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Polarization of Hepatic Macrophages by HBsAg: a means to an end for viral maintenance

Marion Delphin

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**Polarization of Hepatic Macrophages by
HBsAg: a means to an end for viral
maintenance**

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“Time is of no importance. Only life is important.”

Mondoshawan – The fifth element

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Résumé

LA POLARISATION DES MACROPHAGES HEPATIQUES PAR HBSAG COMME MOYEN DE MAINTENANCE VIRALE

Les macrophages hépatiques sont impliqués à la fois dans des mécanismes de tolérances et de clairances des pathogènes. Afin de mieux comprendre leur rôle dans l'infections chroniques des Virus de l'Hépatite B (HBV) et de l'Hépatite Delta (HDV), nous avons caractérisé phénotypiquement l'interaction existant *in vivo* et *ex vivo* entre le HBV et macrophages primaires humains (MPH) ou des monocytes primaires, différenciés en macrophages pro- ou anti-inflammatoires (M1-MDMs ou M2-MDMs respectivement).

Les MPH ou MDMs ont été exposé à différents génotypes du HBV et leur activation a été étudié par ELISA ou RT-qPCR. Des biopsies hépatiques de patient chroniquement infectés par HBV ont été analysé par RT-qPCR ou immunohistochimie. Les paramètres viraux du HBV et HDV dans des Hépatocytes Primaires Humains (HPH) et des HepaRG différenciées ont été suivi par ELISA, qPCR ou RT-qPCR.

Dans des biopsies hépatiques de patients HBV, nous avons montré la présence de la protéine de capsid du HBV (HBc) au contact des macrophages, associé à une augmentation des marques anti-inflammatoires de ces derniers. L'exposition *ex vivo* de PLMs à HBV entraine une diminution de leurs sécrétions de cytokines pro-inflammatoires. L'incubation de MDMs avec HBV de génotype C et D entraine une diminution des sécrétions pro-inflammatoires des M1-MDMs (IL-6 et IL-1 β) ainsi qu'une augmentation des cytokines anti-inflammatoires chez les M2-MDMs exposés à HBV-B, C et D. Des expériences de co-cultures nous ont par la suite permis d'identifier la protéine d'enveloppe du HBV (HBsAg) comme étant la principale actrice des modulations observées. HBV et HDV partageant la même protéine d'enveloppe, des modifications similaires sont observées en présence de virions HDV.

De plus, nous avons montré que les cytokines produites par les M1-MDMs sont capable de diminuer l'établissement de l'infection HBV dans les hépatocytes, mais pas celles qui sont produites par des M1-MDMs exposés à HBV. De plus, une forte diminution dose dépendante de la maintenance des ARNs HBV (génotype A à E) et HDV a été observé dans des hépatocytes infectés, suite à un traitement avec de l'IL-1 β recombinant. Cette inhibition semble dépendante de l'activation de la voie NF κ B et entrainer, au moins en partie, la déstabilisation des ARNs viraux.

En conclusion, nos données suggèrent que la protéine de surface d'HBV module les fonctions des macrophages hépatiques pour favoriser l'établissement et la persistance du virus.

Mots clés : Virus de l'Hépatite B, Virus de l'Hépatite D, Macrophages Hépatiques, Interleukine-1 β , pro-inflammatory.

Abstract

POLARIZATION OF HEPATIC MACROPHAGES BY HBsAg: A MEANS TO AN END FOR VIRAL MAINTENANCE

Liver macrophages can be both involved in pathogen clearance and/or pathogenesis. To get further insight on their role during chronic hepatitis B virus (HBV) and hepatitis delta virus (HDV) infections, our aim was to phenotypically and functionally characterize *in vivo* and *ex vivo* the interplay between HBV, primary human liver macrophages (PLM) and primary blood monocytes differentiated into pro-inflammatory or anti-inflammatory macrophages (M1-MDM or M2-MDM, respectively).

PLM or primary blood monocytes either *ex-vivo* differentiated into M1-MDM or M2-MDM were exposed to HBV from different genotype and their activation followed by ELISA or RT-qPCR. Liver biopsies from HBV infected patients were analysed by RT-qPCR or immunohistochemistry. Viral parameters in HBV-infected primary human hepatocytes (PHH) and differentiated HepaRG cells were followed by ELISA, qPCR and RT-qPCR analyses.

We evidenced the presence of HBc protein within macrophages in liver biopsies from HBV-infected patients and higher levels of anti-inflammatory macrophages markers, compared to non-infected ones. *Ex vivo* exposure of naive PLMs to HBV led to a reduced secretion of pro-inflammatory cytokines. Upon exposure to HBV (from genotype C and D) during differentiation and activation, M1-MDM secreted less IL-6 and IL-1 β , whereas M2-MDM secreted more IL-10 when exposed to HBV (from genotype B, C or D) during activation. Using a co-culture approach, we identified HBV envelope particle (HBsAg) as being responsible for the aforementioned modulations of M1-MDMs. As expected, HDV virions, which also display HBsAg, behaved similarly.

We also showed that cytokines produced by M1-MDM, but not those produced by HBV-exposed M1-MDM, decreased HBV establishment in hepatocytes. Besides, we observed a strong dose-dependent decrease of HBV RNAs (from genotypes A to E) and HDV RNAs maintenance upon treatment of infected hepatocytes with recombinant IL-1 β . This inhibition was shown to be dependent on the activation of the NF κ B pathway and would at least involve mechanism targeting HBV RNAs stability.

Altogether, our data strongly suggest that the surface protein of HBV modulates liver macrophage functions to favor the establishment and persistence of the virus.

Key words: Hepatitis B Virus, Hepatitis D Virus, Hepatic Macrophages, Interleukine-1 β , pro-inflammatory.

Résumé substantiel

LA POLARISATION DES MACROPHAGES HEPATIQUES PAR HBSAG COMME MOYEN DE MAINTENANCE VIRALE.

Travaux de thèse effectués par Marion DELPHIN, sous la direction du Dr. Julie LUCIFORA, au sein de l'équipe HepVir dirigée par le Dr. David DURANTEL. Cette thèse a été réalisée dans le Centre International de Recherche en Infectiologie (CIRI) de Lyon, INSERM U1111, dirigé par le Dr. François-Loïc COSSET.

Introduction

Le Virus de l'Hépatite B (HBV) est un virus hépatotrope infectant chroniquement plus de 250 millions de personnes à travers le monde. Il est responsable d'atteintes hépatiques, tels que la fibrose, la cirrhose et le carcinome hépatocellulaire, qui sont responsables de la mort d'un million de porteur chronique chaque année. A ce jour, il existe 10 génotypes du HBV qui ont été décrits (HBV-A à HBV-J), chacun ayant une distribution géographique et une physiopathologie spécifique. De plus, la co-infection avec le Virus de l'Hépatite Delta (HDV), qui est un virus satellite du HBV partageant son enveloppe virale (HBsAg), est associé à une accélération drastique de la progression de la maladie. Plusieurs traitements sont disponibles actuellement, mais aucun ne permet une éradication totale de ces infections. L'interféron alpha est un immunomodulateur utilisé dans le traitement de nombreuses viroses, dont celles liées au HBV et HDV. Seulement, il est accompagné de nombreux effets secondaires et les patients HBV, et plus particulièrement les HBV/HDV, sont souvent non-répondeurs au traitement. C'est pourquoi nous nous sommes intéressés au développement de nouvelles stratégies thérapeutiques afin d'identifier de nouveaux immunomodulateurs efficaces pour contrer ses infections. A cet effet, nous avons montré que l'interleukine-1 beta, parmi un panel de nombreuses cytokines antivirales, était la plus efficace contre HBV, agissant dans des doses peu élevées et donc proches de celles trouvées dans la circulation de patients sains. Plusieurs études semblent suggérer que le HBV est capable d'inhiber les sécrétions de cette cytokine par les macrophages hépatiques, les principales productrices de l'IL-1 β dans le foie. Nous avons donc décidé d'étudier l'interaction existant entre les sécrétions des macrophages du foie et l'établissement et la maintenance du HBV et HDV dans les hépatocytes.

Objectifs

Les objectifs de mes travaux ont donc été les suivants, (1) étudier l'impact du HBV et de ses différents génotypes sur les sécrétions cytokiniques des macrophages hépatiques, (2) Identifier l'agent responsable de ses modulations, (3) comprendre le mode d'action de l'interleukine-1 beta sur l'infection du HBV et HDV dans les hépatocytes et (4) appliquer les connaissances acquises au développement de nouvelles stratégies antivirales.

Matériels et Méthodes

Dans les deux études réalisées, les cellules résidentes du foie (hépatocytes, cellules sinusoidales et endothéliales, cellules hépatiques étoilées et macrophages hépatiques) ont été purifiées à partir de résections hépatiques de patients non-infectées. Les monocytes ont été purifiées à partir de poche de sang périphérique (provenant de dons EFS) puis polarisés vers un phénotype plutôt pro- ou anti-inflammatoire par l'utilisation de GM-CSF et M-CSF respectivement. Ces deux types cellulaires ont été exposés au HBV lors de leur processus de différenciation/activation ou seulement activation pour les macrophages hépatiques (déjà différenciés). Un protocole de co-culture a également été mis en place entre les macrophages dérivés de monocytes et des cellules sécrétant de façon constitutive le HBV (les HepAD38), ou juste (Huh7-HBsAg) ou le HDV (Huh7-HBsAg-HDV+), ainsi que leurs contrôles respectifs (lignées cellulaires HepG2 et Huh7). Pour toutes ses expériences, la détermination des concentrations en cytokines sécrétées (IL-1 β , IL-6 et IL-10) par les macrophages dans les différentes conditions a été obtenue par test ELISA.

Les Hépatocytes primaires humains (issus de résections hépatiques) ainsi que les HepaRG différenciées ont été infectés à une multiplicité d'infection de 100 équivalents génome par cellules pour le HBV et 10 pour le HDV. Une fois l'infection établie (7 jours pour les dHepaRG, 4 jours pour les hépatocytes primaires humains), les cellules ont été traitées avec différentes molécules (RG7834, interféron alpha, Lamivudine), et plus particulièrement l'interleukine-1 beta, ainsi qu'avec des milieux conditionnés produits par récupération du surnageant de macrophages. Différentes cinétiques ont été effectuées, mais de façon générale les paramètres viraux ont été observés 3 jours après le traitement. Les protéines sécrétées (HBsAg et HBeAg) ont été quantifiées par ELISA, l'ADN d'HBV sécrété et le cccDNA par qPCR, l'ARN intracellulaire du HBV et HDV par RT-qPCR.

Résultats

Tout d'abord, des macrophages primaires humains, purifiés à partir de résections hépatiques de patients non-infectées, ont été incubés *ex-vivo* avec le HBV de génotype B, C et D (HBV-B/C/D). Alors que le HBV-C et HBV-D entraînent une diminution des sécrétions pro-inflammatoire (principalement IL-1 β), le HBV-B permet une augmentation drastique des productions d'IL-10, cytokine anti-inflammatoire. Des macrophages dérivés de monocytes (MDM) ont été ensuite polarisés vers un phénotype plutôt pro- ou anti-inflammatoire puis incubés avec le HBV lors de leur processus de différenciation ou d'activation. Le HBV-C et -D entraînent une diminution de la différenciation des MDMs pro-inflammatoires (moins de production d'IL-1 β et IL-6) et tous (HBV-B/C/D) entraînent une augmentation de l'activation des MDMs anti-inflammatoires (plus d'IL-10 sécrété). Enfin, l'utilisation d'une méthode de co-culture entre les MDMs et des cellules produisant le HBV (traitées afin d'obtenir une dichotomie dans les sécrétions virales) ou juste sa protéine d'enveloppe, ont permis l'identification de cette dernière comme principal acteur de ces modulations. Une polarisation similaire des macrophages a été observé en présence de virions du HDV, qui possèdent la même enveloppe que celle du HBV.

L'effet antiviral du traitement par l'IL-1 β sur le HBV-D a par la suite été observé, en pré- et post-infection, suggérant que la molécule agit à la fois sur l'établissement et la maintenance de l'infection. Par ailleurs, les infections par le HBV-A à -E ainsi que le HDV ont toutes été diminuées de façon dose-dépendent par l'IL-1 β , selon un mécanisme encore inconnu qui implique, au moins en partie, la dégradation des ARNs viraux par une activation de la voie NF κ B. Enfin, des nanoparticules contenant un agoniste du récepteur TLR2, connu pour stimuler la voie NF κ B, ont été testées *in vitro* et *in vivo*, ces dernières démontrant un fort effet antiviral associés à une augmentation des marques pro-inflammatoires (au niveau de l'ARN).

Conclusion et perspectives

Cette étude est la première multi-génotypique concernant les macrophages hépatiques et le HBV. De plus en utilisant un modèle physiologiquement relevant de co-culture, le rôle majeur de la protéine d'enveloppe du HBV dans les modulations observées a pu être confirmé. Cette différente polarisation des macrophages hépatiques en présence du virus a été associé à une diminution de leur action antivirale sur l'infection, notamment par la diminution de sécrétion d'IL-1 β . Un effet sur l'ARN du HDV et HBV (multi-génotypique sur ce dernier) a pu être observé et associé à une activation de la voie NF κ B, suggérant que des stimulateurs de cette dernière feraient de bons traitements. A cet effet, l'impact immunomodulateur et antiviral de nanoparticules associées au ligand de TLR2 a été observé, prouvant son intérêt pour un développement thérapeutique plus poussé.

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Abbreviations:

AAV: adeno-associated virus	ISG: IFN-Stimulated Genes
ALT: Alanine Aminotransferase	ISRE: IFN-Stimulated Regulatory Elements
APC: Antigen-Presenting Cells	iNOS: inducible Nitric Oxide Synthase
Arg1: Arginase-1	IL: Interleukins
BCR: B cell receptor	GM-CSF: Granulocyte MΦ Colony Stimulating Factor
CAM: Capsid Assembly modulators	KC: Kupffer Cells
cccDNA: covalently closed circular DNA	LAG3: Lymphocyte Activation Gene 3
CHB: Chronic B Infection	LAM: Lamivudine
CHD: Chronic D Infection	LBP: LPS Binding-Protein
CHB: Chronic Hepatitis B	LPS: Lipopolysaccharide
CHD: Chronic Hepatitis Delta	LSEC: Liver Sinusoidal Endothelial Cells
cGAS: cytosolic DNA sensing	MAPK: Mitogen-Activated Protein Kinase
CSF: colony-stimulating factors	MCPIP1: Monocyte Chemoattractant Protein-induced protein 1 (or regnase-1)
CTL: Cytotoxic T-Lymphocytes	M-CSF: MΦ Colony Stimulating Factor
CTLA4: Cytotoxic T-Lymphocytes Associated protein 4	MDM: Monocyte Derived MΦ
DAMP: Danger Associated Molecular Pattern	MDSC: Myeloid Derived Suppressive Cells
EGF-R: Epidermal Growth Factor Receptor	MHC: Major Histocompatibility Complex
HBV: Hepatitis B Virus	MΦ: Macrophages
HBc: HBV-Core protein	NA: Nucleos(t)ides Analogues
HBV-Pol: viral polymerase	NAP: Nucleic Acid Polymers
HBsAg: HBV-Surface protein	NK: Natural Killer cells
HCC: HepatoCellular Carcinoma	NKT: Natural Killer T cells
HDV: Hepatitis Delta Virus	NPC: Non-Parenchymal Cells
HSC: Hepatic Stellate Cells	NLR: Nucleotide-binding Oligomerization Domain-like receptor
IFN: Interferon	NTCP: Na ⁺ -Taurochlorate Cotransporting Polypeptide
Ig: Immunoglobulins	
iKC: immortalized KC	

PAMP: Pathogen Associated Molecular Pattern

PBMC: Peripheral Blood Mononuclear Cells

pgRNA: pregenomic RNA

PHH: Primary Human Hepatocytes

PHM: Primary Human M Φ

PRR: Pattern Recognition Receptors

PD1: Programmed cell Death 1

rcDNA: relaxed circular DNA

RNP: Ribonucléoprotein

ROS: Reactive Oxygen Species

SVP: Subviral Particles

ResM Φ : Tissue resident M Φ

TGF- β : Transforming Growth Factor beta (

TAM: Tumor-Associated-M Φ

TCR: T cell Receptor

TIR: Toll-IL-1R

TLR: Toll-Like Receptors

TIM3: T cell Immunoglobulin and Mucin domain 3

WHB: Woodchuck HBV

Scientific Background

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I. Basic insight into immune cells and their liver specificity

Immunity is a highly complex concept involving every organ and most of our body's cells. Thus, for this introduction I will only briefly describe the immune system, focusing on some parts which will be of interest for my thesis project.

1. Innate vs adaptive immunity

Research on immunity began in the XXe century, when **Elie Metchnikoff** discovered that phagocytosis was, in fact, a protective mechanism enabling cells to efficiently kill bacteria (Nathan 2008). This mechanism was first attributed only to “phagocytes cells”, called later macrophages (MΦs), which were found to be non-specific against pathogens. For most of the century that followed, scientist focused rather on another cell type, lymphocytes. Their interests were driven by the peculiar properties of lymphocytes that are highly specific against a given pathogen and able to mount a memory of their encounter. This memory permits to respond quicker upon secondary challenge, and thus drove scientist to the creation and popularization of vaccinology. It is then Janeway and Matzinger that rebranded immunology in the 90ies, creating the concept of **adaptive** and **innate** immunity, to characterize specific and unspecific immune cells, respectively (Clark and Kupper 2005). Since then, knowledge on innate immunity gained in complexity, challenging old concepts and permitting a better understanding of our immune system.

a. Innate immunity

Innate immune cells are the **first line of defense** against pathogens or any cellular misconduct, acting within **hours or days**. They do **not** respond in a **specific** manner to antigen challenge, unlike the adaptive immune system. Instead, they are sensing a broad range of Pathogen Associated Molecular Pattern (PAMPs), such as lipopolysaccharide (LPS) or viral DNA; as well as Danger Associated Molecular Pattern (DAMPs) that are related to dysregulation in cellular functions. Altogether, these highly conserved motifs are recognized by Pattern Recognition Receptors (PRRs), a large family of receptors mostly present on innate immune cells. Sensing by PRRs leads to the activation of various immune pathways (for instance: NFκB, Akt) which, in turn, leads to cytokine/IFN release and/or orchestration of the immune response. Besides, as Metchnikoff described, some cells are able to phagocyte pathogens. This engulfment is followed by proteolytic processing and subsequent presentation of related peptides at the cellular surface on a molecule called Major Histocompatibility Complex II (MHC-II). This process is termed “Antigen Presentation” and cells able to perform it are called Antigen-Presenting Cells (APCs). Encounter of adaptive immune cells with the presented peptide, and other innate cells-mediated stimuli, leads to their activation.

Innate immunity is composed of both **circulating** and **tissue resident** cells that are described above (Figure 1):

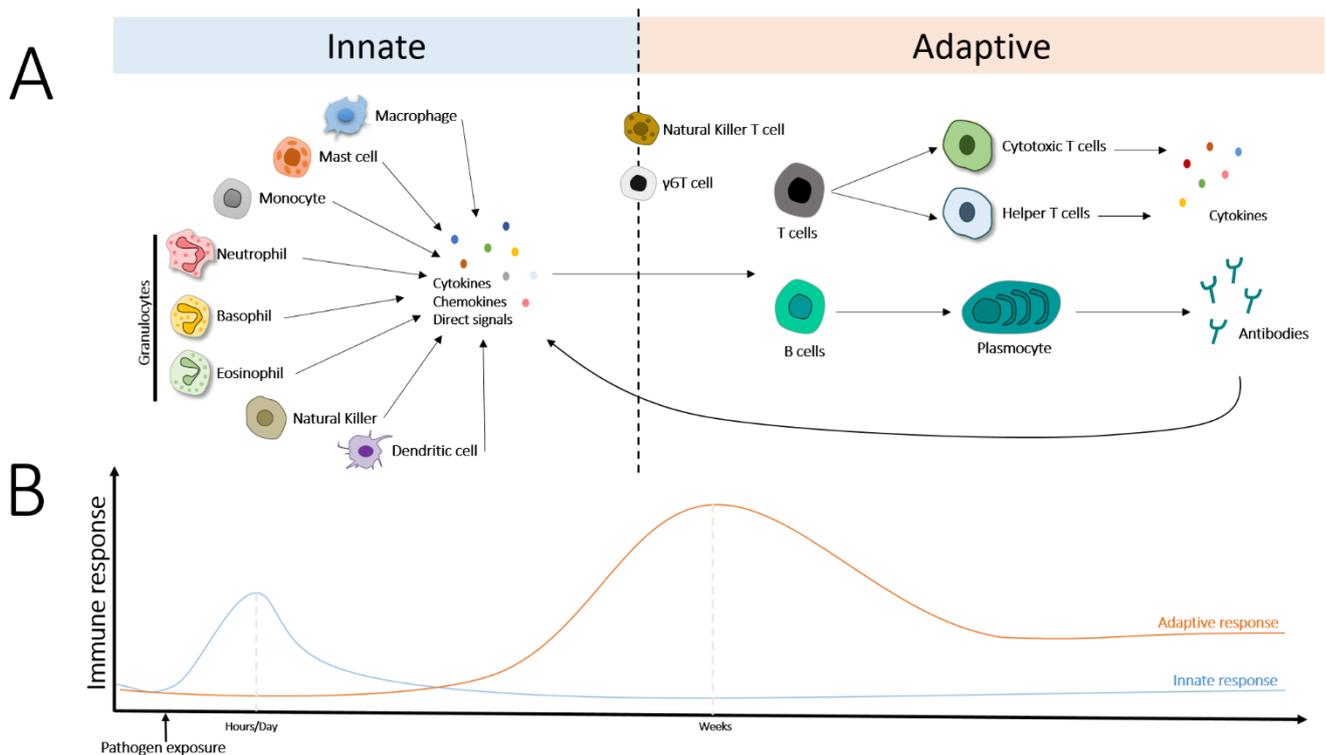


Figure 1: Innate and adaptive immune responses.

(A) Representation of cells composing the innate and adaptive immune system. Innate cells are secreting chemokines, cytokines and sometimes act in cell-to-cell contact, like Dendritic Cells. These molecules trigger T and B cell activation, permitting their differentiation into cytotoxic or helper T cell and plasmacytes, respectively. Cytokines and antibodies secreted by the adaptive immune cells are capable of helping the innate immune cells to resolve the injury/infection. Natural killer T cells and $\gamma\delta$ T cells are in-between cell with lymphocyte phenotype but no or less specific activation. (B) The innate immune response acts within hours or days, this is the first line of defense. Then, they activate and recruit the adaptive immune response at the site, which take between ten days to a few weeks. The adaptive immune response is much stronger and result in specific memory cells.

Monocytes/MΦs

Monocytes and MΦ functions/phenotypes will be discussed further later.

Dendritic cells (DC)

DCs are **professional APC**, mainly identified as HLA-DR^{high}. At an immature state, they are mostly found in tissue and various body surfaces, sensing and sampling broadly self and non-self-antigens (Worbs et al., 2017). Antigen phagocytosis permit their maturation. Upon this process, they migrate to primary and secondary lymphoid organs where they present the antigen to naïve T cells. This interaction ensures T cell differentiation (discussed in the adaptive system part), orchestrating the adaptive immune response. They are also involved in tolerance mechanisms and thus of high importance regarding autoimmune diseases.

Different DCs subsets are known, the main studied are the plasmacytoides DC (pDCs) and conventional DC (cDC1 and cDC2), both originating from a Common DC Progenitor. **pDCs** are mainly involved in immune reactions against intracellular pathogens, such as viral infections or cellular misconducts. They act mainly through the secretion of type I interferon (IFN), inducing IFN-Stimulated Genes (ISGs) (i.e: permitting viral clearance) and leading to immune

recruitment. **cDC1s** are more involved in the surveillance of dying cells and IL-12 and type-III IFN production. **cDC2s** are in charge of regulation and return to homeostasis with a CMH-II^{high}, high IL-12 secretion and T CD4 and CD8 activation.

Natural Killer cells (NK)

They are **large granular lymphocytes** (15-20 µm diameter) identified by CD3⁻CD56⁺NKp46⁺. They are activated by decrease or absence of CMH-I, which is a characteristic of infected and tumorigenic cells. They respond mainly through perforin/granzyme degranulation inducing cytotoxic elimination of the cell.

Mast cells

They are found in a broad range of connective tissues. Their cytoplasm is composed of granules containing inflammatory mediators such as cytokines, growth factors, histamine, heparin, chondroitin sulfate, and neutral proteases; key components of many cells type function (ex: MΦs, DC...). They are involved in vasodilatation, angiogenesis and **bacterial/parasitic** elimination.

Granulocytes

These cells have a specific nucleus shape with three lobes. Granulocytes is a family composed of three cells type. First, **Basophils** are known as the **professional IL-4 producing cells**, as such, they are important orchestrators of the immune response, especially through (1) the tolerogenic polarization of MΦs (2) Th2 polarization and further **helminth elimination** (Chirumbolo et al. 2018). Besides, they are involved in allergy mechanism in an antibody (IgE)-dependent as well as antibody-independent way. Second, **Eosinophils** were first described as cells involved in **anti-parasitic, asthma and allergy** mediated immunity. Their cytoplasm is rich in cationic proteins, stored in granules, that can be secreted and are responsible for most of their functions (Weller and Spencer 2017). In the past few years, eosinophils have been shown to be involved in a broader panel of function, among which tissue repair, development and mediator of both metabolic and immune homeostasis (Weller and Spencer 2017). Third, **Neutrophils** constitute the **first line** of defense against **pathogen in acute infection**. They are the first to be recruited on site, where they phagocyte pathogen and release their granules that are filled with pro-inflammatory cytokines (Kolaczowska and Kubes 2013). Interestingly, they are capable of suppressing T cell proliferation and activation, as well as being capable of presenting the antigen to prime cytotoxic T cells in case of infection (Kolaczowska and Kubes 2013). They are important as well in splenic B cell activation, a process which promote antibody secretion. Thus, they are highly important regarding the orchestration of adaptive immunity.

It is worth noticing that most functions attributed to professional innate immune cells (i.e the ones mentioned above) are shared with non-professional cells. For instance, PRRs are expressed by fibroblast, hepatocytes, mesenchymal and epithelial cells, amongst others (T. M. Schaefer et al. 2004; Bautista-Hernández et al. 2017; Pevsner-Fischer et al. 2006; Faure-Dupuy et al. 2018). Even if less expressed or responding on lower level than professional immune cell, this recognition is essential to mount the appropriate responses against pathogens.

b. Adaptive immunity

Evolutionary speaking, vertebrates emergence as come with the development of another immune system, termed adaptive (Boehm and Swann 2014). Cells composing this system, the **lymphocytes**, are highly **specific** against pathogens and capable of mounting an **immune memory** of the encounter. To reach this peculiar affinity, they are drastically selected in secondary lymphoid organs, according to two factors: they need to (1) avoid any self-recognition that could lead to auto-immunity and (2) have a high affinity for their antigen, which is mediated by their highly specific receptors. To be activated, they need to recognize and interact with the antigen presented by APCs, especially DCs. Of note, some, exception exists, like $\gamma\delta$ T cells that are directly activated by the antigen without the need for presentation by APCs.

This interaction, along with other co-factors, enable lymphocyte activation and further proliferation, termed **clonal expansion**. Once the adaptive immune system is launched, it aids the innate immune system in the resolution of the infection or injury, creating a positive feedback loop (**Figure 1**). As this response is slow to be initiated (around 7-10 days), a memory is created upon first exposure. Thus, some adaptive immune cells become long-lived and are acting as sentinel, ready to be activated upon secondary exposure. This concept is the basis of vaccination were a first encountered with the pathogen is mimicked to ensure sustained and rapid protection in the future (Boehm and Swann 2014).

Very briefly, the adaptive system is mainly composed of B and T lymphocytes.

B lymphocytes possess a B cell receptor (**BCR**) which recognize and internalize a specific antigen. The latter will be presented to T lymphocytes, a process leading to LB differentiation into **plasmocytes** that are antibody-producing cells. These antibodies or Immunoglobulins (Ig) are secreted in the bloodstream and highly specific of the recognized antigen. Different families of antibodies are known, called isotypes: IgA, IgD, IgE, IgG and IgM. Each have different localizations and functions (Schroeder and Cavacini 2010). Antibodies permit various immune mechanisms against pathogens: (1) neutralization by coating, (2) activation of complement molecules facilitating phagocytosis by immune cells and (3) direct activation of immune cells permitting cellular lysis through Antibody Dependent Cellular Cytotoxicity (ADCC). T cell independent activation of B cell has been described in case of foreign polysaccharides and unmethylated CpG DNA recognition by the BCR. While the response is quicker, the antibodies formed have a lower affinity and functionality compared to T-cell dependent activation.

T lymphocytes have a T cell Receptor (**TCR**) on their surface, in charge of specific antigen recognition. They are broadly divided into two distinct subtypes, CD8+T and CD4+T cells. While the first are in charge of killing infected cells through degranulation (perforin/granzyme) or expression of death ligands such as FasL, the latter differentiate in a broad panel of helper T cells (Cano and Lopera 2013). The most well-known Thelper cells were the first to be discovered, the Th1 and Th2, that prompted the M1 and M2 nomenclature (discussed later in this introduction). Since then, a highly complex network of Thelper cell arise (T9, Th17, Th22..) each with specific functions (extensively reviewed here (Cano and Lopera 2013)).

Some lymphocytes are activated in an MHC-independent manner, the **$\gamma\delta$ T cells** (in opposition to other T cell presenting the $\alpha\beta$ TCR chain) and **Natural Killer T cells (NKTs)** (Lawand, Déchanet-Merville, and Dieu-Nosjean 2017). Both are thus more rapidly activated and produce large number of cytokines and cytotoxic components (FasL, perforin/granzyme...). NKTs ligands are

glycolipid loaded CD1d-presenting cells, and as for $\gamma\delta$ T cells, even if poorly characterized, it seems that stress-antigen could be a trigger. Hence, these cells share the characteristics of both immune compartments.

The line between innate and adaptive immune system became thinner and thinner in the past few years, with the discoveries of cells such as innate B cells or innate lymphoid cells, classified as part of the adaptive immune system but demonstrating innate properties (Hillion et al. 2019). Besides, a new kind of innate memory, termed **trained immunity**, was discovered recently after observations of sustained epigenetic modulations in monocytes, leading to more efficient secondary pathogenic challenge (Saeed et al. 2014; Netea et al. 2016; Arts et al. 2018; Netea et al. 2019; Gourbal et al. 2018). Thus, as our knowledge grows, differentiation from innate to adaptive immunity can no longer rely on the presence/absence of a memory or pathogen specificity, as it has been done for the last century.

2. Immune sensing and subsequent response

a. PRRs, IFN and the main pathways downstream of their stimulation

As previously mentioned, PRRs are immune receptor in charge of sensing DAMPs and PAMPs. Different families of PRRs with specific localization and trigger signals are known and recapitulated in the following table:

Table 1: Ligands and cellular localization of PRRs (Kumagai and Akira 2010). *CS: Cell Surface; E: Endosomes; C: Cytoplasm*

PRRs	Ligands	Localization
Toll-Like Receptors (TLRs)	TLR1/2	Triacetylated lipoproteins from bacteria cell wall components
	TLR2/6	Diacetylated lipoproteins from bacteria cell wall components
	TLR3	Double stranded RNA (dsRNA)
	TLR4	LPS
	TLR5	Flagellin
	TLR7	Single stranded RNA
	TLR8	
	TLR9	Unmethylated DNA with CpG motif
	Nucleotide-binding Oligomerization Domain-like receptor (NLRs)	NLRP1
NLRP3		Danger signals, Crystallin substances, Microbial toxins
NLRC4		
NOD1		
NOD2		Peptidoglycan Parasite, viruses, bacteria cell wall
DNA sensors	AIM2	dsDNA of host and pathogen origin
	DHX36	
	DHX9	
	Ku70	
	LRRFIP1	
	IFI16	

	IFIX		
	DAI		
	MRE11		
	DDX41		
C-type Lectin Receptors (CLRs)	Mincle	Damaged cell, fungus	CS
	Clec9a/DNGR	Necrotic cells	CS
	DC-SIGN	High mannose, Fucose	
	Dectin-1	β -glucan	CS
	Dectin-2	High mannose, α -mannan	CS
Retinoic acid-inducible gene I-Like Receptors	LSEctin	High mannose (fungi cell walls), Fucose	CS
	MDA5	dsRNA/ssRNA (long)	C
	RIG-I	dsRNA/ssRNA (short)	C

PRRs activation is a complex mechanism involving a broad range of proteins and signaling pathways, depending on the ligand (PAMP/DAMP type), the PRR triggered and cell type. The most well-known and characterized pathways are displayed in [Figure 2](#). It is worth noticing that others receptors, such as IL-1R and TNFR (cytokine receptors) share the same downstream effectors as most PRRs.

TLRs, at the exception of TLR3, all share the **MyD88 and TIRAP** adaptor proteins (M. S. Lee and Kim 2007) ([Figure 2](#)). Following their activation, IRAK4/1 proteins bind to TRAF6, a process which lead to (1) **IRF5 translocation** in the nucleus and (2) activation of the **TAB/TAK1** complex ([Figure 2](#)). The latter is at the crossroad between two pathways, leading in one hand to NF κ B activation, on the other hand to AP-1. First, the **IKK-complex**, composed of IKK γ , IKK α and IKK β proteins, activation ensure I κ B degradation, releasing NF κ B dimers ([Figure 2](#)). Finally free, NF κ B dimers are able to translocate into the nucleus where they are inducing pro-inflammatory cytokine production. Second, it activates **MAPK** (mitogen-activated protein kinase), **p38 and JNK**, leading to **AP-1 pathway** activation.

Following ligand stimulation, TLR1/2, TLR2/6 and TLR4 are capable of translocating into the endosomes. In this manner, the TLRs use TRAM and not Myd88 as adaptor molecules ([Figure 2](#)). Together with TLR3, endosomal TLR stimulation leads to TRIF activation. TRIF binds to TRAF6, leading to RIP1 activation and TAK1 complex formation, activating the aforementioned pathways. TRIF also binds TRAF3 which further recruit TBK1 and IKKi for efficient **IRF3 activation**. Activation of this complex is also performed through cytosolic DNA sensing (cGAS) and dsRNA sensing (MDA-5 and RIG-I). **TLR7 and TLR9 signaling by Myd88** is a bit different as it activates both pro-inflammatory (NF κ B, AP-1 and IRF5) and IFN (**IRF7**) pathways through formation of a large complex constituted of MyD88-IRAK4-IRAK1-TRAF6-IRF5-TRAF3-IKK α . Activation of **IRF3/IRF7 homodimers** leads to type-I IFN production.

IFNAR (IFN alpha Receptor) binds type-I IFN which subsequently activates the Janus Kinase (Jak)/STAT pathway ([Figure 2](#)) (Lazear, Schoggins, and Diamond 2019). Activated STAT1 and STAT2 complex with IRF9 to form the ISGFR3 transcription factor. The latter binds IFN Stimulated Regulatory Elements (ISRE), promoting the production of ISGs ([Figure 2](#)). These ISGs are key factors in the **antiviral response** and most of them are considered as **restriction factors** (blockers of viral replication and propagation) (Colomer-Lluch et al. 2018). Amongst the most well-characterized are ISG15 (IFN-stimulated protein of 15kDa), the Mx protein (MxA and MxB), RNase-L (Ribonuclease L), PKR (Protein Kinase R) (Mesev, LeDesma, and Ploss 2019). Their antiviral functions are broad; they are, for instance, capable of sensing viral RNA (OAS, PKR), regulating viral mRNA translation (TRIM19, RSAD2, IFIT1/IFIT2), degrading viral RNA (RNaseL, ISG20) and blocking viral secretion (ISG15). In addition, some are upregulating PRRs expression

and other key elements of the IFN pathway (JAK/STAT, IRFs). Of note, type-III IFN are binding a complex formed by IL-10R2 and IFNLR1 that leads to the activations of the same pathway (Jak/STAT) as type-I IFN, (i.e ISGs production). Type-II IFN are rather involved in antimicrobial immunity and binds the IFN γ R (IFN Gamma Receptor).

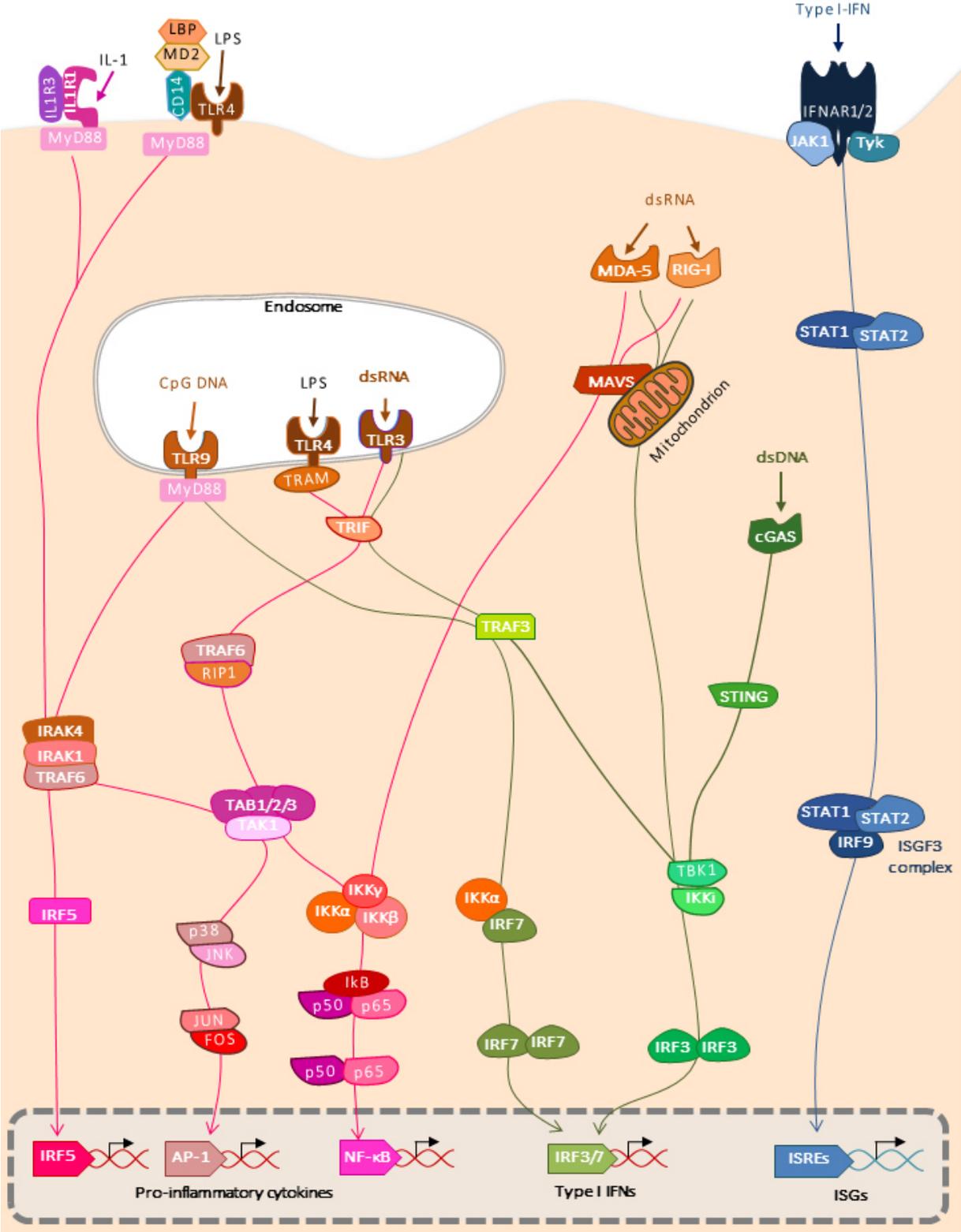


Figure 2: PRRs and IFN downstream pathways, informations are provided in the main text.

b. Cytokines

Cytokines are small (15-20 kDa) and short-lived proteins with key functions in immune signalling (Rose-John 2018). They are produced downstream of the NF κ B or IFN pathway presented above, and are able to act in an autocrine, paracrine or endocrine manner. This family encompasses all interleukins (IL), IFN, some growth hormones (leptin, erythropoietin...) and colony-stimulating factors (CSFs). Binding of these secreted molecule on their receptor leads to additional downstream pathway activation, amongst which the **Akt and JAK/STAT pathways**. They are broadly involved in key functions for immune cells such as **migration, proliferation, cytokine secretion, phagocytosis**, etc. Their mode of activation are extensively reviewed in the literature and will not be discussed in details here (Vara et al. 2004; Vergadi et al. 2017; Villarino, Kanno, and O'Shea 2015; 2017; Weichhart and Saemann 2008). We will however briefly discuss some of the most important cytokines involved in this work, and their downstream pathways.

i. TNF- α

The Tumor Necrosis Factor α is a 17.3 kDa **pro-inflammatory** and **pro-apoptotic** cytokine. While mostly secreted by M Φ s, other immune cells (NK, T and B cells, mast cells) and non-immune cells (endothelial cells, fibroblasts, smooth muscle cells) are also producing it (Aggarwal 2003). TNF- α function are dual and depends mostly on which receptor it binds. Association with **TNFR1**, a receptor ubiquitously expressed, initiates **cellular apoptosis** or **necroptosis** (Ruiz et al. 2021) (**Figure 3**). By binding to TNFR-associated death domain (TRADD), TNFR1 subsequently activates FAS-associated death domain (FADD) and caspase-8, which in turn leads to caspase-3 mediated cellular apoptosis (Ruiz et al. 2021). In some cases, FADD associates with RIPK3 and RIPK1 and the cell undergo another cell death called necroptosis. Finally, both **TNFR1** and **TNFR2** (expressed only on immune and endothelial cells) are recruiting the TNFR associated factor 2 (TRAF2), which activates **AP-1, p38/MAPK** and the **NF κ B pathway** (Ruiz et al. 2021) (**Figure 3**). As TNFR2 is mostly expressed on immune and endothelial cell, TNF- α function is cell-dependent. Finally, depending on TNF- α concentration and other cytokines in the microenvironment, activation of TNFR1 can also lead to cell survival. Abnormal production of TNF- α and TNFR1/2 signaling has been associated with rheumatoid arthritis, Crohn's disease, atherosclerosis, psoriasis, and cancer (Aggarwal 2003).

ii. IL-6

Interleukin-6 is a 26kDa cytokine, mainly involved in **immune activation**, permitting B cell differentiation into plasmocytes, haemopoiesis and acute phase response (Rose-John 2018). It is produced by T and B cells, monocytes, fibroblasts, keratinocytes, endothelial cells, mesangial cells, adipocytes and some tumour cells (Schmidt-Arras and Rose-John 2016). IL-6 production is responsible for many autoimmune diseases (rheumatoid arthritis, castelmans's disease). IL-6 first binds to IL-6R α , permitting the efficient interaction with the transducing subunit of the glycoprotein 130 (gp130) (Rose-John 2018) (**Figure 3**). While gp130 has an almost pleiotropic expression, IL-6R α is solely expressed at the surface of T cells, monocytes, activated B cells, neutrophils, hepatocytes, Hepatic stellate cells (HSCs) and biliary cells (Schmidt-Arras and Rose-John 2016; Rose-John, Scheller, and Schaper 2015). However, IL-6 is able to signal in a broader type of cell as soluble IL-6R α (i.e produced from alternative splicing) efficiently binds to gp130 (Mihara et al. 2011). After efficient heterodimerization of the receptor, JAK will phosphorylate

several tyrosine residues on cytosolic gp130, leading to the activation of the **Jak/STAT** pathway (i.e ISGs activation), and **ERK** (extracellular-signal regulated kinase)/**MAPK** pathway.

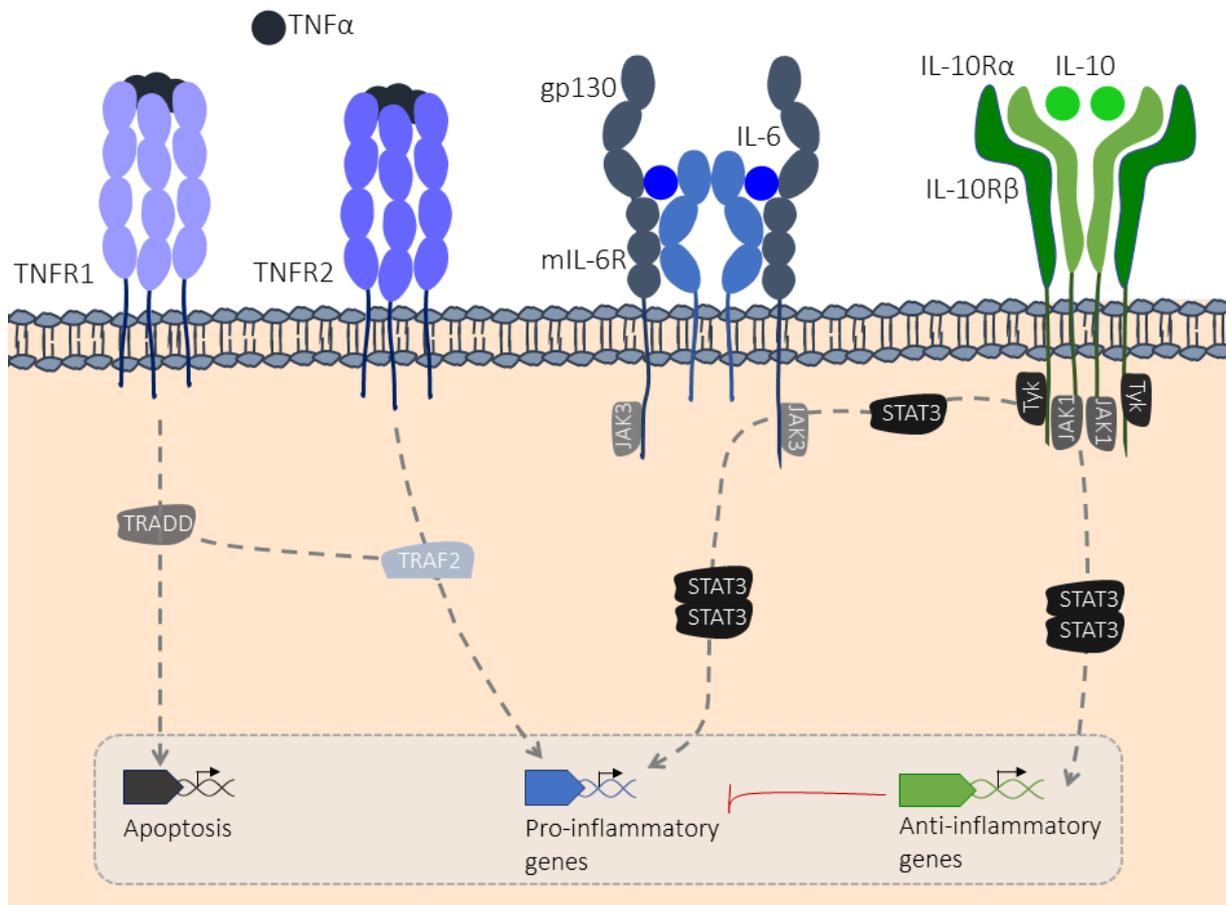


Figure 3: Cytokines, their receptors and main downstream signalling

iii. IL-10

Interleukin-10 is a 18kDa cytokine, mostly characterized for its **anti-inflammatory functions**. It is, indeed, capable of inhibiting pro-inflammatory cytokine production, antigen presentation and the establishment of memory T cells, as well as promoting regulatory T cells differentiation and expansion (Ouyang and O’Garra 2019, 10). Besides, it helps in maintaining homeostasis to gut microbiome and reduces tissue damage. Interestingly, immune stimulation is also, in some cases, mediated by IL-10, such as stimulation of IFN γ production by B cells (Walter 2014).

IL-10 is mainly produced by Th2 cells, but also DCs, MΦs, T cells, NK and B cells. Its production can be induced by several viruses, such as HIV, EBV, CMV, and its downregulation is associated with autoimmunity (Walter 2014). The cytokine binds to a heterodimeric receptor composed of IL-10RA and IL-10RB. Formation of this complex leads to JAK/STAT3 activation (**Figure 3**). Thus, IL-10 stimulation interferes with IL-6 pathway and the resulting signal will mostly depends on the balance between the two cytokines.

IL-1 β being of high importance in our study, we will more substantially discuss this cytokine in the following paragraphs.

c. IL-1 β production and downstream signaling pathway, a peculiar case

i. IL-1 β cytokine

The IL-1 superfamily is composed of 21 members (Fields, Günther, and Sundberg 2019). This family is highly heterogeneous, with some being rather related to pro- (17) or anti-inflammatory (4) functions, 11 being soluble factors and 10 receptors molecules. For the rest of this introduction, we will discuss mostly of the IL-1 β cytokine, however, other IL-1 members are more extensively reviewed here (Dinarello 2018).

IL-1 β is a globular protein of 17.3 kDa with pleiotropic activities.

First, IL-1 β permits the production of chemokines and increase in adhesion molecules promotion necessary for **immune infiltration** (Voronov et al. 2003). It induces the production of pro-inflammatory cytokines, toxic reactive oxygen species (ROS)/nitrogen species, prostaglandins, proteolytic enzymes through IL1R pathway activation (discussed below). All of which lead to a strong **immune activation** (Dinarello 2009).

Besides, it is highly important in T cell differentiation and maturation, though, for instance, induction of **DCs maturation** and consequent Th1 cytokine secretions (Wesa and Galy 2002). It is a growth factor for thymocytes as well as a costimulatory T cell agent (Dinarello 2009). Indeed, it enables IL-2R upregulation leading to **T cell expansion**, and its increase of OX40 induces IL-17 secretion and subsequent **Th17 differentiation** (Dinarello 2009). OX40 increase, along with CD40L upregulation, is also able to enhance T-cell **antibody dependent production** in B cells (Nakae, Asano, Horai, Sakaguchi, et al. 2001). Whether this stimulation is mediated by IL-1 β alone or by IL-1 β -mediated IL-6 production is however still discussed (Nakae, Asano, Horai, and Iwakura 2001; Dinarello 2009).

ii. Production of IL-1 β by the inflammasome machinery

The major source of IL-1 β comes from circulating monocytes, ResM Φ s and DCs.

Among the aforementioned PRRs, NLRP1, NLRP3, NLRC4, AIM2 and IFI16 are part of a peculiar detection system, the **inflammasome**. Formation of this complex is a two-step mechanism. First, a pre-activation through PRR (often TLRs) stimulation (**Figure 4.A1**), inducing the NF κ B pathway. This leads to the production of pro-Caspase-1, pro-IL-1 β (33kDa inactive protein) and pro-IL18 proteins, as well as enhanced production of the inflammasome sensors and its oligomerization (Pellegrini et al. 2017) (**Figure 4.A2-A3**). Then, recognition of specific ligands by the inflammasome sensors itself permit the binding with a common adaptor molecule, called ASC (**Figure 4.A4**). Activation of this macromolecular complex leads to pro-caspase-1 proteolytic cleavage, releasing caspase-1 in the cytoplasm (**Figure 4.5**), which, in turn, is responsible for pro-IL-1 β and pro-IL-18 cleaved maturation (**Figure 4.6**). Once activated, Gasdermin D, another target of caspase-1, forms pores into the cell membrane (**Figure 4.7**), permitting cytokine secretion (J. Shi et al. 2015) (**Figure 4.8**). Of note, the consequent loss of cellular permeabilization can lead to pyroptosis, an immune activating cellular death (Bergsbaken, Fink, and Cookson 2009). The outcome is depending on the stimulator used (Heilig et al. 2018; Rühl et al. 2018).

In recent years, another mechanism of activation has been discovered and termed **non-canonical inflammasome**, in opposition to the canonical one mentioned above (Downs et al. 2020). It relies on internalized LPS sensing (i.e coming from internalized bacteria mostly),

(Figure 4.B1) by caspase-11 (human ortholog: caspase 4/5) in the cytoplasm (Hagar et al. 2013; Kayagaki et al. 2013) (Figure 4.B2). Activated caspase-11/4/5 is inducing the proteolytic cleavage of gasdermin-D, subsequently leading to pore formation (Figure 4.B3), ATP efflux, itself inducing K⁺ efflux (Figure 4.B4) and thus, NLRP3 inflammasome engagement (Rühl and Broz 2015). Of note, caspase-8 activation by Dectin-1 or TLR3/4 stimulation was described as another non-canonical-inflammasome (Gringhuis et al. 2012, 8; Maelfait et al. 2008, 8). To this day, contradicting data have been published and it is unclear if caspase-8 is able to cleave pro-IL-1 β alone or by activating NLRP3 inflammasome (Gringhuis et al. 2012, 8; Maelfait et al. 2008, 8).

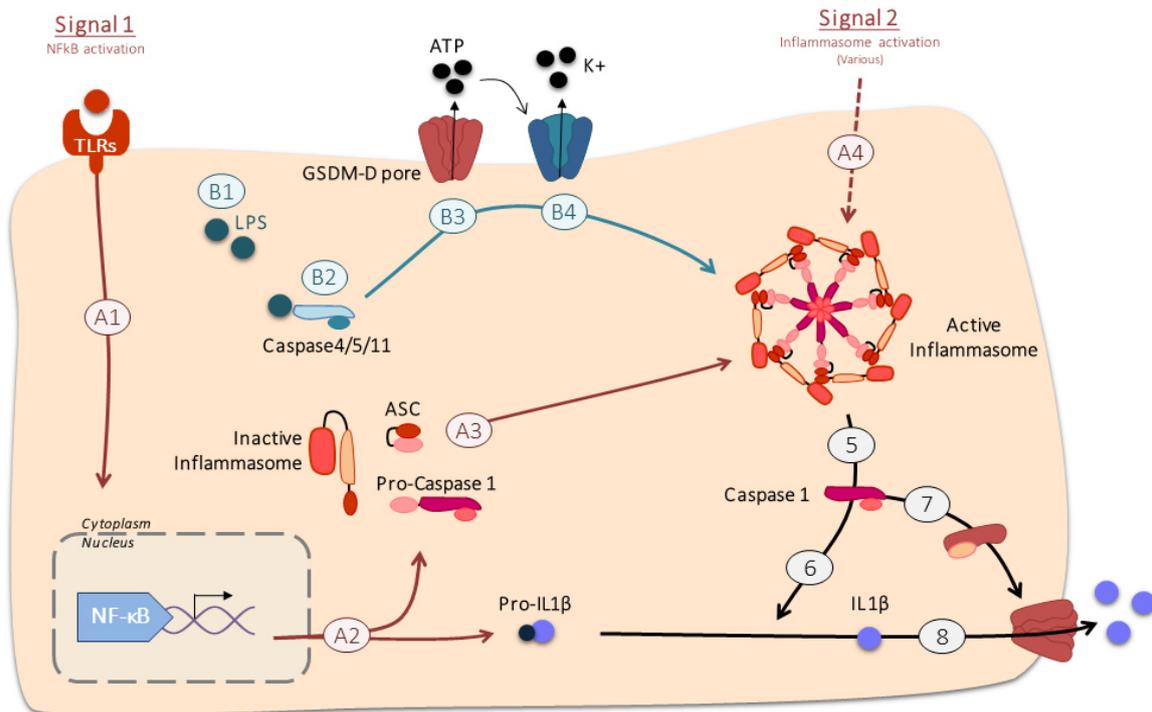


Figure 4: IL-1 β secretion pathway. *Informations are provided in the main text.*
Adapted from (Downs et al. 2020; Swanson, Deng, and Ting 2019)

iii. IL-1R downstream pathway

IL-1 β does not signal under typical PRRs, its receptor is actually a complex, formed with IL-1 Receptor (IL1-R) and the accessory chain IL-1R3. The active IL-1R complex is present in diverse tissues/cell types, such as T cells, myeloid cells, epithelial cells, hepatocytes, amongst others (Boraschi et al. 2018). Of note, this receptor binds also IL-1 α , which has been extensively discussed here (Malik and Kanneganti 2018) and will not be discussed further. Extracellular part of the IL1-R comprises three Ig-like domains (D1,2,3), a transmembrane domain composed of a single helix anchoring the Ig-like part within the plasma membrane, and an intracellular part containing a Toll-IL-1R (TIR) domain, essential for the appropriate transduction of the signal (Boraschi et al. 2018). First, IL-1 β binds to IL-1R, inducing a conformational change permitting the binding of IL-1R to IL-1R3 (D. Wang et al. 2010) (Figure 5.A). Finally, the reunion of TIR domain from IL-1R and IL-1R3 leads to the appropriate recruitment and binding of adaptors molecules. While D1 and D2 from IL-1R are sufficient enough to bind IL-1 cytokines, interaction with D3 is essential for recruitment of IL-1R3 (D. Wang et al. 2010) (Figure 5.A).

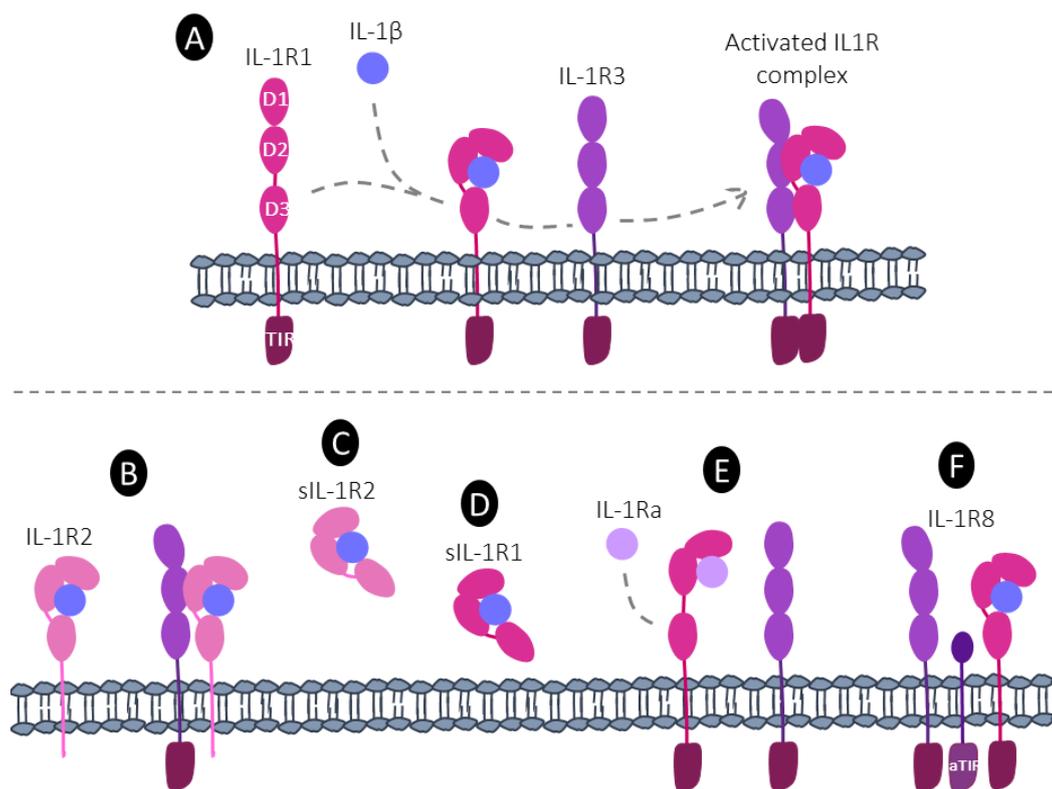


Figure 5: IL-1R and IL-1 β activation pathway

As IL-1 β as **pleiotropic activities**, it is tightly regulated. Thus, negative element of this pathway induction are numerous. First, IL-1 β can bind to IL-1R2, which is a decoy receptor lacking of a TIR domain (**Figure 5.B**). Hence, heterodimerization with IL-1R3 is possible, but abortive. Second, IL-1R2 and IL-1R1 alternative splicing and/or cleavage from the membrane by metalloproteases leads to its secretion in the extracellular milieu, and possible binding to IL-1 β , limiting its systemic functions (Boraschi et al. 2018) (**Figure 5.C-D**). Also, IL-1Ra is an IL-1R antagonist that bear significant sequence and structural homology with IL-1 β , but lack the ability to bind D3 (**Figure 5.E**). Thus, binding of IL-1Ra to IL-1R prevent the formation of IL-1R complex. Finally, IL-1R8, which possess an anomalous TIR domain, interferes with IL-1R pathway by inserting between IL-1R1/R3 (**Figure 5.F**).

