and an uncontrolled IFN response could contribute to immune-pathogenesis {Heim, 2014 #90;Doyle, 2015 #89}.

As for others viruses (Bowie and Unterholzner, 2008; Heim, 2013; Manel and Littman, 2011; Randall and Goodbourn, 2008), HBV has likely evolved mechanisms to prevent and/or escape IFN response. Using rather non-physiological cell culture models and levels of viral protein expression, several groups have pointed out the role of HBx, HBV polymerase, and Hbc as potential intracellular regulator of the induction of the expression of type-I IFN (Jiang and Tang, 2010; Wang and Ryu, 2010; Wang et al., 2010; Wei et al., 2010; Wu et al., 2007b; Yu et al., 2010). Moreover secreted HBV proteins, i.e. HBsAg and HBeAg, were also shown to inhibit TLRs-mediated innate immune responses, yet by an ill-defined mechanism (Wu et al., 2007a; Wu et al., 2009).



**Figure 8: Hbc also inhibits TLR3-L-mediated IFN response in primary Kupffer and immortalized liver endothelial sinusoidal cells.** (A) Immortalized LSEC were mock- or exposed to HBV (1000 vge/cell), and 24h later cells were fixed and permeabilized (2% PFA; 0.5% triton), then immunostained with an anti-Hbc (C1; Abcam). (B and C) Immortalized LSEC or primary KC were either mock-, or exposed to HBV (1000 vge/cell) or recombinant Hbc (500 pg/cell; i.e. equivalent of 1000 vge/cell) for 24h, and stimulated by poly (I:C) (10  $\mu$ g/mL) for 4h. Expression of indicated gene was analyzed by RT-qPCR and compared to the mock control normalized to 100%. (D) ChIP experiment was performed on HBV-exposed (1000 vge/cell) iLSEC and KC, with an anti-Hbc on indicated promoters as in Figure 3. All results are represented as the mean  $\pm$  SEM of at least 2 independent experiments.

In this study, using more relevant models, i.e. proper infection of primary human hepatocytes (PHH) or closely related cells (dHepaRG), we demonstrate that HBc is a key very early negative regulator of the IFN response. Indeed, we have shown that the HBc protein associated to “incoming” HBV virions can inhibit IFN response after trafficking of nucleocapsids (NCs) through the cytosol and translocation of HBc into the nucleus, where it is able to transcriptionally repress IFN-response gene expression. In a previous study, using mainly HepaRG cells, we previously showed (Luangsay et al., 2015b), and we confirmed here using PHH, that this inhibition could be seen as early as 2 hours after the onset of infection, and showed that HBc can be found on the promoter of target genes after only 4h of exposure. Others have found that, after experimental lipo-transfection of NCs to cells, the delivery of HBV genomic DNA into the nucleus takes only few minutes (Rabe et al., 2006), thus confirming that HBc can be rapidly found in the nucleus of neo-infected cells, where it can play its immune-modulatory role.

HBc is capable to bind to dsDNA, including cccDNA as well as host genes (Bock et al., 2001; Guo et al., 2012), and it likely does it via the same domain required for RNA binding, which is a protamine like domain found in mammalian DNA binding protein located at the C-terminus of the protein (Seeger et al., 2015; Zheng et al., 1992). The biophysical aspect of this interaction is yet to be defined, but could involve a dimeric forms of the protein. It is worth noting that all the promoters of targeted genes contained ISRE motifs. EMSA experiment also showed that HBc could bind to ISRE-containing dsDNA (Fernandez et al., 2003).

The early action of HBc could explain why a nascent innate response, as evidenced previously (Luangsay et al., 2015b), could be stopped and why no IFN would not be produced upon infection. We have also shown that when HBV life cycle is launched via a baculoviral vector, therefore in the absence of HBc during inoculation, a strong interferon response, accompanied by eradication of infection, could be observed {Lucifora, 2010 #91}. Altogether our observation show that HBV is indeed a “stealth” virus, not because of absence of detection, but rather because of a very early inhibition of innate immune responses. Such an early inhibition of innate immune responses via a structural protein embarked into virions and readily mobilized into the newly penetrated cells has been shown, to our knowledge, only in the case of the tegument protein (ORF52) of KHSV. This protein was indeed very recently

shown to prevent cGAS DNA sensing just after entry of virions within cells, and in the absence of viral replication and protein neo-synthesis (Wu et al., 2015).

In the nucleus of newly infected cells, we have shown that HBc binds to promoters of IFN response genes, and somehow induce the recruitment of the histone methyl-transferase EZH2, which is in turn responsible for the establishment of the H3K27me3 repressive mark, leading to the impairment of their inducibility upon dsRNA sensors stimulation. The binding of HBc to IFN response gene promoters was also found in liver-humanized HBV-infected FRG mice, thus suggesting that this early-established mechanism, set with incoming HBc, would also be at work in persistent infections. The accumulation of HBc within nucleus is possible throughout the life cycle of the virus thanks to the recycling of neo-synthesized nucleocapsid to nucleus in infected cells. In chronically infected patients, HBc was also found in the nucleus of hepatocytes (Akiba et al., 1987; Chevallier-Queyron and Chemin, 2011; Chu and Liaw, 1987; Nakayama et al., 2001; Ng et al., 2009), in particular in patients who are in the high-replicative, low-inflammatory phase. Here, we show immune-staining data that further support the idea of a correlation between high viremia and nuclear localization of HBc in the liver of patients. The correlation between the nuclear localization of HBc and the low-inflammatory phase might be explained, at least in part, by the inhibitory mechanism we described here.

As we have shown that the virus does not need to replicate to deliver HBc to the nucleus of hepatocytes, HBc could be delivered in other cell types to set similar inhibitory effect. Indeed in the immune tolerance phase, a high amount of infectious particles, containing HBc, circulates in the blood of patients (between  $10^7$  and  $10^{10}$  HBV-genome-copies/mL) (Seeger et al., 2015), and this concentration may be even higher in the liver microenvironment, thus potentially outnumbering liver cells. It has been suggested that HBV could penetrate into LSEC (liver sinusoidal endothelial cells) (Breiner et al., 2001a), Kupffer cells (Esser et al., 2012), and pDC (Vincent et al., 2011), which are three important cell types to mount an efficient innate response. Our data suggest that indeed HBc could be delivered to the nucleus of primary KC and immortalized LSEC, and lead to impairment of IFN gene expression. Therefore, the new concept of IFN response inhibition we described for hepatocytes may also be valid for many other non-parenchymal and/or liver resident immune cells.

Finally, we have shown that prevention of the migration of HBc to the nucleus with nocodazole or CpAMs, restore the IFN response to dsRNA ligands. Does this mean that core

assembly modulators could be used *in vivo* to restore immune function in infected hepatocytes and help reinforced immune response against the virus? As HBc possesses both nuclear localization (NLS) and export (NES) sequences (Li et al., 2010), as it has been proposed that the pool of nuclear cccDNA could be constantly replenished by NCs recycling (Seeger et al., 2015), and despite its long-half life in the nucleus (our data not shown), it is tempting to speculate that a long-term treatment with core inhibitors could lead to such a purge. To substantiate this hypothesis, we have finally shown in this study that a capsid assembly inhibitor, leading to nuclear HBc removal (or aggregation), could potentiate the anti-HBV effect of a TLR3-L.

Many core/capsid inhibitors, also called CpAM (core protein allosteric inhibitor) (Zlotnick et al., 2015), are under current development. Selected on their “canonical functions”, i.e. inhibition of the packaging of HBV pregenomic RNA and subsequent inhibition of capsid reverse transcription, CpAMs could also have additional antiviral effect due to their impact on HBc nuclear function, and should therefore be also selected on this criteria. Our results suggest that CpAMs could be favorably combined to immune stimulators to obtain a greater anti-HBV effect. Among immune-stimulator in current clinical evaluation is the TLR7 agonist GS-9620, which has shown very interesting antiviral effect, leading in particular to the degradation of cccDNA in animal models (Lanford et al., 2013; Menne et al., 2015). As TLR7 is not expressed in infected human hepatocytes (Luangsay et al., 2015a), and TLR7-L likely acts via pDC-expressed IFN- $\alpha$ , it would be of interest to develop PRR agonist that could both stimulate innate cells and induce expression of IFN- $\beta$  and related ISGs within infected hepatocytes. In this respect, our study makes a case for a combinatory approach with CpAMs and PRR agonists in order to progress toward the rupture of tolerance to HBV.

## Material and Methods

**Antibodies and others reagents.** Most of chemicals and control antibodies were purchased from Sigma Aldrich. Poly (I:C) low-molecular weight (LMW), poly (I:C) (LMW)/Lyovec, and other ligands were from Invivogen. Antibodies for ChIP experiments were purchased from Diagenode (anti-H3K27me3 pAb-069-050, anti-H3K4me3 pAb-003-050, anti-H3K27Ac pAb-

196-050, anti-EZH2 pAb-039-050, and control antibodies), or homemade (polyclonal against HBc; (Petit and Pillot, 1985)). A polyclonal rabbit anti-HBc from Dako, as well as a monoclonal from Abcam (Clone C1) were used for immunostainings and Western blots.

***Production of HBV inoculums, Dane particles and non-enveloped HBV nucleocapsids.*** HBV viral stocks were prepared and tittered as previously reported (Luangsay et al., 2015b). Non-enveloped nucleocapsids (density of 1.25 g/mL) were purified from NP40-treated HepG2.2.15. supernatant and characterized, as previously reported (Rabe et al., 2006; Luangsay et al., 2015b). All preparations were tested for the absence of endotoxin (Lonza Verviers, Belgium). *Pichia pastoris*, GMP-produced recombinant HBc (VTI610) was purchased from Meridian Life Science. To standardize the various inocula, the same quantity of HBc was used, and a dose of 100 vge/cell for a full HBV inoculum corresponded to 50 pg of HBc/cell.

***Isolation of primary human cells and cell line culture conditions.*** Primary human hepatocytes (PHHs) and Kupffer cells were prepared from surgical liver resections after informed consent of patients (kindly provided by Pr. Rivoire (CLB, Lyon)) as previously described (Lecluyse and Alexandre, 2010; Zannetti et al., 2016), and cultured in complete William's medium supplemented with 1.8 % of DMSO for PHH, and complete RPMI medium for KC. The human liver progenitor HepaRG cells (Gripon et al., 2002) were cultured and infected as previously described (Luangsay et al., 2015a; Luangsay et al., 2015b). Immortalized liver endothelial sinusoidal cells TRP3/iLSEC cells were cultured as previously reported. (Parent et al., 2014).

***Generation of HBV core and other HBV protein- expressing HepaRG cells.*** To generate recombinant cell line we used lentiviral vectors and procedures from Invitrogen (ViraPower™ T-REx™ Lentiviral Expression System). Stable overexpression of the tetracycline repressor (TR) in HepaRG cells was obtained after transduction with a lentivirus (m.o.i. of 5) produced upon cotransfection of HEK-293 cells with pLenti6-TR plasmid and packaging vectors according to manufacturer's instructions. Blasticidin-resistant HepaRG-TR cells were then transduced with a second lentivirus conferring zeocin resistance and containing either HBc, HBs, HBx, HBe (genotype D, serotype ayw, Galibert's strain) or a T7 phage polymerase transgene, and produced upon cotransfection of HEK-293 cells with packaging vectors and with corresponding pLenti4/TO plasmids, in which transgene are under the control of a tetracycline-regulated promoter. HepaRG-TR-HBc, HepaRG-TR-HBs, HepaRG-TR-HBe, HepaRG-TRx and HepaRG-TR-T7pol were derived by co-selection with blasticidin and zeocin.

**HBV infection and lipo-transfection of recombinant HBcAg and non-enveloped nucleocapsids.** Differentiated HepaRG cells and PHH were infected with HBV or its control mock (depleted from HBsAg, HBeAg and infectious Dane particles) in complete William's medium supplemented with 4% PEG-8000 at 37°C as previously described (Hantz et al., 2009). Recombinant HBcAg and non-enveloped nucleocapsids were lipo-transfected into HepaRG cells using "Pro-Ject Protein Transfection Reagent" kit according to manufacturer's instructions (Thermo Scientific).

**Humanized FRG mouse model and HBV infection.** All animal studies were reviewed and approved by a local ethical comity. The highly immunosuppressed FRG mice ( $Fah^{-/-}/Rag2^{-/-}/Il2rg^{-/-}$ ), deficient for T-, B- and NK-cells were used (Azuma et al., 2007) and maintained in a specific pathogen-free facility. High quality, cryopreserved human hepatocytes were purchased (BD, Biosciences) and injected via an *intra-splenic* route into 2 to 3 month old mice as described previously. Liver humanized  $Fah^{-/-}/Rag2^{-/-}/Il2rg^{-/-}$  mice featuring a seric production of human albumin of at least 5mg/mL were infected with 200  $\mu$ l of HBV inoculums ( $1.10^8$  veg to  $1.10^9$  veg in PBS) via *intra-peritoneal* route (Bissig et al., 2010). Serum was harvested every week by retro-orbital bleeding and stored at -80°C in aliquots for further antigenemia and viremia analysis. Mice were sacrificed 9 weeks post-infection and hepatic tissues were processed for RT-qPCR and CHIP analysis or fixed in formalin and embedded in paraffin for immune-staining.

**PRRs stimulation.** Cells were stimulated with TLR or RLR agonists and harvested for the analysis of the innate gene expression (after 4h) and cytokines production (after 6h). TRL and RLR agonist were: TLR1/2 (Pam3CSK4, 0.8  $\mu$ g/ml), TLR3 (Poly (I:C)-LMH, 10  $\mu$ g/ml), TLR4 (LPS, 0.4 $\mu$ g/ml), TLR5 (Flagellin, 0.1  $\mu$ g/ml), TLR6 (FSL-1, 0.1  $\mu$ g/ml), TLR7/8 (ssRNA40, 10  $\mu$ g/ml), RIGI/MDA5 (Poly (I:C)-LMH-Lyovec 0.2  $\mu$ g/ml).

**RNA extraction and qRT-PCR.** Total RNA were purified with the Nucleospin RNA II kit according to the manufacturer's protocol (Macherey Nagel). cDNA was obtained after reverse transcription using the Superscript® III Reverse Transcriptase (Life technologies) and qPCR was performed using the Express SYBR GreenER qPCR Supermix Universal according to the manufacturer's instructions (Life technologies) and run on the MyiQ Biorad machine. The

relative mRNA expression was analyzed with q-base software (Biogazelle, Belgium) using the comparative cycle threshold ( $2^{-\Delta\Delta Ct}$ ) method with 2 housekeeping genes (RPLP0 and  $\beta$ -Actin). The relative HBV mRNA level was quantified using the same primer pairs used for the HBV PCR quantification. The sequences of primer pairs were listed on **Table 1**.

**Table 1. Primer pairs for RT-qPCR**

GENE	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')
$\beta$ -actin	TGGCATTGCCGACAGGATGC	TCTGCTGGAGGTGGACAGCGA
RPLP0	CACCATTGAAATCCTGAGTGATGT	TGACCAGCCCAAAGGAGAAG
IFN- $\beta$	GCCGCATTGACCATGTATGAGA	GAGATCTTCAGTTTCGGAGGTAAC
IL-29	GTGACTTTGGTGCTAGGCTTG	GCCTCAGGTCCCAATTCCC
OAS-1	AGGTGGTAAAGGGTGGCTCC	ACAACCAGGTCAGCGTCAGAT
IL-6	ACCCCTGACCCAACCACAAAT	AGCTGCGCAGAATGAGATGAGTT

**Western Blotting.** Cells were lysed in TTB buffer (10 mM Tris, 150 mM NaCl, 2 mM EDTA, 0.5% Triton X-100, pH 7.5) for total proteins extraction or in NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific) for nuclear and cytosolic proteins separation. All lysis buffers were complemented with inhibitor cocktail of proteases (Complete EDTA-free tablets, Roche). Extracted proteins were separated electrophoretically on a 10% SDS-PAGE gel and “semi-dry” transferred onto hybond-ECL membranes (Amersham, biosciences). Membranes were blocked with 5% non-fat milk in PBS 0,1% Tween for 1 hr. at RT. Polyclonal rabbit anti-HBc (Petit and Pillot, 1985), anti-actin (13E5, Cell Signaling), anti-tubulin (MAB3408, Millipore) and anti-PARP (Zymed, Invitrogen) antibodies were applied overnight at 4°C under gentle agitation. After washing, HRP conjugated secondary antibodies from Sigma Blots were applied. After extensive washing, blots were revealed using the SuperSignal West Pico Chemiluminescent substrate (Thermo Scientific).

**ELISA.** Commercial immunoassay kits for HBs antigen (Autobio Diagnostics Co., China), HBe/HBc antigens (Autobio Diagnostics Co., China), and IL-6 and IP-10 cytokine (R&D system) detections were used following manufacturer’s instructions.

**Chromatin immunoprecipitation (ChIP) assays.** Cells were fixed with formaldehyde 1% at room temperature with rocking to capture the DNA–protein interaction and cross-linking was stopped with Glycine 0.125M (Euromedex). Cells were washed 3 times with ice-cold PBS 1X then scrapped and lysed in 500µl of lysis buffer per  $10^7$  cells (50 mM Tris HCl pH8, 10 mM EDTA, 1% SDS and protease inhibitor cocktail (Complete EDTA-free tablets, Roche)) for 5 min in ice. Cell lysates were sonicated to reduce DNA length to 200 bp using a Bioruptor system (Diagenode). The sheared chromatin extract was then frozen in aliquots at  $-80^{\circ}\text{C}$  until use. Sheared chromatin (100 µl) was diluted 10-fold in RIPA buffer (10 mM Tris HCl pH5, 140 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 1% Triton, 0.1% SDS, 0.1% sodium deoxycholate, protease inhibitor cocktail) and then pre-blocked with protein A sepharose 4B (Sigma) for 2 hours at  $4^{\circ}\text{C}$  under gentle agitation. Pre-blocked chromatin was incubated overnight with rotation at  $4^{\circ}\text{C}$  with 10µg of specific antibodies. An additional sample of lysate containing no antibody was also incubated overnight at  $4^{\circ}\text{C}$  (no antibody control). Chromatin was then immunoprecipitated with magnetic beads (Dynabeads Protein G, Novex) for 2 h at  $4^{\circ}\text{C}$  with rotation. After several washes with RIPA buffer, cross-linking was reversed at  $68^{\circ}\text{C}$  for 2h under shaking in elution buffer (20 mM Tris HCl pH7.5, 5 mM EDTA, 50 mM NaCl, 1% SDS, 50 µg/ml proteinase K (Eurobio) and inhibitor cocktail proteases). DNA from elution buffer was extracted with Phenol/Chloroform/Isoamyl-Alcohol and precipitated with ethanol supplemented with NaAc 3M and Glycogen 20mg/ml (Sigma) overnight at  $-20^{\circ}\text{C}$ . Extracted DNA from non-immunoprecipitated fraction of no antibody control was used as internal control (total input). Extracted DNA was used as template for qPCR specific on IFN-βp, IL-29p, IL-6p, OAS-1p and GAPDHp. The percentage of input enrichment ( $2^{\Delta\Delta\text{Ct}}$  (total input – antibody input)) was normalized on the Mock condition depending of the experiments (no tetracycline stimulation or no HBV infection) and also on GAPDH housekeeping gene promoter. Results are representative of 3 independents experiments. The list of primer pairs is given on **Table2**.

**Table 2. Primer pairs for CHIP-qPCR**

<b>GENE</b>	<b>Forward primer sequence (5'-3')</b>	<b>Reverse primer sequence (5'-3')</b>
<b><i>GAPDHp</i></b>	CTTCTCCCCATTCCGTCTTC	CCCCAGCTACAGAAAGGTCA
<b><i>IFN-<math>\beta</math>p</i></b>	CTTTCGAAGCCTTTGCTCTG	CAGGAGAGCAATTTGGAGGA
<b><i>IL-29p</i></b>	GCCCAGGGAGTTCTAAGGAT	CTGATGAGGGGAACAGGTGTG
<b><i>OAS-1p</i></b>	TGAAATTCAGCACTG GGATCAC	GGAGGAGCTGTCTTTGCACTT
<b><i>IL-6p</i></b>	GCCCAGGGAGTTCTAAGGAT	CTGATGAGGGGAACAGGTGTG

**Statistical analysis.** Statistical analysis was performed by two-way ANOVA, non-parametric Mann-Whitney, or t-tests using the GraphPad Prism software. For all tests, p-values  $\leq 0.05$  (\*),  $< 0.01$  (\*\*), and  $< 0.001$  (\*\*\*) were considered as significant.

## Acknowledgments

The authors would like to thank Lydie Lefrançois and Judith Fresquet for the isolation of primary human hepatocytes, as well as the staff from Pr Michel Rivoire's surgery room for providing us with liver resection. This work was supported by grants from ANRS (French national agency for research on AIDS and viral hepatitis; several grants from CSS4), FINOVI (Foundation for innovation in infectiology; project call n°4), FRM (Foundation for medical research; DEQ20110421327), and Hoffmann-La-Roche (U.S.A. division) and by INSERM core grants. This work was also supported by the DEVweCAN LABEX (ANR-10-LABX-0061) of the "Université de Lyon", within the program "Investissements d'Avenir" (ANR-11-IDEX-0007) operated by the French National Research Agency (ANR).

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## 5. Third study

### **Characterization of the Inflammasome in Human Kupffer Cells in Response to Synthetic Agonists and Pathogens**

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**Published in 2016 in the Journal of Immunology.**




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This information is current as of February 14, 2018.

Claudia Zannetti, Guillaume Roblot, Emily Charrier, Michelle Ainouze, Issam Tout, François Briat, Nathalie Isorce, Suzanne Faure-Dupuy, Maud Michelet, Marie Marotel, Semra Kati, Thomas F. Schulz, Michel Rivoire, Alexandra Traverse-Glehen, Souphalone Luangsay, Omran Alatiff, Thomas Henry, Thierry Walzer, David Durantel and Uzma Hasan

*J Immunol* 2016; 197:356-367; Prepublished online 25 May 2016;

doi: 10.4049/jimmunol.1502301

<http://www.jimmunol.org/content/197/1/356>

**Supplementary Material** <http://www.jimmunol.org/content/suppl/2016/05/25/jimmunol.1502301.DCSupplemental>

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Print ISSN: 0022-1767 Online ISSN: 1550-6606.



# Characterization of the Inflammasome in Human Kupffer Cells in Response to Synthetic Agonists and Pathogens

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The liver is the largest gland in the human body and functions as an innate immune organ. Liver macrophages called Kupffer cells (KC) constitute the largest group of macrophages in the human body. Innate immune responses involving KC represent the first line of defense against pathogens in the liver. Human monocyte-derived macrophages have been used to characterize inflammasome responses that lead to the release of the proinflammatory cytokines IL-1 $\beta$  and IL-18, but it has not yet been determined whether human KC contain functional inflammasomes. We show, to our knowledge for the first time, that KC express genes and proteins that make up several different inflammasome complexes. Moreover, activation of KC in response to the absent in melanoma 2 (AIM2) inflammasome led to the production of IL-1 $\beta$  and IL-18, which activated IL-8 transcription and hepatic NK cell activity, respectively. Other inflammasome responses were also activated in response to selected bacteria and viruses. However, hepatitis B virus inhibited the AIM2 inflammasome by reducing the mRNA stability of IFN regulatory factor 7, which regulated AIM2 transcription. These data demonstrate the production of IL-1 $\beta$  and IL-18 in KC, suggesting that KC contain functional inflammasomes that could be important players in the innate immune response following certain infections of the liver. We think our findings could potentially aid therapeutic approaches against chronic liver diseases that activate the inflammasome. *The Journal of Immunology*, 2016, 197: 356–367.

The inflammasome is an innate immune pathway by which inflammasome receptors detect pathogens and danger signals, leading to the recruitment of the apoptosis-associated, speck-like protein ASC, which contains a carboxyl-terminal CARD. ASC provides a link between inflammasome receptors and the proform of caspase-1 through its PYD domain at the N-terminal and

its CARD domain, respectively. This signaling cascade is known as the inflammasome complex. The activation of caspase-1 is associated with two events: 1) pyroptosis, a form of programmed cell death distinct from apoptosis, and 2) the production of the proinflammatory cytokines IL-1 $\beta$  and IL-18, produced by prior stimulation from TLR ligands. Once released, mature IL-1 $\beta$  and IL-18 signal their target cells, thus allowing the expansion of innate and adaptive immune responses.

Some of the best characterized inflammasomes are the nucleotide-binding domain and leucine-rich repeat caspase recruitment domain 4 (NLR4) inflammasome and the nucleotide-binding domain and leucine-rich repeat pyrin domain 3 (NLRP3) inflammasome. The NLR4 inflammasome is activated primarily by bacteria, including *Salmonella typhimurium*, via cytosolic flagellin or the type 3 secretion system. However, there is evidence showing that some inflammasomes are also able to detect viruses. Indeed, the NLRP3 inflammasome can detect adenovirus (1), vaccinia virus (2), and hepatitis C virus (HCV) (3). In 2009, the protein absent in melanoma 2 (AIM2) was identified as a cytosolic dsDNA sensor, capable of activating the inflammasome via ASC (4–6). Moreover, this AIM2 inflammasome has been shown to detect vaccinia virus (5) and murine CMV (7). These studies suggest that the AIM2 inflammasome is a surveillance system that can monitor the host cytosol for infection with DNA viruses. Finally, IFI16, another DNA innate sensor belonging to the same family as AIM2, was reported to activate a functional inflammasome in response to Kaposi sarcoma-associated herpesvirus (KSHV) in the nucleus (8).

Inflammasome activation in response to the liver-borne pathogens *Francisella tularensis* and *S. typhimurium* has been well described in myeloid cells such as human monocyte-derived macrophages (9–12). However, the characterization of the inflammasome in

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Received for publication November 2, 2015. Accepted for publication April 27, 2016.

This work was supported by La Ligue Contre le Cancer and the Agence Nationale de Recherches sur le Sida et les Hépatites Virales.

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The online version of this article contains supplemental material.

Abbreviations used in this article: AIM2, absent in melanoma 2; ChIP, chromatin immunoprecipitation; *F. novicida*, *Francisella tularensis* subspecies *novicida*; HBc, hepatitis B core protein; HBe, hepatitis B early Ag; HBs, hepatitis B surface Ag; HBV, hepatitis B virus; HCV, hepatitis C virus; IRF, IFN regulatory factor; KC, Kupffer cell; KSHV, Kaposi sarcoma-associated herpesvirus; LMNC, liver mononuclear cell; MOI, multiplicity of infection; NLR4, nucleotide-binding domain and leucine-rich repeat caspase recruitment domain 4; NLRP3, nucleotide-binding domain and leucine-rich repeat pyrin domain 3; PCHH, primary cultured human hepatocyte; PI, propidium iodide; poly(dA:dT), poly(deoxyadenylic-thymidylic) acid; PVL, Panton-Valentine leukocidin; qPCR, quantitative PCR; WT, wild-type.

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www.jimmunol.org/cgi/doi/10.4049/jimmunol.1502301

resident liver macrophages, that is, Kupffer cells (KC), has not yet been investigated. The liver is constantly exposed to a large variety of Ags from the gastrointestinal tract, including dietary Ags, pathogens, and toxins. KC constitute the largest group of macrophages in humans and play a key, diverse role in the phagocytosis of pathogens, apoptotic cells, food Ags, and toxins that enter the portal vein after crossing the intestinal epithelium, as well as in TLR innate immune activation (13). We sought to characterize the inflammasome and inflammasome response in purified primary KC from liver donors. In this study, we aimed to determine, define, and discuss the level of inflammasome gene expression, as well as NLRP3, AIM2, NLRC4, and IFI16 function, in response to several live and synthetic pathogens in KC.

## Materials and Methods

### Ethics statement

Primary cultured human hepatocytes (PCHH) and liver mononuclear cells (LMNC) were obtained from surgically resected anatomically normal human liver tissue from patients diagnosed with hepatic metastases from colon cancer, thanks to Prof. Michel Rivoire (INSERM U1032, Centre Léon Bérard), and under the ministerial agreement numbers DC-2008-99 and AC-2008-1001 approved by the Hospital Civil Review Board. As resected tissue is considered waste, no patient written or oral consent was required for this study.

### Cell culture

Surgically removed samples from liver donors were perfused with collagenase as previously reported (14), and the resultant cell suspension was centrifuged at 800 rpm for 4 min with no break at room temperature. Pelleted cells constituted the PCHH, composed of  $\geq 85\%$  hepatocytes and 3–15% nonparenchymal cells, mainly KC and liver sinusoidal endothelium. Culturing PCHH for 24 h in serum-free medium resulted in the elimination of most liver sinusoidal endothelium (data not shown). The suspension cells underwent FicolI (PAA Laboratories) density gradient centrifugation and three washes. The retrieved cells constituted LMNC. KC were prepared by negative selection (Miltenyi Biotec). PCHH and HepaRG cells were cultured with Williams medium (Life Technologies), supplemented as described (14). PBMC were obtained from the "Établissement Français du Sang" as buffy coats and purified as previously reported (15). THP-1 cells were differentiated into macrophages by adding PMA (Sigma-Aldrich) for 3 h at 0.5  $\mu\text{M}$ . LMNC, KC, PBMC, and THP-1 were cultured in RPMI 1640 medium (Life Technologies) supplemented with 10% heat-inactivated FBS, 1 mM L-glutamine, and 10  $\mu\text{g}/\text{ml}$  cyproxin. HEK293 cells were maintained as previously described (15).

### Stimuli and inhibitors

LPS (100 ng/ml) and Pam2CSK4 (4 nM) ligands were purchased by Cayla-InvivoGen and were used for 4 h unless indicated otherwise. ATP (5 mM) and poly(deoxyadenylic-thymidylic) acid [poly(dA:dT); 1  $\mu\text{g}/\text{well}$ ] were provided by Cayla-InvivoGen. Nigericin was from Sigma-Aldrich. IFN- $\beta$  (Avonex, Biogenidec, France) was used at 1000 U/ml. Recombinant LukS-PV and LukF-PV (16) were mixed together to obtain Pantan-Valentine leukocidin (PVL) and used at 100 ng/ml for 4 h. Recombinant human IL-18 BP $\alpha$  (R&D Systems) was used at 50 ng/ml. Anakinra (Amgen) was used at 150  $\mu\text{g}/\mu\text{l}$ . The amounts of ligands used have been established to be optimal in our *in vitro* experiments and have been previously described (17). Recombinant human IL-12 was provided by PeprTech. Small interfering RNA and its control were purchased from Life Technologies and used according to the manufacturer's instructions. Actinomycin D was purchased from Sigma-Aldrich.

### Constructs

The human IL-8 promoter luciferase plasmid was transfected and tested with PCHH and KC supernatants as previously described (18). AIM2 promoter linked to luciferase was purchased from Switchgear, and the mutated form was provided by the laboratory of Ruben Tooze (University of Leeds, Leeds, U.K.) (19).

### Cell death assay

Quantification of cell death was performed by analysis of propidium iodide (PI) incorporation. Briefly, primary KC ( $2 \times 10^5/\text{well}$ ) were seeded in black, flat-bottom 96-well plates in phenol red minus medium and infected

with *F. tularensis* subspecies *novicida* (*F. novicida*) and *S. typhimurium* strains. One hour postinfection, PI (5  $\mu\text{g}/\text{ml}$ ) was added to the cells. PI incorporation was measured during a 24-h period using a microplate fluorometer (Tecan).

### Hepatitis B virus production and components

The hepatitis B virus (HBV) is routinely produced in large quantities from HepG2215 cells (20), but it may also be purified from the plasma of patients with chronic HBV infection. The virus is concentrated by polyethylene glycol precipitation or ultracentrifugation as needed, and the various components of the viral inoculum (i.e., hepatitis B surface Ag [HBs], hepatitis B early Ag [HBe], Dane particle nucleocapsids, hepatitis B core protein [HBc]) can be purified after separation on an iodixanol gradient.

KSHV was supplied by the laboratory of Thomas Schulz (Institute of Virology, Hannover Medical School, Hannover, Germany).

### Bacteria

*F. novicida* strain U112 and a mutant lacking the whole FPI were cultured in tryptic soy broth supplemented with 0.1% cysteine. *S. typhimurium* wild-type (WT; 12023 strain) and the mutant lacking SIPB ( $\Delta\text{SIPB}$ ) were grown in Luria-Bertani medium.

### Immunoblotting

Protein extracts were obtained by lysing the cells with a protein/RNA extraction kit (Macherey Nagel) according to the manufacturer's instructions. Abs used for immunoblotting were ASC, caspase-1 p10 (Santa Cruz Biotechnologies), IL-1 $\beta$  (3ZED, NCI), IFN regulatory factor (IRF)7 (Santa Cruz H-246), and  $\beta$ -actin (MP Biomedicals). Western blots were developed using the intelligent dark box (Fuji film).

### Chromatin immunoprecipitation assay

Chromatin immunoprecipitation (ChIP) assays were performed using the Shearing Optimization kit and the OneDay ChIP kit (Diagenode) as described previously (21). Abs for IRF7 (Santa Cruz H-246) and IRF1 (Santa Cruz F-2) were used.

### ELISA

Supernatants from cultured cells were harvested and diluted up to 40 times to perform ELISA on IL-1 $\beta$  (BioLegend), IL-18 (MBL International), and IL-6 (R&D Systems).

### Transfection and luciferase assay

Poly(dA:dT) transfection was performed with Lipofectamine 2000 (Life Technologies) according to the manufacturer's instructions. One microgram poly(dA:dT) and 2  $\mu\text{l}$  Lipofectamine 2000 were used for each well in a 24-well plate. Transfection of HEK293 cells or THP-1 cells was performed in triplicate with FuGENE (Promega), and luciferase assay was performed as previously described (15, 21).

### Real-time quantitative PCR

Total RNA was extracted from cells using the protein/RNA extraction kit from Macherey-Nagel. cDNA synthesis was performed with 1  $\mu\text{g}$  total RNA with a first-strand cDNA synthesis kit (Thermo Scientific). Quantitative PCR (qPCR) was performed using the Mx3000P real-time PCR system (Agilent Technologies) with Mesa Green qPCR MasterMix Plus (Eurogentec).

### Primers

The following primers were used: NLRP3, forward, 5'-CAGA-CCTCTGTGTGGGACTGA-3', reverse, 5'-TCCTGACAACATGCT-GATGTGA-3'; AIM2, forward, 5'-ATGCAGCAGGACTCATTCA-3', reverse, 5'-CGTCTTCAGGAGGAGAAGGA-3'; ASC, forward, 5'-CGC-GAGGGTCACAAACGT-3', reverse, 5'-TGCTCATCCGTCAGGACCTT-3'; IFI16, forward, 5'-ACTGCGTACAACAAAGCCATTTGA-3', reverse, 5'-TTGTGACATTGTCTGTCCAC-3'; caspase-1, forward, 5'-AAAATCT-CACTGCTTCGGACATG-3', reverse, 5'-GGAACGTGCTGTCAGAGGTC-TT-3'; IL-1 $\beta$ , forward, 5'-AATCTGTACCTGTCTGCGTGT-3', reverse, 5'-TGGGTAATTTTTGGGATCTACACTCT-3'; IL-18, forward, 5'-ATCGTCTTCTCGCAACAA-3', reverse, 5'-CTTCTACTGGT-CAGCAGCCATCT-3'; GAPDH, forward, 5'-GCCAAAAGGGTCAT-CATC-3', reverse, 5'-TGCCAGTGAGCTCCCTC-3';  $\beta_2$ -microglobulin, forward, 5'-TGCTGTCTCCATGTTTGTATCT-3', reverse, 5'-TCTGTCTCCCACTTAAGT-3'; and AIM2 ChIP primers, forward, 5'-TACCTTCATTGTGCTTATTC-3', reverse, 5'-ACTGATCT-CAGAGGGTATCA-3'.

### FACS staining

To phenotype KC,  $5 \times 10^5$  harvested PCHH or KC were resuspended in staining buffer (0.5% BSA, 2 mM EDTA in PBS) and preincubated with FcR blocking reagent (Miltenyi Biotec) for 10 min at 4°C to prevent any nonspecific binding. Cells then underwent surface and live/dead cell staining with the Live/Dead fixable dead cell stain kit (Life Technologies) and intracellular staining. For surface staining, PerCP-Cy5.5-conjugated anti-CD11b (BioLegend), FITC-conjugated anti-C5aR (AbD Serotec) Abs, and the respective isotype controls were used. PE-conjugated anti-CD68 and the isotype control (Miltenyi Biotec) were used for intracellular staining. Cells were analyzed using an LSR II flow cytometer (Becton Dickinson). Data were processed using FlowJo software (Tree Star). For intracellular staining in coculture experiments, PBMC or LMNC were incubated for 5 h with monensin. Cells were stained with the following Abs: allophycocyanin-Alexa Fluor 750-conjugated anti-CD3 (Beckman Coulter), electron-coupled dye-conjugated anti-CD3, anti-CD14, and anti-CD19 (Beckman Coulter), allophycocyanin-conjugated anti-CD56 (Beckman Coulter), and PE-conjugated anti-IFN- $\gamma$  (eBioscience). Cells were analyzed using a Navios flow cytometer (Beckman Coulter), and data were processed using Kaluza software (Beckman Coulter).

## Results

### Expression and function of the inflammasome pathway in KC

Two subsets of macrophages have been reported to populate the liver: resident CD68<sup>+</sup>/CD11b<sup>-</sup> KC with high phagocytic activity, and CD68<sup>+</sup>/CD11b<sup>+</sup>-infiltrating macrophages (22). FACS analysis revealed that our purified primary KC were CD68<sup>+</sup>/CD11b<sup>-</sup> and thereby corresponded to the resident KC population. Using >15 donors, we obtained between 85 and 95% purity of CD68<sup>+</sup>/CD11b<sup>-</sup> KC (Fig. 1A). Accordingly, 90–95% of KC were able to phagocytose unopsonized FITC-conjugated zymosan A (Supplemental Fig. 1A, 1B). The NLRP3 gene was expressed at basal levels and this expression rose in response to LPS (TLR4 ligand), and the AIM2 ligand poly(dA:dT) led to the production of pro-IL-1 $\beta$  and AIM2, whereas procaspase-1, ASC, and pro-IL-18 displayed constitutive expression (Fig. 1B, 1C). As expected, KC primed with LPS plus AIM2 or NLRP3 activators triggered the production of mature IL-1 $\beta$  and IL-18 (Fig. 1D). KC that were primed with LPS followed by ATP (another NLRP3 ligand) or nigericin (data not shown) also led to the production of mature IL-1 $\beta$ , as confirmed by immunoblotting (Fig. 1E). We validated our findings using PVL, a pore-forming toxin that mediates cytotoxic effects after binding to the C5a receptor (C5aR/CD88) expressed in KC. PVL treatment killed all CD68<sup>+</sup>/CD11b<sup>-</sup> KC (data not shown) and led to the loss of inflammasome activation via AIM2 and NLRP3 (Supplemental Fig. 1C). Taken together, we show the expression of several inflammasome genes and that functional NLRP3 and AIM2 inflammasomes are present in KC.

### KC AIM2-mediated inflammasome activation of IL-8 transcription and NK cell IFN- $\gamma$ production

IL-1 $\beta$ -induced IL-8 transcription in immune cells promotes chemotaxis and positively regulates the synthesis of proangiogenic factors. The bioactivity of the supernatant from AIM2-activated KC led to the induction of the IL-8 promoter, which was blocked by the IL-1R antagonist anakinra (Fig. 2A). ATP and nigericin were toxic to KC transfected with the IL-8 promoter, meaning that luciferase activity could not be measured (data not shown). In response to IL-18 and IL-12 costimulation, NK cells produce IFN- $\gamma$ , which plays an important role in antiviral immunity. Thus, we cocultured inflammasome-activated KC and PBMC to compare the percentage of intracellular IFN- $\gamma$ -producing NK cells. FACS analysis showed that the percentage of IFN- $\gamma$ -producing NK cells in PBMC increased from 3% in nontreated cells to 24% in inflammasome-activated cells. The addition of IL-18 binding protein  $\alpha$  reduced the percentage of inflammasome-activated NK cells, showing that IFN- $\gamma$  production was mostly dependent on IL-18

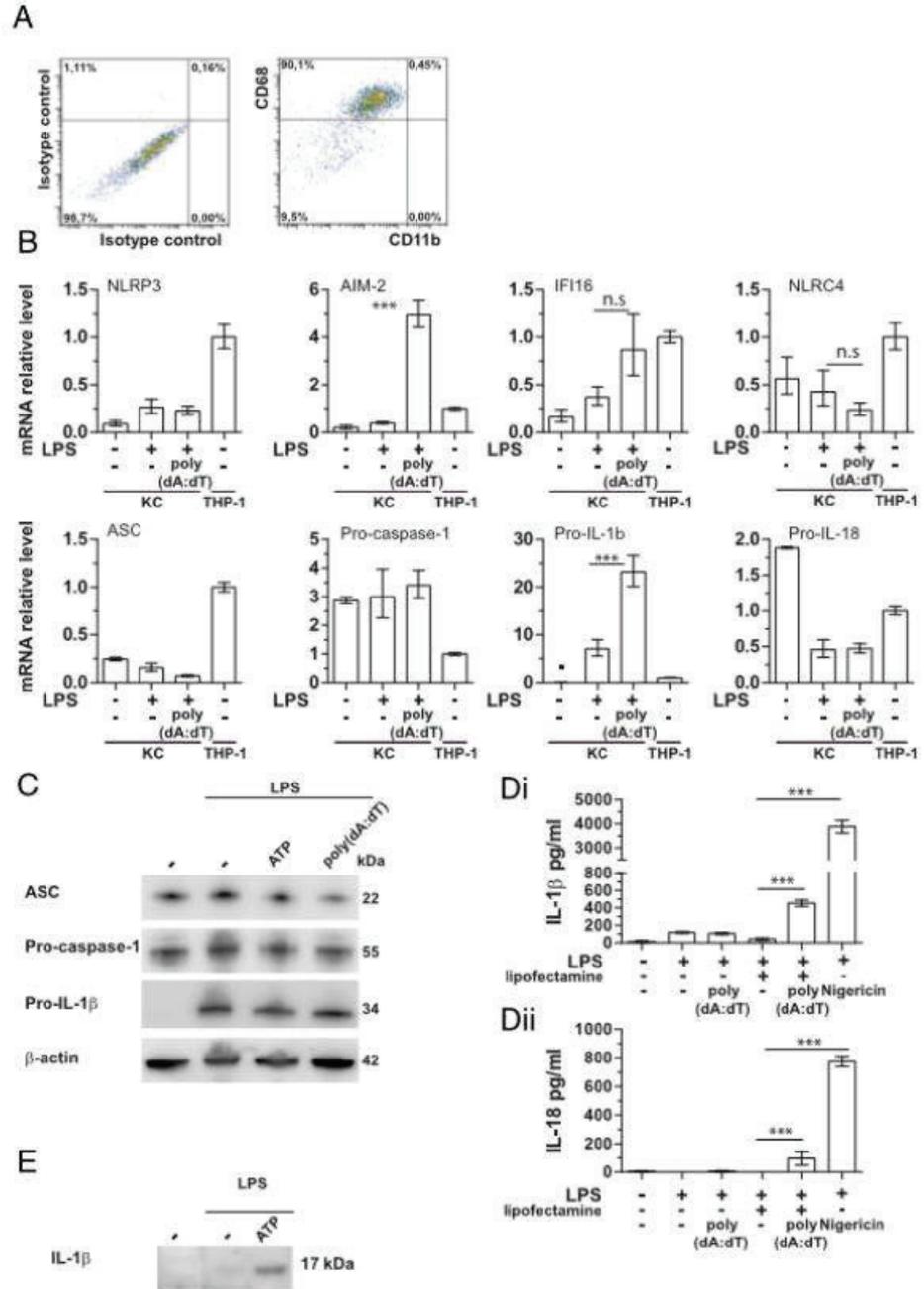
(Fig. 2B). Similar results were obtained from liver NK cells (Fig. 2C). These results show that human KC have functional AIM2 and NLRP3 inflammasomes and that AIM2 production of IL-1 $\beta$  and IL-18 is essential for IL-8 transcription as well as activating liver and peripheral blood NK cells, respectively.

### KC and not primary cultured human hepatocytes have functional AIM2 and NLRP3 inflammasomes

A functional NLRP3 inflammasome in hepatocytes has been reported by some (23) and excluded by others (24). Therefore, we wanted to determine whether PCHH expressed inflammasome genes. PVL treatment was used to remove KC from PCHH, and this removal was confirmed by FACS (Fig. 3A) or phagocytosis of unopsonized FITC-conjugated zymosan A (data not shown). Pro-IL-1 $\beta$  mRNA and protein expression induced by LPS was completely abrogated in KC-depleted PCHH (Fig. 3B, 3C) as were AIM2 transcripts detected upon LPS plus poly(dA:dT) treatment. In contrast, transcript levels of ASC, procaspase-1, pro-IL-18, and protein levels of ASC or procaspase-1 did not change in KC-depleted PCHH (Fig. 3C, 3D). Thus, PVL-mediated KC depletion led to the complete loss of mature IL-1 $\beta$  and IL-18 production upon activation of the AIM2 and NLRP3 inflammasomes in PCHH (Fig. 3D). To determine whether PVL treatment had any toxic effect on hepatocytes, we primed KC-depleted and undepleted PCHH with TLR2-L (Pam3CSK4) and TLR4-L (LPS) (20). Both receptors remained functional following PVL treatment, as determined by IL-6 secretion (Supplemental Fig. 2A). The lack of functional AIM2 and NLRP3 inflammasomes in hepatocytes was further confirmed in HepaRG cells devoid of contaminating immune cells (25). As observed in KC-depleted PCHH, neither IL-1 $\beta$  nor IL-18 production was detected in the HepaRG cells after AIM2 or NLRP3 inflammasome activation (Supplemental Fig. 2B, 2C). These data demonstrate that hepatocytes do not contain functional NLRP3 or AIM2 inflammasomes.

### Several pathogens activate the inflammasome response in KC

We addressed whether an inflammasome response could be induced in KC by liver-related bacterias. *F. novicida* primarily infects macrophages and triggers the AIM2 and NLRP3 inflammasome (26). *S. typhimurium* is a flagellated bacterium that activates the NLRP3 inflammasome in response to flagellin secreted into the host cytosol using the SPI-1-encoded type III secretion system (27). Infection of primary KC with *F. novicida* WT strain U112 led to the production of IL-1 $\beta$  and IL-18 in a multiplicity of infection (MOI)-dependent manner (Fig. 4A). In contrast, the deletion of the *Francisella* pathogenicity island (28) reduced the ability of *F. novicida* to elicit IL-1 $\beta$  and IL-18 production in KC. Infection of primary KC with *S. typhimurium* also led to the production of IL-1 $\beta$  and IL-18. This production was strongly impaired when KC were infected with the *Salmonella* mutant  $\Delta$ SIPB, which lacks the functional SPI-1-encoded type III secretion system (Fig. 4B). We corroborated our data by detecting the cleaved forms of both caspase-1 and IL-1 $\beta$  in the supernatant of KC upon infection by WT strains of *F. novicida* and *S. typhimurium* but not upon infection with mutant strains (Fig. 4C). Activation of caspase-1 is known to activate a form of programmed cell death. As assessed by PI incorporation, infection of KC by WT strains of *F. novicida* (Fig. 4D) and *S. typhimurium* (Fig. 4E) resulted in cell death, which was strongly impaired when using mutant strains. It has been reported that KSHV can activate the IFI6 inflammasome (8). Accordingly, 24 h incubation of KSHV in LPS-treated and untreated KC led to a significant production of IL-1 $\beta$  (Fig. 4Fi) and IL-18 (Fig. 4Fii). Collectively, these results show, in a physiological setting, that the liver-associated pathogens *F. novicida* and *S. typhimurium*, as well as KSHV, activated inflammasome responses in KC.



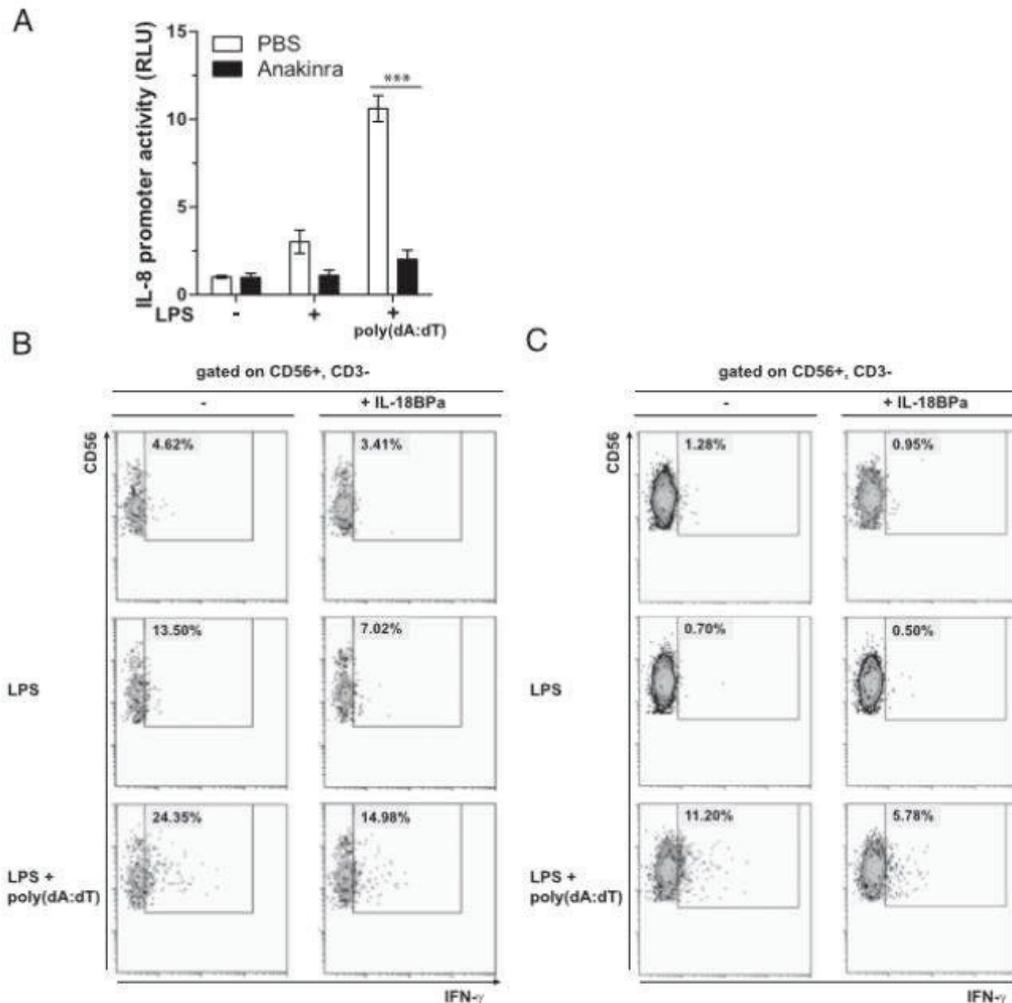
**HBV inhibits the AIM2 inflammasome in KC**

HBV infects the human liver. It is considered a stealth virus that has evolved multiple strategies to evade host immunity, but inflammasome activity in response to HBV has not been examined. The addition of HBV for 3 h on PCHH or purified KC did not induce IL-1 $\beta$  or IL-18 production (Fig. 5A, 5B). Indeed, we observed that HBV virions were able to enter KC as measured by confocal microscopy (Fig. 5C). Moreover, it appeared that HBV inhibited AIM2 inflammasome activation both in PCHH and KC (Fig. 5A, 5B). These results were confirmed in differentiated THP-1 monocyte cells, an established cell line that has been used in previous studies on inflammasomes, using several different batches of HBV (Fig. 5D). The inhibition of IL-1 $\beta$  led to a reduction in IL-8 bioactivity (Fig. 5D). We then examined how HBV affects AIM2 function. Overexpression of AIM2 and the adapter ASC leads to the formation of an inflammasome complex, which is visible as a speck when observed by fluorescence microscopy

(5). The addition of HBV had no effect on AIM2/ASC speck formation (Fig. 6A), nor did it affect AIM2 protein levels, which are driven by an exogenous promoter (Fig. 6B). However, HBV did decrease AIM2 transcripts in KC (Fig. 6C). These results show that HBV can specifically inhibit the AIM2 inflammasome, but not the NLRP3 inflammasome, in KC and differentiated monocytes.