For decades, IL-1R pathway was described as solely inducing the NF κ B pathway (previously described) through Myd88 activation (Boraschi et al. 2018). However, in the past few years, an accumulating body of evidence suggest another properties of the IL-1R pathway, as **an IFN response inducer/amplifier** (Robichon et al. 2020; Orzalli et al. 2018; Aarreberg et al. 2018). In Bone-Marrow derived DCs, IL-1 β treatment led to an early NF κ B activation, followed by a late IRF3/IRF7 gene activation, putatively responsible for West Nile Virus inhibition (Aarreberg et al. 2018). Two studies from independent lab reached the same conclusion of an IRF1-mediated induction by IL-1 β (Orzalli et al. 2018; Robichon et al. 2020). Both laboratories, using hepatocytes and epithelial cells, described an IRF2 mRNA decrease upon IL-1 β treatment which boosted the antiviral response. However, their conclusions diverge. On one hand, Orzalli suggests that, in IL-1 β treated cells, downstream IRF1 activation leads to gp130-JAK-STAT pathway activation and consequent IFN activation (Orzalli et al. 2018). On the other hand,

Aarreberg observes STAT3 phosphorylation and nuclear translocation, in IL-1 β + IFN α treated cells, in hepatoma cell lines (Aarreberg et al. 2018).

iv. IL-1 β in diseases

IL-1 β is widely involved in diseases.

First, it is proven widely efficient as an **antiviral** therapeutic, alone or in combination with IFN α , as observed *in vitro* against Rhinovirus (Piper et al. 2013), HBV (Isorce et al. 2016; M. Li et al. 2020), HCV (R.-J. Lin et al. 2014, 1) DENV (R.-J. Lin et al. 2013), JEV (R.-J. Lin et al. 2013), WNV (Aarreberg et al. 2018), LCMV (Robichon et al. 2020), CMV (Iwata et al. 1999), Vesicular Stomatitis Virus (Orzalli et al. 2018) and Zika Virus (Orzalli et al. 2018). IL-1 β antiviral effect is not well-characterized. In most cases, it has been attributed to RNA degradation mediated by the Monocyte Chemoattractant Protein-induced protein 1 (MCP1P1) (R.-J. Lin et al. 2013; M. Li et al. 2020; R.-J. Lin et al. 2014). However, more relevant models are required to confirm these results as most experiments were performed on over-expressing MCP1P1 cells. Others hypothesize that IL-1 β , by activating or sensitizing to the IFN pathway, leads to ISGs activation, which are well-known antiviral factors (Fensterl, Chattopadhyay, and Sen 2015). Activation of the NLRP3 inflammasome and subsequent production of IL-1 β is often the first response against **bacterial** invasion, such as *M.Tuberculosis*, *S.Aureus*, *F.tularensis*, amongst others (extensively reviewed here (Sahoo et al. 2011)). However, in some cases, IL-1 β secretion is deleterious. **Auto-inflammatory diseases**, for instance, are characterized by a dysfunctional caspase-1 activity and consequent IL-1 β production (extensively reviewed here (Dinarello 2011; Kaneko et al. 2019)). In **cancer**, its production is associated with genetic alterations in *KRAS* and *BRACA1*, correlating with disease progression. It drives Myeloid Derived Suppressive Cells (MDSCs) recruitment and subsequent IL-10 production, in addition to being pro-angiogenic, as mentioned (Rébé and Ghiringhelli 2020). On the other side, IL-1 β effect as a Th1-response inducer is correlated with tumor regression in B cell myeloma and lymphoma (Baker, Houston, and Brint 2019). An insight into the dual effect of IL-1 β in cancer is provided by a recent analysis in ColoRectal Cancer (Dmitrieva-Posocco et al. 2019). In this model, IL-1 β effect is cell-autonomous, as it induces efficient tumor growth on CRC cells, as well as efficient activation of myeloid cells leading to a decrease in cancer progression. Thus, IL-1 β effect, as most cytokines, relies on a specific balance.

Despite this accumulating body of evidence, IL-1 β recombinant proteins or IL-1R agonist are not efficient drugs. IL-1 β is considered one of the most powerful cytokine, as it affects every organs, and thus is prone to induce uncontrolled immune response (i.e cytokinetic storm) in very low doses compared to others cytokines. Intravenous injection (10 to 100 ng/kg) is associated with fever, sleepiness, anorexia, generalized myalgias, arthralgias, headache, some gastrointestinal disturbances and most of all, hypotension at high doses (Veltri and Smith 1996). Besides, high dosage or dose escalation are required as the cytokine as a low half-life in the serum, subsequently inducing strong side effects. Thus, even if intensively investigated at the beginning of the 90's, none of the IL-1 β clinical trials reached phase III (Veltri and Smith 1996). However, to date it was proven efficient as a vaccine adjuvant due to its T-cell activating properties (Lapuente et al. 2018). Its use has since been modulated to cope with the aforementioned side effects. Hence, a specific synthetic peptide (aa 163 to 171) was designed with efficient immunostimulatory activities without adverse side effects (i.e devoid of systemic pro-inflammatory activity) (Tagliabue and Boraschi 1993). Others developed

“immunocytokines” which are fusion proteins with a WT cytokine and a targeting moiety (often an antibody) against a specific surface molecule (Neri and Sondel 2016). Even then, side effects were too high and the delivery seems to lack specificity. Eeckhout and colleagues recently developed the **ActAkin** (Activity-on-target cytokines) technologies with IL-1 β (Van Den Eeckhout et al. 2020). It consists in the designing of an immunocytokine, but the cytokine contains a mutation rendering them inactive. Upon their delivery and the interaction with the appropriate surface molecule, cytokines regain their WT abilities. In *in vivo* trials for Influenza vaccines, this ActAkin retains its efficiency on CD8+T cell stimulation with a significant reduction of side effects (Van Den Eeckhout et al. 2020).

Hence, the duality of IL-1 β effects remains a challenge for its *in vivo* use, which is however slowly and increasingly being overcome.

d. Regulation of the immune system: inhibitory receptors

Once the immune system has efficiently cleared the infection or resolved the insult, there is a need for a return to homeostasis. Cells recruited on site and the lymphocyte clonal expansion generates a large inflammation of the tissue that could be harmful if uncontrolled. Besides, as mentioned earlier, cells generated may be autoimmune, or become such. The immune response must thus be tightly controlled and, if required, terminated. **Immune inhibitory receptors (IRs)** are paired with an activator one (i.e PRRs). They can recognize different or similar patterns, and in case both are stimulated, the balance leans towards the most strongly stimulated (Ravetch and Lanier 2000). Amongst them, the most well-known are Programmed cell Death 1 (PD1), Lymphocyte Activation Gene 3 (LAG3), T cell Immunoglobulin and Mucin domain 3 (TIM3) and Cytotoxic T-Lymphocytes Associated protein 4 (CTLA4). These receptors are mostly expressed on CD8+T lymphocytes and their ligands are broadly expressed by myeloid cells (especially MDSC), regulatory T cells and some cancer cells. Upon chronic infections, where cells are sustainably exposed to antigens, immune regulation leads to the increase of these receptors. T cells expressing IRs are then called “**exhausted**” as they lose their main immune functions (Wherry and Kurachi 2015). They are unable to secrete harmful cytokines and clear pathogens. Besides, tumor cells are often capable of secreting or expressing IR ligands in order to escape from immune surveillance. Some viruses, especially upon chronicity, like Hepatitis B Virus (HBV), are also capable of hijacking these pathways to hide from the immune system (Hang Li et al. 2012b; Nebbia et al. 2012).

3. Focusing on M Φ s

a. Generalities

i. Definition and general functions of M Φ s

In 1908, **Elie Metchnikoff** received the Nobel Prize for its discovery of M Φ s functions (Nathan 2008). More than a hundred years after, scientific across the world are still trying to better understand this fascinating cell type. M Φ s are highly potent immune cells with a broad range of functions, but they were first described for their capacities for **phagocytosis**, which

encompass a large array of complexes and molecules, making them gate keepers of our homeostasis, in many ways (Hirayama, Iida, and Nakase 2017).

Their phagocytic capacities enable them to clear pathogenic content from our system. To do so, they are equipped with a broad range of engulfing mechanisms involving passive (endocytosis, micropinocytosis, etc.) as well as active (Antibody-Dependent) mechanisms (Hirayama, Iida, and Nakase 2017). Two different mechanisms have been described for the handling of antibody-bound pathogenic content, the ADCC and the Antibody Dependent Cellular Phagocytosis (ADCP) (Gül and Egmond 2015). ADCC results in degranulation and consequent lysis of the targeted cell, it is however quite rare in monocyte/MΦs (more related to NK cells). Thus, in most cases, antibody binding on MΦs leads to efficient ADCP (Gül and Egmond 2015).

Following internalization, foreign materials are addressed to lysosomal compartments to be digested by specific enzymes. Finally, as MΦs are equipped with **antigen-presenting mechanisms**, they express on their surface the pathogenic peptides, loaded on their MHC-II. Recognition of this presented peptide by other immune cells further activates the immune system, if required. Phagocytosis is also important in keeping body homeostasis. For instance, spleen MΦs are in charge of clearing old erythrocytes from our circulation, (up to $2 \cdot 10^{11}$ each day), participating in **iron and haemoglobin recycling** (Murray et al. 2014). Organ's integrity is ensured through their capacities to **engulf apoptotic bodies** and/or clear senescence cells, limiting the inflammatory impact of such cellular death. They also secrete extracellular matrix components and pro-angiogenic factors (such as VEGF), making them key players in wound-healing mechanisms.

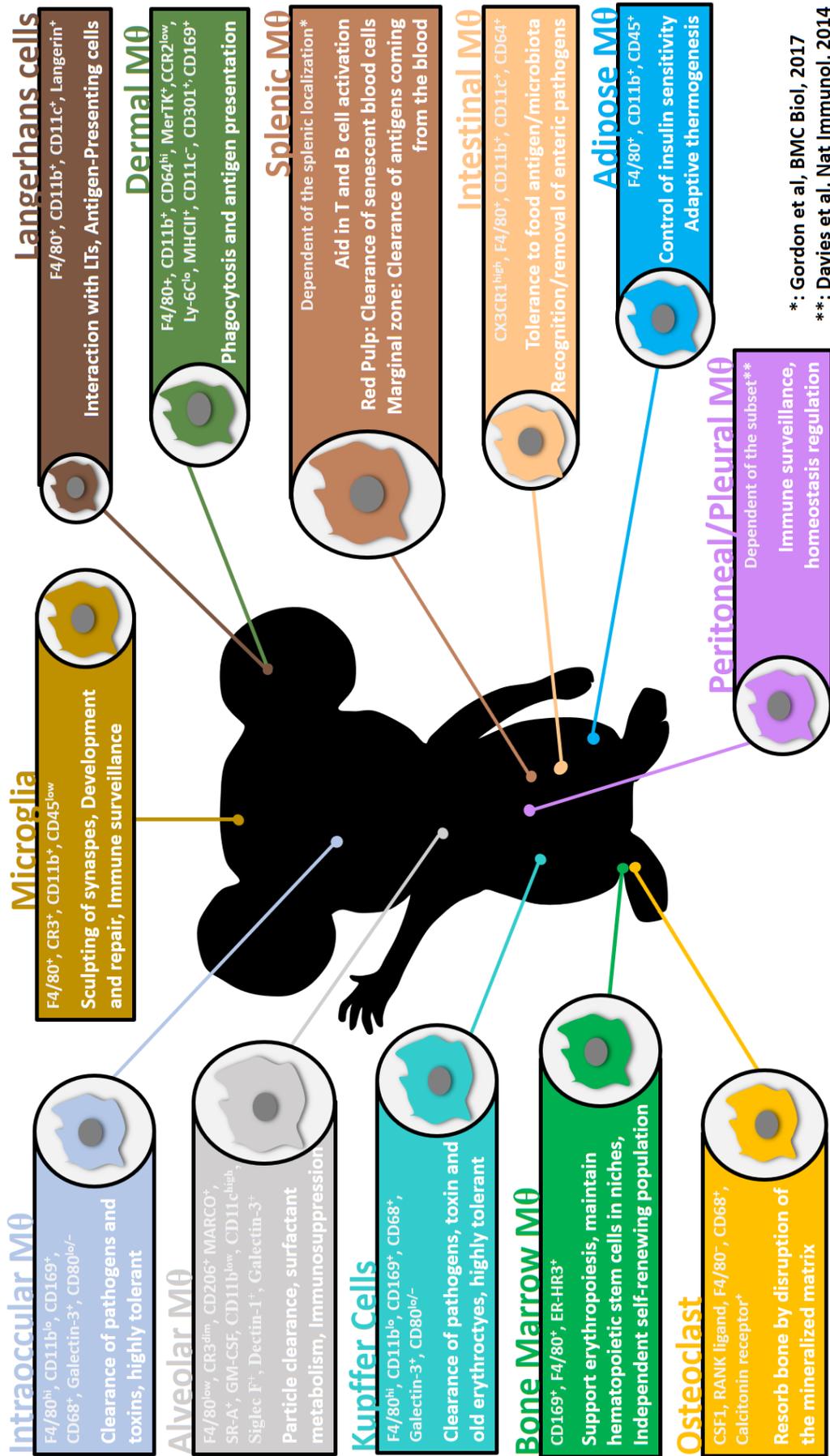
As part of the first wave against most pathogens, they express a large array of PRRs, enabling them to **sense** and **respond** to a broad range of signals. These signals can lead to (1) **chemokine secretion**, recruiting other immune cells, (2) **cytokine** secretion, activating and modulating their response, (3) **phenotype shift/reorganization**, in addition to the (4) phagocytic activities described above.

Finally, MΦs have highly diverse functions depending on their ontogeny, but also their environment, and thus, not all tissue resident MΦ (ResMΦs) have the same functions.

ii. ResMΦs, ontogeny & functions

Deciphering ResMΦs ontogeny has been an important challenge of the past few years in regards of the difficulties to track such cells with a lack of clear markers and models, especially in humans. Here is the most plausible hypothesis to date (Ginhoux and Guilliams 2016).

ResMΦs originate first from the **yolk sac**, at an earlier time point than hematopoiesis, and populate the entire embryo (Ginhoux and Guilliams 2016). Around day 10.5E, fetal liver becomes the major hematopoietic organ, replenishing MΦ pool in all organs, except for the brain. Following birth, the amount of fetal liver MΦ in the heart, pancreas, gut and dermis is slowly replaced by infiltrating monocytes that further differentiate into MΦ. Other ResMΦs from liver, lung, spleen and peritonei maintain themselves independently of monocytes at steady state (Yona et al. 2013). Of note, monocyte replenishment and/or infiltration is observed later in life, in all tissues upon inflammation.



*: Gordon et al, BMC Biol, 2017

** : Davies et al, Nat Immunol, 2014

Figure 6: ResMφs, functions and murine markers.

Recent RNA-seq analysis of mice ResMΦs from different organs and species identified different transcription factors, alone or in clusters, with a tissue-specific distribution (Gosselin et al. 2014; Lavin et al. 2014) (Figure 6). They even concluded that more similarities can be found between ResMΦs from one organ but in different species, than within a single individual (Schultze and Schmidt 2015). Indeed, the **wide range of MΦ functions** is highly modulated, as it depends mostly on the need/requirement of the organ. Recently, Homaira Hamidzada presented similar informations in humans, by comparing and sequencing MΦs from the liver, heart, brain, spleen and kidney (transplant patients) (EMDS 2021 talk, unpublished data). In addition to these variations between organs, and in accordance with their high environment-dependent plasticity, MΦ functions are highly disturbed upon injury or diseases, such as chronic inflammation and cancer.

All together, these data highlight the importance of the environment rather than the ontogeny when looking at MΦs function and phenotype.

iii. From monocytes to ResMΦs

As mentioned above, MΦ can derive from **monocyte**.

Monocyte is a specific circulating immune subset, representing 10% of human peripheral leukocytes. They originate from the adult bone marrow where they share a Common Myeloid Progenitor with erythrocytes, platelets cDCs and granulocytes (Guilliams et al. 2014). Even if, for decades, they were only seen as MΦ and conventional DCs precursor, abundant evidences suggests that there is more than meet the eye.

First, their compartment is diverse, as they can be classified into two distinct subsets with specific functions. The Ly6C^{hi}CD14⁺CD16⁻ “**classical monocytes**” represent 80-90% of the population and are responsible for most of the pro-inflammatory response events (A. A. Patel et al. 2017). They are short-lived (one day), highly plastic and efficient APCs, both in the circulation and in lymph node, helping in T helper maturation (Jakubzick, Randolph, and Henson 2017). Over time, if not recruited, they transform into Ly6C^{low}CD14^{low}CD16⁺ “**non-classical**” monocytes, which are less frequent (10-20%), long lived (7 days) and supposedly more related with vasculature/endothelial cells integrity keepers (A. A. Patel et al. 2017). Two putative new clusters were recently uncovered by RNA-seq (Villani et al. 2017). Of note, as discussed above, as ResMΦs are, in most part, from embryonic origins, monocytes are supposed to be mostly DC precursors in steady state condition.

Monocytes-to-MΦs differentiation process is induced by specific cytokines and chemokines secretions in the bloodstream. Amongst them, two are growth factors of high importance and the main molecules used for monocyte-to-MΦs *in vitro* differentiation into pro- and anti-inflammatory cells, MΦ Colony Stimulating Factor (M-CSF) and Granulocyte MCSF (GM-CSF) respectively (Lacey et al. 2012). These are essential for MΦ development and maintenance. Upon this maturation process, monocytes, that are usually 7-8µm, became larger (15-20µm) and their secretion as well as metabolic profile is modulated.

Once infiltrated in the organ and undergoing differentiation, monocytes join the pool of ResMΦs already present on site. These infiltrating cells, with a more pro-inflammatory phenotype than ResMΦs, help the resident in the resolution of the injury, as it was observed in murine studies (Schiwon et al. 2014). Recently, Aegerter and colleagues, described a specific

timeline of alveolar M Φ replenishment and their conversion in ResM Φ s, by investigating trained immunity (Aegerter et al. 2020). They observed that, one month after their first Influenza Virus encounter, mice challenged with *S.pneumoniae* were less susceptible than the non-infected ones to the bacteria. This was associated with the infiltrating M Φ which conserved a more activated epigenetic profile (500 genes modulated upon re-challenge, vs 60 for the ResM Φ s), as an imprinting of their environment of recruitment. Thus, infiltrating M Φ to ResM Φ s conversion is a slow process during which the **InfResM Φ s** (inflammatory ResM Φ s) have a distinct profile, more plastic and pro-inflammatory than the one of ResM Φ s. With time and return to homeostasis, InfResM Φ s are slowly converted into ResM Φ s (Guilliams and Svedberg 2021; Aegerter et al. 2020). In addition to this conversion, infiltrating monocytes can also egress from the inflammatory site, or die (Scott et al. 2016; Guilliams and Svedberg 2021). The latter are termed “Transient M Φ s” or **TransM Φ s**, in respect to their short-term live (Guilliams and Svedberg 2021).

b. M Φ 's polarization profile

i. Let the Old Nomenclature fall.

M Φ s are highly plastic, integrating a broad range of signals from the environment that shape their phenotype and functions. Deciphering the specific mechanisms that drives M Φ activation and the resulting phenotype took a long time and it is still partly unclear.

This story started in the 60's when the classical phenotype was first described (Mackness 1962) by Mackness and colleagues, as cells that are activated upon Th1 secretome stimulation. Forty years later, another type of M Φ s, activated upon Th2 secretome stimuli, was identified, and named “the alternative phenotype” (Stein et al. 1992). A new nomenclature arised from these discoveries, reflecting the classification of their Th1 and Th2 inductors, M Φ s were termed as **M1 (classical) and M2 (alternative)**. In the following years, many more phenotypes between M1 and M2 were defined (**Figure 7**) (Martinez and Gordon 2014, 2).

This model has been increasingly challenged. One of the first default is the **discrepancies** of differentiation/stimulation protocols in between laboratory that described these populations (Murray et al. 2014). *In vitro* polarization of M Φ s extracted from blood bags or bone marrow are widely used in the community to mimic the *in vivo veritas*. The main setting consisting of using cytokine secreted by T helper 1 or 2, according to each phenotype, to ensure their polarization, (i.e: TLR agonist + IFN γ for M1- M Φ , IL-4 and IL-13 for M2- M Φ). This protocol has many advantages, amongst which cost-effectiveness and simplicity. However, labs are using different stimuli and, even using the same protocol, ligands and/or cytokine doses and markers of each phenotype are not standardized, increasing the already existing discrepancies. Responding to this issue, Xue and colleagues presented a polarization model dependent not on this M1 vs M2 discrimination, but rather on the **stimuli used** (Xue et al. 2014).

Their transcriptomic analysis enabled the definition of at least nine different polarization status that, even if clusters are possible, have their own transcriptomic profiling. Thus, M Φ s can be now termed as M(LPS), M(IL-4), M(IL-13), etc, rather than the M1/M2 old dogma. Anyhow, even if this model encompasses more possibilities than the initial one, it does not surely mirror the *in vivo* reality that is way more complex, as it is a result of a myriad of cytokines and other signals, and the balance between each of them. In the following year, Murray and colleagues presented a summary of all recommendations that one must made when studying M Φ s

polarization status, emphasizing on the importance of environmental cues on stimuli received (Murray et al. 2014). Finally, increased knowledge on *in vivo* MΦ phenotype led to another definition of their polarization status, rather seen as a rainbow of different functions that are intertwined, than defined ones. Hence, even if *in vitro* models are essential for research, they must always be compared with *in vivo* or *ex-vivo* extracted ResMΦs.

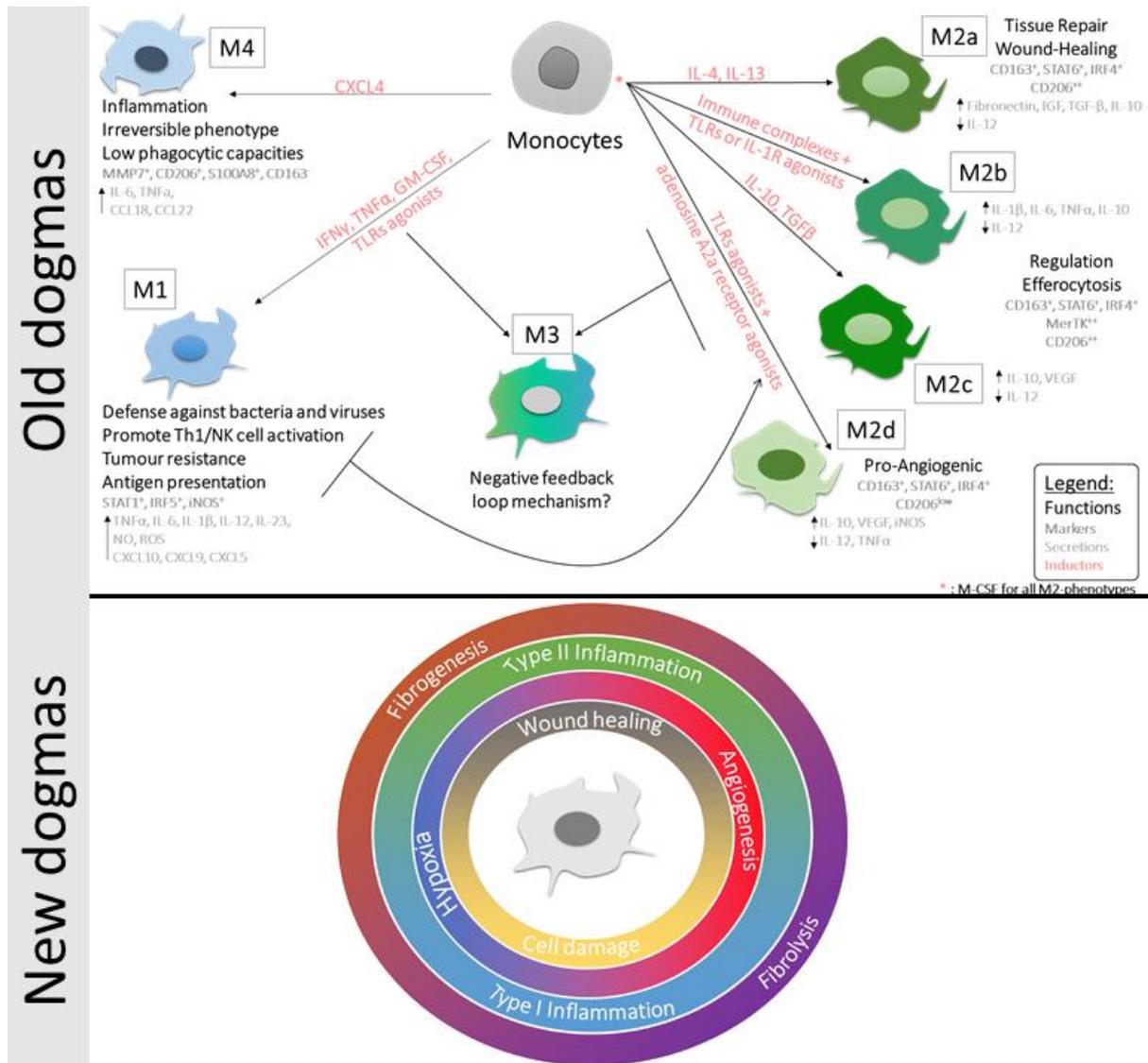


Figure 7: MΦ's polarization profile, old and new dogmas.

ii. MΦs activation programs

These broad spectrums of polarization profile are defined through fine-tuned molecular processes relying mostly on the activation triggers. They depend on (1) MΦ origin and ontogeny, the tissue in which they are resident (already discussed), each tissue having different needs fulfilled by a specific profile of MΦs and (2) the type of tissue injury, every insults inducing different sets of cytokine secretion/protein expression that will differentially shift MΦs (Schultze and Schmidt 2015).

MΦs are sentinels, equipped with a broad range of PRRs to detect and respond to inflammation. Thus, their activation relies mostly on the detection of DAMPs/PAMPs and the downstream pathway involved, as discussed earlier (**Figure 8**). This is the integration of the sum of these signals as well as environmental cues that leads to a specific phenotype (**Figure 8**).

The importance of metabolism in MΦ polarization status:

Metabolism has increasingly become an important feature of immune cell polarization status, especially MΦ. Abundant evidences correlate MΦ functions, their needs, to their metabolic status. Two major metabolic status have been described, relying mostly on the opposition between the metabolic requirements of pro-inflammatory MΦs and their anti-inflammatory counterparts (Stunault et al. 2018).

Upon pathogen-mediated injury or infection, **pro-inflammatory** MΦ are quickly recruited in inflamed tissues, which are often characterized by low levels of oxygen (i.e hypoxia). Anti-pathogenic MΦs obtain energy through **anaerobic glycolysis**, enabling low but rapid production of ATP. Upregulation of glucose transporters expression in these cells enable increased glucose uptake (Freemerman et al. 2014). Glucose is further converted into pyruvate that fuel the TCA cycle (Galván-Peña and O'Neill 2014). Pro-inflammatory MΦ have **two break in their TCA cycle** leading to cytoplasmic accumulation of succinate and malate (Stunault et al. 2018) (**Figure 8**). Enrichment of these substrate promote HIF1 α expression leading to pro-inflammatory cytokine production and increase in inducible Nitric Oxide Synthase (iNOS) expression (Stunault et al. 2018). iNOS mediates L-arginine catabolism into citrulline and nitric oxide (NO), the latter being a well-known anti-bacterial agent. In pro-inflammatory MΦ, the pentose phosphate pathway is induced, leading to NADPH oxidase activation and increase in ROS production. Thus, metabolism is important for pro-inflammatory MΦs to obtain specific features, such as ROS, NO and pro-inflammatory cytokines secretion.

MΦs that are more involved in **wound healing** and **anti-inflammatory** function have different metabolic requirements. These processes require a more sustained response, with a lot of energy and are often occurring in pro-angiogenic environment that are rich in oxygen. That is provided through **aerobic respiration**, which is slower, but enable cells to produce 30 to 32 molecules of ATP, compared to two in anaerobic conditions (Verdeguer and Aouadi, 2017). In that case, this is mostly fatty acid oxidation and oxidative metabolism that is used, leading to pyruvate processing in **an unbroken TCA cycle** (S. C.-C. Huang et al. 2014). Activation of wound-healing MΦ is often mediated through IL-4 and IL-13 stimuli. The downstream pathway activated by these cytokine leads to STAT6 mediated activation of PGC-1 β (Stunault et al. 2018). PGC1 β is responsible for mitochondrial respiration and biogenesis, enabling the production of cytochrome c and ATP. PPAR γ and lambda are both induced by PGC1 β and orchestrate the alternative phenotype by induction of anti-inflammatory factors such as arginase-1 (Arg1) and antagonize the activity of NF κ B and AP-1 (Ricote and Glass 2007). In anti-inflammatory MΦs, L-arginine is processed by Arg1 into urea, ornithine and polyamine that are substrates involved in wound-healing. Glutaminolysis support the alternative phenotype through epigenetic regulation of several genes, such as demethylation of jmjd3, a gene controlling IRF4 expression (Stunault et al. 2018). Upon resolution of the infection, inflammation needs to be tightly controlled to return to an homeostatic states and metabolism is a key feature in this matter. For instance, CARKL, a protein involved in the PPP, is downregulated in pro-inflammatory MΦs (Haschemi et al. 2012). Besides, L-arginine is a crucial substrate for both pro- and anti-inflammatory MΦs metabolism.

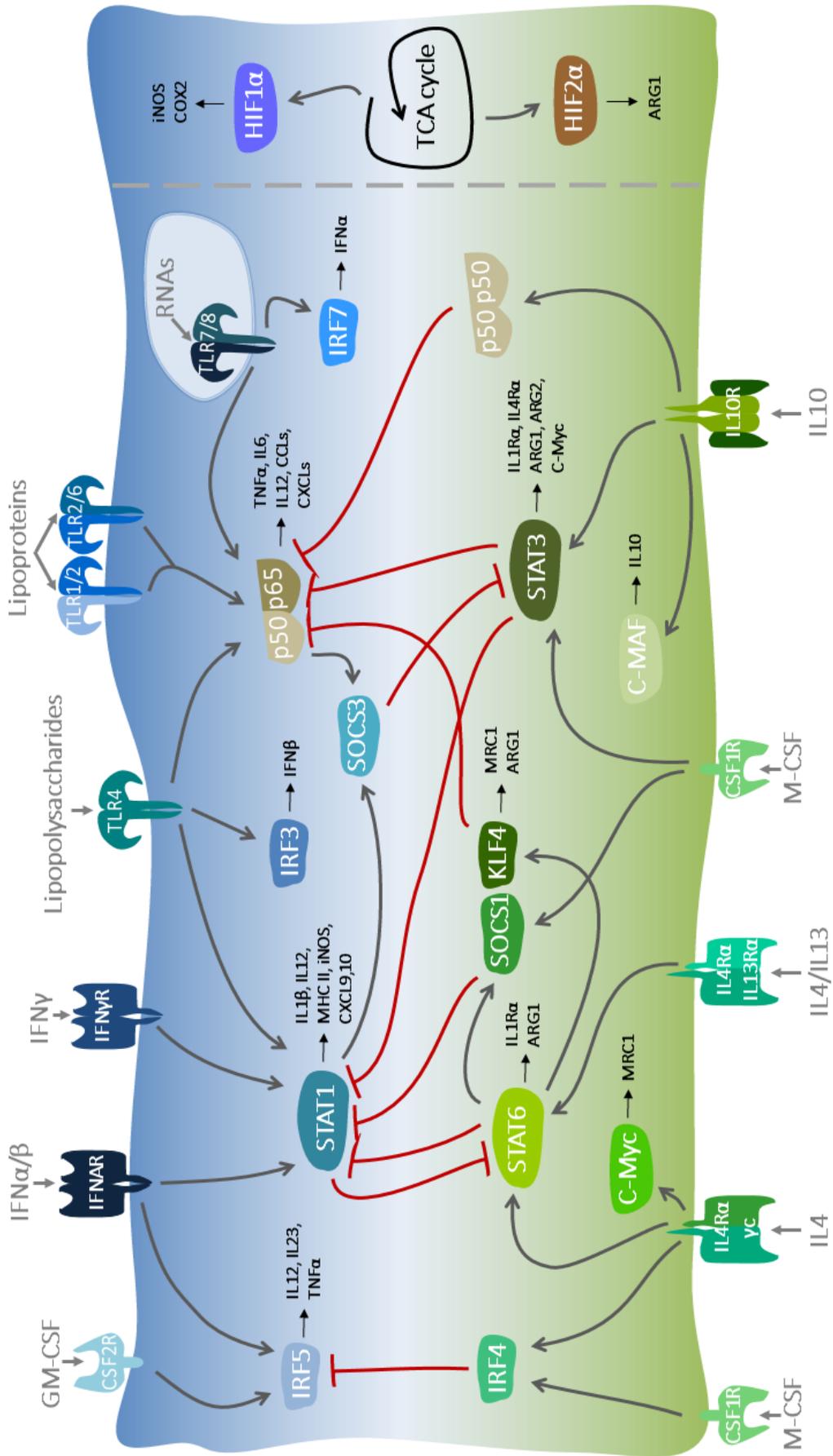


Figure 8: MΦ activation profile. Informations are provided in the text.

Evidence suggest that Arg1 and iNOS are competing for L-arginine processing with different advantages, Arg1 being faster and iNOS having a better affinity for the substrate (Stunault et al. 2018). Thus, the balance between the two enzymes is of high importance for the resulting phenotype. Interestingly, prolonged exposure of classical activators, such as LPS, induces TIPE2 expression that promote Arg1 over iNOS processing of L-arginine, enabling a return to homeostatic condition.

Transcriptional regulation factors involved in MΦs polarization:

Modulation of gene expression is a major step toward shifting MΦ phenotypes. Following PRRs or cytokine stimulation, the resulting downstream pathways are triggering a large array of transcription factors and regulators. More precisely, transcriptomic analysis identify a **set of transcriptional regulators involved in MΦs activation**, independently of their kind of stimuli (NFKB1, JUNB, CREB1, HIVEP1, HBP1, HMGA1, NFE2, ZNF148, SMARCA2, DDX21, MNDA, TBLX1, RELA, IRF1, IRF2, IRF7 and TBP) as well as peculiar clusters of TFs stimuli-dependent (e.g JunD and STAT1 for LPS exposure) (Roy et al. 2015; Xue et al. 2014). They are, nonetheless, activated distinctively. On one hand, strongly but transiently by IFN γ , and on the other hand, in a moderate but continuous manner following IL-4/IL-13 stimuli (Roy et al. 2015) (**Figure 8**). These kinetic differences are in line with the pro and anti-inflammatory metabolism mechanisms discussed above. In addition, they suggest that the same TFs could act in different timelines, once again, shaping the activation profile. These TFs are associated with active histone marks as well as enhancer regions. **Enhancers** are short DNA regions on which TF are able to bind. An elegant study performed by Kaikkonen and colleagues, coupled nuclear run-on and deep sequencing to identify a set of approximately 3000 enhancers that are primed as rapidly as 10 minutes after KLA challenge (a known TLR4 agonist) (Kaikkonen et al. 2013). These regions were enriched in NFkB, PU.1, C/EBP and AP-1, consistent with previous findings. Super Enhancers (SE) regions are recently discovered clusters of enhancers densely occupied by TF. They were described as key player in defining cell identity and fate (Peng and Zhang 2018). Besides, SE are more prone to produce **eRNA**, a newly discovered type of non-coding RNA derived from enhancer regions. These eRNA promote TF binding and gene transcription in tight cooperation with SE. SE can be both tissue specific, like Gata6 for large peritoneal MΦs, and tissue unspecific (Spi1, Cebpa, Csf1r and others) (Gosselin et al. 2014). They enable the specific transcription of gene sets, defining the resulting MΦ phenotype.

Importantly, they presented that, for peritoneal MΦs, loss of retinoic acid signal effectively shifted the transcriptional profile of these cells, especially concerning SEs expression, rendering obsolete *in vitro* studies that do not and cannot encompass all environmental elements (Gosselin et al. 2014). Thus, gaining a better understanding of all environmental signals important in a tissue-specific manner is of high concern for the relevance of *in vitro* studies.

Modulation by non-coding RNA

miRNA are 20 nucleotide long RNA that pair to complementary 3'UTR sequences of specific mRNA. This base-pairing will induce silencing of the targeted mRNA. It has become increasingly known that miRNA play important roles in the regulation of immune functions, especially concerning MΦs (Curtale, Rubino, and Locati 2019). In the past few years, abundant data described miRNA as key modulators of MΦs polarization. They are broadly upregulated (miR21, miR147, miR146a, miR214, miR125b, miR455) or reduced (miR223) following LPS stimulation to orchestrate the immune response. They are involved in several feedback loop mechanisms,

such as the IRAK1/TRAF6 by miR146a and the control of NFκB activity by miR9, miR21 and miR147 (Curtale, Rubino, and Locati 2019). Besides, they aid in the cross-inhibition existing between pro and anti-inflammatory mechanism, such as the BCL6-mediated IL-13 pathway inhibition mediated by miR155.

Long non-coding RNA are 200 nucleotides long non-coding RNA able to bind DNA, RNA and protein, alone or in complex. Their exact role is still under investigation; however, several studies have demonstrated the capacity of DAMPs and PAMPs to induce these lncRNA to modulate the immune response (Atianand et al. 2016; Carpenter et al. 2013; Covarrubias et al. 2017; G. Hu et al. 2016; Krawczyk and Emerson 2014; Zhonghan Li et al. 2014; Scacalossi, van Solingen, and Moore 2019).

Logically, these pathways are targeted by most pathogens in the reduction of MΦ pathogenicity, as observed mostly in liver viruses (*discussed in the annexe "How to get away with liver innate immunity? A viruses' tale - Delphin et al"*), HIV-1 and/or *M.tuberculosis* (Lugo-Villarino et al. 2011), but also extensively reviewed here (Atri, Guerfali, and Laouini 2018).

c. MΦ in pathological context

i. MΦ in cancer: discovery of immune suppressive cell types

In a large panel of solid tumor, infiltration of myeloid cells account for up to 50% of the total tumor cells (Noy and Pollard 2014). These specific MΦ, termed **Tumor-Associated-MΦs (TAMs)**, possess a broad panel of functions. In some settings they are anti-tumoral, supporting cancer cell detection and clearance from the organ. However, in most cases, they are increasing angiogenesis, promoting an anti-inflammatory milieu prone to T cell exhaustion, being a source of specific metabolites needed by tumor cells, and are thus linked to resistance to cancer therapy along with poor outcomes (Noy and Pollard 2014).

Another MΦ subset, the **MDSCs**, is linked to cancer cells. In cancer, the distinction between MDSCs and TAM of a suppressive phenotype is not clear to this day (Corzo et al., 2010; Kumar et al., 2016). As specific markers for both cell types are found in solid tumor, it is highly debated if MDSC are immature TAM or if both cell types are distinct. However, MDSCs possess specific functions and are involved not only in cancer setting but also in chronic inflammation and stress-induced pathologies (Dorhoi et al. 2019; Zhai et al. 2017; Pal et al. 2019a). They are involved in angiogenesis, promotion of tumor escape, immune checkpoint blockade and more importantly, drug resistance for multiple cancer (Gabilovich 2017; S.-M. Park and Youn 2019). They are mainly characterized by poor phagocytic activity, continuous production of ROS, Reactive Nitrogen Species (RNS), NO and secretion of anti-inflammatory cytokines (TGF-β, IL-10, VEGF) (Bronte et al. 2016). MDSCs are surely part of a negative feedback loop in order to protect tissue integrity following continued inflammation, but tumors have found ways to hijack this system at their advantage, increasing MDSC recruitment. Thus, MDSC presence in tumor correlate with **poor prognosis** in a broad range of cancer (Chesney, Mitchell, and Yaddanapudi 2017).

MDSC are similar in morphology and phenotype to monocytes and neutrophils, but possess different biochemical and genomic profile due to their suppressive functions. Thus, they are Polymorphonuclear MDSC (**PMN-MDSC**) defined as CD11b⁺CD14⁻CD15⁺ or CD11b⁺CD14⁻CD66b⁺, or Monocytic MDSC (**M-MDSC**), defined as CD11b⁺CD14⁺HLA-DR^{-/lo}CD15⁻S100a9^{high} according to their resemblance with neutrophils or monocytes, respectively (Bronte et al. 2016). In human, a third type of MDSC, called early stage MDSC (eMDSC), defined as Lin⁻ (including CD3, CD14, CD15, CD19, CD56) HLA-DR⁻CD33⁺, are described as a mixed group of MDSC that encompass different immature progenitors.

MDSC arise from pathological conditions through a low dose but persistent stimulation of the myeloid compartment. More precisely, Condamine and colleagues suggested a two-step mechanism (Condamine, Mastio, and Gabrilovich 2015a). The first set of signals is composed mainly of tumor-derived factors, such as STAT3, IRF8, C/EBP, Notch, Adenosine receptors A2b and NLRP3. They enable the expansion of the myeloid subset without permitting their final differentiation. The second wave consist mainly of pro-inflammatory cytokines (IL-6, TNF α , IL-1 β), PRRs stimulation, STAT1/6, prostaglandin E2, cyclooxygenase 2, the ER stress response and the NF κ B pathway. These factors permit the differentiation from immature myeloid cells into pathologically activated MDSCs.

MDSCs and TAMs characterization and mode of action has been mostly described in cancer setting but the mechanisms discovered is being broaden to other context involving these cells, such as pathogenic invasion (Ugel et al. 2015).

ii. M Φ and viruses

M Φ are the first line of defense against pathogens, as already discussed in this introduction. Interestingly, they also represent a target of choice for more than 30 viruses that uses them as viral target and/or reservoir (Nikitina et al. 2018). Most of the infections begin with a pro-inflammatory response, prone to viral clearance. However, upon chronicity or at later stages, more anti-inflammatory phenotypes are observed, associated with viral establishment and persistence (Atri, Guerfali, and Laouini 2018). Viruses able to hijack monocyte/M Φ (HIV, HCMV, HCV, HBV, amongst others), as well as their strategies are numerous. Of note, these modulations are not solely imputed to an active/functional viral replication, as they are also observed in latent (Lusso 2006) or non-permissive contact (Faure-Dupuy, Delphin, Aillot, Dimier, Lebossé, Fresquet, Parent, Sebastian Matter, et al. 2019).

First, viruses are able to use the circulating ability of monocytes as **Trojan Horse** for difficult to reach organs, such as the brain, as it was described with HIV, HCMV and JEV (Kruize and Kootstra 2019; Nikitina et al. 2018). On the way to a specific organ, interaction with monocytes can lead to delayed apoptosis, providing more time for the infectious agent to reach its target (Chan et al. 2010; Nikitina et al. 2018; Kruize and Kootstra 2019), or modifying monocytes inflammatory phenotype (Kruize and Kootstra 2019; Faure-Dupuy, Delphin, Aillot, Dimier, Lebossé, Fresquet, Parent, Sebastian Matter, et al. 2019). **Increased recruitment and/or migration** of M Φ is observed in HIV-1-Nef transgenic mice and *in vitro* models (Vérollet et al. 2015). In HBV and HCV infected patients, viruses are able to **recruit MDSCs** to favour an immunosuppressive environment (Pallett et al. 2015a; Pal et al. 2019b; Zhai et al. 2017).

Once internalized in M Φ , viruses need to avoid detection and thus some are equipped to **decrease PRRs expression**, as seen in Peripheral Blood Mononuclear Cells (PBMCs) and liver M Φ of HBV infected patients (Y.-W. Huang et al. 2013a; Vincent et al. 2011b; Visvanathan et al.

2007a; van Montfoort et al. 2016a; Vanlandschoot et al. 2002). Cytokine secretion profile is a key aspect of immune cell phenotypes. **Pro-inflammatory cytokines**, such as IL-1 β , TNF α , IL-6, IL-12 or IFN-I, **decreases** were observed *in vitro* upon incubation with HBV, HCV, HHV-6 and ZIKV (Faure-Dupuy, Delphin, Aillot, Dimier, Lebossé, Fresquet, Parent, Matter, et al. 2019; Z. Tu et al. 2010; J. Lang et al. 2018; Lusso 2006). On the other hand, **anti-inflammatory cytokines** (IL-10, TGF- β , M-CSF) and/or **surface markers** (CD163) are **upregulated** in patients with HBV and HIV infection (or *in vitro* models) (Dunn et al. 2009a; S. Wang et al. 2013a; Faure-Dupuy, Delphin, Aillot, Dimier, Lebossé, Fresquet, Parent, Matter, et al. 2019; Osman et al. 2014; Souriant et al. 2019); as well as in patient sera and *in vitro* models of HCV infection (Sène et al. 2010). Finally **increases in inhibitory receptors** (i.e PD-1/Tim-3) and especially their ligands (PD-L1/2, Gal9) have been observed in a large number of infections, namely HIV, HBV, HCV, HSV-1, Influenza, LCMV and RSV (Jeon et al. 2018; Schönrich and Raftery 2019; Xiao et al. 2016; Staples et al. 2015; Nebbia et al. 2012; Xie et al. 2009).

4. The liver, a specific immune organ

The liver is a hub, essential for a broad number of physiological processes.

Its peculiar organization (**Figure 9**), enables it to **filter** blood of the gastrointestinal tract and systemic circulation, that comes through the portal vein and hepatic arteries, respectively (Doherty 2016). It is thus a key player in our body's **detoxification**, through the breakdown and elimination of a number of xenobiotic compounds (toxins, hormones, drugs, alcohol) as well as old red blood cells (Kalra et al. 2021). Moreover, the estimated 1.5L of blood received in the liver every minute, contains a massive amount of harmless dietary residues and bacterial products, all of which should trigger a massive immune response. Instead, the liver is commonly known as a **tolerant organ** (Calne et al. 1969; Crispe 2009a). Its unique immunoregulatory functions are mediated by every cell composing the tissue, both professional and non-professional immune cells. Some pathogens, like hepatitis B and C viruses, take advantage of this tolerance to install their chronicity (Faure-Dupuy, Durantel, and Lucifora 2018). It is, however, possible to break this tolerance to clear harmful pathogen, but this lead often to inflammatory mediated liver damages. Thus, liver immunity is highly peculiar and tightly regulated.

The liver is also responsible for more than 500 processes involving **metabolism** (Trefts, Gannon, and Wasserman 2017). First, it is involved in fatty acid oxidation as well as cholesterol and lipoprotein synthesis (i.e **lipid metabolism**). Second, its production of amino-acid (synthesis and breakdown of protein) accounts for 80-95% of the total circulating protein volume, making it one of the major organ regarding **protein metabolism**. Third, **carbohydrate metabolism** in the liver is essential for energy production of many pathways (Kalra et al. 2021).

It is a **reservoir** for minerals (iron, cooper), vitamins, lipids but also glucose. Indeed, glucose is stored within the liver in its glycogen form, which is released through **glycogenolysis** to provide an essential energy source, when required (i.e upon punctual diets) (Trefts, Gannon, and Wasserman 2017). Besides, the liver is able to use the amino acids and fatty acids produced to form glucose, a process termed **neoglucogenesis**. Thus, it is an important gatekeeper of our blood sugar level. Finally, it is an important producer of growth factors, albumin, but also bile acids. Bile is an essential liquid for **digestion** (Kalra et al. 2021). It is secreted and released in liver's bile duct, to be re-absorbed mostly by enterocytes in the intestine.

The liver is composed mainly of **Hepatocytes**, the parenchymal cells (**Figure 9**). Non-Parenchymal Cells (NPCs) are composed of (1) liver specific cells: the Liver Sinusoidal Endothelial Cells (**LSECs**), **Kupffer Cells** (KCs) and **HSCs** and (2) professional immune cells that are circulating and can infiltrate the liver upon activation such as Monocytes, Lymphocytes, NK, NKT, DCs.

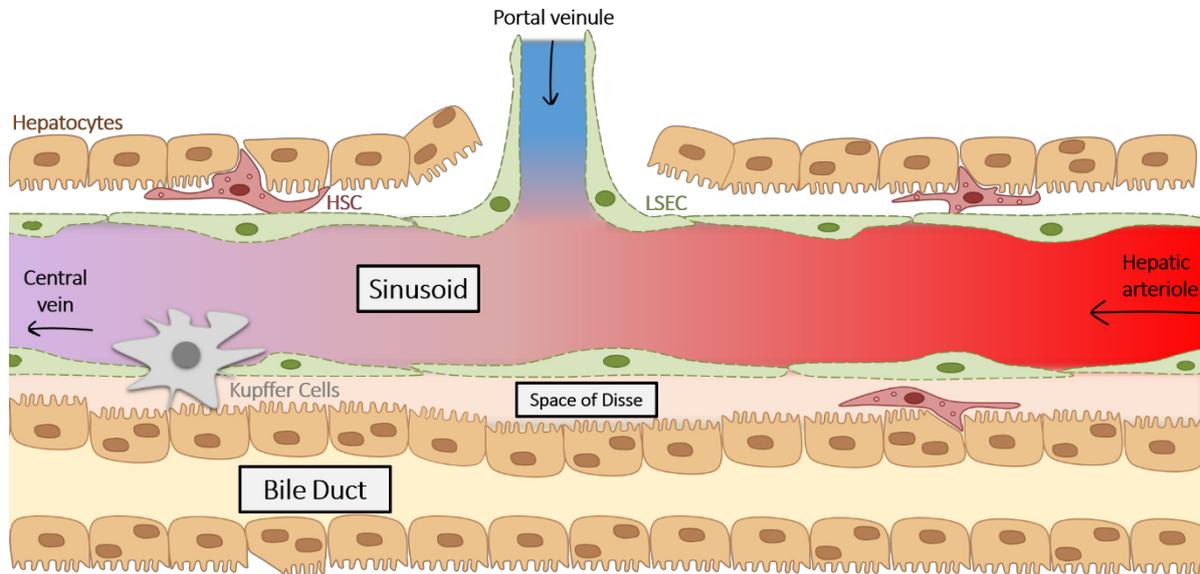


Figure 9: Liver organization, detailed explanations are provided in the text.

a. Liver specific cells

i. Hepatocytes

Parenchymal liver cells, also called hepatocytes, constitute 70-80% of the total liver mass (Crispe 2009a) (**Figure 9**). These cells are of high importance for liver homeostasis, they carry various functions among which (1) drug detoxification (2) urea elimination (3) cholesterol synthesis (4) storage of glucose as glycogen (5) glycogenolysis (Z. Zhou, Xu, and Gao 2016). Their position in the organ and the fact that they are polarized enable them to interact with elements coming from both the blood (apical pole) along with elements from the bile duct (basal pole), allowing them to assure their broad panel of functions.

Hepatocytes are highly involved in the **liver intrinsic immunity**. They express many PRRs (but no inflammasome proteins), enabling them to detect and respond to extra and intra-cellular pathogens (Faure-Dupuy et al. 2018; Zannetti et al. 2016). Besides, they are considered as downstream effectors of the immune system as they express many elements of response to cytokines such as the STAT3 and NF κ B pathway (Bode et al. 2012). Following their intrinsic immune activation, hepatocytes produce and release cytokines, complement and other opsonin proteins, soluble effectors of the TLR4 pathway (sMD-2, sCD14, LBP) and iron-modulating proteins (involved in the inhibition of bacterial growth) (Z. Zhou, Xu, and Gao 2016). They do not express the co-stimulatory molecules CD80/CD86 but are still capable of activating CD4⁺ and CD8⁺ T cells and present antigen through their CMH expression (Bertolino, Trescol-Biémont, and Rabourdin-Combe 1998; Qian et al. 2001). This was only demonstrated in mice model but suggest that this partial activation leads to the loss of the cytotoxic features of CD8⁺T

cell three days post interaction with the hepatocytes and induces apoptosis (Bertolino, Trescol-Biémont, and Rabourdin-Combe 1998).

ii. LSECs

LSECs form the **epithelium of the hepatic sinusoid (Figure 9)**. Fenestration of this cell lining enables free diffusion of small molecules into the space of Disse, that separates LSECs from hepatocytes (Braet and Wisse 2002). LSECs, which constitute 20% of the total liver cells, are highly peculiar endothelial cells as they mediate immune tolerance toward innocuous gut-derived food and microbial antigens, coming from the portal veins (Crispe 2009a). Their high expression of scavenger and mannose receptor render them able to phagocytose material of less than 200nM, leaving the larger one to KCs. They process them and present the antigen to CD4+ and CD8+ T cells through their constitutive expression of CMH class II molecules. In addition, their expression of CD80/86 and CD40 enable them to interact with T cells, like other APCs. Unlike classical APCs, their engagement of CMHII and costimulatory molecules result in adaptive immune tolerance, favoring Treg expansion and induction over CD8+T cell activation (Crispe 2009a).

They present a **restricted TLR activation profile**, leading to lower inflammation. Response to TLR4 stimulation leads to IL-10 secretion and decrease in antigen presentation, ensuring Treg expansion and induction (KNOLLE et al. 1998). They are also able of sequestering CD8+T cell in the liver sinusoid, through enhanced adhesion molecule expression such as V-CAM, I-CAM and VAP, and downregulating their activation through low CD80/86 and high PD-L1 expression. The latter mechanism leads to cell-to-cell contact via CMH-TCR and PD-L1/PD-1 that induces CD8+T cell tolerance. Besides, as they express constitutively FasL, direct contact with lymphocytes leads to their apoptosis (Tokita et al. 2006). They express several C-type lectin amongst which LSECTin that was shown to inhibit T cell proliferation and activation, decreasing the secretion of IL-2 and IFN- γ and leading to apoptosis (F. Xu et al. 2014). Finally, they are capable of decreasing DC-mediated CD8+T cell activation through cell-to-cell contact by downregulating CD80/86 expression and IL-12 secretion by DC.

Nevertheless, in some context of viral infection, LSECs are capable of inducing CD8+T cell immune activation, instead of decreasing/inhibiting it, through the decrease of PD-L1 expression (Kern et al. 2010).

Thus, under physiological conditions, LSECs facilitate the acquisition and maintenance of a tolerogenic phenotype characteristic of the liver environment, but are still capable of aiding in pathogen clearance when required.

iii. HSCs

HSC – also called **Ito cells** – are localized in the subendothelial space of Disse and interact with many liver cells using their multiple dendritic-like cytoplasmic protrusions (Yin et al. 2013) **(Figure 9)**. **Quiescent stellate cells** represent 5-8% of total liver cells. They store fat (Vitamin A and triglycerides), impact sinusoidal blood flow and are the major liver producers of extracellular matrix components (Weiskirchen and Tacke 2014). Upon viral or toxin-induced liver insults, damaged liver cells secrete signal molecules, mainly TGF- β , which causes trans-differentiation of HSCs into activated myofibroblast-like cells. They acquire a flat fibroblast-like shape and secrete massive amounts of extracellular matrix components that protect against further damages and scares the injury (Yin et al. 2013). Prolonged HSC activation shift the balance towards over secretion of **pro-fibrogenic** and pro-inflammatory cytokines, causing

fibrosis, cirrhosis and HepatoCellular Carcinoma (HCC) emergence (Weiskirchen and Tacke 2014). Upon removal of liver injury inducers (i.e mainly hepatitis virus or alcohol consumption), a diminution of activated HSCs is observed, through apoptosis, senescence or return to a quiescent state (Yin et al. 2013).

In the past few years, an increasing body of evidence suggest that HSC have a pivotal role in liver immunity by being capable of either downregulate or orchestrate the immune response (Fujita and Narumiya 2016). HSCs are capable of sensing various pathogens through their expression of several TLRs (Faure-Dupuy et al. 2018). When activated, they secrete a broad panel of chemokines, attracting neutrophils, NK/NKT cells and monocytes to the liver and promoting lymphocytes infiltration (Gupta, Khadem, and Uzonna 2018; Holt et al. 2009).

On the other hand, activated HSC were suggested to possess immune regulatory functions as well. For instance, they inhibit both T and B cell activity, through increase in PD-L1 expression at their surface (Y. Li et al. 2016; M.-C. Yu et al. 2004). Besides, HSC are involved in CD4⁺T cell to Treg differentiation process and monocytes recruitment, shifting MΦs towards an MDSC-like phenotype. Altogether, these data demonstrate HSC capacity to establish immune suppression in the liver, as a participation to liver homeostasis, as well as being capable, when required, to induce immune activation.

iv. hepatic MΦ

Hepatic MΦs are a heterogeneous population consisting of Monocyte Derived MΦs (MDMs), which are recruited upon tissue injury or infection (CD68⁺/TIMD4⁻), and liver ResMΦs, KCs (CD68⁺/ CD32^{high}/MARCO⁺/TIMD4⁺)(Zwicker, Bujko, and Scott 2021) (**Figure 9**). Even if clearly identified in mice, transposition to human is always difficult. However, it seems that, as mentioned earlier, two distincts MDMs populations can be observed within the organ, identified by some as CD68⁺/CD32^{high}/MARCO⁺/TIMD4⁻ for the InfResMΦ and CD68⁺/CD32^{int}/MARCO⁻/TIMD4⁻ for TransMΦ (Zwicker, Bujko, and Scott 2021). Finally, also in mice, another short-lived MDM population have been observed, the capsule MΦ (termed based on its localization), expressing CD64⁺/F4/80⁺/Clec4F⁻/TIM4⁻ (Sierro et al. 2017)

In the past few years, Bonnardel, Guilliams and colleagues investigated this monocyte-to-MΦ transition and discovered the concept of **liver niche** (Bonnardel et al. 2019; Guilliams et al. 2020). They postulate that colonization and imprinting is oriented by the surrounding cells, at least in their mice model (i.e induced loss of KCs, may induce bias). Indeed, TNFα and IL-1β secreted by dying ResMΦs activate HSCs and LSECs to produce increased levels of chemokines (i.e attracting monocytes) and adhesion molecules (i.e trapping monocytes) to specifically attract monocyte to the appropriate place, the niche. Engrafted monocytes are then slowly imprinted into ResMΦs by HSC, LSECs and hepatocyte secretions, as observed with the acquired expression of liver-associated transcription factor of DNA3 (ID3) and Liver X Receptor α (LXRα) (Bonnardel et al. 2019).

KCs are **non-migratory** ResMΦs with a **long life span** (a year) and **slow self-renewal** capacities (Tacke 2017). KCs constitute the largest MΦ population in the body (80% of the total) and are mostly found in the sinusoid, adhering to LSECs (Crispe 2009a). They degrade harmful pathogens entering from the portal veins and remove damaged red blood cells from the circulation. They express scavenger receptors and immunoglobulin receptors, enabling them to perform complement-mediated endocytosis of opsonized particle (Tacke 2017). Following particle engulfment, they are capable of antigen presentation through their expression of

CMHII and co-stimulatory molecules, further orchestrating the adaptive immune response. Besides, they are capable to sense many pathogens through the expression of a **broad range of PRRs**, such as TLR1/2, TLR2/6, TLR3, TLR4, TLR7 and TLR8, RIGI, MDA5 along with fully functional inflammasome (Faure-Dupuy et al. 2018). These features make them indispensable liver sentinels. At steady state, KCs are **tolerogenic**, thus they are mostly responding to bacterial stimuli, such as endotoxin challenge, by trapping CD8+T cell (John and Crispe 2005) and secreting IL-10, the major anti-inflammatory cytokine responsible for liver tolerance (Knoll et al. 1995). However, upon specific PRRs stimuli, hepatic MΦ are capable of mounting an efficient pro-inflammatory response. They can recruit neutrophil (IL-8, TNFa), leukocytes (CXCL8) and monocytes (CCL2), and modulate lymphocyte polarization status through IL-12 and IFN γ cytokine secretion. They secrete ROS and other pro-inflammatory cytokine, enabling **bactericidal as well as antiviral activities**. Their function as APCs allows lymphocyte reactivation in injured sites through presentation of pathogenic debris (Crispe 2009a). Thus, hepatic MΦs are key player in liver immunity.

b. Other immune cells with specialized compartment in the liver

In addition to liver-specific cells, NPCs are constituted by multiple immune cells that are present in the entire body but have a specific phenotype in the liver due to this tolerogenic milieu.

i. DCs

Intriguingly, **plasmacytoides and myeloid DCs** were found in **higher amounts** in the liver than in lymphoid organs, in healthy patients (Crispe 2009a). They are located in the portal area and, less frequently, in the parenchyma.

cDCs from the liver express low level of CMH-II and co-stimulatory molecule and thus are poor T cell inducers compared to non-hepatic DCs (Dou et al. 2018). They have lower endocytic capacities and secrete large amount of PGE₂, inducing IDO upregulation and subsequent IL-10 secretion thus resulting in induction of regulatory T cells (Dou et al. 2018; Lukacs-Kornek and Schuppan 2013).

In a similar manner, pDCs have decreased pro-inflammatory functions, mainly attributed to an upregulation of NOD2, that is well-known for dampening TLR signaling and upregulating PD-L1 expression (Castellaneta et al. 2009, 2). These cells secrete low levels of type I-IFN and diverse pro-inflammatory cytokines (IL-6, IL-12p70...) and have T regulatory inducers function as well.

ii. T Lymphocytes

Most liver T lymphocytes display an **activated state**. Their amount and functions differ in case of infection or liver injury (Crispe 2003). CD8+ T cells are mainly negatively regulated in the liver, as it has already been stated. Concerning CD4+T cells, the liver contains mainly Th1 and Th2 (Klugewitz et al. 2002). In mice liver, inhibition of IFN- γ secretion and other pro-inflammatory cytokines in Th1 cells is observed while Th2 functions were not impaired (Klugewitz et al. 2002).

iii. Natural Killer Cells

NK cells represent more than 50% of liver lymphocytes (Crispe 2009a). KCs production of IL-18 promotes perforin/granzyme cytotoxic release, and IL-12 stimulation enables IFN- γ (B. Gao,

Radaeva, and Park 2009). Unlike peripheral NK cells, liver NK cells are highly cytotoxic against tumor cells, express high levels of TRAIL, perforin, granzyme B...(B. Gao, Radaeva, and Park 2009). Interestingly, they are capable of recruiting T cell towards their activation and not tolerance, unlike many liver cells (Crispe 2003).

In mice, **NK-DCs** account for one third of total liver lymphocytes (B. Gao, Radaeva, and Park 2009). They have increased anti-tumoral responses along with higher IFN- γ secretion. In case of liver infection, they are suggested to be the only NK cells expressing CMH-II and costimulatory molecules (Burt et al. 2008).

NKT, which are rare in the bloodstream of healthy subjects, are strikingly, abundant in the liver (Crispe 2009a).

II. HBV and its satellite, Hepatitis Delta Virus (HDV)

1. HBV-HDV cellular biology

Informations about HDV are adapted from Lucifora and Delphin, AVR, 2020.

a. Discovery of the first hepatitis virus and its satellite

In 1965, Dr. Baruch Blumberg discovered the “**Australia antigen**” (Baruch S. Blumberg and Alter 1965), later associated with patients having acute and chronic hepatitis (B. S. Blumberg et al. 1967; Okochi and Murakami 1968). This antigen was visualized by electron microscopy in 1970 by Dane and colleagues (Dane, Cameron, and Briggs 1970). Predominant forms observed were filamentous (50-100 nm) and spherical (22 nm diameter). A later less frequent form consisted in a larger particle (42 nm) made of an inner body, the viral nucleocapsid (28 nm), surrounded by a 7 nm coat made of component which reacted with antibodies against the Au antigen, as spheres and filament did. This protein was identified as being the HBV surface protein (HBsAg) which can form self-assembled complexes termed Subviral Particles (SVPs) (i.e spheres and filament), as well as coating the infectious virion, termed Dane particle (**Figure 10**). Within the envelope, the nucleocapsid, formed with HBV-Core (HBc) protein dimers, contains the HBV relaxed circular DNA form (rcDNA), a 3.2 kb DNA covalently linked to the viral polymerase (HBV-Pol) (**Figure 10**). Lately, other HBV-related particles have been detected in patient’s blood, such as non-infectious particles (i.e enveloped nucleocapsid without rcDNA or with viral RNA) and naked nucleocapsids (Hu and Liu 2017) (**Figure 10**). The biological relevance of these forms is still misunderstood.

In the late 70s, the study of HBV infected patients experiencing extremely severe hepatitis led to the discovery of a small virus, **HDV** (M. Rizzetto et al. 1977). This virus was later shown to be a **defective satellite** virus that uses the envelope of HBV (**Figure 10**) to egress from and to re-enter into hepatocytes (Smedile et al. 1982). HDV is quite unique amongst animal RNA viruses, but actually shares a lot more with plant viroids.

Beside the HBV envelope, HDV virions contain a ribonucleoprotein (RNP) composed of (1) a circular single-stranded negative RNA genome (**HDV-G**) of almost 1,7 kb (K. S. Wang et al. 1986), presenting an unbranched “quasi” double-stranded conformation, and of (2) both isoform of the viral proteins called **S-HDAg** and **L-HDAg** for Small and Large HD antigen, respectively (**Figure 10**). More precisely, HDV RNA forms an extended quasi-dsRNA containing numerous internal loops and bulges. Its association with HDAg would depend more on its secondary structure than on its primary sequence (Griffin et al. 2014).

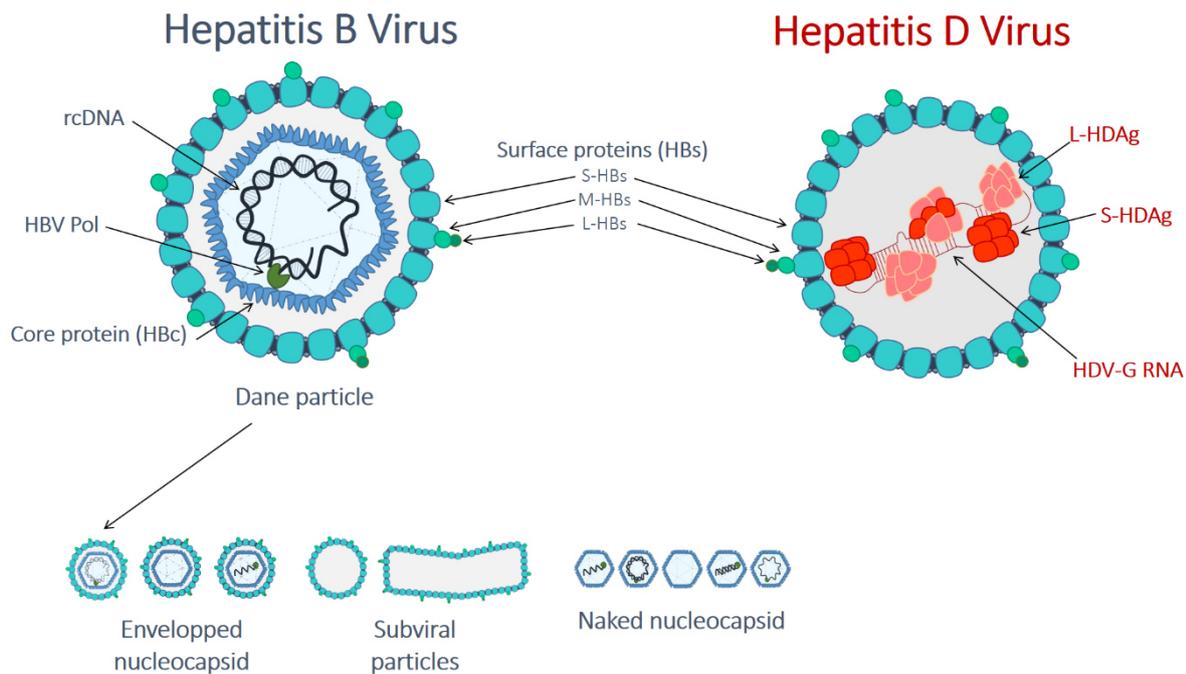


Figure 10: HBV and HDV particles, from Lucifora and Delphin, 2020.

Apart from the entry and egress steps, HDV intracellular RNA replication is independent of HBV. Hence, HDV RNAs can be detected for at least 6 weeks in the liver of experimentally infected HuHep mice in the absence of HBV (Giersch et al. 2014). Contrary to HBV, HDV is able to replicate in different tissues/cell types after transfection of a plasmid containing HDV genomes (head-to-tail dimers or trimers) used to launch the infection cycle (Polo, Lim, et al. 1995; Polo, Jeng, et al. 1995). Moreover, HDV-like viruses were recently identified in birds (Wille et al. 2018), snakes (Hetzl et al. 2019) but also fish, amphibians and invertebrates without association to any partner hepadnavirus (W.-S. Chang et al. 2019). These recent data challenge the hypothesis of HDV solely originated from an escaped human gene (Salehi-Ashtiani et al. 2006) and suggest that HDV might be able to use other helper viruses to egress. Results of a series of *in vitro* experiments also showed that HDV RNP can be packaged with envelopes from several non-HBV related viruses such as *hepacivirus*, *flavivirus* and *vesiculovirus* (Perez-Vargas et al. 2019), broaching the question of HDV also being transmitted by other viruses in humans.

b. Viral life cycle

i. HBV

HBV is a **DNA virus** of 3.5 kb which belongs to the *hepadnaviridae* genus with a **unique tropism for hepatocytes** (H. Yan et al. 2015; Iwamoto et al. 2019). HBV entry is mediated by the Na⁺-Taurochlorate Cotransporting Polypeptide (**NTCP**) receptor, in collaboration with the Epidermal Growth Factor receptor (EGF-R)(Ni et al. 2014; Iwamoto et al. 2019) (**Figure 11.A**). The nucleocapsid, internalized in clathrin endosomes (Macovei et al. 2010; H.-C. Huang et al. 2012; Herrscher et al. 2020), is further released in the cytoplasm, most probably using PreS1 as a fusogenic peptide, though this mechanism is rather poorly understood at the moment (**Figure 11.B**). Then, the nucleocapsid is addressed to the nucleus, where it releases the viral genome (**Figure 11.C**).

The latter is further converted from **rcDNA** into covalently closed circular DNA (**cccDNA**) (**Figure 11.D**), which is used as a template for the synthesis of viral genomic RNA (called pregenomic RNA or **pgRNA**) as well as viral mRNAs (Grimm et al., 2011) (**Figure 11.E**). mRNAs are translated into eight viral proteins (1-3) the Small, Medium and Large HBsAg (**S-HBsAg**, **M-HBsAg** and **L-HBsAg**) (**Figure 10**), (4) **HBc**, (5) **HBeAg** (6) **HBV-Pol**, (7) the X protein (**HBx**) and (8) the Hepatitis B Spliced Protein (**HBSP**) (**Figure 11.F**). **pgRNA** and the viral polymerase are encapsidated by **HBc** dimers (**Figure 11.G**) in which the **pgRNA** goes through reverse transcription into **rcDNA** (**Figure 11.H**). Then, the mature neo-formed nucleocapsid are either (i) addressed back to the nucleus, a process termed “recycling” permitting to amplify the **cccDNA** pool, or (ii) enveloped by the three surface proteins in Multi Vesicular Bodies (MVBs) and secreted as infectious virions (i.e. Dane particles) (**Figure 11.I**).

SVPs egress through the Golgi secretion pathway (**Figure 11.J**). They are secreted in large excess compared to the infectious particles (10^4 to 10^5), as mentioned earlier, and up to 1 mg/mL has been observed in patient bloodstream (Bruss, 2007). Along with the **HBeAg** protein, also secreted by the infected hepatocytes (**Figure 11.K**), they have been described as immune modulators (*see section III-2-b*) (Bruss, 2007).

Of note, in 10% cases, the **pgRNA** is converted into double stranded linear DNA (**dsDNA**), which is released or can be re-addressed to the nucleus, as the mature viral nucleocapsid is (T. Tu et al. 2017). In the nucleus, this **dsDNA** can be inserted within the host genome (**Figure 11.L**). If the **integration** process is rather uncharacterized, to date, it is suggested to happen early upon **HBV** infection. The first observation in patients were estimated at 5 months post primary infection, and as early as three and seven days in *in vitro* infected Huh7-NTCP and HepG2-NTCP, respectively (T. Tu et al., 2018). Integration impacts all **HBV** ORF except for the **HBsAg** one (i.e: **HBsAg** stays intact even if the integrated DNA is replication-defective) (T. Tu et al., 2017).

i. HDV

HDV is the unique member of the *deltavirus* genus. As it uses **HBV** envelope proteins, **HDV** virions enter into hepatocytes via the same mechanisms (i.e binding to **NTCP** and **EGFR**) (**Figure 11.M**). Then, **HDV RNP** translocates to the nucleus, probably because of the **NLS** sequences present in **S-HDAg** (Chou et al., 1998; Tavanez et al., 2002) (**Figure 11.N**). Incoming **HDV-G** serves as template for (1) the synthesis of the antigenomic sense **HDV mRNAs**, coding for **S-HDAg** (Gudima et al., 2000; Hsieh et al., 1990) (**Figure 11.P**) and (2) the synthesis of an antigenomic form of **HDV-G (HDV-AG)**, by a rolling-circle replication strategy, as described for viroids (Branch & Robertson, 1984) (**Figure 11.O**).

Replication starts with the generation of *de novo* synthesized linear transcripts, complementary to **HDV-G**, stabilized by 74% base pairing. Small self-cleaving RNA sequences (*i.e.* ribozyme) identified in **HDV-G** and **HDV-AG** (Kuo et al., 1988) are responsible for the cleavage of these RNA molecules from the multimeric transcript. Cleaved monomers are then circularized and ligated to form **HDV-AG**, through a mechanism that remains elusive and may involve ribozyme self-ligation activity (Kuo et al., 1988) or cellular(s) factor(s) (Reid & Lazinski, 2000) (**Figure 12**). It represents the only enzymatic activity reported for **HDV**, and neither **S-HDAg** nor **L-HDAg** are required for this **HDV** RNAs cleavage, but their presence enhances it (Jeng et al., 1996; Lazinski & Taylor, 1994). Of note, **S-HDAg** is a positive regulator of many steps of the replication cycle of **HDV** and the incoming **S-HDAg** is sufficient to initiate the replication process.

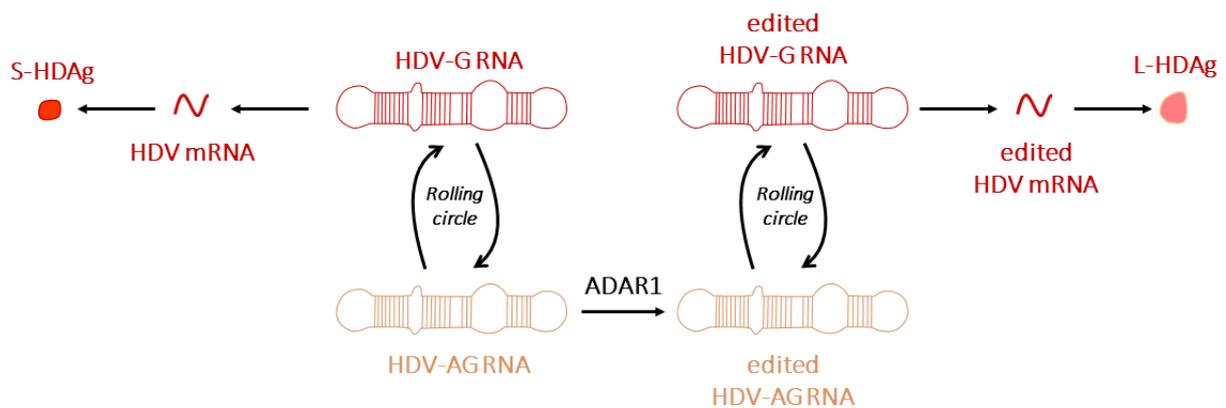


Figure 12: Rolling circle replication machinery of HDV.
Detailed explanations are given in the text.

The neo-formed HDV-AG is then edited by the adenosine deaminase ADAR1 before going through a second rolling-circle cycle, enabling the production, and thus amplification, of HDV-G RNA (Figure 11.R). Even if highly similar to the first mRNA produced, in sequence and length, this new RNA is edited on the amber termination codon (H. Zheng et al., 1992). **The mRNA, resulting from this edited RNA, leads** to the translation of an additional 19 amino acids present on the final protein, which is thus called “Large” HDAg. Using loss and gain of function approaches, the adenosine deaminase ADAR1 has been shown to be responsible for this editing. Whether only the non-inducible form (ADAR1-S) or also the IFN inducible form (ADAR1-L) is implicated in this editing is debated (Hartwig et al., 2006; Wong & Lazinski, 2002). As the structure adjacent to the amber site is suboptimal for editing and interaction with HDAg reduces the access to ADAR1 (Sato, Cornillez-Ty et al. 2004, Hsu, Juang et al. 2019), the expression of L-HDAg is delayed. This delayed expression allows a tuned progress from replicative (i.e more S-HDAg) to morphogenetic (i.e more L-HDAg) phases of the viral life cycle. More precisely, HDV-G RNA synthesis can be inhibited by low amount of L-HDAg whereas the synthesis of HDV-AG and HDV mRNA were found to be inhibited only when L-HDAg are in vast excess over S-HDAg (Modahl et al., 2000). According to the “black sheep” model proposed, L-HDAg would “poison” the homo-oligomeric S-HDAg multimers bound to HDV-AG, thereby potentially disrupting the positive effect of S-HDAg on HDV-G RNA synthesis (Y. P. Xia & Lai, 1992). Conformational differences between S-HDAg and L-HDAg induced by prenylation would be responsible for the differences in their trans-activating and trans-dominant inhibitory biological activities (Hwang and Lai 1994). Thus, HDV replication is first enhanced using incoming and newly produced S-HDAg, before being inhibited by L-HDAg, which actually favor virion egress. Indeed, **L-HDAg** contains a nuclear export signal (NES) (Y.-H. Wang et al., 2005) and a prenylation site (Glenn et al., 1992) that are required for viral assembly. It is thus responsible for the appropriate trafficking of the HDV-RNPs from the nucleus to the trans-Golgi network (C. Huang et al., 2007, 2009; Y.-C. Wang et al., 2009), and its **assembly with HBsAg** (Hwang & Lai, 1993) (Figure 11.S). Of note, the sequences and amount of HBsAg influence the assembly, release (H. H. Shih et al., 2008) as well as infectivity of HDV particles (Freitas et al., 2014).

ii. Interplay between viruses

In a majority of human HBV/HDV co-infection cases as well as in different animal models including chimpanzees, WHV-infected woodchucks and liver-humanized mice, **HDV suppresses HBV replication** (Colombo et al., 1991; Hadziyannis et al., 1985; Lütgehetmann et al., 2012; Negro et al., 1989; Pastore et al., 1990; Pollicino et al., 2011; E. Sagnelli et al., 1991; Smedile et al., 1982). Limited knowledge has been generated to understand how HDV would interfere with HBV. It probably involves mechanisms independent from the adaptive immune system since it is also observed in isolated hepatocytes infected by both viruses (Alfaiate et al., 2016) and upon overexpression of HDAg in hepatoma cell lines (J. C. Wu et al., 1991). Using overexpression system, it has also been suggested that both forms of HDAg might inhibit HBV replication by repressing the HBV enhancer activity (Williams et al., 2009). Moreover, the levels of HBV RNAs but not of cccDNA were decreased in the presence of HDV in non-transformed and immune-competent hepatocytes infected *in vitro*, suggesting an HDV-induced inhibition of HBV RNAs transcription or stability (Alfaiate et al., 2016). This is supported by the fact that besides their association to HDV RNAs, HDV proteins were shown to be able to associate to some cellular mRNA (Chen, Du et al. 2019). Of note, infectious HDV particles can be produced in HBV non-replicating cells producing HBsAg from integrated HBV DNA (Freitas, Cunha et al. 2014) suggesting that chronic HDV infection could persist even though HBV replication would be suppressed (i.e no cccDNA, only integrated HBV) highlighting the need to develop specific treatments for HDV infections.

c. Viral genome and proteins

i. HBV

The viral genome entering hepatocytes is the rcDNA, a partially double stranded DNA form, which needs to be converted into cccDNA, the only template for viral RNA transcription. This process is still poorly characterized and is thought to involve both viral (HBc) and host proteins (extensively discussed here (Y. Xia & Guo, 2020)). HBV genome contains **4** overlapping Open Reading Frames (**ORF**), 4 promoters, 2 enhancer elements and 1 single polyadenylation signal used by all viral RNAs for termination (Gerlich et al., 2020). Of note, regulatory elements are only functional after the conversion from rcDNA to cccDNA is complete.

Six majors HBV RNAs are transcribed from the cccDNA, using the host' RNA-Polymerase II. They all possess a 5' cap and a shared Poly A tail found at the 3' end. Due to peculiar overlapping of the ORFs and the shared PolyA, all viral RNAs contain the HBx transcript at their 3'end (Lamontagne, Bagga, and Bouchard 2016). On these six RNAs, five are only coding for proteins mRNAs (X, PreCore, PreS1, PreS2, S). The last one is the **pgRNA**, used for the translation of both HBc and HBV-Pol proteins, as well as being the RNA intermediates used as a **template for rcDNA synthesis**, a process common to all hepadnaviruses (Lamontagne et al., 2016).

The P ORF and HBV-Pol

Covering more than 70% of the viral genome, it codes for the HBV-Pol (Nassal, 2008) (**Figure 13**). This enzyme, the only one of HBV, is the target of actual therapeutics (*extensively discussed in part IV*). HBV-Pol is a 90 kD protein composed of three domains (from N-term to C-term), the (i) Terminal Protein domain, responsible for the attachment and encapsidation of the pgRNA

(interaction through pgRNA-ε loop) and its reverse transcription, (ii) the Reverse Transcriptase domain, involved in the completion of the rcDNA positive strand as well as the conversion from pgRNA to rcDNA and (iii) RNase-H domain responsible for RNA degradation (i.e pgRNA or small RNA primers still on the DNA after reverse transcription or DNA completion) (Lamontagne et al., 2016). A variable spacer region of unknown function is localized between the terminal protein and reverse transcriptase domain. Errors made by the HBV-Pol in the reverse transcription process are responsible for HBV genome variability (*extensively discussed in part II-4*). Its substitution rate has been estimated around 10^{-3} to 10^{-6} substitution/site/year (Hannoun et al., 2000).

The PreS1/S2/S ORF and HBsAg

It leads to the transcription of three RNAs (a 2.4kb PreS1 mRNA and two 2.1 kb PreS2-S mRNA) and contains three start codons coding for S-HBsAg, M-HBsAg and L-HBsAg (Figure 13-14). They all share the S region at their 3' and possess the same terminal stop codon (Figure 13-14).

This S region alone codes for the S-HBsAg, the smallest form of the envelope protein (24 kD) which is also the most abundant one, indispensable for efficient virion production (Bruss & Ganem, 1991). It contains the transmembrane domain of HBsAg, required for appropriate membrane insertion of the viral protein (Lamontagne et al., 2016) (Figure 14). The M-HBsAg, of 31 kD, is coded by the S and PreS2 region (a 55 residues long region), and the largest one, L-HBsAg (39 kD), by the S, PreS2 and PreS1 region (a 108-119 residues long region, depending on genotypes). L-HBsAg is essential for appropriate nucleocapsid envelopment (Pastor et al., 2019)

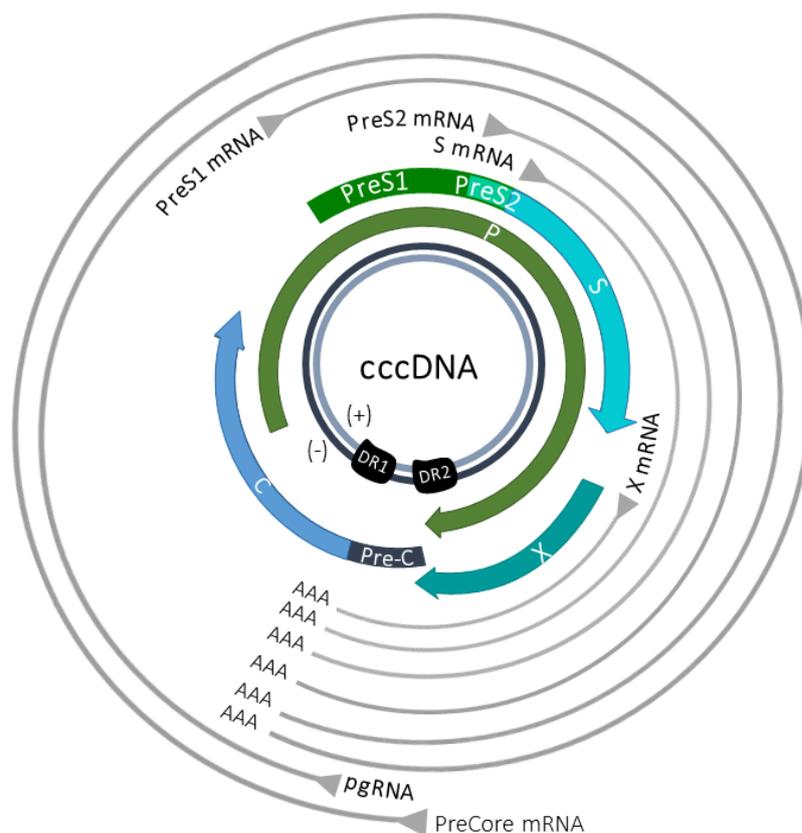


Figure 13: HBV genome organization.
Adapted from (Gerlich et al, 2020; Liang et al 2009).

and its myristoylated N-term region is required for NTCP-R mediated entry (H. Yan et al., 2015) (**Figure 14**). While S- and L-HBsAg are essential for efficient virion production and secretion, M-HBsAg is not and seems to act mainly as a spacer protein (i.e enabling appropriate conformation and layout within HBV envelope) (Venkatakrishnan & Zlotnick, 2016).

All three proteins are differentially found within the Dane particle virions and SVPs. Spheres have a low quantity L-HBsAg, whereas filaments and Dane particles have a similar composition (4:1:1 ratio in S, M and L-HBsAg respectively). Of note, these ratios are modulated with HBV genome variability (*extensively discussed in part II-4*) (Peiffer et al., 2018).

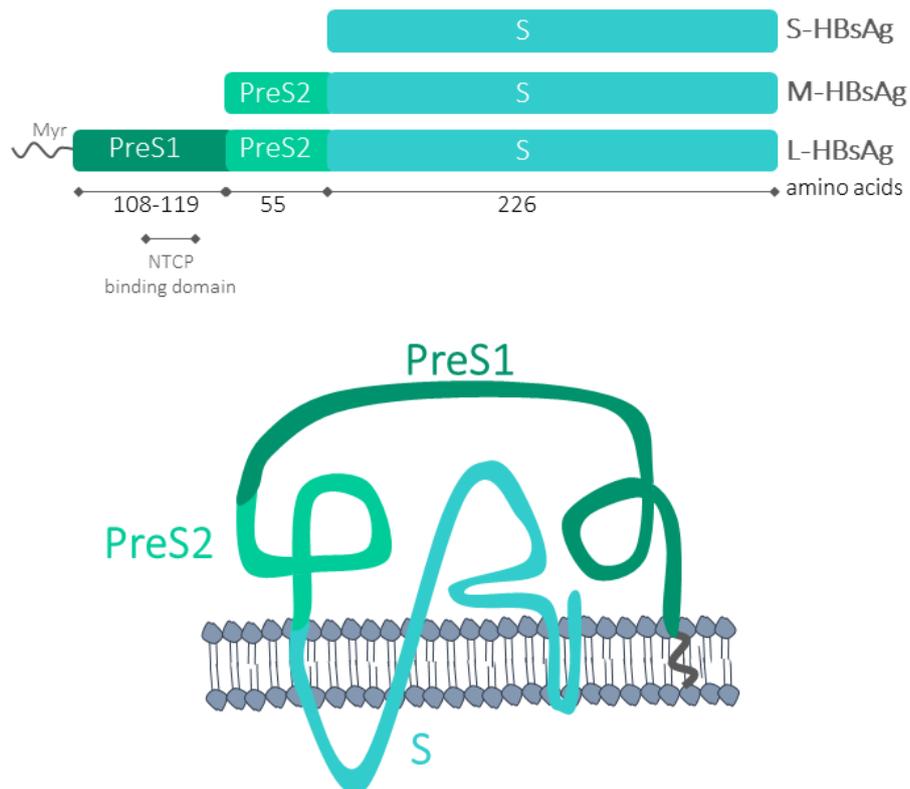


Figure 14: HBsAg protein sequence and conformation within the lipid envelope.
Adapted from (Glebe et al., 2020)

As the sole protein capable of being produced from both the cccDNA and integrated DNA form of the virus, HBsAg is the most robustly expressed HBV protein. It is thus a **serum marker** of HBV infection and its decrease in patients is associated with the functional cure of the disease (*extensively discussed in part IV*)(J. Liu et al., 2014). Recent analysis of L-M-S-HBsAg ratio in infected carrier associated a specific decrease of L-M (vs total HBsAg) in the circulation with a good prognosis (Pfefferkorn et al., 2018, 2021). Interestingly, it is also the protein which has been the most associated with the **induction of immune tolerance** (*extensively discussed in part III-3*).

The PreCore/Core ORF, HBc and HBeAg

The PreCore/Core ORF contains two start codons, leading to HBc and HBeAg production. While HBc is translated from the pgRNA, HBeAg is generated from the Pre-Core mRNA, which as a similar length as the pgRNA, with only few bases more (Figure 13).

HBc is a 21 kD structural protein. Its association in dimers constitutes the **viral nucleocapsid** structural unit. Interaction between HBc proteins is made on its N-term domain, and its C-term domain phosphorylation state regulates its maturation. In 95% cases, the nucleocapsid is made of 120 dimers organized in a T=4 symmetry, while the remaining consist of 90 dimers with a T=3 symmetry (Stannard & Hodgkiss, 1979). In addition to its strictly structural functions, HBc (i) contains a Nucleus Localization Sequence (NLS) on its C-term domain, responsible for its addressing during viral cycle, and (ii) directly binds to pgRNA for subsequent encapsidation and DNA replication (Zlotnick et al., 2015).

PreCore mRNA translation generates a precursor protein of 25 kD which only diverge from the Core protein by a 29 amino-acid addition on its N-term region (Standring et al., 1988). Its C-term region contains an endoplasmic reticulum (ER) targeting sequence, where the newly translated peptide is further processed to its mature 15 kD form, through N- and C-Term sequential cleavages. Concerning its functions, they are still not fully elucidated. To date, we know that it may be involved in various immune evasion mechanisms, from placenta-mediated tolerance to T cell exhaustion (*extensively discussed in part III-3*). Intriguingly, while HBeAg is not required for viral replication, as seen using HBeAg⁻ strains (Parekh et al., 2003), the seroconversion from HBeAg to anti-HBeAg in Chronic HBV infected (CHB) patients has been associated with the end of active HBV replication and is thus used as a surrogate marker (J. Liu et al., 2014).

The X ORF and HBx

This is the smallest ORF, which encodes for the X mRNA, the template for HBx protein translation. HBx, while not packaged within the nucleocapsid, is essential for an efficient establishment and sustained replication of HBV (Lucifora et al., 2011). HBx have been attributed many functions. First, related to the HBV replication cycle, it aids in the epigenetic modulations of the cccDNA for efficient transcription. It acts as a **trans-activator** of all HBV promoters (except on integrated DNA), and also many cellular genes, and is involved in nucleocapsid phosphorylation. Finally, it is able to **degrade the host restriction factor** SMC5/6, ensuring sustained HBV replication (Decorsière et al., 2016; Lamontagne et al., 2016). In addition, it is strongly associated with cell cycle regulation, DNA damage response, cellular apoptosis and calcium flux (Geng et al., 2015; Lamontagne et al., 2016), hence it is a key protein in the development of hepatocarcinogenesis (Geng et al., 2015). Of note, HBx have been shown as a modulator of the immune response (*extensively discussed in part III-3*). Altogether, it is worth noticing that many discrepancies are existing between studies, which can be attributed, at least in part, to the model used (Slagle et al., 2015) thereby, we will not go into the details of each functions.

Splicing and HBSP

Spliced variants of HBV have been described for almost thirty years (H. L. Wu et al., 1991), 17 from the pgRNA alone and four from the PreS1/PreS2/S gene (Candotti & Allain, 2017). Most of them were described in the HBV-A genotype, and not all are expressed in HBV-B/C/D/E, suggesting a genotype-dependent expression of these spliced variants (Candotti & Allain, 2017). It is worth noticing that while 11 of them have been successfully found in patient' serum and/or liver samples, others were described in transfected hepatoma cell line.

To date, it has been shown that only three lead to efficient protein translation, coding for HBV Spliced Protein (HBSP), HBV Double SP (HBDSF) and Polymerase-Surface Fusion Protein (P-S FP) (Candotti & Allain, 2017). HBSP is the most described HBV spliced protein, observed first in 2000 in CHB liver biopsies by Patrick Soussan (Soussan et al., 2000a). HBSP comes from the alternative splicing product of the pgRNA, termed **SP1**, that shares the 46 amino-acids sequences of the HBV-Pol fused to a unique C-terminal sequence of 65 amino acids (transcribed from a new ORF generated by the alternative splicing). Even if HBSP functions are currently poorly characterized, the protein seems to be involved in protection from liver pathogenesis and related immune modulations (Duriez et al., 2017; Soussan et al., 2003). Of note, to date, it is rather unclear if HBSP is activating (Bayard et al., 2012; Duriez et al., 2017; Soussan et al., 2003) or repressing (Pol et al., 2015) the immune response.

ii. HDV

HDV possesses three RNA forms, the HDV-G RNA, HDV-AG RNA and HDV-mRNA. While HDV-G and -AG are highly similar in shape and length (both of 1.7kb with complementary bulges and rhods), the HDV mRNA are smaller (0.8kb) and resemble more the ones of the host. Indeed, it contains a poly(A) tails in 3' and cap structures in 5'(Hsieh et al., 1990).

HDV-G and -AG RNA are coated with HDAg that binds as **multimers** (probably octamers (Cornillez-Ty & Lazinski, 2003; Zuccola et al., 1998) to unbranched quasi-double-stranded HDV RNA segments (with length requirement of about 311 nt) to form nuclease-resistant complexes (Defenbaugh et al., 2009). Depending on the method used, it was reported that 30 to 200 molecules of HDAg would be bound to one molecule of HDV RNA (Defenbaugh et al., 2009; Gudima et al., 2002; Ryu et al., 1993) and therefore between 3 to 25 HDAg octamers per full-length HDV RNA. S-HDAg and L-HDAg, that can exist as either homomultimers or as heteromultimeric structures (Y. P. Xia & Lai, 1992), have been found in equal quantity in circulating HDV virions (Bergmann and Gerin 1986, Bonino, Heermann et al. 1986).

As mentioned earlier, over the course of replication, a fraction of the newly synthesized antigenomic RNA is edited by ADAR1 at the stop codon of S-HDAg ORF. This changes the UAG to UIG and the inosine is recognized rather as a Guanosine, leading to a change from UAG to UGG, which is tryptophan. This increases the length by 19 or 20 amino acids, depending on the genotype, leading to the preferential translation of L-HDAg. S-HDAg (195 a.a) and L-HDAg (214 a.a), despite being both bound to HDV-G and having almost identical sequences, have very distinct roles in the viral life cycle, as previously mentioned. S-HDAg is thus essential for HDV mRNA transcription and replication (Glenn et al., 1992; Harichandran et al., 2019; Kuo et al., 1989), whereas L-HDAg is indispensable for virion assembly but would repress HDV RNA synthesis (Glenn et al., 1992; Hsieh et al., 1990; Y. P. Xia & Lai, 1992).

More precisely, several lines of evidence suggest that S-HDAg would mimic histones and recruit cellular factors to promote HDV RNAs synthesis (Abeywickrama-Samarakoon et al., 2020; Lucifora & Delphin, 2020). As histones on DNA, S-HDAg binds to HDV RNA as octamers (Cornillez-Ty & Lazinski, 2003; Zuccola et al., 1998). Besides, S-HDAg undergoes several post-translational modifications that would be critical for its positive effect on the synthesis of HDV-G RNA and HDV mRNA synthesis but not on HDV-AG synthesis (Lucifora & Delphin, 2020). Beside its possible involvement in the recruitment of transcription factor, and thus enhancement of the transcription machinery, S-HDAg would also promote RNA elongation by removing inhibitory elongation factors (Yamaguchi et al., 2001). Even though, both mRNA transcription and HDV-AG RNA synthesis take place on the same template (HDV-G), they require different post-translational modifications of HDAg (Tseng et al., 2008) and as mentioned above, are probably carried out by different transcription machinery. It was recently suggested that, in the case of HDV-AG, S-HDAg would be more important for post-transcriptional events such as stabilization of the HDV-AG RNAs (to avoid nuclease activity) or promotion of ribozyme activity than directly for synthesis (Harichandran et al., 2019).

2. HBV-HDV infection and related physiopathology

It is estimated that one third of total earth population has been in contact with HBV. In most cases, **immunocompetent adults develop an acute infection**, directly followed by the efficient clearance of the virus from their organisms. However, in 5% cases, resolution of the infection is not achieved and HBV infection becomes chronic (Durantel & Zoulim, 2016). Infants in contact with HBV **before one year of age will develop CHB in 90% cases**, and in up to 50% cases when aged in between one and five. This age-dependency is mostly attributed to the lack of mature immune system in young children and infants. Using an hydrodynamically HBV-injected mice model this prevalence have been linked, at least in part, to a lack of monocyte recruitment on site in younger mice (L.-L. Wu et al., 2019), leading to an unbalanced ratio between recruited inflammatory monocytes (detrimental for HBV) and the highly tolerant ResMΦs which are more favoring HBV establishment (*extensively discussed in part III-3*) (L. Xu et al., 2014). However, the lack of appropriate animal model as well as early HBV diagnostic in patients renders the understanding of these specific mechanism(s) highly challenging.

Although under-diagnosed and underestimated because of the lack of standardized assays (Le Gal et al., 2016), HBV/HDV infection is thought to be the most prevalent co-infection worldwide, leading to the most aggressive chronic form of viral hepatitis (H.-Y. Chen et al., 2019). HDV can co- (i.e simultaneously with HBV) or sur- (i.e infection in CHB patient) infects the liver. Even if co-infection mostly leads to acute hepatitis and/or chronicity, in similar rates as HBV alone, sur-infection of CHB patients leads in 90% cases to HDV chronicity.

To date, even if complete HBV-HDV cure has not been obtained, it is possible to (i) prevent both infections by **vaccination**, which is safe and efficient; and (ii) control HBV infection once installed, using **nucleos(t)ides analogues (NA)** (that block HBV-Pol) or, in certain cases for HBV

and as a standard treatment for HDV, the immunomodulatory **pegylated-IFN α** (*extensively discussed in part IV*).

a. Acute infection

It is the **first encounter** and may last **up to six months** during which the virus replicates in infected hosts, that are thus capable of transmitting it (Jindal et al., 2013). Most of the time, acute infection is asymptomatic, with no signs of liver damage, as seen with no increase in Alanine Aminotransferase (ALT), a common liver injury-related marker. Besides, Acute HBV (AHB) and HDV (AHD) infections are clinically indistinguishable from each other (Tseligka et al., 2021) and HDV super-infection is often mistaken with HBV reactivation or AHB. This renders a putative estimation of AHB and AHB-AHD patient impossible.

In some cases, loss of appetite, joint and muscle pain, low-grade fever and possible stomach pain can occur, with a peak in symptoms at around 3 months of infection. In HBV-HDV co-infection, a second peak is often observed after an improvement in the symptoms (2-5 weeks later), thought to be caused by sequential spreading of HBV and HDV (Tseligka et al., 2021). More **severe symptoms** drive patients to consult a health care provider (and thus being diagnosed) such as jaundice, nausea, or bloated stomach. Finally, in less than 1% cases, aberrant immune responses induce **fulminant hepatitis** (Jindal et al., 2013), a disease with an 80% rate of mortality within 2-10 days after infection. In these patients, 33% to 39% are tested positive for HDV (Govindarajan et al., 1984; Smedile et al., 1982).

As AHD is rarely studied, we will now focus on AHB. It is worth noticing that most mechanisms must be shared in between the two viruses, as the clinical appearance of the disease is indistinguishable.

In most cases of AHB, **IFN response is not detected in patient**, as it was previously observed using animal models (Dunn et al., 2009b; Wieland et al., 2004). Besides, NK and CD8+T cell, which are seen as the innate and adaptive arms against HBV infection, are specifically inhibited during HBV viremia peak (Dunn et al., 2009b). It was positively correlated with HBV viremia and IL-10 (most probably secreted by KCs). Of note, this phenomenon was not observed in patients with mild HBV viremia, suggesting a putative threshold needed for immune inhibition. Nonetheless this decline of NK and T cell response is only transient and viral inhibition is rapidly overcome as viral load decreases, enabling the immune system to finally clear the virus (Stelma et al., 2017). Most well-known mechanisms of NK and T-cell mediated antiviral effect are cytotoxic (perforin/granzyme) and thus can induce immune-mediated liver damage. Sandalova and colleagues elegantly reported that Arginine-1 secretion by dying infected hepatocytes correlates with a suppression of activated CD8+T cell response, enabling a return to homeostasis once viral clearance is achieved (Sandalova et al., 2012). Of note, ALTs are not always elevated in AHB, suggesting the existence of non-cytopathic mechanisms, mostly mediated by IFN- γ and TNF α secretion, as seen in infected chimpanzee (Guidotti et al., 1999) and liver biopsies of CHB patients (Y. Xia et al., 2016). An hypothesis is that decrease of the infection could be attributed, at least in part, with an APOBEC3B-mediated cccDNA deamination and subsequent decay (Lucifora et al., 2014).

Subject resolving the infection ends up with a life-long immunity against HBV.

As most of AHB are asymptomatic or resemble a simple virose, diagnostic is not often made and there is a lack of understanding of the specific steps toward viral clearance, along with many discrepancies between studies due to intrinsic variations between patients as well as the stages of the disease. Hence, many questions remain, amongst which whether the decreased viremia after HBV peak is mediated by or is required for NK and T cell immune response to arise. Thus, raising the question of a maybe underestimated part in HBV clearance for other immune cells.

b. Chronic infection

If AHB and/or AHB-AHD is not resolved within six months, the infected patient is considered as having CHB and/or Chronic HDV (CHD) infection, assessed by a **persistent detection of HBsAg and HDV RNA in patient's blood**, respectively (Bertoletti & Le Bert, 2018).

Upon establishment in infected hepatocytes, HBV is slowly inducing liver complications, namely fibrosis, cirrhosis and/or HCC. The latter being the major cause of death in infected patients. Globally, 8-20% of CHB patients are at risk of developing cirrhosis. Progression of HCC in these subjects is correlated with HBeAg positivity (3.6 more chances) and HBV DNA levels. However, patients (age, gender, genetic background and immune status), viral parameters (viremia, genotype, mutations, putative co-infection) and environment (alcohol and tobacco intake, exposure to aflatoxin, obesity) variability are also at play in this matter (El-Serag, 2012). An accelerated progression towards fibrosis/cirrhosis followed by clinical liver decompensation and related death have also been described as major complications for chronically HDV/HBV infected patients (Buti et al., 2011). The risk of cirrhosis has been evaluated to be 23%, 41% and 77% after respectively 10, 20 and 30 years of co-infection (Yurdaydin, Idilman et al. 2010). Studies reported at least a three-time higher risk of HCC development in chronically HDV/HBV co-infected patients compared to HBV mono-infected ones (Alfaiate et al., 2020; Fattovich et al., 2000; Ji et al., 2012). Moreover, HDV was identified as the main comorbidity factor in pluri-infected HIV patients in the national HIV Swiss cohort (Béguelin et al., 2017).

i. CHB

The natural history of CHB has been sequentially divided into subcategories by the European Association for the Study of Liver (amongst other national/international research agencies) using different markers, some related to HBV virology (serum HBsAg and HBeAg, HBV intrahepatic DNA levels) other related to liver damage (ALT). The last EASL report suggests a categorization of patients between five phases, which are not necessarily successive (**Figure 15**). This new nomenclature is based on HBeAg serum levels and a division of CHB between chronic infection (i.e infection without elevated ALT) and chronic hepatitis B (with elevated ALT and liver damages). Of note, the purpose of this classification is to aid in patient's management and monitoring.

	1	2	3	4	5
	HBeAg-positive chronic HBV infection	HBeAg-positive chronic hepatitis B	HBeAg-negative chronic HBV infection	HBeAg-negative chronic hepatitis B	Resolved HBV infection
HBsAg	High	High / intermediate	Low	Intermediate	Negative
HBeAg	Positive	Positive	Negative	Negative	Negative
HBV DNA	>10 ⁷ IU/mL	10 ⁴ –10 ⁷ IU/mL	<2,000 IU/mL	>2,000 IU/mL	<10 IU/mL
ALT	Normal	Elevated	Normal	Elevated*	Normal
Liver disease	None/minimal	Moderate / severe	None	Moderate / severe	None

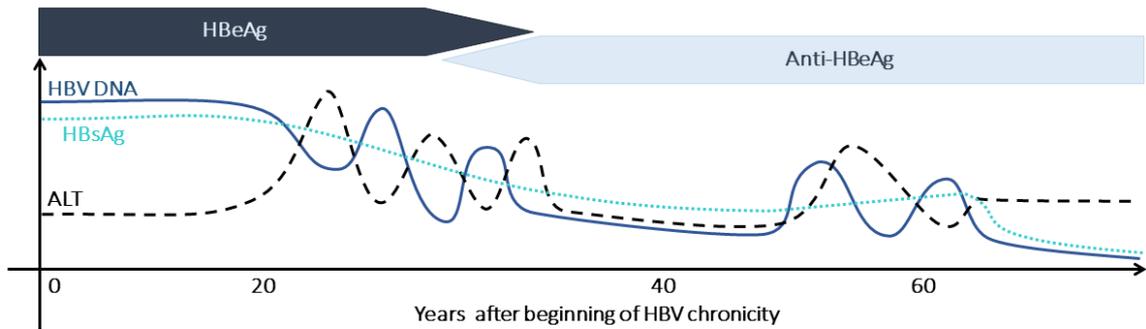


Figure 15: Natural history of CHB.
Adapted from EASL report 2017

Phase 1: HBeAg-positive chronic HBV infection

The first phase is characterized with a very **high viremia** and presence of **serum HBsAg** and **HBeAg**. It is asymptomatic, with no or poor liver damages observed, as seen with **normal ALT** levels, and can last for decades. HBV is often seen integrated in the host genome and an increased clonal expansion of hepatocytes within this phase suggests that hepatocarcinogenesis could begin here (Levrero & Zucman-Rossi, 2016).

Phase 2: HBeAg-positive chronic hepatitis B

Often reached after years of infection, phase two correlates with **liver damages** (ALT elevation), **high viremia** and presence of **HBsAg** and **HBeAg**. Of note, this phase is reached faster in primo-infected adult.

Phase 3: HBeAg-negative chronic HBV infection

Progression in phase three is highly variable in between patients and corresponds to the **beginning of HBV immune control**, consecutive **HBeAg seroconversion** (antibodies against HBeAg in patient's serum) and **undetectable/low viremia**. Patients in phase three have low fibrosis and/or necroinflammation, as seen with **normal ALT** levels. HBsAg loss and seroconversion happen spontaneously in 1-3% cases per year.

Phase 4: HBeAg-negative chronic hepatitis B

Phase four is characterized by **fluctuating and/or persistent viremia** and **HBeAg negativity**, which is mostly attributed to variations/mutations in HBV genome. **Liver damage**, as seen by necroinflammation and fibrosis are observed in histology and thus ALT levels are elevated.

Phase 5: HBsAg-negative phase

This phase, also known as “occult HBV infection” is associated with **HBsAg serum negativity**, production of **anti-HBcAg** and **putative anti-HBsAg**. This is the result of a low replication of HBV which may be attributed to cccDNA epigenetic silencing. Patients have **normal ALT** values and if phase five begin before the onset of cirrhosis, complication (i.e HCC) can be avoided.

ii. CHD

Whether CHD is accompanied with distinct peaks in ALT or activation-inhibition of HDV itself, as it is observed in CHB, is, unfortunately, not clear. Interestingly, a recent cross-sectional study in a small cohort of 109 HBV/HDV infected patients reported HDV dominance in 75% of the cases, HBV dominance in 7 % and no dominance for the rest of the cases. HDV dominance was associated with **down-regulations of some interleukins, chemokines and cytokines** as well as with a delayed in response to treatment with Pegylated IFN alpha (Peg-IFN- α) (Lutterkort et al., 2018). On a virological point of view, patients are mostly HBeAg negativ with low HBV DNA serum levels, and a sustained expression of HDV RNA, HDAg and anti-HDV IgM/IgG (Tseligka et al., 2021). Of note, longitudinal analyses of HBV/HDV co-infected patients showed different profiles of viral dominance over time, emphasizing the complexity of the interactions between the two viruses and their host (Schaper et al., 2010). Besides, HBV reactivation is observed upon HDV clearance (spontaneous or drug-induced).

3. HBV-HDV epidemiology

a. Worldwide prevalence

Even if a safe and efficient vaccine is available and efficient against both viruses, they are still a global health issue. The World Health Organization estimates that 257 million people are currently living with CHB, 5% being HDV positive. This result in one million deaths per year, due to the aforementioned HBV-related disease, one out of five HCC cases being associated with HDV positivity.

In 2015, seroprevalence to CHB, defined as HBsAg carriage, was evaluated at 3.5% of worldwide population (**Figure 16**). Major endemic areas, defined as <7.5 % of global population having CHB, are South Africa (sub-Saharan) and South-East Asia, with some regions reaching up to 22% (El-Serag 2012; European Association for the Study of the Liver, 2017). Of note, as most endemic areas are low-income countries with limited resources, an underestimation of population living with HBV, as for HDV, is highly probable.

At least 25 million people are estimated to be chronically co-infected with HBV and HDV (Wedemeyer and Negro 2018), but two recent meta-analyses proposed two to three times more co-infection worldwide (H.-Y. Chen et al., 2019; Miao et al., 2020). Even if methods used in this study and thus the exact numbers, are still debated (Stockdale et al., 2020), HDV positivity is generally under diagnosed, and thus largely underestimated, because of the lack of standardized assays (Le Gal et al., 2016). Major endemic areas for HDV are Central Africa (15-50%) and West Africa (17-30%), South America (mainly the north, 13-29%), the Mediterranean

basin (27%), the Middle East (7.8% in Iran), Northern (26-60% in adults in Mongolia) and Southeast (15% in Vietnam) Asia, southern Italy and Eastern Europe (20% in Romania) (C. Koh et al., 2019; Stockdale et al., 2020) (Figure 17).

Improvements are observed in some highly endemic areas due to socioeconomic development, better vaccine coverage and drugs efficiency/availability, such as Taiwan that reported it in a 25-year survey of general population, concerning HBV infection (Hsu et al., 2017).

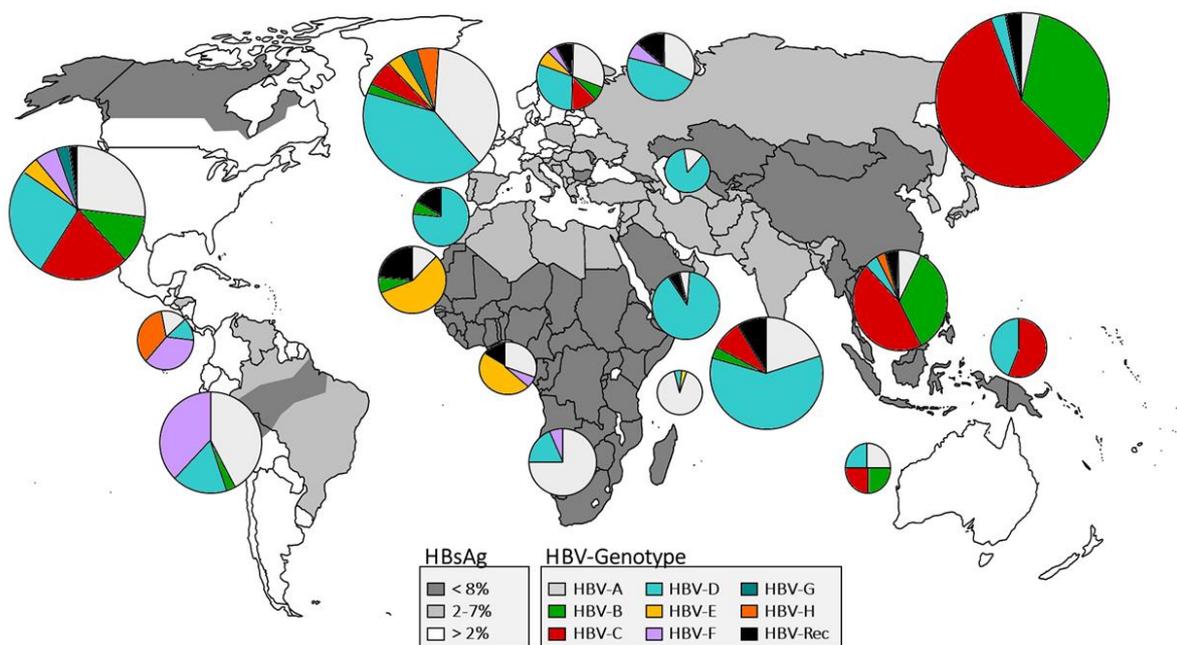


Figure 16: Worldwide prevalence of HBV and global genotype spread.

b. Transmission routes

HBV and HDV are transmitted through contaminated blood or body fluids (saliva, menstrual, vaginal, seminal fluids) (Y.-F. Shih & Liu, 2017). Transmission routes for HBV vary in between low and highly endemic regions. In regions with high prevalence of CHB, viral spread is mostly attributed to mother-to-infant transmission (MTIT), also termed **vertical transmission**, whereas this transmission is rare for HDV. Three possible routes have been elucidated for MTIT: intrauterine transmission, blood exchange during delivery, and postpartum transmission (close contact and breast milk) (Y.-F. Shih & Liu, 2017, p.). Of note, HBeAg is the sole HBV protein capable of passing through the placenta, it is suggested to be responsible for helper T cell tolerance against capsid and HBeAg protein, and thus perinatal transmission is less frequent in HBeAg negative mother than in positive one (Kramvis, 2016).

Other route of infection, called **horizontal transmission**, are preferred by HDV and are related to low endemic HBV regions. They encompass (i) infected family close contact in the first years

of life, (ii) child-to-child transmission, (iii) unsanitary intravenous drug use, (iv) unprotected sexual intercourse with infected partner, (v) unsanitary medical-surgical-dental procedures or infected blood perfusion and (vi) unsanitary tattooing/piercing (non-exhaustive list) (M. H. Nguyen et al., 2020).

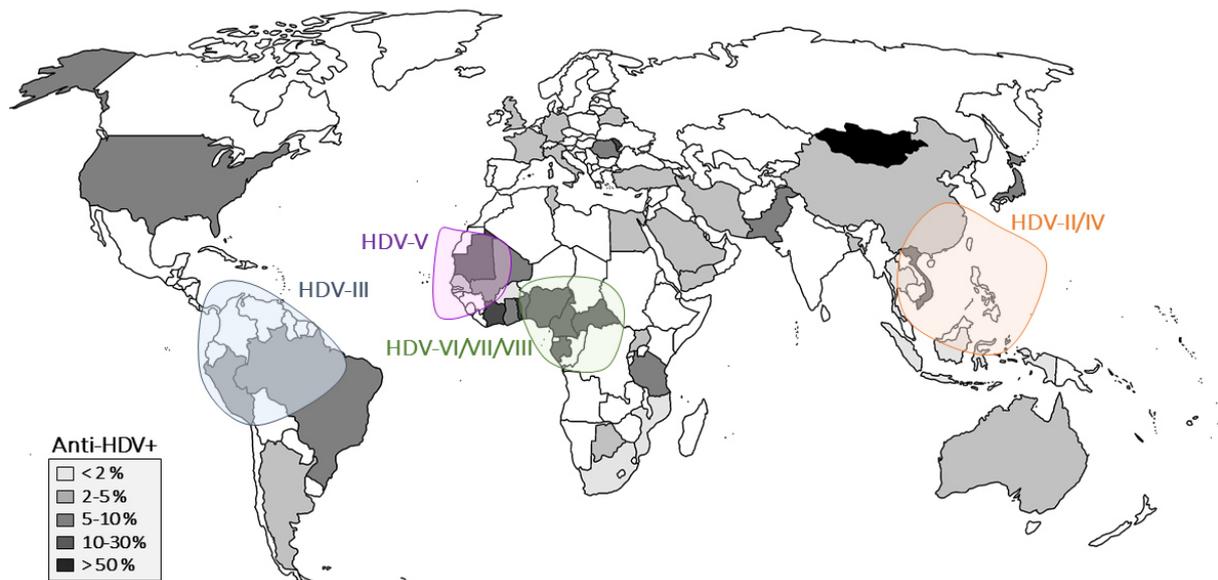


Figure 17: HDV worldwide prevalence and genotypes distribution in general population. As HDV-I is ubiquitous, its area was not delimited. *Adapted from* (Stockdale et al., 2020)

4. Genotypes

a. Natural history of genotype discovery

A decade after its first discovery as the “australian antigen” by Baruch Blumberg (Blumberg & Alter, 1965), studies unraveled polymorphisms in HBsAg sequence, especially in one region termed “a” (Bancroft et al., 1972; Le Bouvier et al., 1972). Le Bouvier and Bancroft initiated a classification based on these variations, and termed the different subclasses “serotypes” (Bancroft et al., 1972; Le Bouvier et al., 1972). These four major serotypes, *ayw*, *ayr*, *adw* and *adr*, were classified based on Lysine to Arginine substitution at residues 122 and 160 respectively (*d/y* and *w/r*) (Bancroft et al., 1972; Le Bouvier et al., 1972). Further sub-classification, using other polymorphisms was undertaken to better characterize HBV strains variations. In 1988, 18 genomes were sequenced, unraveling deeper variations between HBV strains that could not be recapitulated using only HBsAg sequence. Thus, it initiated a new classification, this time based on alphabetical letters (from A to D), which were termed “genotypes”, and possess a genetic divergence of at least 8% (Okamoto et al., 1988). HBV-E, HBV-F, HBV-G and HBV-H were then sequentially discovered in 1993 (Naumann et al., 1993), 1994 (Naumann et al., 1993; Norder et al., 1994), 2000 (Stuyver et al., 2000) and 2002 (Arauz-Ruiz et al., 2002). In 2008, a new recombinant between HBV-A (in the polymerase region), HBV-G and, in large proportion, HBV-C, was discovered in a vietnamese patient, and further defined as a new genotype, HBV-I (Huy et al., 2008). Of note, even if its classification as a putative

genotype has been challenged by Kurbanov and colleagues at first (Kurbanov et al., 2008), further sequencing and expansion of the cohort of HBV-I patient enabled to reach good bootstrap support for its classification as a new genotype. Finally, in 2009, one sequence was found in a single Japanese man with HCC (Tatematsu et al., 2009), and identified as being a new genotype, termed “HBV-J”. Even if largely meeting the criteria for identification as a new genotype, with around 10.7% genetic divergence, it is currently unclear if HBV-J should be. Indeed, later sequence analysis and further comparison suggest that HBV-J can be classified as a recombinant of HBV-C and Gibbon HBV, especially in the S region, suggesting that HBV-J could in fact be the result of a cross-species transmission (Kramvis, 2014).