**Hepatitis B surface Ag blocks IRF7 binding to the AIM2 promoter**

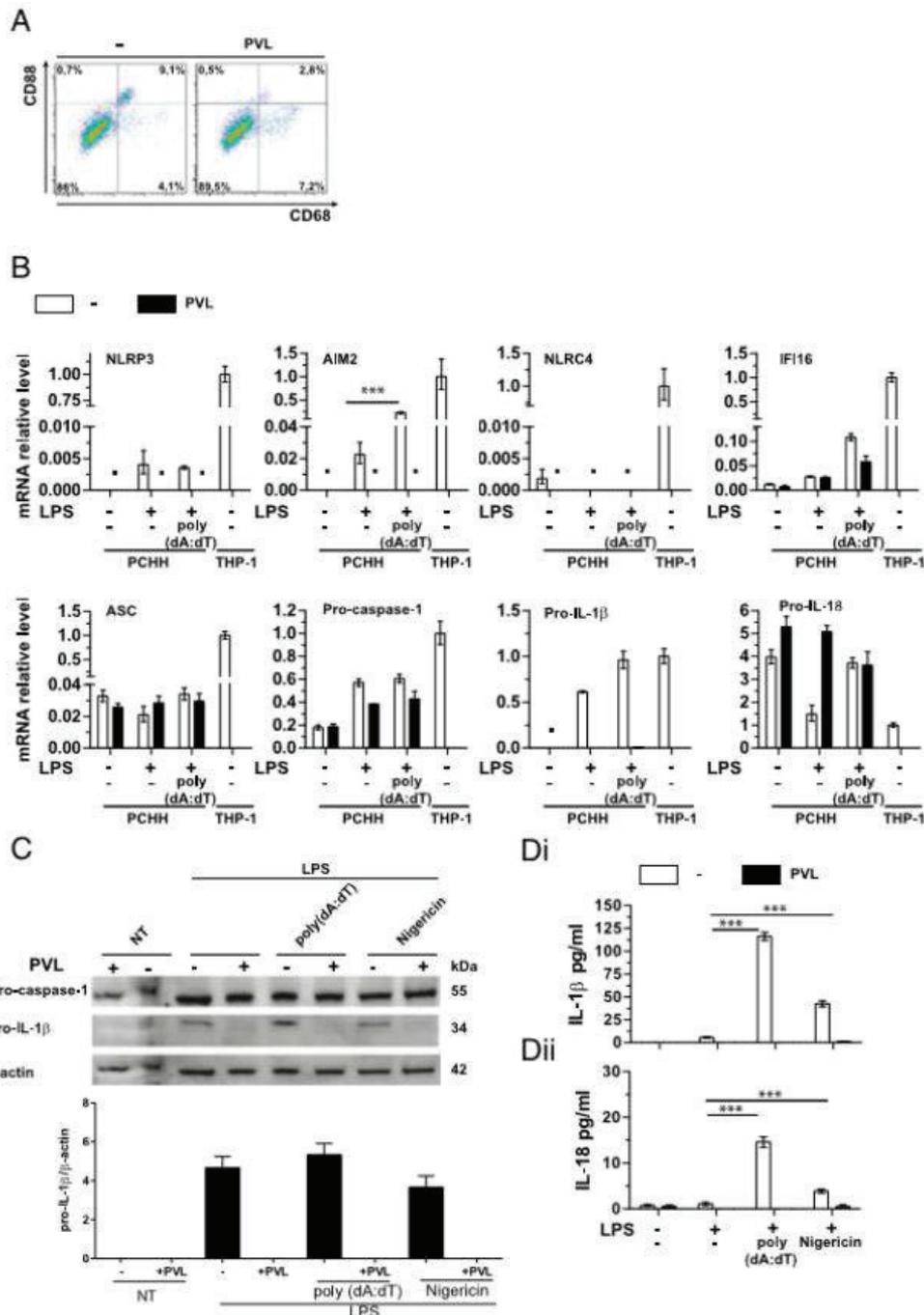
We wanted to determine which viral component was responsible for blocking the AIM2 inflammasome. HBV is a DNA envelope virus that contains HBc, which encapsulates the viral DNA envelope, and HBs, which forms a complete viral or Dane particle. HBV particles HBs and HBe (a truncated form of HBc) are secreted by infected hepatocytes (29). Dane particles and HBe had no effect (Fig. 7B) on AIM2 function, but HBs was able to block IL-1 $\beta$  production in a dose-dependent manner (Fig. 7C). AIM2 transcription is induced



**FIGURE 2.** AIM2 inflammasome activation in KC leads to production of IL-8 and IL-18 and biological activation of NK cells. **(A)** After 24 h the supernatant from KC primed with LPS with and without poly(dA:dT) or left untreated was incubated with PBS or anakinra and added to HEK293 cells transfected with the IL-8 luciferase promoter.  $***p > 0.0001$ . **(B and C)** Freshly purified KC were primed overnight with LPS, washed, and then transfected with poly(dA:dT) or left untreated. After 3 h, PBMC (B) or LMNC (C) were added to each well with and without recombinant IL-18 binding protein  $\alpha$  (IL-18BP $\alpha$ ) and 6 h later were analyzed by FACS following cytokine intracellular staining. FACS plots show cells population following gating on CD4<sup>-</sup>, CD14<sup>-</sup>, CD19<sup>-</sup>, CD3<sup>-</sup>, CD56<sup>+</sup> cells. The percentage of IFN- $\gamma$ <sup>+</sup> NK cells is shown. Results shown are from one experiment representative of three independent experiments.

by type I IFNs (30), and HBV has been shown to inhibit IRFs, which are essential for IFN type I production. Indeed, the suppression of AIM2 mRNA by HBV was reversed when additional IFN- $\beta$  was added (Fig. 8A). HBV may decrease AIM2 by two distinct mechanisms: HBV can affect the stability of AIM2 transcript or it can directly inhibit AIM2 transcription. To discriminate between these two possibilities, we have determined whether HBV could influence the activity of the AIM2 promoter. The isolated AIM2 promoter cloned in front of the luciferase reporter gene was introduced by transient transfection in the THP-1 cell line that expresses endogenous AIM2. High basal luciferase activity was detected in these cells after transient transfection, but HBV as well as HBs inhibited luminescence, indicating that HBV via HBs can block the transcription of the AIM2 gene (Fig. 8B). Furthermore, a mutation at the IRF site that has been shown to regulate AIM2 transcription by IRF1 (19) prevented HBV as well as HBs inhibition of the AIM2 promoter (Fig. 8B). HBe did not inhibit AIM2 promoter activity (data not shown). Indeed, the knockdown of IRF7 reduced the basal levels of AIM2 (Supplemental Fig. 3), indicating that IRF7 in our

system regulates AIM2 expression. We previously reported that HBV can block IRF7 activity (15). We observed that in THP-1 cells treated with IFN- $\beta$ -induced IRF7 mRNA expression (Fig. 8C), this expression was blocked by HBs but was restored when additional IFN- $\beta$  was added (Fig. 8C). ChIP assays in THP-1 cells revealed that IRF7, and not IRF1 (19), was bound to the IRF site on the AIM2 promoter; however, HBs reduced IRF7 binding on the IRF site (Fig. 8D). We also noted that bound IRF7 on the AIM2 promoter was further increased in the presence of IFN- $\beta$  but was abrogated by HBs (Fig. 8E). Additional IFN- $\beta$  treatment overcame IRF7 inhibition by HBs (Fig. 8E). We next corroborated our findings in KC. As seen in THP-1 cells, IRF7 mRNA levels were reduced in KC when exposed to HBs (Fig. 8F). We next hypothesized that HBs may affect the stability of IRF7 mRNA, which influences AIM2 levels. KC were treated with actinomycin D for 24 h and then treated for 3 h with HBs. We observed that the half-life of IRF7 mRNA was reduced by HBs in KC (Fig. 8G). Additionally, IRF7 protein levels were abrogated by HBs, which was correlated with a reduction in IRF7 binding to the AIM2 promoter (Fig. 8H). We show, to our

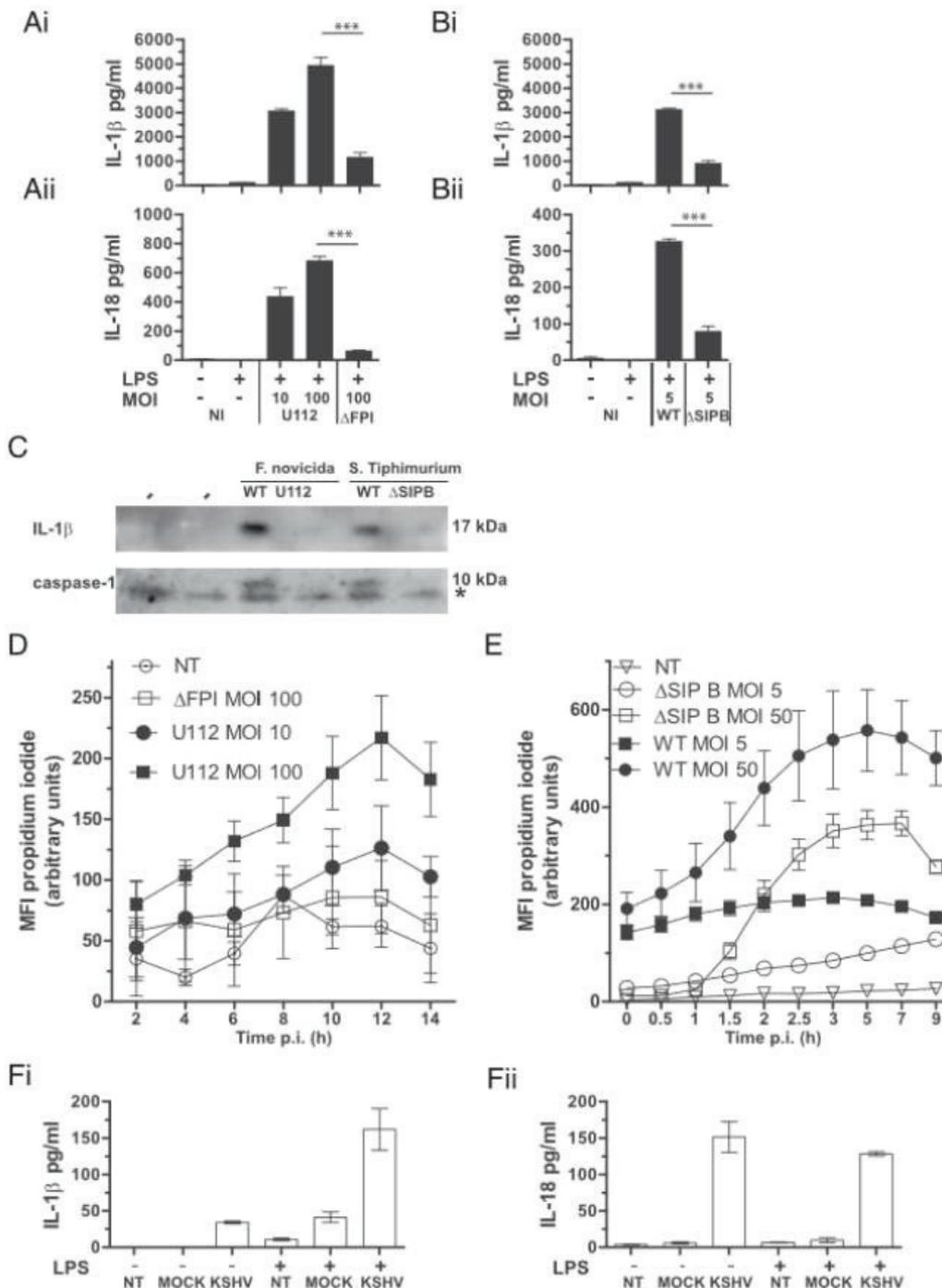


**FIGURE 3.** KC, not hepatocytes, represent the primary source of NLRP3, AIM2, and IL-1 $\beta$  production and display inflammasome functionality. **(A)** KC populations in PCHH cultures were analyzed by FACS using CD68 and CD88 Abs. **(B)** PCHH with and without PVL and/or LPS were treated for 4 h, and cells were transfected with poly(dA:dT) or left untreated for a further 3 h. The mRNA levels of NLRP3, AIM2, NLRC4, IFI16, ASC, procaspase-1, pro-IL-1 $\beta$ , and pro-IL-18 were determined by qPCR and normalized to GAPDH. PMA-differentiated THP-1 served as control. **(C)** PCHH primed with LPS with and without PVL as in (A) were primed with the AIM2 and NLRP3 inflammasome activators for 3 h and harvested and protein extracts were used for monitoring procaspase-1, pro-IL-1 $\beta$ , and  $\beta$ -actin protein levels;  $\beta$ -actin served as the loading control. One out of three experiments is represented (Western blot); densitometry was performed for IL-1 $\beta$  levels on all three experiments and is represented in graph form. **(D)** The supernatant of PCHH treated as in (C) was harvested, and IL-1 $\beta$  (i) and IL-18 (ii) levels were assessed by ELISA. Data are representative of three independent experiments performed in triplicate. The means  $\pm$  SEM are shown. \*\*\* $p$  < 0.0001, based on an unpaired Student  $t$  test.

knowledge for the first time, that different pathogens behave differentially on the same cell type. We demonstrated that human KC have active inflammasomes that respond to liver-borne bacterial infections as well as to KSHV. However, HBV can abrogate AIM2 responses by deregulating IRF7 expression and binding on the AIM2 promoter.

### Discussion

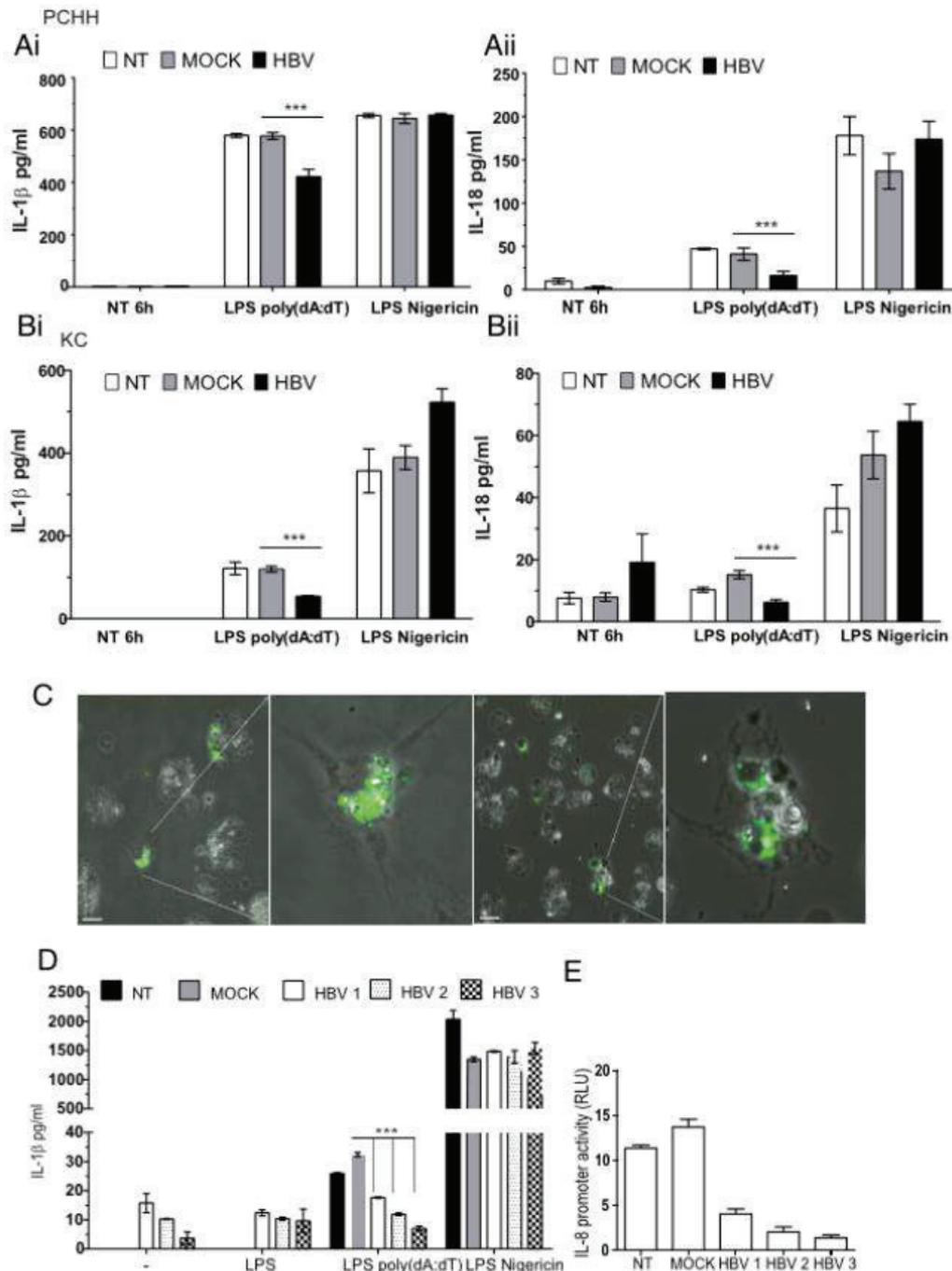
Many different microbial and viral signals activate innate immunity in the presence of liver disease. The inflammasome response may inhibit or contribute to several of these pathologies. This balance was extensively discussed in the review by Szabo and Csak (31), and several recent findings speculate on the role of different liver



**FIGURE 4.** Induction of the inflammasome pathway in purified KC in response to live pathogens. **(A)** KC were infected with *F. novicida* WT or  $\Delta$ FPI mutant strains at the indicated MOI. Eight hours after infection, supernatant was harvested and analyzed for IL-1 $\beta$  **(Ai)** and IL-18 **(Aii)** concentration by ELISA. **(B)** The supernatant of KC infected for 3 h at an MOI of 5 with *S. typhimurium* WT and  $\Delta$ SIPB mutant strains was harvested, and IL-1 $\beta$  **(Bi)** and IL-18 **(Bii)** concentration was assessed by ELISA. **(C)** The supernatant from KC treated as in **(A)** and **(B)** was analyzed by Western blot to determine IL-1 $\beta$  and caspase-1 protein levels. **(D)** and **(E)** Cell death of KC infected with *F. novicida* **(D)** and *S. typhimurium* **(E)** at the indicated MOI and time after infection was assessed by measuring PI fluorescence using a 96-well microplate fluorometer. **(F)** KC were primed with LPS for 4 h and then incubated with KSHV or MOCK (MOI of 25). After 24 h, supernatant was harvested and IL-1 $\beta$  **(Fi)** and IL-18 **(Fii)** concentration was determined by ELISA. The means  $\pm$  SEM are shown. \*\*\* $p < 0.0001$ , based on an unpaired Student *t* test. MFI, mean fluorescence intensity; NT, not treated.

cells in various liver pathologies. However, there is still no clear study on which liver cells contribute to liver inflammasome activity. We deduced that KC would be the major contributor in liver inflammasome responses, as it has been historically characterized in murine bone marrow and human monocyte-derived macrophages. In this study, we characterized the expression of inflammasome

genes and signaling molecules, as well as the inflammasome response to ligands and pathogens in KC. We have shown that IL-1 $\beta$  and IL-18 are produced in response to AIM2, NLRP3, IFI16, and NLRP4 activators. Moreover, our data show inflammasome activation of human KC in response to *F. tularensis* and *S. typhimurium*. Although PCHH did not show functional NLRP3 or AIM2



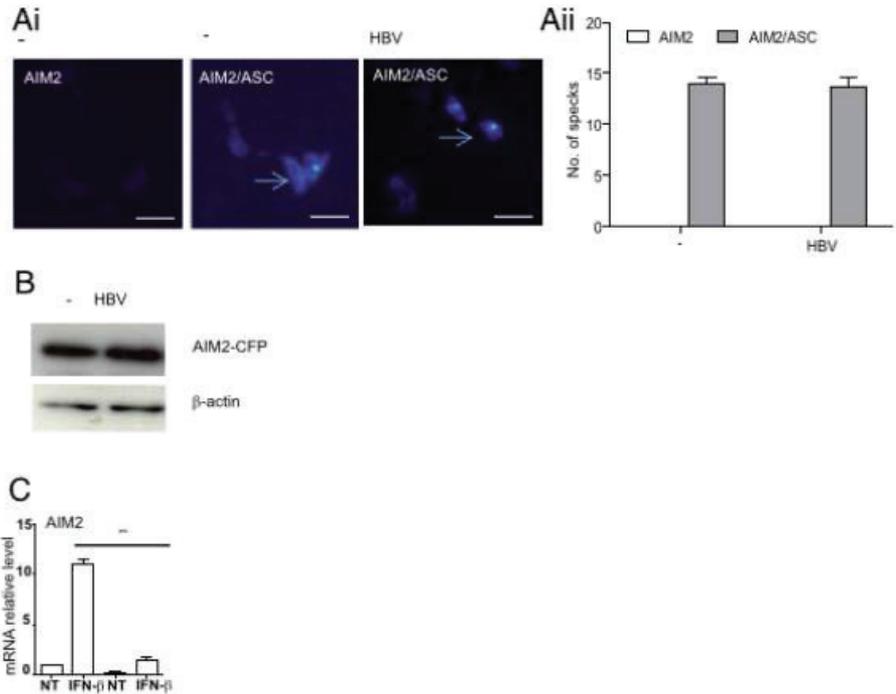
**FIGURE 5.** The AIM2 inflammasome is blocked by HBV. **(A)** PCHH were primed with LPS for 3 h and then incubated with HBV, MOCK, or left untreated for 3 h. Nigericin (ligand for NLRP3) and poly(dA:dT) (ligand for AIM2) were added for 3 h. IL-1 $\beta$  (**Ai**) and IL-18 (**Aii**) was measured by ELISA. **(B)** KC were primed with LPS for 3 h. After 3 h of incubation with HBV or MOCK, nigericin and poly(dA:dT) were added for 3 h. IL-1 $\beta$  (**Bi**) and IL-18 (**Bii**) levels were assessed by ELISA. **(C)** Kupffer cells were purified as described, and  $20 \mu\text{l } 2 \times 10^{10}$  Alexa Fluor 488-labeled HBV virions was added for 2 h. Images were taken 3 h after HBV treatment using the Olympus FV110 confocal microscope. Scale bars, 25  $\mu\text{m}$ . **(D)** THP-1 cells were differentiated and primed with LPS for 3 h, and then three different viral productions of HBV were added for 3 h. Cells were washed three times with PBS, and nigericin and poly(dA:dT) were added for 3 h. IL-1 $\beta$  levels were assessed by ELISA. **(E)** Supernatant from cells primed with LPS with and without HBV with and without poly(dA:dT) or left untreated was incubated with PBS or anakinra and added to HEK293 cells transfected with the IL-8 luciferase promoter for 24 h. Data are representative of three independent experiments performed in duplicate. The means  $\pm$  SEM are shown. \*\*\* $p < 0.0001$ , based on an unpaired Student *t* test.

inflammasomes, we cannot exclude the possibility that other functional inflammasomes exist in hepatocytes, and we think that further investigations in this area are warranted.

KC tightly adhere to sinusoidal endothelial cells and are often in contact with NK cells. This cross-talk between KC and NK cells is essential for immune activation. We demonstrated that stimulation

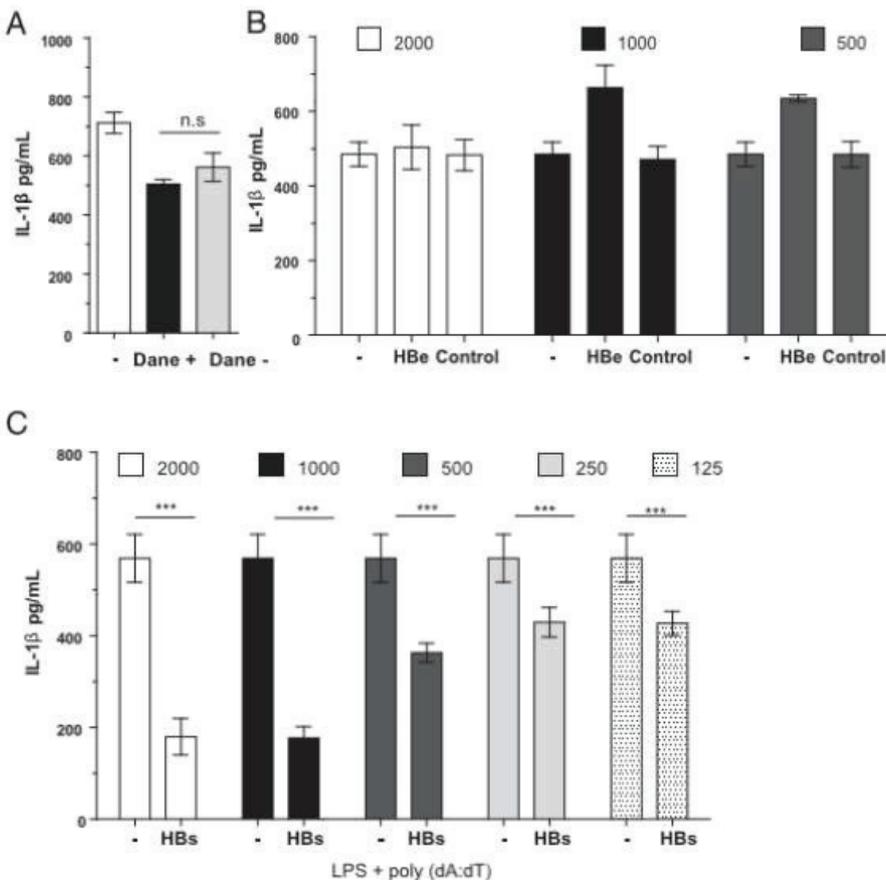
of AIM2 with poly(dA:dT) led to IL-18 production in KC, and coculture with NK cells led to IFN- $\gamma$  production. A recent study linked inflammasome activation to NK cell-mediated tumor attack to suppress metastasis in the liver, demonstrating the importance of the innate immune system in this organ (32). Mice with colorectal cancer who were deficient in NLRP3 inflammasome components

**FIGURE 6.** AIM2/ASC speck formation was not affected by HBV. **(A)** (i) AIM2-CFP and ASC-YFP plasmids were transfected in HEK293 cells for 36 h. HBV was added at a viral genome equivalent of 2000 and speck formation was observed. Scale bars, 20  $\mu$ m. (ii) Specks were counted in five different fields and are represented as a graph. Data are representative of three independent experiments performed in duplicate. **(B)** As in (A), after HBV addition, HEK293 cells were lysed and immunoblotting was performed using a GFP Ab to detect AIM2 protein levels. **(C)** PMA-differentiated THP-1 were primed with IFN- $\beta$  with and without HBV for 6 h and AIM2 mRNA was measured by qPCR and normalized to housekeeping gene expression GAPDH.  $***p > 0.0001$ .



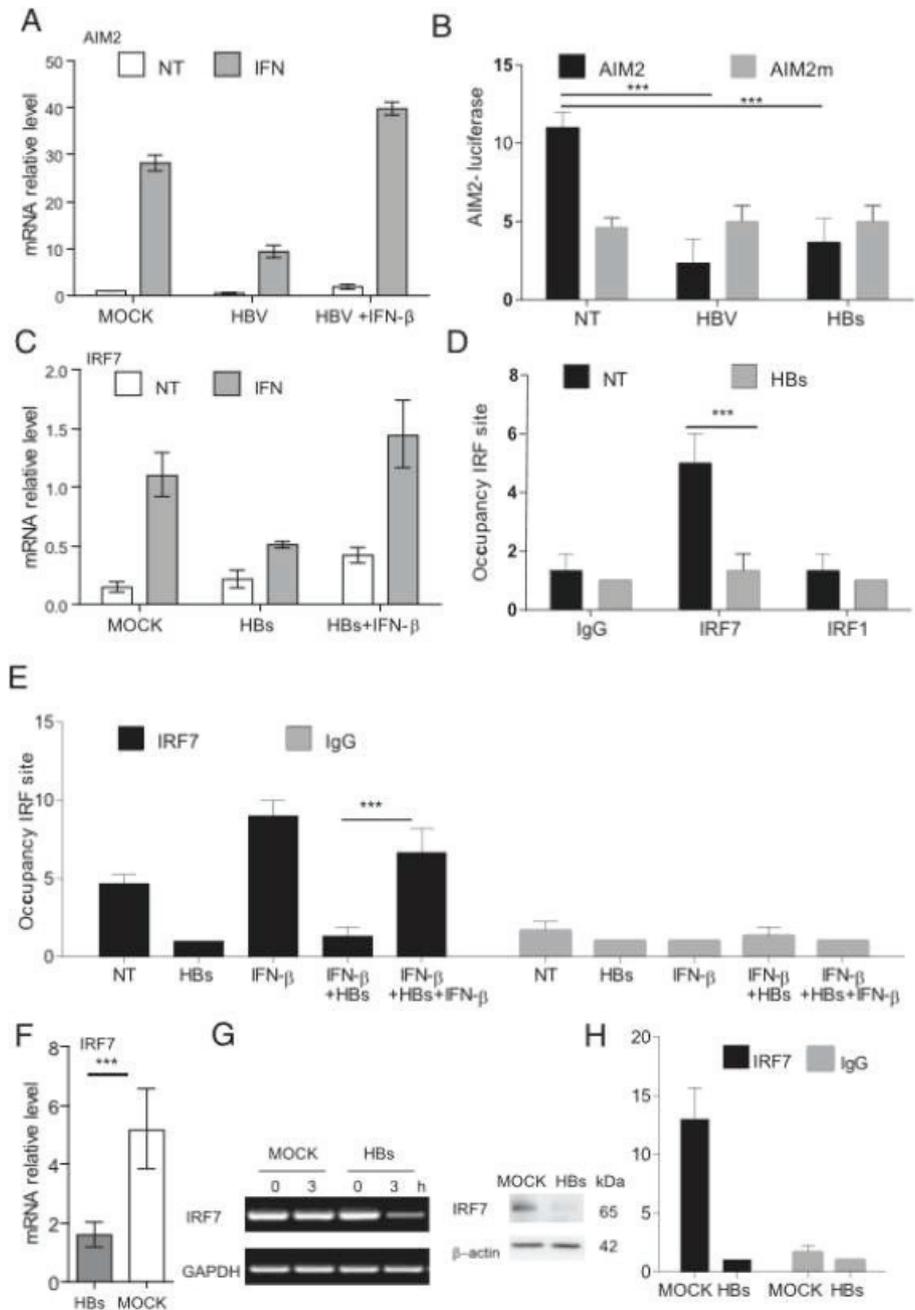
had exacerbated metastatic growth in the liver, which was mediated when the inflammasome production of IL-18 in KC was impaired, reducing the maturation of hepatic NK cells, the surface expression of the death ligand FasL, and the capacity to kill FasL-sensitive tumors. Indeed, the inflammasome might have a crucial role in mediating multiple immune response signals in the liver, which can

vary in their timing, cellular location, and intensity, and can determine the pathological outcome. Recent works described the role of IL-1 $\beta$  in the pathogenesis of several different chronic liver diseases (33–36). Jo et al. (37) revealed a link between hepatic inflammation and disease in patients with chronic HCV, which was attributed to IL-1 $\beta$  secretion following activation of the NLRP3 inflammasome



**FIGURE 7.** HBs inhibits AIM2 mRNA expression. **(A)** THP-1 cells were differentiated and primed with LPS for 3 h, and then Dane particles or a control (ranging from 2000 to 500 viral genome equivalents) at a viral genome equivalent of 1000 were added for 3 h. Cells were washed three times with PBS, and poly(dA:dT) was added for 3 h. IL-1 $\beta$  levels were assessed by ELISA. **(B)** THP-1 cells were differentiated and primed with LPS for 3 h and then exposed to a decreasing concentration of HBs particles or control. After 3 washes with PBS, poly(dA:dT) was added for 3 h. IL-1 $\beta$  levels were assessed by ELISA. **(C)** THP-1 cells were differentiated and primed with LPS for 3 h and then exposed to a decreasing concentration of HBs particles or control. Cells were washed three times with PBS and poly(dA:dT) was added for 3 h. IL-1 $\beta$  levels were assessed by ELISA.  $***p > 0.0001$ .

**FIGURE 8.** IRF7 expression is abrogated by HBV, which prevents AIM2 expression. **(A)** THP-1 cells were differentiated and treated with MOCK or HBV at a viral genome equivalent of 400 for 3 h with and without IFN- $\beta$  (200 ng/ml). Cells were washed three times with PBS and IFN- $\beta$  was added for another 3 h. The mRNA levels of AIM2 were determined by qPCR using GAPDH and/or  $\beta_2$ -microglobulin as the housekeeping gene. **(B)** THP-1 cells were transfected with the AIM2 WT or mutated promoter linked to luciferase gene and processed as previously described (15, 20). **(C)** As in (A), IRF7 mRNA expression was examined by qPCR. **(D)** ChIP assay using anti IRF1, IRF7, or IgG was performed on the IRF site using THP-1 cells treated with HBs at 400 viral genome equivalent for 3 h. **(E)** THP-1 cells were treated as in (A) and ChIP assay performed for IRF7. **(F)** KC were treated with HBs or MOCK for 3 h and mRNA levels for IRF7 were examined by qPCR using GAPDH and/or  $\beta_2$ -microglobulin as the housekeeping gene. **(G)** KC were treated with actinomycin D for 24 h and then treated for 3 h with HBs or MOCK at 200 viral genome equivalent, and an RT-PCR was performed for IRF7 and GAPDH. Immunoblot analysis for IRF7 and  $\beta$ -actin was performed on THP-1 cells treated with HBs or MOCK for 3 h. **(H)** ChIP assay for IRF7 binding to the IRF site on the AIM2 promoter was performed on KC treated for 3 h with HBs or MOCK. The means  $\pm$  SEM are shown. \*\*\* $p$  < 0.0001, based on an unpaired Student  $t$  test. Data are representative of three independent experiments performed in duplicate.



in liver macrophages (CD68<sup>+</sup>/CD14<sup>+</sup>). TLR8 induction of IL-18 and IL-12 was reported in infiltrating liver macrophages and MAIT cells in normal and in HBV- and HCV-infected livers (25).

However, certain works have highlighted the role of IL-1 $\beta$  and other innate cytokines in controlling HBV infection in liver cells. Watashi et al. (38) found that priming with IL-1 $\beta$  and TNF- $\alpha$  remarkably reduced host cell susceptibility to HBV infection. Furthermore, Hösel et al. showed that HBV particles and HBs induce IL-1 $\beta$ , IL-6, CXCL8, and TNF production by human CD68<sup>+</sup> cell-enriched nonparenchymal cells via NF- $\kappa$ B activation (14) and subsequently inhibit HBV replication in primary hepatocytes. This inhibitory effect was mainly ascribed to IL-6, but also to TNF-inhibited HBV replication in a noncytopathic manner. More recently, Boltjes et al. (39) showed that HBs is internalized by KC, leading to NK activity. In contrast, Li et al. (40) demonstrated that rat ED1<sup>+</sup>-adherent KC exposed to HBV virions hardly expressed IL-1 $\beta$ , IL-6, or TNF, but produced the immunoregulatory

cytokine TGF- $\beta$ . Our data suggest that targeting AIM2 prevents the recognition of dsDNA expressed by the HBV, and that the limited innate response observed upon HBV infection may be due to viral-mediated immune evasion. There are several studies that support this hypothesis. HBe has been implicated in the downregulation of TLR2 functionality (41) and expression in hepatocytes, KC, and PBMC. HBs has been reported to inhibit TLR9 functionality and expression in plasmacytoid dendritic cells and PBMC (15). The nonstructural HBx protein encoded by HBV has been shown to affect RIG-I-MDA5 signaling in vitro (42). Furthermore, a previous study showed diminished IL-18 responses and reduced IFN- $\gamma$  of NK cells in differentiated monocytes from PBMC (43). HBV polymerase and HBs have been shown to affect the signaling of transcription factors associated with IFN transcription, such as IRF3 and IRF7 (15, 44, 45). In the present study, we showed that HBV via HBs can block IRF7 mRNA stability, which in turn affects AIM2 expression. These novel findings demonstrate another strategy used

by HBV to evade innate immune responses. Therefore, understanding inflammasome–pathogen interactions in the liver holds promise not only for our understanding of the fundamental aspects of HBV persistence, but also for our understanding of resolving other liver pathologies.

## Acknowledgments

We thank Drs. Nadege Goutagny (INSERM U1052, Centre de Recherche en Cancérologie–Lyon, Lyon, France) and Peggy Parroche (INSERM U1111, Centre International de Recherche en Infectiologie, Lyon, France) for technical support, as well as Viet Hornung (University of Bonn) and Reuben Tooze (University of Leeds) for reagents.

## Disclosures

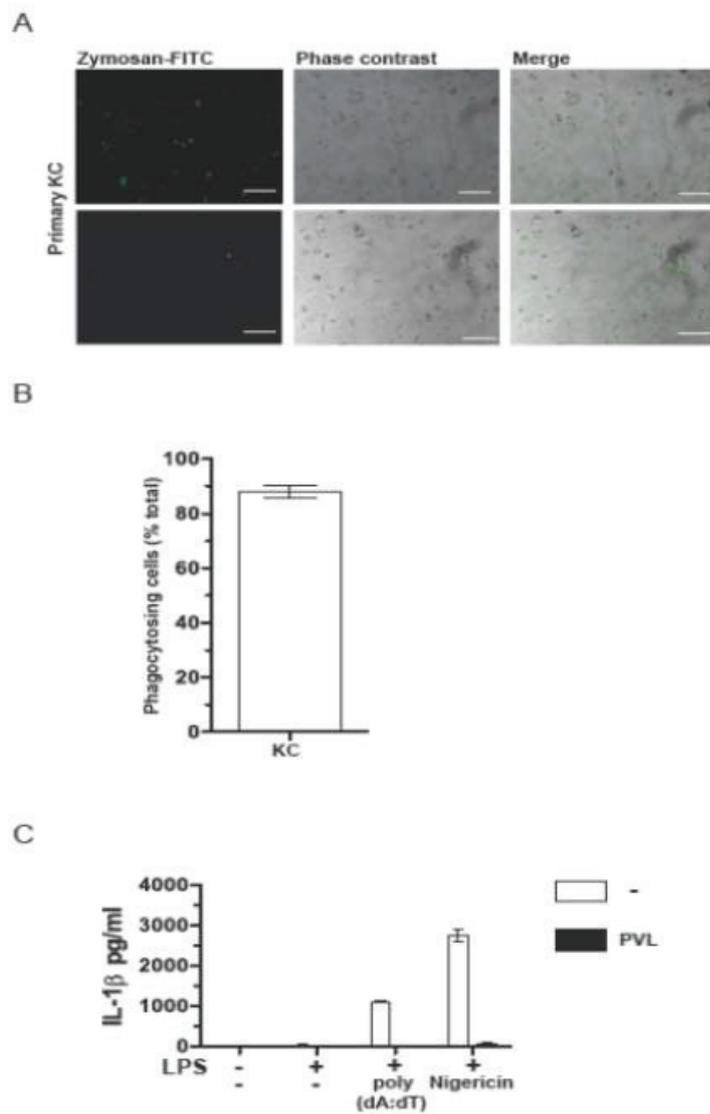
The authors have no financial conflicts of interest.

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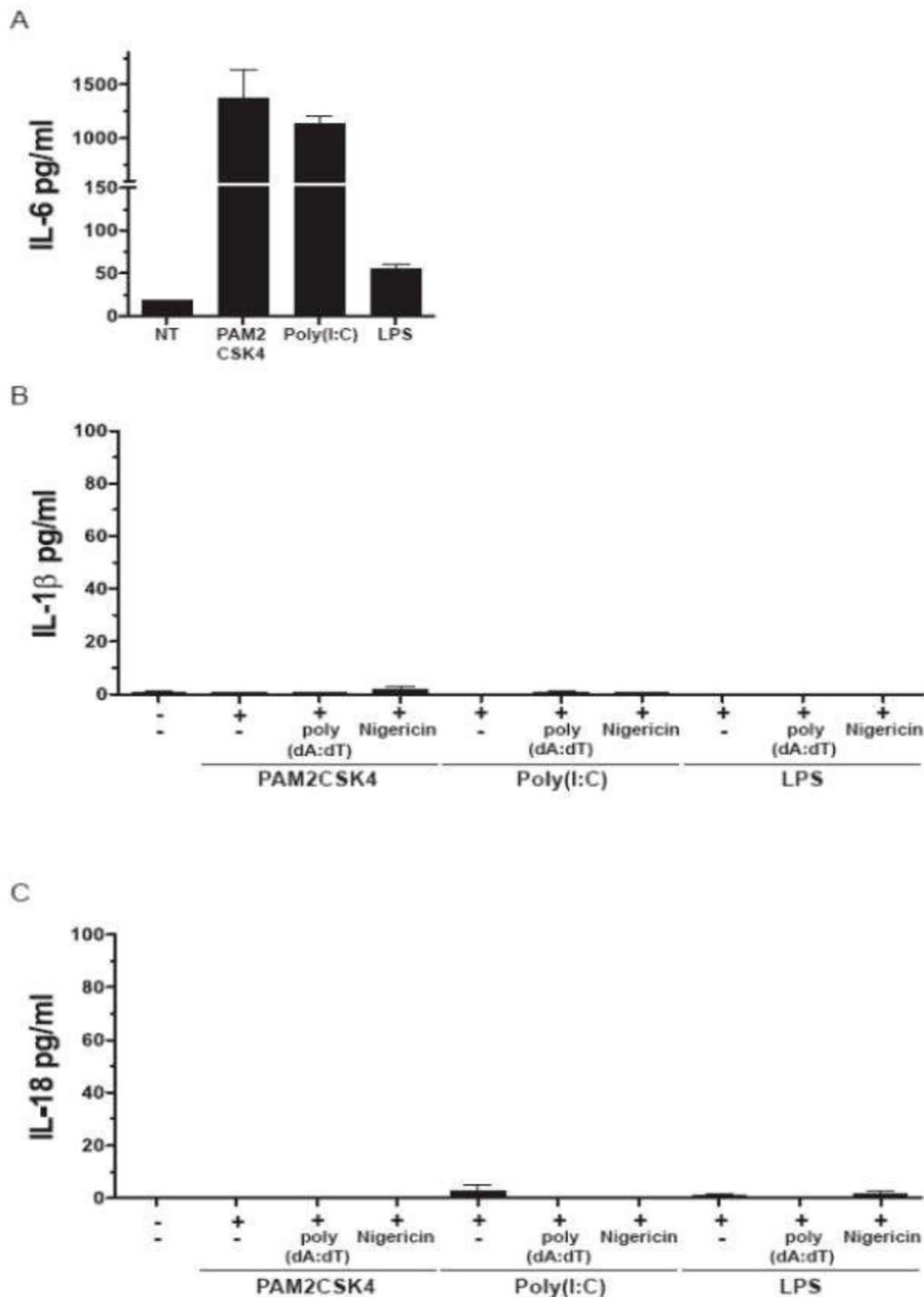
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## Supplementary text :



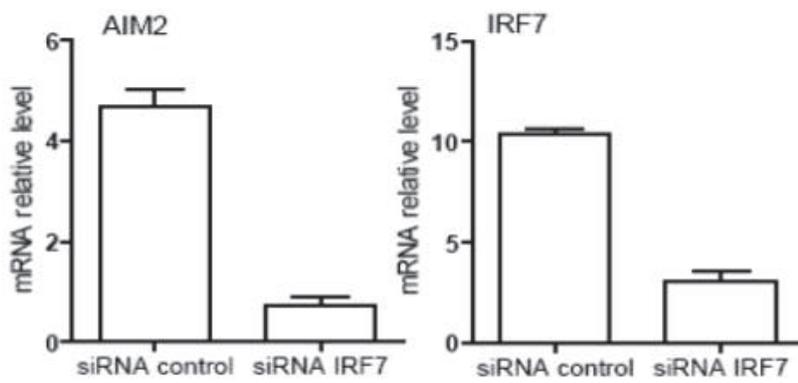
Supplementary Figure 1ABC

**Figure 1.** (A) Phagocytosis of FITC-conjugated zymosan particles by KC. Original magnification,  $\times 20$  (Axio vision microscope). Scale represents 50 $\mu$ m. (B) Graph representation of the number of phagocytosing cells in 5 different fields. (C) PCHH primed with LPS  $\pm$  PVL were stimulated with the AIM2 and NLRP3 inflammasome activators for 3 h and harvested and protein extracts were used for IL-1 $\beta$  secretion by ELISA.



Supplementary Figure 2 ABC

**Figure 2.** (A). HepaRG cells were treated with the indicated TLR ligands for 4 h and then incubated with fresh medium. After 3 h supernatant was harvested and IL-6 concentration was quantified by ELISA. (B, C) HepaRG cells were stimulated with the indicated TLR ligands for 4 h and incubated with inflammasome activators as reported in figure legend. After 3 h supernatants were tested for IL-1 $\beta$  (B) and IL-18 (C) concentration by ELISA.



Supplementary Figure 3

**Figure 3.** KC were treated for 48 h with IRF7 siRNA or scramble control and mRNA levels of the indicated genes were determined by qPCR using GAPDH and/or  $\beta$ 2-microglobulin as the housekeeping gene.



## 6. Fourth study

### Interference of Hepatitis B Virus (HBV) with liver macrophages differentiation and/or activation to promote its establishment

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§ Equal contribution

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**In preparation.**

**Abstract**

The use of immunotherapeutic strategies is currently being investigated as an alternative to the current treatments of chronic Hepatitis B Virus (HBV) infection that only allow replication viro-suppression. IL-1 $\beta$  is a pro-inflammatory cytokine produced upon inflammasomes induction that has a very strong antiviral effect on HBV replication in hepatocytes. In the liver, this cytokine is mostly produced by pro-inflammatory macrophages or related cells, but little is known on the interaction between HBV and these cells. Using primary human liver macrophages, primary blood monocytes differentiated into pro-inflammatory or anti-inflammatory macrophages, liver biopsies (from healthy or HBV-infected donors), primary human hepatocytes and differentiated HepaRG cells, we performed *ex vivo* experiments to further characterize the interplay between HBV and macrophages. We showed that pro-inflammatory macrophages strongly reduced the establishment of HBV infection in hepatocytes through the secretion of cytokines such as IL-1 $\beta$ . Moreover, we demonstrated that HBV escapes the antiviral effect of pro-inflammatory macrophages by interfering with their activation or differentiation respectively. Finally, we observed that HBV promotes the activation of anti-inflammatory macrophages to prevent the triggering of the adaptive immunity. Altogether our data showed that HBV can modulate the liver innate immunity to favour its establishment.

## **Introduction**

Hepatitis B virus (HBV) chronically infects around 250 million people worldwide (WHO data, 2016). Current treatments of CHB patients, mainly based on nucleos(t)ide analogues, induce a viro-suppression, but do not allow virus elimination from the liver (1). The persistence of HBV replication in CHB patients increases their risk of developing cirrhosis and hepatocellular carcinoma (2). New treatments are needed to eliminate HBV infection in these patients.

HBV is a small DNA virus that persists as a covalently-closed-circular DNA (cccDNA) within the nucleus of liver parenchymal cells (hepatocytes). Viral RNAs, including mRNAs and the pre-genomic RNA (pgRNA) are transcribed from the cccDNA. PgrNA is encapsidated within the cytoplasm and converted into relaxed-circular DNA (rcDNA) by an HBV polymerase-mediated reverse-transcription step. Different viral products circulate in the blood of infected patients including HBe antigens (HBeAg), Dane particles (infectious particles) and empty (i.e., nucleocapsid free) enveloped subviral particles (SVPs). The latter two have envelope proteins at their surface and are indistinctly detected as HBs antigens (HBsAg) (1). SVPs, which are produced in large excess compared to virions, are thought to play an important role in terms of immune subversion (3).

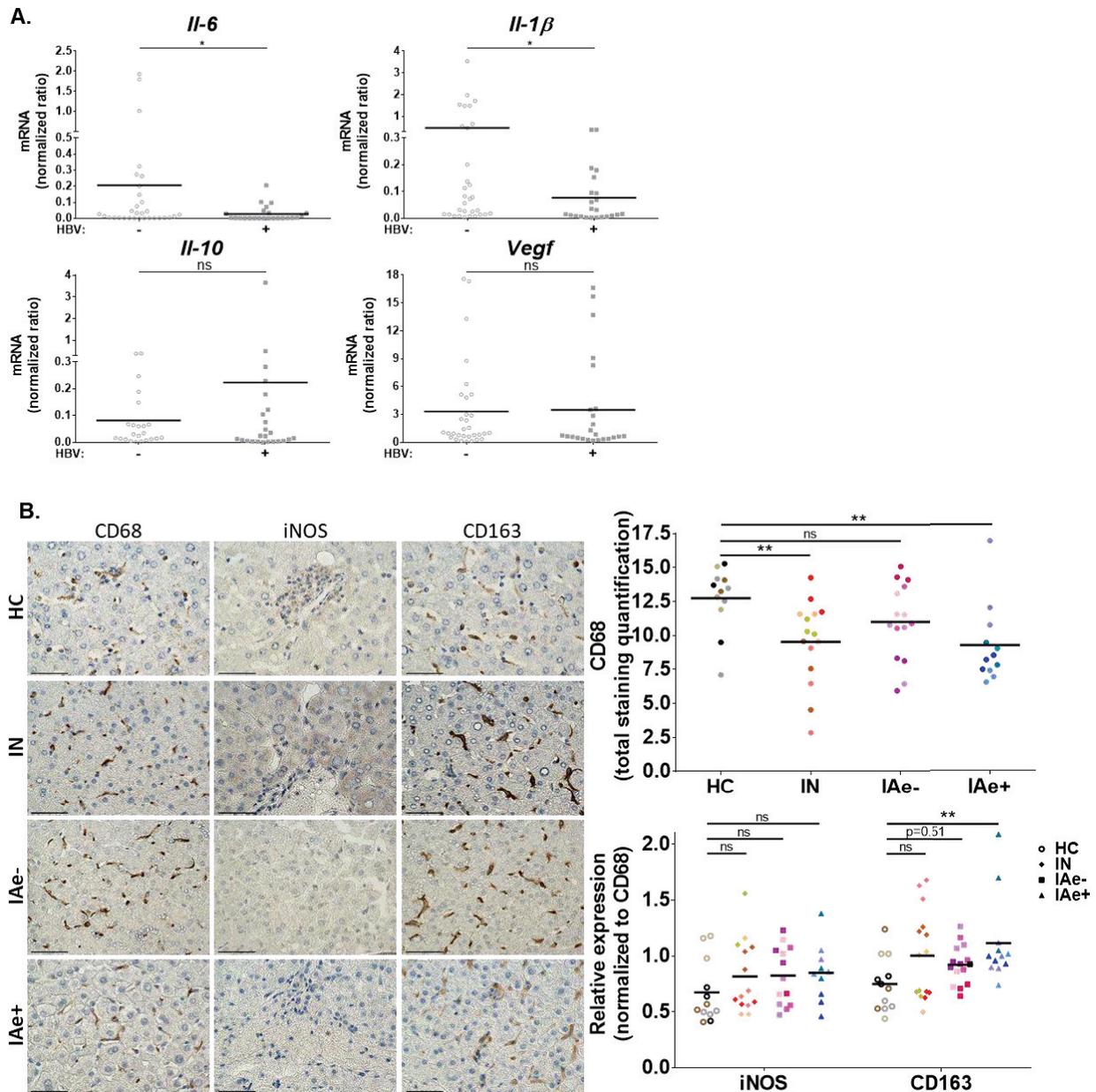
Several cytokines have been shown to induce a strong and direct antiviral effect on established HBV infections in hepatocytes (4–6), with IL-1 $\beta$  being the most efficient one (4). IL-1 $\beta$  is a pro-inflammatory cytokine produced upon inflammasomes induction (7). In the liver, it is mostly produced by macrophages since hepatocytes do not possess functional inflammasomes (8,9). Liver resident macrophages named Kupffer cells (KC) represent 80% of the macrophages count in the body at steady state (10). They are specialized in the detection of pathogens coming from the enteric circulations, as well as in the elimination of aging blood cells, through their high phagocytic capacity (10). Upon inflammation, monocytes from the blood circulation can be recruited in the liver and differentiate into macrophages that are called monocyte-derived-macrophages (MDM) (11). KC and MDM have different embryonic origin and function (12). In vivo, a wide range of different phenotype of macrophages exists depending on their origin, localization, and their micro-environment (13). Inflammatory macrophages, commonly called M1, secrete pro-inflammatory cytokines such as IL-6, IL-1 $\beta$ , IL-12, and TNF- $\alpha$  (13). M1 macrophages have a broken Krebs cycle characterised by the accumulation of citrate, that induce the expression of the inducible nitric oxide synthase (iNOS), and the accumulation of

succinate that induce the accumulation on HIF1 $\alpha$  and IL-1 $\beta$  production (14). M1 macrophages are implicated in inflammation and the elimination of pathogens (13). Anti-inflammatory macrophages, commonly called M2, secrete anti-inflammatory cytokines, such as IL-10 and TGF $\beta$ , as well as angiogenic factors, such as VEGF, and express the high affinity scavenger receptor CD163 (13). M2 macrophages are implicated in the resolution of the inflammation and in tolerance mechanisms (13). M2 macrophages are also found in tumour microenvironment and are called tumour associated macrophages (TAM) (15).