To date, **eight HDV genotypes** have been described. After its discovery in 1977 (Rizzetto et al., 1977), mostly HDV-I were analyzed, until the publication in 1991 of a new sequence of HDV, termed HDV-II, observed in Japan (Imazeki et al., 1991). Then HDV-III (Casey et al., 1993) in Northern South America, HDV-IV in Taiwan (J. C. Wu et al., 1998) (**Figure 17**). In 2004, the genetic variability of African samples of HDV was investigated and three genotypes (from V to VII) were described (Radjef et al., 2004). Finally, in 2006, Le Gal and colleagues extended our knowledge with an eighth HDV clade, also present in central Africa (Le Gal et al., 2006).

It is worth noticing that experiment and cohort related to HBV and/or HDV genotypes are mostly comparisons made between several genotypes (or sometimes even only two) which are endemic to the given areas of study (often, HBV-D with HDV-I). There are still poor data which are pan-genotypic and thus it is difficult to establish the clear characteristics of each genotype. However, in the following part, we will try to compute and discuss the main studies made on the subject.

b. Geographic distribution and worldwide burden

It has been recently estimated that HBV-A to E were responsible for 96.2% of HBV burden worldwide, 2.5% of global population is infected with HBV variants of recombination or co-infection, leaving 1.3% to HBV-F to J (Velkov et al., 2018). This shifted balance has been associated with the genotype worldwide distribution (**Figure 16**), with some being termed as pandemic, such as HBV-D, and others rather endemic, such as HBV-F (Velkov et al., 2018).

HBV-C is the **most prevalent genotype** amongst HBsAg seropositive patient as it represents 26.1% of the total infected population. Infected patients are found in 98.6% of cases in Asia, where HBV-B, -I and -J are also mostly found (Velkov et al., 2018). Of note, HBV-I is widely spread across isolated native Asian populations, in Laos, North India and China, suggesting that it was endemic in a large area of Asia for a long time (Velkov et al. 2018), whereas HBV-J, as previously mentioned, was only found in Japan, to date. Second in terms of HBV infection burden, HBV-D represents 22.1% of total seropositive patient and is mostly found in Asia (61.9%), Africa (22%) and Europe (13.5%). Third and fourth with a total of 17.6% and 16.9% of HBV infected population respectively, HBV-E and -A are mainly found in Sub-Saharan Africa (Okamoto et al. 1988; Velkov et al. 2018; Ito et al. 2018). Finally, HBV-H and HBV-F are endemic to America, with HBV-H mostly found in Mexico (H. Norder, Couroucé, and Magnius 1994; Naumann et al. 1993; Anna Kramvis 2014).

HDV-I is the most prevalent genotype worldwide, with a distribution characterized as “ubiquitous” (Stockdale et al. 2020). It is, however, mostly found in Europe and North America. Genotype II/IV are predominant in East Asia, with some sequence from HDV-II recently found in Egypt and Iran (C. Sagnelli et al. 2021), and HDV-III is endemic to the north of South America (Figure 17). Genotypes V to VIII are endemic of East Africa, with HDV-V being mostly found in the North-East and VI to VIII in the Central-East (Figure 17) (C. Sagnelli et al. 2021). Of note, HDV-VIII sequences were recently found in patients from Northeast Brazil, Maranhão, without known interaction with African peoples. This suggests that HDV spread could have benefited from the slave trade, in accordance with historical knowledge on the region (Santos et al. 2016).

Differences are existing in transmission routes with HBV-B, HBV-C and HBV-I being more related with vertical transmission, whereas HBV-A, HBV-D, HBV-G and, in general, HDV, are rather related with sexual encounter and unsanitary drug injection (i.e horizontal transmission) (Anna Kramvis 2014). Prevalences in transmission routes are suggested to be associated, at least in part, with later HBeAg seroconversion in some genotypes, such as the HBV-C. This leads to increased chances to be HBeAg positive for women bearing child, and thus increased vertical transmission for HBV, as discussed earlier. Besides, genotype-dependent transmission is closely associated with countries of endemicity which do not share the same politics regarding HBV/HDV infection (systematic pregnant women testing and medical support, sanitary drug use, democratisation of sexual protection...).

c. Modulation of virological parameters and genetic variability

i. Inter-genotypic divergences and subsequent modulations of virological parameters

In terms of sequence variability, genotypes differ by at least 7.5%, as mentioned, with an average length of 3.2 kbp, HBV-G being the longest one (3.248 kbp) and HBV-D the smallest (3,182 kbp) (S. Schaefer 2007). Inter-genotypic genetic divergences have been associated with differences in the **concentration of virological parameters** found in patient’s serum, namely HBeAg, HBV DNA and HBsAg (Peiffer et al. 2018). For instance, HBV-B and –C patients have the highest viremia, however HBV-A seems to possess the highest level of secreted HBsAg, as seen in a cohort of HBeAg-positive Japanese patient (Hosaka et al. 2013). Some association can be made regarding **patient’s serotypes**, with *adw* associated with HBV-A, B, F, G and H, *adr* with C and *ayw* with HBV-D and E (Anna Kramvis et al. 2008). Of note, HBV-E displays the unique *ayw4* serotype (Anna Kramvis 2014). These differences in the envelope protein are also impacting **the ratio between HBsAg S/M/L** protein in filament and spheres (Helene Norder et al. 2004; Peiffer et al. 2018), with, for instance, HBV-D having more M-HBsAg than HBV-A to E, and HBV-E having less L-HBsAg compared with the four first genotypes. Altogether, these data suggest that HBsAg composition of spheres, filaments and virions might differ broadly between genotypes.

Some genotypes have been associated with **specific mutations**, or are more prone to develop some. Many are related to the **PreCore/Core region**. Indeed, due to a unique 6 nucleotide

insertion in the C-terminal of the HBeAg PreCore gene, HBV-A HBeAg protein present a variable length which is observed in patient serum (Ito et al. 2018). No specific function has been associated with this addition but some suggest that it could be a mechanism of immune evasion. A low serum HBeAg has been correlated with the high prevalence of G1896A PreCore mutation, which is especially found within HBV-B patient, when compared with HBV-C and D, and lead to the insertion of a stop codon (El-Serag 2012; Suppiah et al. 2015). In HBV-C/D, it is rather the A1762T/G1764A mutation in the basal core promoter, inducing cytoplasmic localization of HBc, also associated with elucidation of the host immune response (C.-L. Lin and Kao 2017). In HBV-G, several mutations are described in the PreCore/Core region which lead to (1) enhanced expression of the capsid protein – 36 nt insertion in Core region – and (2) no ability to secrete HBeAg – nonsense mutation in PreCore region (Zaaijer et al. 2011). However, it is competent in RNA transcription, genome replication and virion secretion, as seen in patients in general and more mechanistically in Huh7 (K. Li et al. 2007). Finally, HBV-E possess a unique 3 nucleotide deletion in the **PreS1 region**, a novel start codon in Met 83 leading to a putative larger M-HBsAg (Anna Kramvis et al. 2005) of a yet unknown interest.

Most of HDV genotypes have around 90% similarities in sequence, except for HDV-III that diverge by 40% (C. Sagnelli et al. 2021). A clear **genotype-specific activity of HDAg** in supporting HDV RNA replication has been established by reporting the inability of HDAg genotype III to support replication of HDV genotype I for instance (Casey and Gerin 1998). HBV genotypes may also influence HDV infection and replication since it was reported that HDV viral load is lower in patients co-infected with HBV-A compared to those co-infected with HBV-D or –F, but co-infection with HDV is more frequent in HBV-F infected patients (Kiesslich et al. 2009).

ii. Intra-genotypic variations: subgenotypes

HBV-A to D, HBV-F and HBV-I possess enough genomic heterogeneity to be further classified into **sub-genotypes**, each having a **subspecific global distribution** and **disease burden**. They are characterized by more than 4% intra-genotype nucleotide divergence (Anna Kramvis, Kew, and François 2005; Miyakawa and Mizokami 2003; Helene Norder et al. 2004). HBV-C, is the most heterogeneous one with sixteen subgenotypes described to date (Okamoto et al. 1988; Anna Kramvis 2014). Then comes HBV-D (7), HBV-B (5), HBV-A (4), HBV-F (4) and HBV-I (2) (McNaughton et al. 2019). Of note, HBV-I has been subclassified into HBV-I1 and I2 subgenotype, with an intragenotypic divergence of 3.40% (i.e less than the 4% required), but exception has been made as each have a different serotype (*adw2* and *ayw2* respectively) (Anna Kramvis 2014).

Interestingly, HBV-B subgenotypes are all made of 93% recombination event with HBV-C in the preCore/Core region, except for B1 and B5 (B1 being suggested as B5 ancestor), demonstrating the divergences existing between subgenotypes (Anna Kramvis 2014). Heterogeneity is directly correlated with the estimated age of each genotype, illustrated by HBV-C, the most ancient one, and HBV-E. Indeed, the latter, with an estimated age of 130 years, is the youngest HBV genotype and thus possess a poor genetic variability (around 1.2% divergence (Anna Kramvis, Kew, and François 2005)) with no subgenotypes (Anna Kramvis 2014). On the contrary, while HBV-H is suggested to date back to the Aztec period, no subgenotypes have been described to date (Anna Kramvis 2014).

HDV-I has the largest intragenotype variability, with four subgenotypes (a to d) known to date (Le Gal et al. 2017). They have a mean intergroup nucleotide divergence ranging between 14.6 to 16%. Two genotypes (HDV-1a and b) are African, whereas HDV-1c is described in Pacific Island and HDV-1d have clearly spread worldwide, with sequences found in every continents. Genotypes II to VIII can all be divided into two subgenotypes of >10% genetic divergence. Of note, even if 16.6% variation in sequence was found in HDV-III infected patients, its categorisation into two subgenotypes needs further sequencing to be confirmed (Le Gal et al. 2017). Interestingly, a recent analysis challenged this classification, with still eight genotypes but 2 subgenotypes I (HDV-Ib to d are merged) and 3 subgenotypes for HDV-III and HDV-VI (Miao et al. 2019).

iii. Recombination

Recombination between strains is a **common phenomenon** for HBV. To date, 24 phylogenetically independent variants have been described with specific distribution and epidemiology, such as HBV-B/C recombinant mostly found in mainland Asia in HBV-B infected people. Some even represent the major type in the area such as Tibet where 96% of HBV isolates are recombinant from HBV-C and D (Cui et al. 2002).

d. Disease-related specificities and treatment sensibility

Concerning the natural course of HBV infection, HBV-A AHB is associated with a lower peak of ALT and an increased peak of HBV DNA, which lead to an increased tendency to chronicity, when compared with HBV-B and HBV-C, but is suggested to be less severe (Ito et al. 2014). HBV-A, HBV-C and HBV-D are displaying the A1762T/G1764A core promoter mutation, leading to advanced liver diseases (A. Kramvis and Kew 2005; Thakur et al. 2002; C.-L. Lin and Kao 2017). Even if pan-genotypic data are scarce, it is widely accepted that **HBV-C** is inducing one of the, if not the most, **severe form of CHB-induced liver pathologies** (Anna Kramvis 2014). This has been imputed to (1) more PreS deletion than HBV-B (Sugauchi et al. 2003), (2) enhanced oxidative stress (still compared with HBV-B) in a chinese cohort (Xianyu et al. 2018) and higher stellate cells activation (i.e liver fibrosis), as seen with increased levels of TGF- β , compared with HBV-B and A, in a humanized mice model. Similar to HBV-C, HBV-D is associated with increased propensity to HCC (Thakur et al. 2002; Livingston et al. 2007) and HBV-B higher risks of severe liver complications (Sunbul 2014). According to two Alaska cohorts papers, comparing HBV-A to D and HBV-F, the latter is related with particular increase in HCC development (Livingston et al., 2007; McMahan et al., 2021), and subsequent higher mortality when opposed to HBV-A to HBV-D (Sánchez-Tapias et al., 2002). Thus, even if data on **HBV-F** are scarce, the american genotype **might even be deadlier than HBV-C**. On the contrary, HBV-H is related with low prevalence of HCC (Roman et al., 2013) which is probably linked to the increased chances of entering phase five (HBsAg negative phase) as observed in a cohort of HBV-H infected children (Escobedo-Melendez et al., 2014). The few existing and relevant clinical studies showed that viral genotypes (and some subtypes) highly influence the diseases outcome in HBV-HDV infection. Indeed, HBV genotype C and HDV genotype I would be associated with lower remission rates and more aggressive outcomes as compared to HBV genotype B and HDV

genotype II and IV respectively (Su et al., 2006). HDV-III, associated with HBV-F, -A and -D, was found strongly associated with a late 80's outbreaks of fulminant hepatitis in the Amazon Basin (Gomes-Gouvêa et al., 2009). HDV-I (originating from European-born patients) and HDV-V were recently associated with an increased risk of developing cirrhosis multiplied by two compared to others (Roulot et al., 2020). However, when comparing the disease outcome, HDV-V seem associated with a better prognosis than HDV-I (still mostly originating from European-born patients), in a different british cohort (Spaan et al., 2020). Finally, in an Asian cohort, HDV-II and -IV were associated with lower cirrhosis and HCC incidence when compared with HDV-I, suggesting, once again, that this genotype is especially aggressive (Mentha et al., 2019). Of note, a genetic variant of HDV-IV infecting 40 patients from the Miyako Island (Japan) was associated with quicker progression to cirrhosis (C. Sagnelli et al., 2021).

In addition to the evolution of liver pathologies, HBV genotypes differs in their serologic evolution. Compared with HBV-A, B, D and F, which all have an estimated time to seroconversion of less than 20 years, HBV-C takes as much as 47.8 years, according to the already mentioned Alaska survey on infected patient (Livingston et al., 2007). Besides, **HBV-D** is associated with **lower chances of serconversion** compared with HBV-A, and the lowest HBeAg positivity was observed in HBV-E Ghanaians patients with CHB, compared with chinese ones infected with HBV-C and HBV-B, suggesting that seroconversion occurs earlier in age in this genotype (Kramvis, 2016). Interestingly, in an untreated spanish cohort, remission and natural clearance of HBV DNA was higher in HBV-A patients than HBV-F and HBV-D (Sánchez-Tapias et al., 2002), suggesting that genotypes may also differ in the infection outcome. HBV genotypes may also influence HDV infection and replication since it was reported that HDV viral load is lower in patients co-infected with HBV-A compared to those co-infected with HBV-D or -F and co-infection with HDV is more frequent in HBV-F infected patients (Kiesslich et al., 2009). Thus, it is of high importance to analyse genotype-dependent vaccine and treatment response.

Vaccine escape has been documented in some cases with genetically distant genotypes (Glebe et al., 2020), and thought to occur mainly in low titer anti-HBsAg individuals (< 100 IU/L). For instance, a large scale blood testing in America led to the discovery of 9 donors which were HBV positive. Amongst them, all unvaccinated patients were infected with genotype A2 (from which the vaccine is made), whereas the vaccinated ones were infected with HBV-B, -C, -D and -F (Stramer et al., 2011). Of note, even if the vaccinated donor had asymptomatic and/or transient infection, low doses of HBV are sufficient enough to transmit (16 virions according to a recent computational estimation) (Candotti et al., 2019), suggesting that they remain a potential threat to others.

No clear data establish a **genetic-dependent response to NAs** (the first-line HBV drug targeting HBV-Pol) (Damerow et al., 2010), which is consistent with the highly conserved features of HBV-Pol in between genotypes. Some studies suggest a better response of HBV-B compared with HBV-C in response to lamivudine, or increased rates of resistance strains, but data are scarce and were not replicated, to date (Rajoriya et al., 2017).

Genotypes	Subgenotypes	Geographic distribution	Predominant transmission route	Tendency to HBeAg seroconversion	Tendency for liver complications development	Sensitivity to pegIFN α treatment
A	A1-4	Europe, Africa	Horizontal	Early	More rapid less severe	Good responders
B	B1-5	North America, South-East Asia	Vertical	Early		Good responders (but relapse)
C	C1-16	North America, South-East Asia	Vertical	Very slow (=47,5 years)	Most severe	Worst responders
D	D1-7	Worldwide	Horizontal	Slow	High	
E	None	Western and Central Africa	Horizontal	Rapid		
F	F1-4	Central and South America	Horizontal	Early	High mortality	Good responders
G	None	Europe, North America	Horizontal	ND	ND	ND
H	None	Mainly Mexico	ND	ND	Low	ND
I	I1-2	Asia	Vertical	ND	ND	ND
J	None	Japan	ND	ND	ND	ND

Table 2: Genotype-dependent differences.

However, it is now established and included in the guidelines that there are **specificities in antiviral responses to peg-IFN α** (second-line drug) (Erhardt et al., 2005). HBV-B and -A infected patients seems to better respond than HBV-C and HBV-D, however, considerable relapse is observed following end of treatment with HBV-B (Sonneveld et al., 2012). For HBV-A, it is associated with a stronger and more sustained effect (Sonneveld et al., 2012), thus suggesting that HBV-A infection, even if leading more often to chronicity, seems to be dealt more efficiently by the host than other genotypes. Of note, HBV-C low response to pegIFN α treatment may be related with its peculiar mutation pattern in the ISRE (Y. Guo et al., 2019). Finally, a South American study with HDV-III infected patients reported undetectable HDV RNA level after 48 weeks of combination of entecavir and pegylated IFN- α (Borzacov et al., 2016), a combination that showed poor efficiency on HDV in the past. Thus, HDV-III might react differently than others to treatments.

e. Genotypes and the immune response

HBV infection could be detected by several immune sensors. However, It has been widely established that HBV infection correlates with **a lack of IFN response** and immune activation in general (Lebossé et al., 2017; Wieland et al., 2004), **except** maybe for **HBV-C** (Sato et al., 2015). Besides, the **A1762T/G1764A** mutation mentioned earlier as increased in HBV-C and HBV-D, is associated with cytoplasmic Hbc localization and further elucidation of the intrinsic immune response (C.-L. Lin & Kao, 2017). Regarding the adaptive immune system, a general decrease in the amount of lymphocytes T follicular helper (**TfH**) cells has been reported in HBV-B and HBV-C patients (of note with 1.5 more in HBV-B than HBV-C patients) compared with control, correlated with lower concentration of serum IL-21 (Xibing et al., 2013). This could impact HBV-B/C seroconversion as TfH aid B cells in their maturation to antibody secreting plasmocytes. Finally, a recent study correlated polymorphisms in the “a” region of HBsAg with vaccine escape, particularly found within HBV-D and HBV-A (Raheel et al., n.d.). In addition, no changes have been observed in vaccinated vs unvaccinated cohort from Gambia regarding HBV-E prevalence, thus, it is believed that HBV-E may be related with vaccine escape as well (Malagnino et al., 2018).

Of note, since the viral genotypes have different geographical distributions, it is not excluded that environmental factor may also influence virus’s replication and diseases outcome. Most of the studies performed *in vitro* or in animal models used HBV genotype D and HDV genotype I, and none reported a systematic comparison between the different viral genotypes.

III. Interplay between the hepatic immune system and HBV-HDV: Doctor Liver and Mister Hepatitis

1. Interplay between liver MΦs and hepatocytes, which model is the most relevant?

a. Human in vitro models

i. MΦs

Transformed cells

Transformed cell lines, especially **THP-1** and **U937**, are widely used in the MΦ field, (Sundström & Nilsson, 1976, p. 937; Tsuchiya et al., 1980) (**Figure 18**). They are monocytic and promonocytic leukaemia cell lines, respectively, capable of differentiation towards a more “MΦ-like” phenotype upon incubation with phorbol-12-myristate-13-acetate (PMA)(Daigneault et al., 2010). To date, there is only one established immortalized KC (**iKCs**), isolated by Julie Lucifora and colleagues (Faure-Dupuy et al., 2018) (**Figure 18**). iKCs were isolated from live resection of uninfected patient and further transformed by transduction of a lentivirus expressing the E6-E7 proteins of Human Papillomavirus, known for its immortalization capacities (Niebler et al., 2013).

These cell lines are easy to use and virtually infinite due to their high proliferative capacities. However, as all transformed cell, they are genetically less stable and thus easily heterogeneous in between laboratories and passages. Besides, carcinogenesis itself comes often with immune modulations, i.e aberrant activation/inhibition of some pathways, and the E6 protein is inhibiting NLRP3-mediated IL-1β release (Niebler et al., 2013), thus iKCs may not be best suited for HBV studies (Wellenstein & de Visser, 2018). Thereby, experiments conducted with transformed cell line on HBV modulations must be carefully conducted and verified using more relevant models such as the ones discussed below.

Primary cells

Monocytes extracted from blood bags are widely used and many protocols are known to differentiate them in MΦs (**MDMs**), presenting different immune features, as discussed previously (Xue et al., 2014) (**Figure 18**). It is however suggested to use stimuli as close as possible of the physiological environment of the MΦ compartment studied (Murray et al., 2014). For instance, for hepatic MΦ, it is thus recommended to use LPS as this compound is found in high levels in the liver due to proximity of the portal vein with the dietary tract and bacterial residues (Crispe, 2009b). **Primary Human MΦs** (PHM) can be isolated from liver resection (Faure-Dupuy et al., 2018) (**Figure 18**). This is, to date, the best suited model for studying hepatic MΦ interaction with HBV.

A major drawback in the use of primary cells is their low availability, especially for PHM that requires collaboration with surgeons, which is not often easy to implement. These cells are not

proliferating, thus novel isolation is constantly needed, and they can be kept in culture for several days (for PHM) to a week or two (for MDMs), depending on the protocol used.

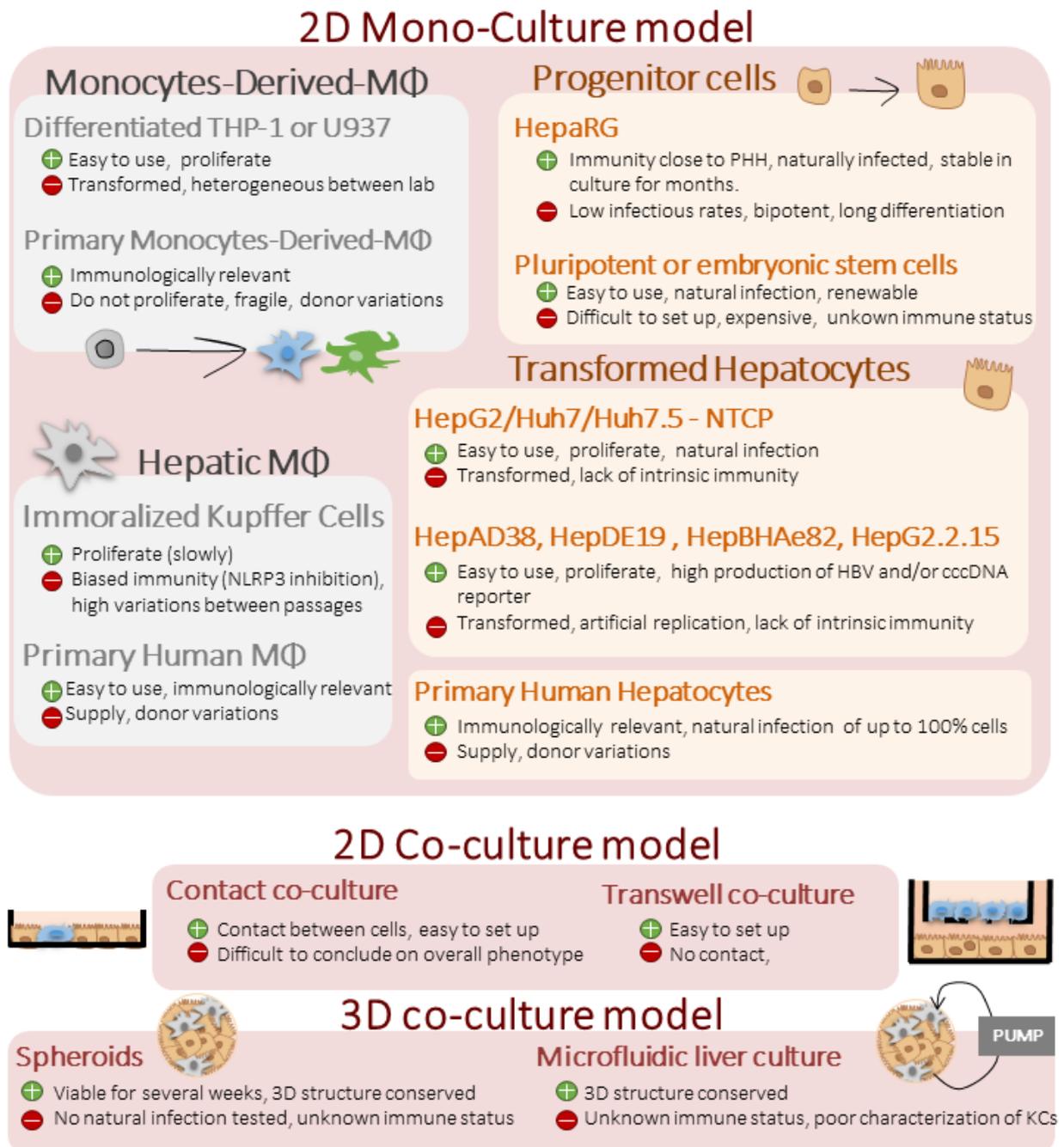


Figure 18: *in vitro* models to study the interplay between HBV infection and MΦ.
Extensive details are provided in the text.

ii. Hepatocytes

Transformed cells

Hepatocytes have been isolated from the HCC of a young afro-american (**HepG2** cells) and a 57 years old Japanese (**Huh7**) (Allweiss & Dandri, 2016) (**Figure 18**). Major issues in the study of HBV-HDV infection in these transformed hepatocytes are the rapid loss of expression of NTCP over carcinogenesis process. Thereby, for years natural infection was not possible and cells

were transiently transfected with HBV DNA and/or HDV RNA (Wose Kinge et al., 2020). These models, highly proliferative, permit also to easily study specific viral protein impact, mutants and genotypes. Stable transfected cell lines were also designed for HBV infection, such as **HepDE19**, **HepBHAe82**, **HepG2.2.15** and **HepAD38** used mainly as cccDNA reporters or for HBV production (Wose Kinge et al., 2020) (**Figure 18**).

We will provide here further details concerning HepAD38 cells which were widely used in our study. To obtain this cell line, HepG2 were stably transduced with two plasmids coding for (1) the pgRNA cDNA of HBV serotype *ayw* under a minimum CMVtet promoter and (2) a trans-acting transcriptional regulator of tetracyclin response (Ladner et al., 1997). Upon 15 days of antibiotic withdrawal, HBV mRNA and proteins are efficiently formed (level similar to what observed in CHB patients) and recycling of nucleocapsid is accompanied with stable cccDNA formation (T. Zhou et al., 2006). Hence, these cells are secreting a full HBV inoculum in a tet-off manner. Of note, tetracyclin induction inhibits all HBV components at the mRNA/protein level; except for HBsAg which is efficiently produced and secreted, and HBx.

To obtain more natural infectious models, stable transfection of NTCP (**HepG2-NTCP**, **Huh7-NTCP**) were set up, leading to efficient HBV and/or HDV replication (H. Yan et al., 2012) (**Figure 18**). However, these models, by being transformed cell, have numerous immune deficiencies compared to Primary Human Hepatocytes (PHH), as discussed earlier, and thus are not the best suited model for intrinsic immunological studies.

Cells differentiated from liver progenitors

The most used liver progenitor is the **HepaRG** cell line (Gripon et al., 2002a) (**Figure 18**). Isolated from an HCV infected female suffering from HCC, this progenitor cell line is bipotent. Upon DMSO treatment, HepaRG can be differentiated into hepatocyte-like cells and cholangiocytes. As they are not transformed, their intrinsic immune properties are close to the one observed in PHH (Faure-Dupuy et al., 2018) and they express NTCP, thereby supporting the study of most of the HBV-HDV life cycle. Nonetheless, it is estimated that only 5% of total HepaRG cells are infected with HBV-HDV, most probably due to the lack of re-infection (for both) and the bipotency of the cells (only 40-50% hepatocyte) (Wose Kinge et al., 2020). Besides, the replication levels are low, most probably due to the absence of nucleocapsid recycling for HBV (i.e no cccDNA amplification).

Human pluripotent or embryonic stem cells can also be differentiated into hepatocyte-like cells (**iHeps**) (**Figure 18**). They are easy to handle once established and come with an unlimited/renewable supply (Y. Xia et al., 2017). They sustainably support HBV infection by inducing a long term hepatocyte differentiation, however, data are still lacking regarding their immune status and they require expensive and high expertise to setup (J. Hu et al., 2019).

Primary cells

PHH are, to date, the gold standard to study HBV/HDV infection and related immunology (**Figure 18**). Isolated from liver resection, thereby with all the drawbacks discussed already in the section on PHM, they support high infection rates by HBV/HDV (Gripon et al., 1988). Depending on the isolation and liver piece, PHH can be viable up to on month post-plating (Lucifora, Michelet, Rivoire, et al., 2020) with appropriate level of immune pathways/receptors (Faure-Dupuy et al., 2018). However, their proliferation is rapidly stopped in culture, which is beneficial for HBV infection, but does not ensure a constant supply.

In addition to all the drawbacks previously mentioned, dedifferentiation occurs very often due to the 2D culture or the lack of supportive stromal cells, hampering the possibility for long-term study of HBV/HDV infection. To cope with these issues and investigate further the interplay of infected cells with their surroundings, 2D and 3D models are increasingly designed (Akbari et al., 2019; Bell et al., 2016; Faure-Dupuy, Delphin, Aillot, Dimier, Lebossé, Fresquet, Parent, Sebastian Matter, et al., 2019; T. V. Nguyen et al., 2015; Ortega-Prieto et al., 2018).

iii. Co-culture of hepatocytes and MΦs to study HBV-mediated modulation(s) of immune responses

2D co-culture

Contact co-culture of both hepatocytes and MΦs from different models is relatively easy-to-handle, especially when using cell lines (Figure 18). However, specific participation of each lineage in the overall phenotype is difficult to establish. Besides, it does not mirror the *in vivo veritas* where liver MΦs and hepatocytes are physically separated by the Space of Disse. Even if cell-to-cell interaction is possible *in vivo*, they are surely not closely intertwined (Crispe, 2009b). Thus, models of **co-culture in transwell**, thereby compartmentalizing both lineages, are used in the laboratory and proved to be very efficient to study the interaction between HBV infection and hepatic MΦs (Faure-Dupuy, Delphin, Aillot, Dimier, Lebossé, Fresquet, Parent, Sebastian Matter, et al., 2019) (Figure 18).

3D co-culture

Liver spheroids of PHHs, KCs and most NPCs were designed and viable for several weeks, but, to date, only a recombinant HBV adenovirus was used in this model and no analysis of immune parameters was performed (Bell et al., 2016) (Figure 18). Of note, a new innovative 3D microfluidic model of PHH spheres was recently presented, however specific characterization of KC-containing spheres is still required (Ortega-Prieto et al., 2018) (Figure 18).

New innovative 3D co-culture models to be implemented to investigate HBV/MΦ interaction

Several highly relevant 3D liver models were designed recently, but have not been tested for HBV/HDV susceptibility (in a context where PHMs are present) yet, to our knowledge. I will briefly present few models of peculiar interest:

- **Organoids**: spheroid assembled from pluripotent stem cells which will be further differentiated into liver cells. Already set up in various system, however none of them with KCs (Akbari et al., 2019).
- **Micropatterned Coculture (MPCCs)**: PHH organized in micro-patterned colonies surrounded by supportive murine embryonic fibroblasts and KCs (T. V. Nguyen et al., 2015). Of note, cytokine secretions were assessed in this model and seem to be polarized toward a more pro-inflammatory state, physiologically far from the highly tolerant polarized status of hepatic MΦ.
- **Liver-on-a-Chip** platforms: a 3D reconstruction of liver acinus containing all NPCs (X. Li et al., 2018).
- **Precision-cut liver slices (PCLS)**: 250µm slices, cutted using vibrating microtome (X. Wu, Roberto, et al., 2018). Even if cytokines and other immune pathways were

increased by the sliced-induced choc, PCLS are still capable of responding to TLR3 and TLR4 agonist (X. Wu, Roberto, et al., 2018).

Hence, *in vitro* models to study the interaction of HBV/HDV with hepatic MΦs are suboptimal. Even if primary cells are the gold standard, the use of cell line is always required as it is more convenient/available, especially to study further the mechanism associated with a phenotype (easier to handle for transfection or else). The difficulty to recapitulate *in vivo veritas*, concerning hepatic MΦs polarization status as well as PHH intrinsic immunology, renders the study of HBV/HDV-mediated modulations highly heterogeneous and thus highly controversial.

b. Animal models

i. Natural infectious model for HDV, HBV and the hepadnavirus genus

Animal infection of diverse hepadnaviruses (same genus as HBV) have gain increased interest with the finding in the last few years of **fish** (White sucker and Bluegill HBV) and **amphibian** (Tibetan frog HBV) strains (Dill et al., 2016; Hahn et al., 2015), in addition to the one already established in various **avian** models (Heron, Ross Goose, Stork and Parrot HBV), **non-human primates** (Chimpanzees, Gorillas, Orangutans, Gibbons, Woolly monkey HBV), **rodents** (Woodchuck, Ground squirrel, Arctic squirrel HBV) and **bats** (btHV) (Locarnini et al., 2013; McNaughton et al., 2019), thereby demonstrating the wide spread and ancient character of the family (**Figure 19**). Besides, endogenous viral elements of woodchuck HBV (WHB), resulting from ancient HBV integration, were described in zebrafish (Katzourakis & Gifford, 2010). Altogether, these new models permit exciting new platforms to investigate HBV evolution; however, they are not suited to study HBV biology. Current model capable of being naturally infected by *hepadnaviridae* family members in laboratory condition encompass the **Pekin Duck** and **Woodchuck**, using Duck HBV (DHBV) and WHB viruses respectively (McNaughton et al., 2019). These natural infectious model have been widely used to study liver pathogenesis and test antiviral, however they only share 70 to 40% similarities with human HBV (W.-N. Guo et al., 2018). Besides, as they are able to naturally envelop human HDV, they are good surrogate to study HBV-HDV infection (Giersch & Dandri, 2021).

Human HBV is capable of naturally infecting various non-human primates; however they develop only mild symptoms in most cases (Allweiss & Dandri, 2016). The **pan troglodytes chimpanzee**, which is susceptible to HBV and HDV, develop acute and chronic infection, accompanied with hepatitis and an immune profile highly similar to what is observed in human (Allweiss & Dandri, 2016) (**Figure 19**). Especially, this model demonstrated that HBV acute infection was not correlated with immune activation (Wieland et al., 2004). However, due to obvious ethical concerns, restrictions have been established by international committee such as the NIH, prompting the need for other model of natural HBV/HDV infection (W.-N. Guo et al., 2018). In 2013, HBV infection was found in small non-human primates, namely **Macaca fascicularis** from Mauritius island (Dupinay et al., 2013) (**Figure 19**). They develop acute hepatitis and, even if poor data have been computed on putative immunomodulation, this model is promising as macaques immune system are one of the closest from ours (Burwitz et al., 2017). Of note, even if natural infection was first reported, laboratory recapitulation and infection with HBV/HDV was not possible without a first infection of AAV-hNTCP (Burwitz et al., 2017; Giersch & Dandri, 2021). Finally, **tupaia belangeri** (tree shrew) are also susceptible to HBV and HDV,

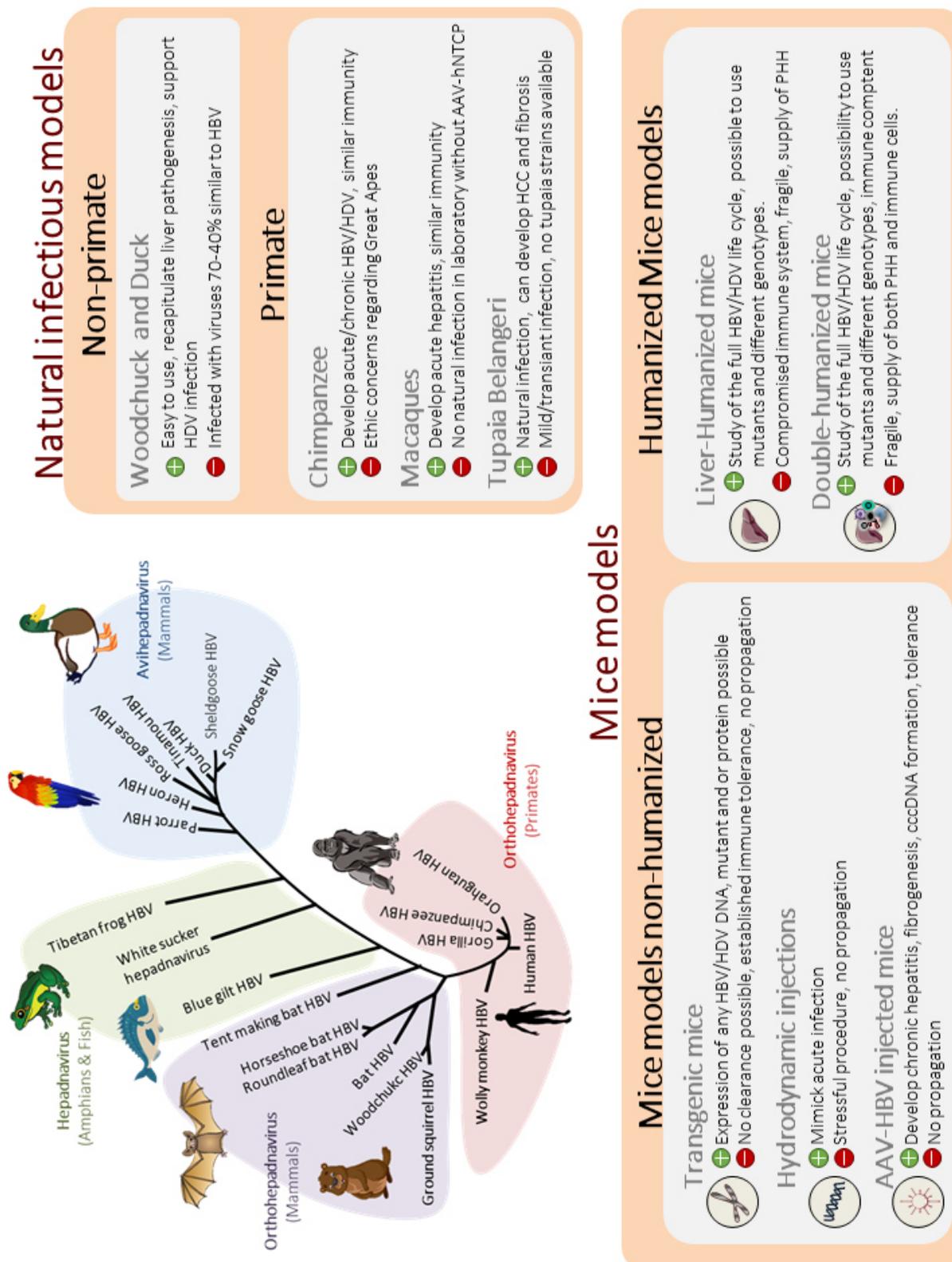


Figure 19: The hepadnaviruses family and existing HBV animal models.

resulting in a mild/transient infection (W.-N. Guo et al., 2018) (Figure 19). Mimicking human infection, tupaia develop HCC and fibrosis, but only if injected in neonates (Allweiss & Dandri, 2016). This model enabled the discovery of NTCP as HBV receptor (H. Yan et al., 2015), nonetheless, it is mostly used for *in vitro* studies.

ii. Designing an immunocompetent mice model susceptible to HBV infection

Mice are the animal most characterized and best suited for laboratory purpose; however, they cannot be naturally infected with HBV due to differences in NTCP-R sequence. To circumvent these issues, several models have been developed. The first attempts to obtain a relevant mice model, achieved in the 80', consisted in **transgenic** mice, genetically expressing HBV DNA or HDV RNA, or specific viral components (Allweiss & Dandri, 2016; Giersch & Dandri, 2021) (**Figure 19**). For HBV, as the genome is integrated, no clearance is possible and the cccDNA is not formed, rendering this model suboptimal for antiviral research toward a functional cure (i.e cccDNA elimination). Besides, as viral proteins (expressed alone or with the full HBV DNA) are expressed since birth, immune tolerance is established and thus, no liver injury is observed (W.-N. Guo et al., 2018). Of note, this observation further suggests that HBV itself is non-cytopathic. Concerning HDV transgenic mice, a first attempt of S/L-HDAg transgenic mice induced no evidence of liver attempts, and HDV-RNA tg mice were not able to produce HDV-AG and mRNAs (Giersch & Dandri, 2021).

Due to the lack of physiological relevance of this model, attempt to infect mice focused then on new viral delivery of HBV DNA or HDV into hepatocyte. First, rapid injection of large volume of liquid containing the naked viral DNA in the animal tail vein, termed **hydrodynamic injection**, permit an efficient uptake of the virus in hepatocytes (Allweiss & Dandri, 2016) (**Figure 19**). Both infection are resolved rapidly after a viral peak and subsequent immune activation. Thereby, it is mainly a model of acute infection, used in antiviral research for compounds targeting the early step of the viral life cycle. Another issue related to this model is the stress intrinsically induced by the procedure and the subsequent liver damages. To cope with the lack of immune-relevant model, low doses of adenovirus or adeno-associated virus (AAV) recombinant with HBV (genotype D) were inoculated in mice (D. Yang et al., 2014). **AAV-HBV infected mice** at early age demonstrate sustained HBV level and cccDNA formation for up to six month, along with chronic hepatitis and fibrogenesis (Lucifora et al., 2017a; L. Ye et al., 2015) (**Figure 19**). Interestingly, no acute inflammation was observed in these animals, which resembled human pathology with a lack of appropriate immune response as seen with no HBsAg antibodies detected and low response of CD8+T cells (D. Yang et al., 2014). **AAV-HDV mice models** were designed in 2017 (Suárez-Amarán et al., 2017; Usai et al., 2020), with a persistence of HDV for no more than 45 days and a decrease in viremia starting day 21. Interestingly, acute liver injury and HDV dominance was recapitulated in this model. To date, they are the most convenient model to study the interaction of HBV/HDV with the immune compartment. However, virions produced in both model are not capable of re-infecting hepatocytes (due to the lack of hNTCP), and the mice immune compartment, even if well characterized, is different from humans. Several attempts were made to establish mice models with humanized NTCP (AAV or transgenic). Even if the models were successfully expressing the HBV-HDV receptor, HBV infection could not be established and HDV was only transient, thus suggesting that species barriers cannot be overcome only by NTCP (Giersch & Dandri, 2021).

The last innovative strategy to obtain hepatocytes susceptible for HBV/HDV infection is the design of **liver humanized mice** (**Figure 19**). First described in 1995 by Rhim and colleagues, it consist in the induction of liver damages in mice, thereby creating an empty space which will serve as a niche for adoptively transferred PHH (Rhim et al., 1995). After a regenerative stimulus, PHH slowly repopulate the mice liver in two months. Adaptive immune deficiency is required to avoid immune-mediated attack of xenogeneic hepatocytes, thereby animals were

crossed with mice which were either NOD, RAG^{-/-}, IL-2R γ chain^{-/-} or SCID (or several combined). Three humanized models have been designed so far, differing in their mode of induced liver failure:

- **uPA:** urokinase-type plasminogen activator (uPA) overexpression in mice induces liver failure (Rhim et al., 1995). Transgenic mice and tetracyclin-induced models are available. Often crossed with RAG^{-/-} or SCID mice.
- **FRG:** Fumaryl acetoacetate hydrolase (Fah) deficiency leads to accumulation of toxic tyrosine catabolites which induced liver failure (Allweiss & Dandri, 2016). This model is inducible through injection of NTBC (2-nitro-4-trifluoro-m ethylbenzoyl)-1,3-cyclohexanedione which protects animal. As a knock-down of both RAG and IL-2R γ is required, the model was termed FRG (for Fah-RAG-IL-2R γ).
- **TK-NOG mice:** This model carries the herpes simplex virus thymidine kinase transgene under the albumin promoter. It is crossed with mice from NSG lineage which is NOD/SCID/IL-2R γ ^{-/-}. Delay of liver failure is possible by the addition of ganciclovir. Of note, male TK-NOG are sterile.

These models permit the study of different genotypes, mutants, strains engineered or isolated from patient serum. In this model, it is possible to study the full HBV life cycle, including integration of HBV genome (W.-N. Guo et al., 2018).

Based on these mice models, Strick-Marchand and colleagues designed the first **immunocompetent liver chimeric double-humanized mice** (Strick-Marchand et al., 2015) (**Figure 19**). In this model, co-transplantation of human hematopoietic stem cells and PHH from the same donor were adoptively transferred in BALB/c mice which were RAG2/IL-2R γ . Even if previous attempt of double-humanization has been performed, it is the first to show a stable engraftment of cells from both myeloid and lymphoid lineage for up to five months. Interestingly, in this model, the immune response was dependent of the viral load, and infected mice had increased level of anti-inflammatory (IL-10) cytokines (Dusséaux et al., 2017).

However, due to the need for supply of both viable PHH and blood cells, double-humanization are even more expensive and difficult to handle than already is the simple humanized model.

Altogether, models to study the interaction between HBV/HDV and M Φ s are suboptimal and thus precaution needs to be taken. In addition to previously discussed limitations, it is worth noticing that HBV/HDV inoculum themselves needs to be carefully characterized as endotoxin contamination (LPS-like components) is often seen in culture supernatant. As PHH as well as hepatic M Φ s express TLR4 (Faure-Dupuy et al., 2018), it is essential to eliminate any contamination to avoid false positive when looking at HBV/HDV activation of the immune system.

However, using these distinct models, paired with studies in human cohorts, we gained understanding of the interplay existing between immunity and HBV/HDV. It is now clear that the chronicity is accompanied by sequential phases of immune activation and tolerance due to immune cells dysfunctions, which lead to hepatocarcinogenesis and difficulty for viral clearance, respectively.

2. HBV/HDV-driven immune-pathogenesis

As previously discussed, HBV and HDV are thought to be mainly non-cytopathic *per se*; yet it is not excluded that some particular genotypes or mutants, which can arise during the natural or on-therapy histories of infections, might directly impact hepatocytes viability. When infections are destined to become chronic, liver flares are relatively rare or of weak intensity in the early months of HBV or HBV/HDV infections. It is generally 10 to 30 years after CHB has started that ALT level increases as the patient enter phase 2 of the disease (European Association for the Study of the Liver. Electronic address: easloffice@easloffice.eu & European Association for the Study of the Liver, 2017), and as mentioned previously, much earlier in CHD (Tseligka et al., 2021). There, repeated CD8-mediated attacks of infected cells leads to the slow appearance of fibrosis, which paves the way for further cirrhosis and/or HCC (Ferrari, 2015) (Figure 20). To date, the specific triggers enabling to pass from a chronic infection in the context of immune tolerance to an active immune-pathogenesis, and the exact mechanisms under HBV/HDV induced carcinogenesis, are poorly understood.

However, studies conducted on CHB patients' immune active phases enabled to gain a better understanding of how immune cells are major component of this liver disease.

Of note, immunity is not the sole factor for fibrosis, cirrhosis and HCC induction, as explained earlier in this introduction.

a. Immune infiltrates of activated cells

The liver is highly tolerogenic. LSECs and KCs secrete significant amount of IL-10 at steady state, to protect the organ from over-activation mediated by dietary residues or bacterial products (Crispe, 2009b). Thereby, as being an immune privileged organ, pro-inflammatory activation of liver cell, if uncontrolled, is highly detrimental.

CHB is associated with **large infiltrates of mononuclear cells**, especially CD8+T cytotoxic cells, CD4+T cells and NK/NKT (Peeridogaheh et al., 2018) (Figure 20.A). When the lymphocyte response is HBV-specific, it is rapidly followed with viral clearance, as seen in acute infection (Stelma et al., 2017), however chronic-associated infiltrates are mostly HBV-unspecific and correlate with elevated ALT and liver damages due to **CTL-mediated hepatocyte apoptosis** (Maini et al., 2000) (Figure 20.B). **Non-cytolytic clearance** of HBV through IFN- γ production by CTLs also activates various immune cells, such as KCs and LSECs (Figure 20.B-C). This leads to a further increased concentration of IFN- γ (feed forward process or vicious circle), and the **production of other inflammatory cytokines** (TNF- α , IL-8, IL-12) **and chemokines** (CCL3, CXCL3, CCL5) in the liver (Rehermann, 2013) (Figure 20.C); further recruiting and activating innate immune cells, mainly monocytes and neutrophils, as well as increasing the amount of CD8+T/CD4+T/NK cells already presents on site. Thereby, it creates a massive infiltrate of activated pro-inflammatory immune cells (Maini et al., 2000; Rehermann, 2013; J.-Y. Zhang et al., 2011) (Figure 20.D). Once arrived on site, matrix metalloproteinases (MMPs) secreted by activated neutrophils and KCs remodel the extracellular matrix (ECMs), facilitating the loss of vascular endothelial cell integrity, permitting immune invasion of the liver parenchyma (J. Chen et al., 2017; C. Yan & Boyd, 2007) (Figure 20.D). The newly recruited immune cells mainly possess a pro-inflammatory phenotype, once again enhancing liver flares. This was nicely presented by Zhang and colleagues which observed a specific increase of CD16⁺CD14⁺, monocytes in the circulation of immune active patients (J.-Y. Zhang et al., 2011). This subset is characterized by a massive secretion of IL-6, IL-1 β and IL-12/IL-23p40 and are responsible for Th17 differentiation, a T cell subset also found especially increased in CHB patients

(Peeridogaheh et al., 2018). Th17 secrete IL-17, amongst other cytokines, which lead to TNF- α and IL-6 production by monocytes and DCs on site (Rehermann, 2013), further facilitating the recruitment of monocytes, mDCs and neutrophils in the liver (H. Q. Sun et al., 2012, p. 17). Neutrophils massively recruited on site (J.-Y. Zhang et al., 2011) release cathepsin G upon TNF- α exposure, leading to platelets activation, which correlates with increased fibrogenesis and elevated ALT (Q. Jiang et al., 2019; Y. Lee & Friedman, 2010; Renesto & Chignard, 1991). Altogether, immune unbalance toward a pro-inflammatory phenotype is observed in CHB during immune active phases, accompanied with massive secretion of pro-inflammatory cytokines and chemokines, inducing a deleterious recruitment and activation of cells, from both the adaptive and innate immune system. This over-activation leads to (i) hepatocyte apoptosis and (ii) liver fibrogenesis, paving the way for cirrhosis and HCC appearance.

b. Specific and unspecific triggers of hepatocytes apoptosis

Persistent inflammation in immune active phases of the disease leads to the death of hepatocytes (Peeridogaheh et al., 2018) through, (i) the **perforin granzyme B** system and (ii) **death receptor-mediated apoptosis** (J. Y. Lee et al., 2004) (Figure 20.B).

This mechanism is of key importance as hepatocyte loss is accompanied by (i) pathological wound-healing leading to fibrosis (Figure 20.F) and/or (ii) compensation through increased division which, if uncontrolled, leads to carcinogenesis (Figure 20.G).

Once CD8+LT have recognized HBV-infected cells, secretory granules are relocalized to the immune synapse to be further released (J. Y. Lee et al., 2004; Voskoboinik et al., 2015). Released vesicles contain perforin, which form large transmembrane pores inside the targeted cells, and granzyme, passing through the pores to induce caspase-mediated apoptosis (J. Y. Lee et al., 2004; Voskoboinik et al., 2015) (Figure 20.B).

In addition, CHB patients show increased liver cells expression of death-receptor and death-receptor ligands, leading as well to caspase-dependent apoptosis (Hou et al., 2017; S. Lin & Zhang, 2017). Indeed, NK, NKT and CD8+T cells of CHB patients show increased **TNF-related-apoptosis-inducing ligand** (TRAIL) (Dunn et al., 2007; S. Lin & Zhang, 2017) and **Fas-death-receptor ligand** (FasL) expression (Hou et al., 2017; S. Lin & Zhang, 2017). Hepatocytes, on the other hands, express increased levels of Fas and TRAIL-Receptor, especially in an HBx-dependent manner (Dunn et al., 2007).

It is worth noting that HBx was suggested to modulate hepatocytes apoptosis (L. Cao et al., 2016). At increased level, **HBx** would mediate (i) NF κ B translocation in the cytoplasm and (ii) induces the MAPK-JNK pathway (i.e **sensitization** to TRAIL and Fas mediated apoptosis) leading to hepatocyte apoptosis (L. Cao et al., 2016). In contrast, at **low rates, HBx would be anti-apoptotic**. It has been suggested that, by modulating p53, HBx favors FasL over Fas expression on infected hepatocytes, thereby inducing immune cell death instead of hepatocytes apoptosis (Cao et al. 2016). Besides, NF κ B activation by HBx leads to increase expression of up-regulated gene 7 (URG7), which block caspase-8 and putatively also caspase-3 (Feitelson et al., 2009). Hence, it is possible that the massive apoptosis observed in CHB patient's liver could be targeted especially on uninfected cells, thereby promoting HBV persistence.

Altogether, these mechanisms lead to the massive presence of apoptotic bodies in the liver, which is a major inducer of immune cell activation (Peeridogaheh et al., 2018), but also a pro-fibrogenesis factor (Peeridogaheh et al., 2018).

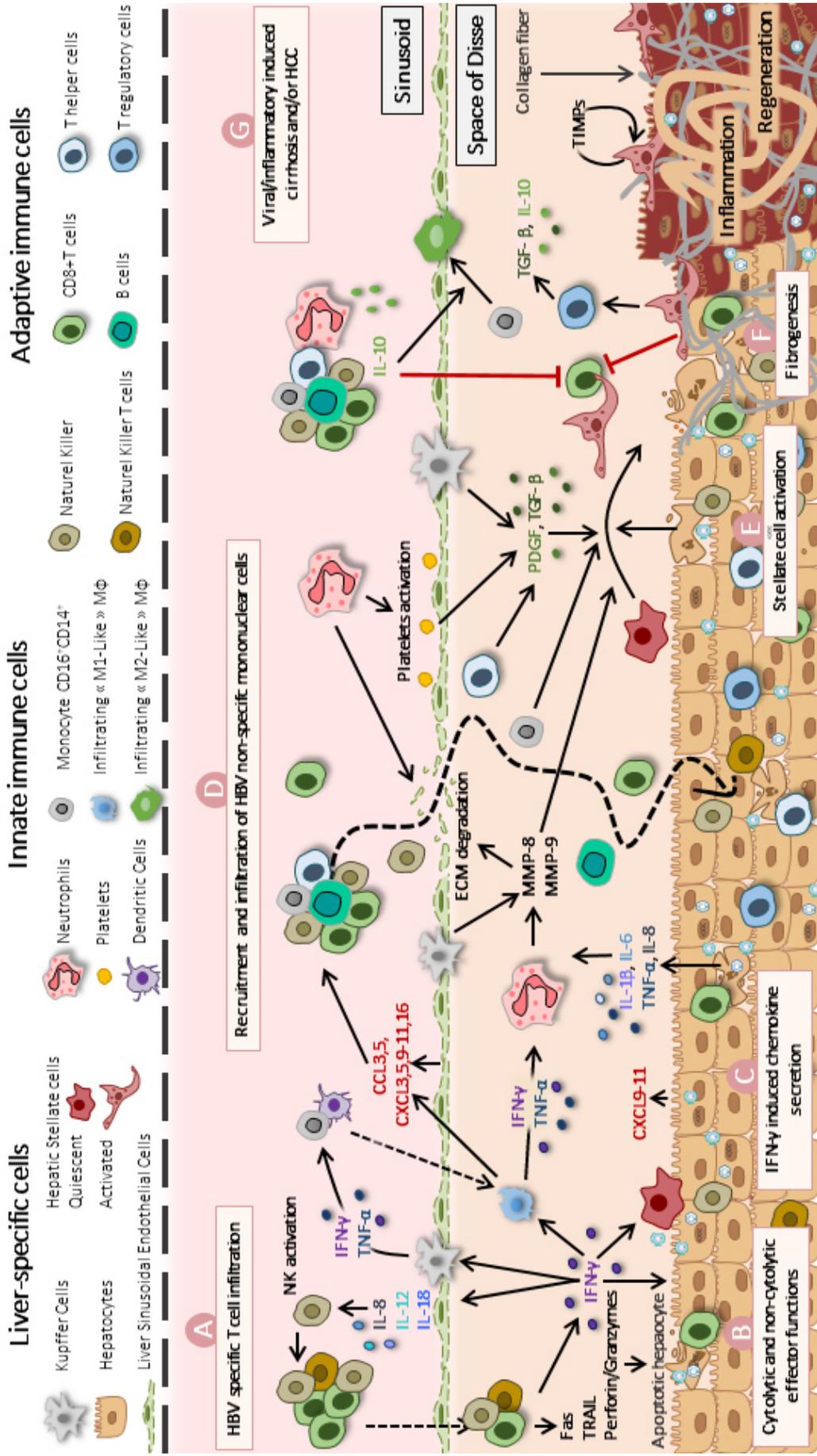


Figure 20: Liver HBV-driven immunopathogenesis

c. Induction of fibrogenesis

Liver fibrosis is the result of HSCs transition from a fat-storing quiescent phenotype to a migratory myofibroblast-like one (Fujita & Narumiya, 2016) (Figure 20.E). This transdifferentiation process is characterized by the slow appearance of the alpha smooth muscle actin (α -SMA), loss of vitamin A droplets, increased cellular proliferation and synthesis of fibronectin, collagen I and tissue inhibitors of metalloproteinases (TIMPs) (Higashi et al., 2017). It relies on various triggers, such as (i) TGF- β and PDGF cytokine secretions amongst other growth factors and cytokines (Higashi et al., 2017), (ii) MMPs production, (iii) phagocytosis of apoptotic bodies, (iv) TLR9 activation by recognition of DNA from dying hepatocytes (Rehermann, 2013) and (v) C5a complement factor (R. Xu et al., 2013).

TGF- β and PDGF are produced by activated KCs (H. Li, Zheng, et al., 2012), infected hepatocytes, Th17, Th9 and platelets, but also HSCs once activated (Y. Lee & Friedman, 2010) (Figure 20.E). In addition, some HBV proteins were shown to favor their secretion as well, such as HBeAg, HBsAg, HBcAg and HBx; however most studies rely on immortalized HSCs cell line and recombinant proteins, thereby data are lacking using more relevant models (Q. Bai et al., 2012; Higashi et al., 2017). Besides, CD16⁺CD14⁺ induces **elevated collagen production** in HSCs, which could not be reversed totally using TGF- β antibody, suggesting a cocktail effects mediated by the secretions of infiltrating monocytes (Zimmermann et al., 2010).

Neutrophils, which are recruited in the liver of CHB patients, are major producers of MMP-8 and MMP-9, amongst other MMPs, which participate in ECM proteolytic degradation, as discussed earlier (Feitelson et al., 2009) (Figure 20.D). Involved in wound-healing at steady state, their activation of HSCs leads to up-regulation of pro-fibrogenic factors (such as TGF- β) and further increase in MMPs secretion (H. Li, Zheng, et al., 2012, 2012). After a first MMP elevation, TIMPs are produced by activated HSCs, and have a dual role of (i) inducing HSCs survival and (ii) leading to MMP inhibition. Hence, a **scarring tissue** arises with **uncontrolled generation of ECM components**, leading to fibrosis (Figure 20.F).

In order to avoid fibrogenesis, HSCs needs to either become quiescent again, which has not been described *in vivo* yet, or enter apoptosis which is thought to be attenuated in CHB patients (Gupta et al., 2018; Sasaki et al., 2016; R. Xu et al., 2013).

d. HDV-induced pathologies

Concerning HDV induced carcinogenesis, even if clearly linked; the exact mechanisms are poorly understood. HDAg proteins were reported to alter autophagy process to promote its genome replication (Khabir et al., 2020), to cause oxidative stress (Williams et al., 2012) as well as modulating the TGF- β (Choi et al., 2007) and NF- κ B (Williams et al., 2012) signaling pathways, all of which are associated factors of fibrogenesis or hepatocarcinogenesis, as previously discussed. Besides, contrary to HBV, HDV is a strong inducer of IFN secretion/production, suggesting an increase in immune activation in co-infected liver. However, most of the studies performed on the interplay between HDV and cellular processes have been performed in artificial overexpressing systems, some even using non-hepatocytes cell lines. Numerous interactions between HDV proteins and cellular proteins have been reported (for review see (Goodrum & Pelchat, 2018)) but most of them need to be confirmed in infectious systems.

3. HBV/HDV interplay with the immune system

Clearance of HBV and HDV infected cells is physiologically performed by activated immune cells through activation of intrinsic immune pathways within hepatocytes. Indeed, activation of such pathways by IFN, pro-inflammatory cytokines (IL-6, IL-12, TNF α and most potent IL-1 β) (Isorce et al., 2016) and PRRs agonists (Lucifora et al., 2018) lead to **efficient decrease of viral replication** through activation of the NF κ B and/or IFN pathway. However, even if these components can be secreted by liver immune cells, both viruses establish efficiently a chronic infection within the organ, suggesting a lack of activation and/or efficient hiding from these mechanisms.

Of note, there is a tremendous lack of understanding of HDV interplay with the immune system, thus most of the following discussion will focus on HBV.

Conflicting results are dividing the scientific community regarding whether HBV is **directly inhibiting immunity** or if the virus is just peculiarly efficient at **hiding from immune sensors**. During HBV life cycle, encapsidation of the viral genome within the nucleocapsid during all the cytoplasmic steps shields the viral genome from being recognized by various PRRs (Grimm et al., 2011; Levrero et al., 2009; T. Tu et al., 2017). Besides, the cccDNA and viral RNAs, are host-like genomes, which interact with host transcription factors and polymerase, respectively. Thus, they are **recognized as “self”** and remain unrecognized. These data are supported by experiments conducted in infected chimpanzees as well as CHB patients' biopsies which show only poor induction of innate immune responses at the RNA level (Fletcher et al., 2012; Lebossé et al., 2017; Wieland et al., 2004). On the other hand, several groups, amongst ours, demonstrated a specific inhibition of immune pathways capable of recognizing HBV (Luangsay, Gruffaz, Isorce, Testoni, Michelet, Faure-Dupuy, Maadadi, et al., 2015; Verrier et al., 2018). These data however are not mutually exclusive as (1) low PRR stimulation favors immune tolerance over activation and (2) poor induction of innate immune genes could be attributed to active inhibition.

It is however worth noticing that divergences between laboratories can be attributed to the lack of appropriate models to study HBV. In all hepatic cell lines, high M.O.I and the use of PEG is required for HBV natural infection (Allweiss & Dandri, 2016); thus increasing the amount and time passed by Dane/Subviral particle at the cell surface, where they could putatively be recognized. In addition, endotoxin (LPS-like component) and HBV naked nucleocapsid (not observed *in vivo*) are known immune activators which can contaminate HBV inoculum (M. Li et al., 2015; Vanlandschoot et al., 2007; Z. Zhang et al., 2020). However, viral inoculum is not always characterized thoroughly. Another concern is the use of recombinant proteins, produced in yeast or bacteria, can (i) also be contaminated with endotoxin (Vanlandschoot et al., 2007) or (ii) are dissolved in DMSO, a well-known immune modulator (Kelly et al., 1994). Besides, it is possible that HBsAg, produced in non-hepatocyte cell lines could differ from its physiological form as it is well-known that the protein is coated in hepatocyte lipids upon egress, which have a highly specific composition putatively not recapitulated in other models (Vanlandschoot et al., 2002). Last but not least, natural infection is not systematically performed; however transduction of viral DNA is inducing the IFN response, whereas physiological infection is not (Luangsay, Gruffaz, Isorce, Testoni, Michelet, Faure-Dupuy, Maadadi, et al., 2015).

In the following part we will thus address the following question: (i) Is HBV recognized by the immune system? (ii) Does HBV inhibit the innate and adaptive immune response?

a. Are HBV/HDV recognized by the immune system?

While it is discussed for HBV, **HDV** has been shown to strongly activate the IFN pathway via **MDA5 recognition**, in primary human hepatocytes and humanized mice models (Alfaiate et al., 2016; Giersch et al., 2014; Williams et al., 2009; Z. Zhang et al., 2018).

Surface receptors, namely **TLR2** and **CD14**, are capable of sensing **HBV capsid** and **surface protein**, respectively ((M. Li et al., 2015, p. 2; Yi et al., 2020; Z. Zhang et al., 2020). TLR2 recognition of HBV nucleocapsid leads to either immune activation (Yi et al., 2020; Z. Zhang et al., 2020) or anti-inflammatory phenotype promotion in KCs (M. Li et al., 2015). One possible origin from naked-nucleocapsid could be their release from dying hepatocytes during immune-active phases, which could participate in the immunopathogenesis described earlier. As TLR2 expression in CHB patients is inhibited in HBeAg positive patients and increased in HBeAg positive ones, compared to control, it further suggests a modulation of the TLR2 pathway by HBV (Visvanathan et al., 2007b). CD14, a TLR4 co-receptor, direct interaction with HBsAg was evidenced, both as a circulating complex and a membrane-bound form (van Montfoort et al., 2016b; Vanlandschoot et al., 2002). sCD14-HBsAg complexes are correlated with BDCA3+ mDCs activation in CHB patients sera (van Montfoort et al., 2016b), whereas HBsAg bound to membrane-form CD14 and LPS Binding-Protein (LBP) on monocytes, leading to anti-inflammatory polarization (Vanlandschoot et al., 2002). It is worth mentioning that HBsAg lipid composition was essential in this interaction, and the sCD14-HBsAg complex was present only in HBeAg positive patients (Phase I and II). Hence, it is possible that changes in the liver environment along CHB phases (i.e fibrosis induction, HCC) could modulate lipid composition within hepatocytes thereby modifying HBsAg particle coating. Unfortunately, to date, no lipidomic study of HBsAg particles composition upon natural CHB infection course have been performed.

Two **cytosolic sensors**, namely **cGAS** and **RIG-I**, are capable of recognizing HBV genome, respectively the rcDNA and the stem-loop secondary structure of the 5'-region of pgRNA (Sato et al., 2015; Verrier et al., 2018). In both cases, transfection of naked HBV genome induced Type I and III IFN response (cGAS) and Type III IFN and pro-inflammatory cytokine (RIG-I) secretion. Upon more physiological HBV infection (i.e natural infection, no transfection), this sensing was not observed, even at high multiplicity of infection, suggesting that the virus could, again, evade this immune sensing. While some suggests that this evasion is solely imputed to the encapsidation of the viral genome within the nucleocapsid during all the cytoplasmic steps, shielding viral genome from being recognized (Grimm et al., 2011; Lauterbach-Rivière et al., 2019; Levrero et al., 2009; T. Tu et al., 2017), others suggest an **active inhibition** of these pathways by the virus (Luangsay, Gruffaz, Isorce, Testoni, Michelet, Faure-Dupuy, Maadadi, et al., 2015; Verrier et al., 2018). Indeed, cGAS related genes (Verrier et al., 2018) and the IFN response (Luangsay, Gruffaz, Isorce, Testoni, Michelet, Faure-Dupuy, Maadadi, et al., 2015) were found sustainably decreased in hepatocytes, and putatively attributed to the Dane particle itself for the latter.

Altogether, it seems that sensors have the capacities to recognize HBV genome, but their *in vivo* activation and role in CHB is still a matter of debate.

Finally, even if presenting a host-like structure, cccDNA epigenetic status and efficient transcription seems to be blocked by the **SMC5/6** complex (Decorsière et al., 2016, p. 6) and **SETDB1** (Rivière et al., 2015, p. 1) respectively, suggesting a putative recognition of the viral

minochromosome. In both cases, this inhibition is rescued by HBx protein (Decorsière et al., 2016; Rivière et al., 2015).

In summary, HBV recognition by various PRRs and host-proteins is still poorly characterized and hampered by the use of suboptimal models. However, it seems that viral proteins are capable of counteracting the recognition/inhibition observed, suggesting that HBV could, indeed, be capable of modulating the immune compartment. We will now get into the detailed literature concerning inhibitory mechanisms mediated by HBV and HDV.

b. Immune inhibition

i. Innate immune tolerance

Tolerance can be induced as soon as during fetal life, as suggested by vertical transmission prevalence rates in infected mothers (Velkov et al., 2018). In most cases, it was especially increased in untreated HBeAg+ mother (84.2%) whereas treated HBeAg- mother transmission rates were quasi null (0.4%) (Z. Li et al., 2015). Besides, HBeAg is the sole HBV antigen capable of crossing placental barrier (J. S. Wang & Zhu, 2000), prompting the hypothesis of its responsibility in fetal immune tolerance (Kramvis et al., 2018). To better understand this mechanism, a model which mimic mother-to-child transmission, designed by Tian and colleagues, was used to observe the susceptibility to HBV chronicity of the offspring (Y. Tian et al., 2016). While normal and HBeAg- mothers cleared efficiently the infection through “M1-like” MΦ and CD8+T cell activation, the progeny of **HBeAg+ mother** developed immune tolerance towards HBV with the rise of “M2-like” MΦ and **CD8+T cell exhaustion** (Kramvis et al., 2018; Y. Tian et al., 2016). Absence of MΦ in the offspring liver (i.e. chlodronate treatment) as well as anti-PD-L1 treatment rescued the phenotype, suggesting that HBeAg positivity in the mother enable the establishment of an immunosuppressive environment. This is in line with several studies which correlates the anti-inflammatory cytokine IL-10 with HBV establishment (Das et al., 2012; Rybicka et al., 2020).

In the last decades, an accumulating body of evidences suggest that the innate immune compartment is modulated towards HBV tolerance, at various levels: (i) PRRs expression and their pathways in infected cells (ii) cytokine secretions and antiviral functions of immune cells and (iii) overall increase in suppressor functions.

Modulation of infected cells: interplay with hepatocytes's intrinsic immunity

In infected hepatocytes, the **IFN response** and **NFκB pathway** seems broadly inhibited by HBV. MMP9 and collagen triple helix repeat containing 1 (CTHRC1), parkin and rubicon are all host factors, increased in CHB patient liver, responsible for IFN/JAK/STAT pathway inhibition (Junbo Chen et al. 2017; L. Bai et al. 2015, 1; Tan et al. 2018). Viral proteins are also directly involved in these modulation, especially **HBx**, **HBeAg**, **HBcAg** and **HBV Pol** (Y. Liu et al., 2015; H. Wang & Ryu, 2010) (**Figure 21.A**).

An **overall decrease of ISGs fold change** in HBeAg+ patients when compared with positive ones were observed (Mitra et al., 2019), consistent with previous data (Lebossé et al., 2017), suggesting a specific impact of the protein on this pathway. Interestingly, while HDV is a known IFN-inducer, Pugnale and colleagues associated the lack of IFN impact on the virus with a blockade of Tyk2 activation and subsequent STAT1/STAT2 activation (Pugnale et al., 2009). Even

if performed in HDV-transfected hepatoma cell lines, this study is consistent with the low rates of patients that are IFN-responders in CHD cohorts.

In vitro studies suggest an impact of the HBeAg intracellular form, p22, on TIR protein Mal and TRAM involved in TLRs cascade (MyD88 co-adaptors) leading to decreased IFN- β production, in highly suboptimal models and thus to be confirmed (T. Lang et al., 2011). Besides, HBeAg association with NEMO (NF κ B classical pathway) in CHB patients biopsies, correlated with a decreased NF κ B activation, probably due to K63 ubiquitination (Y. Wang et al., 2019) (**Figure 21.A**). Also, HBc is capable of recruiting Enhancer of Zeste Homologue 2 (EZH2), a histone-methyl-transferase, on specific gene promoters to epigenetically repress the dsRNA-dependent IFN induction (i.e RIG-I/TLR3/MDA5 pathway) (Gruffaz et al., 2013) (**Figure 21.A**). The dsRNA-sensing pathway is targeted as well by HBx protein through (i) binding to IPS-1 (Kumar et al., 2011) which decreases RIG-I dependent IFN- β production and (ii) deubiquitination of IRF3, RIG I, RIG I-2CARD, TRAF3 and IKKi, leading to subsequent proteosomal degradation (J. Jiang & Tang, 2010) (**Figure 21.A**). HBx suppressed the IFN-mediated expression of TRIM22 (tripartite motif 22), a mediator of the IFN-induced antiviral response, through methylation of a single CpG site in its 5'-UTR in a mouse model, primary human hepatocytes and human liver tissues (Lim et al., 2018). Many other mechanisms for HBx-mediated inhibition of IFN pathway in hepatocytes are known (Wei et al., 2010). It is however worth mentioning that most studies relative to HBx protein are using artificial models of overexpression which are not always physiologically relevant as exemplified by Slagle and colleagues (Slagle et al., 2015) and by the differential induction of NF κ B seen earlier (L. Cao et al., 2016). Finally, HBV-Pol is highly potent in modulating the IFN pathway, as it is capable of inhibiting STING by blocking K63-linked ubiquitination (Y. Liu et al., 2015) and its association with DEAD box RNA helicase (DDX3) leads to inhibition of IRF effector kinase (H. Wang & Ryu, 2010, p. 3) (**Figure 21.A**).

Some proteins are especially targeted, as seen by the impairment of pSTAT activation and translocation by both HBV-Pol (J. Chen et al., 2013) and intracellular HBeAg p22 isoform (Mitra et al., 2019) and HDV (Pugnale et al., 2009). Also, HBc, HBeAg and HBSP were able to downregulate MxA protein expression, an important antiviral kinase, via interaction with the MxA promoter, in Huh7 cells (Rosmorduc et al., 1999; Soussan et al., 2000b).

In addition to their downstream pathways, TLRs themselves are found broadly modulated in CHB patients (Visvanathan et al., 2007b) especially TLR2. As HBV only replicates within hepatocytes, modulations of other cells (i.e NPCs) by the virus has often been understudied. However secreted HBV proteins (HBsAg and HBeAg) have been successfully found internalized within NPCs (Zannetti et al., 2016), modulating their immune response as well.

Modulation of innate immune cell secretions and functions

Previously mentioned studies were conducted on different animals (Wieland et al., 2004; J. Wu et al., 2009), and patients biopsies (Z. Chen et al., 2008; Lebossé et al., 2017; Visvanathan et al., 2007b) with an overall modulation of the immune response. Some experiments were also conducted using PBMCs from infected patients, which showed **especially impaired stimulation of TLR1-2-4-6 transcripts** (Z. Chen et al., 2008; Müller & Zielinski, 1990), in some cases correlating with serum HBsAg levels. Some evidences even suggests a putative genotype-dependent modulation with HBV-C mediated TLR2 decrease (Z. Chen et al., 2008). TLR2 and

2013b). More precisely, HBeAg+ patients biopsies were correlated with downregulation of TLR2 associated with reduced TNF α and IL-6 secretion by PBMCs (Visvanathan et al., 2007b). In PBMCs and Monocytes/M Φ from infected patients, TLR2 downstream pathways is impaired by HBsAg, which inhibits c-Jun N-terminal protein Kinase (JNK), resulting in a default in IL-12 secretion (S. Wang et al., 2013b) (**Figure 21.B**). Evidences from the same study suggest a similar inhibition for the TLR4 pathway resulting in a decrease of IL-12, IL-6 and TNF- α secretions, however only immortalized cells were used and more robust data are needed (**Figure 21.B**). TLR9 stimulation of pDCs seemed also impaired in presence of HBV (Martinet et al., 2012) (**Figure 21.C**). Data from our lab unraveled that HBsAg was actually capable to coat synthetic DNA, (such as CpG OGN, the TLR9-agonist used), thereby diminishing their available quantity for an appropriate TLR9 stimulation in pDCs and subsequent IFN- α production, questioning the *in vivo* relevance of the previous experiments (Aillot et al., 2018). Of note, HBsAg seems also capable of inducing IFN- α inhibition independently of this mechanism (Ludovic Aillot' thesis, unpublished data).

Inhibitions of these pathways are accompanied with **changes in immune cells phenotype**, especially towards anti-inflammatory secretions. In NPCs exposed to HBV, a global increase of TGF- β and IL-10 secretion levels are observed (M. Jiang et al., 2014; H. Li, Zheng, et al., 2012), especially correlated with the M Φ compartment, as seen with secretions of monocytes from CHB patients (H. Li et al., 2018) and Hepatic M Φ (H. Li, Zheng, et al., 2012) (**Figure 21.D**). In murine KCs and LSECs, HBsAg incubation led to a decrease in NF κ B, IRF3 and MAPK pathways, inhibiting IFN production, in favor of IL-10 secretion (M. Jiang et al., 2014), suggesting a role for the surface protein in these modulations (**Figure 21.D**). IL-1 β has been described as a key cytokine against HBV infection, more efficient than IFN- α (Isorce et al., 2016). Its successful secretion requires NF κ B activation, which has already been discussed as shifted by HBV, and the activation of at least one of the inflammasome complex (Broz & Dixit, 2016). However, inhibition of both AIM2 and NLRP3 inflammasome is described, mediated by HBsAg and HBeAg, respectively (X. Yu et al., 2017; Zannetti et al., 2016) (**Figure 21.E**). HBeAg is binding to Mal and TRAM to decrease NF κ B pathway, as previously discussed, and decrease ROS production (X. Yu et al., 2017), while HBsAg destabilize IRF7 mRNA (Zannetti et al., 2016). It is worth noticing that while both phenotypes were observed in *ex-vivo* extracted human KCs, mechanistic insights are coming from THP-1 treated cells using recombinant HBV proteins. In a mice model of HBV hydrodynamic injection, mice KCs and TLR2 were found crucial for viral establishment and recombinant HBcAg led to IL-10 secretion on *ex-vivo* incubated mice KCs, suggesting a putative role for the capsid protein as well (M. Li et al., 2015) (**Figure 21.F**). Increased secretion of IL-10 was recapitulated in double humanized mice (Dusséaux et al., 2017). Interestingly, in the same model, increase of overall M Φ compartment (CD68+) was observed, attributed mostly with a recruitment/polarization into "M2-like" (CD206+) M Φ (Bility et al., 2014). While M Φ recruitment is not always recapitulated in CHB patients, these experiments highly suggest that the virus is, indeed, capable of modulating M Φ phenotype toward anti-inflammatory cytokine secretion which seems beneficial for its establishment.

DCs, which are the professional IFN secreting cells, are impacted by HBV as well. More specifically, cDC1 and pDCs functions are decreased by HBsAg (van der Aa et al., 2016; Vincent et al., 2011a), leading to **reduction of Type III IFN (cDC1) (Figure 21.G)** and **Type I-III (pDCs) secretions (Figure 21.C)**. Besides, their **antigen-presenting functions are decreased** as well, as seen with low DC-SIGN expression in circulating DCs from CHB patients, which could not be rescued through *ex-vivo* TLR stimulation (Sukriti et al. 2016) (**Figure 21.H**), and pDCs seems less capable of activating NK cells (Martinet et al., 2012). Of note, even if DCs have highly potent

antiviral function, the human DCs hepatic compartment is not often studied due to its low concentration and the intrinsic difficulties it rises (i.e scarcity of cells after liver extraction), thus most experiments are conducted on circulating DCs and may differs from what is happening in the liver.

Finally, NK cells, which plays a major role in resolution of acute infection, are impaired in HBV infection, as early as during the first peak, due to IL-10 and TGF- β secretion in the liver (Dunn et al., 2009b; Sandalova et al., 2012; Stelma et al., 2017). This **tolerogenic environment reduces the capacities of NK** cells to produce IFN- γ and TNF- α (J.-F. Li et al., 2015; Peppia et al., 2010), partly due to miR146a overexpression which modulates STAT1 (J.-F. Li et al., 2015). Of note, miR146a expression is dependent of the cytokinic milieu and its downregulation by IL-12, IFN- α and IFN- β reverse the aforementioned phenotype. Besides, in HCC-CHB patients, liver infiltrating NK cells, mainly of the CD11b⁻CD27⁻ subset, are of an inactive/immature phenotype (Q.-F. Zhang et al., 2017). They secrete less IL-12, IL-18 and t-bet, and more IL-10 and TGF- β , further establishing the tolerogenic milieu (H. Li et al., 2018). Regarding their cytolytic capacities, there are conflicting results. Some are pointing towards an exacerbation of cytolytic functions of NK, with Fas/TRAIL overexpression, leading to the previously discussed immunopathogenesis (Maini & Peppia, 2013). Others suggest that, as well as their non-cytolytic capacities (i.e IFN- γ production), their cytolytic mechanisms are dampened by the infection through various mechanisms, amongst which: (i) miR146a decrease of STAT1 (J.-F. Li et al., 2015), (ii) exosome delivery of HBV genome which dampen RIG-I expression and inactivates NF κ B and p38 pathways (Y. Yang et al., 2017) and (iii) TGF- β 1 mediated decrease of NKG2D/DAP10, 2B4/SAP (receptor activator) on NK cells (C. Sun et al., 2012, p.). As most studies supporting the latter hypothesis were conducted with Immune-Tolerant patient samples, it is possible that NK cells have sequential inhibition during CHB, with all functions inhibited during IT phase, and cytolytic functions activated during immune active phases.

Finally, MAIT population in HDV infected patients, but not HBV mono-infected ones, were found dramatically decreased (Dias et al., 2019). As MAIT contribute to tissue remodeling and M2 differentiation process, amongst other functions, it is possible that the general inhibitory phenotype discussed above is different in co-infected patients.

Induction of a suppressive phenotype: role of inhibitory receptors

In addition to the overall tolerogenic state induced in CHB patients livers by anti-inflammatory cytokines and broad impairment of PRRs pathways, HBV infection is associated with an increase in suppressive functions, exemplified with a **broad overexpression of inhibitory receptors (IRs) and their ligands (IR-Ls)**(PD-1, Lag3, 2B4, CD160, Tim-3) (D. Cao et al., 2013; Ju et al., 2010; H. Li, Wu, et al., 2012a; H. Li et al., 2018; Nebbia et al., 2012; Y. Tian et al., 2016) (**Figure 21.I**). IRs everyday purposes are to act as safe guards from over-activation and development of auto-immune diseases, in charge of return to homeostasis when immunity gets carried away (further discussed in Part I). However, they are broadly modulated by viruses towards evasion, and HBV is not an exception (Ong et al., 2016). Their overexpression and role in exhausted CD8⁺T cell is well-characterized, however, an accumulating body of evidence suggests that innate immune cells as well are impacted by these receptors, putatively towards their own suppression, or its overall establishment in the liver. Tim-3 is overexpressed on NK from CHB patients (Ju et al., 2010) and its most well-characterized ligand, galectin-9, on KCs (H. Li, Wu, et al., 2012a; Nebbia et al., 2012), while PD-1 is expressed on NK, CD16⁺ monocytes and KCs, with its ligand PD-L1 ubiquitously found in infected liver biopsies (D. Cao et al., 2013; L. Chen et al., 2007). In monocytes, PD-L1 expression is mostly associated with HBsAg, as seen using surface protein

purified from patient's plasma (H. Li et al., 2018). Interestingly, in mice model of mother-to-child transmission mentioned earlier, the increase of PD-L1 on anti-inflammatory M Φ was associated with HBeAg expression rather than HBsAg, and resulted in sustained HBV viremia (Y. Tian et al., 2016).

Discovered at first in mice (Kong et al., 2014) and validated later in CHB patients (A. Huang et al., 2014; Pallett et al., 2015b), the **recruitment of MDSC** in infected liver was positively correlated with viral load in absence of necro-inflammation. These suppressive cells are thought to participate greatly in the overall suppressive phenotype establishment (**Figure 21.J**). Of note, Pallett and colleagues observed an increase of **PMN-MDSCs** when Huang, Zhang, Yang and Pal described elevation of the **M-MDSCs** subset. These contradictory results could come from discrepancies of HBV genotype used and the lack of established markers and protocols for MDSCs identification and purification, respectively (Bronte et al., 2016). Besides, it is not clear whether HBsAg (Fang et al., 2015; Pal et al., 2019a; W. Zhang et al., 2019) or HBeAg (F. Yang et al., 2019) is responsible for the specific increase of MDSC in infected liver.

Both cell types are producing massive amount of IL-10 and inhibitory receptors' ligands (A. Huang et al., 2014; Kong et al., 2014; F. Yang et al., 2019; W. Zhang et al., 2019) (**Figure 21.I-D**). In addition, they are major secretors of arginase, an enzyme mediating L-Arginine deprivation, generating metabolic modulations in the hepatic environment which (i) could favor anti-inflammatory phenotype of immune cells (Galván-Peña & O'Neill, 2014) and (ii) induces CD8+T cell exhaustion (A. Huang et al., 2014; Pallett et al., 2015b; F. Yang et al., 2019) (**Figure 21.K**). Often recruited upon constant pro-inflammatory secretion, it is possible that MDSCs could, at least partly, be responsible for the establishment of immune tolerance following acute hepatitis phases of the disease. However, the exact mechanism of MDSCs induced differentiation and molecular partner(s) of the virus are not well characterized and further studies are required in this matter.

Altogether, the innate immune compartment seems broadly impaired, displaying tolerogenic functions, which correlates with the absence of viral clearance in CHB patients. In addition to the dampening of innate immunity, this immunosuppression has direct impact on the establishment of immunotolerance of the adaptive system.

ii. Inhibition of the adaptive compartment

Most studies in HBV patients have focused on the adaptive immune compartment, as it was presented of high importance for viral clearance (Ferrari, 2015). The liver environment, characterized by massive secretion of anti-inflammatory cytokines IL-10 and TGF- β , favors the recruitment of CD4+T cells and their polarization toward a regulatory phenotype (Ferrari, 2015). **Treg** are overexpressing CTLA-4, which competes with CD80/CD86 binding on CPA and leads to decreased presentation; and other inhibitory receptors (similar to the ones discussed in the previous part on inhibitory receptor in the innate immune compartment), which participate in the establishment of the immunosuppressive environment. HBV and HDV infections are both associated with increased T helper infiltrates and subsequent IL-10 production (Grabowski et al., 2011). In addition to the constant exposure to HBV particles, it leads to **CD8+T cell exhaustion** (Wherry & Kurachi, 2015; B. Ye et al., 2015). Broadly observed in CHB patients, this exhaustion is characterized by decreased antiviral functions (TNF α and IFN γ secretion), overexpression of inhibitory molecules (PD-1, Lag3, 2B4, CD160, Tim-3) loss of

proliferation (IL-2 production) and even deletion of the subset (increase of TRAIL-R) (B. Ye et al., 2015). In patients undergoing CHD, the CD57 senescence marker was especially upregulated on CD8+T cells, suggesting a similar phenotype between HBV and HBV-HDV infections (Schirdewahn et al., 2017). Interestingly, IL-12 supplementation rather than PD-1 blockade was able to restore lymphocyte compartment (Grabowski et al. 2011).

Finally, antibody production and secretion is also impaired in CHB patients. **B cells** infiltrates are mostly **immature** and produce IL-10 over HBV-specific antibodies (Das et al. 2012).

Even when humoral response is not fully dysfunctional, HBV can evade recognition by (i) the rise of escape mutant of HBV and HDV (Kim et al. 2016; Kefalakes et al. 2019) and (ii) the excess of secretion (10^4 to 10^5 excess compared to infectious particle) of HBV SVPs, which serves as decoy (Ferrari 2015).

IV. How can we eradicate HBV and HDV infection worldwide?

1. Prevention

As HDV is a defective HBV satellite virus, all strategies aiming at preventing HBV also restrain HDV.

The first-line prevention is **vaccination**.

HBV vaccine, designed by Maurice Hillman's team and approved by the FDA in 1981 (Buynak et al. 1976), was primarily made from plasma-derived HBsAg (i.e. purified spheres). Later on, to cope with the low availability of infected plasma, the same team generated the first vaccine using recombinant HBsAg, expressed in yeast (Hilleman 1987). Finally, a third-generation vaccine arose in the early 90s using mammalian cells (transfected Chinese Hamster Ovary – CHO – cells) and consisting of S/PreS1 and PreS2 HBsAg, whereas previous ones only contained S protein (Soulié et al. 1991). More recently, chimeric HBV-HCV subviral particles were designed and successfully elicited a specific antibody response in animal models (rabbit), for both viruses (Beaumont et al. 2013), especially when mixing different HCV genotypes (Beaumont et al. 2020).

Vaccination, which is safe and efficient, has enabled to pass from 8-15% chronically infected children in endemic areas to less than 1% in vaccinated children. The WHO recommends that all infants must be vaccinated, as early as 24 hours of age, followed by 2 to 3 other doses in the first semester ("Hepatitis B Vaccines: WHO Position Paper—Recommendations" 2010). Besides, to **reduce mother-to-child transmission**, pregnant women diagnosed with HBV must be treated, at least in the third semester, to reduce viral loads (provide better efficiency to the vaccination strategy); and vaccine can be accompanied by HBV immunoglobulin injection in newborns (M. H. Nguyen et al. 2020).

HBV/HDV prevalence is thus controlled overall due to immunization strategies, performed mostly in developed countries. However, in endemic areas that are often low-income countries, vaccination is still low and thus both viruses remain a plague (Mario Rizzetto 2016). Thereby, there is also a **need for education** (of both endemic general population and medical staff), for proper sterilization/use of needles and syringes, and a mandatory screening of blood bag for patient transfusion. It is worth noticing that, as for most pandemics, one of the major drawbacks remains unbalanced country incomes.

2. Current Therapeutics

a. Nucleos(t)ides Analogues: first-line treatment for HBV

According to the latest EASL guidelines, and most other international viral-hepatitis related agency, **NA** are the first-line treatment for CHB patients (European Association for the Study of

the Liver. Electronic address: easloffice@easloffice.eu and European Association for the Study of the Liver 2017) (Figure 22.A).

NAs, by mimicking natural nucleos(t)ides, inhibits HBV polymerase by incorporating in newly synthesized HBV DNA/RNAs, causing chain termination. As it is not directly targeted by NAs, cccDNA amounts are decreasing very slowly following treatment (up to decades), and integrated DNAs are not affected. Besides, as HDV relies on host polymerase and is able to survive only using integrated HBV, NA are ineffective (Mario Rizzetto 2016).

NAs, given orally, lead to functional cure (defined with the loss of HBsAg) in less than 1% patients and should therefore be taken life-long (Durantel and Zoulim 2016). Therapy discontinuation is sometimes authorized in patients which have undergone seroconversion or reached undetectable HBV DNA levels, but to observe a long-lasting response, 4 years at least are recommended, and a 7 years period lower the relapse rates to less than 30% (M. H. Nguyen et al. 2020).

To date, several NAs have been authorized by the FDA, Tenofovir Alafenamide (TAF), Entecavir (ETV), Tenofovir Disoproxil Fumarate (TDF), Lamivudine (LAM), Adefovir dipivoxil (ADF), Telbivudine (TBV). Along years of treatment, poor side effects have been described (contrary to IFN α), however, major concerns on NAs use are escape mutant selection (M. H. Nguyen et al. 2020). To this end, EASL favors the use of TDF, TAF and ETV, which have the lowest rate of resistance mutation (TAF and TDF patients show no mutation after 8 years of treatment), contrary to the previously used LAM and ADF. Indeed, treatment with the latter lead to a specific rise of A181T mutation (amongst other resistant variants), associated with cytoplasmic accumulation of a truncated form of HBsAg, inducing activation of the cRAF/MAPK pathway, leading to carcinogenesis (M.-L. Wang and Tang 2016). Of note, these variants confer resistance to LAM/ADF, but also other NAs.

b. Immunotherapy: the case of IFN- α

Alternatively, injection of **pegIFN- α** is also approved for the treatment of chronic HBV and HDV infection (Figure 22.B).

As a major actor in antiviral response, IFN α efficiency against HBV have been evidenced since 1976 (Greenberg et al. 1976), and accepted for CHB and CHD treatment in the nineties. However, the exact mechanism(s) of IFN α effects on HBV/HDV is still not fully understood. Several have been suggested, with direct action on infected hepatocytes as well as activation of the immune response (non-exhaustive list, reviewed recently (Tan et al. 2018)).

Firstly given as conventional IFN α , it was later pegylated to increase its half-life and thereby lead to less frequent dosing and a more sustained viral inhibition (Tan et al. 2018). However, its use has been lowered in patients as it was still associated with strong side effects and poor efficiency (HBV suppressed in only 5-7% patients (Fanning et al. 2019)). A recent study associated the lack of pegIFN α effects with a limited NK cell activation, induced by a strong clearance of the pegylated complexes by KCs (Nishio et al. 2021).

Nowadays, it can be considered as an initial treatment option only for patients with mild-to-moderate HBeAg-positivity, or which are HBeAg negative already (European Association for the Study of the Liver. Electronic address: easloffice@easloffice.eu and European Association for the Study of the Liver 2017). Its efficiency is highly correlated with patient's immune status (especially active phases of hepatitis, i.e increased ALT), HBV genotype, and HBeAg status.

Unfortunately, it is, to date, the gold standard (only drug) for CHD patients. Its use is highly suboptimal as only 25% patients are responders and more than 50% of them relapse after treatment discontinuation (Mario Rizzetto 2016). Before 2020, no treatment options were available for IFN non-responders.

c. Entry inhibitor, the bulevirtide

Bulevirtide (Hepcludex) is a synthesized myristoylated derivative of PreS1 peptide of L-HBsAg (Kang and Syed 2020) (Figure 22.C). The peptide is competing with the Dane particle binding for entry, as it irreversibly blocks NTCP receptor.

Subcutaneous injection of Bulevirtide has been extensively tested, first in clinical trial for HBV, but also HDV infected patients with encouraging results. Excellent safety was reported in phase II clinical trial associated with a reduction in HDV circulating levels (Loglio et al. 2019). However, combination with pegIFN α or NAs is under investigation as monotherapy does not enable HBsAg decrease and have no effect on established cccDNA.

Facing the lack of appropriate drugs to deal with CHD patients and the absence of strong side effects, the European Medical Agency (UE) and Food and Drug Administration (USA) approved its use in 2020 for the treatment of CHD patients with compensated liver disease.

d. Others

Patients with decompensated cirrhosis must undergo **liver transplantation**. They are treated with NAs and HBV Immunoglobulin prior to the operation to achieve undetectable serum HBV DNA and thus reduce the risk of re-infection. NAs are given post-operation as well, with or without HBV immunoglobulin injections, to prevent re-infection (European Association for the Study of the Liver. Electronic address: easloffice@easloffice.eu and European Association for the Study of the Liver 2017). The pipeline for HBV/HDV co-infected patients is the same, as decreasing circulating HBV particles ensures the inhibition of HDV propagation as well.

Altogether, current treatments are not sufficient to achieve HBV/HDV functional cure in most patients, and are coming with either strong side effects, or are given life-long. Novel antiviral, permitting efficient HBsAg loss, low side effects, and given in a limited time course, are the current goal for viral hepatitis cure.

3. New therapeutics in development

a. Novel antiviral drugs targeting the infection in hepatocytes

Now that we achieved control of HBV infection with NAs-dependent decrease of viremia in CHB patient, efforts are made towards total viral eradication and HDV clearance. Different strategies have been developed: (1) inhibits viral replication or secretion, (2) blocking cccDNA formation or/and promoting its degradation (or at least silence it) to avoid re-infection after therapy discontinuation.

i. HBsAg release inhibitors, the Nucleic Acid Polymers (NAPs)

NAPs are a large class of antiviral polymers, amongst which can be found attachment or entry factors inhibitors. NAPs belong to the class of phosphorothioate oligonucleotides (PS-ONs) which can be safely given to human where they naturally accumulate in the liver and enter hepatocytes. In HBV infected cells, NAPs are very efficiently inhibiting HBsAg assembly and egress through a yet unknown mechanism(s) (Figure 22.D). As only HBsAg levels (SVPs) decreases, and also HDV RNA in coinfection settings, without touching viral DNA, it is suggested that NAPs could act by modulating HBsAg trafficking within the Golgi apparatus (Vaillant 2016). **REP2139** is the most promising NAP. It has been tested in HBV/HDV coinfecting patient in combination with pegIFN α with promising results. HBsAg loss was observed in 60% patients and 56% were anti-HBsAg positive. The lack of HBsAg in patient serum enabled a return of the immune control beneficial for HBV clearance (Vaillant 2019). Out of the 12 patients tested, 11 became seronegative for HDV, and 7 remain that way after treatment discontinuation.

A recent study using NAs and REP2139 in the Pekin Duck infected with DHBV and HDV suggested that this combination could also be highly efficient (Quinet et al. 2018). Thus, NAPs combination could brought a very interesting alternative for CHD patients.

ii. Farnesylation inhibitor for HDV: Lonafarnib

As mentioned earlier, farnesylation of the L-HDAg Cys211 residue is crucial for appropriate recruitment of HBsAg and thus HDV secretion. Fortunately, farnesylation inhibitor were designed in the beginning of the century as anti-tumoral drug and already tested in phase I to III clinical trials (Morgillo and Lee 2006). Even if they were not efficient enough to be implemented in cancer therapy, their use on CHD patients was found successful with the first proof of concept in a phase 2a clinical trial in 2015 (C. Koh et al. 2015) (Figure 22.E). They presented a dose-dependent decrease of HDV RNA serum levels. While being highly promising, high doses are required to obtain sufficient HDV decreases, and such concentration comes with strong side effect in patients (Yurdaydin et al. 2018). Future clinical trials are thus focusing on combination with already known therapeutics (NAs, IFN α) to decrease Lonafarnib concentration without impacting efficiency (Asselah et al. 2020).

The following treatments are developed mainly against HBV. However, inhibiting HBV, and thus HBsAg secretion, could have similar impact as NAPs on HDV propagation. Hence, even if no clinical trials are existing in CHD patients for these drugs, they are clear areas of interest in both fields.

iii. Inhibition of HBV RNA

siRNA is one of the most widely used biochemical techniques *in vitro* to silence RNAs (Figure 22.F). Even if its administration in humans has been for long an issue, nowadays, liver-targeted subcutaneous injections are efficiently delivering metabolically stable siRNAs (M. H. Nguyen et al. 2020). Arrowheads developed and tested the first siRNA in clinical trials, the ARC520, designed to reduce all RNA transcripts from cccDNA (Man-Fung Yuen et al. 2019). Clinical evaluation showed good reduction of viral parameters in general, low side effects, but only a

moderate reduction of HBsAg, probably due to integrated HBV DNA mediated HBsAg production. Thereby, it prompted the design of a second-generation siRNA, encompassing the full HBV genome this time (integrated or not). Lately, a third generation siRNA clinical trial on 40 infected patients led to a drastic decrease of all viral parameters, and a mean reduction in HBsAg of 2 logs (88% patients had <100 UI/mL by the end of the three-month treatment), without harmful side effects. A phase II clinical trial is ongoing to establish the effects of the HBV RNAi JNJ-3989 (developed by Arrowheads and J&J) in HBV/HDV co-infected patients.

RNA destabilizers, such as RG7834, are also capable of decreasing HBV viremia in a specific manner *in vitro*, and, interestingly, also HBsAg, in mice models (Fanning et al. 2019) (Figure 22.F). However, clinical trials were inconclusive.

iv. HBV- Capsid Assembly modulators (CAMs)

CAMs are targeting HBV core protein, leading to blockade of Dane particle secretion and/or formation (Figure 22.G). Besides, it also impacts cccDNA maintenance by avoiding recycling and thus cccDNA primo-formation from rcDNA (M. H. Nguyen et al. 2020).

CAMs are divided into two categories, depending on their effect on HBV capsid (Mak et al. 2017):

- Formation of **aberrant nucleocapsid** at low doses, and capsid degradation when used at increased dose.
- Acceleration of **capsid** assembly leading to normal nucleocapsid formation but **without pgRNA**.

First clinical study on CAMs presented a 1 to 2 log reduction in HBV DNA, RNA and HBeAg levels using NVR3-778 in HBeAg positive patients, with no changes in HBsAg (Mak et al. 2017). Many more potent CAM are currently investigated in pre-clinical studies and combination strategies are favored (M. H. Nguyen et al. 2020). Of note, CAM resistance has already been demonstrated by the increase of signature mutation in the capsid pocket region (T128I, T33N) but can be prevented by combination with NAs (polymorphisms susceptible to antiviral therapy) (M. H. Nguyen et al. 2020).

v. Inhibition of the established pool of cccDNA

Several strategies can be used to degrade, decrease or just silence HBV cccDNA (Figure 22.H). Here are some of the new drugs/antiviral related strategies in development, even if far from clinical trials, to date:

- **Epigenetic modulators** such as chromatin-modifying enzymes (Levrero et al. 2009) that will permit cccDNA transcriptional repression/silencing. These strategies are however not favored as off-target effects on host genes are most probable.
- **Degradation of cccDNA** pool through Zinc-finger nucleases and CRISPR/Cas9 system. No clinical trials has been launched for now, due to the unknown side effects of this technology, even if deep and rapid reduction is observed *in vitro* (Ramanan et al. 2015, 9).

Altogether, there is a large number of new antivirals that will be tested or passing through clinical trials in the next few years. However, as it is promptly suggested in the last EASL report, antiviral drugs, such as the ones discussed above, will often not be sufficient enough to clear HBV and HDV from their host. Indeed, reactivation of a correct immune response mediated by immunomodulatory agents and leading to unblocking the established immunosuppression will

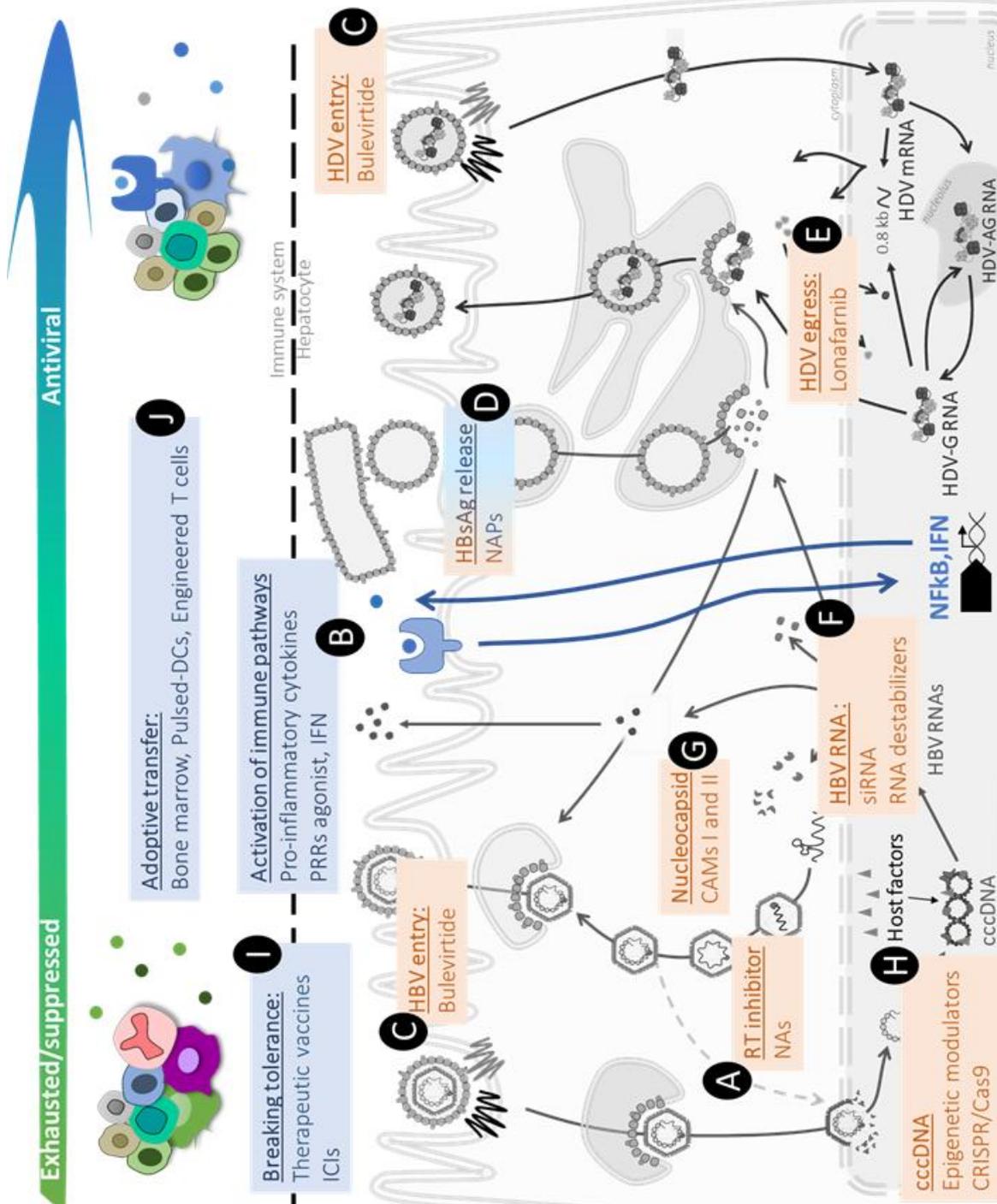


Figure 22: current and novel therapeutic options for HBV-HDV

be needed. This area is highly challenging as both diseases come with immune active phases ensuring strong immunopathogenesis. Thus, it is more a question of re-educating the immune response towards an appropriate targeting and clearance of the viruses, than only a question of activation. Unfortunately, no immune-mediated drugs have entered clinical trials for HDV, to date.

b. Immunomodulatory treatment towards viral clearance

As explained, treatments for CHB must take in account the immune context, i.e the high tolerance induced by the virus and the subsequent exhausted/suppressed immune status. Strategies developed towards reversion of such phenomenon are the following: (i) the use of cytokines, IFNs and PRRs agonists to activate immune pathways; (ii) immune checkpoint inhibitors to reverse the immune-suppressed phenotype, (iii) therapeutic vaccine to boost T cells and even (iv) replace the suppressed cell through adoptive transfer.

i. Direct activators of immune pathways: cytokines, IFNs and PRRs agonists

A major area of HBV drug design is the induction of **pro-inflammatory cytokines** (namely IL-6, TNF α , IL-1 β) and/or **IFNs** (not only IFN α , but also IFN β) by immune cells (Dusheiko 2020) (**Figure 22.B**). A large screening identified IL-6 and, most importantly, IL-1 β , has being peculiarly potent at inhibiting HBV replication, even more than IFN- α (Isorce et al. 2016). The main advantage of such molecule is their dual action as they can both impair HBV replication in infected cells as well as reactivating the immune compartment. However, it is currently impossible/poorly advised to inject systematically such molecules. It would lead to a systemic immune over-activation, which may end in a septic shock caused by the cytokinetic storm.

As they are secreted naturally by immune cells in response to pathogen sensing, another strategy would be to artificially activate such immune sensors towards their secretion. Indeed, the efficiency of diverse immune receptors agonists on HBV infection has been largely documented in the past few years, amongst which **agonists of TLRs** (Julie Lucifora et al. 2018), **Lymphotoxin- β receptor** (Julie Lucifora et al. 2014), **STING and RIG-I** (M. H. Nguyen et al. 2020). **TLR7 and 8 agonists** were of the first to enter clinical trials in the past few years (E. J. Gane et al. 2015; Janssen et al. 2018; Boni et al. 2018, 7). TLR7 is expressed on myeloid and lymphoid cells, thus its agonisation permits only indirect clearance of HBV through general immune activation (Bertoletti and Le Bert 2019, 7; Boni et al. 2018, 7). In animal models, a loss of both HBV DNA and HBsAg was observed, even accompanied with HCC rate decrease. Unfortunately, clinical trials using TLR7 agonists, did not lead to a meaningful loss of HBsAg (Janssen et al. 2018). Interestingly, this has been correlated with a lack of immune modulation in patients, rather than antiviral potency (Bertoletti and Le Bert 2019). As TLR8 is expressed on myeloid cells, its agonisation is highly potent in activating pro-inflammatory cytokine secretion, leading to subsequent inhibition of HBV as seen *in vitro* (Embrechts et al. 2018, 8; Mackman et al. 2020) and *in vivo* in the woodchuck models (Mackman et al. 2020, 9688). Selgantolimod (TLR8 agonist developed by Gilead) is currently in phase II clinical trial with first reports suggesting a safe and

efficient activation of immune cells in 48 CHB patients; however, similar to TLR7 agonists, with only poor impact on HBV infection (Gilead report). SB-9200 (inargivir), a **RIG-I agonists**, which was highly potent in mammalian models and resulted in an IFN-mediated antiviral immune response (Korolowicz et al. 2016) entered clinical trial. First reports suggested a low toxicity profile associated with a reduction of HBV parameters (HBV DNA and RNA) in a cohort of 40 CHB patients (M.-F. Yuen et al. 2018). However, the first death was recently reported and subsequently ended the clinical trial. **Thymosin α 1** ($T\alpha 1$), a 28 amino acids long peptide bearing immunomodulatory functions is suggested to acts via TLR2 and TLR9 in myeloid cells and pDCs, leading to IFN- γ production and IL-2 mediated T cell activation. While $T\alpha 1$ treatment alone led to fewer side effects than IFN- α , combination with ETV in CHB patients yielded similar results than ETV monotherapy, with only a higher tendency for HCC inhibition (Xiaoning Wu et al. 2018). Finally, even if poorly evaluated in CHB patients to date, immunomodulators aiming at switching M Φ phenotypes from M2-like to M1-like are already in advanced clinical trials in cancer patients (amongst which HCC) (Degroote et al. 2018; Z. Tian et al. 2019).

Of note, **metabolic modulations** of the immune system is the current trend in cancer and autoimmune diseases therapeutics options (C. H. Patel et al. 2019). In the past decades, many compounds were developed, highly interesting in regard to the re-shaping of an appropriate T cells and/or myeloid compartment. A lot are currently undergoing advanced clinical trials for these diseases. Thus, it is my profound opinion that such metabolite should be tested in CHB/CHD patients. To date, the sole metabolite in advanced clinical trial is the Farnesoid X Receptor(FXR) agonist EYP001, currently in phase I for CHB patients (Erken et al. 2018). FXR is indeed an highly interesting target as it was described as a proviral factor for HBV replication, and is a well-known anti-inflammatory inducers (NF κ B inhibition) (Fiorucci et al. 2018). Recently Schmidt and colleagues demonstrated the potential of Acyl-CoA:cholesterol acyltransferase (ACAT) inhibitors has an antiviral and anticancer therapeutic for the management of CHB patients (Schmidt et al. 2021). This compound was able to reduce both HBV parameters and enhance T cell responsiveness to PD-1 blockade or engineered T cell therapy.

ii. Immune checkpoint inhibitors

Immune checkpoint, are inhibitory receptors (IRs) highly expressed on exhausted/regulatory lymphocytes (Wherry and Kurachi 2015). IRs and their ligands (IR-Ls), which are expressed on granulocytes, neutrophils, cancer and myeloid cells, participate largely in the immune inhibition/tolerance observed in cancer (Darvin et al. 2018) and some viral infection (P. Gao et al. 2019; E. Gane et al. 2019; Nebbia et al. 2012, 3; Schönrich and Raftery 2019, 1). In cancer therapy, **immune checkpoint inhibitors** (ICIs) are highly efficient, however, they are less potent in viral setting where, even if beneficial effect have been observed, it is often transient (Pauken et al. 2016) and may lead to viral reactivation and/or over activation of pro-inflammatory mechanisms and therefore cytotoxicity (Lombardi and Mondelli 2019). So far, only nivolumab (PD-1 neutralizing molecule) was tested in CHB patients, with unsatisfying results (**Figure 22.I**). While the drug was considered safe (El-Khoueiry et al. 2017), it does not led to a significant decrease of HBV parameters and an appropriate control cohort is currently lacking (E. Gane et al. 2019). One of the main reason of viral persistence following ICI treatment is the lack of direct antiviral effect and thus maintenance of viral particles and protein in the infected hepatocytes, but also blood circulation (Nebbia et al. 2012, 3; Pauken et al. 2016, 1; Shayan et al. 2017).

Indeed, as HBsAg and HBeAg bear immunomodulatory activities that are involved in the observed immunosuppression (Fang et al. 2015; F. Yang et al. 2019; Zannetti et al. 2016), it is essential to dampen their expression before aiming at reactivating the immune compartment.

iii. Therapeutic vaccination

Therapeutic vaccine is a novel strategy to boost the immune system (Figure 22.J). Contrary to the preventive vaccine, its purpose is not solely to avoid the infection, but to boost the effective CD4/CD8+T cell response against HBV (Hoogeveen and Boonstra 2020). However, clinical trials, performed mostly in immunotolerant CHB patients, are disappointing for both HBsAg (Dikici, Bosnak, et al. 2003; Dikici, Kalayci, et al. 2003; Yalcin et al. 2003; Fontaine et al. 2015), HBcAg (Heathcote et al. 1999) alone or in combination (Lok et al. 2016). Even if broadly well-tolerated, and often accompanied with an efficient mounting of the CTLs response, vaccine therapy did not lead to efficient HBV decrease. This failure has been mostly attributed to the establishment of tolerance by viral proteins and the subsequent lack of immune recruitment/response. Thus, other strategies, validated to date only in murine models, encompass the designing of DNA vaccines encoding HBsAg and HBcAg, the use of viral vectors as a vaccine platforms, specific adjuvant and/or combination with direct antivirals (Meng, Chen, and Lu 2020). First, in Tg mice, addition of a saponin-based **adjuvant** (ISCOMATRIX) to a therapeutic vaccine based on recombinant protein (HBsAg and HBcAg) was found successful in inducing HBsAg antibody production (Buchmann et al. 2013). In the same model, a **Modified Vaccinia virus Ankara** (MVA) expressing HBsAg from different subtypes (ayw/adw) and HBcAg, in combination with an efficient adjuvant (PCEP), managed to break CD8+T cell tolerance (Backes et al. 2016). In the wudchucks model, **Adenovirus DNA prime-boost** ensured efficient WHsAg antibody production, leading to sufficient immune response to eliminate the WHV (Dembek, Protzer, and Roggendorf 2018). However, as it is not always sufficient to clear HBV in mice models (Tg and AAV-HBV injected mice), **combination with siRNA** targeting HBV was successfully tested. It led to sufficient reactivation of the immune repertoire, to induce HBV clearance, which was not observed in the group treated only with siRNA (Michler et al. 2020). Finally, even if only minor decrease in HBsAg levels were observed, an adenovirus based vaccine expressing a **fusion protein** between HBV-Pol, Hbc and HBsAg (TG1050) successfully passed phase 1 clinical trial (Zoulim et al. 2020). The vaccine was able to efficiently reduce HBcAg levels, as well as inducing HBV-specific immune response, especially when combined with **NA** (Zoulim et al. 2020).

iv. Immune cell transfer

New strategies aim at **transferring activated immune cells** and/or direct **ex-vivo reactivation of the deficient immune cells**. First, **bone-marrow** transfer from donor with resolved HBV infection permitted efficient immune control in CHB patients, as seen with the development of a specific B and T cell memory and HBsAg negativity (Ilan et al. 2000). Also, directly incubating HBsAg on *ex-vivo* purified mDCs have been tested in mice (Farg et al. 2012) and CHB patients (Akbar et al. 2011) (Figure 22.J). mDCs from CHB patients were incubated for 8 hours with the viral protein before being re-injected. These **HBsAg-pulse DCs** were well-tolerated and induced an HBV-specific immune response in 3 out of the 5 patients, suggesting the putative efficiency of such a strategy and the need for validation in a larger cohort. However, in both human and mice, this DC transfert (considered also as a therapeutic vaccination) was not sufficient to decrease viral parameters.

More specifically, as T cell response is low and mainly exhausted in CHB patients, adoptive transfer of **engineered T cells** have been designed (Meng, Chen, and Lu 2020). The first proof of concept of this strategy was performed in 2011 when Gehring and colleagues isolated CD8+T cells from HBV infected patients and transduced them with retrovirus coding for a TCR against HBc or HBsAg (Gehring et al. 2011). Co-culture of HCC lines expressing HBV with these engineered T cells led to efficient lysis of infected cells and subsequent HBV clearance (Gehring et al. 2011). Thus, *ex-vivo* isolated uPA-SCID mice CD8+T cells were modified accordingly (Wisskirchen et al., n.d.). After efficient adoptive transfert, the engineered T cells were sufficient to reduce drastically HBV infection, inducing specific lysis of the infected hepatocytes (Wisskirchen et al., n.d.). A more direct recognition of the antigen was designed, termed **Chimeric Antigen Receptor (CAR)**. These CARs are composed of an extracellular antigen-receptor domain, a spacer, a transmembrane domain and an intracellular signaling domain. The latter, composed of TCR costimulatory molecules, enables efficient TCR downstream signaling upon antigen recognition. In Tg mice, adoptive transfer of CAR-T cells specific for HBsAg (S-CAR) (Krebs et al. 2013) led to a significant reduction in HBV parameters (HBV DNA in serum, HBV DNA in the liver). However, in both models, lytic viral clearance is associated with increased inflammation and subsequent tissue injury (already discussed in part III-2-b). Thus, recently, in a succesfull effort to obtain non-lytic viral clearance, resting T cells were electroporated with HBV-TCR mRNA (S. Koh et al. 2018). Because T cells were not in an activated state already, the perforin-granzyme response (i.e lytic clearance) was reduced and viral clearance was mainly attributed to APOBEC3B activation in infected PHH and uPA-SCID mouse. Lately, pluripotent stem cells were efficiently differentiated into HBsAg-specific CD8+T cells, with highly potent effect in reducing HBV infection of hydrodynamically injected mice (Haque et al. 2020).

This strategy which seems efficient in murine models and come with great therapeutic potential seem however technologically too complicated to implement as the main HBV therapy, when the already present drugs (NAs) are already too expensive in most endemic countries.

Altogether, immune treatments are impaired by our lack of understanding of the immune inhibition established by HBV, and thus unraveling these mechanisms is crucial. Besides, current immunomodulatory treatments are only poorly or partially efficient in patients where the infection is not controlled, illustrating the need for combination therapy.

A correlation between viral hepatitis and HBV, at this time known as the “Australian antigen”, has been made since 1967 (B. S. Blumberg et al. 1967). Patients undergoing extremely severe hepatitis were discovered as HDV seropositive in 1977 (M. Rizzetto et al. 1977). However, it is not until May 2010 that the WHO officially acknowledged HBV as a global health problem in recognition of the increasing burden of viral hepatitis worldwide. Thereby, they launched in 2011 the WHO Global Hepatitis Program to coordinate global efforts against viral hepatitis. Signed by 194 governments, its objectives are to eradicate HBV by 2030 by (1) reducing incidence of viral hepatitis by 90%, (2) increasing treatment availability, (3) reducing by 65% the hepatitis-related morbidity, and (4) having 90% of people living with hepatitis diagnosed. Since then, the WHO report in 2019 estimates that on the 94 million individuals eligible for treatment, only 4.8 million received therapy (so 10.5%, versus 90% needed by 2030), treatments are still too expensive and not often available in certain area (especially Latin America). Regarding vaccine coverage, even if large programs have been launched, only 46% of infants born from infected mother received the birth dose of HBV vaccine needed to avoid transmission. Besides, many countries, even in European Union, did not include HBV vaccine in their national program, and in 2015, 39 countries were still not routinely screening blood transfusion for transmissible pathogen. Finally, an estimated 9 out of 10 people infected with HBV are unaware of such. To date, most international agency does not have specific recommendation for HDV, while CHD is widely recognized as the most aggressive form of viral hepatitis.

Hence the following question: will the design of a functional cure be sufficient enough to eradicate HBV and/or HDV?

Research projects.