Several studies showed that HBV can influence macrophages (16), but few have been performed using resident or infiltrating primary human cells. Using highly relevant models, our aim was to further characterize the interplay between HBV and liver macrophages in order to determine their roles in the establishment of HBV infection in hepatocytes. Ex vivo and in vitro experiments were mainly performed; these approaches were possible because the modification of macrophage phenotype was readily measurable and could be recapitulated upon relatively short exposure with HBV.

## Results

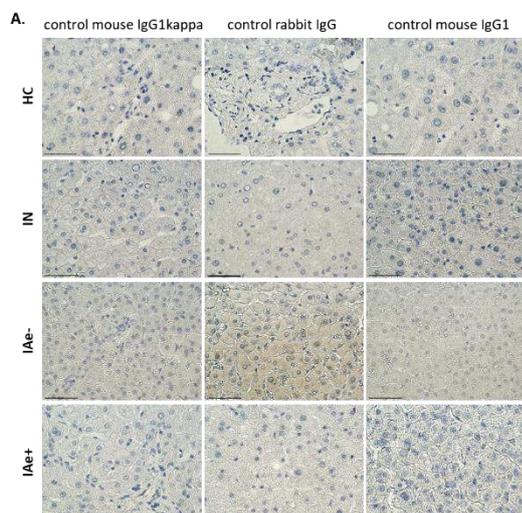
### HBV affect liver macrophages (liver MΦ) phenotype in CHB patients.



**Figure 1: Pro and anti-inflammatory markers in HBV-infected liver macrophages.** (A) Total RNAs were extracted from HBV-infected or non-infected liver biopsies and the levels of the indicated mRNA were assessed by RT-qPCR. (B) Liver biopsies from healthy controls (HC), inactive carrier (IN; HBeAg negative, low viral load), immune active (IA; HBeAg negative, high viral load) and immune tolerant (IT; HBeAg positive, high viral load) patients were stained by IHC for the expression of CD68 (total macrophage), iNOS (M1 macrophage) and CD163 (M2 macrophage). Representative pictures as well as quantification of the levels of CD68, iNOS and CD163 are presented.

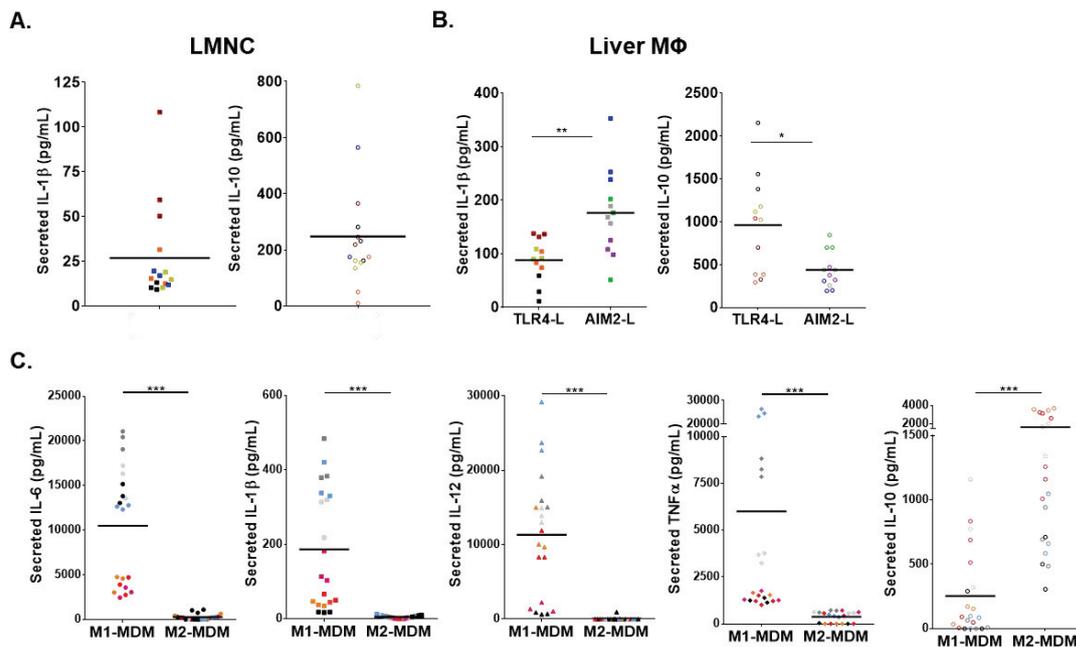
To initiate our analysis using human samples, we used liver biopsies from a cohort of 24 HBV patients and 31 control patients with different aetiologies (**Table S1**) to analyse the levels of

livers mRNAs from two pro-inflammatory cytokines (IL-6 and IL-1 $\beta$ ) and two anti-inflammatory cytokines (IL-10 and VEGF). No correlation was found between the levels of these mRNAs and the levels of HBV mRNAs (data not shown), but the levels of liver IL-6 and IL-1 $\beta$  mRNAs were found to be significantly lower in HBV patients compared to controls (**Figure 1A**). Although not significant, we also observed a slight increase in the levels of liver IL-10 mRNA in HBV patients compared to controls, but no difference in the levels of VEGF mRNA (**Figure 1A**). Second, to assess the consequences of HBV infection on the number of pro-inflammatory and anti-inflammatory liver M $\Phi$ , we analysed liver biopsies by immunohistochemistry. CD68 was used a marker for total amount of M $\Phi$ , iNOS for pro-inflammatory M $\Phi$  (13), and CD163 for anti-inflammatory M $\Phi$  (13). Another cohort of 18 patients was divided into four groups (**Table S2**): healthy controls (HC; not infected by HBV), inactive carrier (IN; HBeAg negative with low viral load), HBeAg-negative immune active (IAe-; HBeAg-neg with high viral load), and HBeAg-positive immune active (IAe+; HBeAg-pos with high viral load). Strong signals were observed for CD68 and CD163 in the liver of HC, whereas iNOS signals were lower (**Figure 1B**) and no signal were observed with control antibodies (**Figure S1**). No correlation between the levels of CD68, iNOS or CD163 expression was observed for any of the viral parameters available (data not shown). However, the overall levels of CD68 staining decreased in HBV infected patients with a significant reduction of 25% in IN and 30% in IAe+ (**Figure 1B**). Moreover, among the total M $\Phi$  (CD68 positive cells), the number of anti-inflammatory M $\Phi$  (CD163 positive cells) were higher in HBV patients (**Figure 1B**). Altogether, these data suggest that HBV may affect liver M $\Phi$  phenotypes. We therefore performed several ex vivo analyses presented thereafter to investigate the influence of HBV on liver cells with a focus on liver M $\Phi$  that include Kupffer cells (KC) and monocyte derived macrophages (MDM) (8,9).



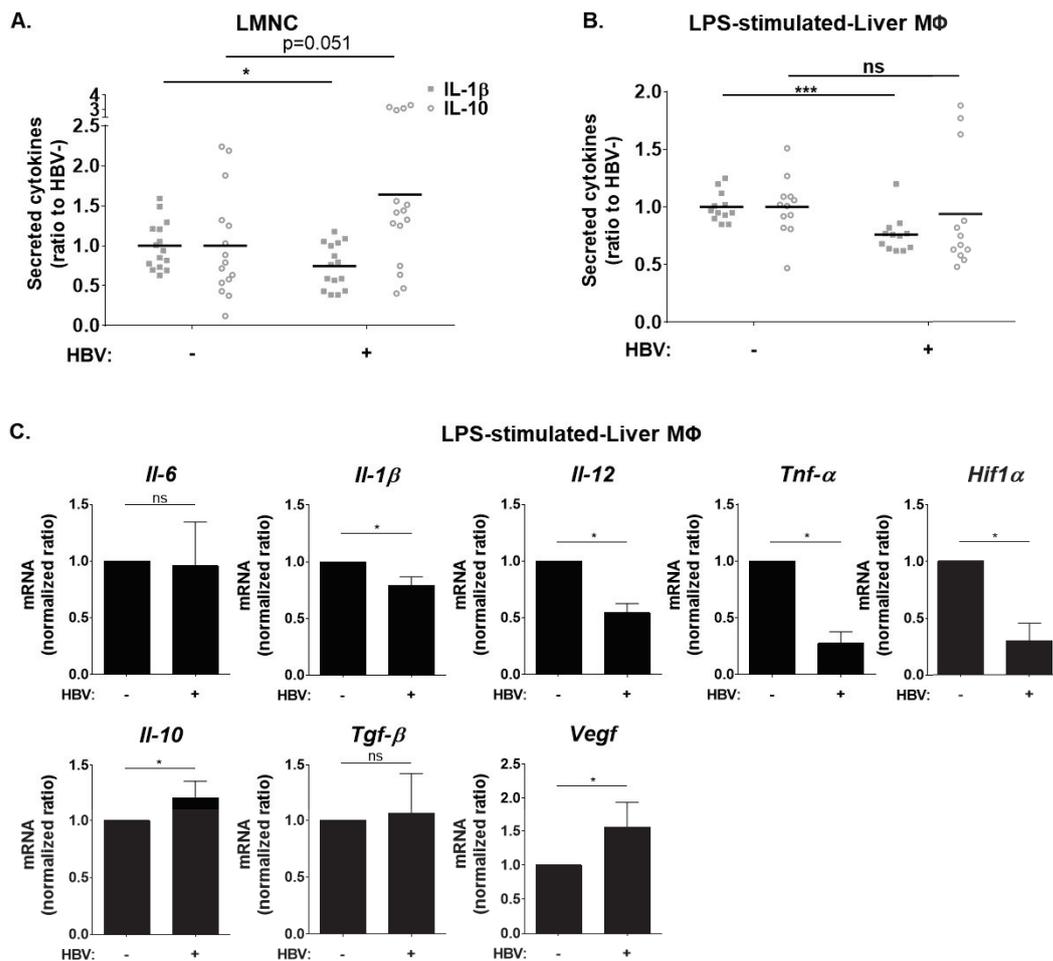
**Supplementary figure 1: Control antibodies for immunohistochemistry analyses.** Liver biopsies from healthy controls (HC), inactive carrier (IN; HBeAg negative, low viral load), immune active (IA; HBeAg negative, high viral load) and immune tolerant (IT; HBeAg positive, high viral load) patients were stained by with control antibodies.

## HBV inhibits activation of primary liver macrophages



**Supplementary figure 2: Levels of cytokines secreted by LMNC, liver M $\Phi$ , and MDM from different donors.** (A) Total LMNC were isolated from liver resection and cultivated for 24h. (B) Liver M $\Phi$  were isolated from liver resection and stimulated 24h later with 100 ng/ml of LPS (TLR4-L) or 100 ng/ml of LPS + 100 ng/ml of Poly (dA:dT) (AIM2-L) for another 24h. (C) Monocytes were purified from peripheral blood mononuclear cells, differentiated in M1-MDM or M2-MDM for 6 days, stimulated with 10 ng/mL LPS for 3h, washed and further cultured for 24h with a medium exchange 3h after washing. (A, B, C) Supernatant were collected and the levels of the indicated secreted cytokines were assessed by ELISA. For each cell type, three biological replicates per donors (one colour per donor) have been analysed.

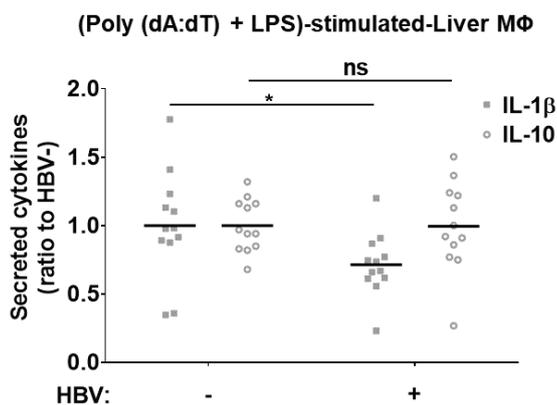
To show that HBV could modulate the phenotype in ex vivo experiments realised in short-time exposure of naïve cell to the virus, and demonstrate that phenotypes can be recapitulate in this setting, we isolated non-parenchymal primary human liver mononuclear cells (LMNC) from liver resections (17) and assessed the effect of a 24h exposure to HBV on the secretions of several cytokines. We used HBV inoculum (genotype D, serotype ayw, multiplicity of infection varying from 100 to 1000 vge/cell as indicated in legends) produced by either HepG2.2.15 or HepAD38 for these experiments. Despite high variations in the activation of cells from one donor to the other (**Figure S2A**), a significant 25% decreased of IL-1 $\beta$  and a slight increase of IL-10 secretions were measured in supernatant from LMNC exposed to HBV compared to non-exposed cells (**Figure 2A**). As IL-1 $\beta$ , in the liver, is mainly produced by activated macrophages, purified primary liver M $\Phi$  were stimulated with LPS (for TLR4



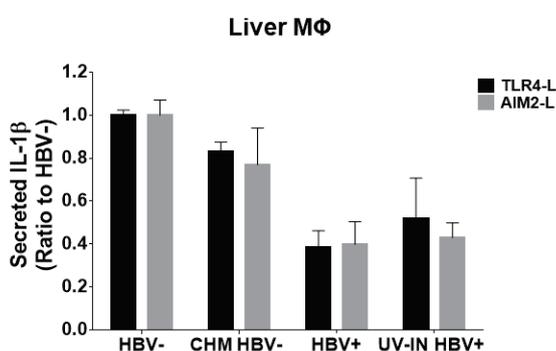
**Figure 2: Levels of pro-inflammatory markers decreased in liver macrophages exposed to HBV.** (A) Total LMNC were isolated and exposed or not to HBV for 24h. Supernatants were collected and the levels of secreted IL-1 $\beta$  and IL-10 were assessed by ELISA and analysed as ratio to non-exposed cells. Results are the mean of five independent experiments (with five different donors) each performed with three biological replicates. (B, C) Liver M $\Phi$  were isolated, exposed or not to HBV for 24h before stimulation with 100 ng/ml of LPS for (C) 3h or (B) 24h. Supernatants were collected and the levels of secreted IL-1 $\beta$  and IL-10 were assessed by ELISA and analysed as ratio to non-exposed cells. Total RNAs were extracted and the levels of the indicated mRNA were assessed by RT-qPCR. Results are the mean +/- standard deviation of four independent experiments (with four different donors) each performed with three biological replicates.

stimulation) or LPS + poly(dA:dT) (for AIM2 stimulation). LPS stimulation triggers non-canonical activation of the NLRP3 (NOD-like receptor family, pyrin domain containing 3) inflammasome (18) leading in our conditions to a slight induction of IL-1 $\beta$  (90 pg/ml) and a strong induction of IL-10 (1000 pg/ml) (**Figure S2B**). LPS + poly(dA:dT) stimulation triggers the

AIM-2 (absent in melanoma 2) inflammasome leading here to a stronger secretion of IL-1 $\beta$  (190 pg/ml) and a more modest secretion of IL-10 (450 pg/ml) (**Figure S2B**). When exposed to HBV for only 24h, primary liver M $\Phi$  secreted equal amount of IL-10, but 25% to 35% less IL-1 $\beta$  in response to inflammasome stimulations (**Figure 2B and S3**). Accordingly, mRNA levels of most of the tested pro-inflammatory markers (IL-1 $\beta$ , IL-12, TNF $\alpha$ , and HIF1 $\alpha$ ) were decreased in primary liver M $\Phi$  exposed to HBV (**Figure 2C**), whereas levels of the anti-inflammatory IL-10 and VEGF mRNAs were slightly increased (**Figure 2C**). Of note, concentrated supernatant from naïve hepatocytes (that does not produce HBV) did not affect primary liver M $\Phi$  activation showing that the effects observed above are due to HBV and not to any factors co-secreted by infected hepatocytes (**Figure S4**). In addition, UV-inactivated HBV also decreased IL-1 $\beta$  secretion of exposed liver M $\Phi$  (**Figure S4**), and HBV-exposed liver M $\Phi$  or MDM did not secrete HBeAg or HBsAg (**Figure S5**), highlighting that the effect of HBV on M $\Phi$  is independent of viral replication within cells. Altogether these data suggest that HBV may interfere with activation of primary liver M $\Phi$ .

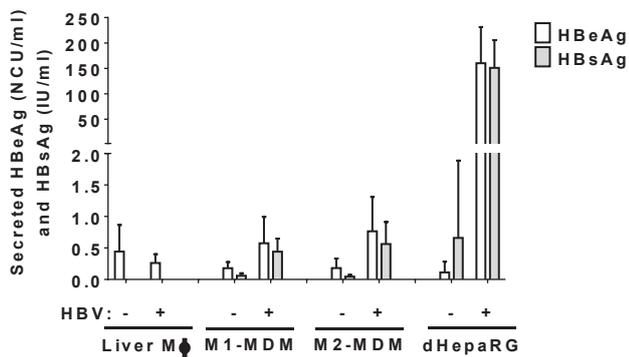


**Supplementary figure 3: Levels of IL-1 $\beta$  decreased in liver macrophages exposed to HBV.** Liver M $\Phi$  were isolated, exposed or not to HBV for 24h before stimulation with 100 ng/ml of LPS and 100 ng/mL of poly(dA:dT) for (C) 3h or (B) 24h. Supernatants were collected and the levels of secreted IL-1 $\beta$  and IL-10 were assessed by ELISA and analysed as ratio to non-exposed cells. Results are the mean +/- standard deviation of four independent experiments (with four different donors) each performed with three biological replicates.



**Supplementary figure 4: Concentrated medium from hepatocytes does not affect liver M $\Phi$  secretions and UV-inactivated HBV has similar effect as HBV.** Liver M $\Phi$  were isolated from liver resection, exposed to medium (HBV-), concentrated supernatants from non-infected hepatocytes (CMH HBV-), HBV (HBV+) or to an UV-inactivated HBV (UV-IN HBV+) for 24h, and stimulated with 100 ng/ml of LPS (TLR4-L) or 100 ng/ml of LPS + 100 ng/ml of Poly (dA:dT) (AIM2-L) for

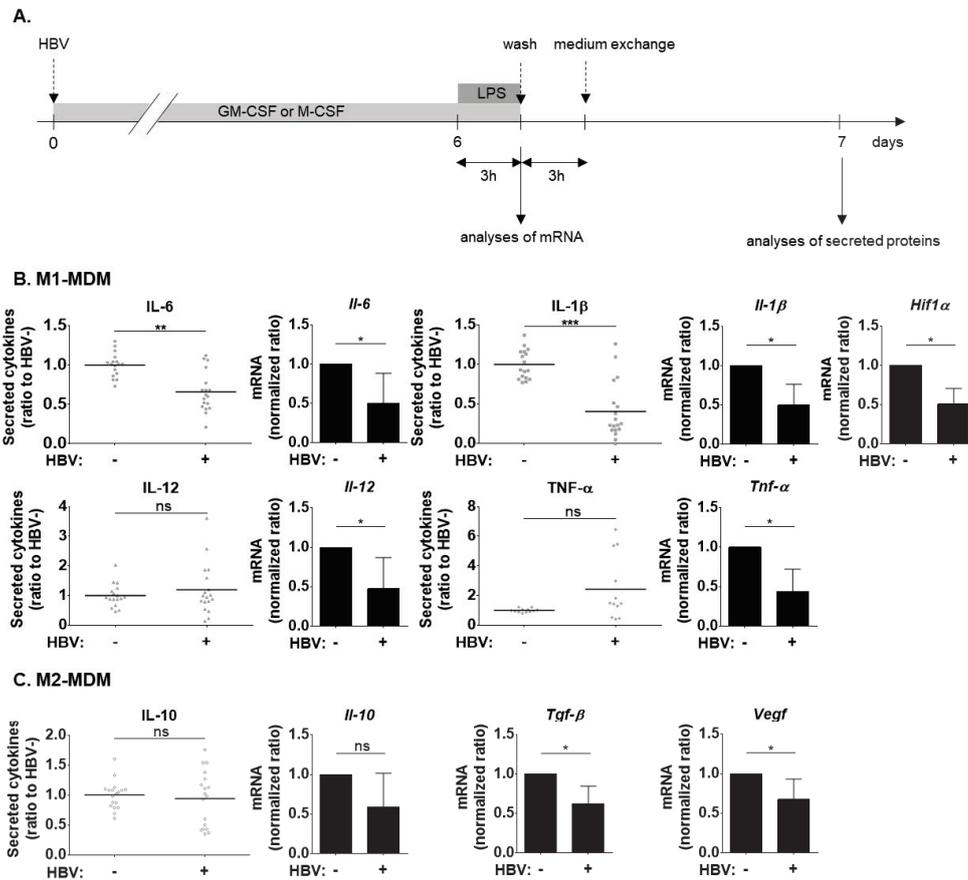
another 24h. Levels of IL-1 $\beta$  were assessed by ELISA and analysed as ratio to non-exposed cells. Results are the mean +/- standard deviations of one experiment performed with three biological replicates.



**Supplementary figure 5: HBV did not replicate in macrophages.** Liver MΦ and dHepaRG were exposed to HBV for one day and M1-MDM and M2-MDM for six days. Supernatant were collected and levels of HBeAg and HBsAg were determined by ELISA seven days post exposure. Data are presented as mean +/- SD of three different experiments each performed in three biological replicates.

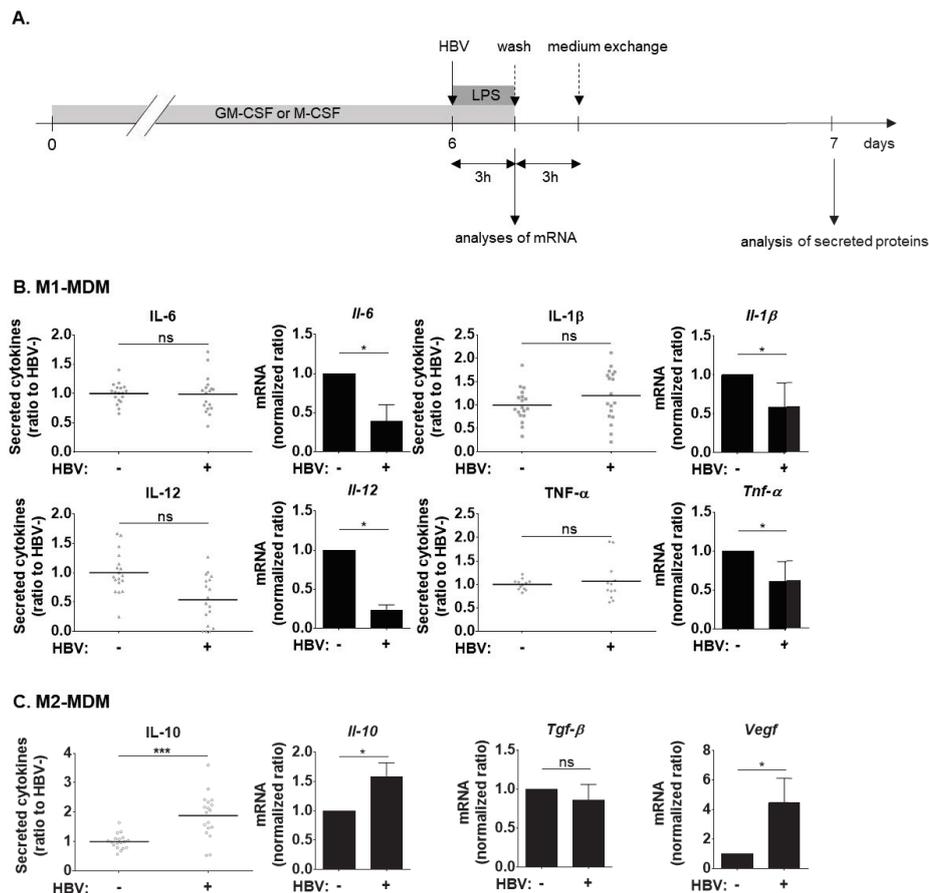
### HBV interferes with blood monocytes differentiation and activation

During inflammation or injury, a high quantity of immune cells, among which monocytes, are recruited to the inflammation/injury site to mount a strong pro-inflammatory response and contribute to infection control (19). This response is thereafter limited by anti-inflammatory response, also involving infiltrating monocytes, in order to start the scarring processes and prevent chronic inflammation (19). Infiltrating monocytes differentiate into MΦ within the tissue (13) and, in the case of the human liver, cannot be fully distinguished from resident MΦ. To assess the effect of HBV on infiltrating-like MΦ, monocytes were purified from peripheral blood mononuclear cells (PBMC) and differentiated into pro-inflammatory MΦ (M1-MDM) or anti-inflammatory MΦ (M2-MDM) (20) that express and secrete pro-inflammatory cytokines (IL-6, IL-1β, IL-12, TNF-α) or anti-inflammatory cytokines (IL-10) respectively (**Figure S2C**). Upon exposure to HBV during differentiation and activation (i.e., 6 days exposure to HBV) (**Figure 3A**), M1-MDM (i.e., monocyte intended to become M1 according to chemokine treatment) secreted 35% and 60% less IL-6 and IL-1β, respectively (**Figure 3B**). Accordingly, we detected less IL-6, IL-1β, IL-12, TNF-α, and HIF1α mRNAs (**Figure 3B**). No significant effect was observed on the levels of secreted IL-12 and TNF-α, whereas mRNA level were significantly lowered (**Figure 3B**), thus suggesting that post-transcriptional events are likely involved for the final production of those cytokines. M2-MDM exposed to HBV during differentiation and activation secreted as much IL-10 as non-exposed cells but displayed lower IL-10, TGF-β and VEGF mRNAs (**Figure 3C**).



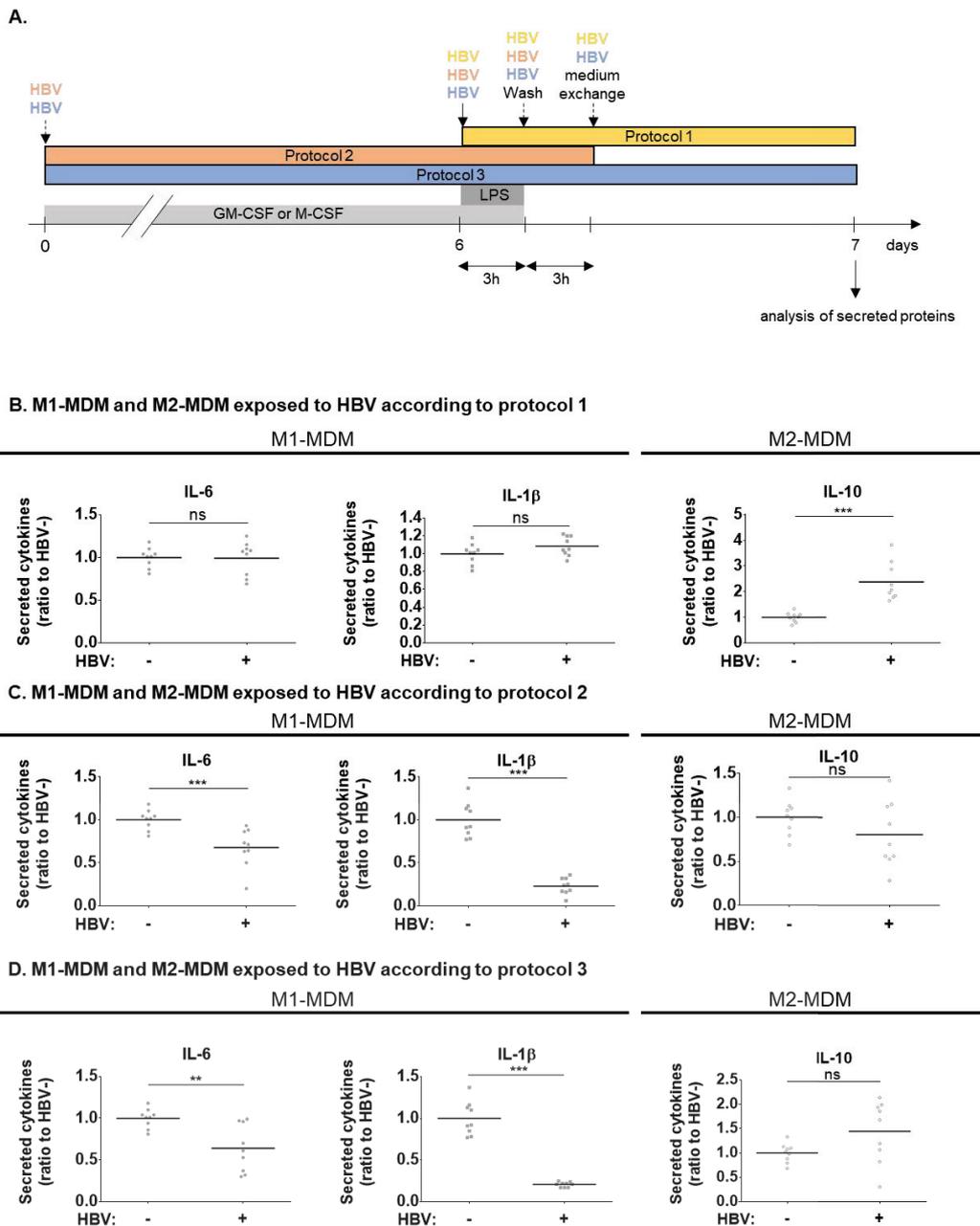
**Figure 3: HBV interfered with M1-MDM differentiation.** Monocytes were purified from peripheral blood mononuclear cells, differentiated into (B) M1-MDM (with the use of GM-CSF) or (C) M2-MDM (with the use of GM-CSF) in the presence or not of HBV before stimulation with 10 ng/ml of LPS for 3 hours. Media removals are indicated by dotted arrows in (A) the schematic representation of the experiment. Levels of the indicated mRNA were assessed by RT-qPCR at the end of the stimulation. Results are the mean +/- standard deviation of three independent experiments (with three different donors) each performed with three biological replicates. Levels of the indicated secreted cytokines were assessed by ELISA and analysed as ratio to non-exposed cells. Results are the mean of six independent experiments (with six different donors) each performed with three biological replicates.

We further investigated the effect of HBV on MDM activation by exposing cells (i.e., M1-MDM or M2-MDM) to the virus once already differentiated by 6 days exposure to either GM-CSF or M-CSF (**Figure 4A**). Although, levels of IL-6, IL-1 $\beta$ , IL-12, TNF- $\alpha$ , and HIF1 $\alpha$  mRNAs were lower in M1-MDM activated in the presence of HBV compared to non-exposed cells, no difference was observed in the levels of the secreted cytokines (**Figure 4B**). However, a 2-fold increase of IL-10 secretion and mRNA expression as well as a strong increase of VEGF mRNA were observed in M2-MDM activated in the presence of HBV (**Figure 4C**). These results were confirmed with many donors and different time of exposure to HBV (**Figure S6**).



**Figure 4: HBV increased M2-MDM activation.** Monocytes were purified from peripheral blood mononuclear cells, differentiated into (B) M1-MDM (with the use of GM-CSF) or (C) M2-MDM (with the use of GM-CSF) and stimulated with 10 ng/ml of LPS for 3 hours in the presence or not of HBV before. Media removals are indicated by dotted arrows in (A) the schematic representation of the experiment. Levels of the indicated mRNA were assessed by RT-qPCR at the end of the stimulation. Results are the mean +/- standard deviation of three independent experiments (with three different donors) each performed with three biological replicates. Levels of the indicated secreted cytokines were assessed by ELISA and analysed as ratio to non-exposed cells. Results are the mean of six independent experiments (with six different donors) each performed with three biological replicates.

In previous experiments, we used free HBV virus produced by either HepG2.2.15 or HepAD38 and concentrated by filtration/retention to allow exposure of cells to high, yet relevant (as high viral load are encountered in patients) levels of infectious HBV particles. It has been already shown in particular for HCV that differences could exist between phenotype changes induced by free virus or cell-produced one (i.e. experiment made in co-culture) (21,22). To assess if the physiologic secretion of viral components by infected cells could also interfere

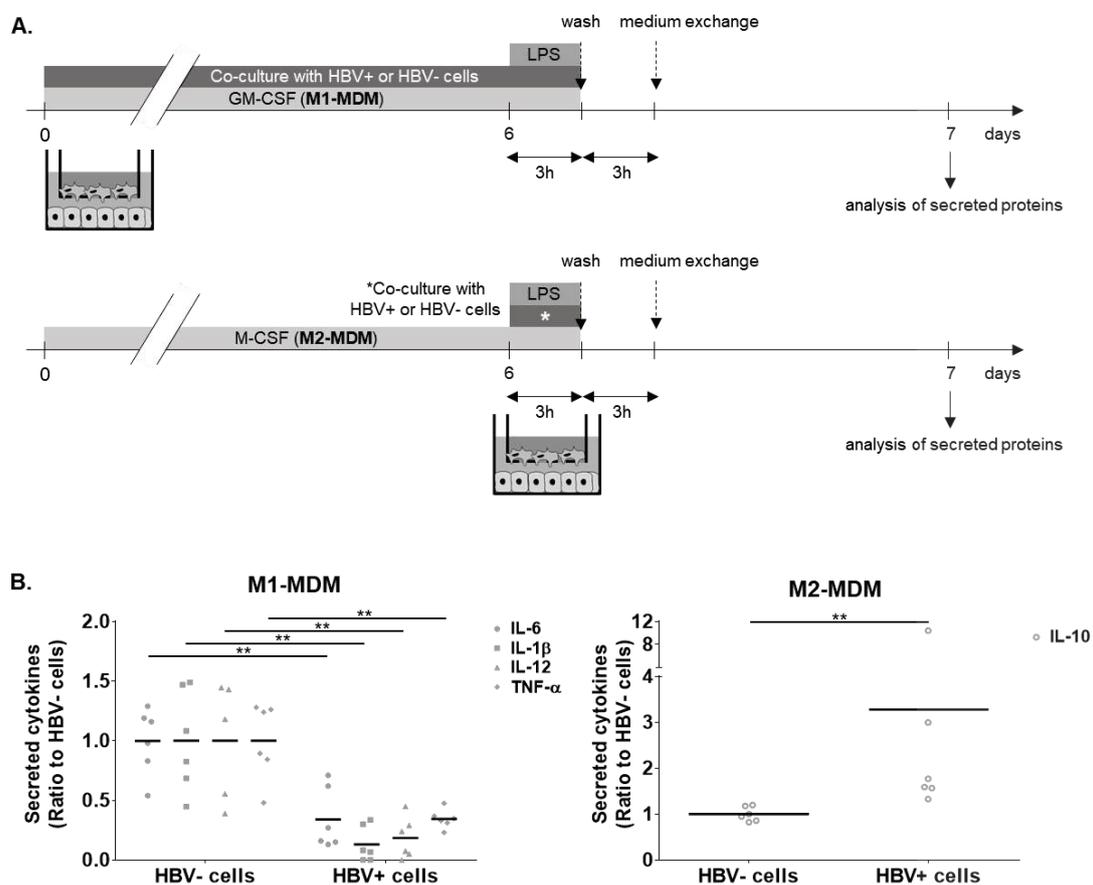


**Supplementary figure 6: HBV interfere with M1-MDM differentiation and increase M2-MDM activation.** Monocytes were purified from peripheral blood mononuclear cells, differentiated into M1-MDM or M2-MDM, stimulated with 10 ng/ml of LPS for 3 hours (A) in the presence or not of HBV. Levels of the indicated secreted cytokines were assessed by ELISA and analysed as ratio to non-exposed cells. Results are the mean of three independent experiments (with six different donors) each performed with three biological replicates.

with M1-MDM differentiation, monocytes were exposed to either non-infected hepatocytes (i.e. HepG2-NTCP; HBV negative cells) or HBV producing hepatocytes (i.e. HepAD38; HBV positive cells) during their differentiation and activation. M1-MDM exposed during their

differentiation to HBV producing cells secreted less IL-6, IL-1 $\beta$  but also less IL-12 and TNF- $\alpha$  (**Figure 5**). As described above (**Figure 3, 4 and S6**), exposition to concentrated virus did not modify IL-12 and TNF- $\alpha$  secretion. This difference was not due to cell cytotoxicity (data not shown) but might come from the fact that, in this co-culture model, M1-MDM are exposed to constantly renewed amounts of viral components. Similarly, M2-MDM exposed to HBV producing cells during their activation secreted more of IL-10 (**Figure 5**).

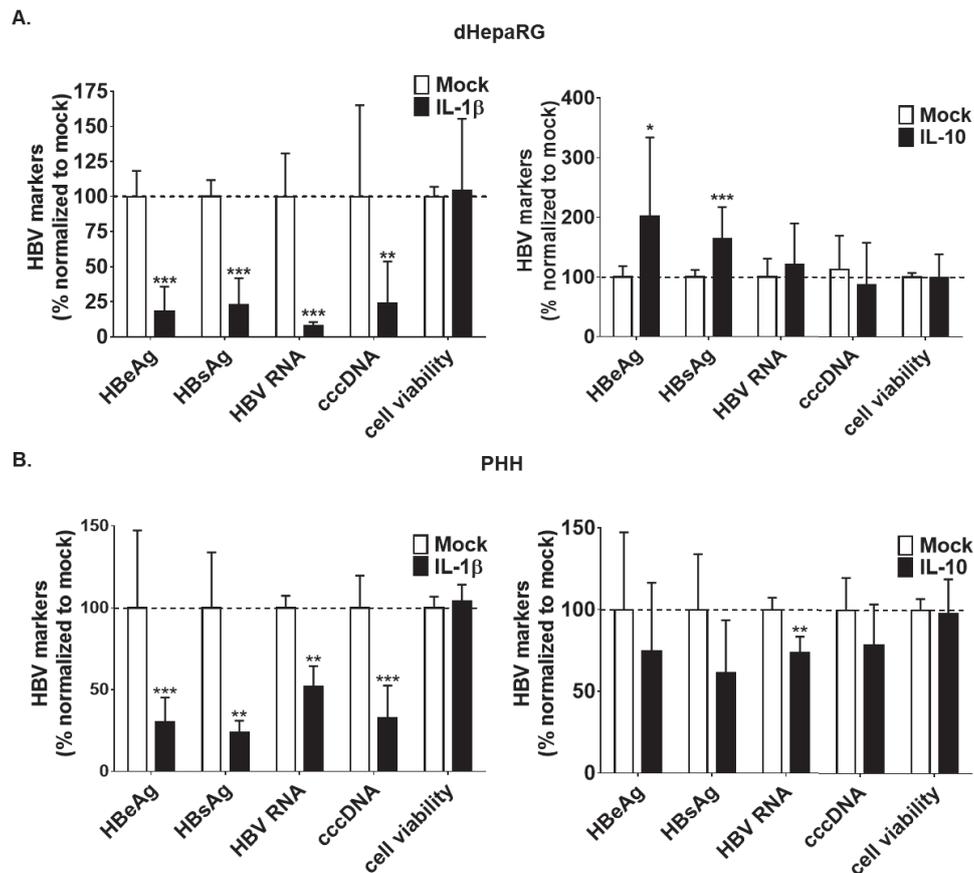
Altogether these results demonstrate that on one hand, HBV interferes with M1-MDM differentiation, and thereby with the secretion of IL-1 $\beta$ , and on the other hand, HBV promotes the secretion of IL-10 by M2-MDM.



**Figure 5: HBV producing cells impaired M1-MDM differentiation and enhanced M2-MDM activation.**

Monocytes were purified from peripheral blood mononuclear cells, differentiated into M1-MDM (with the use of GM-CSF) or M2-MDM (with the use of GM-CSF) and stimulated with 10 ng/ml of LPS for 3 hours, in the presence of non-infected HepG2-NTCP (HBV- cells) or HepAD38 (HBV+ cells). Levels of the indicated secreted cytokines 18h after the last media exchanges were assessed by ELISA and analysed as ratio to non-exposed cells. Results are the mean of two independent experiments (with two different donors) each performed with three biological replicates.

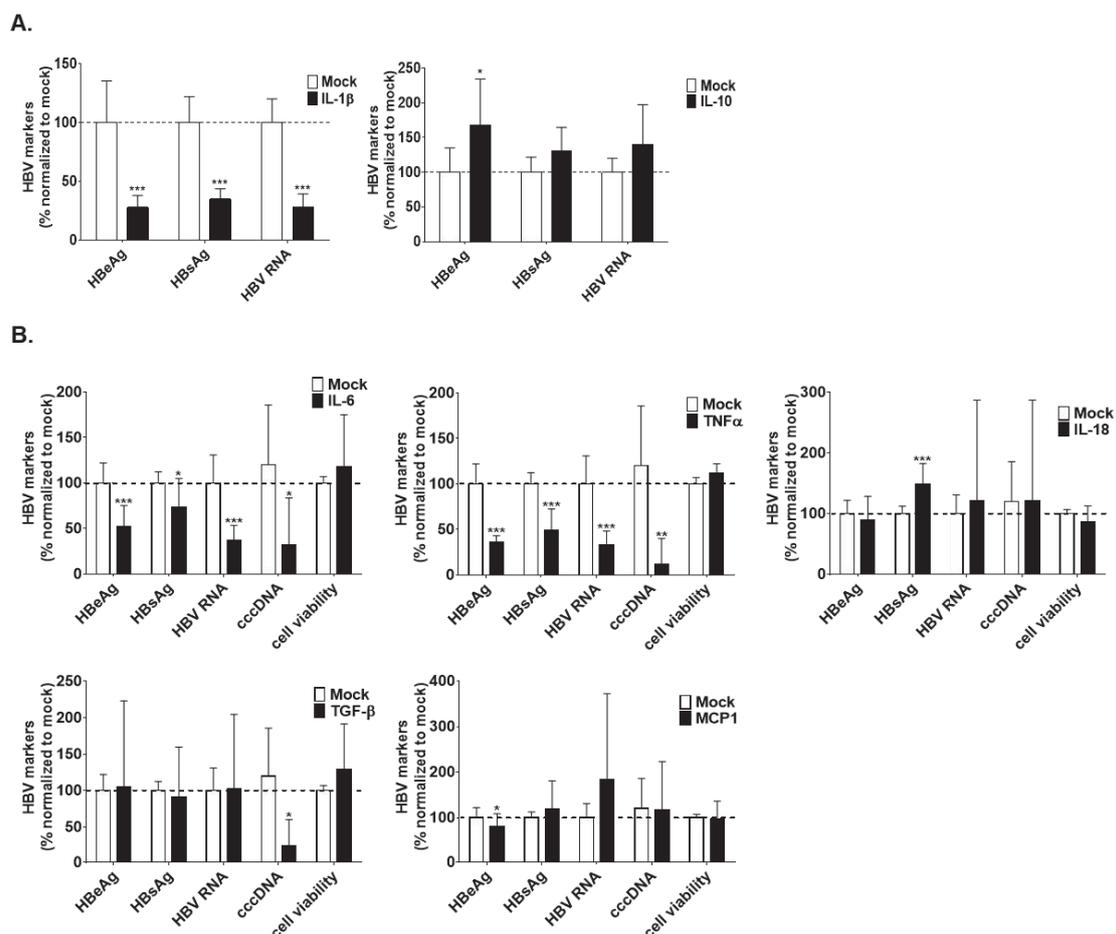
### Pro-inflammatory cytokines reduce establishment of HBV infection in hepatocytes.



**Figure 6: IL-1 $\beta$  strongly inhibited establishment of HBV infection in hepatocytes.** (A) dHepaRG cells or (B) PHH were treated with recombinant IL-1 $\beta$  or IL-10 24h before infection and during infection with HBV. Seven days post infection, supernatants were collected and levels of HBeAg and HBsAg were quantified by ELISA. Cells were harvest, total RNA or DNA were extracted and levels of HBV RNAs and cccDNA were quantified by RT-qPCR or qPCR analyses. Cell viability was assessed by neutral red uptake assay. Results, presented as ratio to non-treated cells, are the mean +/- standard deviation of three independent experiments each performed with three biological replicates.

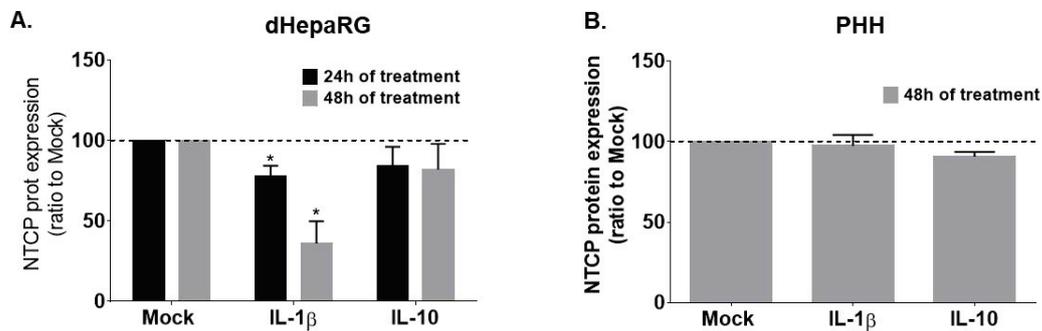
We previously showed that pro-inflammatory cytokines can directly block HBV replication in cells chronically infected in vitro and IL-1 $\beta$  was the most efficient one (4). To investigate the effect of cytokines secreted by M $\Phi$  on the establishment of HBV infection, dHepaRG cells or primary human hepatocytes (PHH) were treated 24h before and during the infection with recombinant IL-1 $\beta$ , IL-6, TNF- $\alpha$ , IL-18, IL-10, TGF $\beta$  and MCP-1 (**Table S3**). IL-1 $\beta$  treatment led to a 70-80% decrease of HBeAg and HBsAg secretion, HBV mRNA and cccDNA, without any cytotoxic effect in both dHepaRG cells and PHH (**Figure 6**). Of note, a 24h treatment with IL-

1 $\beta$  before HBV infection, was already sufficient to decrease HBeAg and HBsAg secretion and HBV mRNA by 75% in dHepaRG (**Figure S7A**). IL-6 and TNF- $\alpha$ , led to a 50% decreased of all the measured viral parameters without cytotoxicity, with the exception of HBsAg which is only decreased by 25% by IL-6 (**Figure S7B**). IL-18 had no direct effect on the establishment of HBV infection (**Figure S7B**). IL-10 treatment led to a slight increase of HBeAg and HBsAg secretion in dHepaRG cells, but not in PHH. A slight decrease of HBV RNA was also observed in PHH treated with IL-10 before and during infection (**Figure 6B**). Other tested anti-inflammatory cytokines, TGF- $\beta$  and MCP-1, had no significant effect on the establishment of HBV infection (**Figure S7B**). The level of NTCP (sodium-taurocholate cotransporting polypeptide), HBV receptor, decreased in dHepaRG after 24h or 48h of treatment with IL-1 $\beta$  (**Figure S8A**) but was not affected in PHH (**Figure S8B**). Altogether, these data indicate that IL-1 $\beta$  is very efficient to inhibit the establishment of HBV infection in hepatocytes.



**Supplementary figure 7: Effect of recombinant cytokines on the establishment of HBV infection.** dHepaRG cells were treated with the indicated recombinant cytokines (A) 24h before or (B) 24h before infection and during infection with HBV. Seven days post infection, supernatants were collected and levels of HBeAg and HBsAg were quantified by ELISA. Cells were harvest, total RNA or DNA were extracted and levels of HBV RNAs and cccDNA

were quantified by RT-qPCR or qPCR analyses. Cell viability was assessed by neutral red uptake assay. Results, presented as ratio to non-treated cells, are the mean +/- standard deviation of three independent experiments each performed with three biological replicates.



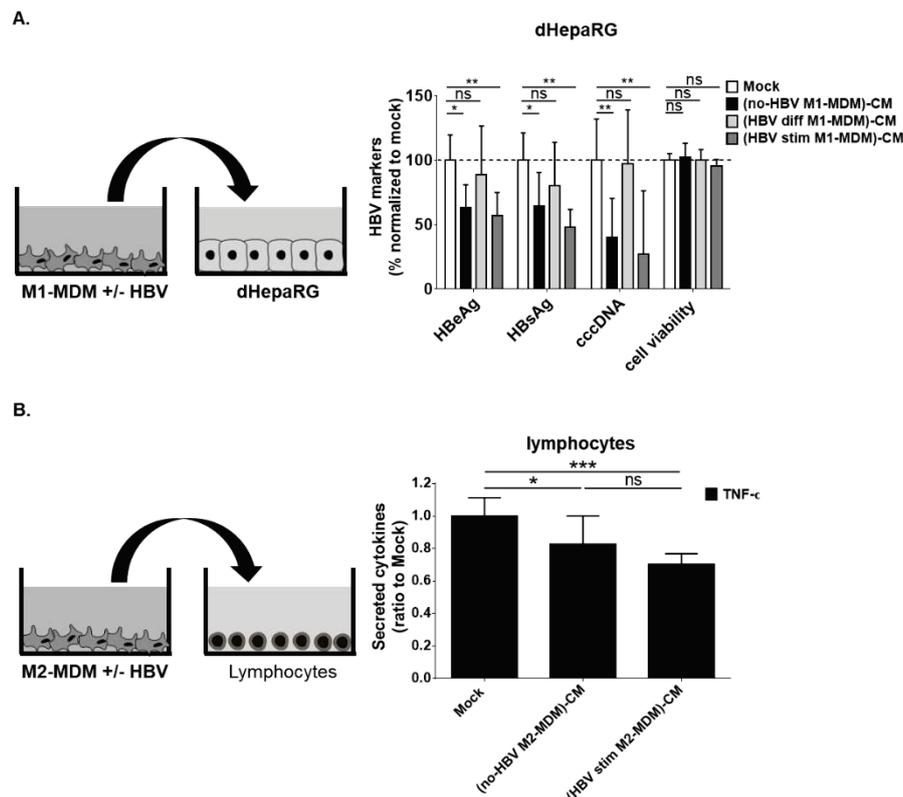
**Supplementary figure 8: NTCP levels in dHepaRG and PHH treated with IL-1 $\beta$ .** (A) dHepaRG cells were treated with recombinant IL-1 $\beta$ , and IL-10 for 24h or 48h. (B) were treated with recombinant IL-1 $\beta$ , and IL-10 for 48h. NTCP protein expression was analysed by flow cytometry. Results are presented in percentage normalised to the non-treated cells. Data are presented as mean +/- SD of three independent experiments.

### **HBV escapes the antiviral effect of cytokines secreted by pro-inflammatory macrophages and impairs lymphocyte activation.**

In accordance to the latest data, concentrated supernatants from M1-MDM (containing 106.3 ng/ml of IL-6, 4.1 ng/ml of IL-1 $\beta$ , 293.3 ng/ml of IL-12, 182.9 ng/ml of TNF $\alpha$ , and 3.9 ng/ml of IL-10 as shown in **Figure S2 and table S4**) also reduced HBV infection of dHepaRG (**Figure 7A, (no-HBV M1-MDM)-CM**). As HBV inhibits IL-6 and IL-1 $\beta$  secretion of M1-MDM (**Figure 3B and Table S4**), we assessed if this inhibition was sufficient to prevent the antiviral effect M1-MDM secretions. Indeed, dHepaRG treated with concentrated supernatants from M1-MDM exposed to HBV during differentiation showed similar levels of HBV markers to those measured in non-treated dHepaRG (**Figure 7A, (HBV diff M1-MDM)-CM**). Accordingly, dHepaRG treated with concentrated supernatants from M1-MDM exposed to HBV during activation, that contains similar levels of pro-inflammatory cytokines to non-exposed M1-MDM (**Figure 4B and Table S4**), showed a 40% decrease of secreted HBV antigens and a 60% decreased of cccDNA amounts (**Figure 7A and TableS4, (HBV stim M1-MDM)-CM**).

Moreover, as IL-10 is known to impair lymphocytes activation (23), we assessed if the increase of IL-10 secretion induced by HBV (**Figure 4, 5 and S6B**) would further impair this activation. Total lymphocytes were purified from peripheral blood and exposed to M2-MDM conditioned

media for 2h before their activation with Phorbol 12-Myristate 13-Acetate (PMA) + ionomycin. As expected, total lymphocytes exposed to M2-MDM supernatants secreted 20% less TNF- $\alpha$  than non-exposed ones (**Figure 7B, (no-HBV M2-MDM)-CM**). Interestingly, supernatants from M2-MDM exposed to HBV during their activation containing higher amounts of IL-10 (**Figure 4C and Table S5, (HBV stim M2-MDM)-CM**) lead to a 30% inhibition of TNF- $\alpha$  secretion by total lymphocytes (**Figure 7B**).



**Figure 7: HBV prevented the antiviral effect of M1-MDM on HBV establishment and enhanced the inhibition of lymphocytes activation by M2-MDM.** (A) dHepaRG cells were treated 24h before and during HBV infection with concentrated supernatants from LPS-stimulated M1-MDM exposed to HBV during their differentiation or during their stimulation (CM; conditioned medium pooled from at least 5 independent experiments). Seven days post infection, supernatants were collected and levels of HBeAg and HBsAg were quantified by ELISA. Cells were harvest, total DNA were extracted and levels of cccDNA were quantified by specific qPCR analyses. Cell viability was assessed by neutral red uptake assay. Results, presented as ratio to non-treated cells (mock), are the mean +/- standard deviation of three independent experiments each performed with three biological replicates. (B) Lymphocytes were extracted from peripheral blood mononuclear cells and exposed to media (mock) or LPS-stimulated M2-MDM exposed or not to HBV during their stimulation (CM; conditioned medium) for 2h before stimulation with 20 ng/ml of PMA and 500 ng/ml of ionomycin. Supernatants were collected 22h later and the levels of TNF- $\alpha$  were assessed by ELISA. Results, presented as ratio to non-treated cells (mock), are the mean +/- standard deviation of three independent experiments (lymphocytes from 3 different donors) each performed with three biological replicates.

Altogether, our data indicate that HBV could escape the antiviral effect of pro-inflammatory macrophages by interfering with their differentiation into IL-1 $\beta$  producing cells and prevent the activation of a functional adaptive immune response (i.e. lymphocytes activation) by enhancing IL-10 secretion from anti-inflammatory macrophages thereby favouring its establishment in hepatocytes.

## Discussion

Pro-inflammatory macrophages have been defined as crucial mediators of the defense against pathogens, whereas anti-inflammatory macrophages are mainly implicated in the resolution of inflammation, as well as in immune-tolerance and may also contribute to immune virus-infected or cancerous cells escape (13). As dendritic cells, macrophages, in particular in the liver, are thought to play a major role in the orchestration of adaptive responses. But more directly they also produce cytokines that may directly affect HBV replication after binding to hepatocyte-expressed receptors. As already reported in animal models of HBV infections (22–24), we confirmed here that cytokines secreted by pro-inflammatory macrophages (including IL-1 $\beta$  and IL-6) strongly inhibit HBV establishment (**Figure 6 and 7**) in addition to our previously reported antiviral effect in already infected human hepatocytes (4) (i.e. dHepaRG cells and PHH). As also reported, we observed a decrease in the level of NTCP (25) in dHepaRG treated with IL-1 $\beta$  (28), but not in PHH (**Figure S8**) hence ruling-out that IL-1 $\beta$  prevent HBV establishment exclusively by influencing the amount of HBV entry receptor.

Similar to several studies (8,29,30) and contrary to some others (29–31), HBV itself did not induce the secretion of any cytokine by primary human liver macrophages or MDM in our experimental settings (data not shown), thus suggesting either a lack of or an HBV-mediated evasion to the detection by innate sensors, including PRRs and inflammasomes. The discrepancies in the “sensing” of HBV by liver macrophages may be due to the quality and quantity of the HBV inoculum used to perform experiments. Indeed, culture conditions for HBV production and concentration may lead to different amounts of contaminating non-enveloped nucleocapsids, recognized by TLR2 (34) or endotoxins, recognized by TLR4. This highlights the importance to characterize the HBV inocula used in each study (**Figure S9**), as well as to investigate the effect of HBV particles concentrated from HBV-infected sera of patients since peptide, lipid, glycan, and associated host-factors composition of virions and SVPs might change during the infection course.

Our data support the hypothesis of HBV being a stealth virus (35,36), not by passive mechanism, but by actively inhibiting liver innate responses. In particular, many studies using animal models or macrophage cell lines reported an inhibition of macrophages responses to HBV (8,24,25,37). Here, using human cells, we observed (i) lower levels of IL-1 $\beta$  and IL-6 mRNAs in liver biopsies from HBV-infected patients compared to non-infected ones (**Figure**

**1A**), (ii) that primary liver macrophages ex vivo exposed to HBV secreted less pro-inflammatory cytokines upon activation (**Figure 2**) confirming our previously published data (8), (iii) that M1-MDM also secreted less IL-1 $\beta$  and IL-6 upon activation if exposed to HBV during differentiation (**Figure 3, 4 and S6**) and (iv) that the antiviral effect of M1-MDM was lost when cells were differentiated in the presence of HBV (**Figure 7A**). These data are also in accordance with those reporting a down-regulation of TLR2 and TLR3 in PBMC and KC from chronically infected patients (38,39) but are opposed to a very recently published study that used ex vivo stimulated liver biopsies (38). Regarding the latter, even though, the model allows the maintenance of the liver 3D organization, as well as the natural proportion of all liver resident cells, the culture conditions used by the authors lead to a dramatic reduction of the hepatocytes viability after 24h of culture (40), and viability and functionality of non-parenchymal cells (including liver macrophages) were not assessed. Moreover, the high doses of TLR4 ligands (20  $\mu$ g/mL compared to the 10 to 100 ng/mL that we used here) might activate the immune response to such a high extent that the HBV-induced inhibition is not sustained. Alternatively, the inhibition of M $\Phi$  by HBV could be highly reversible and therefore lost ex vivo in a medium without any HBV particles and/or antigens. Hence in a model where phenotype changes could be due to a constant interaction between viral components and the targeted cell, purification procedure and ex vivo conditions cultures could have an impact and lead to the reversibility of a phenotype. Our data showing an increased inhibition of the secretion of pro-inflammatory cytokines by M1-MDM upon constant exposure to HBV via co-cultures with HBV producing cells (**Figure 5A**) compared to a single exposure to concentrated virus (**Figure 3B**) support the latest hypothesis. It would be important to test if the addition of HBeAg/HBsAg/HBV virions in the culture medium of ex vivo cultured liver biopsies would recapitulate the inhibitory phenotype we described here.

Importantly, we observed an increase in the number of anti-inflammatory macrophages (CD68+ CD163+ cells) in liver biopsies from HBV-infected patients compared to those from non-infected patients (**Figure 1B**) and an increase in the levels of IL-10 secreted by M2-MDM upon exposure to HBV during activation after ex vivo differentiation (**Figure 4 and S6**). These data suggest that HBV may reinforce the already described liver immune tolerance (39) to establish and/or maintain its infection. This is in accordance with studies reporting an increase of anti-inflammatory cytokines secretion (especially IL-10) induced by HBV in animal models

and patients (30,42–44) and a positive role of anti-inflammatory liver macrophages in HBV persistence (42). Here, we reported that HBV-induced increase of IL-10 secretion by M2-MDM might further impair the activation of lymphocytes (**Figure 7B**). As others had previously described (29,42,44), the increased IL-10 expression during HBV infection could favor a tolerogenic environment and the inhibition of functional adaptive immune response, compromising HBV elimination. As these anti-inflammatory macrophages are also implicated in cancerization processes (through the secretion of angiogenic factors, such as VEGF, and the impairment of an anti-oncogenic response by the secretion of regulatory mediators, such as IL-10 (45)), it should be investigated if the HBV-induced modification of macrophages phenotypes may also play a role in HCC initiation and promotion.

Which viral components and by which mechanisms HBV influences the secretion of cytokines by macrophages remain so far unclear. Few data suggest that HBV can be internalized by macrophages (29,44). HBeAg (9), HBsAg (8,37) and the HBV capsid protein (47) have been suggested to contribute to the inhibition of macrophages responses. Of note, the use of recombinant viral proteins may be suboptimal, as post-transcriptional modifications associated to a given protein produced in a given cell system (bacteria, yeast or mammalian cells) could be different as compared to those found in viral proteins from infected hepatocytes and therefore have a different effect on immune cells. In addition, these modifications may vary during the different phases of HBV infection, giving the numerous functions of the viral proteins. HBV probably interfere with activation of macrophages (and thereby cytokine secretion) at different levels. Indeed down-regulation of innate sensors such as TLR (36,37,46) or inhibition of cell different signaling pathways (23,27,35) have been reported. Epigenetic modulations induced by the fixation of one viral protein to gene promoters, as we reported in hepatocytes (47) and as also reported for other viruses (47–50) might also be implemented in macrophages even though HBV do not replicate in those cells.

In addition, it should be investigated if, besides affecting secretion of cytokines, HBV also disturbs other macrophages functions such as phagocytosis or antigen presentation in order to prevent, for instance, viral recognition and/or local reactivation of lymphocytes. Cell metabolism was recently evidenced as a key player in the implementation of an efficient immune response (53) and HBV was shown to hijack or promote enzymes involved in lipids or amino acids metabolism in hepatocytes or myeloid cells (54–56). We observed here that

the level of the *hif1 $\alpha$*  transcript, a marker of succinate accumulation and broken Krebs cycle in pro-inflammatory M $\Phi$ , is lower in liver M $\Phi$  and M1-MDM (**Figure 2, 3, 4**). The significance of these decreases for the cell function and the consequences for HBV infection need further investigation. One tempting hypothesis is that metabolism modulations of macrophages may promote the secretion of factors necessary to compensate the increased need of hepatocyte in amino acid and lipids to cope with HBV replication.

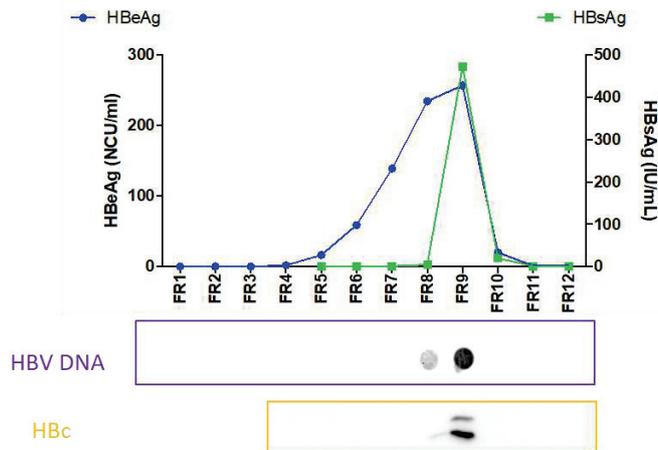
With therapeutic implications, we confirmed here that IL-1 $\beta$  is a very efficient cytokine to inhibit HBV establishment and replication in hepatocytes (**Figure 6 and S7**), thus highlighting a potential more direct contribution of IL-1 $\beta$ -producing cells in HBV control. However, recombinant IL-1 $\beta$  cannot be used systemically without risking severe side effects due to cytokine storm. Thus, new anti-HBV therapeutic options should aim at inducing the local and endogenous secretion of IL-1 $\beta$  to prevent global inflammation. Different mechanisms could be considered to promote pro-inflammatory over anti-inflammatory phenotype in liver resident and infiltrating macrophages. For example, specific inflammasome inducer delivered in the liver and/or specifically to liver macrophages, may induce a boost of IL-1 $\beta$  secretion and ultimately an antiviral phenotype (57). Moreover, studies on cancer model showed that the tumour delivery of GM-CSF, an inducer of pro-inflammatory macrophage, induced tumor regression (58,59). Another recent study showed that modifying the succinate/ $\alpha$ -ketoglutarate balance in macrophages, with inhibitor of glutaminolysis, led to an anti- to pro-inflammatory phenotype switch (60). In summary, we showed that HBV can modulate the resident and transiting-macrophage phenotypes to favor its establishment, and likely its maintenance in the liver. On the one hand, HBV escapes the antiviral effect of pro-inflammatory liver resident and infiltrating M $\Phi$  by interfering with their activation or differentiation respectively. And, on the other hand, it promotes the activation of liver anti-inflammatory M $\Phi$  to prevent the launching of the adaptive immune response. Our data also suggest that therapeutic strategies promoting the differentiation of pro-inflammatory liver macrophages over anti-inflammatory ones should be tested to reverse immune tolerance and cure chronic HBV infections.

## **Material and methods**

**Primary cells purification and cells culture.** Peripheral blood mononuclear cell (PBMC) from blood donors were isolated by Ficoll gradient (Histopaque®-1077, Sigma) as previously described (61). Lymphocytes were separated from total PBMC by a Percoll gradient and further cultured in macrophage medium (RPMI medium supplemented with 10% of decomplemented FBS and 50 U/ml of penicillin/streptomycin). Monocytes were purified by Percoll gradient followed by a negative selection with the Monocyte Isolation Kit II (Miltenyi Biotec) and cultured in macrophage medium. Monocytes were exposed during 6 days to 50 ng/ml of GM-CSF (R&D) or 50 ng/ml of M-CSF from (Peprotech) for M1-MDM or M2-MDM differentiation respectively. MDM were activated by a 3-hours stimulation with 10 ng/ml of LPS (Invivogen). Cells were washed three times with PBS and cultured in fresh medium for another 3 hours before a last medium exchange. Supernatants and cells were collected 24 h post stimulation (i.e. 18h accumulation). Liver cells were isolated from hepatic resections obtained in collaboration with three surgical departments of Lyon (Centre Léon Bérard, Hôpital de la Croix Rousse and centre hospitalier Lyon-Sud) with the French ministerial authorizations (AC 2013-1871, DC 2013 – 1870, AFNOR NF 96 900 sept 2011). After a two-step collagenase perfusion, the liver extract was filtered and centrifuged, as previously described (62). Primary Human Hepatocytes (PHH) were cultured on collagen layer and maintain in PHH medium (Williams medium supplemented with 5% of fetal clone II serum, 50 U/ml of penicillin/streptomycin, 1X glutamax, 5 µg/ml of bovine insulin, 5x10<sup>-5</sup> M of hydrocortisone, and 2% of DMSO). Liver mononuclear cells (LMNC) or only liver macrophages were purified from the non-parenchymal cells mixture by respectively Ficoll gradients or a two phase iodixanol gradient (63). Liver macrophages were isolated by negative selection using pan monocyte isolation kit (Miltenyi Biotec) and cultured in macrophage medium. HepaRG cells were cultured and differentiated as previously described (64).

**Viral infection.** Differentiated HepaRG (dHepaRG) or PHH were cultured and infected by HBV as previously described (65). Blood monocytes, LMNC and liver macrophages were exposed to HBV at a multiplicity of infection of 1000 vge/mL HBV inocula are prepared by concentrating supernatant from HepAD38 (66) by ultrafiltration (Merk Millipore; UFC710008). All virus preparations were tested for the absence of endotoxin (Lonza) and characterized by analyses of the fractions from a 5.6-56% iodixanol gradient and analysed by ELISA, dot blot with HBV

dig labelled probe (67) and western-blot (DAKO, B0586) (**Figure S9**). Virus was inactivated by a 30 min exposition to UVB.



**Supplementary figure 9: Characterization of HBV inoculum.** Supernatants from HepAD38 were concentrated by ultracentrifugation and characterized by analysis of the fractions from a 5.6-56% iodixanol gradient. Viral parameters were assessed in each fractions by ELISA (HBeAg and HBsAg), dot blot (HBV DNA), and western-blot (HBc) analyses.

**Cytokine treatment.** Cytokines references and used concentration are indicated in **Table S3**.

Cytokine	Reference	Concentration
TNF $\alpha$	Life Technologies PHC3016	50 ng/ml
IL-1 $\beta$	MBL International Corporation JM4128-10	1 ng/ml
IL-6	R&D Systems 206-IL/CF	10 ng/ml
IL-18	MBL International Corporation B003-5	10 ng/ml
IL-10	Biovision # 4155-10	20 ng/ml
TGF- $\beta$ 1	Biovision # 4342-5	5 ng/ml
MCP-1	R&D 279-MC-010	1 ng/ml

**Table S3.** Cytokine references and working concentration

**Quantification of secreted proteins by ELISA.** HBeAg and HBsAg secretion were quantified using chemiluminescence immunoassay kit (Autobio) following the manufacturer's instructions. Cytokines secretion were analysed using Duoset ELISA (R&D system) following manufacturer's instructions.

**RNA extraction and RT-qPCR.** Total mRNA from hepatocytes or macrophages were extracted with NucleoSpin<sup>®</sup> RNA II or NucleoSpin<sup>®</sup> RNA XS respectively (Macherey-Nagel). cDNA were synthesized using the SuperScript<sup>®</sup>III Reverse Transcriptase (Life technologies) according to the manufacturer's instructions. qPCR analyses were performed using "Express SYBR GreenER<sup>™</sup> qPCR SuperMix Universal" (Invitrogen). mRNA expression was assessed by comparative cycle threshold (Ct) method ( $2^{-\Delta Ct}$ ); GUS and RPLP0 were used as housekeeping

genes for hepatocytes and macrophages respectively. Primers sequences are presented in **table S6**.

GENE	FORWARD	REVERSE
GUS	CGTGGTTGGAGAGCTCATTGGAA	ATTCCCCAGCACTCTCGTCGGT
HBV	GGAGGGATACATAGAGGTTCCCTTGA	GTTGCCCGTTTGTCTCTAATTTC
HBV cccDNA	ATGGTGAGGTGAACAATGCT	GACTCTCTCGTCCCCTTCTC
RPLP0	CACCATTGAAATCCTGAGTGATGT	TGACCAGCCCAAAGGAGAAG
IL-6	TCGAGCCCACCGGGAACGAA	GCAACTGGACCGAAGGCGCT
IL-1 $\beta$	AATCTGTACCTGTCCTGCGTGTT	TGGTAATTTTTGGGATCTACACTTC
IL-12	CAAGCAAGGCTGCAAGTACA	TGCGTTCCCATCCATCACAA
TNF $\alpha$	CCTGCTGCACTTTGGAGTGA	GAGGGTTTGCTACAACATGGG
HIF1 $\alpha$	CCACCTCTGGAATTGCCTTT	CTCCATGGTGAATCGGTCCC
IL-10	AAGACCCAGACATCAAGGCG	AATCGATGACAGCGCCGTAG
TGF- $\beta$	GCACGTGGAGCTGTACCAGAAATA	AAGGCGAAAGCCCTCAATTTCC
VEGF	AGGGCAGAATCATCACGAAGT	AGGGTCTCGATTGGATGGCA

**Table S6. Primers sequences**

**DNA extraction and cccDNA quantification.** Total DNA were extracted using the NucleoSpin<sup>®</sup> Tissue kit (Macherey-Nagel) following the manufacturer's instructions. Total intracellular DNA was digested for 45 min at 37°C with T5 exonuclease (epicentre) to remove rcDNA (relaxed circular HBV DNA) followed by a 30 min heat inactivation. cccDNA amount was quantified by qPCR analyses as previously described (2).

**Cytotoxicity assay.** Seven days post treatments, hepatocytes cell viability was assessed by quantification of neutral red uptake by living cells, as previously described(4).

**Lymphocytes activation.** Lymphocytes were cultured in different M2-MDM conditioned media at a density of 300,000 cells/cm<sup>2</sup> for 2 hours. Cells were activated by a 22h exposition to 20 ng/ml of Phorbol 12-Myristate 13-Acetate (Sigma) and 500 ng/ml of Ionomycine. Activation was measured by assessment of TNF- $\alpha$  secretion.

**Macrophage supernatants concentration.** M1- or M2-MDM supernatants were pooled and concentrated by ultrafiltration (using 10 kDa Amicon ultra-15 column; Merk-Millipore) following the manufacturer's instruction. Cytokines concentration of the concentrated supernatants were assessed by ELISA and amounts are presented in **Table S4**. Concentrated supernatants were diluted to 1/100.

MDM	M1		
	HBV -	HBV + protocol 1	HBV + protocol 2
Cytokines (ng/ml)			
IL-6	106,3	58,5	82,8
IL-1 $\beta$	4,1	0,4	4,2
IL-12	239,3	145,9	226,3
TNF $\alpha$	182,9	149,2	174,9
IL-10	3,9	2,6	4

**Table S4.** Supernatant from LPS-activated M1-MDM from at least 3 different donors were pooled, concentrated by ultrafiltration and concentrations of the indicated cytokines were determined by ELISA.

MDM	M2	
	HBV -	HBV + protocol 2
Cytokines (ng/ml)		
IL-6	0	0
IL-1 $\beta$	0,02	0,03
IL-12	0	0
TNF $\alpha$	0	0
IL-10	3,3	5,7

**Table S5.** Supernatant from LPS-activated M2-MDM from one donor was collected and the indicated cytokines were determined by ELISA.

**Immunohistochemistry.** Paraffin was removed from FFPE samples by successive alcohol bathes and epitopes were unmasked as previously described (68). Endogen peroxidases were blocked by a 15 min exposition at room temperature with Dual Endogenous enzyme Block (DAKO S2003). Saturation was performed by two successive incubations at RT of 20 min with horse serum (VECTOR S-2012 Za0328) and 15 min with DAKO antibody diluent (DAKO S3022). Antibodies (iNOS (ab15323), CD163 (ab74604), and CD68 (ab955)) were incubated overnight at 4°C in DAKO antibody diluent. Samples were incubated for 15 min at RT with secondary antibodies (Anti-Rabbit IgG/HRP (P0448; DAKO) and anti-mouse IgG/HRP (P0447; DAKO)). Coloration was performed with DAB substrate (DAKO K3468) and counterstaining with haematoxylin (SIGMA MHS1). Negative controls were performed using control IgGs (IgG rabbit (cell signalling 27295), IgG 1 mouse (Biolegend 400143), IgG 1 mouse (ab18448)), and no staining was observed (Figure S1). Optical density (OD=log (max intensity/mean intensity)) of the DAB staining was quantified using Fiji (ImageJ) and the results are presented as 1/OD.

**Patients' samples.** Samples were used under the French IRB 'CPP Sud-Est IV' approval #11/040 (2011) from the 'Biobanque INSERM CRCL Hépatologie (U1052)', France #DC2008-235.

Written informed consent was obtained from each patient. Patients' characteristics are presented in **table S1** for biopsies mRNA analysis, and **table S2** for IHC staining cohort.

ID	HBV status	Type	Age	Sexe	Hbv mRNA (2 <sup>^-</sup> ΔCT)	inflammation	score fibrose	Il-6 mRNA (2 <sup>^-</sup> ΔCT)	Il-1β mRNA (2 <sup>^-</sup> ΔCT)	Il-10 mRNA (2 <sup>^-</sup> ΔCT)	Vegf mRNA (2 <sup>^-</sup> ΔCT)
P#1			44	M	1,2612	1	3	0,0018	0,0118	0,0002	0,4939
P#2			62	M	5,3926	2	3	0,0006	0,0314	0,0052	0,6301
P#3			37	M	78,5729	3	3	0,0340	0,1533	0,1219	3,5286
P#4			45	M	3,2916	1	2	0,2058	0,3816	0,5063	8,2914
P#5			23	M	1,0073	1	2	0,0013	0,0063	0,0035	0,3960
P#6			43	M	0,0510	1	2	0,0287	0,0960	0,0480	0,7457
P#7			32	M	14,0500	1	2	0,1016	0,6465	3,6577	9,0906
P#8			63	M	0,4765	0	2	0,0036	0,0612	0,0361	3,6375
P#9			42	M	22,1076	2	2	0,0026	0,0357	0,0243	1,9459
P#10			56	M	1,5789	0	1	0,0710	0,1793	0,2287	15,7056
P#11			54	M	1,7238	1	1	0,0174	0,0692	0,0757	0,8622
P#12			15	M	10,7613	1	1	0,0007	0,0049	0,0027	1,2968
P#13			58	M	0,5162	0	1	0,0014	0,0163	0,0082	0,6605
P#14			42	M	9,6725	1	1	0,0962	0,3810	0,1044	13,7163
P#15			50	F	0,1198	0	1	0,0010	0,0151	0,0113	0,3934
P#16			17	M	0,4993	1	0	0,0004	0,0035	0,0020	0,2462
P#17				M	0,0031			0,0033	0,0104	0,0155	0,5262
P#18				M	1,8628			0,0019	0,0136	0,0128	2,8710
P#19			54	M	0,0744			0,0486	0,1877	0,2818	16,6380
P#20			68	M	0,3611	1	4	0,0001	0,0066	0,0237	0,2793
P#21				M	1,8024	1	4	0,0315	0,0938	0,1791	0,6080
P#22				M	0,0000	0	4	0,0030	0,0086	0,0052	0,6812
P#23			62	M	0,0057			0,0005	0,0025	0,0027	0,3412
P#24				M	0,1525			0,0011	0,0030	0,0040	0,1999
P#25			50	M	0	0	0	0,0006	0,0080	0,0013	0,7907
P#26			45	M	0		0	0,0328	0,1375	0,0644	17,5915
P#27			43	M	0		0	0,0121	0,0728	0,0614	0,9790
P#28		metabolic syndrome	32	M	0		0	0,2731	1,4822	0,9540	6,2778
P#29			30	M	0		0	0,2008	0,6520	0,2466	2,3703
P#30			51	M	0		0	0,2623	1,4828	2,4196	0,0610
P#31			41	M	0		0	0,0025	0,0136	0,0139	0,9601
P#32			50	M	0		0	0,3240	1,7095	1,7376	4,8164
P#33			64	M	0		0	0,1005	0,4609	0,3868	0,9564
P#34			44	M	0		0	0,0438	0,1251	0,0666	5,1597
P#35			34	M	0		0	0,0015	0,0079	0,0122	1,0378
P#36		alcoholic	45	M	0		0	1,9293	1,9685	7,3501	17,3497
P#37			51	M	0		0	0,0274	0,0789	0,0674	13,2938
P#38			58	M	0		0	0,0014	0,0079	0,0055	0,5531
P#39			47	M	0		1	0,0001	0,0038	0,0030	0,3648
P#40			45	M	0		1	0,0040	0,0185	0,0180	0,7914
P#41			30	M	0	0	1	0,0224	0,0570	0,0162	0,1616
P#42			40	M	0	1	1	0,0053	0,0254	0,0243	2,8957
P#43			17	M	0	0	1	0,0054	0,0297	0,0299	2,5218
P#44		auto-immun	30	M	0	0	1	0,0032	0,0272	0,0605	0,6246
P#45			26	M	0	0	2	0,0326	0,0826	0,1895	0,8319
P#46			49	M	0	1	2	0,0021	0,0150	0,0149	1,0689
P#47			48	M	0		2	1,0130	3,5396	6,0162	1,5715
P#48			49	M	0		0	0,0753	0,5392	0,1492	8,7835
P#49			30	M	0		0	0,0458	0,2007	ND	0,1320
P#50			62	M	0		0	0,0144	0,0315	0,0350	5,1586
P#51		other	26	M	0		0	0,0020	0,0144	0,0116	1,4334
P#52			25	M	0		0	0,0028	0,0157	0,4022	0,3248
P#53			45	M	0		0	0,1475	0,1136	ND	0,3005
P#54			33	M	0		0	1,7972	1,5441	3,5068	2,9964
P#55			30	M	0		0	0,0007	0,0086	0,0077	0,6816

Table S1. Patients' characteristic (livers biopsies analyzed by RT-qPCR)

ID	Group	Age	Sexe	Treatment	HBsAg (IU/mL)	HBeAg (N or P)	HBV DNA (IU/mL)	cccDNA/cell	Total HBV DNA/cell	rcDNA/cell	Replicative activity	ALAT (IU/ml)	Fibrosis (1 to 4)	Necro-inflammatory activity
P#1	Healthy control (HC)	84	F	None	-	-	-	-	-	-	-	-	-	-
P#2		66	M	None	-	-	-	-	-	-	-	-	-	-
P#3		76	M	None	-	-	-	-	-	-	-	-	-	-
P#4		49	M	None	-	-	-	-	-	-	-	-	-	-
P#5	Inactive carrier (IN; HBeAg negative Low viral load)	23	F	None	6511	N	31	0,02	0,74	0,72	31,59	15	1	1
P#6		36	M	None	1555	N	50	0,09	12,09	11,99	127,86	42	1	0
P#7		42	F	None	53,13	N	86	0,03	0,71	0,68	20,50	35	0	1
P#8		59	M	None	5926	N	440	0,02	1,89	1,87	89,17	27	1	0
P#9		40	M	None	10681	N	541	0,08	5,54	5,45	65,07	65	1	0
P#10	Immuno-active (IA; HBeAg negative High viral load)	42	M	None	3250	N	25 238	0,41	3,09	2,68	6,58	78	3	1
P#11		42	M	None	1052	N	27 343	0,80	38,57	37,76	46,93	47	1	1
P#12		45	M	None	1205	N	17 263	0,18	2,33	2,15	11,74	62	1,5	0
P#13		32	M	None	26910	N	93 709	0,13	11,12	10,98	82,79	115	1	1
P#14		60	M	None	7170	N	1 511 762	0,34	3,75	3,41	10,12	45	1	1
P#15	Immuno-tolerant (IT; HBeAg positif High viral load)	28	M	None	8069	P	32 000 000	6,35	4005,87	3999,52	629,75	68	1	1
P#16		34	M	None	10539	P	139 181	0,18	28,61	28,43	155,61	61	3	1
P#17		32	M	None	66820	P	68 275 329	2,04	1975,58	1973,55	968,95	106	1	1
P#18		30	M	None	8051	P	15 800 000	4,47	5734,49	5730,02	1281,31	75	2	1

Table S2. Patients' characteristic (livers biopsies analyzed by IHC)

**Flow cytometry.** Cells were washed extensively with PBS and removed from plate with 150  $\mu$ l of versene-EDTA at 37°C for 5 min before centrifugation at 1300 rpm for 5 min at 4°C. Cells were incubated for 30 min at 4°C with primary antibody (ab175289, 1/50). After extensive washes, cells were incubated with secondary antibody in the dark for 30 min at 4°C. After another wash, staining was measured by Flow Cytometry (BD FACSCALIBUR).

**Statistical analysis.** Results are presented as mean  $\pm$  standard deviation and analysed for statistical significance by a Mann-Whitney test. Significance is represented as follow:  $p < 0.05$ : \*;  $p < 0.01$ : \*\*;  $p < 0.005$ : \*\*\*.

### **Acknowledgments**

The authors would like to thank Maud Michelet, Lydie Lefrançois, Jennifer Molle, Océane Floriot, Anaëlle Dubois for help with the isolation of primary human hepatocytes as well as the staff from Prof Michel Rivoire's surgery room for providing us with liver resection. We also thank the medical staff of Prof. Zoulim as well as Dr. Bancel and Dr Subic (Croix Rousse Hospital, Lyon) for collecting tissue material. We thank the French blood institute (EFS) and all the donors that consented to give their blood. This work was supported by grants from ANRS (French national agency for research on AIDS and viral hepatitis), FINOVI (Foundation for innovation in infectiology), ARC (French Agency for Research against Cancer), Novira Therapeutics and INSERM. This work was also supported by the DEVweCAN LABEX (ANR-10-LABX-0061) of the "Université de Lyon", within the program "Investissements d'Avenir" (ANR-11-IDEX-0007) operated by the French National Research Agency (ANR).

### **Author contributions statements**

- study concept and design: SFD, DD, JL
- acquisition of data: SFD, MD, LA, LD, JF
- analysis and interpretation of data: SFD, AL, KK, DD, JL,
- drafting of the manuscript: SDF, DD, JL
- statistical analyses: SFD
- material support: MR, NBV, FL, RP, FZ

### **Competing financial interest**

SFD, MD, LA, LD, JF, FL, RP, MR, NBV have nothing to declare. FZ, JL, and DD received a research grant from Novira Therapeutics to perform experiments. AL and KK were employees of Novira Therapeutics.

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## 7. Fifth study

### **Characterisation of Pattern Recognition Receptors (PRR) expression and functionality in liver primary cells and derived cell lines**

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**Submitted to the Journal of Innate Immunity.**

**ABSTRACT**

Different liver cell types are endowed with immunological properties, including cell-intrinsic innate immune functions that are important to initially control pathogen infections. However, a full landscape of expression and functionality of the innate immune signalling pathways in the major human liver cells is still missing. In order to comparatively characterize these pathways, we purified primary human hepatocytes (PHH), hepatic stellate cells (HSC), liver sinusoidal endothelial cells (LSEC), and Kupffer cells (KC) from human liver resections. We assessed mRNA and protein expression level of the major innate immune sensors, as well as checkpoint-inhibitor ligands in the purified cells, and found TLR, RLR, as well as several DNA cytosolic sensors to be expressed in the liver microenvironment. Amongst the cells tested, KC were shown to be most broadly active upon stimulation with PRR ligands confirming their predominant role in immune shaping the liver microenvironment. By KC immortalization we generated a cell line that retained higher innate immune functionality than THP1 cells extensively used to study monocyte/macrophages functions. Our findings and the establishment of the KC line will help to understand immune mechanisms behind antiviral effects of TLR agonists or checkpoint inhibitors that are in current preclinical or clinical development.

## INTRODUCTION

Infection by microorganisms leads to the activation of the host immune response through a sensing mediated by innate pattern recognition receptors (PRRs). PRRs include Toll-like receptors (TLRs), C-type lectin receptors (CLRs), RIG-I-like receptors (RLRs), NOD-like receptors (NLRs), and DNA sensors, such as IFI16 (Gamma-interferon-inducible protein 16), cGAS or AIM2 (absent in melanoma 2)[1]. Each PRR detects specific pathogen-associated molecular patterns (PAMPs) derived from viruses, bacteria, mycobacteria, fungi and parasites that initiate the recruitment of distinct sets of adaptor molecules such as Myd88 (Myeloid differentiation primary response gene 88), TRIF (TIR-domain-containing adapter-inducing interferon- $\beta$ ), MAVS (Mitochondrial antiviral-signalling protein), and STING (Stimulator of interferon genes), among others [1]. Activation of those signalling pathways lead to the secretion of many inflammatory cytokines, including interferons (IFN), but also different chemokines and antimicrobial peptides.

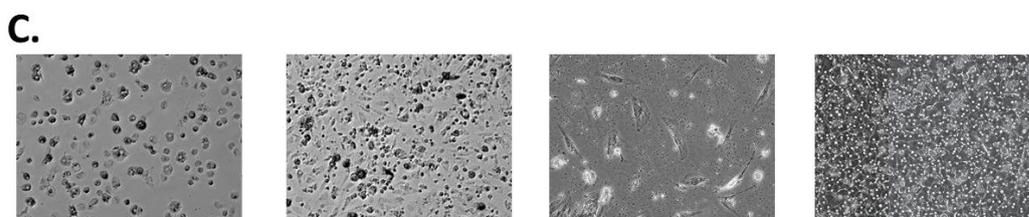
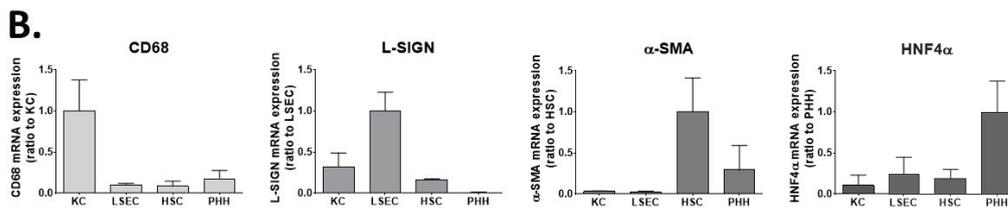
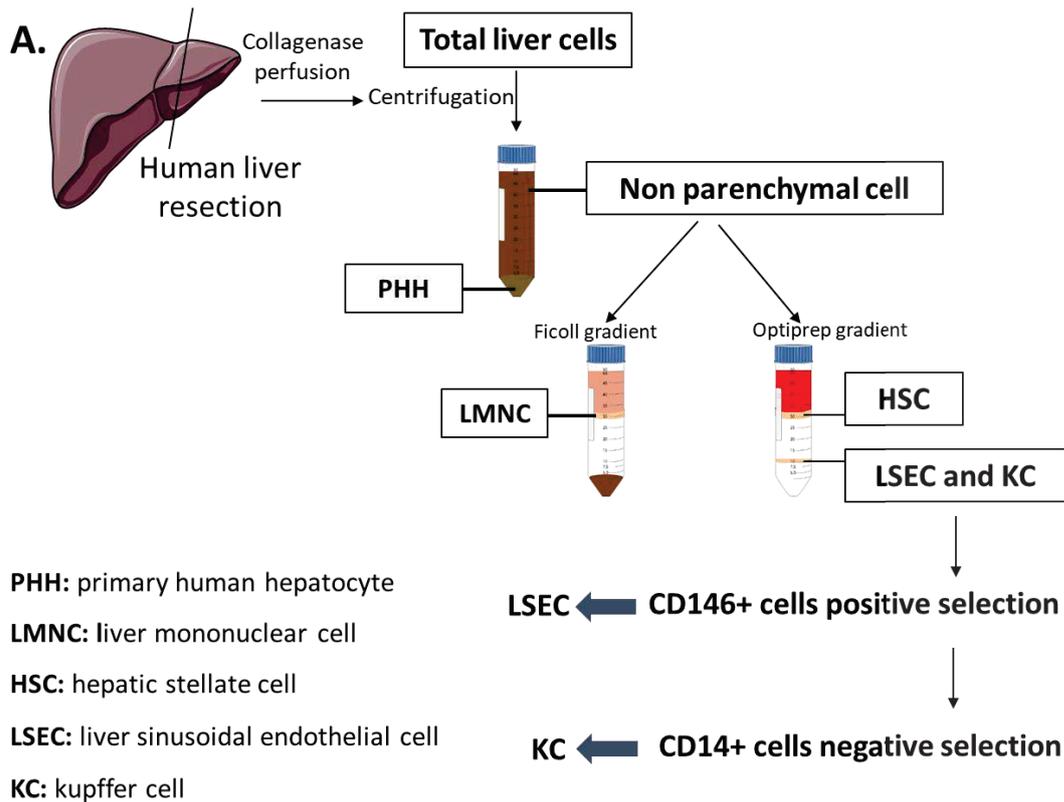
The liver is located at the crossroads of the systemic and enteric circulations and carries out important metabolic functions such as detoxifications, glucose and lipid metabolism. In addition, the liver performs many essential immune tasks and is considered a secondary lymphoid organ due to the number of flowing-through, infiltrating and resident immune cells it contains [2].

Mechanisms of physiologic tolerogenicity are in place in the liver in order to prevent a persistent inflammation in reaction to permanent exposure to gut-derived “microbial degradation products” or even live bacteria, which can pass-through gut mucosa [2,3]. Among tolerogenic mechanisms, there are checkpoint ligand-receptor systems (i.e. PD-1/PD-L1, CTLA-4/B7-1or2...) that modulate T-cell receptor (TCR)-mediated T cells activity [4,5]. This physiologic tolerogenicity represents a sort of Achilles’ heel of the liver, which consequently can be the target of various pathogens establishing chronic infections [6].

The liver is, however, also capable of mounting a potent antimicrobial response. The liver tissue environment is composed of highly specialized cell types, including parenchymal and a number of non-parenchymal cells that play a key role in regulating hepatic immune functions. Parenchymal cells, called hepatocytes, account for 80% of liver mass and respond to different type of stimuli [2,3,7]. Liver sinusoidal endothelial cells (LSEC) are also well known to

participate in liver immune response by secreting cytokines upon pathogenic stimuli [8]. These cells also play a key role upon danger signal leading to fibrosis since upon shear stress, they will undergo cytoskeletal remodelling, leading to a loss of fenestration [8,9]. Hepatic stellate cell (HSC), the liver fibroblasts, and producers of extracellular matrix, are localised in the space of Disse, a perisinusoidal space between hepatocytes and sinusoids, and thus not directly exposed to the bloodstream. These cells normally represent 5 to 8% of the total number of the liver cells. However, upon chronic inflammation, HSC undergo transformation to become myofibroblasts (MFs), the activated state of HSC [9-11]. Once activated, these cells proliferate and start secreting numerous components of the extracellular matrix creating a scar-like tissue [12]. However, during uncontrolled inflammation and scarring/healing process, the overproduction of extracellular matrix induces fibrosis, which can ultimately lead to cirrhosis and favour the development of hepatocellular carcinoma (HCC) [9,12]. Finally, Kupffer cells (KC), the liver resident macrophages, represent 80% of total macrophages population within the body [13]. As macrophages, they form the first line of defence against pathogens and are specialized in pathogen recognition [14]. In response to stimulation, they produce a large spectrum of cytokines and chemokines that attract other immune cells such as neutrophils or infiltrating monocytes that will differentiate into macrophages upon entering the liver. KCs have a high phagocytic capacity and are implicated in the elimination of aging blood cells and pathogens. KC can subsequently present associated antigens to lymphocytes to reactivate them at the site of injury or infection [13].

Successful liver pathogens evolved strategies to either passively or actively evade innate and adaptive immunity. Indeed, Hepatitis B Virus (HBV) and Hepatitis C Virus (HCV) can persistently infect the hepatocytes. HBV and HCV, chronically infect around 70 million and 250 million people worldwide respectively, leading to more than 1.2 million deaths per year (WHO 2017). While curative treatments have been recently developed for HCV [15], leading to viral clearance in more than 95% of cases, HBV cure is not achievable yet, as available treatments (i.e. nucleoside analogues) only allow the control of viral replication and require lifelong administration [16]. New treatments that could lead to a “functional cure” are currently under evaluation [16]. Among them, TLR7 agonists are currently evaluated in clinical phase-II study after showing promising antiviral effects in preclinical models of HBV infections [17,18].



**Supplementary Figure 1: Isolation method and purity of liver cells.** (A) Schematic representation of the experimental procedure to purify PHH, LMNC, HSC, LSEC, and KC from liver resection. (B) PHH, LSEC, HSC and KC were purified from three different donors and cultured for 24h. RNAs were extracted and expression level of the indicated mRNA were analysed by RT-qPCR. Data are presented as mean +/- standard deviation of three different experiments (three different donors). (C) Representative photos of the different type of purified liver cells.

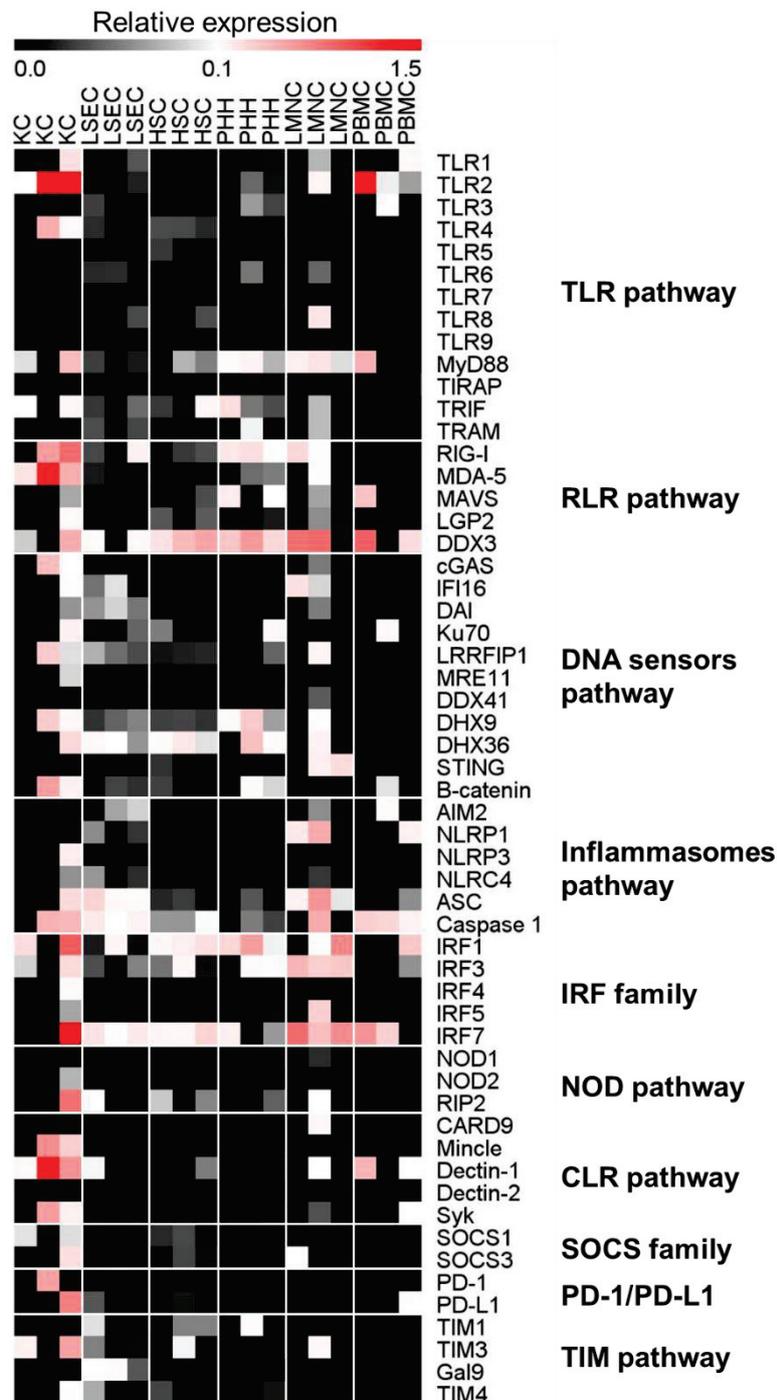
Moreover, PD-1/PD-L1 inhibitors are considered to combat the development of several types of cancers [19,20], including HCC in chronic HBV infection [21,22]. Interestingly, they also are currently in clinical trial to treat CHB patients without signs of HCC. In this context, a better

knowledge of the expression and functionality of innate sensors in liver cells would help developing novel PRR agonists, with antimicrobial or anticancerous properties, or other strategies to revert immune inhibitory processes. This lead us to examine the expression and functionality of some PRR in freshly isolated liver cells, as well as in cell lines derived thereof.

## RESULTS and DISCUSSION

PHH, LSEC, HSC and KC were purified from different liver resections (**Figure S1A**). RT-qPCR analyses using specific markers (i.e. HNF4 $\alpha$  for PHH, L-SIGN for LSEC,  $\alpha$ -SMA for HSC and CD68 for KC) revealed a high enrichment for each cell type (**Figure S1B-C**). The expression level of 53 mRNAs that are known to be involved in pathogens sensing and immune cells regulation was assessed in non-stimulated cells (i.e. basal state) by microfluidic high-throughput quantitative RT-qPCR (Fluidigm, Biomark) assays. As controls, we used total peripheral blood mononuclear cells (PBMC) and total non-parenchymal liver mononuclear cells (LMNC) from different healthy donors [23].

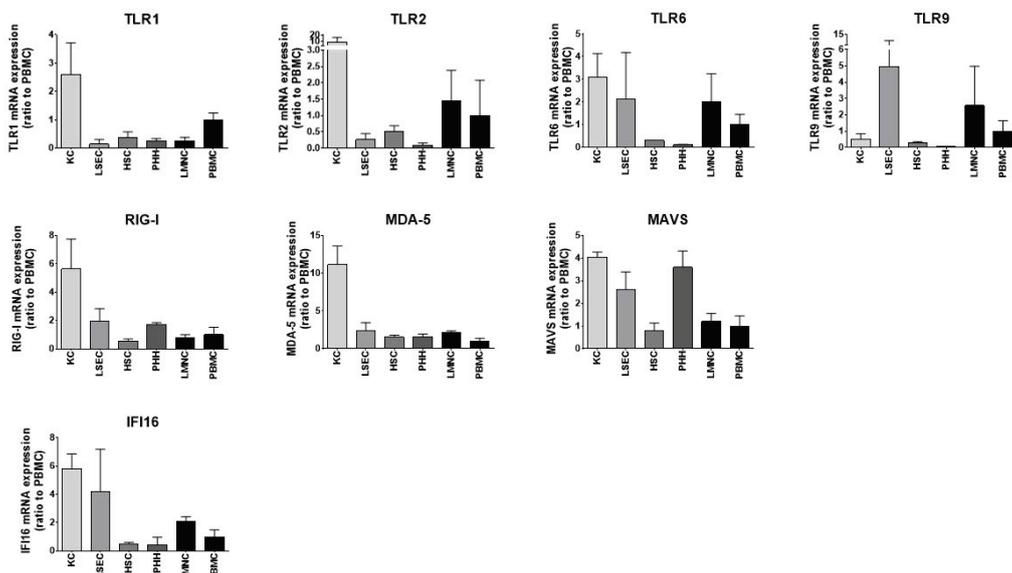
Interestingly, resident LMNCs showed, overall, higher expressions of the tested genes at basal state compared to PBMC supporting the fact that the liver is important for host immunity [2]. Non-stimulated liver cells showed high expression of cytosolic DNA (i.e. DDX3, Ku70, DHX9 or DHX36) and RNA sensors (i.e. RLR, RIG-I and MDA-5) compared to PBMC (**Figure 1**). Except for TLR2 and TLR4, which are highly expressed at basal level in KCs, TLRs mRNA levels were relatively low in liver cells. In contrast, MyD88, TRIF, and TRAM, the main TLR adaptors showed high basal expression level in all the different primary liver cells (**Figure 1**) and IRF1, 3, and 7, the transcription factors involved in TLR signalling, were detected in almost all tested cells. Similarly, expression of NOD1 and NOD2 (previously described to be functional in hepatocytes [24,25]) was barely detectable at basal level but RIP2, a NOD adaptor protein was detected in almost all cell tested (**Figure 1**). Inflammasome receptor mRNA (NLRP1, NLRP3, NLRC4, AIM2) were also barely detected, whereas mRNA coding for their signalling proteins (ASC and caspase 1) were readily detected in all liver cells. Finally, CLR, SOCS family, PD-1 and PD-L1, and TIM pathways were barely detected in the liver at basal state using microfluidic high-throughput quantitative RT-PCR (**Figure 1**).



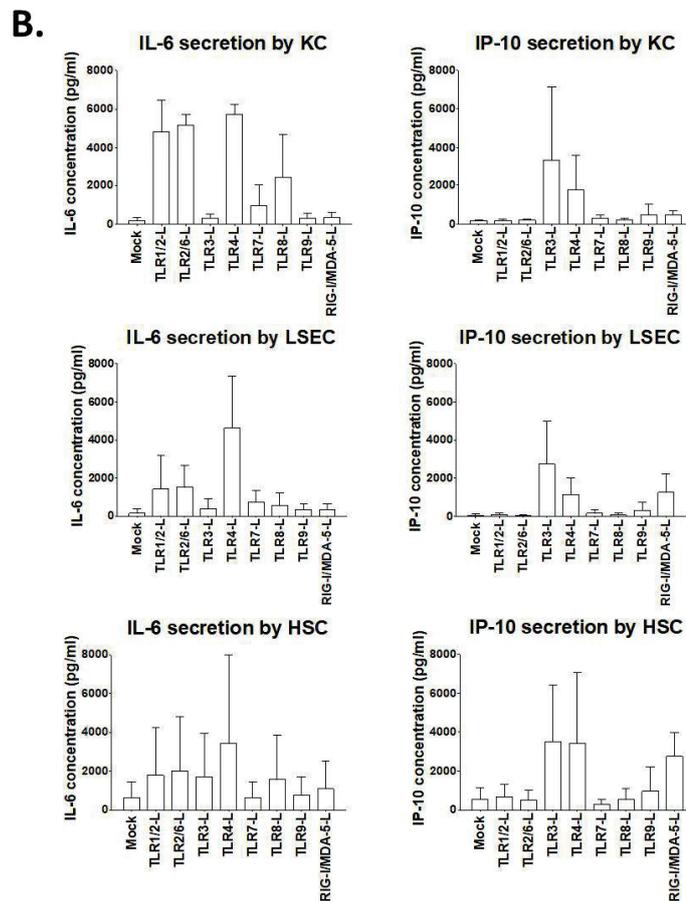
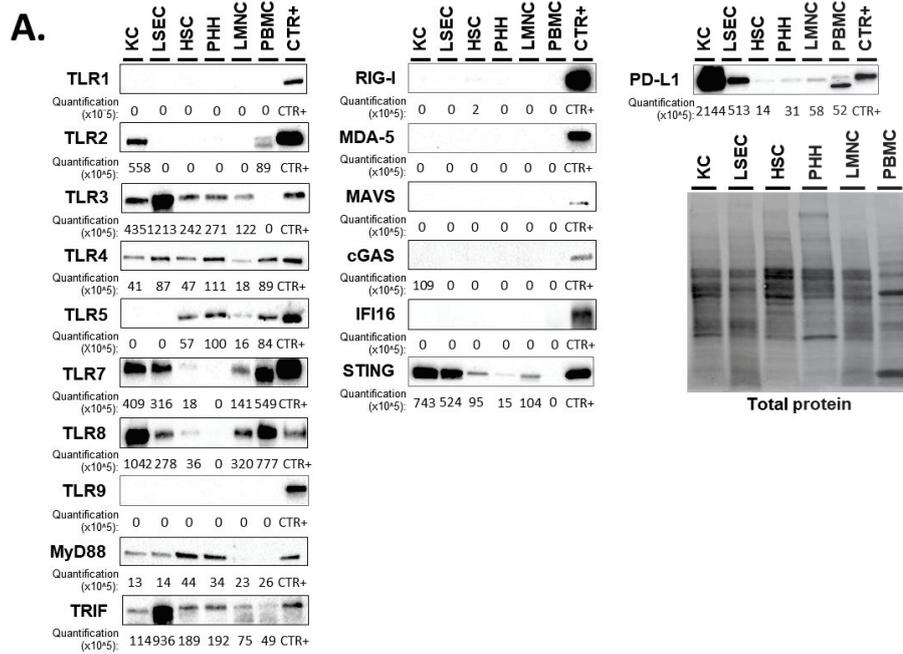
**Figure 1: Liver cells expression of innate sensors and related molecules.** PHH, LSEC, HSC, KC, LMNC and PBMC were purified from different donors and cultured for 24h. RNAs were extracted and expression level of 53 mRNAs was assessed by microfluidic high-throughput quantitative RT-PCR (Fluidigm, Biomark) assays. Data are presented as relative expression to two housekeeping genes (RPLP0 and GUS). Black square indicates an absence of expression, nuances of grey a low expression, and nuances of red a higher expression. TLR: Toll Like Receptor; RLR: Retinoic acid Inducible Gene (RIG) Like Receptor; IRF: Interferon Regulatory Factor; NOD: Nucleotide-binding Oligomerization Domain (NOD) Like Receptor; CLR: C-type Lectin Receptor; SOCS: Suppressor Of Cytokine Signalling protein; PD-1: Programmed cell Death one; PD-L1: Programmed cell Death Ligand one; TIM: T-cell Immunoglobulin and Mucin domain.