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I. Hypothesis and objectives

Hepatitis B Virus (HBV) chronic infection leads to the death of 1 million people per year due to the induced liver complications (Durantel and Zoulim 2016). Co-infection with the Hepatitis Delta Virus (HDV), a satellite virus of HBV, is associated with a drastic acceleration of the aforementioned liver diseases. Hence, even with a safe and efficient vaccine, an estimated 250 million people are currently chronically infected worldwide. Current treatments are able to control HBV (NA), by blocking viral RNA to DNA conversion, or cure the infection (pegIFN- α) but only in 10-20% of cases. For HBV/HDV co-infected patients, NA are not effective and the only treatment option is pegIFN- α . However, it comes with strong side effects and most patients are resistant. Ten genotypes of HBV have been described to this day (HBV from A to J) each having specific geographic distribution and physiopathologic specificity (Anna Kramvis, Kew, and François 2005). In some studies, differences were observed regarding antiviral therapy, such as a better response to IFN α in genotypes A and B compared to C and D (A. Kramvis and Kew 2005).

To this day, no explanations have been found to these differences/resistance mechanisms, and very **few studies are investigating genotype-related immunity**.

A major area of HBV-HDV drug design is the development of immune modulators to replace or most probably act in combination with current treatment (Dusheiko 2020). Indeed, the efficiency of diverse immune receptors activators and cytokines has been largely documented in the past few years, especially NF κ B-inducers (Julie Lucifora et al. 2014; 2018; Isorce et al. 2016). Their main advantage being that they can act both directly on the infected hepatocytes, and indirectly by modulating immune cells, present as well in the liver. Unfortunately, some molecules, which were promising *in vitro*, such as agonists for TLR7, did not lead to a meaningful loss of HBsAg (a key feature of the functional cure for HBV) contrary to what was observed in animal models (Janssen et al. 2018). Interestingly, this has been correlated with a lack of immune modulation in patients, rather than antiviral potency (Bertoletti and Le Bert 2019).

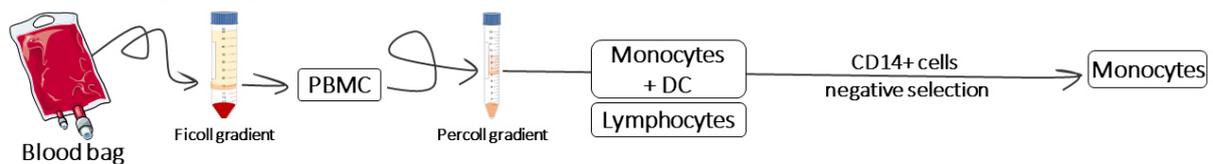
An important cell type in HBV-related immunity is liver ResM Φ . Indeed, several studies conducted in mice models demonstrate that depletion of liver ResM Φ prevented HBV establishment (L. Xu et al. 2014). Besides, CHB is correlated with an increase in tolerogenic M Φ and inhibitory mechanisms, as seen with the recruitment of M-MDSCs (Fang et al. 2015; A. Huang et al. 2014; Nebbia et al. 2012).

The **aims of this research project** are to (1) study on HBV-mediated modulation of M Φ and see if it could differ according to the HBV genotypes or HDV; (2) investigate and confirm which viral component(s) is/are responsible for these modulations and how; (3) investigate new immune modulators as a therapeutic for HBV and HBV-HDV infections.

II. MATERIALS AND METHODS

Primary cells purification and cells culture. 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 (Julie Lucifora, Michelet, Rivoire, et al. 2020). 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, 5x10⁻⁵ M of hydrocortisone, and 2% of DMSO). Liver mononuclear cells (LMNC) or only liver MΦ were purified from the non-parenchymal cell's mixture by respectively Ficoll gradients or a two phase iodixanol gradient (Figure 23). Liver MΦ were isolated by negative selection using pan monocyte isolation kit (Miltenyi Biotec) and cultured in macrophage medium (RPMI medium supplemented with 10% of decompemented FBS and 50 U/ml of penicillin/streptomycin). Peripheral blood mononuclear cell (PBMC) from blood donors were isolated by Ficoll gradient (Histopaque®-1077, Sigma) as previously described (Combes et al. 2017). Monocytes were purified by Percoll gradient followed by a negative selection with the Monocyte Isolation Kit II (Miltenyi Biotec) and cultured in macrophage medium (Figure 23).

Monocytes purification



Liver cells purification

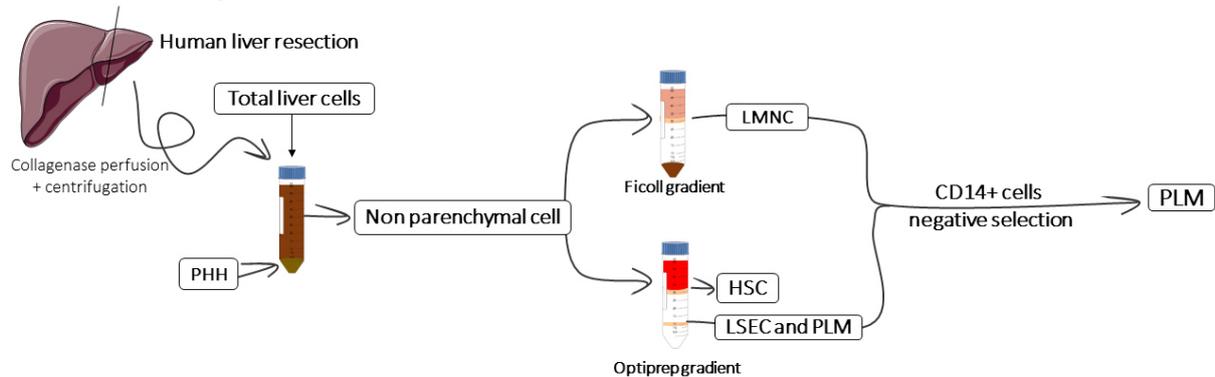


Figure 23: Monocyte and liver cells extraction method, schematic view.

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 (Figure 24). 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). HepaRG cells were cultured and differentiated as previously described (Philippe Gripon et al. 2002b).

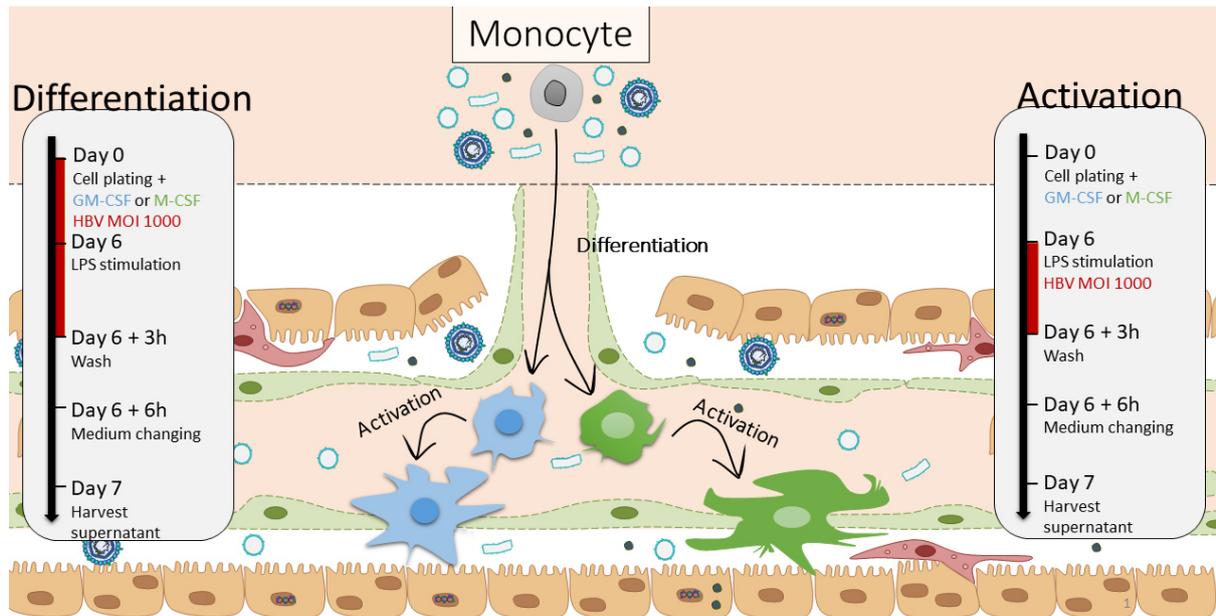


Figure 24: Schematic representation of MDMs differentiation and activation protocol.

Viral inoculation. Differentiated HepaRG (dHepaRG) or PHH were cultured and infected by HBV and or HDV (PEG-precipitated) as previously described (Luangsay, Gruffaz, Isorce, Testoni, Michelet, Faure-Dupuy, Ait-Goughoulte, et al. 2015; Alfaiate et al. 2016).

As PEG is not compatible with immune cells (uncontrolled activation), blood monocytes and liver MΦ were exposed to HBV inocula (or control medium) prepared by concentration of supernatant from HepAD38 (Iwamoto et al. 2014) by ultrafiltration. HBV genotype viral inocula were similarly prepared from the supernatant of a newly developed stably-transformed HepG2 cell lines. Briefly, cell lines were obtained by transfection of a linearized pcDNA3Neo-HBV plasmid containing 1.35 genome unit of a consensus sequence of HBV genotype A, B, C, D and E (obtained from HBV database: <https://hbvdb.lyon.inserm.fr/>;; sequences are available upon request) and a double-round selection under G418 (500 ng/mL) by colony cell cloning (very low density seeding in large flasks). All virus inocula 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 (Julie Lucifora et al. 2017b) and western-blot (HBc, DAKO, B0586). This allowed to rule out the presence of non-enveloped nucleocapsids that may activate the immune cells (Cooper et al. 2005). Blood monocytes and liver MΦ were exposed to at least three different batches of HBV at a multiplicity of infection of 1000 vge/mL (if not stated otherwise).

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.

Cytokine and drugs. Molecule references and concentrations used are provided in table 1.

Table 1: Molecules used

MOLECULE	SUPPLIER	REFERENCE	CONCENTRATION
TYPE-I IFN	ROCHE	ROFERON-A	500 UI
hIL-1 β	MBL	JM4128-10	100 μ g/mL
Anakinra (IL-1Ra)	SOBI	Kineret	0,4 mg/mL
3TC	SIGMA-ALDRICH	L1295	1 μ M
RG7834	ARBUTUS BIOPHARMA	-	1 μ M
TRIPTOLID	SIGMA-ALDRICH	T3652-1MG	1:10.000 ^e
TPCA1	SIGMA-ALDRICH	T1452	1 μ M

cccDNA quantification. Total DNA MasterPure™ Complete DNA and RNA Purification Kit (Epicentre, Lucigen distributed by Euromedex, Souffelweyersheim, France) following the manufacturer's instructions, without the protein K. cccDNA amount was quantified by qPCR analyses as previously described (Werle-Lapostolle et al. 2004).

RNA extraction and RT-qPCR. Total RNA from hepatocytes or M Φ were extracted with RNAzol or NucleoSpin® RNA XS (Macherey-Nagel), respectively. cDNA were synthesized using the Maxima RT (Thermo Scientific™, Life Technologies, Villebon-sur-Yvette, France) according to the manufacturer's instructions. qPCR analyses were performed using Luna (Invitrogen). mRNA expression was assessed by comparative cycle threshold (Ct) method ($2^{-\Delta Ct}$); RPLP0 was used as housekeeping genes. Primers sequences are provided in table 2 (human) and 3 (mouse).

Table 2: human primers sequences

GENE	FORWARD	REVERSE
Total HBV	GGAGGGATACATAGAGGTTCCCTTGA	GTTGCCCGTTTGTCTCTAATTC
3.5 KB HBV	GTGAGTGGGCCTACAAA	CTCCTCCAGCTTATAGACC
HDV	CGGGCCGGCTACTCTTCT	AAGGAAGGCCCTCGAGAACA
RPLP0	CACCATTGAAATCCTGAGTGATGT	TGACCAGCCCAAAGGAGAAG
IL-1 β	AATCTGTACCTGTCTGCGTGTT	TGGGTAATTTTTGGGATCTACACTTC
IL-6	TCGAGCCCACCGGAACGAA	GCAACTGGACCGAAGGCGCT
A20	TCCTCAGGCTTTGTATTTGAGC	TCTCCCGTATCTTACAGCTT
OAS	AGGTGGTAAAGGGTGGCTCC	ACAACCAGGTCAGCGTCAGAT
MXA	GGTGGTCCCCAGTAATGTGG	CGTCAAGATTCCGATGGTCTCT

Table 3: mouse primers sequence

GENE	FORWARD	REVERSE
IL-1 β	TGATGTGCTGCTGCGAGATT	TGCCACCTTTTGACAGTGATG
IL-10	TGCCTGCTCTTACTGACTGG	CTCTAGGAGCATGTGGCTCTG
IL-6	CTATACCACTTCACAAGTCGGAGG	TGCACAACCTCTTTTCTCATTTC
TNF α	ACGCTCTTCTGTCTACTGAACT	ATCTGAGTGTGAGGGTCTGG
F4/80	CTGAACATGCAACCTGCCAC	TTCACAGGATTCGTCCAGGC
iNOS	ACTACTGCTGGTGGTGACAA	GAAGGTGTGGTTGAGTTCTCTAAG
Arg1	CTGGGGATTGGCAAGGTGAT	CCCGTCGACATCAAAGCTCA
CD206	ATGGATTGCCCTGAACAGCA	CTCGTCAGCACCCCAGTTAG

Northern Blot. NB was performed as previously described (Alfaiate et al. 2016; Sureau 2010; J. Lucifora et al., n.d.).

Western Blot. WB procedure was the same as previously described (Julie Lucifora, Michelet, Salvetti, et al. 2020). Antibody used are the following: anti-HBc (HBc, DAKO, B0586), anti-HDag (produced in house, see (Julie Lucifora, Michelet, Salvetti, et al. 2020)) and anti-actin (a2228, Sigma-Aldrich).

NanoP production. Poly(Lactic)Acid nanoparticles were fully characterized by Myriam Lamrayah and Fanny Charriaud, as previously published (Lamrayah et al. 2019).

Mice Experiments. All animal were housed in the A3 animal facility of the IBCP in Lyon. The procedures were approved by the local ethic committee and authorized by the French ministry of research. Twenty-four 5-week-old C57BL6/N male mice were injected intravenously with 1.10^{11} VGE of AAV8-HBV and treated weekly with Lamivudine 5 (100 mg/kg/days), TLR2-L or NanoP-TLR2-L. For TLR2-L and NanoP-TLR2-L, a dose escalation was performed with 20 and 2.5 μ g on week 5, 40 and 5 μ g for weeks 6-7 and 80-10 μ g for week 8-9, respectively (Figure 25). Mice were then left untreated for seven days before being sacrificed. Liver pieces were flash frozen in liquid nitrogen and kept at -80°C before further processing.

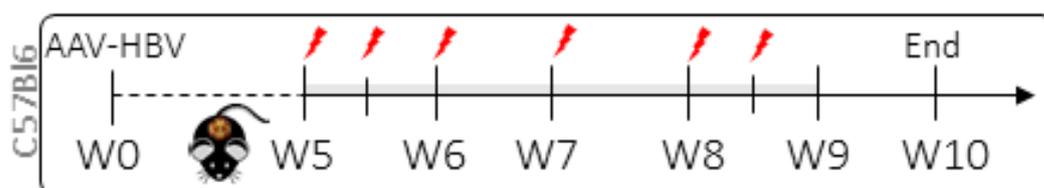
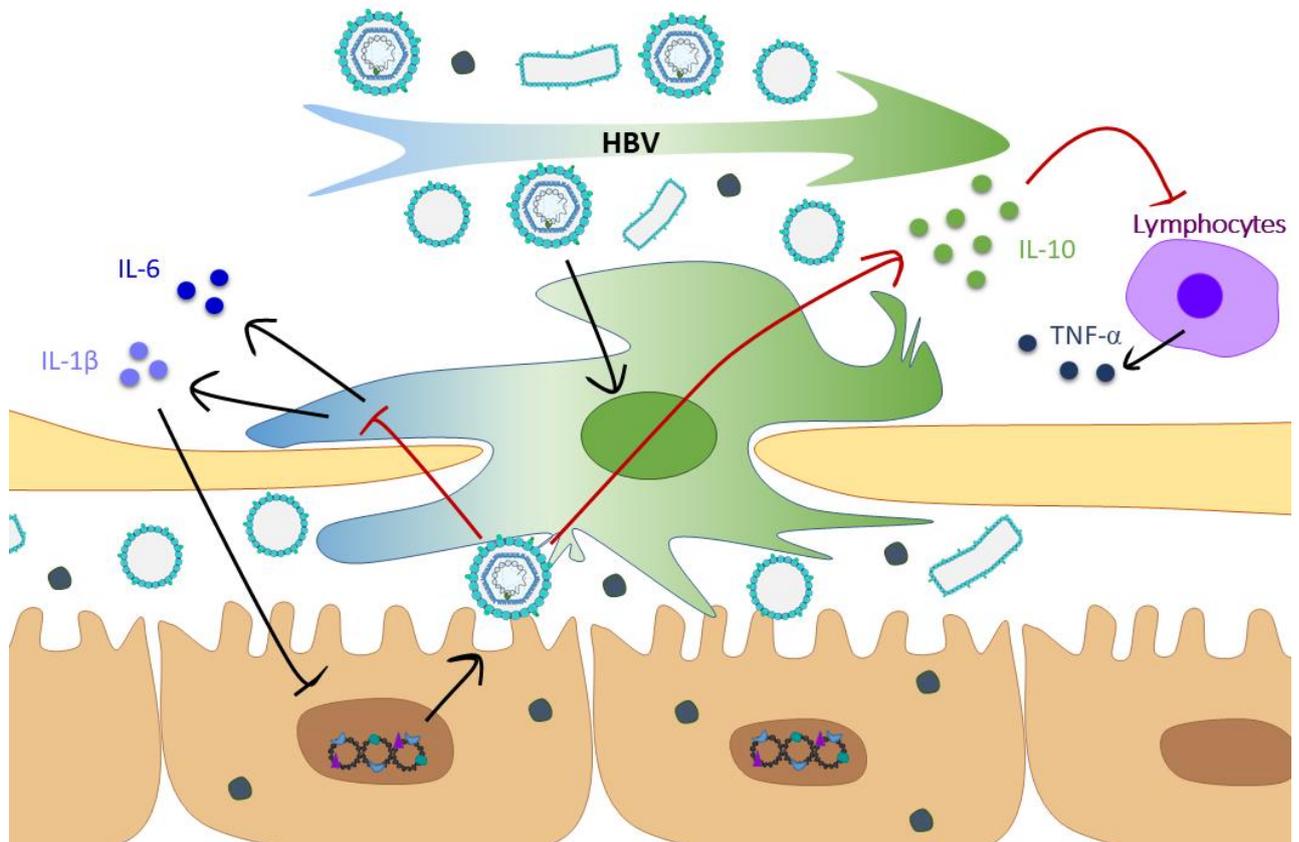


Figure 25: Schematic representation of the mice experiment

Statistical analysis. Results are presented as mean \pm standard deviation and analysed for statistical significance using the Mann-Whitney test with Prism software. P values are indicated.

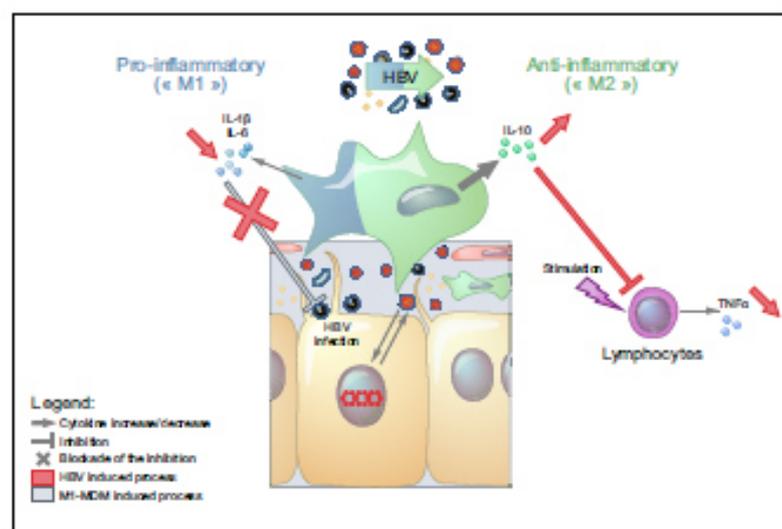
III. Research Article

Hepatitis B Virus-induced modulation of liver macrophage function promotes hepatocyte infection



Hepatitis B virus-induced modulation of liver macrophage function promotes hepatocyte infection

Graphical abstract



Highlights

- Hepatitis B virus proteins are observed in liver macrophages from patients.
- Hepatitis B virus impairs pro-inflammatory macrophage secretion.
- Hepatitis B virus increases anti-inflammatory macrophage secretion.
- Impairment of pro-inflammatory secretions favours the establishment of hepatitis B virus infection.
- Increase of IL-10 secretion further impairs lymphocyte activation.

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Lay summary

Hepatitis B virus modulates liver macrophage function in order to favour the establishment and likely maintenance of infection. It impairs the production of the antiviral cytokine IL-1 β , while promoting that of IL-10 in the microenvironment. This phenotype can be recapitulated in naive liver macrophages or monocyte-derived-macrophages *ex vivo* by short exposure to the virus or cells replicating the virus, thus suggesting an "easy to implement" mechanism of inhibition.

Hepatitis B virus-induced modulation of liver macrophage function promotes hepatocyte infection

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Background & Aims: Liver macrophages can be involved in both pathogen clearance and/or pathogenesis. To get further insight on their role during chronic hepatitis B virus (HBV) infections, our aim was to phenotypically and functionally characterize *in vivo* and *ex vivo* the interplay between HBV, primary human liver macrophages (PLMs) and primary blood monocytes differentiated into pro-inflammatory or anti-inflammatory macrophages (M1-MDMs or M2-MDMs, respectively).

Methods: PLMs or primary blood monocytes, either *ex vivo* differentiated into M1-MDMs or M2-MDMs, were exposed to HBV and their activation followed by ELISA or quantitative reverse transcription PCR (RT-qPCR). Liver biopsies from HBV-infected patients were analysed by RT-qPCR or immunohistochemistry. Viral parameters in HBV-infected primary human hepatocytes and differentiated HepaRG cells were followed by EUSA, qPCR and RT-qPCR analyses.

Results: HBc protein was present within the macrophages of liver biopsies taken from HBV-infected patients. Macrophages from HBV-infected patients also expressed higher levels of anti-inflammatory macrophage markers than those from non-infected patients. *Ex vivo* exposure of naive PLMs to HBV led to reduced secretion of pro-inflammatory cytokines. Upon exposure to HBV or HBV-producing cells during differentiation and activation, M1-MDMs secreted less IL-6 and IL-1 β , whereas M2-MDMs secreted more IL-10 when exposed to HBV during activation. Finally, cytokines produced by M1-MDMs, but not those produced by HBV-exposed M1-MDMs, decreased HBV infection of hepatocytes.

Conclusions: Altogether, our data strongly suggest that HBV modulates liver macrophage functions to favour the establishment of infection.

Lay summary: Hepatitis B virus modulates liver macrophage function in order to favour the establishment and likely maintenance of infection. It impairs the production of the antiviral cytokine IL-1 β , while promoting that of IL-10 in the microenvironment. This phenotype can be recapitulated in naive liver macrophages or monocyte-derived-macrophages *ex vivo* by short exposure to the virus or cells replicating the virus, thus suggesting an "easy to implement" mechanism of inhibition.

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Introduction

Hepatitis B virus (HBV) chronically infects around 250 million people worldwide (World Health Organization data, 2016) and increases the risk of developing cirrhosis and hepatocellular carcinoma.¹ Current treatments, mainly based on nucleos(t)ide analogues, reduce blood viremia to undetectable levels in the majority of patients, but do not achieve virus elimination from the liver.² New treatments, including immune-therapeutic components, are therefore needed in order to progress toward a functional cure for HBV.

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. The pgRNA is encapsulated within the nucleocapsid 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), HBV RNA containing particles and empty (*i.e.*, nucleocapsid free) enveloped subviral particles (SVPs). The latter 3 have envelope proteins at their surface and are indistinctly detected as HB surface antigens (HBsAg).² SVPs, which are produced in large excess compared to virions, are thought to play an important role in terms of immune subversion.³

Several pro-inflammatory cytokines (IL-6, IL-1 β and TNF α) and interferons (IFN α and IFN γ) were shown to induce a direct antiviral effect on HBV replication in hepatocytes,⁴⁻⁷ with IL-1 β

Keywords: Liver macrophage; Hepatitis B virus (HBV); Phenotypic immune-modulation; IL-1 β ; IL-10; Anti-inflammatory; Anti-viral effect.

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being one of the most efficient at inhibiting already established HBV infections *in vitro*.⁴ IL-1 β is a pro-inflammatory cytokine produced upon inflammasome activation.⁸ In the liver, it is mostly produced by macrophages (M Φ) since hepatocytes do not possess functional inflammasomes.^{9,10} Liver resident M Φ , named Kupffer cells (KCs), represent 80% of the M Φ count in the body at steady state.¹¹ They are specialised in the detection of pathogens coming from the enteric circulation, as well as in the elimination of aging blood cells, through their high phagocytic capacity.¹¹ Upon inflammation, monocytes from the blood circulation can be recruited in the liver and differentiate locally into M Φ that are called monocyte-derived-macrophages (MDMs).¹² KCs and MDMs have different embryonic origins and functions.¹³ *In vivo*, a wide range of different phenotypes of M Φ exist, depending on their origin, activation status, localisation, and their micro-environment.¹⁴ Inflammatory M Φ , commonly called M1, which are phenotypically described as CD40⁺ CD86⁺ HLA-DR⁺, are characterised by their capacity to secrete pro-inflammatory cytokines/chemokines, such as IL-1 β , produce various antimicrobial factors, including nitric oxide, and are therefore implicated in inflammation and elimination of pathogens.¹⁴ Anti-inflammatory M Φ , commonly called M2, express the arginase 1, mannose receptors, and the high affinity scavenger receptor CD163, and secrete anti-inflammatory cytokines, such as IL-10 and TGF β , as well as angiogenic factors, such as VEGF.¹⁴ M2 M Φ are involved in the resolution of inflammation and in tolerance mechanisms.¹⁴ They are also found abundantly in the tumour microenvironment and are called tumour-associated M Φ (TAMs).¹⁵

Several studies showed that HBV can influence the phenotype and biology of M Φ ,¹⁶ but few have been performed using liver resident or infiltrating primary human cells. To get further insights into the role of liver M Φ in the establishment of HBV infection in hepatocytes, we used highly relevant models such as primary human liver M Φ , pro-inflammatory/M1-like or anti-inflammatory/M2-like M Φ differentiated *in vitro* from primary blood monocytes, liver biopsies, primary human hepatocytes (PHHs), differentiated HepaRG cells (dHepaRGs), and co-culture models.

Materials and methods

Patient samples

Two different cohorts were analysed. The collection and analysis of human patient samples of the first cohort (Swiss/German cohort) was approved by the ethics committee of Northern Switzerland (EKNZ, Study ID: PB_2018-00308, 310/12), confirmed to the ethical guidelines of the 1975 Declaration of Helsinki, and was provided by the Department of Pathology, Basel, Switzerland. The biopsies were stained in Germany. Patients' characteristics are presented in Table S1. Samples from the second cohort were used under the French IRB 'CPP Sud-Est IV' approval #11/040 (2011) from the 'Biobanque INSERM CRCL Hépatologie (U1052)', France #DC2008-235 and stained in France. Written informed consent was obtained from each patient. Patient characteristics are presented in Table S2.

Immunohistochemistry

Paraffin was removed from formalin-fixed paraffin-embedded samples by successive alcohol baths and epitopes were unmasked as previously described.¹⁷ Endogen peroxidases were blocked by a 15 min exposition at room temperature (RT) with

Dual Endogenous enzyme Block (DAKO S2003). Saturation was performed by 2 successive incubations at RT of 20 min with horse serum (VECTOR S-2012 Za0328) and 15 min with DAKO antibody diluent (DAKO S3022). Antibodies (Table S3) were incubated overnight at 4 °C in DAKO antibody diluent. Samples were incubated for 15 min at RT with secondary antibodies (Table S3). Coloration was performed with DAB substrate (DAKO K3468) or permanent red substrate (DAKO K0640), and counter-staining with haematoxylin (SIGMA MHS1). Negative controls were performed using control IgGs (Table S3) or non-infected patients, and no staining was observed (Fig. 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. For the CD68, HBcAg double staining, a BONDMax robotic staining machine (Leica) was used.

Primary cell purification and cell culture

Peripheral blood mononuclear cells (PBMCs) from blood donors (Etablissement Français du Sang, EFS; code 895/1052) were isolated by Ficoll gradient (Histopaque[®]-1077, Sigma) as previously described.¹⁸ Lymphocytes were separated from total PBMCs by a Percoll gradient and further cultured in M Φ 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 M Φ 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. MDMs were activated by a 3 h stimulation with 10 ng/ml of lipopolysaccharide (LPS) (InvivoGen). Cells were washed 3 times with PBS and cultured in fresh medium for another 3 h before a last medium exchange. Supernatants and cells were collected 24 h post stimulation (*i.e.* 18 h accumulation). Liver cells were isolated from hepatic resections obtained in collaboration with 3 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, APNOR NF 96 900 sept 2011). After a 2-step collagenase perfusion, the liver extract was filtered and centrifuged, as previously described.¹⁹ PHHs were cultured on the collagen layer and maintained in PHH medium (Williams medium supplemented with 5% of foetal clone II serum, 50 U/ml of penicillin/streptomycin, 1 \times glutamax, 5 μ g/ml of bovine insulin, 5 \times 10⁻⁵ M of hydrocortisone, and 2% of DMSO). Liver mononuclear cells (LMNCs) or only liver M Φ were purified from the non-parenchymal cells mixture by respectively Ficoll gradients or a 2 phase iodixanol gradient.²⁰ Liver M Φ were isolated by negative selection using pan monocyte isolation kit (Miltenyi Biotec) and cultured in M Φ medium. HepaRG cells were cultured and differentiated as previously described.²¹

Viral infection

Differentiated HepaRG (dHepaRG) or PHHs were cultured and infected by HBV (PEG-precipitated) as previously described.²² As PEG-concentrated HBV virions cannot be used for exposition to immune cells, because PEG activates cells, blood monocytes, LMNCs and liver M Φ were exposed to HBV inocula (or control medium), which were prepared by concentrating supernatant from HepAD38²³ (or from non-infected HepG2-NTCP respectively) by filtration/retention centrifugation using column with a cut-off retention of 100 kDa (Merk Millipore; UFC710008).

All virus inocula were tested for the absence of endotoxin (Lonza) and characterised by analyses of the fractions from a 5.6–56% iodixanol gradient and analysed by ELISA, dot blot with HBV DIG-labelled probe²⁴ and western-blot (DAKO, B0586) (Fig. S2). This allowed us to rule out the presence of non-enveloped nucleocapsids that may activate immune cells, as HBc was shown to be a TLR2 ligand.²⁵ Blood monocytes, LMNCs and liver MΦ were exposed to at least 3 different batches of HBV (full inoculum concentrated by ultrafiltration) at a multiplicity of infection of 1,000 vge/ml (if not stated otherwise). Viral titre superior to 10¹⁰ vge/ml were routinely obtained and uniquely used in order to allow a dilution of 100- to 1,000-fold when preparing “infection medium” at a multiplicity of infection of 1,000 vge/cell. This way, we limited the impact of “carried-away” contamination. For some experiments, virus was inactivated by a 30 min exposition to UVB. Stocks of adenovirus vector (AAV) particles were generated by the vector Core of the University of Nantes by calcium phosphate transfection of HEK-293 cells as described previously using pDG8 (as a helper plasmid). The vector particles were purified on caesium chloride gradients as previously described²⁶ and genome particles/ml (gp/ml) were titered by quantitative PCR (qPCR). MΦ were exposed to AAV at a multiplicity of infection of 1,000 gp/ml.

Cytochalasin D and cytokine treatments

M2-MDMs were exposed to 1 μg/ml of Cytochalasin D (Sigma Aldrich; C8273-1MG) during their activation. As Cytochalasin D is diluted in DMSO, monocytes were exposed to a similar dose of DMSO as control. Cytokines references and concentrations used are indicated in Table S4.

Quantification of secreted proteins by ELISA

HBeAg and HBsAg secretion were quantified using chemiluminescence immunoassay kit (Autobio) following the manufacturer's instructions. Cytokine secretion was analysed using Duoset ELISA (R&D system) following manufacturer's instructions.

RNA extraction and RT-qPCR

Total mRNA from hepatocytes or MΦ were extracted with NucleoSpin[®] RNA II or NucleoSpin[®] RNA XS respectively (Macherey-Nagel). cDNA was synthesised using the SuperScript[®] III Reverse Transcriptase (Life technologies) according to the manufacturer's instructions. qPCR analyses were performed using “Express SYBR GreenER[™] qPCR SuperMix Universal” (Invitrogen). mRNA expression was assessed by comparative cycle threshold (Ct) method ($2^{-\Delta\Delta Ct}$); GUS and RPLP0 were used as housekeeping genes for hepatocytes and MΦ respectively. Primers sequences are presented in Table S5.

DNA extraction and cccDNA quantification

Total DNA were extracted using the NucleoSpin[®] 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 HBV rcDNA, followed by a 30 min heat inactivation. The amount of cccDNA was quantified by qPCR analyses as previously described.¹

Cytotoxicity assay

Seven days post treatment, hepatocyte 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/cm² for 2 h. Cells were activated by a 22 h 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-α secretion.

MΦ supernatant concentration

M1- or M2-MDM supernatants from at least 5 different donors were pooled and concentrated by ultrafiltration (using 10 kDa Amicon ultra-15 column; Merk-Millipore) following the manufacturer's instruction. The cytokine concentrations of the concentrated supernatants were assessed by ELISA before dilution to 1/100 in hepatocyte media (experiments from Fig. 9) or dilution to 1/2 in MΦ media (experiments from Fig. 7). Final concentrations are presented in Tables S6 and S7.

Flow cytometry

Cells were washed extensively with PBS and removed from plates with 150 μl of versene-EDTA at 37 °C for 5 min before centrifugation at 1,300 rpm for 5 min at 4 °C. Cells were incubated for 30 min at 4 °C with primary antibody (Table S3). 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 or median ± standard deviation. Statistical significance was analysed using Prism software. *P* values are indicated and non-significance is indicated by “n.s.”.

Results

HBV is associated with phenotypic changes in liver MΦ in infected patients

To assess if HBV may closely interact with liver MΦ *in vivo*, levels of CD68 expression (identifying MΦ), CD163 (identifying anti-inflammatory MΦ¹⁴) and HBc (identifying the HBV core protein) were analysed by immunohistochemistry on liver biopsies from 2 distinct cohorts of patients (see Tables S1 and S2 for details on patients). As expected, signals for CD68 and CD163 were detected in the sinusoidal space (i.e., in between hepatocytes where myeloid cells are), and no signals were observed with control antibodies (Fig. 1 and S1). Co-localised signals were observed for HBc and CD68 in liver samples from HBV-infected patients (Fig. 1A and zoom in Fig. 1B), demonstrating the presence of this HBV protein within liver MΦ. Of note, the proportion of MΦ co-stained with HBc varied from 1 patient to the other; approximately 20% of all patients analysed lacked co-localisation between HBc and MΦ, 50% displayed less than 5% co-localisation, and 30% displayed between 5% to 20% co-localisation (Fig. 1A, zoom in Fig. 1B). Furthermore, quantification of the signals highlighted a 1.8-fold increase in CD163⁺ myeloid cells in HBV-infected patients (+79%, *p* = 0.0271) compared to non-infected patients in the Swiss/German cohort and a 1.3-fold increase in the French cohort (Fig. 1C, 1D, S1B). Interestingly, a positive correlation between the level of HBc staining and the increase of CD163⁺ cells was observed (Fig. 1E). Altogether, these data suggest that HBV interacts with liver MΦ and may affect their phenotype and functions. We therefore performed several *ex vivo* analyses to investigate the

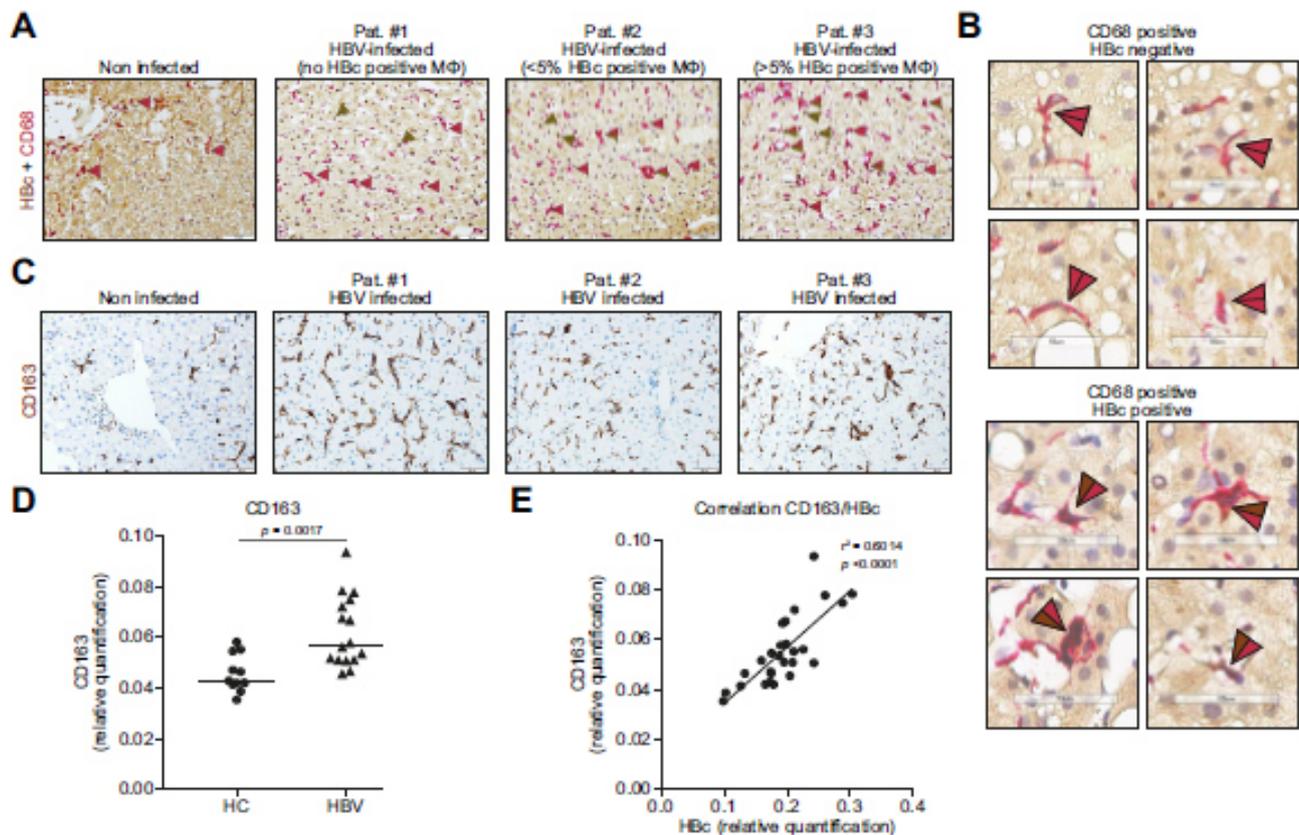


Fig. 1. An HBV protein co-localized with liver M Φ and the proportion of anti-inflammatory M Φ is higher in CHB liver biopsies. HBc (HBV capsid protein), CD68 (total M Φ) and/or CD163 (M2 M Φ), in liver biopsies from non-infected or HBV-infected patients (Table S1), were stained by IHC. (A,B) Representative photos of HBc (brown arrows) and CD68 (pink arrows) stainings. (C) Representative photos of CD163 stainings. (A,B,C) Bars represent 50 μ m. (D) Quantification of CD163 stainings. Horizontal bars represent the median of values and data were submitted to Kruskal Wallis test. n.s., not significant. (E) Correlation curve between CD163 and HBc stainings. CHB, chronic hepatitis B; HBV, hepatitis B virus; IHC, immunohistochemistry.

influence of HBV on liver cells with a focus on liver M Φ , including KCs and MDMs.^{9,10}

Ex vivo, HBV inhibits the activation of primary liver M Φ by direct exposure

First, we investigated the impact of HBV particles and antigens (concentrated by filtration/retention from HepAD38 cell supernatants and characterised as shown on Fig. S1) on the secretion of cytokines by non-parenchymal primary human liver mononuclear cells (LMNCs) freshly isolated from hepatic resections (5 donors) as previously described.²⁷ Of note, we used a virus concentrated by filtration/retention rather than PEG concentration, as PEG *per se* activates M Φ . The basal levels of 2 prototypic cytokines, namely IL-1 β and IL-10, in non-stimulated LMNCs from individual donors are shown in Fig. 2A. These cells were either left untreated or exposed for 24 h to total HBV inoculum immediately after their isolation, and the same cytokines were dosed in the supernatant (Fig. 2B). The mean concentration of secreted IL-1 β was 25% lower in HBV-exposed LMNC preparations compared to controls, whereas the mean IL-10 level was 64% higher (Fig. 2B).

Purified primary liver M Φ (PLM Φ) exposed to HBV or not were then stimulated with synthetic pathogen-associated molecular patterns (PAMPs); LPS was used to stimulate TLR4 and the NLRP3 (NOD-like receptor family, pyrin domain containing 3) inflammasome,²⁸ whereas LPS + poly(dA:dT) was

used to stimulate TLR4 and the AIM-2 (Absent In Melanoma-2) inflammasome⁹ (Fig. 2C). The mean level of secreted IL-1 β was 31% lower in LPS-stimulated PLM Φ exposed to HBV, compared to the unexposed condition (Fig. 2D). Similar results were obtained with HBV-exposed, LPS and poly(dA:dT)-stimulated PLM Φ , for which the mean level of secreted IL-1 β was 35% lower (Fig. 2E). Interestingly, the expression of *HIF1 α* mRNA, which encodes a protein tightly associated to IL-1 β production and secretion in M Φ ,²⁹ was 70% lower in LPS-stimulated-PLM Φ exposed to HBV compared to control (Fig. S3). While the mean level of secreted IL-10 was not affected by HBV with either type of stimulation in our experimental conditions (Fig. 2D and 2E), the mRNA levels of VEGF, an angiogenic factor expressed by anti-inflammatory cells,³⁰ was 56% higher in LPS-stimulated-PLM Φ exposed to HBV, compared to control cells (Fig. S3). Altogether these data suggest that HBV may interfere with the activation of PLM Φ .

Ex vivo, HBV interferes with blood monocyte differentiation and activation by direct exposure

During viral infections, a high quantity of immune cells, including monocytes, are recruited to the inflammation/injury site to mount a strong pro-inflammatory response and contribute to infection control.³¹ This response is subsequently limited by an anti-inflammatory response, in order to start the scarring processes and prevent chronic inflammation.³¹ Infiltrating

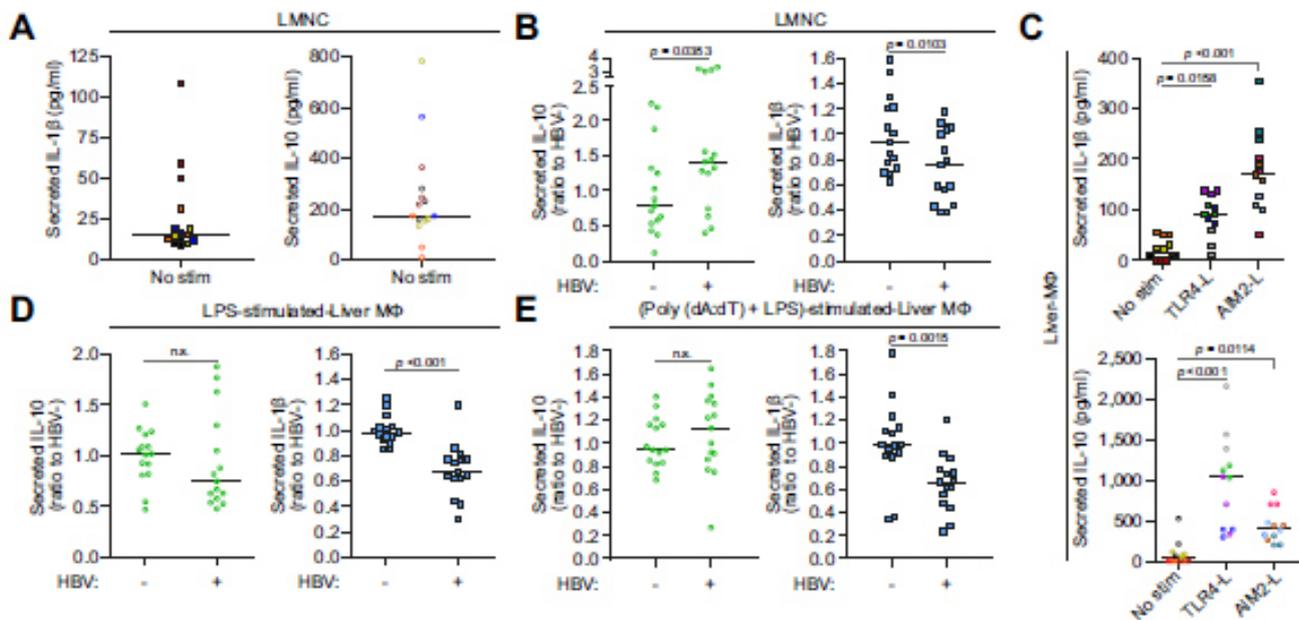


Fig. 2. Levels of pro-inflammatory markers decreased in liver MΦ exposed to HBV. (A,B) Total LMNCs were isolated from liver resection and exposed or not to HBV for 24 h. (C,D,E) Liver MΦ were isolated from liver resection, exposed or not to HBV for 24 h and stimulated 24 h later with (C,D) 100 ng/ml of LPS (TLR4-L) or (C,E) 100 ng/ml of LPS + 100 ng/ml of Poly (dA:dT) (AIM2-L) for another 24 h. (A-E) Supernatant were collected and the levels of IL-1 β and IL-10 were assessed by ELISA. Data are presented either as non-normalised secretion or as ratio to non-exposed cells. Results are the median of 5 independent experiments (with 5 different donors) each performed with 3 biological replicates. Horizontal bars represent the median and data were submitted to Wilcoxon test. HBV, hepatitis B virus; LMNCs, liver mononuclear cells; LPS, lipopolysaccharide; ns., not significant.

monocytes differentiate into MΦ within the tissue¹⁴ and, in the case of the human liver, cannot be fully distinguished from resident MΦ.

To assess the effect of HBV on MΦ that experimentally mimic those derived from liver-infiltrating monocytes, monocytes were purified from PBMCs and differentiated, with well-established polarizing *in vitro* conditions, into M1-like MΦ (M1-MDMs) upon GM-CSF or M2-like MΦ (M2-MDMs) upon M-CSF stimulation.³² The differentiation process is schematically presented in Fig. 3A. M1-MDM cells derived from peripheral monocytes expressed and secreted, under a physiologic exposure to LPS (10 ng/ml), pro-inflammatory cytokines IL-6, IL-1 β , and TNF- α , but not IL-10 (Fig. 3B, 3C, 3D, and 3E). In contrast, *in vitro* differentiated M2-MDM cells expressed only residual levels of IL-6, IL-1 β , and TNF- α , but secreted high levels of the anti-inflammatory cytokine IL-10 in the same condition of physiologic LPS exposure (Fig. 3B, 3C, 3D, and 3E). Upon exposure to HBV during differentiation and activation (*i.e.*, 6 days exposure to HBV) (Fig. 3A), GM-CSF-treated monocytes (*i.e.*, intended to be differentiated into M1-MDMs) activated with low LPS stimulation, secreted 34% and 60% less IL-6 and IL-1 β , respectively (Fig. 3F). Accordingly, a decreased level of *HIF1 α* mRNA was detected (Fig. S4A). Under these conditions, no significant effect of HBV incubation was observed on the level of secreted TNF- α (Fig. 3F). M2-MDMs exposed to HBV during differentiation and activation secreted similar levels of IL-10 as unexposed cells (Fig. 3G) but expressed lower *VEGF* mRNA levels (Fig. S4A).

We further investigated the effect of HBV on the activation of already differentiated M1-MDMs or M2-MDMs in response to physiologic LPS stimulation (Fig. 4A). No difference was

observed in the mean levels of secreted IL-6, IL-1 β , or TNF- α (Fig. 4B). In contrast, lower mean mRNA level of *HIF1 α* was observed (Fig. S4B). Differentiated M2-MDMs showed a 2-fold increase of mean secreted IL-10 levels and an increase of mean *VEGF* mRNA levels in the presence of HBV (Fig. S4B). These results were confirmed with different donors and durations of exposure to HBV (Fig. S5). Moreover, increasing doses of HBV inoculum revealed an increasing effect i) on the inhibition of IL-6 secretion by M1-MDMs exposed to the virus during their differentiation and activation, and ii) on the increase of IL-10 secretion by M2-MDMs exposed to the virus during their activation (Fig. 5A and 5B).

Interestingly, the presence of cytochalasin D (Cyt D, an inhibitor of endocytosis and phagocytosis³³), during the activation of M2-MDMs, in the presence of HBV, did not modify IL-10 secretion (Fig. 5C), suggesting that the effect of HBV on M2-MDM activation might be independent of virus productive entry within MΦ. It is also worth noting that UV-inactivated HBV has a similar effect on PLMΦ (Fig. S6A) and on M1- and M2-MDM (Fig. S6B) secretions as replication-competent HBV, and that HBV-exposed liver MΦ or MDMs did not secrete HBeAg or HBsAg (Fig. S7), highlighting that the effects of HBV on MΦ are independent of viral replication within cells. Moreover, no significant modification of cytokine secretion was observed when M1- or M2-MDMs were exposed to concentrated supernatants from non-infected HepG2-NTCP during, respectively, their differentiation and activation or activation only (Fig. S6C and S6D), suggesting that the effect is specific to HBV and not to a factor secreted by hepatocytes in the absence of HBV. Finally, AAV, either expressing GFP or the HBV genome, had no effect on M1-MDM secretion (Fig. 5D), suggesting that the

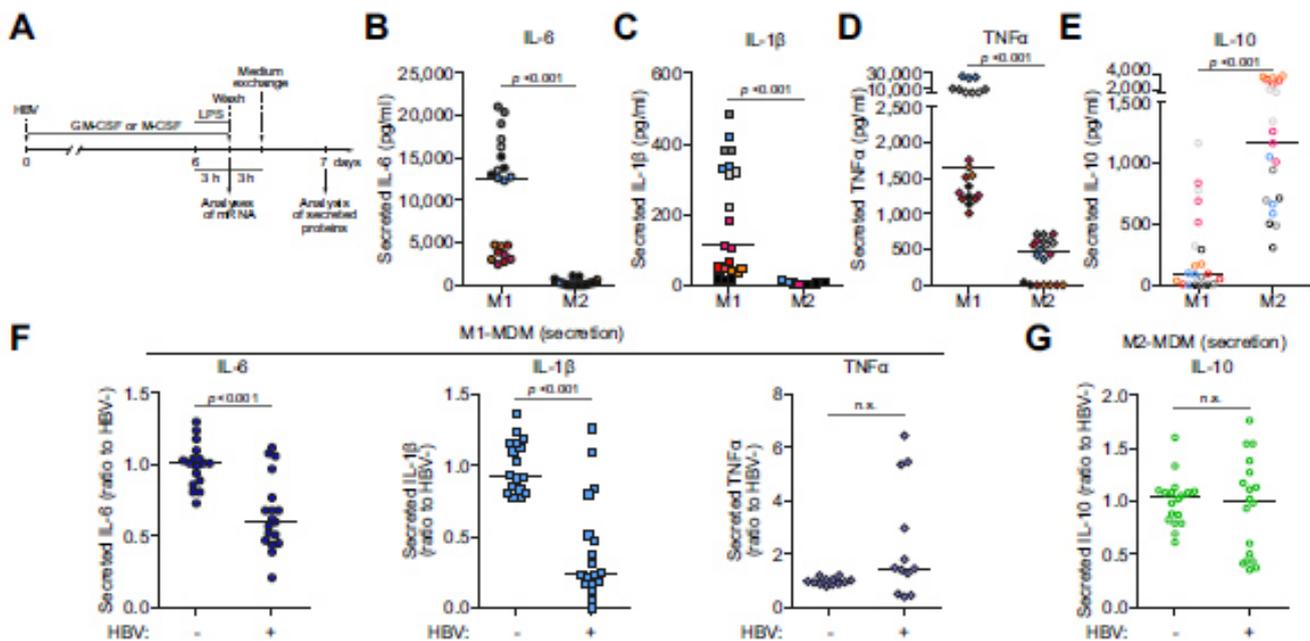


Fig. 3. HBV interferes with M1-MDM differentiation. Monocytes were purified from peripheral blood mononuclear cells, differentiated into (B-F) M1-MDM (upon GM-CSF culture) or (B-E,G) M2-MDM (upon M-CSF culture) in the presence or not of HBV before stimulation with 10 ng/ml of LPS for 3 h. (A) Schematic representation of the experiment. Medium change is indicated by dotted arrows. (B-G) Levels of the indicated secreted cytokines were assessed by ELISA and analysed either as non-normalised secretion or as ratio to non-exposed cells. Results are the mean of 6 independent experiments (with 6 different donors) each performed with 3 biological replicates. Horizontal bars represent the median and data were submitted to Wilcoxon test. HBV, hepatitis B virus; LPS, lipopolysaccharide; M1-MDM, M1-like monocyte-derived macrophages; M2-MDM, M2-like monocyte-derived macrophages; n.s., not significant.

observed effect is specific to HBV particles and/or antigens. Of note, the cells exposed to AAV-HBV did not secrete HBeAg or HBsAg (data not shown).

HBV interferes with blood monocyte differentiation and activation in co-culture conditions

To ensure that the effect of secreted HBV components on M1-MDM differentiation and M2-MDM activation was not due to the high amount of HBV used in previously presented *ex vivo* experiments, we set up co-culture assays that allowed us to study the effect of HBV components physiologically secreted from infected hepatocytes. Monocytes, which were intended to differentiate into M1-MDMs and M2-MDMs, were co-cultured with either non-infected HepG2-NTCP cells (as HBV-negative control cells) or with HBV-producing HepAD38 cells during their respective differentiation (for M1-MDMs) or activation (for M2-MDMs) (Fig. 6A). We confirmed the results obtained with cell free-derived viruses and observed an even stronger effect of HBV since monocytes co-cultured with HBV-producing cells during their differentiation into M1-MDMs secreted between 46% and 85% lower mean levels of IL-6, IL-1 β , and TNF- α (Fig. 6B). In contrast, M2-MDMs co-cultured with HBV-producing cells during their activation secreted 2.25-fold more IL-10 (Fig. 6C).

Altogether these results suggest that exposure of peripheral monocytes to cell-free HBV and also cell-transmitted HBV components can interfere with their differentiation into M1-MDMs, as indicated by the significantly reduced secretion of pro-inflammatory cytokines, including IL-1 β . Moreover, an exposure to HBV during M2-MDM activation led to an increased secretion of the anti-inflammatory cytokine IL-10.

HBV impairs lymphocyte activation

IL-10 is well-known to impair lymphocyte activation.³⁴ Therefore, we assessed if an increase of IL-10 secretion induced by HBV (Fig. 4C) would further impair this activation. Total lymphocytes were purified from peripheral blood and exposed to M2-MDM-conditioned media for 2 h before their activation with PMA (phorbol 12-myristate 13-acetate) + ionomycin. Even though not significant, total lymphocytes exposed to M2-MDM supernatants secreted 17% less TNF- α than unexposed lymphocytes (Fig. 7, [no-HBV M2-MDM]-CM). Supernatants from M2-MDMs that were exposed to HBV during their activation, and contain higher levels of IL-10 (Fig. 4C and Table S6, [HBV stimulated M2-MDM]-CM), significantly decreased TNF- α secretion (30%) by total lymphocytes (Fig. 7).

Pro-inflammatory cytokines reduce the establishment of HBV infection in hepatocytes

As previously shown, pro-inflammatory cytokines can directly block HBV replication in cells persistently infected *in vitro* and IL-1 β was the most efficient.⁴ To investigate the effects of M Φ -derived cytokines on the establishment of HBV infection in hepatocytes, dHepaRG cells or PHHs were treated 24 h before and during the inoculation of cells with HBV with various recombinant pro-inflammatory cytokines (rhIL-1 β , rhIL-6, rhTNF- α , rhIL-18), anti-inflammatory cytokines (rhIL-10, rhTGF β), as well as with rhMCP-1, which is known to be secreted by both M1- and M2-M Φ (Table S4).³⁵ RhIL-1 β treatment led to a 70–80% decrease of HBeAg and HBsAg secretion, HBV mRNA, and cccDNA formation, under conditions where cell viability of dHepaRG cells and PHHs were not affected (Fig. 8). Of note, a 24 h pre-treatment with rhIL-1 β , followed by a

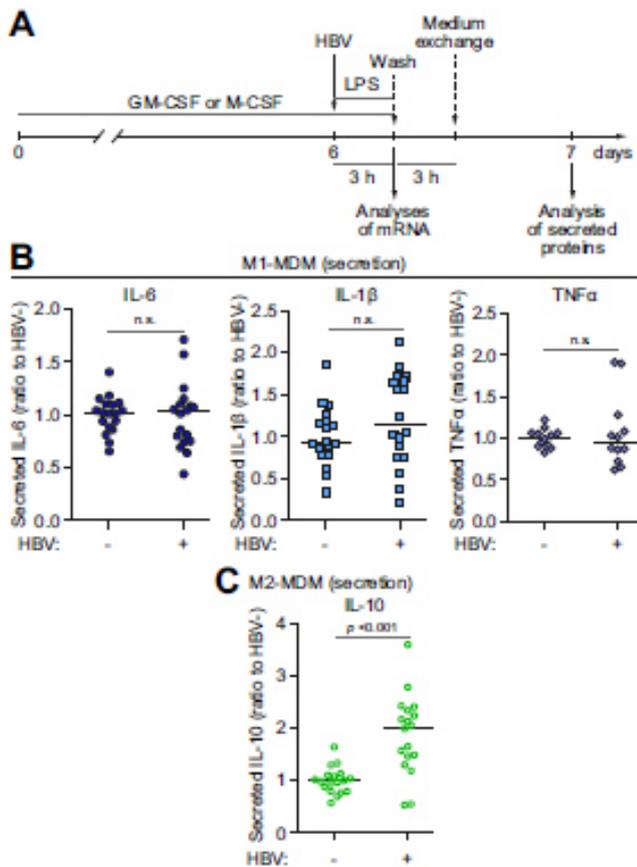


Fig. 4 HBV increases M2-MDM activation. Monocytes were purified from peripheral blood mononuclear cells, differentiated into (B) M1-MDM (upon GM-CSF culture) or (C) M2-MDM (upon M-CSF culture) and stimulated with 10 ng/ml of LPS for 3 h in the presence or not of HBV. (A) Schematic representation of the experiment. Medium exchange is indicated by dotted arrows. (B-C) Levels of the indicated secreted cytokines were assessed by ELISA and analysed as ratio to non-exposed cells. Results are the mean of 6 independent experiments (with 6 different donors) each performed with 3 biological replicates. Horizontal bars represent the median and data were submitted to Wilcoxon test. HBV, hepatitis B virus; LPS, lipopolysaccharide; M1-MDM, M1-like monocyte-derived macrophages; M2-MDM, M2-like monocyte-derived macrophages; n.s., not significant.

withdrawal during HBV inoculation, was sufficient to decrease HBeAg and HBsAg secretion and HBV mRNA by 75% in dHepaRG (Fig. S8A). In addition, treatment with rhIL-6 and rhTNF- α before and during HBV inoculation resulted in an approximately 50% decrease of HBeAg, HBV RNA, and cccDNA formation. HBsAg was lowered by ~50% with rhTNF- α and by ~25% with rhIL-6 treatment (Fig. S8B). rhIL-18 had no effect on the establishment of HBV infection under these conditions (Fig. S8B). Upon rhIL-10 treatment, we observed a slight increase of mean HBeAg and HBsAg levels in the supernatant of dHepaRG cells, but not in PHHs. HBV RNA was modestly reduced in PHHs treated with IL-10 before and during infection (Fig. 8B). Other tested cytokines, TGF- β and MCP-1, had no significant effect on the establishment of HBV infection (Fig. S8B). Of note, we calculated that around 250 pg/ml of rhIL-1 β and 25 ng/ml of rhTNF α are necessary to obtain a 50% decrease in the establishment of HBV infection (Fig. S9), whereas no further decrease was obtained with 20 ng/ml of rhIL-6 (Fig. S9) and there was no effect with increasing doses of rhIL-10 (Fig. S9). Importantly, if the

level of NTCP (sodium-taurocholate co-transporting polypeptide), the HBV receptor, was decreased in dHepaRG after 24 h or 48 h of treatment with IL-1 β (Fig. S10A), it was not affected in PHHs (Fig. S10B), indicating that the measured anti-HBV effect was not uniquely due to modulation of hNTCP expression. Collectively, these data indicate that pro-inflammatory cytokines, and IL-1 β in particular, are very efficient at inhibiting the establishment of HBV infection in hepatocytes.