By Western blot analyses, we assessed more specifically the expression patterns of PD-L1 as well as a subset of proteins belonging to the TLR, RLR and DNA cytosolic sensor families in liver cells. Despite the presence of mRNA (**Figure S2**), TLR1, TLR9, members of the RLR family (RIG-I, MDA-5, and MAVS) and the DNA sensor IFI16 and cGAS proteins could not be detected in any of the tested cells (**Figure 2A**) suggesting that those proteins are expressed in liver cells at very low levels in steady state but may be up-regulated upon specific stimulation as already described [26,27]. Surprisingly, we could not detect TLR9 proteins neither in PMBCs nor in LMNCs (**Figure 2A**) that should contain pDCs and B cells known to express TLR9 [28] and as confirmed with pDC purified from PBMC (**Figure 2A**, TLR9, CTR+). This might be due to the heterogeneity of cells proportion between donors. TLR4, TLR5, TLR7, TLR8 and TRIF proteins were detected (although at various levels) in both LMNC and PBMC (**Figure 2A**) whereas TLR3 and STING proteins were only detected in LMNC suggesting that percentage of the different immune cells populations are slightly different in blood compared to the liver and/or that the liver microenvironment may influence the phenotype of immune liver cells. TLR3, TLR4, MyD88, TRIF and STING are ubiquitously expressed by KC, LSEC, HSC and PHH (**Figure 2A**) implying that the liver is particularly well equipped to detect lipopolysaccharides as well as cytosolic RNA and DNA [29]. Whereas TLR2 mRNA was present in all cells (**Figure S2**), TLR2 protein was detected only in KCs, suggesting that those cells are specialized in sensing bacterial cell wall components. In accordance with their phagocytic capacities [3], KC and LSEC express TLR7 and TLR8 on protein level (**Figure 2A**), both TLRs involved in intracellular ssRNA detection [30]. Interestingly, TLR5 protein was only detected in HSC and PHH (**Figure 2A**). Since these cells are not directly exposed to blood streams, this specific basal expression could be an evolutionary way to detect infiltrating pathogens and avoid persistent inflammation due to the exposition of bacterial components coming from the enteric circulation. Finally, KC(Folkl and Bienzle, 2010) but also LSEC highly express PD-L1 protein (**Figure 2A**), highlighting those cells as the main drivers of liver tolerogenic activity. Altogether, we observed that all mRNAs from the tested TLR, RLR, DNA sensors and their respective adaptors are at least expressed by one of the liver resident cell types. While adaptors (Myd88, STING, TRIF, and STING) are detected at the protein level in all cells, a large number of proteins from these pathways are not detected. These results suggest that the majority of sensors are regulated to be expressed at relatively low levels probably to prevent chronic inflammatory stimulation at basal state [31]. However, in the presence of pathogens, parenchymal and non-parenchymal liver cells

might mount an efficient immune response since all the adaptor molecules and the transcription factors are highly expressed already at basal state (**Figure 1 and 2A**). For instance, we previously showed that primary human hepatocytes (PHH) and differentiated HepaRG cells (dHepaRG) express a number of PRR and that their agonisation can efficiently inhibit hepatitis B virus replication [32]. PHH responded to TLR1/2, TLR3, TLR4, TLR5, TLR2/6, and RIG-I/MDA-5 stimulation [32] despite the undetectable basal level of expression of several of those PRRs, such as TLR1/2 and RLR proteins (**Figure 2A**). Functionality of TLR and RLR were also assessed in KC, LSEC and HSC by stimulating cells from three to four different donors with the respective ligands. As expected from the protein expression data (**Figure 2A**), KC produced IL-6 and/or IP-10 in response to TLR1/2, TLR3, TLR4, TLR7 and TLR8 ligands (**Figure 2B**). Similarly, LSEC did also respond to TLR3 and TLR4 but not to TLR7 or TLR8 stimulations (**Figure 2B**) suggesting that the amount of those proteins detected by Western blot analysis (**Figure 2A**) was too low to derive a measurable response upon ex vivo stimulation with agonists. In contrast, LSEC produced IL-6 and IP-10 in response to TLR2 and RIG-I/MDA-5 ligands respectively (**Figure 2B**), despite the fact that TLR2, RIG-I or MDA-5 proteins were not detected (**Figure 2A**) and only low amounts of mRNAs were detected in those cells (**Figure S2**). HSC mostly produced IP-10 in response to ligands for TLR3, TLR4 and RIG-I/MDA5 whereas IL-6 production was very variable from one donor to the other (**Figure 2B**).



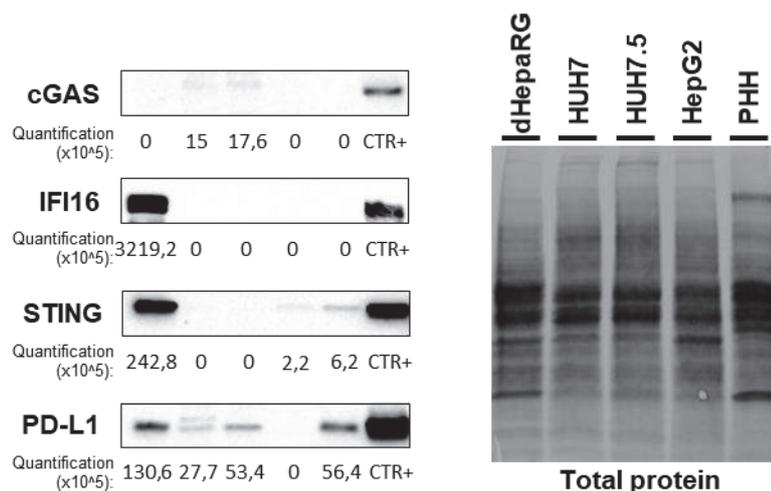
**Supplementary Figure 2: TLR1, TLR2, TLR6, TLR9, RIG-I, MDA-5, and IFI16 mRNA expression in liver primary cells.** PHH, LSEC, HSC, KC, LMNC and PBMC were purified from different donors and cultured for 24h. RNAs were extracted and expression level of the indicated mRNA were analysed by RT-qPCR. Data are presented as mean +/- standard deviation of three different experiments (three different donors).



**Figure 2: TLR, RLR, cytosolic DNA sensors and PD-L1 expression in non-stimulated primary liver resident cells and in responses to PRR stimulations.** (A) PHH, LSEC, HSC, KC, LMNC and PBMC were purified from at least 3 different donors and cultured for 24h. Proteins were extracted, pooled and TLR1 to TLR9, MyD88, TRIF, RIG-I, MDA-5, MAVS, cGAS, IFI16, STING, and PD-L1 protein expression was assessed by western blot analysis. Target protein levels are normalised to total protein quantification assessed by stain-free staining. Stimulated cells were

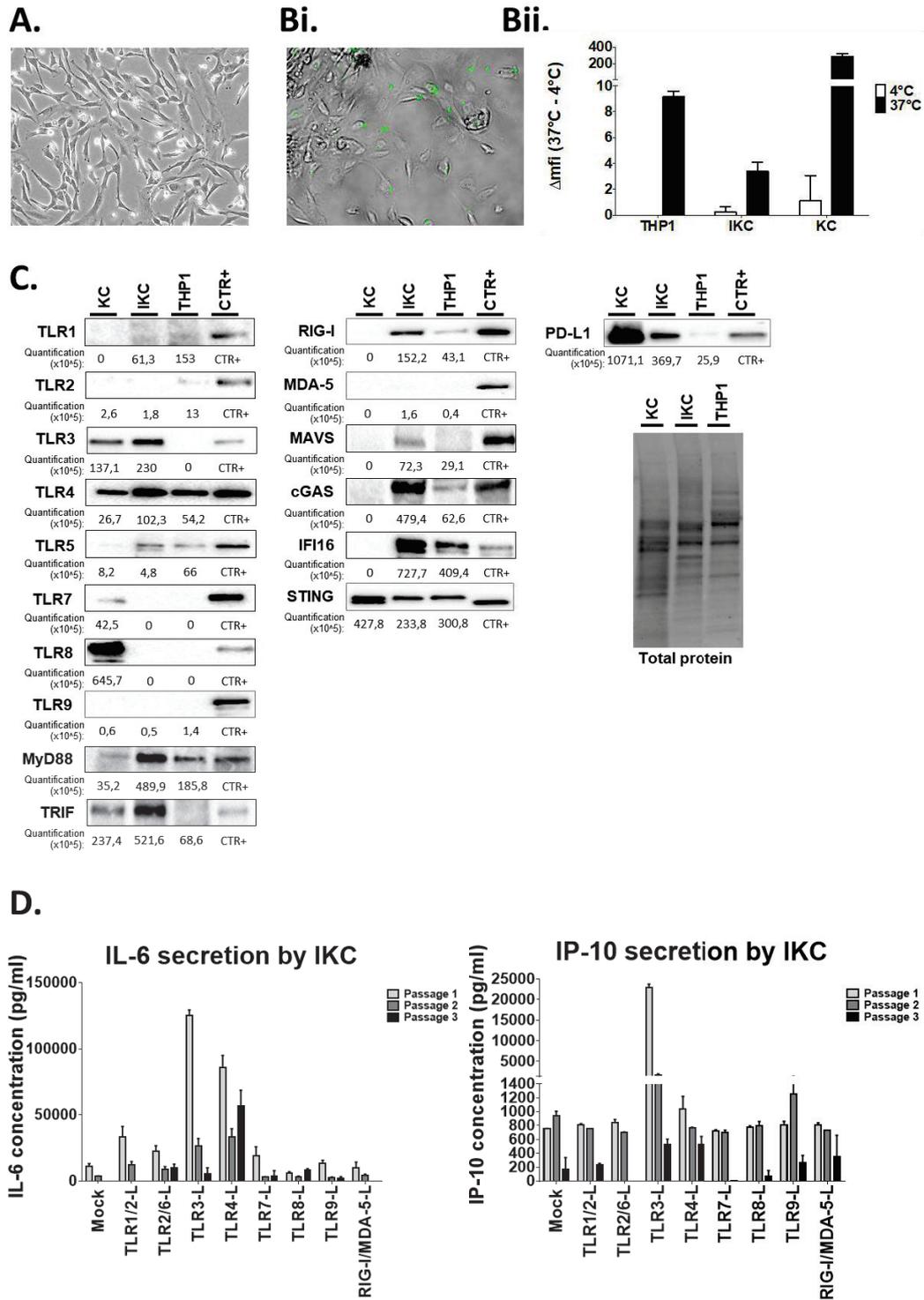
used as controls (CTR+) for primary antibodies efficiencies as indicated in Table S2. (B) KC, LSEC and HSC from different donors were exposed to the indicated ligands at the concentration indicated in Table S3 for 24 hours. Supernatants were collected and IL-6 and IP-10 secretions were analysed by ELISA. Data are presented as mean +/- standard deviation of three biological replicates.

Access to liver resection and therefore primary liver cells is limited. Moreover, donors have different genetic backgrounds, environmental backgrounds and histories in term of diseases, treatment, food habits, etc that may influence metabolism, physiology and responses to pathogens of the liver cells. It is therefore complementary to test antimicrobial strategies in well-characterized cell lines, that can be in addition genetically engineered to perform mechanistic studies. We previously showed that, compared to Huh7 or HepG2 cells, dHepaRG cells were closest to PHH in term of TLR and RLR pattern expression [33] and therefore a suitable model to test antiviral effect of TLR and RLR ligands. Here we decided to extend our analysis to other immune signalling pathways than the ones we had previously characterized. cGAS protein basal level are not detected in the different hepatocyte *in vitro* models (**Figure S3**). Regarding IFI16 and STING level of expression, we observed a higher basal expression in dHepaRG cells compared to the other *in vitro* models (**Figure S3**). In addition, PD-L1 protein levels in dHepaRG cells are similar to PHH, compared to HuH, HuH7.5, and HepG2 (**Figure S3**). Altogether these results highlight the importance to choose the proper *in vitro* model according to the scientific question addressed.



**Supplementary Figure 3: cGAS, IFI16, STING and PD-L1 expression in hepatocytes.** PHH were purified from at least three different donors and cultured for 24h. HuH7, HuH7.5, HepG2-NTCP cells were seeded and cultured until reaching confluence. HepaRG cells were differentiated for four weeks. Proteins were collected and expression level of the indicated proteins were analysed by WB. Stimulated cells were used as controls (CTR+) for primary antibodies efficiencies as indicated in Table S2.

KCs are a unique macrophage population differentiated from myeloid progenitor during embryogenesis and have specific functions, such as self-renewing capacity, which distinguish them from circulating monocytes [34]. THP1 cell line is the most commonly used model for monocyte and macrophages [35-37] but to our knowledge no KC derivative cell line has been described so far. We therefore immortalized Kupffer cells (IKCs) by expressing the E6 and E7 proteins of the papilloma virus [38]. The resulting polyclonal cell line consisted of elongated cells (**Figure 3A**). IKC acquired proliferative capacity, far beyond the very self-renewing capacity of KC. To stop IKC proliferation, cells were treated with 2% DMSO during 48h for the following experiments. Although to a lower extent compared to primary KC, IKC retained their ability to phagocytose bacteria (**Figure 3Bi-3Bii**). KC, IKC and THP1 had similar pattern of protein expression for TLR1, TLR4, MDA-5 and STING (**Figure 3C**). Similarly to the abolishment of TLR9 expression by HPV [39], TLR7 and TLR8 proteins were not detected in IKC (and THP1) as compared to KC, whereas higher amounts of TLR5, MyD88, RIG-I, cGAS, and IFI16 proteins were found in both cell lines (**Figure 3C**). MAVS protein was only detected in IKC (**Figure 3C**). Even though, KC and IKC express different amounts of TLR7, TLR8, Myd88, RIG-I and MAVS proteins, their responses to the respected stimulations were overall similar (**Figure 3D**). Whereas a low basal signal was detected in both KC and THP1, TLR2 protein was not detected in IKC (**Figure 3C**) and response to TLR1/2 agonisation was much lower in IKC than in KC (**Figure 3D, to be compared to Figure 2B**). Interestingly, TLR3, TRIF and PD-L1 protein expression pattern in IKC was closer to KC than THP1 (**Figure 3C**). Furthermore, KC and IKC strongly responded to TLR3 and TLR4 stimulations (**Figure 3D**). Of note, amounts of secreted IL-6 upon IKC stimulations were 10 times higher than those detected in KC suggesting that immortalization processes potentiate these pathways (compare **Figure 2B** and **Figure 3D**). Of note, IL-6 and IP-10 secreted amounts (**Figure 3D**), as well as proliferating capacity (data not shown), decreased with cell passaging of IKC highlight the importance to perform experiments on low passage cells.



**Figure 3: Characterization of Immortalized Kupffer Cells (IKC).** IKC from three different passages were seeded and cultured for 48h with 2% DMSO. KC purified from three different donors were seeded and cultured for 24h. THP1 were seeded and cultured for 48h with PMA. Cells were then (A, C) left untreated, (B) exposed to pHrodo™ bacteria for 1 hour 4°C or 37°C or (D) exposed to the indicated ligands at the concentration indicated in Table S3 for 24 hours. (A, Bi) Microscopic analyses of cells (x20 magnification) were performed. (Bii) Bacteria phagocytosis

was assessed by flow cytometry analysis. (C) Proteins were extracted, pooled and TLR1 to TLR9, MyD88, TRIF, RIG-I, MDA-5, MAVS, cGAS, IFI16, STING, and PD-L1 protein expression was assessed by western blot analyses. Target protein levels are normalised to total protein quantification assessed by stain-free staining. Stimulated cells were used as controls (CTR+) for primary antibodies efficiencies as indicated in Table S2. (D) Supernatants were collected and IL-6 and IP-10 secretions were analysed by ELISA. Data are presented as mean +/- standard deviation of three biological replicates.

To summarize, we provided here a comprehensive analysis of innate immune signalling capacity of the most important liver cell populations and demonstrate their ability to respond to pathogens or PRR ligand stimulation. This knowledge will be useful to understand mechanisms behind antiviral effects of TLR agonists or check-point inhibitors [16,19-21,40,41], whose development as direct effectors or “adjuvant” in more complex immune-therapeutic strategies are currently investigated to treat cancer and chronic viral infection. For instance, TLR7, TLR8, RIGI, and STING agonists are currently developed to fight chronic HBV infections [16] but our work highlights other ligands such as TLR2 and TLR3 agonists that are highly expressed in KC and LSEC and have the best direct anti-HBV properties in HBV-replicating PHH [42].

## MATERIAL AND METHODS

**Liver primary cells purification and cells culture.** Peripheral blood mononuclear cell (PBMC) from at least 3 blood donors were isolated by Ficoll gradient cultured in RPMI medium supplemented with 10% of decompemented FBS and 50 U/ml of penicillin/streptomycin. Liver cells were isolated from liver resections (**Figure S1A**) obtained from three surgical departments in Lyon (Centre Léon Bérard, Hôpital de la Croix Rousse and centre hospitalier Lyon-Sud) with the French ministerial authorizations (AC 2013-1871, DC 2013 – 1870, AFNOR NF 96 900 sept 2011). After collagenase treatment, the liver extract is filtered and centrifuged, as previously described [43]. Pellets contain PHH whereas supernatants contain remaining non-parenchymal and other liver resident cells. PHH were cultured on collagen layer and maintain in PHH medium (Williams medium supplemented with 5% of fetal clone II serum, 50 U/ml of penicillin/streptomycin, 1X glutamax, 5 µg/ml of bovine insulin, 5x10<sup>-5</sup> M of hydrocortisone, and 2% of DMSO). The supernatants (containing all liver cells except PHH) were either used to purify total liver mononuclear cells (LMNC) by Ficoll gradient or used to

purify hepatic stellate cells (HSC), Kupffer cells (KC) and liver sinusoidal endothelial cell (LSEC), by a two phase iodixanol gradient [44] (**Figure S1**). HSC were further cultured in Williams medium supplemented with 10% of fetal clone II serum, 50 U/ml of penicillin/streptomycin, 1X glutamax, 5 µg/ml of bovine insulin, and  $5 \times 10^{-5}$  M of hydrocortisone). LSEC were purified by positive selection with CD146 microbeads (Miltenyi Biotec) and culture in LSEC medium (MCDB131 supplemented with 20% of fetal clone II serum, 50 U/ml of penicillin/streptomycin, 5X glutamax, 35 mM of hydrocortisone, and 10 mg/ml of cAMP). KC were isolated by negative selection using pan monocyte isolation kit (Miltenyi Biotec) and cultured in in RPMI medium supplemented with 10% of decompemented FBS and 50 U/ml of penicillin/streptomycin. At least 3 batches of each primary cells were used to perform the experiments. Huh7, Huh7.5 and HepG2 cell lines were cultured as previously described [45-47]. HepaRG cells were cultured and differentiated as previously described [48]. THP1 were cultured and differentiated as previously described [49]. Immortalised Kupffer cell (IKC) were immortalized by transduction with lentiviruses expressing the HPV E6E7 proteins, cultured in macrophage medium and differentiated by a 48h treatment with 2% DMSO to stop proliferation.

**RNA extraction, reversion transcription, dPCR and qPCR.** Total RNA were extracted with Nucleospin RNA II (Macherey-Nagel) and cDNA synthetized using the SuperScript<sup>®</sup>III Reverse Transcriptase (Life technologies) according to the manufacturer's instructions. cDNA were first analysed by digital PCR (dPCR), on a 96x96 Biomark HD system (Fluidigm) using EvaGreen<sup>®</sup> dye according to the manufacturer's instructions. Primers sequences are presented in Table S1. qPCR analyses were performed using "Express SYBR GreenER<sup>™</sup> qPCR SuperMix Universal" (Invitrogen). mRNA expression was assessed by comparative cycle threshold (Ct) method, by normalising the mount of target cDNA on housekeeping genes: RPLP0 and GUS for dPCR and RPLP0 for qPCR ( $2^{-\Delta Ct}$ ).

**Heat map construction and analysis.** Primary results obtain with Biomark HD system were analysed with manufacturers software (Fluidigm Real-Time PCR Analysis). Construction of the Heat map was performed on MEV software.

**Immunoblot Analysis.** Cells were lysed in RIPA buffer supplemented with protease inhibitors for 30 minutes on ice, sonicated and boiled in Laemmli buffer complemented with DTT. Protein concentration was measured by BCA protein assay kit (Thermo Scientific). Equal

amounts of protein from total cell lysates (30 to 40  $\mu\text{g}$ ) were loaded onto SDS-PAGE Stain-Free precast gels (BioRad) and transferred onto nitrocellulose membranes (BioRad). The membranes were blocked with TBST (1 $\times$  TBS with 0.1% Tween 20) + 5% milk at room temperature for 1h and incubated overnight at 4°C with primary antibodies. Primary antibodies and their corresponding positive controls used in this study are described in Table S2. Membranes were washed and incubated with horseradish peroxidase (HRP)-coupled secondary antibody (SIGMA) for 1h at room temperature. Activity was visualized by chemiluminescence and the signal was quantified by ImageLab software. Stain-Free analysis was performed following manufacturer's instructions. Western blot analyses were performed on a pool of protein samples obtained from at least three different donors. The nature of positive control samples are specified in Table S2.

Target protein	Name	Reference	Stimulated Control
<b>TLR1</b>	Polyclonal anti-TLR1 antibody	PA5-11589 (Thermo Fisher)	THP1 + PAM3CSK4
<b>TLR2</b>	Monoclonal anti-TLR2 antibody	#12276 (Cell Signaling)	THP1 + PAM3CSK4
<b>TLR3</b>	Monoclonal anti-TLR3 antibody	#6961 (Cell Signaling)	LMNC + Riboxsol
<b>TLR4</b>	Monoclonal anti-TLR4 antibody	sc-293072 (Santa Cruz)	THP1 + LPS
<b>TLR5</b>	Monoclonal anti-TLR5 antibody	Ab168382(Abcam)	THP1 + LPS
<b>TLR7</b>	Monoclonal anti-TLR7 antibody	#5632 (Cell Signaling)	THP1 + Imiquimod (R837)
<b>TLR8</b>	Monoclonal anti-TLR8 antibody	#11886 (Cell Signaling)	LMNC + ssRNA40/Lyovec
<b>TLR9</b>	Monoclonal anti-TLR9 antibody	#13674 (Cell Signaling)	pDC
<b>RIG-I</b>	Monoclonal anti-RIG-I antibody	#3743 (Cell Signaling)	HepaRG + Poly(I:C) (LMNC)/Lyovec
<b>MDA-5</b>	Monoclonal anti-MDA5 antibody	#5321 (Cell Signaling)	HepaRG + Poly(I:C) (LMNC)/Lyovec
<b>cGAS</b>	Monoclonal anti-cGAS antibody	#15102 (Cell Signaling)	THP1 + STING
<b>STING</b>	Monoclonal anti-STING antibody	#13647 (Cell Signaling)	THP1 + STING
<b>PD-1</b>	Polyclonal anti-PD1 antibody	PA5-20350 (Thermo Fisher)	THP1 + PAM3CSK4
<b>PD-L1</b>	Polyclonal anti-PDL1 antibody	PA5-28115 (Thermo Fisher)	THP1 + PAM3CSK4
<b>IFI16</b>	Monoclonal anti-IFI16 antibody	Ab169788 (Abcam)	THP1 + Imiquimod (R837)
<b>MyD88</b>	Monoclonal anti-Myd88 antibody	#4283 (Cell Signaling)	THP1 + PAM3CSK4
<b>TRIF</b>	Polyclonal anti-TRIF antibody	#4596 (Cell Signaling)	LMNC + Riboxsol
<b>MAVS</b>	Polyclonal anti-MAVS antibody	#3993 (Cell Signaling)	HepaRG + Poly(I:C) (LMNC)/Lyovec

Table S2: Antibodies and controls used for western blot analysis.

**PRR stimulation and ELISA.** TLR or RLR ligands from Invivogen or Riboxsol life science are listed in Table S3. Cells were exposed to the ligands, supernatants were collected 24h later and IL-

6/IP-10 concentration were assed using DuoSet ELISA (R&D systems) according to the manufacturer's instructions.

<b>PRR</b>	<b>Ligand</b>	<b>Reference</b>	<b>Concentration</b>
<b>TLR1/2</b>	PAM3CSK4	tlr1-pms	0,5 µg/ml
<b>TLR2/6</b>	FSL-1	tlr1-fsl	1 µg/ml
<b>TLR3</b>	Poly(I:C) (HMW)	tlr1-pic	5 µg/ml
<b>TLR3</b>	Riboxsol	A-00102	5 µg/ml
<b>TLR4</b>	LPS SM	tlr1-smmps	0,5 µg/ml
<b>TLR7</b>	Imiquimod (R837)	tlr1-imq	5 µg/ml
<b>TLR8</b>	ssRNA40/Lyovec	tlr1-lrna40	1 µg/ml
<b>TLR9</b>	ODN 2395	tlr1-2395	5 µM
<b>RIGI/MDA5</b>	Poly(I:C) (LMW)/Lyovec	tlr1-picwlv	0,025 µg/ml
<b>STING</b>	2'3'-cGAMP	tlr1-cga23	5 µg/ml

**Table S3: Ligands and concentration used for PRR stimulation.**

**Phagocytosis assay.** THP1, IKC and KC phagocytosis capacity were assessed using pHrodo™ Green E. coli Bioparticles™ conjugates for phagocytosis. Cells were exposed for 1h to the bioparticles at either 4°C or 37°C. After 1h, cells were collected by scrapping and phagocytosis was assessed by flow cytometry using FACScalibur flow cytometer and analysed using CellQuestPro software (BD Biosciences).

## **ACKNOWLEDGMENTS**

The work was supported by grants from INSERM, ANRS (French national agency for research on AIDS and viral hepatitis; grants from CSS4 and CSS7 study committees), the DEVweCAN LABEX (ANR-10-LABX-0061), and the FINOVI foundation. The authors would like to thank Maud Michelet, Jennifer Molle, Loïc Peyrot, Anaëlle Dubois, Océane Floriot, and Marie-Laure Plissonnier for their help in the isolation of primary human hepatocytes, as well as Pr Michel Rivoire's, Pr. Jean-Yves Mabrut's and Dr. Guillaume Passot's staffs for providing liver resections. The authors would also like to thank Maëlle Locatelli, Léa Monnier, Brioux Chardès and Hélène Chabrolles for providing the hepatocyte cell lines. Finally, the authors thank the EFS (French establishment for blood donation) for providing the blood bags.

Gene	Full name	Forward	Reverse
<b>RPLP0</b>	ribosomal protein lateral stalk subunit P0	CACCATTGAAATCCTGAGTGATGT	TGACCAGCCCAAAGGAGAAG
<b>GUS</b>	$\beta$ -glucuronidase	CGTGGTTGGAGAGCTCATTGGAA	ATTCACCCAGCACTCTCGTCGGT
<b>TLR1</b>	Toll like receptor 1	ACAAGCAGGTTGCTTTGTGT	GAGGGCTGGTACCCTTATT
<b>TLR2</b>	Toll like receptor 2	CTCTGGTGCTGACATCCAATGGAA	GGGCTTGAACCAGGAAGACGATAA
<b>TRL3</b>	Toll like receptor 3	AGAAGGTTTTGGGCCAGCTTT	TGACAGCTCAGGGATGTTGGTATG
<b>TLR4</b>	Toll like receptor 4	CACACCAGAGTTGCTTCAATGGC	AGAGAGTCCAGGAAGGTCAAGTT
<b>TLR5</b>	Toll like receptor 5	CCTGACCTTATAGTTGCCAGCTT	TCCGACATCTCCCTGGATGCTAA
<b>TLR6</b>	Toll like receptor 6	ACCCATTCCACAGAACAGCATTCC	TCCTTGGGCCACTGCAAATAAGTC
<b>TLR7</b>	Toll like receptor 7	CCAACTGTTCCAGAACCTCAAGA	CCAGATATCGCAACTGGAAGGCAT
<b>TLR8</b>	Toll like receptor 8	AGTTTCTCGGCTCGAGTTGCTTG	AGAGGGTAGGTGGAAATCCTGTT
<b>TLR9</b>	Toll like receptor 9	AACTGGCTGTTCTGAAGTCTGTG	AGTGGGCAAAAGTCAGAATCATGG
<b>MyD88</b>	myeloid differentiation primary response 88	AGAAAAGAGTCCCCAGCATCCTGA	CAAGGCGAGTCCAGAACCAAGATT
<b>TIRAP</b>	TIR domain containing adaptor protein	GCGCAGGCCTTACATAGGAA	CTGGCCAGGAGGATATTCGG
<b>TRIF</b>	TIR-domain-containing adapter-inducing interferon- $\beta$	AGAAAACAGCACCAACTACCCA	GGTCTTTGACAGAGCAGGGTTT
<b>TRAM</b>	translocation associated membrane protein	TGGGTCTGCATGGACAATCT	GGCCGCATGGGTATAACAGA
<b>RIG-I</b>	retinoic acid inducible gene 1	TTCTGATTGCCACCTCAGTTGC	TCCTCTGCCTCTGGTTTGGATCAT
<b>MDA-5</b>	melanoma differentiation-associated protein 5	AGGTCTGGGCATGGAGAATAACT	TGCCCTGAATCAGTCCCATGTT
<b>MAVS</b>	mitochondrial antiviral-signaling protein	TGAACACAGTGGCCCTGAAAGT	ACGGGTTGAGTTGATGGCAAT
<b>LGP2/DHX58</b>	DEXH-box helicase 58	GGCACCCACCATGCAATG	CCCAGACCTCCCCACAGTT
<b>DDX3</b>	DEAD-box helicase 3	CAGTTCAGGTTGGAGTTCTAGCAA	CTGCCAATGCCATCGTAATCACTC
<b>cGAS</b>	cyclic GMP-AMP synthase	GAAGGCCTGCGCATTCAAACCT	GAGAGAAGGATAGCCGCCATGTTT
<b>IFI16</b>	gamma interferon inducible protein 16	GACCAGCCCTATCAAGAAAGAGGA	TCTGGGAGTTACCTGACATTTGGC
<b>DAI</b>	DNA-dependent activator of IFN-regulatory factors	GGTGAAGGAATGCCAAGCAC	CCTTCAGGATCAGTCCCGC
<b>Ku70</b>	-	GCATGCCGTGGAATGTCGTC	TAGTCTCCACTTGCCTCAAGGT
<b>LRRFIP1</b>	LRR binding FLII interacting protein 1	GAGTCCGCTCTGCATTGGATAAA	CAAGAGTGCCTCCGATTTGCT
<b>MRE11</b>	meiotic recombination 11 homolog	GGGTCTCAAAGAGGAAGAGACAC	GACATTTCCGGGAAGGCTGCT
<b>DDX41</b>	DEAD-box helicase 41	GCAGTCTGAGGAGTGAGCTTC	CTACCCCTCAGATCAAGCCG
<b>DHX9</b>	DEXH-box helicase 9	AGCAATGCTGCCAGAGACTT	GCGGAGATGTACCCCAAAA
<b>DHX36</b>	DEXH-box helicase 36	AGTGCCATTTAGTTGCGGA	TGTTTCTTGGCAACCGACT
<b>STING</b>	stimulator of interferon genes	CCATGGGCTGGCATGGTCATATTA	AATATACAGCCCTGGCTCACT
<b>B-catenin</b>	-	TTGTGCGGCCCATTTTAAG	TCCTCAGACCTTCTCCGTC
<b>AIM2</b>	absent in melanoma 2	TCGGCACAGTGGTTTCTTAGAGG	TCGGGGTTTACCAGCTTTTCT
<b>NLRP1</b>	NLR family pyrin domain containing 1	CTACGTTGGCCACTTGGGAT	AGGTGAAGGTACGGCTATGC
<b>NLRP3</b>	NLR family pyrin domain containing 3	AAAGGAAGGCCGACACCTTGAT	TGGCTGTGTCTCAGAACTGAAA
<b>NLRC4</b>	NLR family CARD domain containing 4	GAGGTCCCAACTCGTCAA	GATTTCCCGCAAATTCAC
<b>ASC</b>	apoptosis-associated speck-like protein containing a CARD	CGGAGGGTCAAAAACGT	TGCTCATCCGTCAGGACCTT
<b>Caspase 1</b>	-	AAAATCTCACTGCTTCGGACATG	GGAACGTGCTGTCAGAGGCTCT
<b>IRF1</b>	interferon regulatory factor 1	TTGTGCCGGACAGCACCAGT	TCCACGTTTGTGGCTGCCACT
<b>IRF3</b>	interferon regulatory factor 3	ACCCAGCCGTGGACCAAGAG	TACCAAGCCCTGAGGCAC
<b>IRF4</b>	interferon regulatory factor 4	GACCGAAGCTGGAGGACTA	TGTCACCTGGCAACCATTTC
<b>IRF5</b>	interferon regulatory factor 5	TGTCAGTGCAAGGTGTTCTGGA	TGTTGGTCTGGCCCTTTTGAA
<b>IRF7</b>	interferon regulatory factor 7	TGTTCTGGTGAAGCTGGAA	GATGTCGTCATAGAGGCTGTTGG
<b>NOD1</b>	nucleotide-binding oligomerization domain-containing protein 1	AGAGGCTCTGCGGAACCA	TGTGGAGATGCCGTTGGA
<b>NOD2</b>	nucleotide-binding oligomerization domain-containing protein 2	CCCTGCAGCTGGACTACAACCT	AGATGCCTCGGCTGAGGATATTG
<b>RIP2</b>	receptor interacting serine/threonine kinase 2	CGTGCTCGACAGTGAAAGA	TCAGGCTCATTGCAAATTC
<b>CARD9</b>	caspase recruitment domain-containing protein 9	TTGAGAACTACCGCAGGAAGCG	GGCTAGGAGCCCTCAGTGTC
<b>Mincle</b>	macrophage inducible Ca <sup>2+</sup> -dependent lectin receptor	ACGAGAAATTTGTGCGTGA	CTGGGAAGAGGACCTGAGACT
<b>Dectin-1/CLECL7A</b>	C-type lectin domain family 7 member A	TCAGGGGCTCTCAAGAACA	GAGGAGATGCAGCACAGGAT
<b>Dectin-2/CLEC6A</b>	C-type lectin domain family 6 member A	AACACAGGGAGCCTGCATAAT	ATCCCAGCCACAGACCAGAG
<b>Syk</b>	spleen tyrosine kinase	TGAGCCTCACCAAAACAGG	ACAGCAAACTGAAATCAATGGGT
<b>SOCS1</b>	suppressor of cytokine signaling protein 1	CACCTCCGCACATTCCGTTT	CAGTAGAATCCGACGGGCTC
<b>SOCS3</b>	suppressor of cytokine signaling protein 3	CCATTCCGGAGTTCCTGGAC	TTGGCTTCTGTGCTTGTGC
<b>PD-1</b>	programmed cell death 1	AAACCCTGGTGGTGGTGTGTC	CTCCTATTGTCCCTCGTGGC
<b>PD-L1</b>	programmed cell death ligand 1	CTGAACGCATTTACTGTCACGG	AGACAATTAGTCGACCCAGGT
<b>Tim1</b>	T-cell immunoglobulin and mucin domain 1	ACTGGAGAAAGCCGACGTG	GGACCTCTGGGTTGAAATGA
<b>Tim3</b>	T-cell immunoglobulin and mucin domain 3	CTACTGCTGCCGGATCCAAA	GTCCCTGGTGGTAAGCATC
<b>Gal9</b>	galectin 9	ACAGCCAAGTTCGTTTGGTTT	AAAGGGGACAGCTGGACTCA
<b>Tim4</b>	T-cell immunoglobulin and mucin domain 4	GTCCTGCTGACATCCAAAGAGT	TTGTTTTGTTCTGCTCAGGAACTG
<b>CD68</b>	Cluster of differentiation 68	AGGCTGGCTGTGCTTTTCTC	CTCTGTAACCGTGGGTGCA
<b>L-SIGN</b>	liver/lymph node-specific ICAM-3-grabbing integrin	GACTGCATTTGAACGCCTGT	TGACGGAGTTGTGCCAGTTC
<b><math>\alpha</math>-SMA</b>	$\alpha$ -smooth muscle actin	GTGTTGCCCTGAAGAGCAT	GCTGGGACATTGAAAGTCTCA
<b>NTCP</b>	sodium-taurocholate cotransporting polypeptide	TGACCACCTGCTCCACCTTC	GAATGAGAAGCAGGACCAAGTAT

Table S1: Primers sequences

## AUTHOR CONTRIBUTIONS STATEMENT

J.L, S.F.D., S.V. and D.D. designed the study; S.F.D., S.V., J.L., L.A., L.D. and M.Bo. performed and/or analysed experiments; S.F.D., K.E., M.Br., U.P., N.B.V. contributed to the establishment of protocols for liver cells isolation; M.R., G.P., M.L., J.Y.M. and C.D. provided liver resections. F.Z and A.S provided material. J.L., S.F.D., S.V. and D.D. wrote the manuscript. U.P., F.Z. and N.B.V. did a critical reading of the manuscript.

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## 8. Discussion and perspective

Most viruses are detected/sensed early after infection by both immune and/or infected cells mainly via Pathogen Recognition Receptors (PRR) expressed at the surface or inside cells (Wilkins and Gale, 2010). It is still debated if HBV is recognized by the innate immune system and/or if the virus can actively suppress or avoid antiviral responses that could impair its establishment and/or maintenance in hepatocytes.

### **Is HBV recognized by the host innate immune system?**

A few years ago, our team found that a strong intracellular HBV replication, experimentally launched by a recombinant baculovirus vector and thus bypassing the viral entry step, was sensed by innate receptors of hepatocytes and induced potent antiviral responses (Lucifora et al., 2010). The recent studies, in which I participated in, further confirmed these results and observed only a very modest and transient response, with no detectable production of cytokines in cell supernatant when HBV replication was launched in dHepaRG cells or PHH through a proper infection with cell-culture-produced HBV inocula (study#1; (Luangsay et al., 2015b)). Moreover, in our different experimental settings, we did not detect any cytokine secretion by LMNC, primary human liver macrophages or MDM exposed to cell-culture-produced HBV inocula or co-cultured with HBV producing cells (HepAD38) even though the virus was observed within these cells ((Zannetti et al., 2016) and data not shown). This is in sharp contrast with chronic HCV or HIV infections that induces strong host responses (Doyle et al., 2015; Heim and Thimme, 2014; Shi et al., 2017a; Soper et al., 2017; Wieland et al., 2004b) but in accordance with studies that reported an absence of measurable innate immune responses in patients, animal or *in vitro* models upon primary HBV infection (Dunn et al., 2009; Fletcher et al., 2012; Mutz et al., 2018; Stacey et al., 2009b; Suslov et al., 2018; Tan et al., 2010; Wieland et al., 2004b) and contributed to define HBV as a stealth virus (Wieland and Chisari, 2005).

Of note, several groups have reported an induction of innate responses following exposure to HBV (Cooper et al., 2005; Hösel et al., 2009c, 2017; Sato et al., 2015). For instance, an activation of myeloid cells and the subsequent secretion of pro-inflammatory mediators were observed after exposure to HBsAg (Boltjes et al., 2014, 2015; van Montfoort et al., 2016). It

was also shown that HBV activated inflammation related genes in hepatocytes, including CXCL10, a chemokine implicated in the recruitment of pro-inflammatory cells (Yoneda et al., 2016). The induction of IL-6 and other pro-inflammatory cytokines was also reported in primary human hepatocytes/non-parenchymal cells co-cultures (Hösel et al., 2009c). Intriguingly, a recent publication described that HBV did not elicit any immune responses in hepatocytes, but a large quantity of the virus activated macrophages (Cheng et al., 2017).

Different hypotheses could explain the discrepancies reported between the different studies concerning the potential induction of cellular responses after *in vitro/ex vivo* exposure to or cellular replication of HBV. First, the quality of the HBV inoculum used to perform the *ex vivo* analyses is probably very different from one study to the other since no standard inocula are available and only very few manuscript reported the characterization of their inocula. However, different HBV producing cells (HepG2.2.15 (Ni et al., 2014), HepAD38 (Ladner et al., 1997b), etc.), different culture conditions (different media as well as different concentration of supplements such as FCS/FBS or DMSO) and different concentration procedures (PEG precipitation, ultrafiltration, Heparin columns, etc) have been reported, which may all lead to different ratios of viral antigens, subviral particles and Dane particles as well as contaminating non-enveloped nucleocapsids, recognized by TLR2 (Cooper et al., 2005) or endotoxins, recognized by TLR4 (Park and Lee, 2013) in the inocula. Similarly, HBsAg can be produced in yeast, purified from cell cultures or from patients' sera. The resulting different HBsAg might have different ratios of surface proteins with post-translational modifications and lipid compositions that may vary in their interactions with host cells/proteins. For instance, phospholipids have been shown to be essential for HBsAg's interaction with CD14 (Vanlandschoot et al., 2002). Of note, those parameters (ratios of the surface proteins, post-translational modifications of these proteins and lipid composition) might be modified during the course of HBV infection, and therefore may modify their impact on the immune responses during the different phases of the disease. Thus, the study of the protein and lipid composition of virions and SVPs during HBV infection from different genotypes could bring to light any varying effect of HBsAg on immune responses.

Second, most of the *in vitro* studies are performed with HBV genotype D. However, clinical studies showed that viral genotypes highly influence the disease outcome (Kramvis and Kew, 2005; Mayerat et al., 1999). For instance, HBV genotype C is associated with lower remission rates and more aggressive outcomes as compared to HBV genotype B (Su et al., 2006). Viruses

from different genotypes might therefore have different interplays with their host. Interestingly, the strongest hepatocyte response was described in experiments using HBV genotype C (Sato et al., 2015) whereas HBV genotype D did not elicit any responses in chimpanzees (Wieland et al., 2004b). Our team is currently producing HBV inocula from genotype A, B, C, D, E, H, and G and their ability to be detected by liver cells will be investigated.

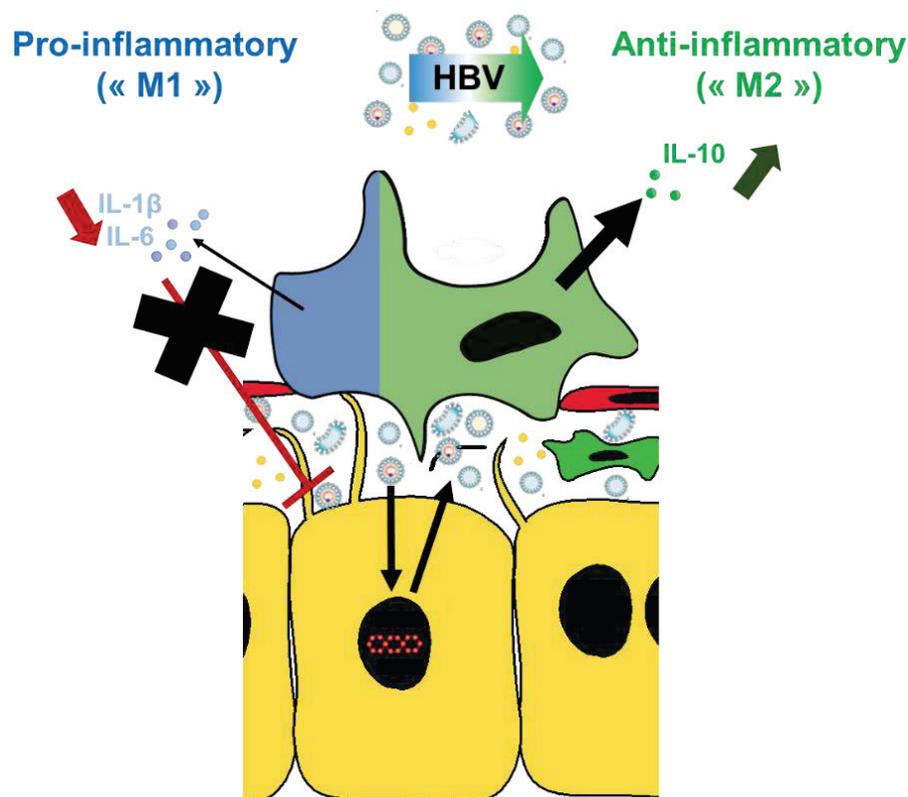
### **Does HBV inhibit the host innate immune system?**

A stealth virus can be a virus that i) does not induce measurable innate responses by passively evading innate immune detection by PRR or ii) a virus that is able to actively inhibit nascent innate responses. The viral particles' composition (enveloped virus), as well as features of the HBV life cycle (capped viral mRNA, retro-transcription within the capsids), which both limit the recognition of potential HBV PAMPs, favor the first hypothesis. However, many studies reported that HBV could not only modulate innate immune responses in hepatocytes but also in immune cells (Faure-Dupuy et al., 2017), and innate immune genes (TLRs, cytokines...) was shown to be down-regulated in chronically infected HBV patients (Lebossé et al., 2017). Indeed, even though HBV is only replicating in hepatocytes (i.e., virus progeny originates only from hepatocytes), the secreted infectious virions, as well as other secreted non-infectious viral components, including enveloped nucleocapsids (either containing viral RNA or no viral nucleic acids), subviral particles (HBsAg spheres and filament; SVPs), and proteins (HBeAg and HBSP) may interact with the liver resident and circulating blood cells and modulate their physiology.

To decipher if HBV was able to modulate innate immune responses, we studied the interaction of the virus with several cells types: the infected cells, i.e. hepatocytes (studies#1 and 2), two types of non-parenchymal cells, the LSEC and the liver M $\Phi$  (studies#2, 3 and 4), and one type of infiltrating cells, the monocytes-differentiated-macrophages (study#4).

First, we assessed if HBV could inhibit the induction of an innate immune response upon TLR/RLR stimulations of hepatocytes. A TLR3 or RIGI/MDA5 (sensors implicated in the recognition of dsRNA) stimulation that normally leads to the activation of interferon (IFN- $\beta$ , and subsequently the interferon stimulated gene, OAS1) and pro-inflammatory (IL-6) responses was indeed shown to be impaired (study#1 (Luangsay et al., 2015b) and study#2). We observed that the type I interferon response (IFN- $\beta$  and OAS1) was inhibited by HBV

before the onset of viral replication (i.e. after 2h of exposure, and 8h for pro-inflammatory response (IL-6)). We therefore first concluded that the viral components implicated in this inhibition should be present in the viral inoculum (Luangsay et al., 2015b) and further identified the structural HBV capsid protein (HBc) as a key regulatory protein (study#2). Indeed, several experiments showed that HBc could bind to the host DNA and this binding was associated with an increase of repressive epigenetic marks on several innate immune genes promoters such as *ifn-β* and *oas1*.



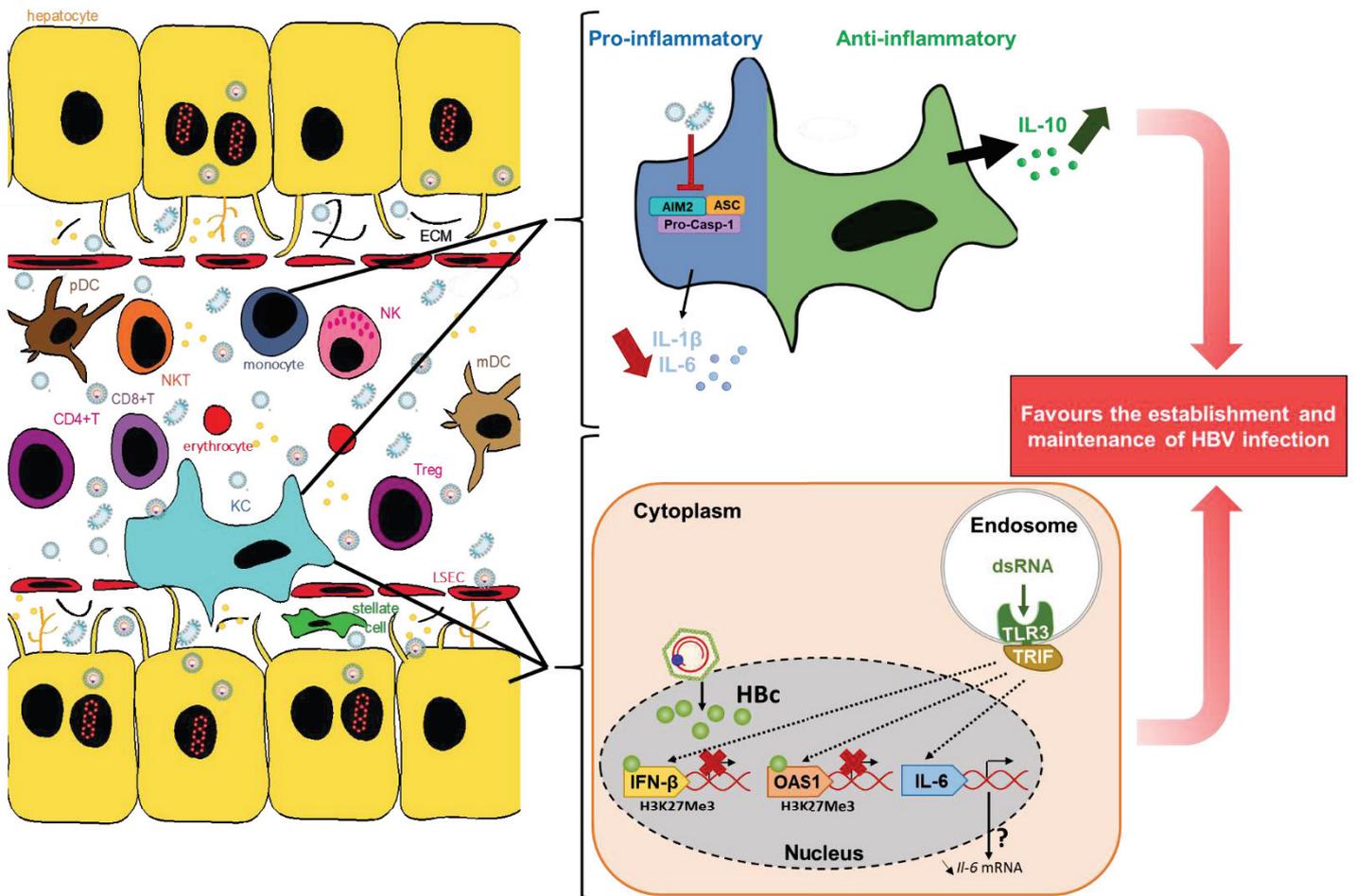
**Figure 29. HBV modifies MΦ phenotype towards the establishment of its infection.** Schematic representation of the results obtained in the fourth study. HBV interferes with the differentiation of monocytes into M1-MDM preventing the anti-viral effect of M1-MDM secretions, while impairing adaptive immune responses by increasing IL-10 secretion by M2-MDM.

Second, we hypothesized that if the inhibitory effect of HBV occurs without viral replication, this might be found in other cell types that are able to internalize the virus (for instance by endocytosis or phagocytosis). Indeed, we observed that HBV is able to impair several immune pathways in LSEC and liver MΦ. The TLR3-mediated induction of IFN-β and OAS1 was also

inhibited in those cells by HBV through an Hbc-dependant mechanism (study#2). In addition, using primary liver M $\Phi$ , we showed that HBV is able to inhibit the secretion of IL-1 $\beta$  induced by different stimulations (study#3 (Zannetti et al., 2016) and study#4). We also demonstrated that HBV interferes with the differentiation of blood monocytes from healthy donor into pro-inflammatory macrophages, thus preventing IL-6 and IL-1 $\beta$  secretions, while promoting an anti-inflammatory phenotype. In addition, we assessed whether the differentiation of purified monocytes from chronically infected patients into pro-inflammatory M $\Phi$  was impaired. However, the high inter-donor variations observed in the ability of monocytes to be differentiated *ex vivo* have not allow any definitive conclusions to be drawn yet. Interestingly, the modification of pro-inflammatory macrophages secretions by HBV was sufficient to abolish the antiviral effect of those M $\Phi$  secretions (**Figure 29**). The anti-inflammatory response was also amplified in presence of HBV during the activation of anti-inflammatory MDM.

Taken together, our *ex vivo* experiments demonstrate that HBV can actively inhibit the induction of interferon and a pro-inflammatory response by liver resident cells (hepatocytes, LSEC and macrophages) and infiltrating M $\Phi$  (MDM). This modification of innate immune responses might favour the establishment as well as the maintenance of the infection especially through the inhibition of antiviral effectors such as IFN (Konerman and Lok, 2016) and IL-1 $\beta$ /IL-6 (Isorce et al., 2016). Moreover, the increase of anti-inflammatory cytokine secretion, IL-10, might have an impact on the implementation of an adaptive immune response, and, thus, favour the development of chronic infection and the progression to HCC (**Figure 30**).

In contrast to our results, several studies have described that HBV infection does not inhibit the innate immune response. Niu *et al.* showed that the IFN $\alpha$  response induced by TLR7 agonisation (GS-9620) of total PBMCs strongly inhibited HBV established infection in hepatocytes, without encountering any inhibition of innate immune responses in HBV infected cells (Niu et al., 2017). In addition, recently, Suslov *et al.* using fresh biopsies from control or HBV-chronically-infected patients did not observed any inhibition of liver responses to TLR stimulations (Suslov et al., 2018). Even though, the model allow the maintenance of the liver 3D organisation as well as the natural proportion of all liver resident cells, the raw culture



**Figure 30. Effect of HBV on hepatocytes, LSEC and liver MΦ innate immune responses.** Schematic representation of the results from the first to the fourth studies, showing that HBV modifications of liver resident innate immune responses favor the establishment and the maintenance of the infection. Top right panels: HBV prevent the secretion of IL-1 $\beta$  by liver and infiltrating pro-inflammatory M $\Phi$ , through the inhibition of the AIM2 inflammasome and impairment of monocytes' differentiation respectively. In addition, HBV increases IL-10 production by M $\Phi$ , which prevents the induction of an adaptive immune response against the virus. Bottom right panels: HBV prevent the induction of TLR3-mediated interferon and pro-inflammatory responses in hepatocytes, LSEC and liver M $\Phi$ , through the binding of HBc on innate immune genes promoters which is associated to the increase of repressive epigenetic marks (H3K27Me3) on the promoters.

conditions used by the authors lead to a dramatic reduction of the hepatocytes viability after 24h of culture and viability and functionality of NPC were not assessed (Suslov et al., 2018).

It is important to note that, in those two studies, the levels of innate immune stimulation used may have been sufficient to breach the modest HBV-mediated inhibition of innate immune signalling (Allweiss et al., 2014; Luetgehetmann et al., 2011). Indeed, the inhibition we have described (study#1 (Luangsay et al., 2015b) and study#2) was robust, but weak, and was not sufficient to prevent the induction of these pathways by a very strong stimulus (e.g. super-infection with hepatitis D virus (HDV); (Alfaiate et al., 2016)). HBV probably actively suppresses the innate immune response induced by viral infection, but cannot counteract the strong response induced by innate immune activators or other potent inducer pathogens. An alternative hypothesis is that the inhibition of M $\Phi$  by HBV could be highly reversible and therefore lost *ex vivo* in a medium without any HBV particles and/or antigens. It would be interesting to test if the addition of HBeAg/HBsAg/HBV virions in the culture medium would recapitulate, in these latter model, the inhibitory phenotype we described.

Interestingly, beside HBV, other viruses have been described to preferentially induce the anti-inflammatory phenotype in M $\Phi$ . For example, the porcine reproductive and respiratory syndrome virus (PRRSV) has been shown to increase CD163 expression in porcine M $\Phi$  (Patton et al., 2009). In addition, human immunodeficient virus (HIV) chronic infection is associated with an increased proportion of CD16<sup>+</sup>CD163<sup>+</sup> monocytes (Fischer-Smith et al., 2008). The secretion of CD163 soluble form has even been described as a marker of active HIV infection (Burdo et al., 2011). Moreover, hepatitis C virus (HCV) infection can inhibit several TLR signalling pathways, among which is TLR4 (Abe et al., 2007). In a macrophage cell line expressing NS5A, a non-structural HCV protein, a decrease of TLR4 mRNA was observed. Upon stimulation with LPS, NS5A expressing cells secreted less IL-6 and had less phosphorylated ERK1/2. This phenotype was associated to the interaction of NS5A with Myd88 (TLR4 intracellular adaptor) that inhibited Myd88 interaction with IRAK and subsequently the induction of the TLR4 signalling pathway. It should be investigated if similar mechanisms occur in the inhibition of pro-inflammatory M $\Phi$  differentiation and TLR/PRR signalling induced by HBV.

### **How does HBV inhibit the host innate immune system?**

Our results have shown that HBV is able to block innate immune responses in parenchymal (i.e. hepatocytes), non-parenchymal (i.e. LSEC and liver M $\Phi$ ) and infiltrating cells (i.e. MDM). HBc was identified as the key mediators of the early inhibition of the type I interferon

response in hepatocytes, liver M $\Phi$  and LSEC (study#2). In M $\Phi$ , we further demonstrated that HBsAg was able to inhibit the AIM-2-mediated induction of IL-1 $\beta$  (study#3 (Zannetti et al., 2016)). However, which viral protein and the mechanism(s) involved in IL-6 inhibition upon TLR3-L agonisation and in M $\Phi$  phenotype modifications are still unknown.

In hepatocytes, in which a complete viral life cycle can be observed, all of the viral proteins could be implicated in immune modulation. Several groups have pointed out the role of HBx, HBV polymerase, and HBc as potential intracellular regulators of the induction of the expression of type-I IFN in hepatocytes (Jiang and Tang, 2010; Wang and Ryu, 2010; Wang et al., 2010; Wei et al., 2010; Wu et al., 2007b; Yu et al., 2010). Moreover secreted HBV proteins, i.e. HBsAg and HBeAg, were also shown to inhibit TLRs-mediated innate immune responses (Wu et al., 2007a; Wu et al., 2009).

In the immune tolerance phase, a large amount of infectious particles, containing HBc, circulates in the blood of patients (between  $10^7$  and  $10^{10}$  HBV-genome-copies/mL) (Seeger et al., 2015), and this concentration may be even higher in the liver microenvironment, thus potentially out-numbering liver cells. It has been suggested that HBV could penetrate into LSEC (Breiner et al., 2001a), Kupffer cells (Zannetti et al., 2016), and pDC (Vincent et al., 2011), which are three important cell types to mount an efficient innate response. However, due to the absence of replication in those cells (study#4), only the viral proteins secreted (HBeAg, HBSP), or embedded in SVPs (HBsAg) and virions (HBsAg, HBcAg, Pol) could influence the innate immune response. Of note, HBx might also be implicated in this modulation as the mRNA of this viral protein has recently been shown to be detected at highly early time point post infection in hepatocytes, suggesting that the X mRNA is encapsidated with the viral genome (Niu et al., 2017). Whether this viral mRNA would be translated in liver non-parenchymal cells and in sufficient amounts to show an effect remains to be determined.

In non-parenchymal cells, the delivery mechanism of virion/HBc inside the cells still remains unsolved. One hypothesis could be that HBV enters M $\Phi$  either by a non-specific or a specific entry (i.e. another viral receptor as NTCP is not expressed by M $\Phi$  (data not shown)) and escapes the phagolysosomes. Indeed, another virus, HIV1, has been shown to impair phagolysosome fusion preventing anti-fungal activities in PBMCs through a gp120 dependent mechanism (Pietrella et al., 1998; Pittis et al., 1993). It should be determined if HBV could impair the acidification of phagosomes to prevent the destruction of virions, allowing viral delivery in the cells. Moreover, the internalisation of HBV virions/proteins might not even be

necessary to induce the antiviral effect observed, as several surface receptors implicated in the down-regulation of pro-inflammatory responses, such as tyrosine kinase TAM receptor (Lemke, 2013), could be, for instance, targeted by circulating HBV particles and/or antigens. Different molecules such as high molecular weight hyaluronate or Latrunculin A (Forrester and Balazs, 1980; de Oliveira and Mantovani, 1988) could be used to inhibit phagocytosis in M $\Phi$  and thereby to assess if the internalisation of HBV virions/proteins is necessary to modulate the M $\Phi$  phenotype.

The molecular mechanisms of the modulation of innate immune responses by HBV still remain largely unclear. We showed that HBc contributes to the inhibition of the IFN response by the modulation of epigenetic marks on innate immune gene promoters both in PHH and non-parenchymal cells (study#2). HBc is capable to bind to dsDNA, including the viral cccDNA, as well as to host genes (Bock et al., 2001; Guo et al., 2012), and it likely does so via the same domain required for RNA binding, which is a protamine like domain found in mammalian DNA binding proteins, located at the C-terminus of the protein (Seeger et al., 2015; Zheng et al., 1992). The biophysical aspect of this interaction has to be defined, but could involve dimeric forms of the protein. It is worth noting that all the promoters of targeted genes contained ISRE motifs. EMSA experiment also showed that HBc could bind to ISRE-containing dsDNA (Fernandez et al., 2003). Such an early inhibition of innate immune responses via a structural protein embarked into virions and readily mobilized into the newly penetrated cells has only been shown in the case of the tegument protein (ORF52) of KHSV. This protein was indeed shown to prevent cGAS DNA sensing just after entry of virions within cells, and in the absence of viral replication and protein neo-synthesis (Wu et al., 2015).

We showed that the inhibition of IL-1 $\beta$  was dependant on HBsAg (study#3). However, the possible role of HBc in the modulation of IL-1 $\beta$  expression and other pro- and anti-inflammatory factors has not been fully investigated yet. Using recombinant viral proteins, we performed several experiments on MDM to determine which HBV component is responsible for the observed effect but the results are not conclusive yet (data not shown). It is likely that several HBV proteins are implicated to different degrees in the modifications of innate immune responses both in hepatocytes and non-parenchymal cells. Therefore, the use of single proteins might not be sufficient to recapitulate the effects observed with a "full" HBV inoculum. In addition, the delivery of the recombinant viral proteins into M $\Phi$  is probably different from the delivery of viral proteins circulating within viral particles. For example, non-

enveloped capsids and Dane particles (that will both deliver the HBc protein) will probably be internalized by M $\Phi$  using different pathways. Moreover, as naked HBc can be recognized by TLR2 (Cooper et al., 2005), it will trigger a pro-inflammatory response, not observed with the enveloped counterpart. Thus, the study of the role of HBc using recombinant protein might not be possible without bioporting the protein inside the cells, as done with hepatocytes in the study#2. In addition, in hepatocytes, TLR pathway signalling was shown to be impaired by HBsAg (Jiang et al., 2014b). More surprisingly, it was also suggested that HBsAg inhibits this pathway by a mechanism involving the inhibition of the c-Jun N-terminal protein Kinase (JNK), which in turn, would prevent IL-12 production (Wang et al., 2013). To specifically study the role of HBsAg without the HBc component, the use of HDV virions, which share the envelope of HBV, should be envisaged (Alfaiate et al., 2015).

Even though one copy of the viral polymerase is encapsidated in each virion, it is unlikely that this protein will have an effect of innate immune modulation in M $\Phi$  given the limited copies that will enter those cells. Thus, the two proteins remaining that might modulate immune responses are the secreted HBeAg and HBSP. HBeAg has already been described to inhibit NLRP3-mediated induction of IL-1 $\beta$ , through an inhibition of ROS production (Yu et al., 2017), but this protein might have other effects on M $\Phi$  modulation. Moreover, HBeAg was shown to impair TLR pathway signalling (Wu et al., 2009b), and to bind to Myd88 interfering with the TLR2 signalling pathway (Lang et al., 2011). HBSP has been hypothesised to promote HBV infection through the down-regulation of immune responses and to reduce liver inflammation during chronic infection (Pol et al., 2015). Recently, HBSP expression in mice liver was shown to decrease with the recruitment of inflammatory monocytes/macrophages, thus limiting liver damage (Duriez et al., 2017b). Authors further described that this phenotype was correlated with an inhibition of C-C motif chemokine ligand 2 (CCL2) expression in hepatocytes. However, HBSP might also be implicated in other methods of regulating macrophages' phenotypes that will need future investigations.

In summary, even though several studies have shown that HBV can modify the immune response of macrophages, further investigations are required to precisely dissect the involved mechanisms. For instance, a full evaluation of the synthesis and secretion of the different cytokines/chemokines upon HBV exposure should be performed since modification of chemokines' secretion might interfere with the recruitment of other immune effectors and,

hence, HBV elimination. Moreover, it should also be investigated if HBV modulates other functions attributed to macrophages such as phagocytosis or antigen presentation in order to prevent for instance, viral recognition and/or local reactivation of lymphocytes.

### **Does HBV affect liver cells' metabolism?**

Cell metabolism was recently demonstrated to be a key player in the implementation of an efficient immune response (Sinclair et al., 2013) and HBV was shown to hijack and/or promote enzymes involved in lipids or amino acids metabolism in hepatocytes or myeloid cells (Esser et al., 2018; Pallett et al., 2015a; Schurich et al., 2016). We observed that the level of the *HIF-1 $\alpha$*  transcript, a marker of succinate accumulation and broken Krebs cycle in pro-inflammatory M $\Phi$  (O'Neill, 2015), is lower in liver M $\Phi$  and M1-MDM exposed to HBV (study#4). Interestingly, HIV-1 was also shown to modulate M $\Phi$  metabolism through a Vpr-HIF-1 $\alpha$  axis to induce the expression of hexokinase (HK), glucose-6-phosphate dehydrogenase (G6PD) and pyruvate kinase muscle type 2 (PKM2) that facilitates viral replication and biogenesis, and long-term survival of macrophages (Barrero et al., 2013). It would be interesting to investigate if HBV can further modulate metabolic pathways in M $\Phi$  and what are the consequences for the cell function and for HBV infection in hepatocytes.

One tempting hypothesis is that the modulation of macrophage metabolism may promote the secretion of factors necessary to compensate the increased need of hepatocytes in amino acids and lipids to cope with HBV replication. Indeed, it has been shown that the large amount of viruses produced by hepatocytes during acute and chronic infection requires amounts of deoxyribonucleotide triphosphates (dNTPs) that are not available in resting hepatocytes (Cohen et al., 2010). On the one hand, a study has described that HBV is able to increase level of dNTPs through a viral-dependent transcription activation of R2, the key component of ribonucleotide reductase, which is critical for the HBV life-cycle (Cohen et al., 2010). On the other hand, a study on a large cohort of patients' highlighted that HBV chronic infection is associated with an increase in the frequency of circulating gMDSC (Pallett et al., 2015a). During immune-tolerant phases, gMDSCs secrete more arginase-I, leading to peripheral and hepatic deprivation in L-arginine, impairing T cell function (Pallett et al., 2015a). Thus, it appears that even though HBV is in increasing need of nucleotides and amino acids to cope with its

replication rates, not all HBV-induced modifications of metabolism are in favor of promoting the secretion/production of the needed metabolites.

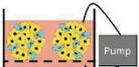
### **Do liver macrophages play a role in the establishment and/or maintenance of HBV infection?**

Liver M $\Phi$  were shown to play a crucial role in the establishment of HBV infection as their depletion resulted in a loss of chronicity in a mice model (Xu et al., 2014). The authors described that IL-10 secretion by M $\Phi$  was the key player to prevent an adaptive immune response against the virus and, thus, the maintenance of the infection. However, HBV might also translocate through liver macrophages to be delivered to hepatocytes as suggested by the group of Ulrike Protzer (personal communication) and shown for LSEC in the case of DHBV (Breiner et al., 2001). If this is so, the delivery of HBV to hepatocytes might be compromised when KC are depleted. To assess the role of liver M $\Phi$  in the establishment and persistence of the infection, it would be interesting to add or deplete those cells (by a clodronate treatment, (Weisser et al., 2012)) before, during or after HBV infection, in different *ex vivo* or *in vivo* models that will be discussed below and presented in [Table VI](#). Of note, the absence of liver M $\Phi$  might also explain the very inefficient rate of HBV infection *in vitro* compared to *in vivo*. Indeed, one viral particle is sufficient to chronically infect chimpanzees (Shikata et al., 1977), whereas at least 100 particles per cell in the presence of PEG are needed to infect hepatocytes cultured *in vitro* in 2D. *In vitro* infection of hepatocytes should therefore be performed in the presence of liver M $\Phi$  to confirm their role in the establishment and/or maintenance of HBV infection. Several *in vitro* models of hepatocytes have been used over the years to study the HBV life cycle such as HuH7, HepG2, dHepaRG and PHH. However, each model has its limitations for the study of the HBV interactions with the immune cells and more precisely with macrophages. For instance, transformed cells (HuH7.5, HepG2) have an impaired innate pathways (Luangsay et al., 2015a). Differentiated HepaRG and PHH do have functional immune pathways, but need to be cultured in the presence of DMSO to prevent their dedifferentiation. However, the use of DMSO triggers M $\Phi$  activation and thereby induces the secretions of cytokines such as IL-6 that were described to have an anti-viral effect (Isorce et al., 2016) (data not shown).

Co-culture of M $\Phi$  and hepatocytes with inserts spatially separating the cells and allowing the use of two different types of media (i.e. containing DMSO for hepatocytes and without for M $\Phi$ ) (**Table VI**) is useful to determine the effect of hepatocyte secretions on macrophages and vice versa. However, the absence of contact between the two cell types prevents any cell-to-cell virus propagation/modification. Interestingly, the IKC cell line presented in the fifth study can be cultured in the presence of DMSO (up to 2%). During my thesis, we co-cultured PHH and liver M $\Phi$  or dHepaRG and IKC in monolayers (**Table VI**) using different cell ratios in order to assess the role of macrophages in HBV infection establishment. However, control conditions (hepatocytes only vs hepatocytes + M $\Phi$ ) were difficult to implement because of the necessity to maintain confluency in the cultures (to keep hepatocytes polarization and avoid dedifferentiation) as well as because of the different durations of the cell sorting procedures (for primary cells).

Alternatively, co-culture of human M $\Phi$  and hepatocytes could be performed in spheroids; such a system has been reported to resemble the *in vivo* human liver (Bell et al., 2016) (**Table VI**). Preliminary results obtained in our team showed that HBV infection can be detected in human hepatocyte spheroids (PHH or dHepaRG cells) cultured without DMSO and the formation of spheroids containing hepatocytes and M $\Phi$  is currently being set up in our team. A “3D microfluidic liver culture” system that allows very efficient HBV infection of 3D spheroids of PHH (in the absence of DMSO and PEG) has also been very recently described (Ortega-Prieto et al., 2018) (**Table VI**). Interestingly, slight increases of HBeAg secretion and pgRNA levels were observed when liver M $\Phi$  were co-cultured with PHH in this system (Ortega-Prieto et al., 2018). Of note, M $\Phi$  that can contaminate PHH cultures were not depleted so it will be necessary to assess in the future if this might have an impact on the establishment and/or persistence of the infection in this system. Moreover, the role of M $\Phi$  might be underestimated when using such systems because of the absence of circulating cells that could be recruited by M $\Phi$  secretions. Culture of liver slices has also been reported in very elegant studies from the team of Pr Stanislas Pol. Such a system presents the advantage of keeping the 3D organisation of the liver as well as a natural proportion of all the liver resident cells (**Table VI**). Compared to cultured biopsies mentioned previously, which cannot be kept in culture for more than 24h (Suslov et al., 2018), liver slices were still viable after ten days (Lagaye et al., 2012) and could be *ex vivo* infected with HCV (Lagaye et al., 2012, 2016). The same culture conditions could be used to test the *ex vivo* susceptibility of the liver slices to

HBV infection. If confirmed, liver macrophages could be depleted to assess their role in HBV the establishment and maintenance of infection. One major drawback to all these *ex vivo* models is the absence of circulating cells that are a major component of the immune responses. Finding a good *in vivo* surrogate gold standard model for HBV infection is still an outstanding challenge. The use of small animals has been compromised by the fact that the mouse and human NTCP sequences are too different to enable infection of mouse hepatocytes by HBV (Yan et al., 2013). Different strategies have been used to launch HBV replication in mice. For instance, mouse hepatocytes can be depleted in the  $Fah^{-/-}$   $Rag2^{-/-}$   $Il2rg^{-/-}$  (FRG) mouse and human hepatocytes engrafted to allow the formation of a humanised liver (Azuma et al., 2007) (**Table VI**). These mice support HBV replication and represent a potent model for the evaluation of HBV targeting drugs (Diab et al., 2017), but they have a compromised immune system that strongly limits studies on the interplay between HBV and immune cells. However, the addition of purified human NPC and/or liver M $\Phi$  to hepatocytes during the formation of the humanised liver should be performed to assess the role of those cells in HBV establishment. On the contrary and to address the same question, liver M $\Phi$  could be depleted (i) in HBV-infected mice in which both the immune system of the mouse and the liver are humanised (Dusséaux et al., 2017) (HIS-HuHEP mice) (**Table VI**) or (ii) in AAV-HBV transduced immune-competent mice (Dion et al., 2013; Lucifora et al., 2017) or macaques (Burwitz et al., 2017) in which the viral entry step is bypassed through the use of the AAV vector (**Table VI**). In summary, in the past decades a lot of new models have been implemented for the study of HBV infection either *ex vivo* or *in vivo*. Those 3D models and/or animal models should lead to a leap forward in the understanding of HBV interactions with the host immune responses, and liver macrophages in particular, as well as for pre-clinical testing of potential modulators of the immune responses.

Model	Schematic representation	Question that can be addressed regarding MΦ	Intervention on liver MΦ	Presence of circulating cells	Possibility of <i>ex vivo</i> HBV infection	Advantages	Drawbacks	Reference
Co-culture with inserts		<ul style="list-style-type: none"> <li>Modulation of MΦ by HBV</li> <li>Modulation of HBV by MΦ</li> </ul>	Added	No	Yes	Co-cultures of PHH and MΦ without a DMSO-induced activation of MΦ	No contact between cells	Faure-Dupuy <i>et al.</i> , in preparation
Contact co-culture		<ul style="list-style-type: none"> <li>Modulation of MΦ by HBV</li> <li>Modulation of HBV by MΦ</li> </ul>	Added	No	Yes	Contact between cells	Need of MΦ cultured with DMSO or hepatocytes cultured without DMSO	Hösel <i>et al.</i> , 2009
Human hepatocytes spheroids		Effect of liver MΦ on HBV establishment and maintenance	Added or Depleted	No	Yes	3D culture without DMSO	<ul style="list-style-type: none"> <li>Not the natural proportion of liver cells</li> <li>No infiltrating cells</li> </ul>	Bell <i>et al.</i> , 2016
3D microfluidic liver culture		Effect of liver MΦ on HBV establishment and maintenance	Added or Depleted	No	Yes	3D culture without DMSO	<ul style="list-style-type: none"> <li>Not the natural proportion of liver cells</li> <li>No infiltrating cells</li> </ul>	Ortega-Prieto <i>et al.</i> , 2018
Liver biopsies		Effect of liver MΦ on HBV maintenance	Depleted	No	No* *biopsies are already infected	3D architecture and natural proportion of liver cells	<ul style="list-style-type: none"> <li>Poor viability after 24h</li> <li>No infiltrating cells</li> </ul>	Suslov <i>et al.</i> , 2018
Liver slices		Effect of liver MΦ on HBV establishment and maintenance	Depleted	No	To be determined	3D architecture and natural proportion of liver cells	No infiltrating cells	Lagaye <i>et al.</i> , 2012
FRG mouse		Effect of liver and infiltrating MΦ on HBV establishment and maintenance	Added or Depleted	Yes	Yes	3D architecture and recruitment of infiltrating cells	Compromised immune system	Azuma <i>et al.</i> , 2007
AAV-HBV infected C57/B6 mice		Effect of liver and infiltrating MΦ on HBV establishment and maintenance	Depleted	Yes	Yes	3D architecture, natural proportion of liver cells and recruitment of infiltrating cells	Mouse immune system is different from the human one	Dion <i>et al.</i> , 2013
HisHuHEP mouse		Effect of liver and infiltrating MΦ on HBV establishment and maintenance	Depleted	Yes	Yes	3D architecture, natural proportion of liver cells and recruitment of infiltrating cells	<ul style="list-style-type: none"> <li>High cost</li> <li>Difficulties of handling</li> </ul>	Dusséaux <i>et al.</i> , 2017
AAV-hNTCP in macaques		Effect of liver and infiltrating MΦ on HBV establishment and maintenance	Depleted	Yes	Yes	3D architecture, natural proportion of liver cells and recruitment of infiltrating cells	<ul style="list-style-type: none"> <li>High cost</li> <li>Highly restricted access</li> </ul>	Burwitz <i>et al.</i> , 2017

**Table VI. Possible HBV *ex vivo* and *in vivo* models that could be used to assess the role of liver macrophages in the establishment and/or maintenance of HBV infection.**

### How activation of the innate immune system may lead to the cure of HBV infections?

Toll-like receptors are important molecular mediators linking innate and adaptive immunity, and their stimulation by cognate agonists induced an antiviral response in animal models of HBV infection. Indeed, treatments of chimpanzees or woodchucks with TLR7 or TLR9 agonists led to reduction of HBV replication markers (Lanford *et al.*, 2013; Lawitz *et al.*, 2015; Meng *et al.*, 2016; Menne *et al.*, 2015). The strongest antiviral phenotype was obtained with GS-9620, a TLR-7 agonist, in the woodchuck model, with a strong effect of treatment on cccDNA levels and a long-lasting efficacy associated with an anti-HBsAg seroconversion. These antiviral effects probably resulted from the activation of non-parenchymal cells and subsequent production of anti-HBV cytokines/IFNs, since hepatocytes do not express TLR7 or TLR9 and cannot therefore be directly activated by these agonists (Luangsay *et al.*, 2015a).

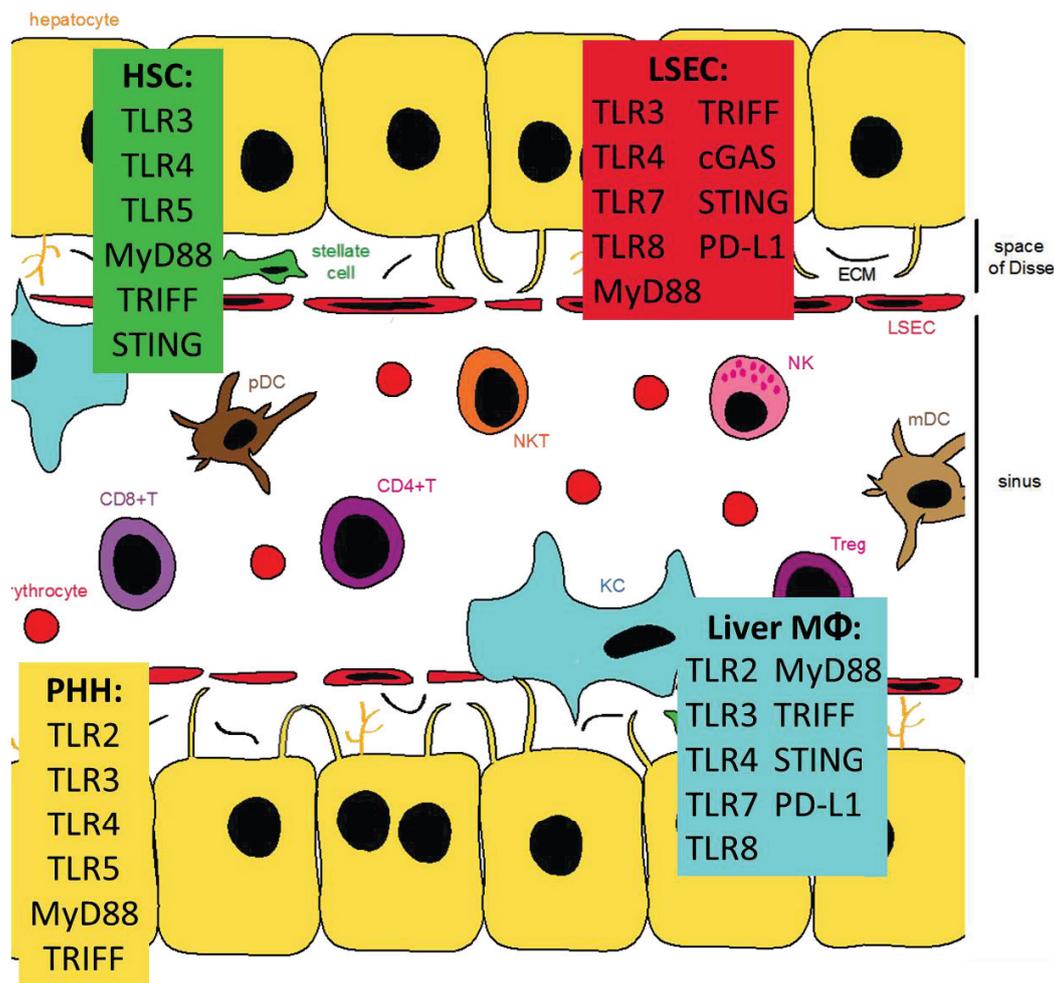
Thus, the knowledge of which resident cells are able to respond to a TLR agonisation and to which extent, is necessary to develop new agonists targeting specific liver immune responses, in hepatocytes and/or non-parenchymal cells. We therefore analysed the expression and functionality of the major immune receptors (study#5). Using primary human HSC, LSEC, hepatocytes and liver M $\Phi$ , we observed that at least one liver resident cells is able to mount a pro-inflammatory response in response to a given TLR agonisation, with the exception being TLR9 which is not expressed in the liver resident cells tested (**Figure 31**). This study highlights the necessity to take into account NPC in the design of new agonists targeting the activation of the immune systems.

Different anti-viral effects mediated by TLR agonisation have been described using mouse models. The team of Pr. Schlaak showed that supernatants from mouse NPCs (liver M $\Phi$  and LSEC) stimulated with different TLR agonists (TLR3 or TLR4 agonists for liver M $\Phi$  and TLR3 agonists for LSEC) strongly inhibited the maintenance of HBV infection in hepatocytes (Wu et al., 2007). However, pro-inflammatory cytokines' secretions were reduced in PBMCs from patients or in murine NPC purified from mice exposed to viral components (virion, HBsAg, or HBeAg) compared to controls (Jiang et al., 2014b; Wu et al., 2009b). Huang and colleagues have described a specific population of inflammatory monocytes that can be activated and aggregated upon TLR9 agonisation to induce cytotoxic T lymphocytes expansion (Huang et al., 2013a). The resulting formation of iMATES (intrahepatic myeloid-cell aggregates for T cell population expansion) enables the control of chronic liver infections after vaccination in chronically infected mice (Huang et al., 2013a). Of note, iMATES formation in humans has not been reported yet.

Our team showed that TLR2 and TLR3 agonisation of human hepatocytes efficiently inhibits HBV infection (Lucifora et al., in revision). As macrophages are also able to respond to TLR2 agonisation, it is tempting to hypothesise that they might amplify this antiviral effect. But a very recent study showed that TLR2 agonisation of intrahepatic myeloid cells resulted in an amplification of KC and an increased IL-10 secretion inducing tolerance in naive CD8 T cell in C57Bl6 mice (Liu et al., 2018).

Thus, even though TLR agonists might offer new therapeutic options for the treatment of HBV infection, the impairment of innate immune responses induced by HBV in NPC and circulating cells might limit TLR agonist efficacy. Local and specific delivery of TLR agonists will probably be required to specifically and strongly stimulate the liver cells without inducing systemic

cytokine storms. In this respect, our team is currently evaluating the use of a functionalized-particulate TLR2 ligand.



**Figure 31. Basal protein expression of tested immune sensors and/or sensors triggered by agonisation in the different liver resident cells.**

We showed that HBV can modulate hepatocytes and liver MΦ to favour the establishment of infection. On the one hand, HBV escapes the antiviral mechanisms launched in hepatocytes, LSEC, pro-inflammatory liver resident and infiltrating MΦ by interfering with their activation and/or differentiation. On the other hand, HBV promotes the activation of liver infiltrating anti-inflammatory MΦ to prevent the triggering of the adaptive immunity. Moreover, we identified IL-1 $\beta$  as the most efficient cytokine to inhibit virus establishment and maintenance in hepatocytes (Isorce et al., 2016). Alternatively to recombinant IL-1 $\beta$  (that cannot be used systemically without risking severe side effects due to the triggering of cytokine storms), specific inflammasome inducer delivered in the liver and/or specifically to liver macrophages,

may induce a boost of IL-1 $\beta$  secretion and ultimately an antiviral phenotype (Demento et al., 2009). Moreover, the use of GM-CSF, that promotes pro-inflammatory macrophages and induced tumour regression after delivery in the tumour (Kim et al., 2006; Mastrangelo et al., 1999), could be tested in the context of chronic HBV infection. Similarly, the efficiencies of inhibitors of glutaminolysis that modify the succinate/ $\alpha$ -ketoglutarate balance in macrophages and led to a switch from an anti-inflammatory to a pro-inflammatory phenotype (Liu et al., 2017) could also be evaluated.

**In conclusion, the understanding of the host-virus interactions and the mechanisms that underlie the regulation of innate responses of (parenchymal and non-parenchymal) liver cells is an essential step for the development of future treatments aiming at reactivating a functional immune response and curing chronic HBV infections.**

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## Acknowledgements

First, I would like to thanks Dr. Hosmalin, Dr. Soussan and Dr. Woltman for agreeing to judge my thesis work.

Je souhaite remercier le **Pr. Zoulim** pour m'avoir accueillie dans son laboratoire, pour ses commentaires constructifs en réunion d'équipe et pour avoir accepté de présider mon jury de thèse.

**David**, merci pour tout ce que tu as fait pour moi, depuis le fait d'avoir choisi en stage une toulousaine qui n'avait jamais croisé un virus de sa vie, jusqu'à tes conseils pour choisir et trouver un post doc. Tu m'as appris beaucoup de chose au cours de ces quatre années et demie. Ton inépuisable inspiration pour toujours trouver de nouvelles idées m'aura donné du fil à retordre quand il fallait canaliser ce trop-plein de pistes mais cela m'aura surtout appris à penser en dehors des cadres pré-établis. Je finis cette thèse grandie par ton enseignement et je ne t'en remercierai jamais assez.

**Julie...** Je pense qu'en cent pages de remerciements je n'aurais toujours pas assez de mots pour te dire à quel point tu as été mon mentor durant ma thèse. Sur le plan professionnel, tu m'as appris comment être une scientifique, comment devenir une chercheuse et comment me faire ma place dans ce monde qu'est la recherche académique. Sur le plan personnel, tu as été mon modèle, mon amie, ma maman, ma sœur, l'épaule sur laquelle pleurer, la confidente, la personne avec qui partager les ragots, se plaindre, critiquer, mais surtout apprendre, rigoler, positiver et aimer. Je sais que les liens qui nous unissent ne sont pas près de s'effiloche. Aujourd'hui, je pense enfin pouvoir te regarder en tant qu'égale et te dire à quel point ce fut un honneur d'être ta première thésarde. Je pourrais encore m'étendre pendant des heures mais j'ai cru comprendre qu'il fallait essayer d'être concise. DU COUP, je vais conclure en te disant (chose promise, chose due), merci pour le tabagisme passif.

**Laura**, sans toi, tout cela n'aurait pas été possible. J'ai eu l'immense chance et honneur de pouvoir travailler avec toi pendant ces trois dernières années. Je sais très bien où j'en serais si tu n'avais pas été là et le 20 avril, quoique les gens pensent, c'est aussi toi qui passe ta thèse. Merci d'avoir toujours été là pour moi autant sur le plan professionnel, quand il me fallait des mains en plus pour les manip, que sur le plan personnel, pour toutes les fois où tu t'es dressée

entre moi et le reste du monde. Tu as été et tu resteras bien plus que la meilleure AI du monde. Ma Lolo, je ne sais pas comment te faire comprendre à quel point je te remercie pour tout ce que tu as fait pour moi.

**Ludo**, le caillot pour les intimes (désolée il fallait bien que ça reste gravé quelque part), merci pour ton aide pour les manips, pour nos grandes discussions sur l'immunité, pour les heures d'extraction de cellules, pour les coups de gueules, pour ta chevelure incroyable, et pour ton amitié. On n'a pas toujours été sur la même longueur d'onde tous les deux, mais on a fait une belle équipe. Ca va presque me manquer de plus t'entendre râler tous les jours. J'ai dit presque ! PS : pour tes derniers mois de thèse, on ne met pas le feu au labo !

**Marion**, je sais que je n'ai pas toujours été facile et j'en suis désolée. Il n'empêche que ces derniers mois tu as su répondre présente quand j'en avais besoin, et ça compte pour moi bien plus que tu ne peux t'en rendre compte. Continue à apprendre petit Padawan. Comme je te l'ai déjà dit ce qui compte ce n'est pas d'où on part mais comment on progresse.

#### **Merci à ceux qui ont marqués ma vie au labo :**

**Alexia**, merci d'avoir fait de moi ta deuxième ou troisième maman de stage. Ça a été un honneur ! **Anaëlle**, merci pour ta bonne humeur même face aux épreuves. **Audrey**, à toi je te dis pas merci, tu as déjà vu la raie ça suffit comme ça. **Aurore**, ma sestra, merci d'avoir pensé à moi en toutes circonstances, pour les soirées sushis et le pinot gris, pour ta bonne humeur, pour ton amitié, et pour tout ce qui est à venir. **Barbara**, merci pour ton aide avec les ChIPs et les stats, tes conseils, tes tiramisus (même si c'est Max qui les fait), pour tes encouragements, et pour la confiance que tu as placée en moi. **Clothilde**, merci pour les brunchs, les gâteaux, le soudolement d'internes, et les vacances au Puy du Fou (mais sans les oiseaux). **Damien**, merci pour la forêt et le blanchiment. **Dulce**, un jour tu m'as dit « Petite, tu verras ce que ça fait quand tu finiras ta thèse ». Aujourd'hui, je fais le point sur ma thèse et je peux dire que tu auras marqué ma thèse et ma vie de ta présence. Merci Dulce d'avoir été la maman, le modèle, la compagne de vacances et l'amie. Le 20 avril, j'espère faire aussi bien que toi ! **Emma**, mon amour, merci d'avoir été la lumière de mes jours et de mes nuits et d'avoir enrichi mon univers de ta présence. Je me languis de ne plus avoir l'occasion d'admirer ton divin corps tous les jours à partir de maintenant. Mais sache que même si la distance nous sépare, nos cœurs

resteront à jamais unis sous la même lune. **Fleur**, merci pour tellement de chose que je ne sais pas où commencer. Bien sûr pour le support sans faille (moral et alimentaire), pour les bons moments, pour les fous rires mais surtout pour ton amitié. Merci au **JAD (Adrien, Anna, Brieux, Héloïse, Manon, Rayan, Thomas)** pour votre aide, vos conseils, les rigolades et les bons moments. **Janet**, thanks for many things from correcting my thesis English, to long discussion about everything and nothing; from the Christmas passion, to the secret of eternal youth (i.e. a glass of water between two glasses of alcohol). **Jennifer**, merci pour l'écoute, pour la voiture, pour les rigolades, les coups de gueules et surtout le P2. **Judith**, merci d'avoir fait de moi une des rares personnes qui pourront dire qu'ils ont passé une soirée avec toi. Tu m'as accordé ta confiance. Tu m'as donné ton amitié. Tu m'as aidée. Tu m'as appris. Tu m'as consolée. Tu m'as écoutée. On a rigolé. On a comméré. On a partagé. Et pour tout ça et plus encore, merci du fond du cœur. **Hélène**, elle s'appelle Hélène, et je la remercie pour avoir été l'oreille discrète qui a su écouter sans juger mais surtout pour les câlins !!! **Laetis**, merci pour avoir toujours été là, à m'écouter, à rigoler avec moi, à m'aimer malgré mes défauts, et à être la confidente des plus noirs secrets. Merci d'avoir été mon co-lutin pendant trois ans, et pour tous ces moments où il a fallu éponger les larmes. Merci ma Laetis ! **Léa**, je ne te dis pas merci pour le poisson mais merci pour tous les bons moments passés dans le bureau, pour les rires et les pleurs, pour le soutien, le fromage, le naturel et l'amitié. **Loïc**, merci pour ces deux petites protubérances qui ont fait de ma vie un fou rire à chaque fois que je te croise. Parfois un geste vaut mieux que mille mots alors à bon entendeur... **Marie-Agnès**, merci d'avoir répondu à toutes mes questions du jour et autant dire qu'en quatre ans, il y en a eu quelques-unes ! Merci également pour ta patience et ton aide sans faille au quotidien. **Marie-Laure**, merci d'avoir toujours été toi, sans filtre. Tu m'as beaucoup appris et je dois dire, tirée vers le haut. J'ai parfois eu mal mais j'ai surtout beaucoup ri. Ne change rien maman MLP ! **Mathieu**, merci pour ta bonne humeur contagieuse, pour les câlins et pour avoir fait une place dans ta vie à des scientifiques un peu fous. **Maud**, merci pour avoir partagé tes connaissances, pour ta bonne humeur, et pour ton écoute même quand tu es en train de courir. **Mina**, merci pour ton enthousiasme et ta réactivité face à un projet difficile à réaliser en une si courte période de temps. **Moms**, merci beaucoup pour ton sang car je sais que tu as très peur des aiguilles ! **Morgane**, merci pour ton naturel et le pimpage de pirogue qui après quatre ans et toujours dans mon bureau. **Nathalie**, merci Nat pour la bonne humeur, les rigolades et la transmission de savoir. **Océane**, merci pour avoir été là ces quatre dernières années et pour les moments

où tu dances avec deux parties indépendantes dans ton corps. **Petite Nat**, merci pour ta bonne humeur, tes petites attentions et tes sourires. **Romain B**, merci pour ce merveilleux weekend à Amsterdam qui restera à tout jamais dans mon cœur, pour ces quelques mois à avoir pu partager ton bureau et pour le reste de notre vie qui s'annonce riche en couleurs. Enfin surtout riche pour moi dès que j'aurai trouvé le trésor des Barnault ! **Romain P**, merci pour avoir toujours pris le temps d'écouter et de conseiller, ainsi que pour ta façon d'envisager la science qui est un atout précieux. **Serena**, merci pour m'avoir écoutée, pour t'être lancée sur des projets impossibles avec nous, pour ton humour indéfectible et pour ton tempérament à l'italienne qui pousse toujours à en faire plus.

### **Merci à ceux qui m'ont construite :**

**Camille**, même si on ne se voit pas souvent, je sais que tu es toujours à un coup de fil près et ça, ça veut dire beaucoup. **Cey, Flo, Lily, et Marie**, merci d'avoir été là depuis le début. Sans vous je ne serais pas là personne qui écrit ces mots aujourd'hui. J'ai grandi avec vous, je vis avec vous et je vieillirai avec vous. Vous m'avez appris que l'amitié n'a pas de limite et que quand on aime, on fait des choses qu'on n'aurait pas crues possible. Cey, merci pour les fous rires. Flo, merci pour le naturel. Lily, merci pour la présence indéfectible. Marie, merci pour la joie de vivre. Je vous aime ! **Dédé**, merci pour l'étendue de ton amitié qui arrive toujours à me surprendre. Mais pas merci pour les « minouchats » ! **Delphine**, merci pour les apéros qui ne manquent jamais d'être hauts en couleurs (par exemple, jade c'est pas mal comme couleur). **Eva**, merci pour ta présence qui m'a permis d'obtenir en ce jour le prix Nobel... Ah non, attends ça c'est le discours que tu m'as écrit en prévision. Merci pour les soirées b\*\*\*\*age et pour ton humour toujours plus improbable. **Floriane**, merci de m'avoir supporté en coloc, la tablette de chocolat toujours dans le frigo, et pour la tequila. **J.B.**, merci pour les grandes discussions, pour les soirées, pour avoir grandi avec moi, et l'amitié sans failles. **Jérémy**, merci pour avoir été mon soutien pendant ma première année à Lyon et pour toutes nos soirées films. **Marie**, merci pour les photos, il faut bien que quelqu'un les prenne. Merci pour la crème fraîche et pour « a thousand miles ». **Pauline**, merci pour ton improbabilité (je maintiens, c'est toi la plus bizarre de nous deux !), pour l'amitié qui ne se tarit pas et le caca bien sûr. **Poppy**, merci pour avoir été la main qui dessine les conneries que j'ai en tête et le support de celle qui a besoin d'aide. **Raph**, merci pour ta folie qui amène l'inspiration. Merci pour l'écoute qui amène la

compréhension. Merci pour l'amitié qui amène une belle histoire. **Tiffany**, merci pour les heures au téléphone, pour ta personnalité qui va droit au but, et pour l'amitié qui ne fait que commencer.

**Merci à ceux qui ont toujours était là :**

**Maman**, merci pour m'avoir appris que je pouvais faire et pouvais être qui je voulais dans la vie. Tu m'as appris à être forte. Tu m'as appris à aimer. Tu m'as appris que même quand tout semble sombre, il faut se relever et chercher la lumière. Maman, je t'aime. **Papa**, merci de m'avoir appris qu'il n'y a pas de limite à l'amour. Merci de m'avoir enseigné à ne pas me laisser marcher sur les pieds. Et surtout merci de m'avoir donné l'amour de la musique, sans quoi je n'aurais pas tenu ces dernières années. **Mamie et Papi**, merci pour votre soutien et votre amour. **Mona**, merci d'avoir été la sœur que je n'ai jamais eue. Merci de m'avoir toujours acceptée et aimée telle que je suis. Bien que je n'aie pas ta prose, je voulais te dire que tu m'as appris à penser hors de ma zone de confort et que de là est née ma tolérance. **Neal**, thanks for the never ending interesting talks, and for making me the daughter you did not chose to have. **Yoyo**, merci pour les discussions scientifiques, pour les moments improbables qui créent les belles histoires d'une vie, pour m'avoir appris à ouvrir une bière avec un briquet et pour ta confiance dans les moments de doutes.

Pour se lancer, c'est important de savoir que ceux qui vous aiment seront toujours là pour vous rattraper. Merci ma famille de fous. Je vous aime.

**Et le meilleur pour la fin :**

**Maele**, pour citer les derniers remerciements que j'ai écrit pour toi : « il y a certaines rencontres qui vous change une vie. ». Trois ans et quelques plus tard, ces mots sont toujours vrais. Merci pendant ces quatre années d'avoir été mon amie, mon putching ball, ma sœur, mon coach, ma critique, ma confidente, mon guide, et ma cuisinière. Les gens ne comprendront jamais l'étendue de ce que tu as fait pour moi. Tu es la personne dans l'ombre qui est toujours là, que ça soit dans les bons comme dans les mauvais moments. Tu es la personne qui me pousse hors de mes retranchements pour me montrer que je suis toujours capable de plus. Tu es la personne qui crée la beauté dans les moments de doutes. Aujourd'hui

c'est le premier jour du reste de ma vie, et même si nos chemins vont diverger, je sais que l'amitié qui nous unit ne se désintègrera jamais. Je t'aime Mama. Et promis à partir de maintenant plus de coquille !

# Appendices

## 1. Published comments and reviews

### 1.1. gMDSCs act as metabolic regulators of hepatitis B virus immunopathology (article in French)

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Comment published in 2016 in *Medecine Science* from the paper of Pallett et al., *Nature Medicine*, 2015.

**NOUVELLE**

### Régulation de l'immunopathogenèse du virus de l'hépatite B par des cellules myéloïdes suppressives

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> Le virus de l'hépatite B (VHB) est communément considéré comme un virus non cytopathique, c'est-à-dire n'altérant pas les fonctions métaboliques, biochimiques ou morphologiques des cellules

infectées. Les dommages au foie qui sont observés à la suite de l'infection par le VHB sont en fait probablement la conséquence de la réponse immunitaire adaptative de l'hôte. La sévérité

de la maladie hépatique varie selon les individus. Dans plus de 90 % des cas, les adultes infectés par le VHB développent une hépatite aiguë et sont capables de contrôler et d'éliminer le virus sans effets

Phases de l'hépatite B chronique	Réplication du VHB	Dommages au foie (transaminases élevées)	Traitement indiqué
Immunotolérante (IT)	Forte	Non	Non
Immunoactive (eAg+ active)	Forte	Oui	Oui
Inactive (IN)	Faible	Non	Non
Réactivation (eAg- active)	Faible/moyenne	Oui	Oui

Tableau 1. Caractéristiques des phases d'une infection chronique par le virus de l'hépatite B.

indésirables à long terme. En revanche, certains individus ne parviennent pas à éliminer le virus mais restent infectés de façon chronique [11] (→). Ils présentent alors un risque accru de développer des maladies du foie sévères telles que

(→) Voir la Nouvelle de J. Lucifora et al., m/s n° 8-9, août-septembre 2014, page 724

la cirrhose ou le carcinome hépatocellulaire [1]. Selon l'Organisation mondiale de la santé (OMS), 240 millions d'individus seraient porteurs chroniques du VHB à travers le monde. Ces patients souffrent d'une hépatite B chronique (HBC) généralement divisée en 4 phases dont la durée et l'ordre peuvent varier (Tableau 1) : la phase d'immunotolérance (IT), la phase immuno-active (eAg+ [hepatitis B e-antigen] active), la phase inactive (IN) et la phase de réactivation virale (eAg- active). Lors de la phase immuno-active, une réponse lymphocytaire T inadéquate peut conduire à d'importants dommages hépatiques dus à l'infiltration de lymphocytes T non spécifiques [2]. A contrario, au cours de la phase d'immunotolérance, une forte charge virale ne conduit pas nécessairement à une forte réponse immunitaire. Dans une étude très intéressante, Pallet et ses collaborateurs [3] ont cherché à comprendre les raisons de ce paradoxe, c'est-à-dire à déterminer comment se réalisent les immunorégulations lors d'une hépatite chronique.

Le rôle central du milieu métabolique dans la régulation de l'immunité et, notamment, le besoin accru en acides

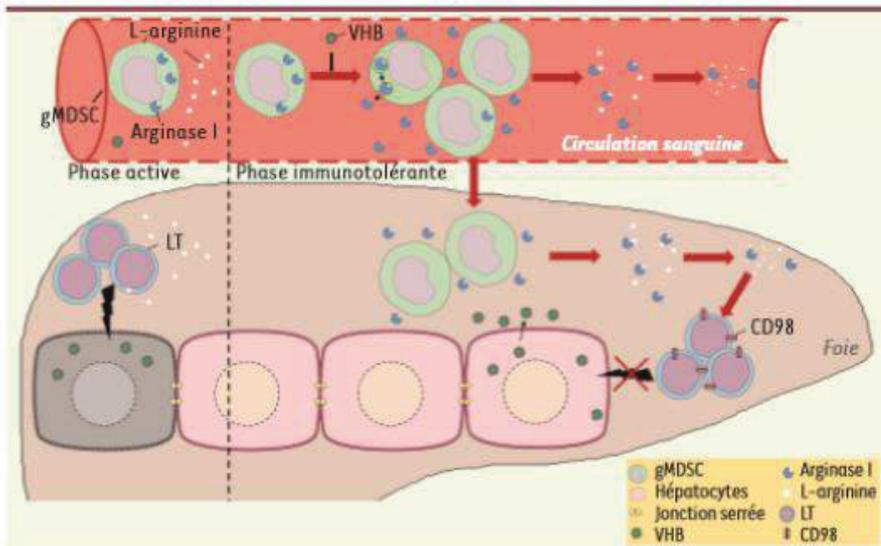
aminés pour mettre en place une réponse immunitaire efficace, a été récemment mis en évidence [4]. Précédemment, l'équipe dirigée par Mala Maini avait décrit, chez les patients atteints d'une hépatite B chronique, un défaut de prolifération des lymphocytes T qui était concomitant avec une perte de l'expression de CD3- $\zeta$ , une des sous-unités constituant le TCR-CD3 (*T cell receptor*) [5]. Cette perte d'expression avait été décrite comme un marqueur caractéristique d'une privation en L-arginine (un acide aminé qui joue un rôle important dans la réponse des macrophages). Faisant suite à ces travaux, et pour comprendre les mécanismes sous-jacents, Pallet et al. ont étudié une population hétérogène de cellules immunitaires suppressives myéloïdes (MDSC, *myeloid-derived suppressor cell*) impliquées dans les régulations métaboliques. De façon intéressante, les auteurs ont montré que ces MDSC étaient impliquées dans l'inhibition de la réponse lymphocytaire T contre le VHB [3]. Les MDSC, et plus précisément les gMDSC (*granulocytic MDSC*, des MDSC qui possèdent des caractéristiques de granulocyte immature et qui expriment l'arginase<sup>1</sup>), sont retrouvés en plus grand nombre dans le sérum et le foie des patients présentant une hépatite B chronique. Cette augmentation, plus prononcée dans le foie [3], est spécifiquement observée chez les patients dont la maladie se situe dans des phases non inflammatoires (phases IT et IN, Tableau 1) et elle est

inversement corrélée avec le score de nécro-inflammation hépatique. Chez les patients infectés de façon aiguë par le virus, le nombre de gMDSC est inversement proportionnel au niveau de transaminases sériques (marqueurs de dommages hépatiques). Ces données suggèrent que les gMDSC pourraient supprimer l'inflammation et donc avoir un rôle de protection contre les dommages hépatiques lors d'une infection par le VHB.

Le (ou les) mécanisme(s) impliqué(s) dans la prolifération des gMDSC lors de ces phases, ainsi que les facteurs et les récepteurs responsables de leur recrutement au niveau hépatique, restent cependant à identifier. Pallet et al. ont comparé, sur les gMDSC sériques et les gMDSC hépatiques, l'expression de différents chimiorécepteurs pouvant intervenir dans le recrutement hépatique de ces cellules. La relocalisation hépatique des gMDSC pourrait ainsi résulter de multiples modifications : une diminution de CXCR4 (*chemokine [C-X-C motif] receptor 4*), ce qui induit une mobilisation des cellules en dehors de la moelle osseuse, et une augmentation de CXCR3 (*chemokine [C-X-C motif] receptor 3*), conduisant à une interaction avec les cellules hépatiques étoilées<sup>2</sup> [3]. Un facteur viral pourrait également participer au recrutement des gMDSC. Auquel cas, le traitement des patients immunotolérants (qui n'est pas préconisé à l'heure actuelle), visant à la réduction

<sup>1</sup> L'arginase hydrolyse l'arginine en ornithine et urée.