Next, we investigated whether cell culture supernatants from M1-MDMs exposed or not to HBV would interfere with the establishment of HBV infection in hepatocytes. Even though the final concentrations of IL-1 β and IL-6 were respectively 25-fold and 10-fold lower than the concentration of recombinant cytokines used above (Fig. 8), treatment of dHepaRG cells with supernatants from differentiated M1-MDM cells (Tables S4 and S7) inhibited HBV establishment, as shown by a reduction of the mean HBeAg and HBsAg levels by ~25% (Fig. 9, [no-HBV M1-MDM]-CM). Accordingly, dHepaRG treated with supernatants from M1-MDMs exposed to HBV during activation, which contained similar amounts of pro-inflammatory cytokines to unexposed M1-MDMs (Fig. 4B and Table S7), showed a 40% decrease of secreted HBV antigens (Fig. 9, [HBV-stimulated M1-MDM]-CM). In contrast, dHepaRG cells treated with concentrated supernatants from M1-MDMs exposed to HBV during differentiation, containing less pro-inflammatory cytokines (Fig. 3F and Table S7), showed similar levels of HBV markers to those measured in non-treated dHepaRG cells (Fig. 9, [HBV diff M1-MDM]-CM).

Altogether, these data suggest that the pro-inflammatory secretome derived from M1-MDMs can inhibit the establishment of HBV infection in hepatocytes. In addition, this antiviral effect is lost when monocytes are exposed to HBV during their differentiation.

Discussion

Dendritic cells and M Φ in the liver are thought to play a major role in the orchestration of adaptive responses and can also produce cytokines that may directly affect HBV infection.³⁶ Herein, we confirmed that cytokines secreted by pro-inflammatory M Φ (including IL-1 β and IL-6) strongly inhibit the establishment of HBV infection in hepatocytes. These results are in agreement with our previously reported data showing the antiviral effects of these cytokines in already infected human hepatocytes⁴ and emphasise the potential direct role of pro-inflammatory cytokines in the control of HBV. Of note, even though we observed a decrease in the level of NTCP in dHepaRG cells treated with IL-1 β as previously reported,³⁷ NTCP levels were not changed by IL-1 β treatment in PHHs under conditions that were sufficient for HBV inhibition. These results suggest that IL-1 β can inhibit HBV establishment through an NTCP-independent but still ill-defined molecular mechanism.

Considering the antiviral potential of liver M Φ -derived cytokines, the main scope of this study was to investigate if HBV could interfere with such M Φ pro-inflammatory functions to facilitate the establishment of hepatocyte infection. Using human primary cells, we observed (i) that PLM Φ exposed *ex vivo* to HBV secrete less pro-inflammatory cytokines upon synthetic PAMP stimulation, thus confirming our previously published data,⁸ (ii) that M1-MDMs also secrete less IL-1 β and IL-6 upon activation if exposed to HBV or HBV-producing cells during differentiation and (iii) that the antiviral effect of

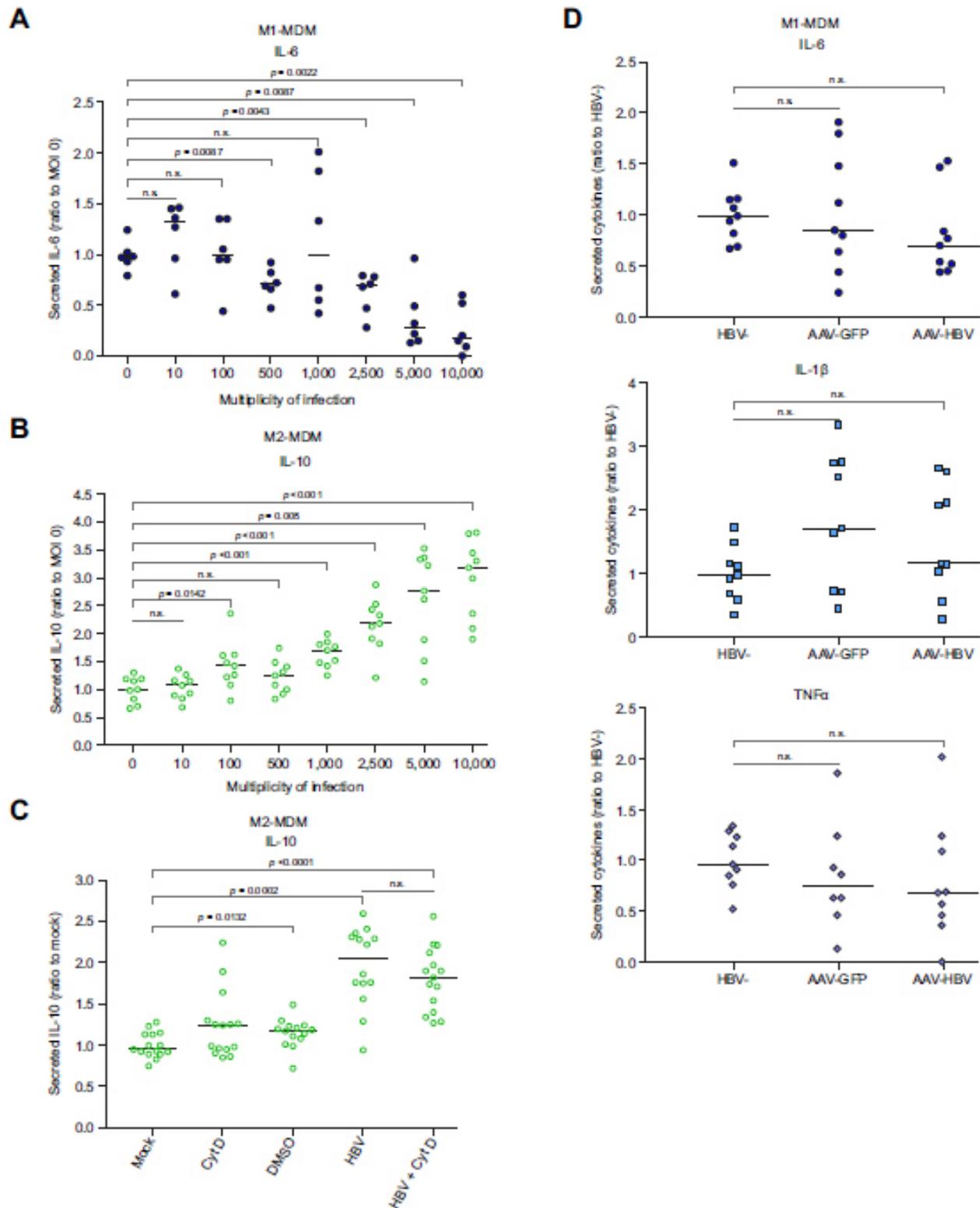


Fig. 5. HBV effect on M Φ is dependent of virus proteins quantity but independent of viral entry and replication. (A-D) Monocytes were purified from peripheral blood mononuclear cells, then differentiated into M1-MDM (upon GM-CSF culture; panels A and D) or M2-MDM (upon M-CSF culture; panels B and C) and stimulated with 10 ng/ml of LPS for 3 h. Cells were exposed or not to HBV, Cytochalasin D, AAV-GFP or AAV-HBV during their differentiation and activation (A,D), or their activation (B,C). (A-D) Levels of the indicated secreted cytokines were assessed by ELISA and analysed as ratio to non-exposed cells (A-B; MOI 0; C: Mock; D: HBV-). Results are the mean of 2 to 5 independent experiments (with 2 or 5 different donors) each performed with 3 biological replicates. Horizontal bars represent the median and data were submitted to Wilcoxon test. AAV, adenovirus vector; HBV, hepatitis B virus; LPS, lipopolysaccharide; M1-MDM, M1-like monocyte-derived macrophages; M2-MDM, M2-like monocyte-derived macrophages; n.s., not significant.

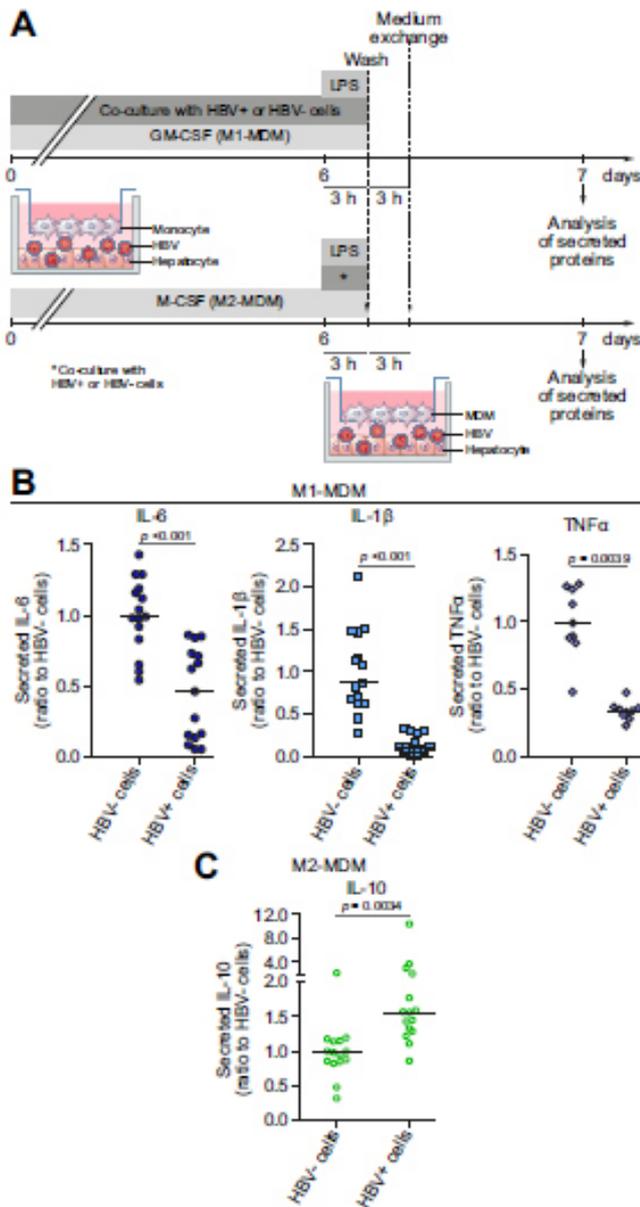


Fig. 6. HBV-producing cells impair M1-MDM differentiation and enhance M2-MDM activation. Monocytes were purified from peripheral blood mononuclear cells, differentiated into (B) M1-MDM (upon GM-CSF culture) or (C) M2-MDM (upon M-CSF culture) and stimulated with 10 ng/ml of LPS for 3 h, in the presence of non-infected HepG2-NTCP (HBV- cells) or HepAD38 (HBV+ cells) during their differentiation or activation, respectively. (A) Schematic representation of the experiment. Medium change is indicated by dotted arrows. (B, C) Levels of the indicated secreted cytokines were assessed by ELISA and analysed as ratio to cells exposed to HBV- cells. Results are the median of 5 independent experiments (with 5 different donors) each performed with 3 biological replicates. Horizontal bars represent the median and data were submitted to Wilcoxon test. HBV, hepatitis B virus; LPS, lipopolysaccharide; M1-MDM, M1-like monocyte-derived macrophages; M2-MDM, M2-like monocyte-derived macrophages; n.s., not significant.

M1-MDM-conditioned medium is lost when cells were differentiated in the presence of HBV. These results are in accordance with those from previously published studies using animal models or M Φ cell lines that reported an ability of HBV to inhibit M Φ responses,^{9,38–41} and further demonstrate that HBV has

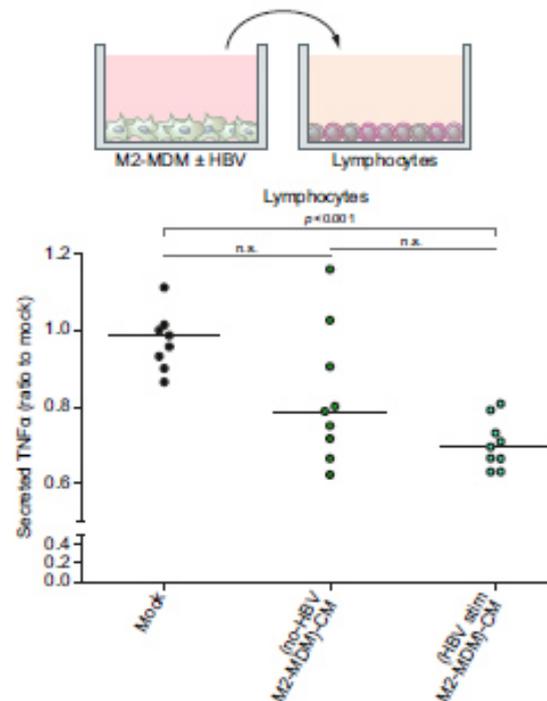


Fig. 7. HBV induces further immunosuppressive properties to M2-MDM. Lymphocytes were purified from peripheral blood mononuclear cells and exposed to media (mock) or LPS-stimulated M2-MDM in the absence (no-HBV M2-MDM) or presence (HBV stimulated M2-MDM) of HBV during their stimulation (CM; conditioned medium) for 2 h before stimulation with 20 ng/ml of phorbol 12-myristate 13-acetate and 500 ng/ml of ionomycin. Supernatants were collected 22 h later and the levels of TNF- α assessed by ELISA. Results, presented as ratio to non-treated cells (mock), are the mean \pm standard deviation of 3 independent experiments (lymphocytes from 3 different donors) each performed with 3 biological replicates. Data were submitted to Wilcoxon test. HBV, hepatitis B virus; LPS, lipopolysaccharide; M1-MDM, M1-like monocyte-derived macrophages; M2-MDM, M2-like monocyte-derived macrophages; n.s., not significant.

evolved strategies to interfere with liver myeloid cell functions, in the absence of any *bona fide* replication (i.e. neo-synthesis of viral genome and proteins) in these cells.

Importantly, this phenotype was similar in naive liver M Φ or MDMs following a short *ex vivo* exposure to the virus or cells replicating the virus, thus suggesting an “easy to implement” mechanism of inhibition, irrespective of a proper replication of HBV in these cells. In addition, we showed that the inhibition of pro-inflammatory cytokines and increase of anti-inflammatory cytokines is dependent on the quantity of virus. However, to be maintained, such an inhibitory phenotype would require a constant exposure to the virus and/or viral components. In this respect, our data showing increased inhibition of the secretion of pro-inflammatory cytokines by M1-MDMs upon constantly renewed exposure to HBV via co-cultures with HBV-producing cells, compared to a single exposure to concentrated virus, support the latest hypothesis. In contrast, the recent study by Suslov and colleagues convincingly showed that the stimulation with synthetic PAMPs (including TLR4 ligands) was not antagonised in *ex vivo* cultivated HBV-positive biopsies.⁴² In this case, the purification procedure and *ex vivo* conditions cultures in the absence of HBV virion or viral components might have reversed the HBV-inhibitory phenotype. Moreover, additional experimental

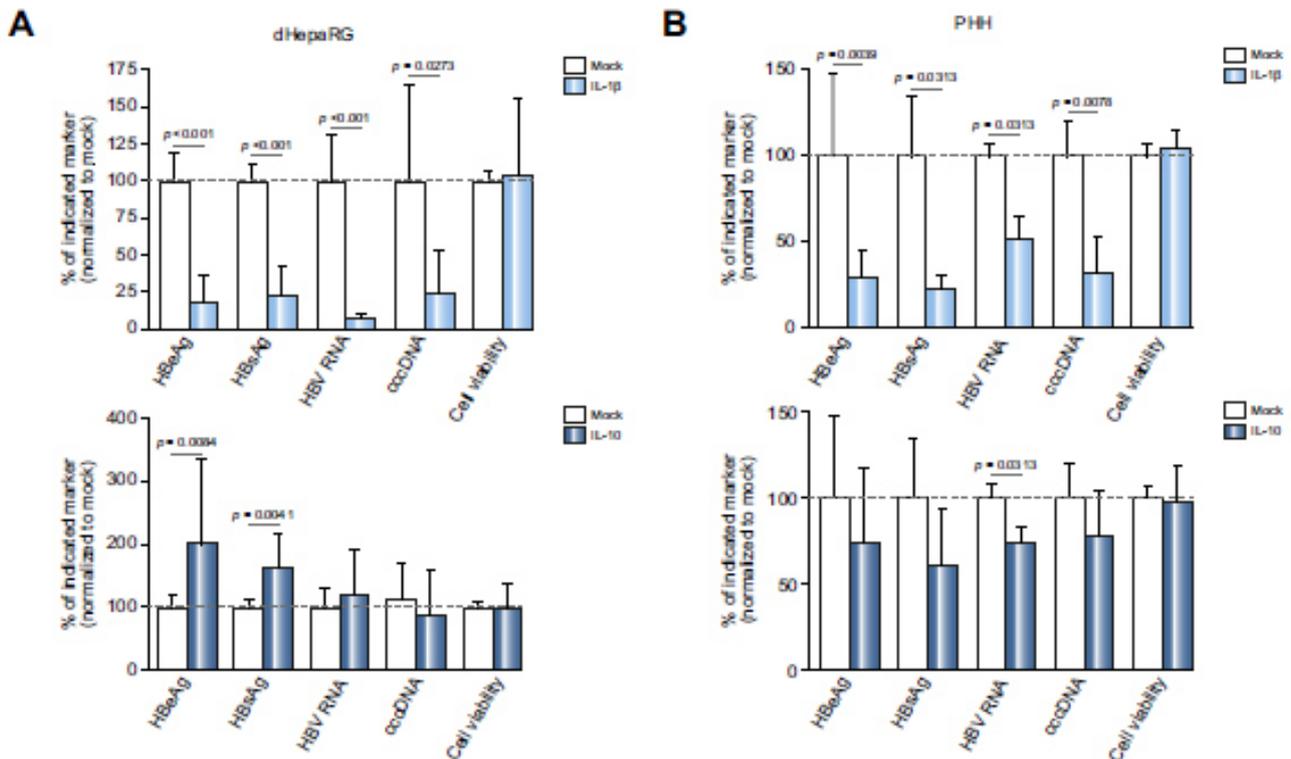


Fig. 8. IL-1 β strongly inhibits establishment of HBV infection in hepatocytes. (A) dHepaRG cells or (B) PHHs were treated with 1 ng/ml of recombinant IL-1 β or 20 ng/ml of recombinant IL-10, 24 h before and during the infection with HBV. Seven days post infection, supernatants were collected and levels of HBeAg and HBsAg quantified by ELISA. Cells were harvested, total RNA or DNA extracted, and levels of HBV RNAs and cccDNA quantified by RT-qPCR or qPCR analysis. Cell viability was assessed by neutral red uptake assay. Results, presented as ratio to non-treated cells, are the mean \pm standard deviation of 3 independent experiments (3 batches of dHepaRG cells and 3 donors of PHH) each performed with 3 biological replicates. Data were submitted to Wilcoxon test. cccDNA, covalently closed circular DNA; HBV, hepatitis B virus; PHH, primary human hepatocytes; qPCR, quantitative PCR; RT-qPCR, reverse transcription quantitative PCR.

pitfalls could explain these opposite results. First, the concentration of TLR4 ligands used in Suslov *et al.*'s study was far higher than those used herein (20 μ g/ml vs. 10 to 100 ng/ml) and probably beyond physiologic conditions. Moreover, despite the commendable effort of Suslov *et al.* to work with highly relevant biological materials, the viability of hepatocytes in these *ex vivo* maintained biopsies was limited and the viability, as well as the functionality, of other liver cells such as liver M Φ were not assessed. It would, therefore, be important to test if the addition of HBeAg/HBsAg/HBV virions in the culture medium of *ex vivo* cultured liver biopsies and the use of physiologic levels of stimulation would recapitulate the inhibitory phenotype we described here.

Importantly, we observed an increase of anti-inflammatory M Φ markers (CD68⁺ CD163⁺ cells) in liver biopsies from HBV-infected patients compared to those from non-infected patients of 2 different cohorts, as well as an increase in the levels of IL-10 secreted by M2-MDMs upon exposure to HBV during activation after *ex vivo* differentiation. These data suggest that HBV may reinforce the previously described liver immune tolerance,¹⁵ helping to establish and/or maintain infection. This is in accordance with studies reporting an increase of anti-inflammatory cytokine secretion (especially IL-10) induced by HBV in animal models and patients^{43–46} and a positive role of anti-inflammatory liver M Φ in HBV persistence.⁴³ Here, we observed that HBV-induced increases in IL-10 secretion by M2-MDMs might impair the activation of lymphocytes. As others had previously described,^{43,47,48} the increased IL-10 expression during

HBV infection could favour a tolerogenic environment and the inhibition of functional adaptive immune responses, compromising HBV elimination. As these anti-inflammatory M Φ are also implicated in affecting distinct oncogenic processes (through the secretion of angiogenic factors, such as VEGF, and the impairment of an anti-oncogenic responses by the secretion of regulatory mediators, such as IL-10⁴⁹), it should be investigated if the HBV-induced modification of M Φ phenotypes may also play a role in hepatocellular carcinoma initiation and promotion.

We did not precisely determine which viral components and which HBV-infection related mechanisms influence cytokine secretion by M Φ . The secreted viral proteins (HBeAg, HBsAg) or viral proteins contained in virions (HBsAg, HBc, viral polymerase) may play a role in the modification of M Φ phenotype. Few data suggest that HBV can be internalised by M Φ (29,44). HBeAg,¹⁰ HBsAg^{9,40} and the HBV capsid protein⁵² have been suggested to contribute to the inhibition of M Φ responses. In the present study, analyses of liver biopsies from HBV-infected patients revealed a co-localisation between HBc and an M Φ marker (CD68; HBcAg). How HBc is delivered to liver M Φ remains to be addressed; it could be through a specific receptor, unspecific engulfment, or by phagocytosis of dying infected hepatocytes. Moreover, it is not yet clear in which subcellular compartment (e.g. organelles; lysosomes) stained HBcAg is located.

Of note, the use of recombinant viral proteins to clarify the involvement of viral proteins in the modification of M Φ

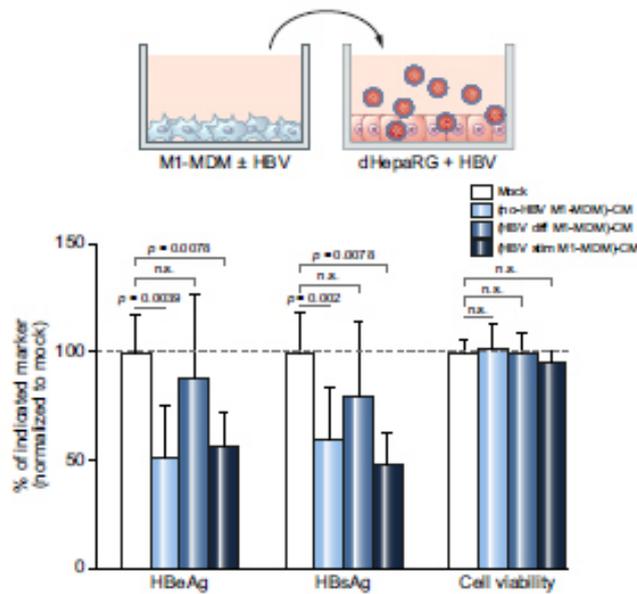


Fig. 9. HBV prevents the antiviral effect of M1-MDM on HBV establishment. dHepaRG cells were treated 24 h before and during HBV infection with concentrated supernatants from LPS-stimulated M1-MDM (CM: conditioned medium pooled from at least 5 independent experiment) exposed to HBV during their differentiation or during their stimulation. Seven days post infection, supernatants were collected and levels of HBeAg and HBsAg quantified by ELISA. Cells were harvested, total DNA extracted and levels of cccDNA quantified by specific qPCR analysis. Cell viability was assessed by neutral red uptake assay. Results, presented as ratio to non-treated cells (mock), are the mean \pm standard deviation of 3 independent experiments each performed with 3 biological replicates. Data were submitted to Wilcoxon test. cccDNA, covalently closed circular DNA; HBV, hepatitis B virus; LPS, lipopolysaccharide; M1-MDM, M1-like monocyte-derived macrophages; M2-MDM, M2-like monocyte-derived macrophages; n.s., not significant; qPCR, quantitative PCR.

responses may be suboptimal, as post-transcriptional modifications associated with a given protein produced in a given cell system (bacteria, yeast or mammalian cells) could be different 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 interferes with the activation of M Φ (and thereby cytokine secretion) at different levels. Indeed downregulation of innate sensors such as TLR^{41,53,54} and inhibition of cell signalling pathways^{30,40,47} have been reported. Epigenetic modulations induced by the fixation of 1 viral protein to gene promoters, as reported in hepatocytes⁵² and as also reported for other viruses,^{55–57} might also be implemented in M Φ even though HBV do not replicate in those cells. Importantly, the different effects observed are specific to HBV as AAVs had no impact on M1- and M2-MDM secretions. Our data suggest that HBV productive entry into cells (i.e. with fusion of the membrane between viral particles and cells) may not be absolutely required to induce phenotypic changes in M Φ . One of several viral proteins present in the inoculum or circulating in the blood of patients (HBsAg and HBeAg) may bind to receptor(s) of M Φ involved in modulation of inflammatory responses. As described before, the activation of specific regulatory receptors at the surface of M Φ can trigger inhibition of pro-inflammatory cytokines secretion.^{58–60} It will therefore be important to determine the target(s) of HBV, on the surface of M Φ , which are responsible

for the modulations observed, in order to develop specific molecules targeting this interaction.

Importantly, in our different experimental settings, we did not detect any cytokine secretion by IMNCs, primary human liver M Φ or MDMs exposed to cell-culture-produced HBV inocula or co-cultured with HBV producing cells (data not shown). This is in sharp contrast with chronic HCV or HIV infections that induce strong host responses,^{61–64} 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,^{42,45,62,65–67} leading to the definition of HBV as a stealth virus.⁶⁸ Of course, as also shown here, this stealthy character could be due to active evasion and not complete passivity. In contrast, several groups have reported an induction of innate responses following exposure to HBV.^{7,25,50,69,70} The quality of the HBV inoculum used to perform the *ex vivo* analyses is probably very different between studies, since no standard inocula are available and only very few manuscripts reported the characterisation of their inocula as we did here (Fig. S2). Indeed, different HBV-producing cells, different culture conditions and concentration procedures may lead to different ratios of viral antigens, subviral particles and Dane particles, as well as contaminating non-enveloped nucleocapsids, recognised by TLR2,²⁵ or endotoxins, recognised by TLR4,⁷¹ in the inocula, which may explain the discrepancies between studies.

From a therapeutic point of view, we herein confirmed that IL-1 β inhibits HBV establishment and replication in hepatocytes very efficiently. However, recombinant IL-1 β cannot be used systemically without risking severe side effects such as cytokine storms. New anti-HBV therapeutic options should aim at inducing the local and endogenous secretion of IL-1 β to induce antiviral activity and prevent the development of pathogenesis. Different strategies may be considered to promote pro-inflammatory over anti-inflammatory phenotypes in liver resident and infiltrating M Φ . For instance, the delivery of GM-CSF in cancerous tissue has been shown to induce tumour regression^{72,73} by promoting pro-inflammatory M Φ . Inversely, a monoclonal antibody against CSF-1R (colony stimulating factor 1 receptor) showed promising results in reducing the number of anti-inflammatory and tumour-associated M Φ .¹⁵ Alternatively, inhibitors of glutaminolysis, that modify the succinate/ α -keto glutarate balance in M Φ ⁷⁴ or specific inflammasome and/or pathogen recognition receptor inducers could be evaluated.⁷⁵

In summary, we showed that HBV can modulate the resident and transiting- M Φ phenotypes to favour its establishment, and likely its maintenance in the liver. HBV escapes the antiviral effect of pro-inflammatory liver resident and infiltrating M Φ by interfering with their activation/differentiation and inhibiting the production of cytokines endowed with anti-HBV activities. Besides, HBV infection promotes the activation of anti-inflammatory M Φ and the production of IL-10 in the microenvironment, likely favouring tolerance. Our data also suggest that therapeutic strategies promoting the differentiation of pro-inflammatory liver M Φ over anti-inflammatory ones or the shift from one to the other should be tested to help break immune tolerance and promote the functional cure of chronic HBV infections.

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Conflicts of interest

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

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

Study concept and design: SFD, MD, JL, DD. Acquisition of data: SFD, MD, LA, LD, JF, DH *et al.* Analysis and interpretation of data: SFD, MD, AL, KK, JL, DD. Drafting of the manuscript: SFD, JL, DD. Critical review of the manuscript: AL, KK, NBV, FZ, MH. Statistical analyses: SFD. Material support: MR, NBV, FL, RP, MSM, FZ.

Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jhep.2019.06.032>.

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IV. Supplementary Results

The data we generated raised some questions.

First, we demonstrated that HBV is capable of decreasing IL-6/IL-1 β cytokine secretion in MDMs and liver PLMs but we do not know which viral(s) component(s) is responsible for the observed modulations. In addition, we used only HBV-D genotype in this article. However, as mentioned already, 10 genotypes of HBV are known, with some associated with differential severity of the disease and sensitivity to IFN α treatment, for instance. We thus wanted to address if the HBV-induced modulations of M Φ s might differ from one HBV genotype to another. Finally, as CHB patients can be co-infected with HDV, an analysis of HDV inocula impact on M Φ s is essential, especially as both viruses share the same envelope.

Second, we demonstrated that treatments with cytokines such as IL-1 β (that are produced upon stimulation of pro-inflammatory macrophages) decreased HBV infection, strongly suggesting that HBV modulations of M Φ may favor its establishment within the liver. We next wanted to address how exactly HBV is inhibited by IL-1 β when infection is already established, if this is similar according to the genotypes and if HDV would also be affected.

In the following section, we will try to answer or provide clues to the aforementioned questions.

1. HBV from different genotypes modulates the levels of cytokine secreted by MΦs.

To assess if the HBV-induced modulations of macrophage is genotype dependent, we produced HBV inocula from genotypes B, C and D. HBV-D was produced by concentration of supernatants from HepAD38 (Ladner et al. 1997). HBV-B and HBV-C were produced by concentration of supernatants from cell lines (constructed in our team from the HepG2 backbone) stably expressing HBV genotype B or C. Concentrated supernatants were characterized for their content on HBV particles and HBV antigens by ELISA and qPCR analyses (Figure 1.A). Quality of the inocula were also assessed by analyses of fractions from iodixanol gradients (Figure 1.B, C and D). HBV-B inocula contained 20 to 70 more HBsAg/ and HBeAg (respectively) than viral genome compared with HBV-C and HBV-D.

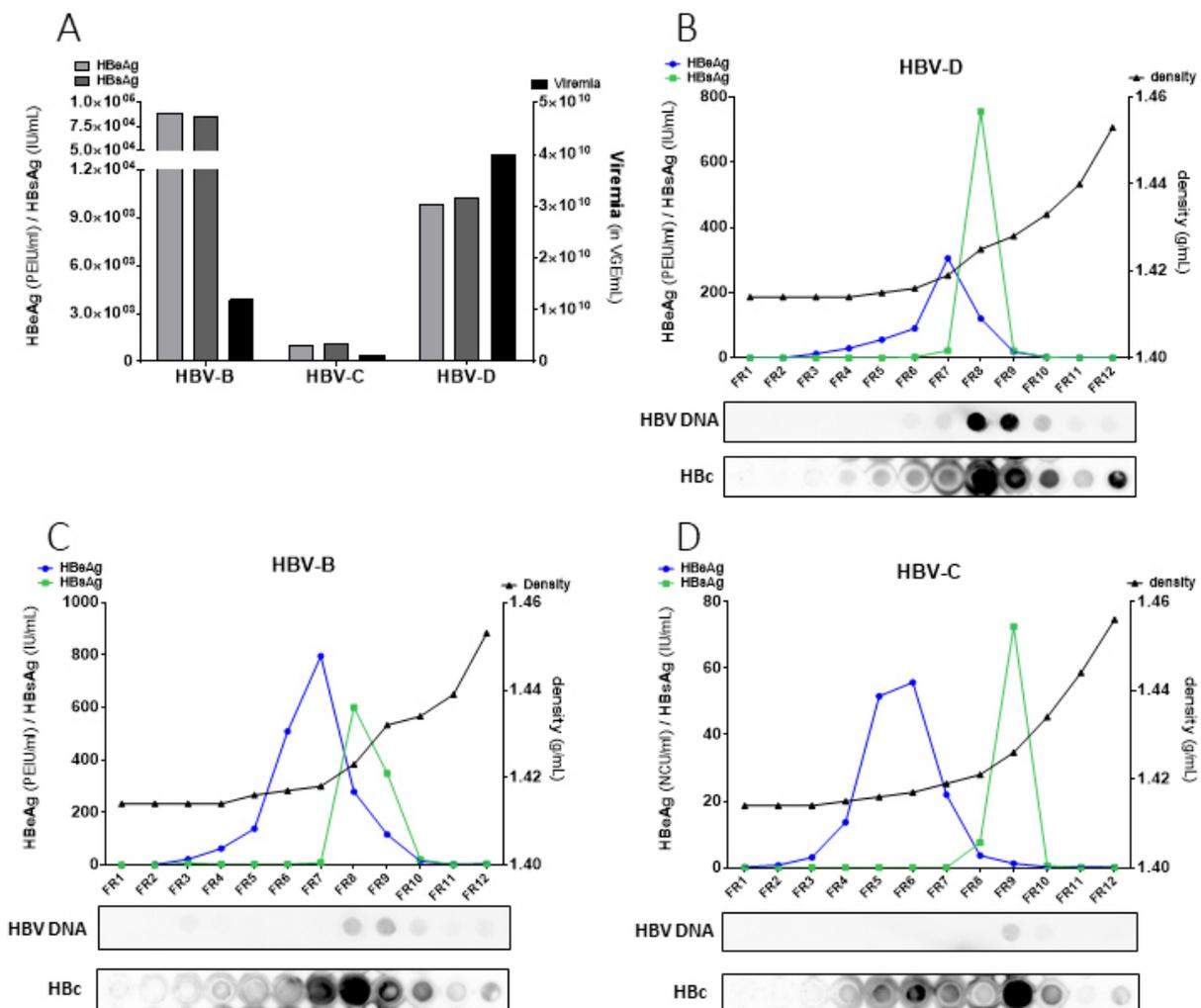


Figure 1: Characterization of HBV-B/C/D inocula

Supernatants from HepG2-HBV-B/C and HepAD38 were concentrated by ultracentrifugation. (A) Levels of HBeAg and HBsAg were quantified by ELISA whereas levels of HBV genomes were quantified by qPCR analyses. (B-D) Concentrated supernatants were submitted to 5.6-56% iodixanol gradients and fractions were analyzed for their content in HBeAg and HBsAg (by ELISA), in HBV DNA (by dot blot analyses) as well as in HBc (by western blot analyses).

PLMs, purified from non-infected hepatic resection (protocol described in (Faure-Dupuy, Delphin, Aillot, Dimier, Lebossé, Fresquet, Parent, Sebastian Matter, et al. 2019)), were incubated with equal amount of viral HBV genomes (1000 VGE/cell) from genotypes B, C and D before stimulation with LPS (Figure 2.A). As described before, (Faure-Dupuy, Delphin, Aillot, Dimier, Lebossé, Fresquet, Parent, Sebastian Matter, et al. 2019), *ex-vivo* short-term exposure of HBV-D led to a low (15-20%) but significant decrease of the level of IL-1 β secreted by PLMs compared to the levels of IL-1 β secreted by mock cells (non exposed to HBV) (Figure 2.A).

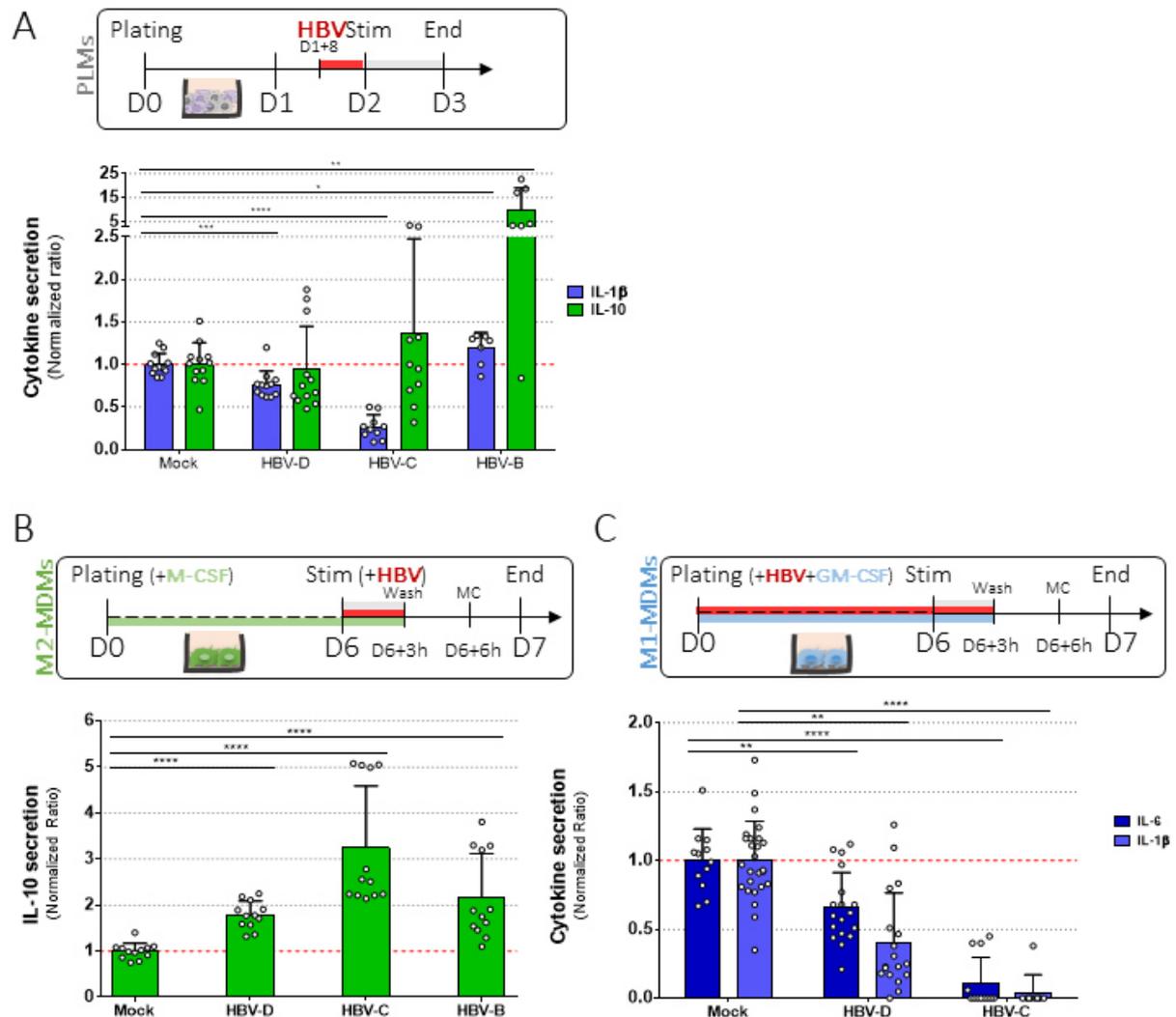


Figure 2: HBV from different genotypes modulates the levels of cytokine secreted by primary liver macrophages as well as differentiated MDMs.

(A) PLMs isolated from liver resections were exposed to 1000 VGE/cell of HBV-D, -C and -B overnight, before LPS stimulation (100 ng/mL) for 24 hours. (B-C) M1- and M2-MDMs purified from blood bags were differentiated for 6 days with 50 ng/mL of GM-CSF or M-CSF respectively. MDMs were incubated with HBV (1000 VGE/mL) during their activation (M2-MDMs) or their differentiation (M1-MDMs) process. On day 6, all cells were stimulated with 10 ng/mL of LPS for 24 hours. In all experiments, harvested supernatants were submitted to ELISA for quantification of IL-1 β , IL-6 and IL-10 secretion levels. Results are the mean \pm SD of at least four independent experiments (i.e four different donors) each performed with four biological replicates and statistical analyses were performed using Mann-Whitney tests.

Exposure of PLMs to HBV-B had poor effect on IL-1 β secretion whereas exposure to HBV-C lead to 80% reduction of the levels of secreted IL-1 β (**Figure 2.A**). Inversely, exposure of PLMs to HBV-D or HBV-C had no major impact on IL-10 secretion while a 10 to 20-fold increase of IL-10 secretion was observed when cells were exposed to HBV-B compared to naive cells (**Figure 2.A**). As a model to mimic infiltrating macrophages, we then used Monocyte-Derived-Macrophages (MDMs) polarized toward pro-or anti-inflammatory phenotype using GM-CSF or M-CSF (**Figure 2. B and C**), as previously described (Faure-Dupuy, Delphin, Aillot, Dimier, Lebossé, Fresquet, Parent, Sebastian Matter, et al. 2019). We already demonstrated that HBV-D impaired the differentiation of M1-MDM, decreasing the secretion of IL-6 and IL-1 β , and increasing the activation of M2-MDM, which secreted more IL-10 (Faure-Dupuy, Delphin, Aillot, Dimier, Lebossé, Fresquet, Parent, Sebastian Matter, et al. 2019). Interestingly, M2-MDMs exposed to HBV-C or HBV-D during their activation process all secreted significantly more IL-10 than non exposed cells (**Figure 2.B**). As already observed (Faure-Dupuy, Delphin, Aillot, Dimier, Lebossé, Fresquet, Parent, Sebastian Matter, et al. 2019) and confirmed here again for HBV-D, we observed a decrease of IL-1 β and IL-6 secretion by M1-MDMs when cells were incubated with the virus during their differentiation (**Figure 2.C**). Interestingly, HBV-C incubation led to stronger decrease than HBV-D, with most of the values under the detection threshold for both IL-6 and IL-1 β (**Figure 2.C**). Due to limitations in the production of HBV-B and issue with MDM differentiations (that varies from donor to donor), the effect of HBV-B on M1-MDM differentiation could not be assessed. Even though modulation may vary in their intensity, overall, these data suggest that HBV-induced modulations of macrophages is not genotype dependent and all the ones we tested seem efficient in inducing a tolerogenic secretive phenotype in M Φ .

2. M Φ s exposure to HBV increased the expression of inhibitory receptors.

As described in the introduction part, immune checkpoint proteins are stimulatory or inhibitory regulators that play a key role in maintaining immune homeostasis and preventing the onset of autoimmunity. Also, they are found increased in HBV infected patients (Nebbia et al. 2012; Jin et al. 2010, 1; A. Huang et al. 2014, 1; Shayan et al. 2017, 1). In order to get first hints into the mechanisms of HBV-induced M Φ s modulations, we assessed the expression of inhibitory receptors in macrophages exposed to HBV or not (**Figure 3**).

We observed an increase of inhibitory receptors Tim-3 and its ligand Gal-9, as well as PD-1 ligand (PD-L1) at the mRNA (**Figure 3.A**) and protein levels for Tim-3 and PD-L1 (**Figures 3.B**) in M1-MDMs exposed to HBV-D during their differentiation process, compared to naïve cells. Of note, since Gal9 is secreted in the supernatant, its protein expression was not assessed by western blot like the others. These results are thus in line with the literature (Nebbia et al. 2012; Jin et al. 2010, 1; A. Huang et al. 2014, 1; Shayan et al. 2017, 1).

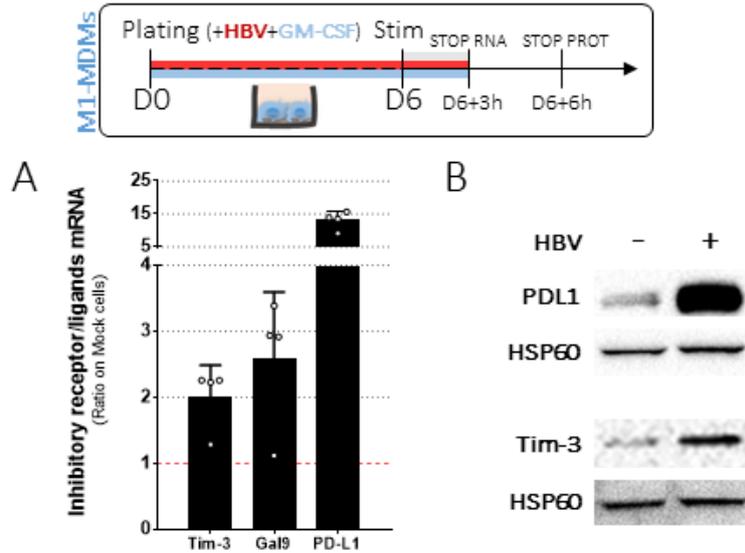


Figure 3: Exposure of M1-MDMs to HBV increased the expression of inhibitory regulators. Schematic view of the protocol. M1-MDMs were differentiated in presence of HBV-D and stimulated with 10 ng/mL of LPS. Cells were harvested 3 and 6 hours post stimulation. **(A)** Total intracellular RNAs were extracted and levels of the indicated mRNAs were analyzed by RT-qPCR. Results are from one experiment performed with four biological replicates. **(B)** Cell lysis were submitted to Western Blot analyses for PDL1, Tim3 and HSP60 proteins (control).

3. HBV modulates cytokine secreted by MΦ through HBsAg

Next, we aimed to identify the HBV protein(s) responsible for its modulations of cytokine secretions in MΦ. Indeed, this question is debated (Faure-Dupuy et al. 2018; M. Jiang et al. 2014; X. Yu et al. 2017) and it is worth noticing that most of the studies used recombinant proteins or made correlations with HBeAg and HBsAg positive/negative patients to conclude in the immune modulatory effect of HBV. In our hands, such experiments were inconclusive (data not shown). We decided to use a co-culture system in transwell (excluding cell to cell contact), between MDMs and HepAD38 cells (Faure-Dupuy, Delphin, Aillot, Dimier, Lebossé, Fresquet, Parent, Sebastian Matter, et al. 2019). In our previous study (Faure-Dupuy, Delphin, Aillot, Dimier, Lebossé, Fresquet, Parent, Sebastian Matter, et al. 2019), we showed a stronger effect on MDMs secretion modulations in this co-culture setting compared to exposure of MDM with HBV inocula, most probably because co-culture led to constant exposure of MDM to viral particles. Moreover, because of the inducible and endogenous promoters for HBV production in HepAD38, HBV genome containing particles and HBeAg secretions can be abrogated without affecting HBsAg release (Ladner et al. 1997) as we recapitulated here (**Figure 4.A**). As a control, cells were also transfected with siRNAs targeting all HBV RNAs (in the HBx region of the HBV genome) and thereby abrogating secretions of all HBV particles and antigens (**Figure 4.A**).

M1-MDMs were incubated with HepAD38, which were previously treated or not with tetracyclin and/or siRNA against HBV. We confirmed (Faure-Dupuy et al. 2019) the impairment of IL-1β and IL-6 secretions by M1-MDMs co-cultured with HepAD38 during their differentiation

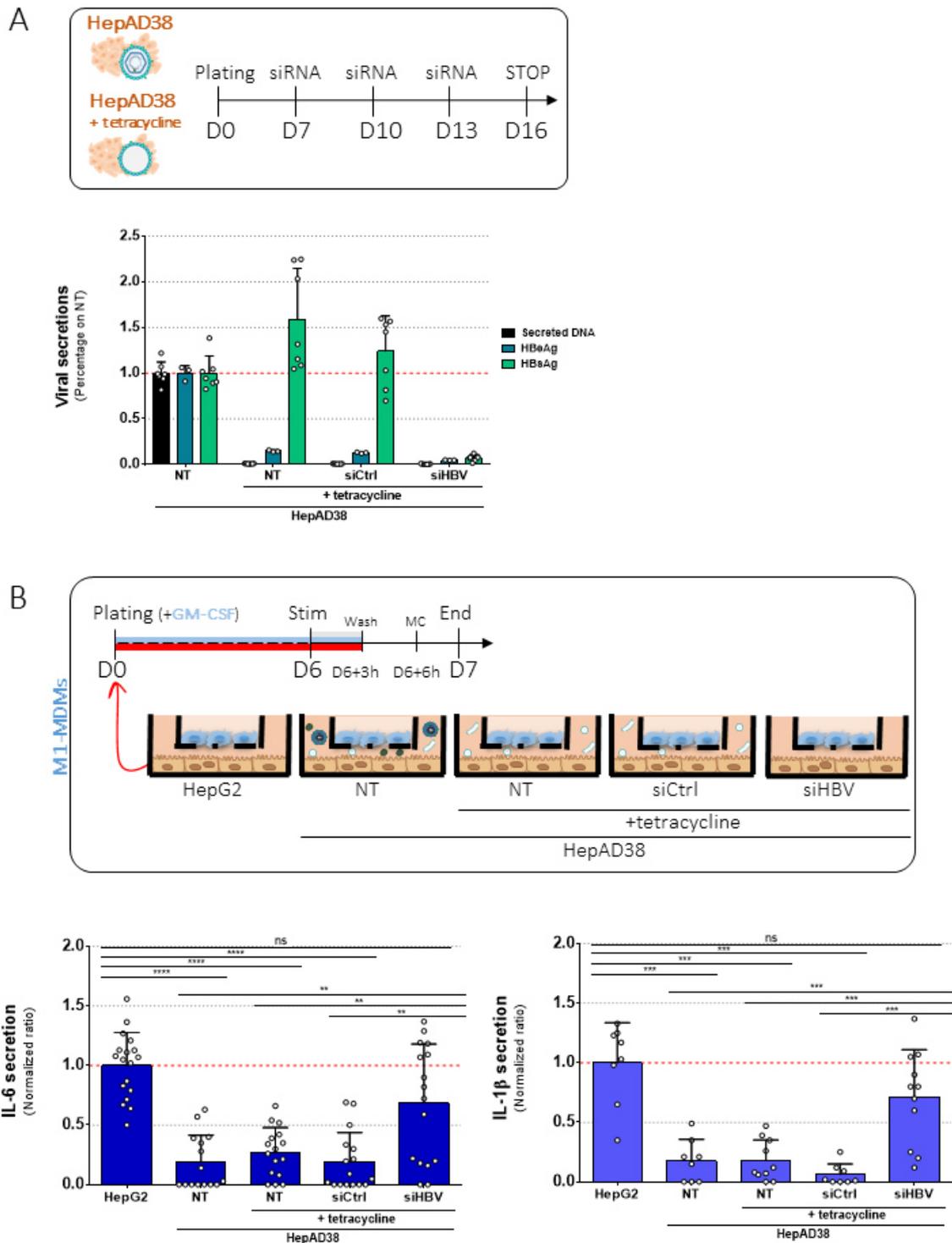


Figure 4: Exposure to HBsAg alone is able to decrease the levels of IL-6 and IL-1 β secreted by M1-MDMs. **(A)** Schematic view of the protocol. HepAD38 cells were treated or not with tetracycline (1 ng/mL) for 30 days before plating to fully abrogate HBV particles and HBeAg secretion. Seven days post-plating, cells were transfected or not with the indicated siRNA (three times with three days between transfections). Supernatants were harvested three days after the final transfection to quantify HBV secreted DNA by qPCR analyses as well as HBeAg and HBsAg by ELISA. **(B)** M1-MDMs purified from blood bags were co-cultured in transwell during their differentiation process (6 days with 50ng/mL of GM-CSF) with HepG2 or HepAD38 treated or not with tetracycline and/or siRNA. Supernatants were collected 24 hours after LPS stimulation (10 ng/mL) and IL-1 β and IL-6 were quantified by ELISA. Results are the mean \pm SD of at least four independent experiments (i.e. four different donors) each performed with four biological replicates and statistical analyses were performed using Mann-Whitney tests.

compared to M1-MDMs co-cultured with HepG2 (the parental cell line that does not express HBV) (Figure 4.B). Interestingly, we observed the same reduction in the levels of IL-1 β and IL-6 secretions by M1-MDMs co-cultured with HepAD38 only producing HBsAg (treated with tetracycline) and a reversion of this reduction when M1-MDMs were co-cultured with HepAD38 cells transfected with siRNA against HBV (Figure 4.B). Altogether, these data strongly suggest that HBsAg alone is responsible for the HBV-induced modulations of M Φ s.

To further confirm our results, we used another cell type, this time Huh7 integrated or not with HBsAg. Besides, since HDV is a satellite virus of HBV, which shares its envelope protein, we investigated whether HDV virions (i.e HDV ribonucleoprotein enveloped within HBsAg) would have a similar impact on M Φ phenotype, using the same cell type. Thus, we set up co-culture experiments, with Huh7, Huh7-HBsAg and Huh7-HBsAg-HDV+ cells (stably producing HBsAg or HBsAg and HDV particles, respectively; obtained through a collaboration with E. Verrier in the team of T. Baumert) (Figure 5). Co-culture with Huh7-HBsAg and Huh7-HBsAg-HDV+ cells led to a strong reduction (90%) in the levels of IL-6 and IL-1 β secreted by M1-MDMs, compared with M1-MDMs exposed to Huh7 (Figure 5).

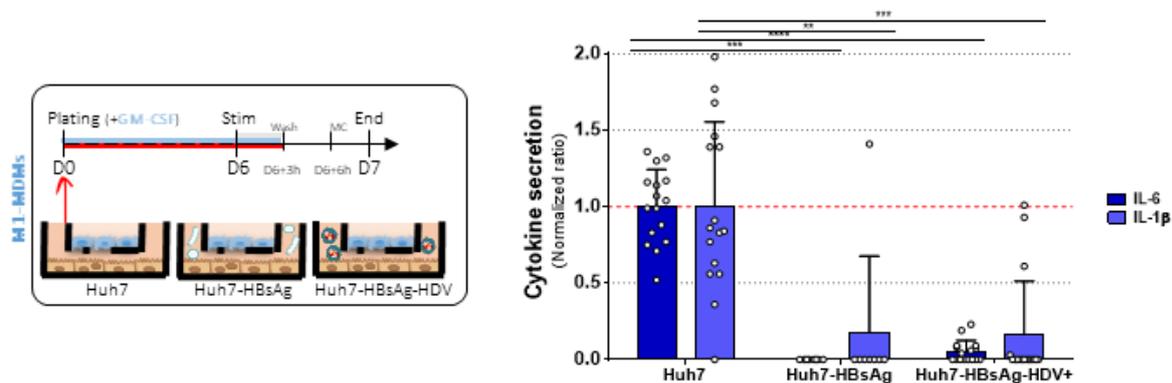


Figure 5: Exposure to HBsAg and possibly HDV particles decreases the levels of IL-6 and IL-1 β secreted by M Φ s.

Schematic view of the co-culture protocol used. M1-MDMs purified from blood bags were co-cultured in transwell during their differentiation process (6 days with 50ng/mL of GM-CSF) with Huh7, Huh7-HBsAg and Huh7-HBsAg-HDV+ cells. Supernatants were collected 24 hours after LPS stimulation (10 ng/mL) and IL-1 β and IL-6 were quantified by ELISA. Results are the mean \pm SD of four independent experiments (i.e two different donors) each performed with four biological replicates and statistical analyses were performed using Mann-Whitney tests.

4. Levels of HBV RNAs (from different genotypes) and HDV RNAs are decreased in dHepaRG cells treated with IL-1 β

IL-1 β was suggested to be the most potent antiviral cytokine against HBV, compared to the panel of interleukin/interferon tested, with an IC₅₀ of 50 pg/mL, without any observed cytotoxicity (Isorce et al. 2016). As it was one of the main cytokines decreased by HBsAg, we decided to screen the effect of IL-1 β treatment on different HBV genotypes as well as on HDV.

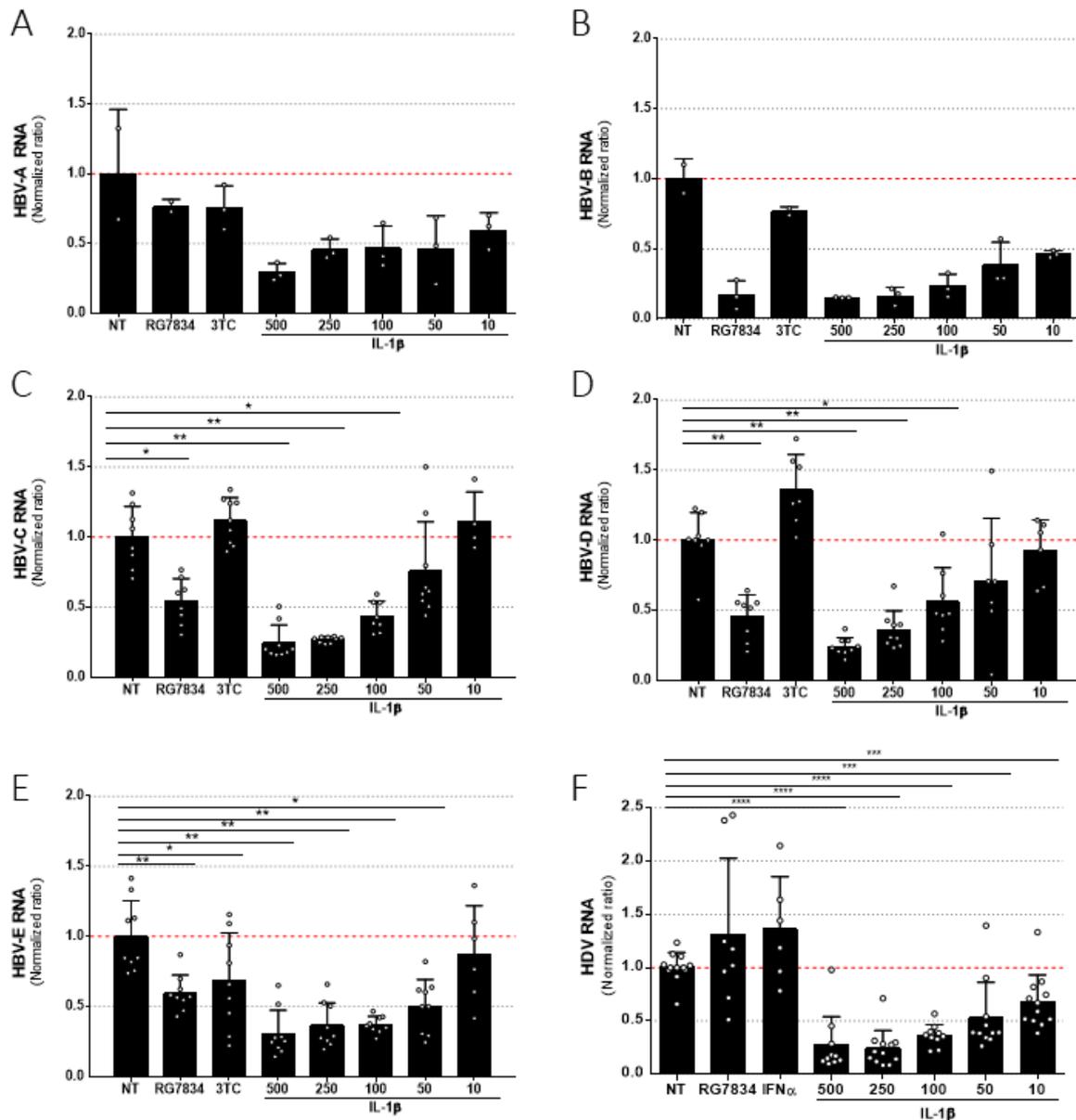
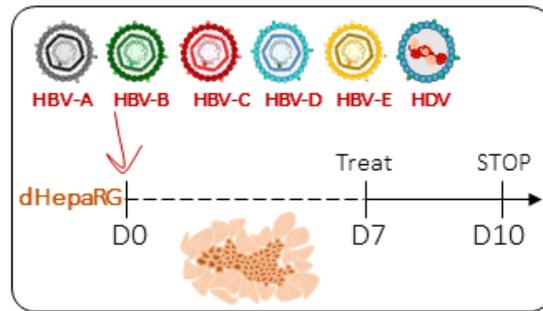


Figure 6: HBV-A to -E and HDV are susceptible to IL-1 β treatments.