<sup>2</sup> Les cellules étoilées acquièrent, après activation, des propriétés myofibroblastiques et synthétisent du collagène.



**Figure 1.** Les gMDSC régulent l'immunopathogénèse liée au virus de l'hépatite B grâce à des changements métaboliques. En phase active, les lymphocytes T CD8 détruisent les cellules infectées. En phase immunotolérante, le VHB active la prolifération des gMDSC et les recrute dans le foie, conduisant à une augmentation des taux sérique et hépatique de l'arginase I. Cette augmentation a pour conséquence une déplétion en L-arginine, carencant les lymphocytes T en cet acide aminé, les rendant ainsi inactifs. En compensation, le niveau d'expression de CD98 augmente sur les lymphocytes T. gMDSC : granulocytic myeloid-derived suppressor cells ; VHB : virus de l'hépatite B ; LT : lymphocyte T ; CD98 : transporteur neutre des acides aminés.

des facteurs viraux sanguins, pourrait permettre d'empêcher le recrutement des gMDSC et donc de maintenir une réponse antivirale active.

Plusieurs études avaient montré que les MDSC pouvaient inhiber les réponses immunitaires par la production d'arginase I, enzyme dégradant la L-arginine, privant ainsi les cellules immunitaires de cet acide aminé, dans les infiltrats tumoraux [6]. Par une série d'expériences élégantes, Pallet *et al.* ont montré que l'augmentation des gMDSC est associée à une accumulation de l'arginase I et à une diminution de la L-arginine sérique. *A contrario*, un ajout de L-arginine permet de supprimer les symptômes associés à la réplication virale (c'est-à-dire une diminution des transaminases sériques) [3]. Parallèlement, une corrélation inverse est observée entre le nombre de gMDSC et l'expression du récepteur CD3- $\zeta$  sur les cellules lymphocytaires T. Ce qui démontre, de façon indirecte, qu'une carence en L-arginine inhibe effectivement la fonction des

lymphocytes T [3]. Par ailleurs, compensant la déplétion en L-arginine, une augmentation de l'expression de CD98 (transporteur neutre d'acides aminés) est observée sur les lymphocytes T CD8 hépatiques, ce qui permet d'améliorer le transport des acides aminés en défaut [3]. Dans ce contexte, il serait intéressant de tester *in vitro*, dans des expériences de co-culture avec les gMDSC, l'effet d'un inhibiteur de l'arginase I sur la fonction des lymphocytes T. En effet, si un inhibiteur de l'arginase I permet de restaurer les fonctions des lymphocytes T, un traitement pourrait alors être envisagé *in vivo*. Bien que les données obtenues semblent robustes, il ne faut pas oublier que les MDSC pourraient avoir d'autres fonctions aux niveaux sérique et hépatique lors de l'infection par le VHB. Une étude a en effet montré que les MDSC hépatiques pouvaient inhiber la production d'interféron gamma (IFN- $\gamma$ ) par les lymphocytes T et ainsi inhiber l'expression de CD40-L (le ligand de CD40) sur les lymphocytes [7]. Or, cette

molécule participe à la formation de la synapse immunologique entre lymphocytes et cellules présentatrices d'antigènes. Elle est, en particulier, nécessaire au changement de la classe d'immunoglobuline [7].

De façon intrigante, Pallet *et al.* ont montré que l'inhibition immunitaire qu'ils avaient mise en évidence n'était pas spécifique des lymphocytes T dirigés contre le VHB mais qu'elle pouvait être également observée, *in vitro*, sur des lymphocytes T dirigés contre d'autres virus comme le virus d'Épstein-Barr ou le cytomégalovirus [3]. Si la carence en L-arginine entraînait une inhibition de tous les lymphocytes T activés, une immunodépression devrait donc être observée lors d'une hépatite B chronique. Or, le système immunitaire des patients infectés reste fonctionnel [8]. L'inhibition des lymphocytes T *in vivo* reste donc, au moins partiellement, spécifique des lymphocytes T dirigés contre le VHB, le mécanisme sous-jacent restant cependant à déterminer. La compréhension de ce(s) mécanisme(s) est donc nécessaire pour parvenir à induire une réactivation de la réponse T chez les patients chroniquement infectés.

En conclusion, Pallet *et al.* ont montré que le nombre de gMDSC exprimant l'arginase I est accru lors d'une hépatite B chronique. Ceci entraîne une augmentation de l'arginase I sérique et hépatique, conduisant à une déplétion en L-arginine et donc à l'inhibition de l'activité des lymphocytes T (Figure 1). Ce travail a permis d'identifier une nouvelle population de cellules immunitaires impliquée dans la régulation des réponses immunitaires chez des patients chroniquement infectés par le VHB, les gMDSC, et d'expliquer un mécanisme d'inhibition de la réponse lymphocytaire T. Cette étude suggère que la phase d'immunotolérance est due à une inhibition forte des réponses inflammatoires plutôt qu'à une inaptitude du système immunitaire à induire une réponse T fonctionnelle. Or, le VHB a longtemps été considéré comme un virus silencieux [9] contre

lequel aucune réponse immunitaire n'était mise en place puisque le système immunitaire du patient était supposément incapable de détecter ce virus. Depuis quelques années, de plus en plus d'études montrent que, plutôt que d'être un virus silencieux, le VHB peut mettre en place de nombreux mécanismes d'inhibition de l'immunité, à la fois innée et adaptative [10]. Les cellules immunitaires des patients infectés par le VHB seraient donc capables de détecter le virus et pourraient, en cas de levée de l'inhibition induite par le virus, mettre en place une réponse immunitaire efficace. Les données obtenues par Pallet *et al.* sont encourageantes pour le développement de thérapies innovantes visant

la réactivation du système immunitaire chez les patients infectés. ♦

### gMDSCs act as metabolic regulators of hepatitis B virus immunopathology

#### LIENS D'INTÉRÊT

Les auteurs déclarent n'avoir aucun lien d'intérêt concernant les données publiées dans cet article.

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## 1.2. Interplay between the Hepatitis B Virus and Innate Immunity: From an Understanding to the Development of Therapeutic Concepts.

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**Review published in 2017 in Viruses.**

# Interplay between the Hepatitis B Virus and Innate Immunity: From an Understanding to the Development of Therapeutic Concepts

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Academic Editors: Ulrike Protzer and Michael Nassal

Received: 12 March 2017; Accepted: 24 April 2017; Published: 28 April 2017

**Abstract:** The hepatitis B virus (HBV) infects hepatocytes, which are the main cell type composing a human liver. However, the liver is enriched with immune cells, particularly innate cells (e.g., myeloid cells, natural killer and natural killer T-cells (NK/NKT), dendritic cells (DCs)), in resting condition. Hence, the study of the interaction between HBV and innate immune cells is instrumental to: (1) better understand the conditions of establishment and maintenance of HBV infections in this secondary lymphoid organ; (2) define the role of these innate immune cells in treatment failure and pathogenesis; and (3) design novel immune-therapeutic concepts based on the activation/restoration of innate cell functions and/or innate effectors. This review will summarize and discuss the current knowledge we have on this interplay between HBV and liver innate immunity.

**Keywords:** HBV; liver immunity; innate immunity; viral escape; immune therapeutic concepts

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## 1. Role of Liver Innate Immunity in Pathogen Clearance and Immune-Driven Pathogenesis: Generalities

The liver, which is made up of approximately 80% hepatocytes, is often considered a secondary lymphoid organ due to the amount of flowing-through, infiltrating, and resident immune cells it contains [1,2]. Notably, innate immune cells, as well as non-parenchymal/non-professional cells endowed with innate functions (e.g., liver sinusoidal endothelial cells (LSEC), hepatic stellate cells (HSC)) [3], are particularly enriched in this solid organ. By order of decreasing abundance, innate cells from: (1) lymphoid origin (natural killer and natural killer T-cells (NK/NKT) but also innate lymphoid cells (ILC), and mucosal-associated invariant T-cell (MAIT)); (2) myeloid origin (e.g., Kupffer cells (KCs), myeloid-derived suppressive cells (MDSC), monocytes, neutrophils); and (3) various types of dendritic cells (DCs; mDC-BDCA1+, mDC-BDCA3+, plasmacytoid DCs; pDCs), can be found in a healthy liver. These cells, as well as the cytokinic/chemokinic effectors that they produce, can be beneficially involved in the early containment of pathogen infection and the orchestration of pathogen-specific adaptive responses [1,3]. However, they can also be involved in immune-driven pathogenesis, if a return to homeostasis is impaired by a pathogen or a recurrent exposure to xenobiotics/alcohol [4–6]. With respect to the latter, sterile or pathogen-driven chronic inflammation, as well as non-specific and uncontrolled cytotoxic activities, can be associated with the development of steatohepatitis, fibrosis, cirrhosis, and hepatocellular carcinoma [4–6].

Many liver innate immune cells are involved in the so-called tolerogenic microenvironment, which prevails and protects this organ against permanent exposure to gut-derived microbial degradation products or a low concentration of living bacteria [7]. Incidentally, the liver houses many chronic infections, including type B and C viral hepatitis, as well as parasitic infections [3]. Despite this

intrinsic immune tolerance, the liver is well equipped to mount a potent antimicrobial response, and successful pathogens have also evolved strategies to either passively or actively evade innate and adaptive immune responses in order to persist.

For the hepatitis B virus (HBV), it has been clearly shown that adaptive responses are needed for an efficient and persistent control of infection [8,9]. However, the role of innate immunity has often been overlooked, as HBV infection is usually diagnosed several weeks after the onset of infection, when the virus has already escaped from early immune responses and viremia is high [10,11]. For this reason, the role of innate immune cells and their effectors, in HBV persistence and associated-pathogenesis, has yet to be actively investigated. This review will discuss our current knowledge of the interplay between HBV and innate immune cells/effectors, as well as envisaged strategies to develop immune therapeutic concepts, particularly involving innate cells/effectors, and to achieve a functional cure (i.e., loss of the HBV surface antigen (HBsAg), with or without anti-HBs seroconversion).

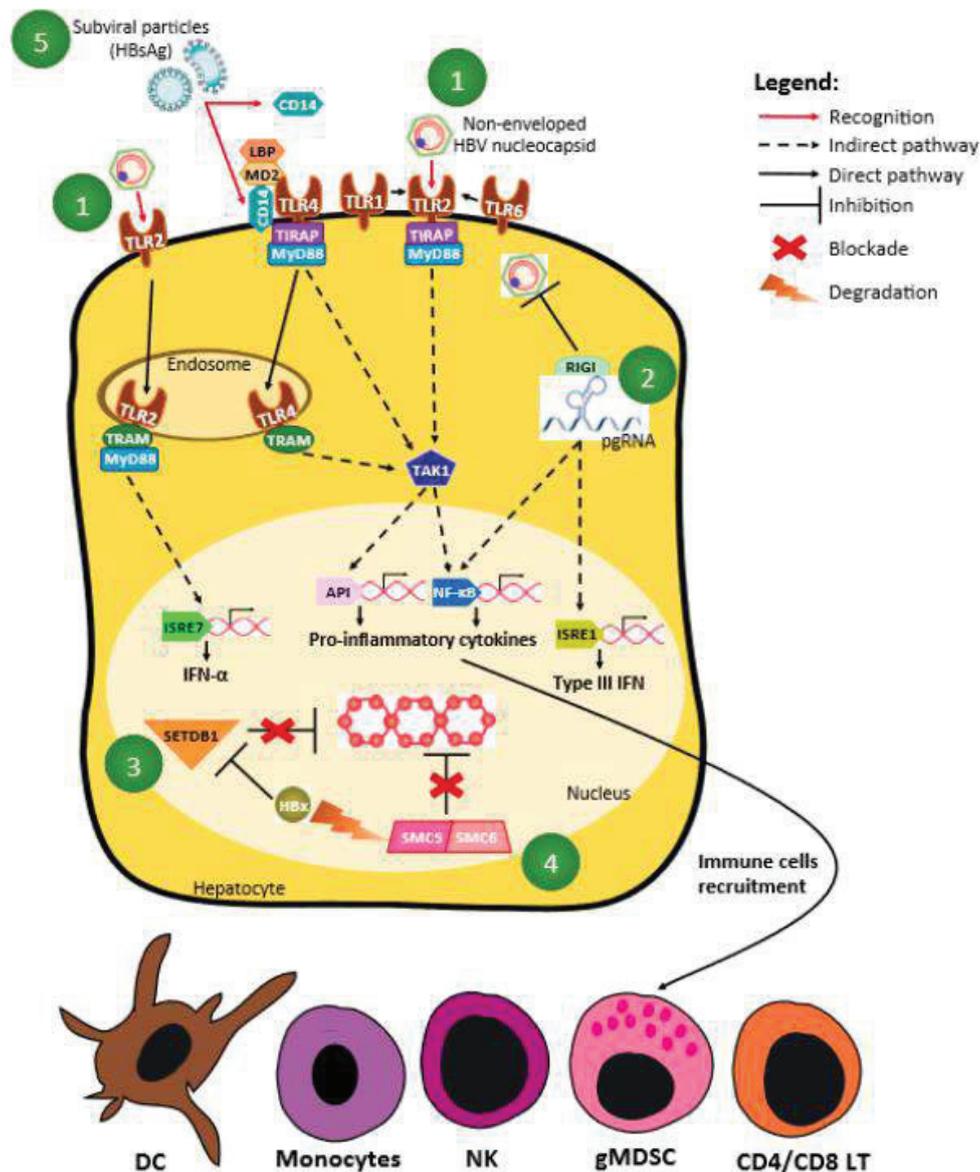
## 2. Interplay between HBV and Innate Immunity: Basic Insights

### 2.1. HBV Recognition by Innate Sensors

The early and non-specific detection of pathogens generally occurs, at subcellular/molecular levels, via the recognition of Pathogen-Associated Molecular Patterns (PAMP) by innate immunity sensors, also called Pathogen Recognition Receptors (PRR). Amongst PRR, there are toll-like (TLR), Retinoic acid-Inducible Gene I (RIG)-like, nucleotide-binding oligomerization domain-containing protein (NOD)-like, C-type Lectin, and DNA-sensing receptors, which are differentially or ubiquitously expressed in various types of epithelial/endothelial cells, as well as professional and non-professional immune cells [12].

Upon interaction between a PRR and its cognate PAMP, various downstream signaling pathways are activated and sequentially involve: (1) adaptor/co-adaptor molecules (e.g., myeloid differentiation primary response gene 88 (MyD88), TIR-domain-containing adapter-inducing interferon- $\beta$  (TRIF), etc.); (2) kinases (e.g., TANK binding kinase 1 (TBK1), transforming growth factor  $\beta$ -activated kinase 1 (TAK1), etc.) and (3) transcription factors (e.g., interferon regulatory transcription factors (IRFs), nuclear factor kappa-B (NF $\kappa$ B), c-fos/c-jun) [12] (Figure 1). This leads to the expression of effector genes, including interferon-stimulated-genes (ISG) and NF- $\kappa$ B-inducible or pro-inflammatory genes. Primary effectors include the various interferons (IFN; IFNs- $\alpha$  (13 different alleles), IFN- $\beta$ , IFNs- $\lambda$  (four alleles), and IFN- $\gamma$ , as well as pro-inflammatory cytokines/chemokines (e.g., interleukin (IL)-6, tumor necrosis factor (TNF- $\alpha$ ) which collectively have direct or indirect anti-microbial actions, and/or are involved in the recruitment of innate and adaptive immune cells to the infected or endangered site (Figure 1).

Other innate detection systems rely on canonical or non-canonical inflammasomes [13,14]. They are induced by PAMP or Danger-Associated Molecular Patterns (DAMP) and lead to the activation of the Caspase-1, which in turn cleaves precursor molecules (e.g., pro-IL-1 $\beta$  and pro-IL-18) to liberate active IL-1 $\beta$  and IL-18 in the infected or endangered microenvironment. In normal/non-transformed hepatocytes, it has recently been shown that most PRR are expressed and functional [15–17], with the potential exception of the cyclic GMP-AMP Synthase/STimulator of Interferon Gene (cGAS/STING) axis [18], and the lack of inflammasome activities [19].



**Figure 1.** Hepatitis B virus (HBV) detection by innate immune sensors and regulation by host factors. (1) Non-enveloped HBV nucleocapsid composed of HBV core/capsid antigen (HBcAg) can be recognized by Toll-Like Receptor 2 (TLR2), triggering pro-inflammatory cytokine secretion in TLR2-positive liver cells via TRIF-Related Adaptor Molecule/ Myeloid Differentiation primary response gene 88 (TRAM/MyD88) or TIR domain-containing Adaptor Protein (TIRAP)/MyD88 adaptation and Activator Protein 1 (AP1) and Nuclear Factor kappa-B (NFκB)-dependent pathways; (2) RIG-I could be a sensor of the “epsilon (ε) stem-loop” present in pregenomic RNA (pgRNA). This recognition leads to the production of type-III interferon (IFN) via both interferon regulatory transcription factor (IRF) 3 and NFκB-dependent pathways, as well as to the sequestration of pgRNA and a subsequent decrease of relaxed circular DNA (rcDNA) synthesis; (3) SETDB1, a histone-methyl-transferase, impairs covalently closed circular DNA (cccDNA) transcription, which can be reverted by HBx; (4) Structural Maintenance of Chromosome 5 and 6 (SMC5/6) complex inhibition of cccDNA transcription can be reverted by HBx, which induces SMC5/6 degradation in a DNA-Damage-Binding 1 (DDB1)-Cul4-E3-ubiquitin-ligase-dependent manner; (5) Subviral particles (i.e., HBV surface antigen or HBsAg) could interact with CD14, leading to their internalization and cell activation in TLR4 positive cells.

Whether HBV is genuinely detected by PRR upon very short exposure (i.e., contact between viral particles/incoming materials and PRR), or after the amplification of its genome and expression of its proteins during its replicative life-cycle in hepatocytes, is a matter of debate. One difficulty comes from the fact that *in vitro* models used to study HBV replication are suboptimal. Indeed, to obtain a strong and productive infection in primary human hepatocytes, 100 to 1000 virus-genome-equivalents/cell of recombinant HBV virions are needed [20], whereas, as a basis for comparison, a multiplicity of infection of <0.1 colony-forming-unit/cell is only needed to infect hepatoma cells with the hepatitis C virus (HCV). Moreover, for an unknown reason, HBV is not capable of spreading/diffusing into a monolayer of hepatocytes *in vitro*, thus indicating that there are impairments in virion egress or/and re-entry, which prevent the spreading, and consequently, the possibility of an increased exposure to PRR. Another problem relies on the fact that the “quality” of HBV inoculum used for *in vitro* experiments needs to be optimal to generate genuine results. Most researchers use HBV produced by HepG2.2.15 [21] or HepAD38 cells [22], in order to efficiently infect primary human hepatocytes (PHH) [23], differentiated HepaRG [20], or Na<sup>+</sup>-taurocholate-cotransporting-polypeptide (NTCP)-expressing hepatoma cells [24]. Various levels of non-enveloped nucleocapsids may contaminate these inocula (supplementary material in [25]) and be at the origin of artefactual results. Moreover, an HBV core/capsid antigen (HBcAg) synthesized in bacteria may be contaminated with lipopolysaccharide (LPS)-like ligands, thus further confusing the matter [26]. Nevertheless, it has been suggested that the HBV nucleocapsid is a ligand for TLR2 [27] and that TLR2-engagement by cognate ligand leads to the strong production of pro-inflammatory cytokines by hepatocytes and/or other TLR2-positive cells [16,25,27–29]. However, the existence of this non-enveloped HBcAg ligand *in vivo* is still a matter of debate. The use of HBV virions purified from a patient would guaranty the relevance of experiments; however, so far, there is no clear demonstration that they can be used to reproducibly and efficiently infect hepatocytes *ex vivo/in vitro*.

In contrast to other viral entities, HBV is a poor inducer of innate immunity because of the intrinsic characteristics of its life cycle [30]. The viral relaxed circular DNA (rcDNA), as well as single-stranded DNA (ssDNA) or linear double-stranded DNA (dsDNA; intermediates/side products of reverse transcription), are likely recognized as damaged, non-methylated, and non-self DNAs. In contrast, the other viral nucleic acids, including covalently-closed-circular DNA (cccDNA; i.e., the nuclear template of transcription) and various viral RNAs, are “host-like” molecules, and are therefore non-differentiable from “self”. Being synthesized within nucleocapsids, rcDNA is protected from a potential recognition by DNA sensors. One possible HBV PAMP could be the ternary complex formed by the pregenomic RNA (pgRNA) HBc and polymerase proteins [30]. In this respect, RIG-I was proposed as a sensor of the “Epsilon ( $\epsilon$ ) stem-loop” within pgRNA, leading to both a measurable production of type-III IFN in PHH, and a “sequestration of pgRNA” associated with an effector-independent antiviral decrease in rcDNA synthesis (i.e., intrinsic-restriction-like mechanism) [31].

If covalently closed circular DNA (cccDNA) ends up being a minichromosome-like structure, likely indistinguishable from self-DNA, it appears that its initial establishment from rcDNA, as well as its early transcription after the onset of infection, are modulated by host proteins, which could be defined as “intrinsic restriction factors”. Hence, it was shown that both the histone-methyl-transferase SETDB1 [32] and the complex SMC5/6 [33] are capable of impairing cccDNA transcription early-on after the onset of infection, and that HBx is needed to either counteract the action of the former [32] or induce the degradation of the latter in a DDB1-Cul4-E3-ubiquitin-ligase-dependent manner [33].

Besides the potential detection of the different HBV nucleic acids, it has also been reported that subviral particles could interact with CD14, a co-receptor of TLR4 in myeloid cells, via their phospholipid moiety [34]; this could lead to the internalization of HBsAg into these cells and their activation [35]. In fact, the soluble fraction of CD14, bound to HBsAg, could be responsible for this activation [36]. The fact that the lipid moiety of HBsAg subviral particles is instrumental for this activation represents an interesting finding. Indeed, the lipidomic of viral particles and its evolution during the natural course of infection in a human is completely unknown; potential changes in lipid

composition, with the incorporation of “toxic lipid” (e.g., peroxydated lipid), could be associated with changes in the immune characteristics of HBV subviral particles.

To summarize, the molecular determinants of a potential recognition of HBV PAMPs by PRRs are still poorly defined and further studies are necessary. This understanding is instrumental in the context of the development of PRR agonists, which could be used in immune-therapeutic combinations.

## 2.2. Mechanisms of HBV-Driven Inhibition of Innate-Signaling Pathways (Figures 1 and 2)

An acute exposure to/infection by HBV, *in vitro*, in an animal model and in a human, does not lead to a strong activation of IFN and pro-inflammatory responses. The first demonstration came from a microarray study performed with the biopsies of experimentally-infected chimpanzees, showing that, in comparison with HCV, the exponential increase in HBV viremia was not associated with a significant/measurable gene expression change in their liver, including innate immunity genes [37]. In agreement with this analysis, no (or a weak) seric production of IFNs and cytokines/chemokines was found in acutely HBV-infected patients (i.e., patients who consulted before clearing acute infection or progressing to chronic stage), as compared to what could be seen with similar patients infected by HIV or HCV [38]. A production of the immunosuppressive cytokine IL-10 was, however, evidenced during the viral load increase and was associated with a transient inhibition of NK functions [39]. This increased production of IL-10 correlates with virus replication, and was also evidenced during virus-induced flares occurring in long-term chronically infected patients [40]. This points toward a virus-mediated manipulation of cytokines/chemokines production in favor of immune suppression.

When studied *ex vivo/in vitro*, the acute exposure of hepatocytes or other liver cell types to HBV is also not globally associated with a strong induction on IFNs and pro-inflammatory cytokines/chemokines. Differentiated HepaRG and PHH infected by “qualitatively relevant” recombinant HBV inoculum did not secrete a measurable amount of IL-6 or IFN- $\beta$ , despite a very weak and transient activation of gene expression [25]. Similarly, KCs exposed to the virus only transiently released a very weak amount of IL-6 [41] or IL-1 $\beta$  [19], suggesting that HBV could be detected/sensed, but could also readily impair nascent responses. This potential lack of measurable events could either be due to: (1) the viral genotype used, as an HBV genotype-C led to a measurable secretion of IFN- $\lambda$  by infected hepatocytes (in contrast to genotype D used in other studies [31]); or (2) to the cell cultivation conditions, as micro-patterned PHH on fibroblast feeder cells exposed to HBV showed a significant induction of type-III IFN gene expression [42].

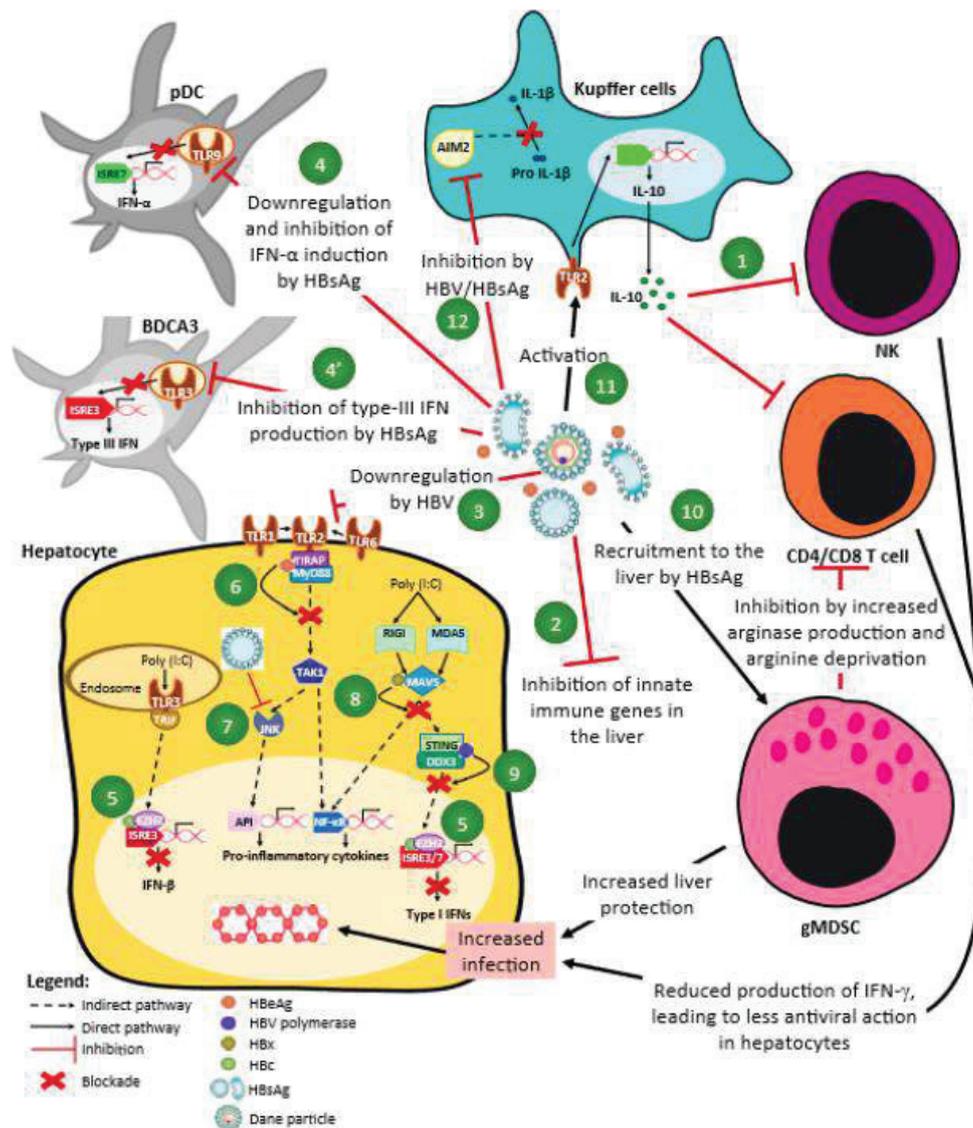
The weak response or lack of IFN and/or pro-inflammatory responses, could also be due to viro-implemented active mechanisms. In this respect, the recently performed analyses in Chronic Hepatitis B (CHB) patient biopsies have shown that many genes of innate immunity were down-regulated when compared to control patients, and the magnitude of this inhibition was dependent upon the HBeAg status and seric levels of HBsAg [43]. Even if these results were only obtained at RNA levels, they confirm previous studies that had shown a down regulation, at the protein level, of innate sensors such as TLR2 [44] or TLR9 [45]. These results are in sharp contrast with what has been observed in the woodchuck model, in which an increase of the expression of the innate immunity gene was observed [46]. Moreover, in liver-humanized mice, which are immune-deficient, HBV infection led to a very weak increased expression of some innate immune genes, but not a significant decrease [47]. This emphasizes that animal models may not always be predictive of what happens in humans.

In contrast to HDV [47,48], HBV infections seem to be associated with a lack of induction or even a decreased expression of ISGs or other pro-inflammatory genes, thus pointing toward mechanisms of active inhibition. The first demonstration that HBV could inhibit PRR signaling pathways came from a study, which showed that both HBeAg and HBsAg could antagonize the antiviral action of PRR agonists in murine parenchymal and non-parenchymal cells [49]. Despite its interest, the results of this study were challenged due to the murine nature of the cell types used, as well as because of the apparent lack of specificity of the observed inhibitory phenotype (i.e., all viral forms led to the inhibitory phenotype) and the lack of insights on the underlying mechanism of action.

More recently, we have shown that HBV could be sensed by hepatocytes, leading to an early (i.e., between 2 h and 8 h post infection) and moderate elevation of some innate immunity gene expression [25]. However, we observed that this response was transient, and that incoming HBV virions were capable, in the absence of neo-synthesized viral proteins, of blocking the induction of IFN genes triggered in trans by cognate ligands of TLR3, RIGI, or Melanoma Differentiation-Associated protein 5 (MDA5). We then found that the core/capsid/HBc protein was responsible for this inhibitory phenotype through the recruitment of a histone-methyl-transferase, i.e., Enhancer of Zeste Homologue2 (EZH2), on targeted gene promoters, which was in turn capable of establishing repressive epigenetic marks to prevent their inducibility [50]. If the capacity of HBc to bind to synthetic dsDNA, the host genome, or cccDNA was already known [51–53], its ability to recruit epigenetic modifying enzymes and modulate gene expression is a novel discovery, and upgraded this otherwise structural protein to a potential innate immunity regulator in infected hepatocytes.

Other viral proteins have been suggested to interfere with innate signaling pathways in hepatocytes [54]. The two which are secreted, HBsAg and HBeAg, are thought to be involved in many immune evasion processes. As previously discussed, they are capable of impairing the activation of all TLR pathways [49,55]. However, regarding their potential role in the inhibition of intrahepatic signaling pathways, experimental data are less clear. It has been proposed that HBe or/and a related intracellular form called p22, could bind to the co-adaptor of Myd88, TIRAP (also called MAL2), and therefore interfere with the TLR2 signaling pathway [56]. More surprisingly, it has also been suggested that HBs could inhibit this pathway by a mechanism involving the inhibition of the c-Jun N-terminal protein Kinase (JNK), which in turn, would prevent IL-12 production [57]. The HBx protein has also been endowed with many biological activities, including the regulation of innate pathways. Hence, it has been proposed that HBx could inhibit the dsRNA-mediated IFN response by either competitively interacting with host-factors of these pathways (e.g., Mitochondrial Anti-Viral-Signaling protein (MAVS), TRIF, IRF3) and/or inducing their degradation by proteasome [58–61]. However, it is worth noting that the overexpression of this protein has often been associated with many un-relevant activities [62]. Finally, the HBV Pol has also been described to inhibit the dsRNA-mediated IFN response by interfering with STING and Dead box protein 3 (DDX3) functions [63,64].

To conclude, there is a lot of published data, however there are still many caveats in demonstrations and an overall lack of knowledge of underlying mechanisms. It seems appropriate to indicate that if the HBV-driven strategies to inhibit immune responses are genuine, they are only adapted to what HBV itself would be able to trigger as a weak inducer of innate responses; indeed, it seems that HBV-mediated inhibitory phenotypes are not very potent and are likely not capable of fully counteracting a more aggressive induction of innate responses, like that mediated, for instance, by HDV [48].



**Figure 2.** HBV modulation of innate immune sensors. (1) Interleukin-10 (IL-10) production can impair Natural Killer (NK) cell function, including the non-cytotoxic anti-HBV action of IFN- $\gamma$  in infected hepatocytes; (2) HBV inhibit innate immune gene inductions in the liver according to HBeAg and HBsAg status; (3) TLR2 is down-regulated by HBV in hepatocytes, leading to a reduced production of pro-inflammatory cytokines/chemokines; (4) HBsAg down-regulates TLR9 in pDC, leading to the inhibition of IFN- $\alpha$  production and; (4') HBsAg inhibits type-III IFN production upon TLR3 stimulation, hence preventing a relevant NK cell activation; (5) Hbc can block the production of IFN upon dsRNA recognition receptor stimulation by the recruitment of EZH2 and immune promoters methylation; (6) HBeAg binds to TIRAP/MyD88 complex to inhibit the TLR2 signaling pathway; (7) HBsAg inhibits JNK pathway preventing IL-12 production; (8) HBx inhibits the dsRNA-mediated IFN response by interacting with host-factors (MAVS, TRIF, IRF3) and/or inducing their degradation by proteasome; (9) HBV polymerase inhibits the dsRNA-mediated IFN response by interfering with the STING and DDX3 function; (10) HBsAg can recruit and trigger the expansion of granulocytic Myeloid-Derived Suppressive Cells (gMDSC), which in turn, specifically inhibit CD4<sup>+</sup> and CD8<sup>+</sup> T-cell by the production of a large amount of arginase, leading to metabolic deprivation; (11) HBV can activate Kupffer cells (KCs) by the TLR2 pathway, leading to the production of IL-10 and the subsequent inhibition of the lymphocyte response; (12) HBsAg inhibits the AIM2-inflammasome and blocks the production of IL-1 $\beta$ , which has a strong antiviral effect on HBV replication in hepatocytes.

### 2.3. Modulation of Innate Cell Number/Frequency by HBV

One way for HBV to initially and persistently escape innate immune cell antiviral functions would be to modulate their numbers, ideally within the infected liver. There are no robust and convincing data showing that the number of CD14<sup>+</sup> monocytes, resident KCs, pDC (BDCA2<sup>+</sup> cells), mDC-BDCA3<sup>+</sup>, and NK/NKT cells within the infected liver, or in the peripheral blood, is affected by HBV throughout the natural history of infection [35,65–69].

Therefore, HBV infections may instead be associated with the impairment of functions rather than the number/frequency of cells. There is at least one convincingly described exception to this, which is the number of myeloid-derived suppressive cells in CHB patients [70]. Indeed, it was recently reported that the number of granulocytic subsets of MDSC is increased in the low-inflammatory (immune tolerant) phase of the HBV natural history. Their number is inversely proportional to the level of liver inflammation, thus suggesting that these cells contribute to the protection of the liver, while likely favoring HBV replication. Although the mechanism has not been completely elucidated, HBV is likely to induce their recruitment/expansion in the liver to its own benefit. In the infected organ, MDSC inhibit HBV-specific CD4<sup>+</sup> and CD8<sup>+</sup> T-cell responses via metabolic means. Indeed, MDSC secrete a very high amount of arginase in the liver microenvironment, which in turn, decreases the amount of available arginine, an amino acid crucial for lymphocyte physiology and growth. This leads to their starving and functional inhibition. HBsAg could trigger their recruitment/expansion by acting through the ERK/Interleukin-6/Signal Transducer and Activator of Transcription 3 (ERK/IL-6/STAT3) signaling pathway [71] (Figure 2).

### 2.4. Modulation of Dendritic and NK/NKT Cells Functions by HBV (Figure 2)

More than affecting their numbers, HBV seems to impair the functions of many innate immune cells. An investigation on HBV-driven inhibitory phenotypes and underlying mechanisms is not easy in the absence of a good animal model and a low access to human liver samples. Moreover, studying liver phenotypes is more relevant than studying peripheral blood ones, as intimate contact/interaction between infected hepatocytes and immune cells can only take place within the liver. Unfortunately, most of the studies performed so far were with blood-derived cells, thus limiting their meaningfulness and feeding the debate around the “systemic perturbation” of innate functions by HBV.

pDCs are a unique dendritic cell (DC) subset specialized in IFN- $\alpha$  production, representing 10% of total DC in the liver (our unpublished data), and are very important antiviral innate cells [72]. Their number in CHB patients seems to be unchanged in both the blood and liver compartments [67,73]. However, it was shown that blood- and liver-derived pDC from CHB patients in an ex vivo study were less capable of producing IFN- $\alpha$  upon TLR9 agonisation [45,67,73], less capable of cross-talking and activating the cytolytic activity of naive NK cells [67], and more prone to induce the generation of regulatory T-cells [74]. HBsAg was suggested to be the main driver of these modulations, recapitulating these phenotypes ex vivo [75]. It may act by interacting with the regulatory receptor BDCA2 and triggering the downstream inhibitory SYK/MEK-ERK1/2 pathway [76], or by inducing the down expression of TLR9 itself by an unknown mechanism [45].

BDCA3<sup>+</sup>/Clec9A<sup>+</sup> myeloid DCs [77], which are responsible for IFN- $\lambda$  production through the activation of TLR3 in particular and represent 10% of total DC in the liver (unpublished data), were also recently shown to be functionally impaired in the blood and liver compartment of CHB patients [69]. HBsAg would also be involved in the inhibition of these cells, as the exposition of naive cells to this antigen recapitulates the inhibitory phenotype. Thus, HBV would be capable of reducing the production of type-I and III IFNs, which are two the main classes of antiviral cytokines, by targeting both subsets of DC and therefore inhibit relevant NK cell activation.

NK cells are particularly enriched in the liver and are a major source of IFN- $\gamma$ , a cytokine with direct anti-HBV activity [78,79] and immune-stimulatory properties. They likely play an important role in the resolution of acute HBV infection [39,80–82] and, not surprisingly, their impaired functionality in CHB patients, in particular regarding their reduced capacity to produce IFN- $\gamma$ , has been shown [83,84].

IL-10 and TGF- $\beta$  present in an HBV-infected liver would be responsible for this inhibition [84]. Regarding their cytotoxic activities in CHB, data are more in favour of an exacerbation of NK-mediated liver damages [85,86]. Interestingly, these features of preserved NK cell cytotoxicity and impaired IFN- $\gamma$  production are found in both chronic HBV and HCV infections [87], thus pointing toward convergent mechanisms in chronic hepatitis. This complexity of the HBV-driven modulation of NK functions has important implications regarding therapeutic manipulation.

### 2.5. Interplay between HBV and Monocytes/Macrophages (Figure 2)

Resident macrophage cells are thought to play a central role with respect to the peculiarities of liver immune functions [88,89]. They can have opposite functions, according to physiological or pathological settings. They largely contribute to the basal liver immune tolerance, but can also be involved in antiviral responses. They can either hasten recovery after liver injury [90], or be involved in immune-pathogenesis [88,89]. Echoing this complexity, the ontology of human liver resident macrophages, also called KCs, remains a very active field of research and is not well known, as compared to what is known for mice; this is largely due to the lack of accessibility to normal and pathologic human liver, which prevents relevant phenotypic and functional immune studies [88,89]. Phenotypically, it is very difficult to distinguish genuine liver resident macrophages (i.e., long-lived, self-renewing KCs originating from embryonic progenitor) from monocyte-derived macrophages. Indeed, with the onset of inflammation, monocytes are readily recruited to the liver, where they can differentiate into macrophages to replace killed resident ones, thus further complicating analyses [89]. It is therefore not surprising that data on the effect of HBV on macrophages/monocytes and the role of these cells in the natural history of the disease have been generated with sub-optimally characterized immune entities.

Despite these considerations, it has been shown in mice hydrodynamically injected/transduced with AAV-HBV, that the depletion of liver macrophages/monocytes, or the transduction of TLR2-deficient mice, was associated with a faster clearance of infection via a specific CD8<sup>+</sup> T-cell response [91]. This suggests a positive proviral role of these TLR2-positive cells for the establishment of a persistent infection in immune-competent mice. This came as a surprise, as these myeloid cells are the main producers of IL-1 $\beta$ , a cytokine bearing strong direct anti-HBV properties [78,92]. However, early contact of HBV with these cells could help prevent the production of this cytokine and promote the production of immune-suppressive ones. Using freshly isolated KCs from human resections, it was indeed shown *ex vivo* that HBV was capable of blocking the AIM2-inflammasome-mediated production of IL-1 $\beta$  via an HBsAg-mediated mechanism [19]. Moreover, we have shown that HBV can favor an alternative differentiation and promote the production of IL-10 by both HBsAg- and HBeAg-driven mechanisms, while inhibiting the production of IL-1 or IL-6 (unpublished data). Knowing that KCs in resting livers are already prone to produce IL-10 and TGF- $\beta$  [93], it may be that HBV further amplifies this phenotype and further reinforces local immune tolerance to promote its early spreading. Related to this, the group of Tian has shown that KCs contributed to HBV-specific T-cell exhaustion in a mouse model, through a core/HBeAg-antigen-TLR2 interaction mechanism [94].

In the context of chronic infection, there is currently no comprehensive study reporting data on the changes in frequency or altered functions of liver KCs/macrophages/monocytes. Again, all available data derive from analyses performed with blood-derived macrophages/monocytes. In this setting, several groups have shown that there was no alteration of the numbers of the different monocyte populations (CD16<sup>-</sup>/CD14<sup>+</sup>) relative to the amount of HBV DNA, HBeAg, and HBsAg [35,65]. In contrast, it was reported that inflammatory CD16<sup>+</sup> intermediate monocytes are increased in frequency in immune active patients [95], and correlates with ALT elevation, hence suggesting a contribution to liver immune-pathogenesis. The latter was particularly obvious in a double-humanized mouse model, with both a human immune system and human liver cells, in which HBV was capable of inducing a strong immune-pathogenesis, which was mediated by M2-like macrophages.

To conclude, the interplay between HBV and macrophages/monocytes is rather complex, and this picture is further complicated by conflicting studies performed with poorly characterized immune cells. Better markers for a better phenotyping of these cells are awaited to further investigate the beneficial or detrimental (or both) roles of liver macrophages in HBV natural history. However, as clearly established in cancer biology, it is likely that tumor-associated macrophage (TAM)-like macrophages, endowed with M2-like functions, could play a role in both the maintenance of HBV replication and pathogenesis.

### 3. Interplay between HBV and Innate Immunity: Therapeutic Insights

#### 3.1. Can We Improve the Efficacy and Use of IFN- $\alpha$ -Based Therapy?

The past and current clinical use of recombinant IFN- $\alpha$  as an anti-HCV and anti-HBV drug is the best proof of concept that innate immune effectors can be therapeutically valuable, despite their intrinsic low safety profile. The treatment of CHB patients with Peg-IFN- $\alpha$  leads to less than 10% of HBsAg loss (i.e., in a setting of trials; even less in real life), which is currently recognized as a good marker of a functional cure [96]. The reasons why some patients benefit from such a treatment are unclear. Besides virologic and biochemical reasons (i.e., pre-therapeutic viral load, viral genotype, level of pre-therapeutic inflammation), the genetics of the host is likely involved. It would be great to identify biomarkers associated with sustained virologic response (SVR) off-treatment to prevent the unnecessary exposure of CHB patients to Peg-IFN- $\alpha$ , which is responsible for multiple side effects and is not well-tolerated [96].

Virus-related mechanisms of resistance to IFN- $\alpha$  at subcellular levels are currently unknown. It could be that some viral proteins impair the Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway, as shown *in vitro* or in liver-humanized mouse models [97,98]. Recently, it was shown that IFN- $\alpha$  could induce, by epigenetic remodeling, the silencing of cccDNA (i.e., inhibition of transcription [99]), which may account for the antiviral effect of the drug in humans, and even in some circumstances, to its degradation via an APOBEC3A-dependent mechanism [100].

If IFN- $\alpha$  administration is meant to boost immunity and help break tolerance to the virus, it has nevertheless been shown that a strong exposure to IFN- $\alpha$  in CHB patients was associated with a NK/NKT-mediated increased killing of HBV-specific T-cells overexpressing the TNF-Related Apoptosis-Inducing Ligand (TRAIL) death receptor [101,102]. As the use of nucleoside analogs (NA) in CHB was associated with a restoration of T-cell functions [103], there was a rationale for combining Peg-IFN- $\alpha$  and a potent second generation NA [104]. The results of a large open-label active-controlled study aiming at determining the benefit of a combination between Peg-IFN- $\alpha$  and Tenofovir, indeed showed that the long-term combination of these two drugs increases the HBsAg loss rate [105], thus encouraging further clinical studies in this respect. In particular, it would be interesting to have biomarkers to identify patients that would benefit the most from IFN-based therapies. In the future, it would be interesting to block the TRAIL-dependent killing of HBV-specific T-cells by monoclonal antibodies, as for other check-point inhibition strategies, to further improve the Peg-IFN- $\alpha$ /NA combination by correcting NK/NKT altered functions [11].

#### 3.2. Is There a Place for Other Innate Immune Stimulators?

Beside IFN- $\alpha$ , the use of the less toxic IFN- $\lambda$  was also proposed to combat viral hepatitis. Indeed, the pattern of expression of the IFN- $\lambda$  receptor was more restricted to epithelial cells and fewer immune cells, and this IFN has been shown to induce less side effects, as compared to IFN- $\alpha$ . In the clinical trial, it was recently shown that Peg-IFN- $\lambda$  is as efficient as, but not superior to, Peg-IFN- $\alpha$  for treatment [106]. If further development for CHB is unlikely, the clinical evaluation of Peg-IFN- $\lambda$  is pursued in the context of co-infection with the hepatitis delta virus.

Recently, a large body of evidence has shown that many IFNs/cytokines, including IFN- $\alpha$ , IFN- $\gamma$ , IFN- $\beta$ , IFN- $\lambda$ , IL-6, TNF- $\alpha$ , and IL-1 $\beta$  were capable of inhibiting HBV replication in hepatocytes,

in the absence of immune cells [78,79,100,107]. These innate effectors are capable of inhibiting the transcription of cccDNA (thought to be through distinct molecular mechanisms; unpublished data), and some of them are also capable of inducing the degradation of cccDNA in vitro, as initially shown with LT- $\beta$ R agonists, which were particularly potent in this respect [100]. If the development of LT- $\beta$ R agonists (i.e., monoclonal antibodies and genetically engineered agonists) is going to be pursued, the use of injectable recombinant pro-inflammatory cytokines in humans, other than IFNs, is unlikely. A sound approach to take advantage of the direct antiviral characteristics of these molecules would be to allow their endogenous, local, and timely controlled de novo synthesis in CHB patients. For this, further fundamental studies are needed to determine, in particular, how HBV is capable of blocking their production and/or antiviral effect in vivo. In this respect, works aiming at a better understanding of the mechanism of the blockade of IL-1 $\beta$  production (IL-1 $\beta$  being the most active cytokines tested in vitro [78]), by liver macrophages/monocytes would be instrumental.

### 3.3. Development of PRR Agonists for Combinational Therapeutic Approaches

Currently, no PRR agonists have been approved for the treatment of a chronic infection. Some PRR agonists are currently developed as adjuvant for prophylactic or therapeutic vaccinations in the field of infectiology and oncology. In the HBV field, the antiviral properties of TLR agonists have been described more than ten years ago in HBV-transgenic mice [108]. More recently, we reported another comparative screen of the in vitro activity of TLR and RLR agonists [109]. In this latest screen, TLR2 and TLR3 ligands were shown to be of particular interest, as compared to TLR7 or TLR9 agonists for instance, as they could not only stimulate immune cells, but also have a direct effect in infected hepatocytes, through the activation of IRFs and/or NF $\kappa$ B pathways. The TLR2 ligand Pam3CSK4 was found to be extremely potent (activity in the nM range) when used in primary hepatocytes [109], as compared to its reported efficacy in hepatoma cell models [110]. Interestingly, it was shown in the woodchuck model that a treatment with NA leading to a decrease in Woodchuck hepatitis virus (WHV) viremia was associated with a re-expression of TLR2 in the liver. Hence, a combination between the TLR2 agonist and NA, or other Direct-Acting-Agents (DAAs) developed against HBV, could be a way forward to circumvent the HBV-induced inhibition of TLR2 expression observed in CHB patients [44,111]. An obstacle to the therapeutic development of TLR2 agonist is the fear of a “cytokine storm” that could occur upon systemic administration. The vectorization of TLR2 agonist into nanoparticles, which could be specifically delivered to the liver, could help circumvent this problem and would be useful to study.

TLR9 agonists have a theoretical interest as they are the only ones capable of inducing the formation of iMATEs in a mouse liver, which are a tertiary immune structure composed of myeloid cells providing a niche for the proliferation and maturation of T-cells [112]. Interestingly, the HBV genome contains CpG motifs that have been shown to be either inhibit or activate TLR9 [45], thus suggesting that HBV could naturally modulate the TLR9 pathway. In this respect, it was also shown that TLR9 expression in the Peripheral Blood Mononuclear Cell (PBMC) of infected patients was reduced; the demonstration of this is pending in liver-derived mononuclear cells. This would have consequences on the use of the TLR9 agonist in therapy. However, TLR9 ligands have been successfully encapsulated into nanoparticles and have shown an improved efficacy in boosting prophylactic HBV immunization [113]. This could be helpful to improve the efficacy of TLR9 agonists in vivo, as TLR9 agonists have so far been shown to be less active as compared to those of TLR2 or TLR7 in the woodchuck model [114]. Other PRR agonists, including those working through cGAS and STING [115–117], or those working through RIG-I/NOD2 [118], are being investigated for their potential anti-HBV properties.

The current clinical development of TLR7 agonists for CHB is more opportunistic than based on a demonstrated superiority of action on the virus. Indeed, amongst all PRR agonists, TLR7 ligands, which are small heterocyclic molecules, are the only ones to be deliverable orally, which is a tremendous advantage over other ligands. The best-characterized and advanced TLR7-L agonist is

GS-9620. This agonist has demonstrated, in mono-therapy, an extremely potent anti-HBV activity in infected chimpanzees and woodchucks, with a very strong, yet unexpected, effect on the cccDNA level in the latest model [119,120]. These results have prompted phase-1b/2 clinical evaluations and official results regarding virologic aspects are awaited. One critical point is related to the doses that have been selected for administration; it is possible that they would end-up being suboptimal, as safety was the main concern of these trials. Mechanistically, GS-9620 works by inducing the production of IFN- $\alpha$ , mainly by pDC, or related mucosal cells, in the upper gut [121,122]. An interesting path forward will be to test this molecule in association with either NA or other DAA (e.g., core assembly inhibitors) in current development, as well as with therapeutic vaccines. Indeed, PRR agonists are mainly meant to have adjacent activity in the context of combination therapy. In this respect, the excellent results obtained in monotherapy in animal models are more indicative than predictive of what will happen in humans.

#### *3.4. Manipulation of the Numbers or Biological Activity of Innate Immune Cells with Impaired Functions in CHB Patients to Help Restore HBV Immune Control*

Therapeutic concepts, based on processes such a manipulation, are more complex to implement, as we have to keep in mind that immune responses in CHB patients are a subtle equilibrium between the lack of antiviral activity and excessive pro-inflammatory or unspecific killing activities, which could worsen immune-pathogenesis.

Beside unspecific innate immune activation via immune-stimulators (IFN- $\alpha$  or PRR agonist that was discussed above), one could envisage to specifically restore beneficial or inhibit detrimental innate immune cell functions. Myeloid cells endowed with M2-like functions, similar to those of TAMs, are likely to be involved in the persistence of HBV replication. In this respect, MDSC were clearly shown to be associated with a dampening of HBV-specific and bystander T-cell responses in CHB. The depletion (or re-differentiation into antiviral myeloid cells) of such cells or an inhibition of their functions could help to restore the immune function, particularly in low-inflammatory/immune tolerant patients with high viremia, no ALT elevation and, yet, a relevant number of HBV-specific T-cells (as compared to a later stage of the disease). This concept was exemplified by the use of anti-CSF-R1 antibodies to inhibit TAM function in cancer biology [123] and a similar strategy could be implemented by targeting a specific marker of MDSC. On the biochemical side, Tadalafil, a FDA-approved small molecule of the PDE5 inhibitor family, was shown to inhibit the suppressive functions of MDSCs in cancer patients, notably by decreasing arginase expression [124]. As for other check-point inhibitors (Ipilimumab, Nivolumab, Pembrolizumab), the repositioning of these drugs to chronic infections is only a matter of time and carefully implemented clinical trials.

If the stimulation of immune cells capable of locally and temporally producing either IFN- $\alpha$  (via pDC), IFNs- $\lambda$  (via BDCA3<sup>+</sup> cells) or IL-1 $\beta$  (via myeloid cells), which have direct anti-HBV properties [107], which is a sound approach theoretically, and could be achieved for instance by PRR agonists, a cell-therapy based on pDC or BDCA3<sup>+</sup> DC loaded with HBV-derived peptides could also be an interesting approach. Indeed, these cells could both produce cytokines and elicit T-cell functions via their APC properties. A proof-of-concept study was recently reported in the context of HBV, by Martinet and colleagues [125].

#### **4. Conclusions**

In the last 10 years, our understanding of the interplay between HBV and innate immune functions has greatly improved and opened novel avenues of research, in particular regarding the potential use of immune-therapeutic components in the setting of expected combination therapies. However, due to the anticipated difficulties in terms of implementation, more fundamental and translational researches are needed to pave this long way to success. In contrast to what has been achieved in the field of HCV, where the sole use of DAAs has led to viral eradication, it is likely that the restoration of immune control of HBV replication, by means of Host-Targeting-Agents (HTA), will be instrumental to achieve

a functional cure in CHB patients. In this respect, an IFN- $\alpha$  based regimen will likely not disappear from the therapeutic landscape in the near future. Hence, current clinical investigations with novel DAA/HTA do contain therapeutic arms featuring a combination with Peg-IFN- $\alpha$ . This combinational rule also holds true for the potential development of other immune-stimulators, including PRR agonists or any other strategy to restore the transient production of antiviral cytokines.

One awaited demonstration is whether a therapeutically induced drop of HBsAg antigenemia (by whatever means) would help restore endogenous immune functions. Indeed, mechanistically speaking, it seems that HBsAg is often involved in HBV-driven immune subversion. One could think that its loss would be a step toward recovered immune functions and the in vivo demonstration of this theory is eagerly awaited. Another viral target, which could be of interest to help restore the immune response by other means, is HBc. If underlying mechanisms are still to be defined, a combination of the TLR7 agonist or Peg-IFN with core/capsid/HBc assembly inhibitors seems to synergistically improve HBsAg loss in the AAV-HBV immune competent mouse model [126,127] (see also patent number: WO-2016146598), thus warranting further clinical studies in humans.

**Acknowledgments:** D.D. and J.L. have received academic grants from the French National Institute of Health and Medical Research (INSERM; core institutional grants), the French National Agency for Research on AIDS and Viral Hepatitis (ANRS; several grants from CSS4), the Foundation for Innovation in Infectiology (FINOVI), the Foundation for Medical Research (FRM; DEQ20110421327), as well as industrial support, through collaborative research contracts, from Hoffmann-La-Roche (pRED, Basel, Switzerland), Gilead Sciences (Foster city, CA, USA) and Novira Therapeutics (Doylestown, PA, USA). S.F.D. is the recipient of an ANRS funding (CSS4) for her thesis work. D.D. was also supported by the DEVweCAN LABEX (ANR-10-LABX-0061) of the “Université de Lyon”, within the program “Investissements d’Avenir” (ANR-11-IDEX-0007), operated by the ANRS.

**Author Contributions:** S.F.D., J.L., and D.D. analyzed the literature; D.D. drafted the first version of the manuscript; S.F.D. and J.L. drafted the figures; S.F.D., J.L., and D.D. finalized the writing and contributed to the revision process.

**Conflicts of Interest:** D.D. and J.L. declare they have received a grant from Hoffmann-La-Roche (pRED, Basel, Switzerland), Gilead Sciences (Foster city, CA, USA) and Novira Therapeutics (Doylestown, PA, USA) for some of their research activities. S.F.D. declares no conflicts of interest.

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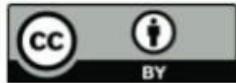
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### 1.3. Kupffer cells: friend or foe of hepatitis B infection

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Submitted to Liver International.

#### Abstract

The Hepatitis B virus chronically infects the liver of 250 million people worldwide. Over the past decades, major advances have been made in the understanding of Hepatitis B virus life cycle in hepatocytes. Beside these parenchymal cells, the liver also contains resident and infiltrating cells involved in immune responses to pathogens and much less is known about their interplay with Hepatitis B virus. In this review, we summarized and discussed the current knowledge of the role of Kupffer cells, the liver resident macrophages, in HBV infection.

- 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.

## Introduction

Hepatitis B virus (HBV) is a hepatotropic virus that chronically infects around 250 million people worldwide (WHO, 2017). Upon exposure to the virus, 90-95% of immune-competent adults will clear the infection, whereas 5-10% will develop a chronic infection. In these chronically HBV-infected (CHB) individuals, the risk of developing, in the long-term, cirrhosis and hepatocellular carcinoma is greatly increased (Testoni et al., 2017). Despite an efficient prophylactic vaccine that protects from infection and treatments that induce a viro-suppression (i.e., reduction of viral load in blood), a complete cure of CHB individuals is not achievable yet, due to the persistence of an episomal viral DNA template, called cccDNA (i.e., covalently-closed-circular DNA), inside the nucleus of infected cells (Nassal, 2015). The development of new treatments is therefore still necessary. One major cause of HBV chronicity comes from a non-optimal immune response against the virus and infected cells during the acute infection phase and after progression to a persistent infection (Fletcher et al., 2012; Wieland et al., 2004a). Why and how the virus interacts with and impairs immune cell functions still remains to be fully understood. In this review, we focus on the interaction between the virus and one specific type of immune cells: the macrophages.

## Macrophages phenotypes and functions

Macrophages (M $\Phi$ ) were first described, by Ilya Metchnikoff at the end of the 19<sup>th</sup> century, as evolutionary conserved phagocytes that have evolved for more than 500 million years (Gordon, 2016). Over the last 2 centuries, a lot has been learnt about M $\Phi$  embryonic origins, phenotypes of different subpopulations, and their various functions.

In solid organs, M $\Phi$  can have two origins. Resident macrophages comes from the yolk sac whereas infiltrating macrophages, which are recruited upon pathogen contamination and/or injury, are derived from the differentiation of peripheral blood circulating monocytes, themselves primarily coming from the bone marrow (Ginhoux and Guilliams, 2016). Depending on the tissue, it is not always possible to distinguish resident M $\Phi$  from infiltrating ones. For

example, in the human liver, no specific marker has been identified to differentiate these two types of MΦ.

Tissue resident MΦ represent the first line of defence against pathogens such as bacteria and viruses, and are therefore an important component of the innate immune response to infections. In addition to their physiologic phagocytic capacity, which allows the elimination of physiologically aging, or infected, or cancerous cells, MΦ can importantly also contribute to adaptive immunity by processing and presenting antigens after pathogen engulfment and their recognition through Pattern Recognition Receptors (PRR)(Hume, 2015). Upon activation, MΦ can secrete a large spectrum of pro-inflammatory cytokines involved in different activities such as lymphocyte polarization (IL-1, IL-12, IFN- $\gamma$ ...) or neutrophil recruitment (IL-8, TNF $\alpha$ ...), and also anti-inflammatory cytokines involved in inflammation regulation (IL-10 and TGF $\beta$ ), as well as chemokines involved in leukocyte recruitment to the sites of injury (CXCL8...)(Martins-Green et al., 2013) and others factors, such as VEGF implicated in angiogenesis(Pollard, 2008).

MΦ can have a large variety of phenotypes that can be classified by specific marker expression and/or secreted cytokines(Hume, 2015; Murray et al., 2014). Several classifications have been proposed over the years, but a consensus is yet to be found. Nevertheless, they can be divided into two major phenotypes: pro- and anti-inflammatory. Of note, the pro-/anti-inflammatory dichotomy is a simplified view since each MΦ expresses a set of genes that on its own could define a new type of MΦ(Hume, 2015).

Pro-inflammatory MΦ, called M1, have been defined as mediators of the defence against bacterial and viral pathogens. They are implicated in inflammation, tumour resistance and killing of intracellular pathogens(Mantovani et al., 2004). M1 are commonly described as STAT1<sup>+</sup>, IRF5<sup>+</sup>, and iNOS<sup>+</sup>, and can secrete a large panel of pro-inflammatory cytokines (IL-6, IL-1 family, IL-12, IL-23, TNF $\alpha$ ...), chemokines (CXCL9, CXCL10, CXCL11, CCL3, CCL5...), and inflammatory mediators (i.e. reactive oxygen species or ROS)(Mantovani et al., 2004). M1 MΦ have a “broken Krebs cycle” leading to the accumulation of citrate and subsequently NO production, as well as the accumulation of succinate, HIF1 $\alpha$  and IL-1 $\beta$  secretion(O’Neill, 2015). M1 MΦ are reported to be able to activate tumour-killing mechanisms and to amplify Th1 responses, inducing a positive feedback in the anti-tumour response(Biswas and Mantovani, 2010). Indeed, M1 macrophages can present antigens to induce an adaptive immune response but can also reactivate lymphocytes locally, at the injury/infection site. Although pro-

inflammatory M $\Phi$  are mainly implicated in the elimination of noxious signals or microorganism invaders, M1-like macrophage-derived cytokine production have also been identified as key factors in several autoimmune and chronic inflammatory diseases, including Crohn's disease, multiple sclerosis, and autoimmune hepatitis(Murphy et al., 2003; Smith et al., 2009).

Anti-inflammatory M $\Phi$ , commonly called M2, are mainly implicated in the resolution of inflammation and wound healing, but also, in some circumstances and by mechanisms not fully understood, in the defence against parasites(Chávez-Galán et al., 2015). M2 are commonly described as STAT6<sup>+</sup>, IRF4<sup>+</sup>, and CD163<sup>+</sup>, and can secrete anti-inflammatory cytokines (IL-10, TGF $\beta$ ), chemokines (CCL1, CCL17, CCL24...) and growth/angiogenic factors (PDGF, VEGF, EGF). In contrast to M1, M2 M $\Phi$  have a "functional Krebs cycle" allowing production of ATP and glycosylation of M2-associated receptors(O'Neill, 2015). M2 macrophages are mandatory for the resolution of inflammation, and tissue repair after an injury and/or cytotoxic activity(Mantovani et al., 2004). However, M2 are also associated with tumour progression through the secretion of negative immuno-modulators, and are called, in the tumour micro-environment, TAM for "tumour associated macrophages"(Tang et al., 2013). TAM have been associated to the progression of numerous cancers and as such are potential new target for therapeutic purposes (Murray and Wynn, 2011; Ries et al., 2014; Tang et al., 2013).

### **Kupffer cells: the liver macrophages**

Kupffer cells (KC), the liver resident M $\Phi$ , was first described by Fahimi in 1970 as peroxidase positive cells(Fahimi, 1970). KC are the largest M $\Phi$  population in the human body as they represent 80% of the total human M $\Phi$  count under normal physiological conditions(Crispe, 2009; Seki et al., 2000). KC are localized in liver blood vessels, seeded on liver sinusoidal endothelial cells and their fenestration, where they can phagocytose debris, aging blood cells or pathogens. After phagocytosis, pathogens can be processed into phagolysosomes and antigens can be presented to lymphocytes to activate or recall a local immune response(Crispe, 2009; Liaskou et al., 2012). As resident M $\Phi$ , KC express all common M $\Phi$  markers (i.e. CD68, CD14...)(Ginhoux and Guilliams, 2016) and have more precisely been

defined as CD14<sup>+</sup>, HLA-DE<sup>+</sup>, HLA-ABC<sup>+</sup>, CD86<sup>+</sup>, DC-SIGN<sup>+</sup> with low expression of CD1b, CD40 and CD83(Tu et al., 2008). As previously mentioned, in humans, no specific marker has been identified to differentiate KC from infiltrating macrophages, whereas in the mouse (i.e. Clec4F)(Scott et al., 2016) and rat (i.e. ED-1 and ED-2)(Zeng et al., 2013) markers have been described. In addition, KC have a long life-span (more than a year) and are self-renewing cells with a slow replicating activity. Thus the liver MΦ pool can be renewed, unlike monocyte derived macrophages which have a limited life-span(Naito et al., 1997). Interestingly, a recent study by Scott and colleagues described that in mice monocyte derived MΦ can, with time, acquire KC phenotype (including self-renewal capacity) after depletion of the resident MΦ in a model of KC expressing the diphtheria toxin receptor(Scott et al., 2016).

KC play a critical role in liver tolerance. Indeed, the liver is at the crossroad between systemic and enteric circulation, with 80% of the blood reaching the liver coming from the hepatic portal vein. Thus, liver cells are constantly exposed to pathogens. Indeed, lipopolysaccharide (LPS) endotoxin is found in measurable amounts (few nano grams) in the enteric circulation(Prytz et al., 1976). This phenomenon should theoretically lead to a constant inflammation within the liver but none is observed in healthy individuals. The liver is indeed described as an immune-tolerant organ(Crispe, 2009). This tolerance has been defined as a systemic or “oral” tolerance of enteric pathogens, which depends on the normal connection between the liver and gut(Yang et al., 1994). Yet, the full role of KC in this tolerance phenomenon still need further investigation, especially by assessing KC phenotype/state in healthy liver. Indeed, the anti- or pro-inflammatory phenotype of KC at steady state is yet to be properly determined. Depending on their localization in the liver, MΦ may have different phenotypes. It could be hypothesised that near the enteric circulation, where the exposition to bacteria is constant, KC will be more likely to be M2 MΦ, the MΦ implicated in tolerance, through the secretion of immune-regulatory mediators(Ju and Pohl, 2005). In contrast, near the systemic circulation, where the presence of pathogens is a sign of infection, KC would be more likely to be in a pre-pro-inflammatory state in order to quickly eliminate any blood infections. Moreover, other factors could play a role in the fate of KC phenotype. The oxygen gradient in the liver is linked to the expression of hypoxia regulated genes (HIF family), among which is HIF1 $\alpha$ , a factor linked to the accumulation of IL-1 $\beta$  in M1 MΦ(Kietzmann, 2017).

As previously described, even in this tolerant environment, KC are able to respond to the detection of specific pathogens (Knolle et al., 1995a; Seki et al., 2000; Tu et al., 2008). KC express a large spectrum of PRR, among which, TLR1/2, TLR2/6, TLR3, TLR4, TLR8 and RIGI/MDA5 (Faure-Dupuy et al., 2018; Sun et al., 2016), leading, after stimulation, to the secretion of a large spectrum of immune factors such as ROS, cytokines (IL-6, IL-10, IL-12, TNF $\alpha$ ...), and chemokines (CCL3, CCL5...) among others (Knolle et al., 1995b; Sica et al., 2014; Tacke, 2017; Tu et al., 2008). KC have also been described to be functional for several inflammasomes (AIM2, NLRP3), which, upon stimulation, induce the production, maturation and secretion of IL-1 $\beta$  and IL-18 (Yu et al., 2017; Zannetti et al., 2016). Chemokines and cytokines secretion will further recruit and activate monocytes from the circulating blood that will locally differentiate and further reinforce the M $\Phi$  population and inflammation (Tacke, 2017). Thus in order to chronically infect the liver, pathogens (such as hepatitis viruses) must have evolved strategies to escape or prevent recognition by the immune system and liver inflammation.

In summary, the liver is both an immune-tolerant (Crispe et al., 2006) and immune-competent organ (Crispe, 2009), in which Kupffer cells play a central role in preventing the circulation in the blood of pathogens and aberrant cells.

### **Interaction between KC and HBV**

The study of HBV infection often focuses on the hepatocytes in which the virus replicates. Viral antigens are defined as secreted viral components which can be protein (hepatitis B e antigen; HBeAg), subviral particles (hepatitis B surface antigen embed in lipid membrane; HBsAg), and virions containing an icosahedral capsid enveloped by surface protein (empty virions, containing no viral genome or Dane particles, the infectious particles). However, the effect of the production of infectious particles and subviral particles in the liver and in the systemic circulation is still largely unknown. To note, several studies have shown that HBV replicative intermediate can be found in peripheral blood mononuclear cells (Gao et al., 2017; Loustaud-Ratti et al., 2013; Shi et al., 2017b), yet, HBV is not able to replicate inside KC (i.e. no secretion of HBeAg and HBsAg, and no detection of HBV RNA) (our unpublished data). It has been described that HBV infection can modify the functions of several immune cells such as natural

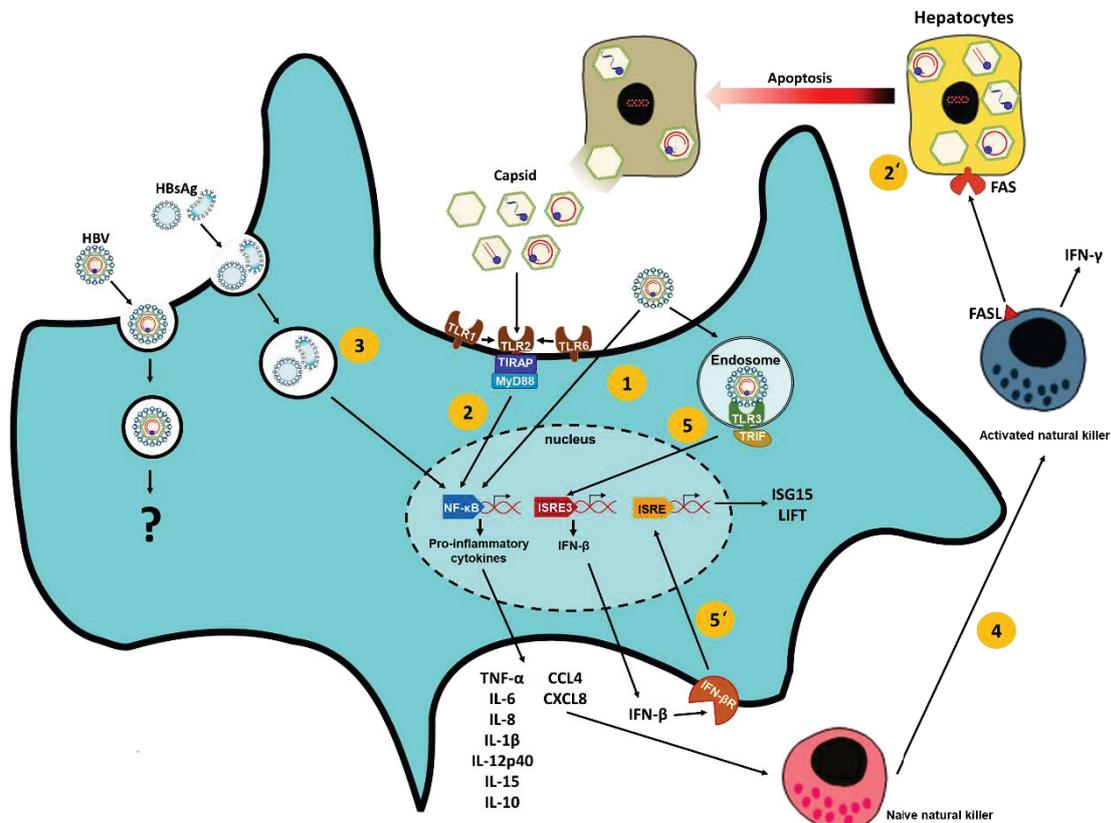
killer (NK) cells, T cells, and granulocytic myeloid derived suppressive cells (gMDSC)(Pallett et al., 2015b). We will now focus on the interaction of HBV and macrophages from four different angles: i) Can macrophages recognize HBV? ii) Can HBV modify the phenotype and function of MΦ? iii) Do MΦ play a role in the establishment or persistence of the infection? iv) Do MΦ play a role in the development of pathologies associated with HBV infection?

### **Can liver macrophages recognize hepatitis B virus?**

Liver MΦ are equipped to recognize pathogens and mount an efficient pro-inflammatory response if needed to prevent, control and delay infections that could become chronic, as described above. A study in Pekin duck revealed that minutes after inoculation HBV was taken up by KC, highlighting their interaction *in vivo*(Tohidi-Esfahani et al., 2010). As a DNA virus with RNA intermediates, HBV could theoretically be recognized by several PRR expressed by KC such as TLR3 or RIGI/MDA5 as well as some cytosolic DNA sensors(Faure-Dupuy et al., 2018). However, the recognition of HBV nucleic acids may be impaired by (i) the physical separation of encapsidated rcDNA (relaxed circular DNA, a partially double stranded DNA) from cytosolic DNA sensors, and (ii) the resemblance between HBV RNAs and host mRNAs as viral RNAs are transcribed by the host polymerase. Indeed, several studies have shown that in animal models, primary exposition to HBV elicits little to no immune responses(Fletcher et al., 2012; Wieland et al., 2004a). Even though the authors did not specifically analyse the responses in macrophages, the lack of immune responses in liver biopsies may be also interpreted as an absence of response in macrophages.

In the KC, the situation may be different as although they do not support viral replication (i.e. no neo formation of HBV RNAs), viral components could be present after phagocytosis and viral epitopes could be recognised by immune receptors. Indeed, after purification of different subsets of human liver cells, Hösel *et al.* showed that non-parenchymal cells (i.e., all liver cells except hepatocytes) and especially KC were activated after a 3h of exposure to HBV. They observed a transient activation of nuclear factor kappa B (NF-κB) and the secretion of pro-inflammatory cytokines (IL-6, IL-8, TNF-α, and IL-1β) without any induction of an interferon response(Hösel et al., 2009b) (Figure 1, path#1). This results were recently confirmed by Cheng and colleagues who showed that in monocyte derived macrophages exposed to high

quantities of HBV, a transient (between 0.5 to 6h) increase of IL-6, IL-1 $\beta$ , and TNF- $\alpha$  mRNAs was observed(Cheng et al., 2017) (Figure 1, path#1).



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

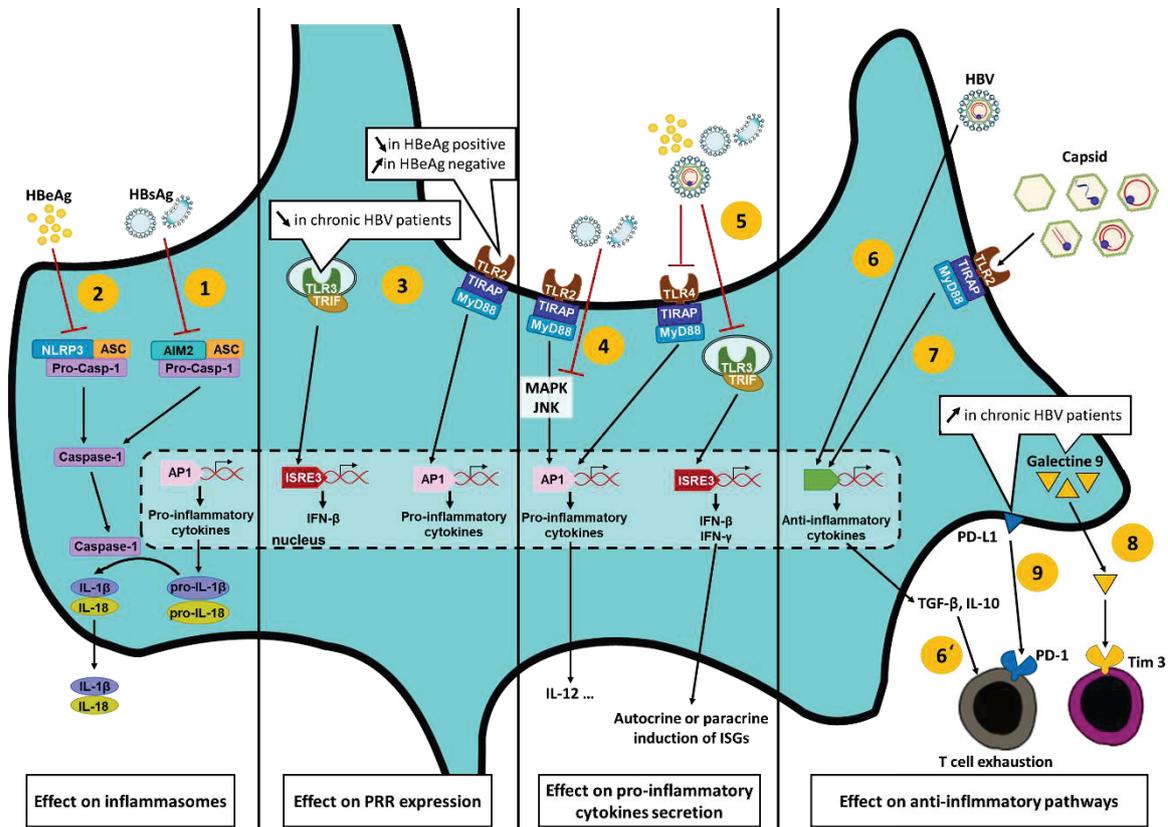
Unexpectedly, TLR2, a PRR implicated in the recognition of bacterial components, has been described to recognize HBV capsid/core protein (Hbc)(Cooper et al., 2005). Cooper and colleagues found that, in a monocyte cell line, THP1, a full-length Hbc was bound to monocyte receptors, leading to their differentiation into M $\Phi$ . Moreover, this binding strongly induced the production of the pro-inflammatory cytokines TNF- $\alpha$ , IL-6, and IL-12p40(Cooper et al., 2005) (Figure 1, path#2). These inductions were dependent of NF- $\kappa$ B, ERK-1/2, and p38 MAPK activation. The authors demonstrated the necessity of binding between the arginine-rich C terminus of Hbc and membrane heparin sulphate for a recognition by monocytes(Cooper et al., 2005). However, the relevance of this model is to be questioned as capsid proteins are normally enveloped in the virion when circulating outside of the hepatocytes, which should prevent recognition by TLR2 ligands, and M $\Phi$  activation. Nevertheless, in the case of cell

death, it may be possible that naked capsid could be released out of the hepatocytes. For example, a study by Arzberger *et al.*, showed that apoptosis of HBV infected hepatocytes led to the release of naked nucleocapsid(Arzberger *et al.*, 2010) which could theoretically induce TLR2-mediated M $\Phi$  activation (Figure 1, path#2'). In addition, a study performed with samples from HBV-positive patients showed that KC stained positive for HBsAg and were more activated than those from control livers(Boltjes *et al.*, 2015). Internalization of HBsAg was also observed *in vitro* using purified KC or monocyte-derived M $\Phi$  and led to a strong induction of pro-inflammatory cytokines such as IL-6, TNF- $\alpha$ , and IL-15 as well as the anti-inflammatory cytokine IL-10, the chemokines CCL4 and CXCL8(Boltjes *et al.*, 2015) (Figure 1, path#3) and the subsequent activation of IFN- $\gamma$  production by NK(Boltjes *et al.*, 2015) (Figure 1, path#4). Moreover, a study in HBsAg-transgenic mice showed that KC secretes IL-12 upon TLR9 ligand inducing liver injury caused by NK(Hou *et al.*, 2016). In this model, an increase of Fas ligand (FasL) and Fas proteins were observed respectively on NK and PHH, leading to an induction of a cytotoxic response against PHH(Hou *et al.*, 2016) (Figure 1, path#2').

Some studies have shown that HBV could also induce an interferon-dependent immune response, which should have a direct antiviral effect on HBV infection in hepatocytes, indeed Peg-IFN $\alpha$  is currently used to treat HBV infection(Durantel and Zoulim, 2016). Using a transgenic mouse model allowing high levels of HBV replication, Real *et al.* recently showed that HBV replication lead to a TLR3 dependent interferon response in non-parenchymal liver cells(Real *et al.*, 2016) (Figure 1, path#5). A significant up-regulation of IFN- $\beta$ 1 and subsequently ISG15 and LFIT1 mRNAs was observed in mouse model in which the small HBsAg is not produced (Figure 1, path#5'). Of note, HBsAg has previously been described to attenuate immune responses facilitating chronic infection (see section on HBV modification of KC phenotype)(Jiang *et al.*, 2014a; Wu *et al.*, 2009a; Zannetti *et al.*, 2016).

In summary (Figure 1), contradictory results regarding the potential activation or lack of activation of an immune response in M $\Phi$  by HBV have been reported. These discrepancies might come from the differences in model (chimpanzees, woodchuck, transgenic mice...) and virus (genotypes, purification, naked nucleocapsid...) used in different experimental approaches. The lack of responses in HBV-infected animal models(Fletcher *et al.*, 2012; Wieland *et al.*, 2004a), compared to results demonstrating that macrophages seems to be equipped to recognize HBV(Boltjes *et al.*, 2015; Cheng *et al.*, 2017; Cooper *et al.*, 2005; Hösel

et al., 2009b; Hou et al., 2016; Real et al., 2016), raises the possibility of an active HBV-driven evasion mechanism which could contribute to the establishment and persistence of infections *in vivo*.



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

### Can HBV modify macrophage phenotype and function?

Several studies have described inhibitory mechanisms used by HBV to block the induction of a potential pro-inflammatory/anti-viral response, including inflammasome inhibition, immune receptor down-regulation, pro-inflammatory secretion inhibition, increase of anti-inflammatory secretion, and negative immune checkpoint induction.

First, HBV has been shown to interfere with inflammasome activation. Zannetti *et al.* determined that KC production of IL-1 $\beta$  by AIM2 inflammasome engagement was blocked by HBV, through an HBsAg-dependent mechanism (Zannetti et al., 2016) (Figure 2, path#1). This inhibition seemed to be specific to the AIM2 inflammasome, as HBV was not capable of blocking IL-1 $\beta$  production driven by other inflammasomes, including NLRP3 (Zannetti et al., 2016). However, Yu *et al.* recently showed that HBV could also inhibit NLRP3 induced IL-1 $\beta$

secretion by KC and that this inhibition was HBeAg dependent (Yu et al., 2017) (Figure 2, path#2). This difference in results may come from differences in the HBV inocula used and especially the quantity of HBeAg in the viral preparations. Although mechanisms involved may differ, both studies described an inhibition of IL-1 $\beta$  secretion by KC after exposure to HBV components. HBV has likely evolved several strategies to block the production of IL-1 $\beta$ , which has been recently described as the most efficient cytokine, amongst several tested (including IFNs) against the replication of HBV *in vitro* (Isorce et al., 2016).

In addition, HBV has been shown to prevent pro-inflammatory cytokine secretion by influencing PRR expression in M $\Phi$ . A down-regulation of TLR2 and TLR3 was found in peripheral blood mononuclear cells (PBMC) and KC of chronically HBV-infected patients compared to healthy controls (Huang et al., 2013b; Visvanathan et al., 2007) (Figure 2, path#3). As TLR2 and TLR3 have been shown to, respectively, recognize HBc protein (Cooper et al., 2005) and HBV genome replication in non-parenchymal cells (Real et al., 2016), a down-regulation of their expression in hepatocytes, KC and PBMCs may contribute to the lack of an efficient immune response against HBV (Real et al., 2016). However, another study showed a higher secretion of IFN- $\alpha$  and - $\beta$ , as well as IL-6 and IL-10 upon TLR3 stimulation in PBMC from HBV patients compared to healthy controls, contradicting the results of a down-regulation of TLR3 in monocytes (Jiang et al., 2014a). Moreover, in HBV patients, TLR2 expression on KC and peripheral monocytes was reduced in HBeAg-positive chronic HBV patients (i.e. immune-tolerant/immune active patients) compared to controls individuals, whereas it was significantly increased in HBeAg-negative chronic HBV patients (e.g., in inactive HBV carrier) compared to controls (Visvanathan et al., 2007). This modification of expression was specific to TLR2, as TLR4 expression did not differ between groups. It appears that the effect of HBV on TLR expression may be differentially regulated during the different phases of HBV chronic infection that would partially explain the difference of immune responses observed during those phases.

HBV may also directly target pro-inflammatory cytokines secretion. For example, in CHB patients, HBsAg induced an inhibition of IL-12 secretion by monocytes/macrophages after TLR2 stimulation through the interference of HBV with the JNK/MAPK pathway (Wang et al., 2013) (Figure 2, path#4). To further decipher what happens in the liver, the team of Pr Schlaak purified murine KC and exposed them to some TLR-ligands in the presence or absence of

HBsAg, HBeAg or HBV virions. Upon exposition to any of these viral components, expression of interferon sensitive genes and pro-inflammatory cytokines was inhibited in KC (Jiang et al., 2014a) (Figure 2, path#5). In addition, Wu *et al.* have shown that stimulating KC with TLR3 or TLR4 ligands induced the production of IFN $\beta$  and the inhibition of viral replication in hepatocytes in mice (Wu et al., 2007). However, this antiviral effect was lost when KC were pre-incubated with HBV (Wu et al., 2009a) (Figure 2, path#5). Among the mediators inhibiting HBV infection, IFN- $\alpha$  and IFN- $\gamma$  were identified and shown to directly inhibit HBV replication in patients (Isorce et al., 2015). Moreover, a recent study showed that HBV virions hijack enzymes involved in lipid metabolism to infect hepatocytes (Esser et al., 2018). It could be hypothesised that the same pathways could be also hijack by HBV in M $\Phi$  to enter or pass-through these cells to be delivered to hepatocytes.

Aiming at the same goal, others studies have shown that in addition to preventing pro-inflammatory responses, HBV also increases anti-inflammatory responses. In purified rat KC exposed to different quantities of HBV virions, no morphological changes were observed on KC, but, compared to non-infected animals, TGF- $\beta$  secretion was increased from 5.38 at day-3 to 7.75 fold at day-7 post-exposure (Li et al., 2012) (Figure 2, path#6). In this study, no modification in the levels of pro-inflammatory cytokines IL-1, IL-6 and TNF- $\alpha$  was observed compared to non-HBV-exposed cells. However, the authors neither indicated the secretion level of those cytokines in both controls and HBV exposed KC nor did they examine if the pro-inflammatory cytokines could be secreted by KC after TLR stimulation and modified by incubation with HBV (Li et al., 2012). In addition, Jiang *et al.* demonstrated that in non-parenchymal liver cells exposed to HBV, the expression of anti-inflammatory cytokines (IL-10 and TGF $\beta$ ) was enhanced (Jiang et al., 2014a) (Figure 2, path#6). This phenotype was associated with an inhibition of NF- $\kappa$ B, IRF-3 and MAPKs pathways in these cells. Specific T-cell activation by KC was also impaired when cells were exposed to HBsAg, but this phenotype could be reverted by a treatment with IL-10 antibodies, suggesting that the effect of HBV on KC impairment of T cell response was mediated by this cytokine (Jiang et al., 2014a) (Figure 2, path#6'). Furthermore, in an established HBV carrier mouse model, Li *et al.* showed that TLR2 deficiency improved HBV elimination, thus indicating that TLR2 could modulate HBV persistence (Li et al., 2015). Liver M $\Phi$  were shown to have an increased TLR2 expression in HBV-carrier mice and to produce more IL-10 upon TLR2 activation by an HBcAg stimulation

leading to a poor induction of CD8<sup>+</sup> T cells (Li et al., 2015; Xu et al., 2014) (Figure 2, path#7). Indeed, IL-10 deficiency or anti-IL-10R treatment resulted in a functional CD8<sup>+</sup> T cell response and HBV elimination in this mice model (Li et al., 2015; Xu et al., 2014). To get further insights into the context of a primary infection, Dunn *et al.* analysed a cohort of 21 patients with acute HBV, including 8 patients analysed before the peak of viremia, when the infection is still being established (Dunn et al., 2009). At the peak of viremia, during the inhibition of lymphocyte activation, a peak of IL-10 was observed in the serum. Moreover, in chronically infected HBV patients an increase of CD68<sup>+</sup> CD86<sup>+</sup> MΦ was observed, promoting a specific Th2 response, thus promoting of phagocytic-independent inflammation (Said et al., 2016). Lastly, Nebbia *et al.* described that in CHB patients there is an up-regulation of Galectin-9 on KC as well as an up-regulation of HBV specific Tim3<sup>+</sup> CD8 T cell (Nebbia et al., 2012) (Figure 2, path#8). This Tim-3/Galectin-9 pathway up-regulation could be the cause of T cell exhaustion during chronic infection (Nebbia et al., 2012).

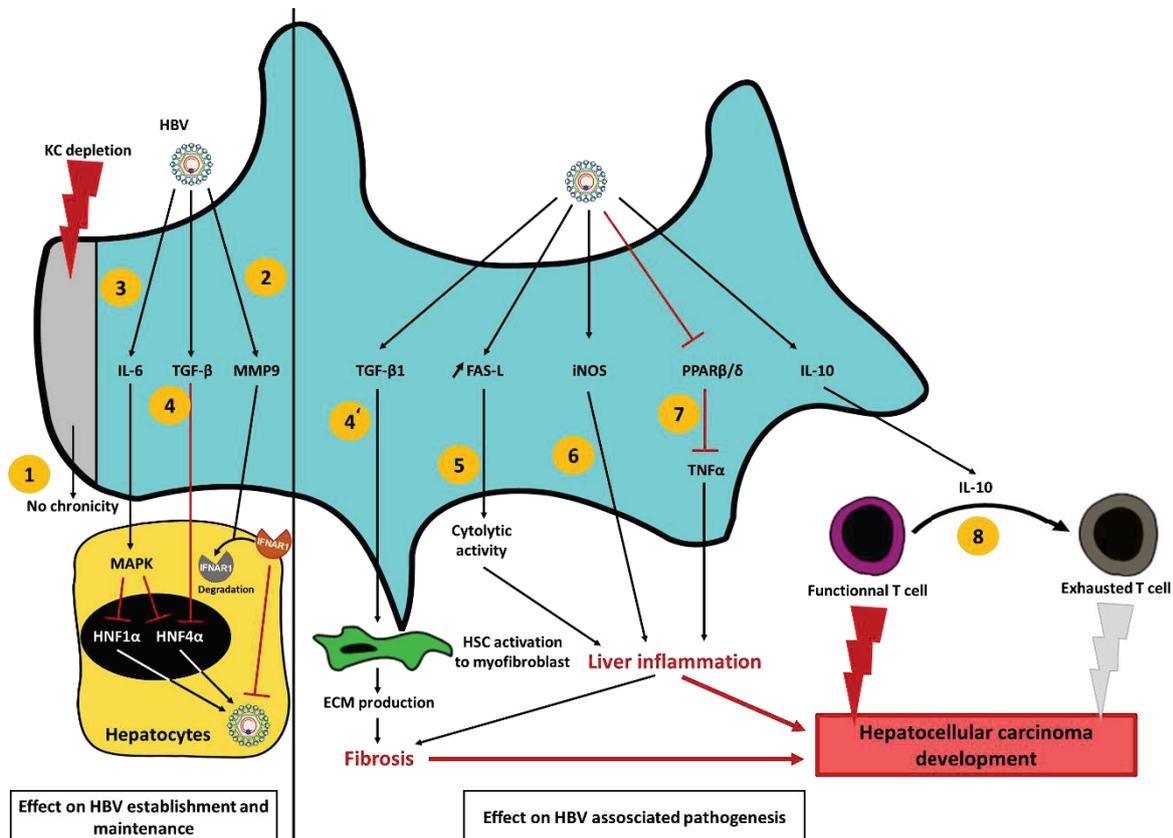
Finally, Programmed Death-1 (PD-1) and its ligands (PD-L1), proteins implicated in tolerance (Wang et al., 2016), have been described to be essential to balance antiviral immunity and inflammation in acute HBV patients (Kassel et al., 2009). A study carried-out with 32 chronic HBV patients, showed an up-regulation of PD-1 and PD-L1 in liver biopsies compared to healthy controls (Xie et al., 2009) (Figure 2, path#9). The same results were found in a chronic carrier mouse model (Tian et al., 2016). PD-1/PD-L1 up-regulation correlated with hepatic inflammation and increased alanine transaminase levels in patients (Xie et al., 2009) and with impaired cytotoxic T lymphocytes response in mice (Tian et al., 2016). However, PD-L1 expression was lower in liver residential antigen presenting cells, including KC (Xie et al., 2009), during the inactive carrier phase. Moreover, in mice, PD-L1 blockade or KC depletion led to an inhibition of HBV viral parameters, as well as an increase of IFN-γ<sup>+</sup> CD8<sup>+</sup> cells (Tian et al., 2016). Another study further showed that PD-L1 expression was up-regulated in CD14<sup>+</sup> circulating cells during chronic HBV infection, liver cirrhosis and hepatocellular carcinoma (Huang et al., 2017). PD-1/PD-L1 interaction may therefore play an important role in balancing liver damage during chronic HBV infection (Xie et al., 2009).

In summary (Figure 2), HBV seems to block pro-inflammatory responses in KC and peripheral monocytes/macrophages, correlating with a poor elimination of the virus. On the other hand, HBV strongly activates anti-inflammatory cytokines secretion (IL-10 and TGF-β) as well as

inhibitory checkpoint factors (e.g., PD-1/PD-L1). Overall, it seems that HBV concomitantly inhibits a pro-inflammatory/anti-viral M1-like phenotype, and activates an anti-inflammatory/pro-tolerogenic M2-like phenotype.

**Do macrophages promote the establishment or persistence of the infection?**

As the first line of defence against pathogens, KC should counteract infection of hepatocytes by HBV. A study performed in ducks infected with DHBV (duck hepatitis B virus) showed that endotoxin stimulation could inhibit DHBV replication in primary duck hepatocytes (i.e., accumulation of viral proteins and amplification of the nuclear extrachromosomal DHBV DNA templates)(Klöcker et al., 2000). The authors further observed that this inhibition was due to non-parenchymal cells (NPC)-mediated cytokine secretion, and more probably to KC, which contaminates isolated hepatocytes. Nevertheless, HBV is able to chronically infect the liver, and counteract/escape these immune responses. The role of KC in the establishment and persistence of the infection remains unclear.



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

Interestingly, in a mouse model the depletion of KC prior to HBV infection prevented the establishment of a chronic infection (Xu et al., 2014) (Figure 3, path#1). This effect was correlated with the secretion of IL-10 by KC, which impaired the humoral response against HBV infected hepatocytes (Xu et al., 2014). Moreover, Chen *et al.* have shown that the matrix metalloproteinase 9 (MMP-9) is up-regulated in PBMCs of chronic hepatitis B patients and can be activated by HBV in PBMCs and macrophages *in vitro* (Chen et al., 2017). This up-regulation may favour the infection as the authors further showed that MMP-9 enhanced HBV replication in hepatocytes through binding to IFNAR1 (IFN $\alpha$  receptor 1), inducing its ubiquitinylation and degradation (Chen et al., 2017) (Figure 3, path#2). IFNAR1 degradation prevents the binding of type I IFN and the associated antiviral effects.

In contrast, some studies have shown that KC may play a role in HBV elimination. Hösel *et al.* showed that KC exposed to HBV secreted more IL-6, inducing the activation of the mitogen-activated protein kinases (MAPK) in PHH, leading to the inhibition of hepatocytes nuclear factor (HNF) 1 $\alpha$  and 4 $\alpha$ , two transcriptional factors essential for HBV replication (Hösel et al., 2009b) (Figure 3, path#3). Another study conducted with HepG2 cells, showed that a treatment with TGF- $\beta$ 1 (cytokine secreted by KC exposed to HBV (Li et al., 2012)) could suppress HBV replication (Hong et al., 2012). TGF- $\beta$ 1 specifically reduced HBV core promoter activity, and, subsequently, viral pre-genomic RNA, core protein, and HBV replication, through repression of HNF 4 $\alpha$  expression (Hong et al., 2012) (Figure 3, path#4).

In summary (Figure 3), it is still unclear if KC plays a role in the establishment and persistence of the infection and further studies are required. The discrepancies between results may be due to the phenotype (pro- or anti-inflammatory) of KC during primary infection and could be one of the key factors involved in the infection outcome.

### **Do macrophages play a role in the development of pathologies associated with HBV infection?**

KC have been described to play a role in several liver associated pathologies including aberrant responses (Ju and Pohl, 2005; Seki et al., 2000) and may be associated with of HBV-associated liver pathologies including fibrosis, cirrhosis and the progression to hepatocellular carcinoma (Levrero and Zucman-Rossi, 2016).

As described above, Li *et al.* have shown an increase of TGF- $\beta$ 1 secretion by KC in the presence of HBV(Li et al., 2012). TGF- $\beta$ 1 can directly promote fibrosis by inducing the differentiation of hepatic stellate cells into myo-fibroblasts, subsequently stimulating the production of extracellular matrix components(Wynn and Barron, 2010) (Figure 3, path#4').

In addition to liver fibrosis, KC plays an important role in liver inflammation/tolerance and injury associated to uncontrolled inflammation. Different populations of macrophages have recently been described in the liver of chronically infected animal models and patients. A study showed that end-stage HBV liver disease is associated with an accumulation of CD14<sup>+</sup>HLA-DR<sup>hi</sup>CD206<sup>+</sup> macrophages(Tan-Garcia et al., 2017). These cells expressed spontaneously pro-inflammatory markers (TNF- $\alpha$ , GM-CSF, Caspase 1...) and had a low expression of anti-inflammatory markers (arginine 2, PPARG...) with the exception of IL-10 which was increased(Tan-Garcia et al., 2017). This population has been described as resistant to LPS tolerization. Another population of CD205<sup>+</sup> macrophages has been described in the liver of HBV transgenic mice and chronic hepatitis B patients(Yong et al., 2017). This population presented a higher expression of activation markers, pro-inflammatory cytokines, chemokines/chemokine receptors, and phagocytosis related genes(Yong et al., 2017). Moreover, in patients with high ALT levels, a large decrease of CD8<sup>+</sup> T lymphocytes and increase of Fas-L positive cells, especially KC, were shown(Tang et al., 2003). This observation led the authors to the hypothesis that during chronic infection, liver injury could not be the result of CD8<sup>+</sup> T cell response but an increase of Fas-L expression on KC and, through an increased cytolytic activity(Tang et al., 2003) (Figure 3, path#5). Another study performed in liver biopsies from 74 chronically infected patients revealed that iNOS intrahepatic expression was significantly higher in patients with chronic HBV(Wu et al., 2008). In these samples, Kupffer cells stained positive for iNOS, a marker of pro-inflammatory M $\Phi$ , which level correlated with ALT level, and with the grading of liver inflammation and staging of liver fibrosis(Wu et al., 2008) (Figure 3, path#6). Moreover, as previously described, peroxisome proliferator-activated receptor- $\beta/\delta$  (PPAR $\beta/\delta$ ) activation was shown to inhibit steatosis and inflammation, well-known cancer risks factors(Nagasawa et al., 2006). In a model of HBV transgenic mice, activation of PPAR $\beta/\delta$  reduced steatosis and tumour multiplicity(Balandaram et al., 2016). PPAR $\beta/\delta$  activation was also correlated with reduced *Tnfa* mRNA levels, as well as, reduced ALT level but increased apoptotic signalling(Balandaram et al., 2016) (Figure 3,

path#7). Ligand activation of PPAR $\beta/\delta$  inhibited LPS signalling and through *Tnfa* expression, which could be prevented in a model of KC non-expressing-PPAR $\beta/\delta$ (Balandaram et al., 2016). This data suggested a role of KC in liver inflammation during chronic HBV infection through TNF- $\alpha$  secretion. Finally, tumour progression in chronic HBV infection could be related to the increased of IL-10 secretion described by several teams (Dunn et al., 2009; Li et al., 2015; Xu et al., 2014). Indeed, a tolerogenic environment in the liver could lead to a non-efficient lymphocyte response and consequently, to cancer progression(Trehanpati and Vyas, 2017) (Figure 3, path#8). However, a study by Sitia *et al.* showed that in a chronic HBV carrier mouse model, KC, through scavenging receptors, removed apoptotic PHH, blocking the release of high-mobility group box 1 (HMGB1) and neutrophil infiltration in the liver(Sitia et al., 2011). The authors hypothesized that this may prevent liver inflammation and damage during chronic infection.

Taken together these data show that HBV may modify KC phenotype in favour of the development of liver-associated pathologies such as fibrosis, particularly through TGF- $\beta$ 1 secretion, and, ultimately, HCC, through aberrant expression of factors implicated in carcinogenesis. Moreover, KC could play an important role in liver inflammation during chronic HBV and prevent a functional humoral response by largely increasing IL-10 secretion by macrophages.

## Conclusion

Contradictory data has been published with respect to the lack or activation of immune responses in M $\Phi$  during HBV infection. The absence of responses seen in HBV-infected animal model(Fletcher et al., 2012; Wieland et al., 2004a), despite the fact that liver macrophages are capable of recognising HBV(Boltjes et al., 2015; Cheng et al., 2017; Cooper et al., 2005; Hösel et al., 2009b; Hou et al., 2016; Real et al., 2016), raises the possibility of an active HBV-driven immune evasion mechanism, which could contribute to the establishment and persistence of infections *in vivo*. Several studies have concluded that HBV does modulate M $\Phi$  phenotypes by: i) inhibiting pro-inflammatory responses in KC and peripheral monocytes/macrophages, ii) promoting anti-inflammatory cytokines secretion (IL-10 and TGF- $\beta$ ), and/or iii) increasing inhibitory checkpoint factors expression (e.g., PD-1/PD-L1). Taken together, HBV would

concomitantly inhibit the pro-inflammatory/anti-viral M1-like phenotype, and promote an anti-inflammatory/pro-tolerogenic M2-like differentiation. This phenotype switch may initially favour viral replication but in the long term may also promote the pathogenesis of liver disease, such as fibrosis, cirrhosis and, ultimately, HCC as collateral damage.

A specific modulation of the function of macrophages to restore more favourable T-cell-mediated immune responses against HBV should be tested within the development of complex immune therapeutic strategies to defeat HBV (Bertoletti and Bert, 2018). Therefore a substantial effort should be made to better understand the complex interplays between liver macrophages and HBV infections in order to determine whether a therapeutic intervention aiming at reversing the phenotype switch induced by HBV could favour the inhibition of HBV infection (i.e. by direct mechanisms for example through IL-1 $\beta$  secretion (Isorce et al., 2016), or by indirect mechanisms through the activation of a functional adaptive response) and prevent disease progression.

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## 2. Congress presentations

### a. ANRS AC-31 « Work in progress »

Paris, April 2016.

**Oral:** Interaction between Kupffer cells, hepatocytes and Hepatitis B virus.

Faure-Dupuy S, Aillot L, Dimier L, Zoulim F, Lucifora J\* and Durantel D\*.

### b. 4<sup>th</sup> CRCL day

Lyon, May 2016.

**Poster:** Interaction between Kupffer cells, hepatocytes and Hepatitis B virus.

Faure-Dupuy S, Aillot L, Dimier L, Zoulim F, Lucifora J\* and Durantel D\*.

### c. BMIC doctoral school day

Lyon, December 2016.

**Oral:** Early function modulation and reprogramming of Kupffer cells promotes establishment and maintenance of HBV infection.

Faure-Dupuy S, Aillot L, Dimier L, Zoulim F, Lucifora J\* and Durantel D\*.

### d. 1<sup>st</sup> international symposium on Immune Responses in Cancer and Infection

Lyon, February 2017. **Best poster award.**

**Poster:** Early function modulation and reprogramming of Kupffer cells promotes establishment and maintenance of HBV infection.

Faure-Dupuy S, Aillot L, Isorce N, Dimier L, Testoni B, Lam A, Klumpp K, Zanetti C, Bendriss-Vermare N, Hasan U, Zoulim F, Lucifora J\* and Durantel D\*.

### e. 17<sup>th</sup> meeting of national hepatitis network

Paris, March 2017.

**Oral:** Early function modulation and reprogramming of Kupffer cells promotes establishment and maintenance of HBV infection.

Faure-Dupuy S, Aillot L, Isorce N, Dimier L, Testoni B, Lam A, Klumpp K, Zanetti C, Bendriss-Vermare N, Hasan U, Zoulim F, Lucifora J\* and Durantel D\*.

#### f. **EASL international liver congress**

Amsterdam, April 2017.

**Poster:** Early function modulation and reprogramming of Kupffer cells promotes establishment and maintenance of HBV infection.

Faure-Dupuy S, Aillot L, Isorce N, Dimier L, Testoni B, Lam A, Klumpp K, Zanetti C, Bendriss-Vermare N, Hasan U, Zoulim F, Lucifora J\* and Durantel D\*.

#### g. **International HBV meeting**

Washington, September 2017.

**Oral:** Early function modulation and reprogramming of Kupffer cells promotes establishment and maintenance of HBV infection.

Faure-Dupuy S, Aillot L, Isorce N, Dimier L, Testoni B, Lam A, Klumpp K, Zanetti C, Bendriss-Vermare N, Hasan U, Zoulim F, Lucifora J\* and Durantel D\*.

#### h. **31st Annual meeting of the European Macrophage and Dendritic Cell Society**

Madrid, September 2017.

**Poster:** Early function modulation and reprogramming of Kupffer cells promotes establishment and maintenance of HBV infection.

Faure-Dupuy S, Aillot L, Isorce N, Dimier L, Testoni B, Lam A, Klumpp K, Zanetti C, Bendriss-Vermare N, Hasan U, Zoulim F, Lucifora J\* and Durantel D\*.

#### i. **7<sup>th</sup> seminar on functional genomics in the liver**

Lyon, March 2018.

**Oral:** Early function modulation and reprogramming of liver resident and infiltrating macrophages promote the establishment of HBV infection.

Faure-Dupuy S, Delphin M, Aillot L, Dimier L, Fresquet J, Testoni B, Rivoire M, Lam A, Klumpp K, Zoulim F, Lucifora J\* and Durantel D\*