Schematic view of the protocol. dHepaRG infected with HBV-A to -E (100 VGE/cell) or HDV (10 VGE/cell) were treated with increasing doses of IL-1 β (10 to 500 pg/ml). RG7834 (1 μ M), 3TC (1 μ M) and IFN α (500 UI/ml) were used as control. dHepaRG were harvested three days later and total RNAs were extracted. Levels of (A) HBV-A, (B) HBV-B, (C) HBV-C, (D) HBV-D, (E) HBV-E or (F) HDV RNAs were quantified by RT-qPCR analysis. Results are the mean \pm SD of (A-B) one or (C to F) three independent experiments each performed with three biological replicates and statistical analyses were performed using Mann-Whitney tests.

dHepaRG were infected with HBV-A to -E or HDV for seven days before being treated for three days with different doses of IL-1 β as well as with RG7834, 3TC and IFN α as controls (Figure 6). The levels of HBV RNAs from all genotypes as well as the levels of HDV RNAs were dose-dependently reduced by IL-1 β with an IC50 around 50-100 pg/mL (Figure 6. A-F). Of note, as mentioned before, due to technical difficulties for viral productions, HBV-A and HBV-B infection were only performed once and thus require further experiments to conclude.

5. The levels of viral RNAs are decreased very early after IL-1 β treatment

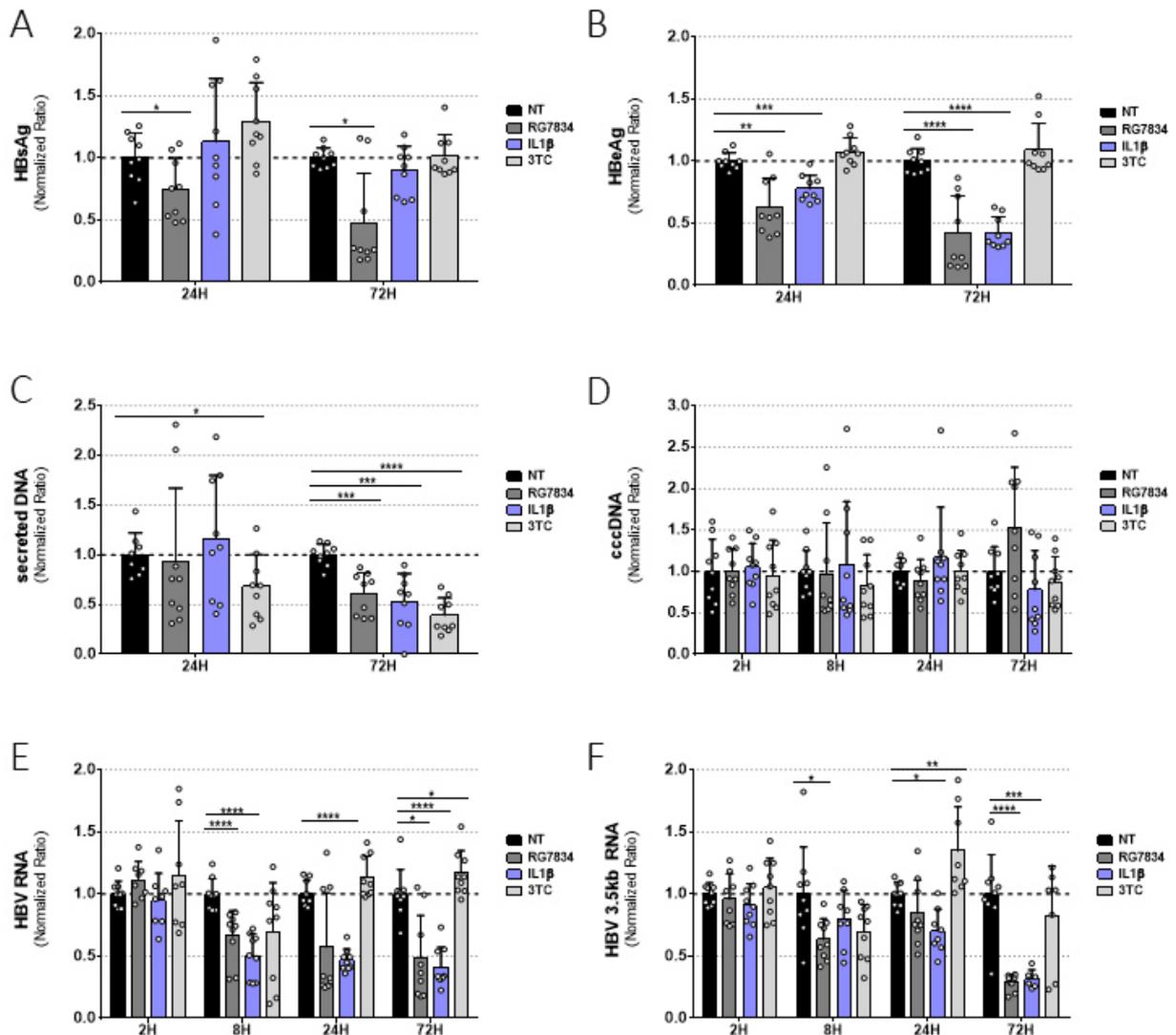
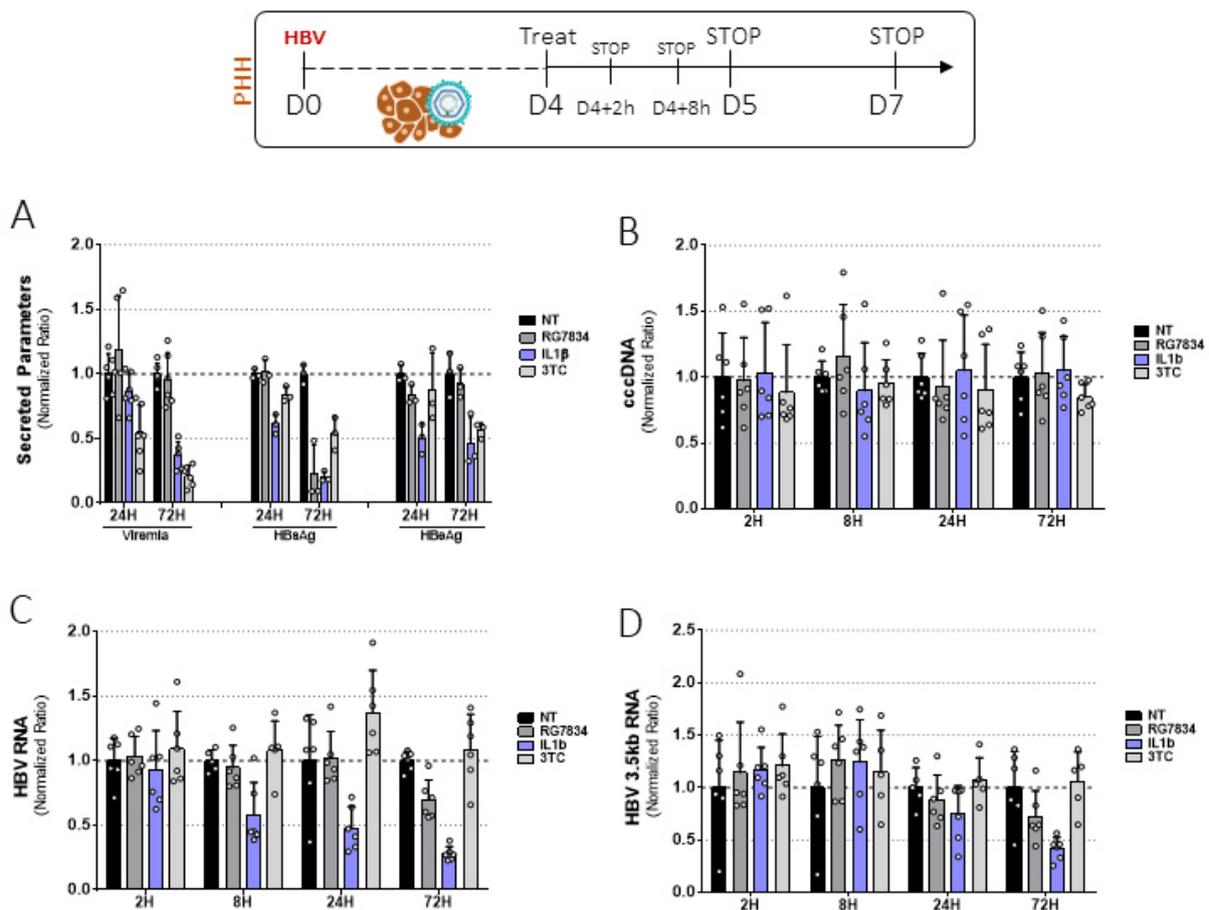


Figure 7: The levels of HBV RNAs are decreased very early after IL-1 β treatment in dHepaRG. Schematic view of the protocol. dHepaRG infected with HBV-D (100 VGE/cell) were treated with IL-1 β (100 pg/mL), RG7834 (1 μ M) or 3TC (1 μ M) for 2h, 8h, 24h and 72h. (A-B-C) Supernatant was harvested at 24h and 72h post treatment. Quantification of (A) HBsAg and (B) HBeAg by ELISA, or (C) HBV secreted DNA by qPCR. (D-E-F) dHepaRG cells were harvested at 2h, 8h, 24h and 72h post-treatment and total RNAs or cccDNA were extracted and analyzed by qPCR or RT-qPCR for (D) cccDNA, (E) HBV total RNA or (F) 3,5kb HBV RNA. Results are the mean +/-SD of three independent experiments each performed with three biological replicates and statistical analyses were performed using Mann-Whitney tests.

To try to unravel the mechanism behind the antiviral effect of IL-1 β treatment, we followed the levels of HBV parameters very early after treatment. HBV infected dHepaRG or PHH were treated once with IL-1 β , RG7834 or 3TC as controls. Supernatants and cells were collected 2h, 8h, 24h or 72h after treatment and viral parameters were analyzed at the extracellular (Figure 7. A, 7.B and 7.C, 8.A) and the intracellular level (Figure 7.D, 7.E, 7.F, 8.B, 8.C, 8.D). Of note, extracellular parameters were only analyzed at 24h and 72h to ensure sufficient accumulation in the supernatant for detection (Figure 7.A, 7.B, 7.C, 8.A).



As expected, because HBsAg is well-known to be especially difficult to decrease in dHepaRG cells, the viral envelope protein was not reduced upon IL-1 β treatment, whereas RG7834 did (as expected from the literature (Han et al. 2018)) (Figure 7.A). In PHH, the levels of secreted HBeAg and HBsAg were decreased faster following treatment with IL-1 β compared to treatment with RG7834 (Figure 8.A). Even though with a slower kinetic than 3TC, IL-1 β decreased HBV secreted DNA as efficiently at 72h post-treatment (Figure 7.C and 8.A). Levels of cccDNA were not affected at all in any conditions (Figure 7.D, 8.B) whereas the levels of total HBV RNAs were already significantly decreased by 50% starting 8h, and the 3.5kb RNA by 50% at 72h (Figure 7. E, 7.F, 8.C, 8.D). These reductions were similar to the ones observed with RG7834 in dHepaRG and even stronger in PHH. Of note, as expected, 3TC did neither decrease the levels of RNA nor the levels of cccDNA (Figure 7.E, 7.F, 8.C, 8.D).

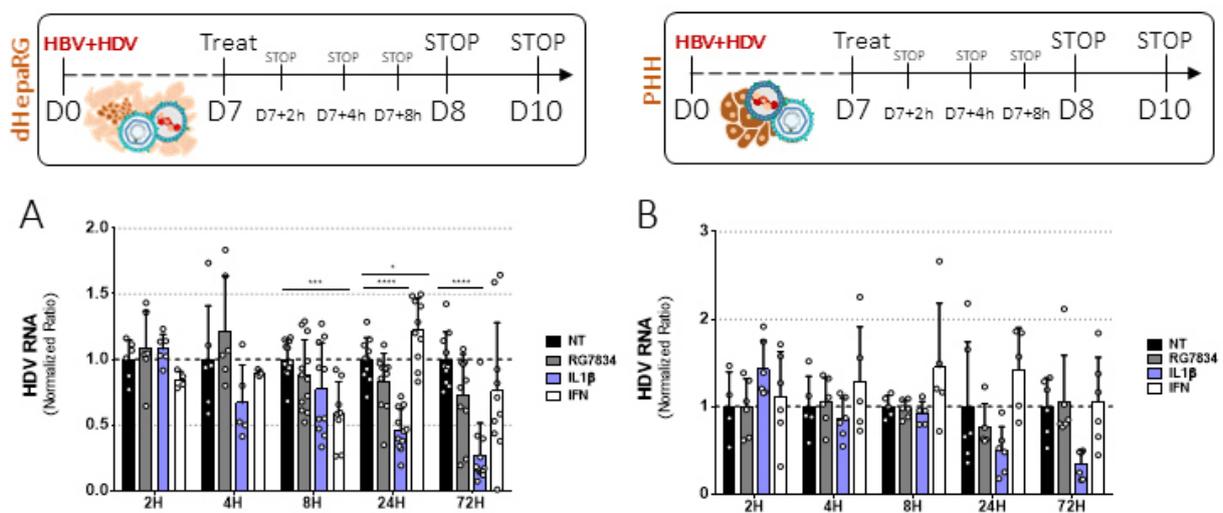


Figure 9: The levels of HDV RNAs are decreased very early after IL-1 β treatment in dHepaRG and PHH. Schematic view of the protocol. (A) dHepaRG and (B) PHH co-infected with HBV-D (100 VGE/cell) and HDV (10 VGE/cell) were treated with IL-1 β (100 pg/mL), RG7834 (1 μ M) or IFNa (500 IU) for 2h, 4h, 8h, 24h and 72h. Total RNA from dHepaRG and PHH were harvested at 2h-4h-8h-24h-72h post-treatment. Total RNAs were extracted and levels of HDV RNAs analyzed by RT-qPCR. Results are the mean \pm SD of three independent experiments each performed with three biological replicates and statistical analyses were performed using Mann-Whitney tests.

A similar approach was used to investigate IL-1 β mediated HDV parameters decrease (Figure 9). We observed a significant decrease of HDV RNA at 24h post treatment in both dHepaRG and PHH (Figure 9.A).

6. IL-1 β treatment leads to induction of the NF κ B pathway

Next, we investigated if IL-1 β was inducing the NF κ B pathway, as established previously (Boraschi et al. 2018) or if it activates the IFN pathway, as suggested by others (Orzalli et al. 2018). Thus, we analyzed the expression of some prototypic genes of both pathways, namely IL-6/A20, for NF κ B, and MxA/OAS1, for the IFN.

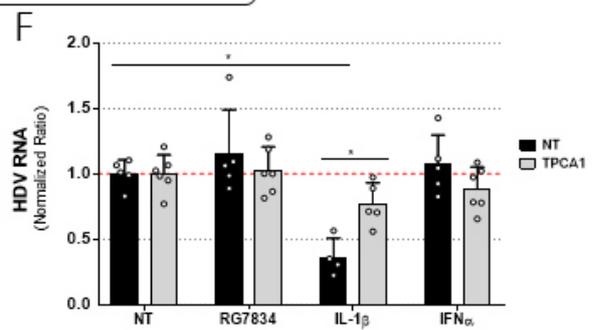
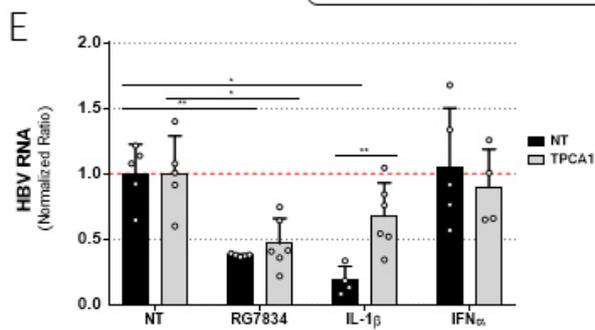
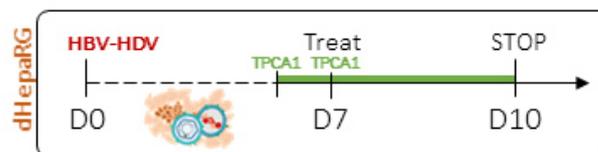
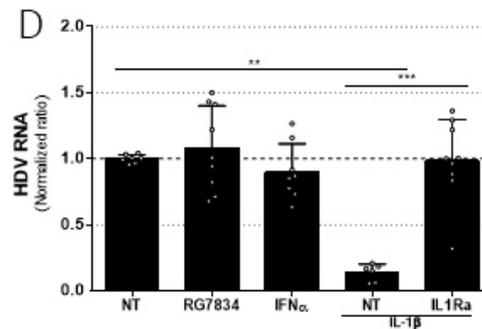
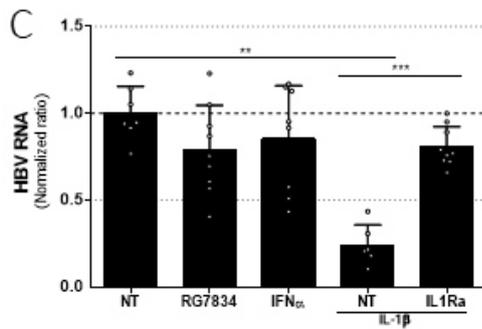
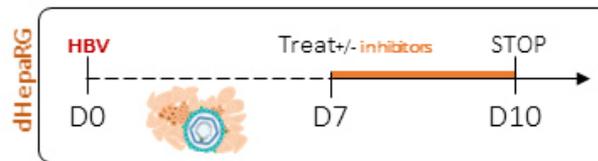
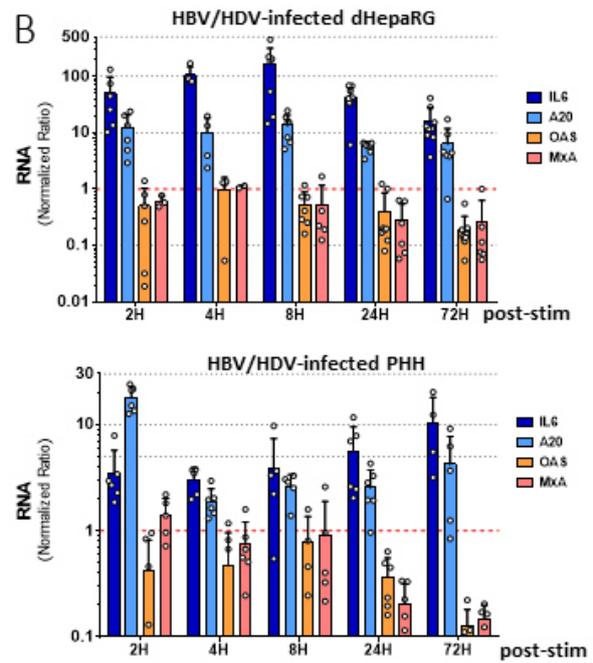
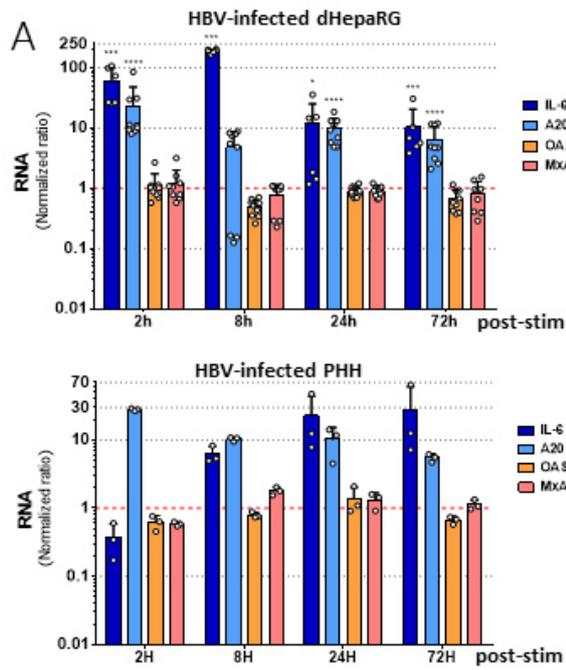


Figure 10: IL-1 β decreases viral RNA by IL-1R mediated NF κ B pathway activation.

(A-B) dHepaRG or PHH infected with (A) HBV-D (100 VGE/mL) or (B) HBV-D (100 VGE/mL) and HDV (10 VGE/mL) were treated 7- and 4-days post-infection, respectively, with IL-1 β (100 pg/mL) for 2h, 8h, 24h and 72h. Cells were harvested and total RNA extracted. IL-6, A20, OAS and MxA RNA were quantified by RT-qPCR analyses. (C-D) Schematic view of the protocol; dHepaRG co-infected with HBV-D (100 VGE/mL) and HDV (10 VGE/mL) were treated with IL-1 β (100 pg/mL) in presence or not of IL1Ra (0.04 mg/mL). (E-F) Schematic view of the protocol; dHepaRG co-infected with HBV-D (100 VGE/mL) and HDV (10 VGE/mL) were treated six days post-infection with TPCA1 (1 μ M). One day later, cells were treated with IL-1 β (100 pg/mL) in presence or not of TPCA1 (1 μ M). (C-F) RG7834 (1 μ M) and IFN- α (500 UI/mL) were used as controls. Three days later, cells were harvested, total RNA extracted and (C, E) HBV and (D, F) HDV RNAs were quantified by RT-qPCR analyses. Results are the mean \pm -SD of three independent experiments (C-D) or two (A, B, E, F) each performed with three biological replicates and statistical analyses were performed using Mann-Whitney tests

To do so, we used the RNAs collected from the previous experiments (Figure 7-8-9). We observed an increase of mRNA from NF κ B prototypic genes (IL-6) upon IL-1 β treatment of more than 100-fold in dHepaRG compared with untreated cells at 2h, and more than 200-fold at 8h (Figure 10. A-B). At 24 and 72h post-treatment, the levels of IL-6 mRNAs decreased compared with earlier time points, but was still upregulated by 10-fold compared with untreated cells. While the levels of A20 mRNA was less increased than IL-6 mRNA, it was however increased by 10-fold all along the kinetic, suggesting a more constant induction (Figure 10. A-B). On the contrary, mRNAs from IFN-prototypic genes, were not modulated upon IL-1 β treatment, or even decreased in HBV-D/HDV treated cells (Figure 10. A-B). Similar observations were found in PHH regarding ratios between the NF κ B and IFN pathway (Figure 10. A-B). The differences in IFN prototypic genes expression upon HBV-D/HDV co-infection can be explained by the differences in IFN genes basal expression (i.e induction in HBV-D/HDV infected cells vs no induction in HBV-D mono-infected cells) (Alfaiate et al. 2016). To finally confirm our results, we treated HBV-D/HDV infected cells with IL-1 β in presence or not of a IL1R inhibitor (Anakinra, IL1Ra) or TPCA1 (IKK β inhibitor) (Nan et al. 2014) (Figure 10. C-F). As expected, IL-1 β effect on HBV-D and HDV RNA were reversed independently upon IL-1R and NF κ B inhibition, without any impact on RG7834 and IFN α treated cells, suggesting that its antiviral pathway is mediated by an IL-1R dependent activation of the NF κ B pathway (Figure 10.C-F).

7. IL-1 β seems to act in a direct antiviral manner on viral RNA

Since NF κ B induction leads to the production of several antiviral cytokines, we wondered if the effect of IL-1 β was due to the production of cytokine(s) that may act through autocrine/paracrine mechanisms and/or directly induced by antiviral proteins induced by the IL-1R pathway (exo/endonuclease, or modulation of transcription factors for instance). As IL-6 and TNF α are potent inhibitors of HBV (Isorce et al. 2016), we treated HBV-D infected dHepaRG with IL-1 β and IL-1Ra (Anakinra, inhibitor of IL-1R), neutralizing antibodies against the aforementioned cytokines, or the appropriate IgG control (Figure 11. A). Besides, TPCA1 is also an inhibitor of STAT3 phosphorylation (activated downstream of IL-6R) and thus could have blocked both pathways (Nan et al. 2014, 1). As expected, IL-1Ra treatment led to a total rescue of HBV RNA inhibition, confirming that IL-1 β was, indeed, acting through IL-1R (Figure 11. A). Even tough, slightly less efficient, treatment of dHepaRG cells with IL-1 β still decreased

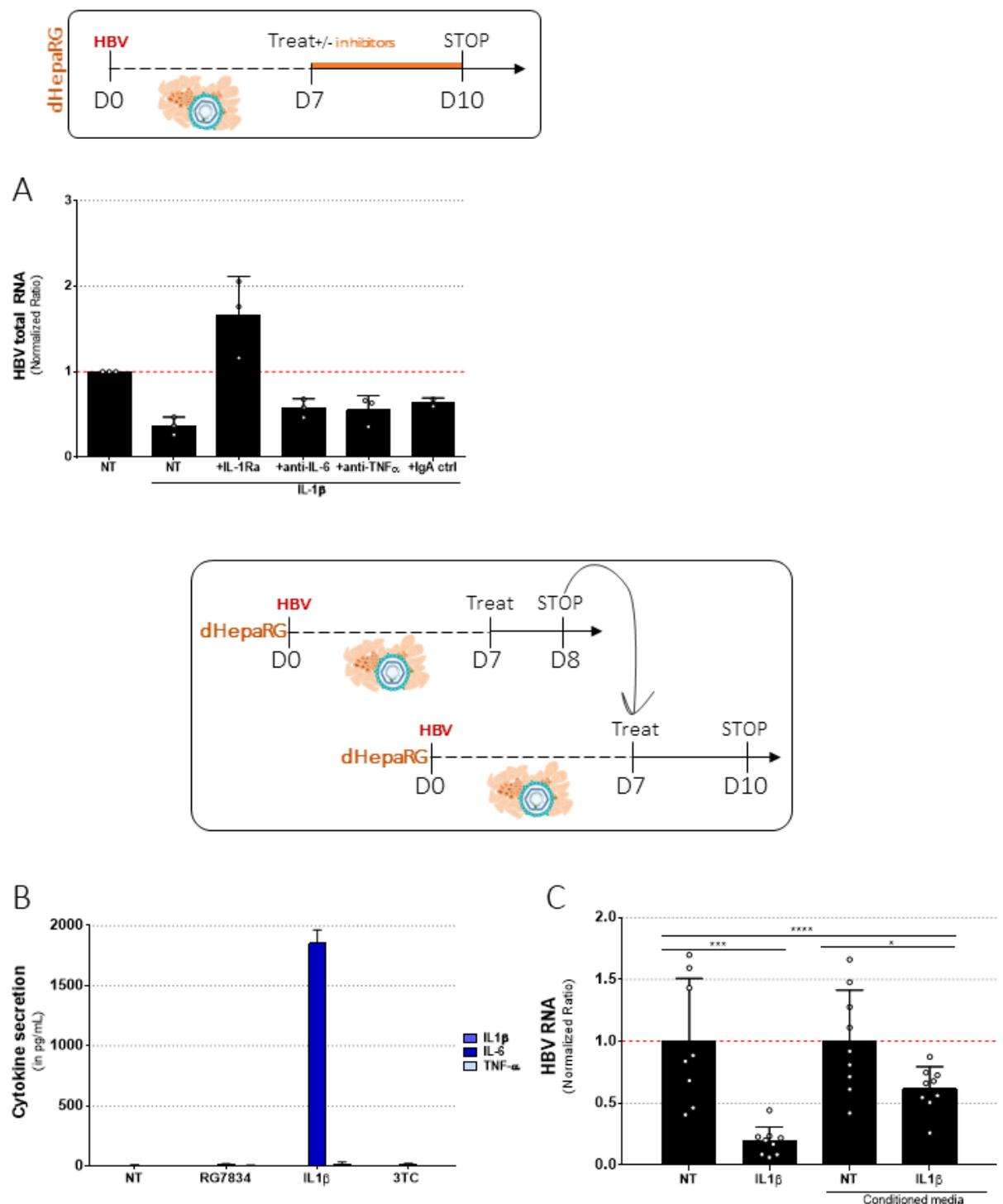


Figure 11: The antiviral effect of IL-1 β on HBV is partially due to secreted cytokines. (A) Schematic view of the protocol. dHepaRG infected with HBV-D (100VGE/mL) were treated with IL-1 β (100 pg/mL) in presence of IL-1Ra (0.4 mg/mL) or anti-IL6, anti-TNF- α and IgA Ctrl. Cells were harvested, total RNAs were extracted and levels of HBV RNAs analyzed by for RT-qPCR. (B-C) Schematic view of the protocol. dHepaRG infected with HBV-D (100 VGE/mL) were treated or not with IL-1 β (100 pg/mL) for 24h. The harvested supernatants were used to treat dHepaRG cells infected with HBV-D (100 VGE/mL). rhIL-1 β (100 pg/mL) was used as a control. (B) Cytokines levels (IL-1 β , IL-6 and TNF α) in the supernatant were quantified by ELISA. (C) Total RNAs were harvested, extracted and HBV RNAs were quantified by RT-qPCR analyses. Results are the mean +/-SD of three independent experiments each performed with three biological replicates and statistical analyses were performed using Mann-Whitney tests.

the levels of HBV RNAs in the presence of neutralizing anti-IL-6 and anti-TNF- α antibodies (Figure 11.A), suggesting that these cytokines are not responsible for the entire antiviral effect. To further confirm these results, we used conditioned media from dHepaRG cells treated or not with IL-1 β (24h accumulation) (Figure 11.B-C). The harvested supernatants were submitted to ELISA for quantification of several inflammatory cytokines, including IL-6, TNF- α and IL-1 β (Figure 11.B). First, no IL-1 β was detected in the supernatant, as this cytokine has a short half-life and is not produced by hepatocytes that lack the proper machinery to do so (i.e inflammasome) (Figure 11.B). TNF- α was detected only at low levels (30 pg/mL) but IL-6 concentration was similar to the ones obtained in MDMs (i.e 2 ng/mL) (Figure 11.B). HBV-D infected dHepaRG were treated with rhIL-1 β , as before, or with conditioned media from untreated or IL-1 β treated dHepaRG cells (Figure 11.C). No modifications were observed on HBV infection when dHepaRG were treated with the CM from untreated cells, compared with control. Interestingly, while low doses of IL-1 β led to an 80% reduction of HBV RNA, CM from IL-1 β treated dHepaRG cells, containing approximately 20-fold more IL-6, led to a decrease of 20-30%, compared with untreated cells (Figure 11.C) suggesting that the antiviral effect of IL-1 β on HBV may be partially due to secreted cytokines, but not entirely.

8. IL-1 β decreases the half life of HBV RNAs

To determine if the decrease in the levels of HBV RNAs following treatment with IL-1 β was due to RNAs degradation, we used triptolide, a well-known RNA Polymerase-I and II inhibitor and followed RNAs decay (Figure 12.A). dHepaRG cells were infected with HBV and treated with IL-1 β , RG7834 and 3TC, in presence of triptolide for 12 and 24 hours. Upon RNA transcription blockade, as expected from the literature (Han et al. 2018), treatment of cells with RG7834, but not with 3TC induced a faster decay of the levels of HBV RNAs compared to untreated cells (Figure 12.A). Similarly, the half-life of HBV RNAs is reduced of about 12h in cells treated with IL-1 β , which is not the case for RPLP0 RNA, suggesting that IL-1 β induces specific HBV RNAs degradation. Of note, as Pol-I and II are involved in various processes of HDV replication (Julie Lucifora and Delphin 2020), triptolide assays are not as discriminant and were thus not conducted. Then, as IL-1 β and RG7834 behaved similarly, we wondered if both molecules could share the same mechanism of action on HBV RNA. RG7834 is known to preclude the establishment of mixed tail on HBV mRNA, inducing its shortening and subsequent degradation by exo/endonucleases (Han et al. 2018). We used RNA from previously extracted experiment (Figure 8) and submitted them to Northern Blot analysis (Figure 12.B). As expected, HBV RNAs from RG7834-treated cells were shifted compared with untreated cells, as a consequence of tail-shortening. In contrast, RNAs from 3TC and IL-1 β -treated cells had similar length than untreated cells (Figure 12.B), suggesting that IL-1 β impact on HBV RNA is independent of mRNA shortening.

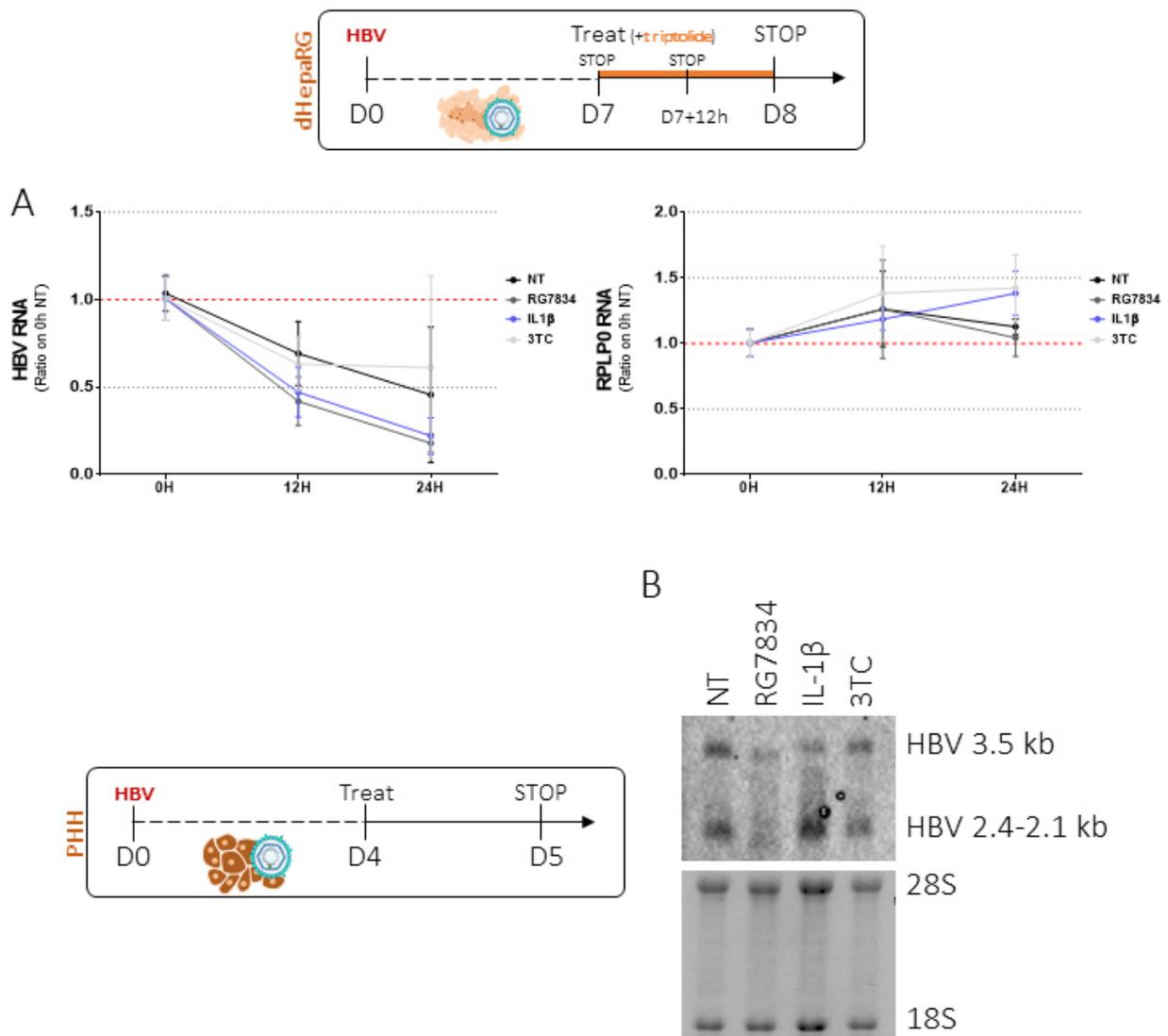


Figure 12: IL-1 β decreases the half-life of HBV RNAs

(A) Schematic view of the protocol. dHepaRG infected with HBV-D (100 VGE/mL) were treated or not with IL-1 β (100 pg/mL), RG7834 (1 μ M) and 3TC (1 μ M) for 12h or 24h in presence of triptolid. (B) PHH infected with HBV-D (100 VGE/mL) were treated or not with IL-1 β (100 pg/mL), RG7834 (1 μ M) and 3TC (1 μ M) for 24h. Cells were harvested, total RNAs extracted and levels of HBV RNA were quantified by RT-qPCR analyses. Results are the mean \pm SD of two independent experiments each performed with three biological replicates.

An accumulating body of evidence suggests that MCPIP1, an endonuclease induced by IL-1 β , could be responsible for the decrease of several viral RNAs, amongst which HCV, DENV, JEV and, more recently, HBV (in cell lines overexpressing MCPIP1) (M. Li et al. 2020; R.-J. Lin et al. 2014; 2013). Here we constructed KO cell lines (HepaRG) for MCPIP1 using the a CRISPR/Cas9 technology. We confirmed that dHepaRG-Cas9-MCPIP1 $_{KO}$ did not express MCPIP1 even after IL-1 β stimulation (Figure 13.A) and we did not observe any modulation in the antiviral effect of IL-1 β in dHepaRG-Cas9-MCPIP1 $_{KO}$ cells infected with HBV, HDV or both viruses compared to the antiviral effect of IL-1 β in dHepaRG (Figure 13. B-E), suggesting that, at least in our conditions, MCPIP1 is not responsible for the decrease of the levels of HBV RNAs and HDV RNAs (Figure 13.C-F).

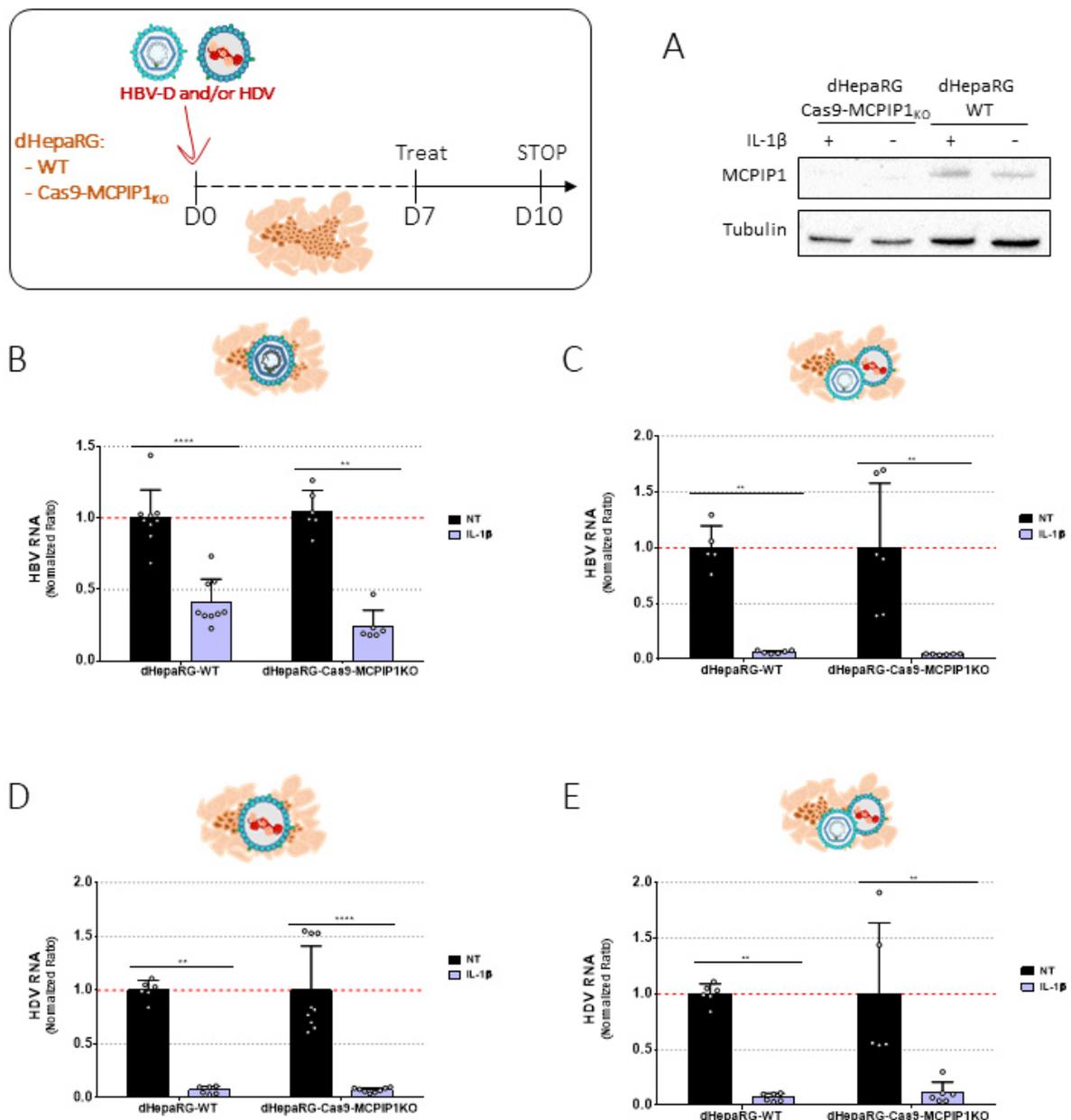


Figure 13: IL-1 β -mediated antiviral effect on HBV and HDV is independent of MCP1P1 expression
dHepaRG or dHepaRG-Cas9-MCPIP1_{KO} were infected with HBV-D (100 VGE/mL) or HDV (10 VGE/mL) or co-infected with both before being treated or not with IL-1 β . **(A)** Protein lysates were submitted to western blot analyses using anti-MCPIP1 and anti-B-tubulin antibodies. **(B-C)** Total RNAs were extracted and levels of **(B-C)** HBV and **(D-E)** HDV RNAs were quantified by RT-qPCR analyses. Results are the mean +/-SD of two independent experiments each performed with three biological replicates.

Altogether, we demonstrated that IL-1 β , through the activation of the NF κ B pathway, is a potent inhibitor of both HBV and HDV. We thus gained interest in NF κ B inducers in general to test their potency as antiviral drugs *in vivo*. Indeed, NF κ B induction should lead to viral inhibition but might also counteract the HBV-induced M ϕ modulations.

9. NanoP-TLR2-L, a useful tool for *in vivo* MΦ activation

Thanks to a collaboration with Bernard Verrier's team (IBCP, Lyon France), we were able to access Poly(Lactic Acid) nanoparticles coated with TLR2-L (Pam3CSK4) (Lamrayah et al. 2019). These NanoP-TLR2-L were designed and characterized *in vitro* and *in vivo* by Myriam Lamrayah and Fanny Charriaud (PhD student in B. Verrier's team), their impact on HBV infection in hepatocytes was studied by Manon Desmares (PhD student in our team). She observed an NFκB-dependent reduction of HBV RNA and cccDNA, depending on the doses used (data not shown – see Manon Desmares's PhD). Of note, while TLR2-L alone and encapsulated within NanoP (i.e NanoP-TLR2-L) had similar impact on the infection, NanoP alone had no impact on HBV.

As TLR2 is present on both hepatocytes and innate immune cells, we investigated the impact of such NanoP-TLR2-L on liver immune cells. We thus extracted Liver MonoNuclear Cells (LMNC) or PLMs alone and exposed them to LPS or increased concentrations of TLR2-L, or NanoP-TLR2-L, or the equivalent in NanoP alone (Figure 14.A-B). In both TLR2-L and NanoP-TLR2-L conditions, cytokines secretions were increased compared with non-treated cells, which was not the case for NanoP alone at low doses (before 500 ng/mL) (Figure 14.A-B).

Compared with LPS (TLR4-L), TLR2-L induces two to three time less IL-10, IL-6 and IL-1β secretion in LMNCs and PLMs. However, in both purified cells, high doses of NanoP-TLR2-L (starting 100 ng/mL) secreted two times less IL-10, and equivalent (IL-6) or more (IL-1β) pro-inflammatory secretions compared with LPS condition. NanoP alone had a similar profile than NanoP-TLR2-L regarding cytokine secretion in the highest doses, but starting at an equivalent of 500 ng/mL. Besides, IL-10 secretion induced by NanoP alone was especially low (400 pg/mL) compared with all other conditions (between 1000 to 2000 pg/mL).

As NanoP were primarily designed for a better *in vivo* delivery (i.e better/quicker addressing to the liver), we decided to investigate NanoP-TLR2-L impact in C57Bl6 injected with of AAV-HBV. Once the infection established (4 weeks), mice were treated or not with increasing doses of TLR2-L, NanoP-TLR2-L or Lamivudine (NA - 3TC) for 4 weeks and sacrificed one week after end of treatment (Figure 15.A). HBV parameters were monitored and total RNA extracted for virological and immune characterization. Of note, one mouse died in the control untreated group (i.e not related with NanoP or TLR2-L treatment) and other mice behaved normally, as monitored with mice weight (data not shown). Other read-out should/will be implemented to better monitor future cohort.

Unfortunately, due to the complexity of the injection (intra-orbital), our first *in vivo* experiment only led to a low infection level of HBV (10^{4-5} VGE/mL when 10^{6-7} at least is required to consider that mice are sufficiently infected) and large inter-mice variability. Thus, no significant modifications of HBV RNA levels were observed in between the different groups, compared with untreated mice (Figure 15.A). Of note, viremic levels in NanoP-TLR2-L treated mice dropped drastically and were undetectable by the end of treatment, even though no statistical significance was reached due to a large inter-mice variability (data not shown). Thus, proof of concept of NanoP-TLR2-L efficiency on HBV infection is still pending *in vivo*.

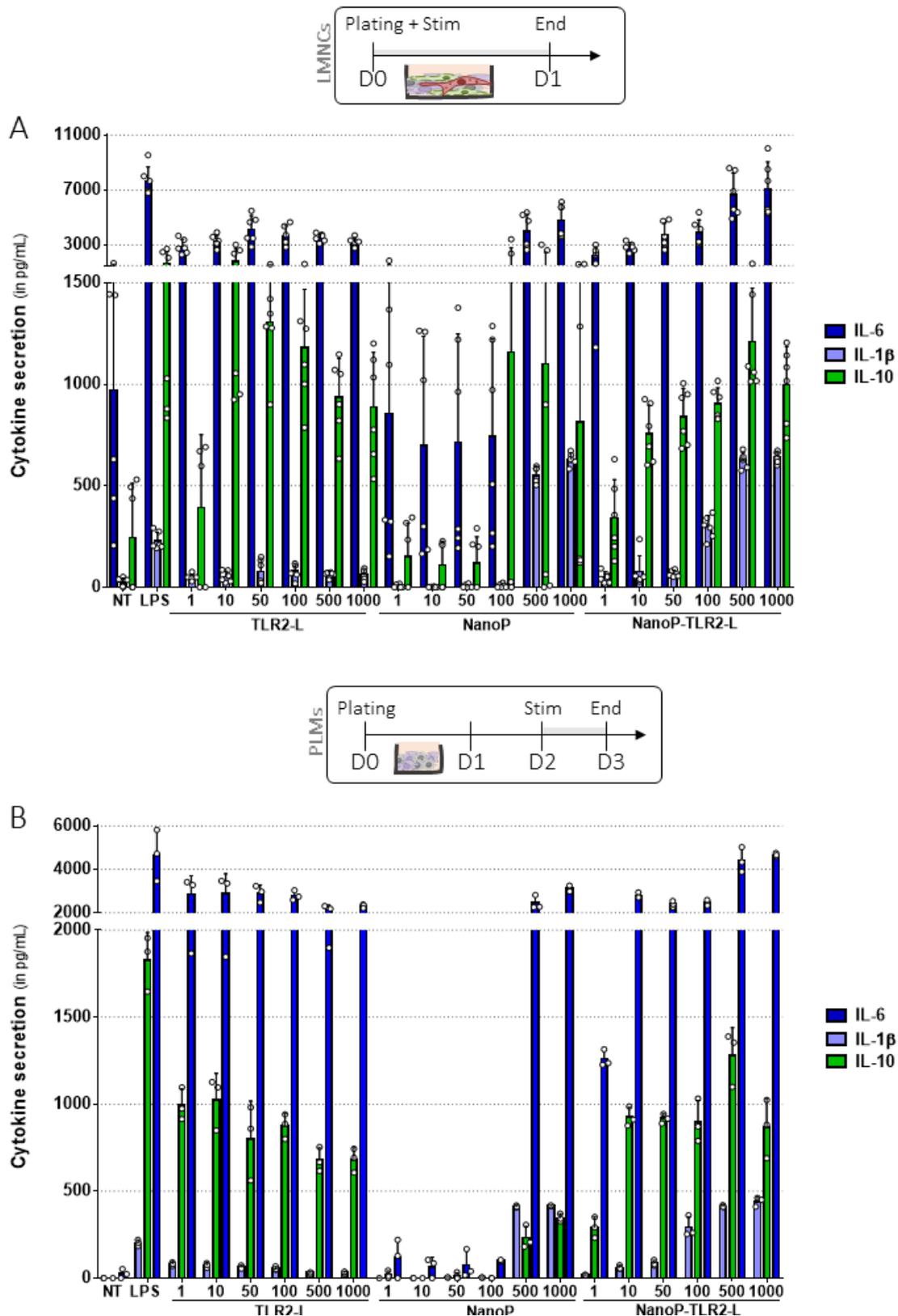


Figure 14: NanoP are more prone than TLR2-L to induce pro-inflammatory cytokine secretions. (A) LMNC and (B) PLMs isolated from liver resections were exposed for 24 hours to LPS (100ng/mL) or increasing doses of TLR2-L, NanoP or NanoP-TLR2-L (from 1 to 1000 ng/mL), or left untreated. In all experiments, harvested supernatants were submitted to ELISA for quantification of IL-1 β , IL-6 and IL-10 secretion levels. Results are the mean \pm SD of (B) one or (A) two independent experiments (i.e two different donors) each performed with three biological replicates.

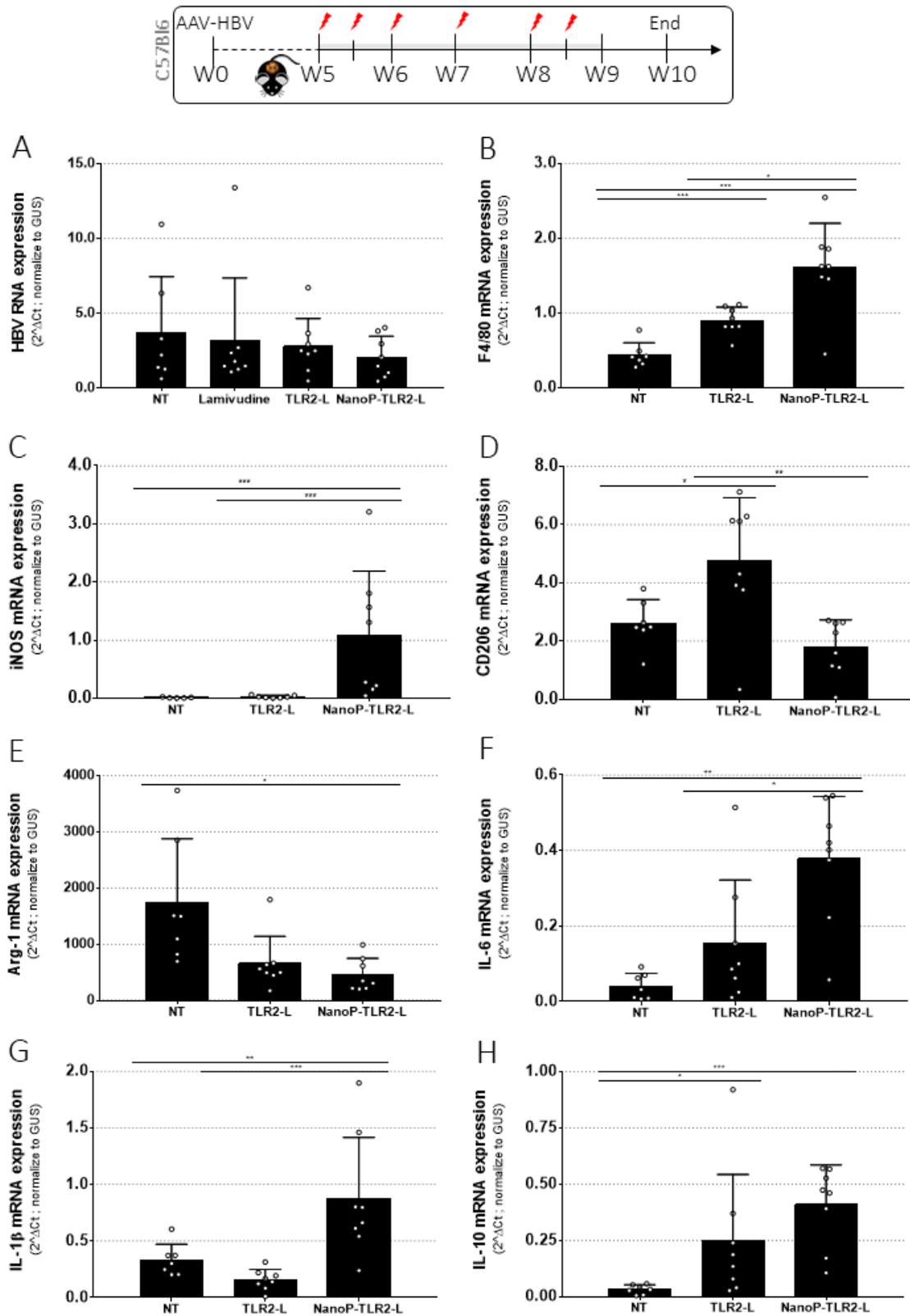


Figure 15: *in vivo* injection of NanoP-TLR2-L leads to preferential increase in pro-inflammatory RNA. C57Bl/6 mice were injected with 1.10^{11} VGE of AAV-HBV. 4 weeks later, mice were treated or not with TLR2-L, NanoP or NanoP-TLR2-L with increasing doses, every week, until week 8. Sacrifice was performed on week 9. Extraction of total RNA from the liver was performed and (A) HBV, (B) F4/80, (C) iNOS, (D) IL-6, (E) IL-1 β , (F) IL-10, (G) CD206 and (H) Arg-1 RNA expression analyzed by RT-qPCR. Results are the mean \pm SD of the eight mice from one experiment and statistical analyses were performed using Mann-Whitney tests.

However, we decided to investigate if our NanoP-TLR2-L could have any impact on the mice' immune system, independently of the infection, and confirm the increased *in vivo* potency of NanoP-TLR2-L compared with TLR2-L alone. Interestingly, even if inter-mice mRNA expression was especially broad, differences reached statistical significances between groups regarding immune-related parameters (Figure 15.B-H). Indeed, F4/80 (general Liver MΦ marker) was increased in TLR2-L, and even more in NanoP-TLR2-L compared with NT mice (Figure 15.B). Besides, iNOS (pro-inflammatory mark) was significantly increased (Figure 15.C) and anti-inflammatory marks, Arg-1, was decreased (only a tendency observed for CD206) (Figure 15.D-E) in NanoP-TLR2-L treated mice. On the contrary, TLR2-L treated mice had no induction of iNOS RNA and an increased expression of CD206 (Figure 15. C-D). Regarding cytokines' expression RNA level, IL-6 and IL-1β were both significantly upregulated, only in NanoP-TLR2-L condition, while IL-10 was increased in both TLR2-L and NanoP-TLR2-L condition.

Altogether, even if encouraging, these results are not sufficient to conclude as total liver RNA extraction do not purely reflect expression in liver MΦ. Thus, FACS analysis will be performed to (i) identify the different myeloid populations present within the liver and (ii) specifically observe liver MΦ inflammatory profile.

Discussion

The antiviral roles of myeloid cells are both direct (secretions of cytokines, phagocytosis...) and indirect (activation of the adaptive immune system). By orchestrating the immune response, they are key elements for every host-pathogen interaction, and HBV and HDV infections are not exceptions. In the present study, we demonstrated that HBV and HDV virions, due to the presence of the envelope protein (HBsAg), are able to change MΦ differentiation towards a more tolerogenic phenotype, i.e less secretion of pro-inflammatory cytokines such as IL-1β and more secretion of anti-inflammatory cytokines such as IL-10. These observations were recapitulated using different HBV genotypes (HBV-C, -B and -D). Besides, we showed that treatment with IL-1β leads to a decrease of the levels of HBV and HDV RNAs in infected hepatocytes. Even if the exact mechanism remains unclear to date, IL-1β seems to act through the induction of the NFκB pathway.

Preamble on the macrophage model used:

In the immunology field, a lot of diverse ways can be found to differentiate and polarize MDM. However, the complexity of ResMΦ phenotypes that have been described *in vivo* is difficult to mirror *in vitro*. Murray and colleagues in 2014 made a series of recommendations of interest for the study of MΦ profile *in vitro*, highlighting the importance of relevant environmental cues on stimuli received (Murray et al. 2014). In our study, we designed a model of MDM differentiated upon GM-CSF and M-CSF incubation for six days, shifting MΦ towards an M1 or M2 phenotype, according to old dogmas (Martinez and Gordon 2014). Activation is then performed using LPS, a well-known component of bacterial cell wall (Lu, Yeh, and Ohashi 2008) present in large quantities in the liver sinusoid due to commensal bacteria proximity. Thus, activation by LPS is a rather physiological phenomenon within the liver. Using this model, we were able to obtain a good dichotomy between the secretive phenotype of M1-MDMs and M2-MDMs ((Faure-Dupuy, Delphin, Aillot, Dimier, Lebossé, Fresquet, Parent, Sebastian Matter, et al. 2019) Figure 3). We also demonstrated that M1-MDMs secretions were able to decrease the infection, whereas M2-MDMs did not ((Faure-Dupuy, Delphin, Aillot, Dimier, Lebossé, Fresquet, Parent, Sebastian Matter, et al. 2019) Figure 9), suggesting that our model could efficiently mirror antiviral vs tolerogenic responses against viruses. However, monocyte to macrophage differentiation using this protocol is challenging since cells are poorly adherent for the first 6 days after seeding and are highly sensitive to DMSO (that is the main diluent of most drugs but lead to uncontrolled pro-inflammatory cytokine secretion by MDM and primary MΦ). Interestingly, cellular adherence and differentiation were much better in the co-culture setting. This is in accordance with the concept of niche suggesting that liver ResMΦ profile and survival is favored by other liver cells, such as hepatocytes (Guilliams et al. 2020). Thus, it should be of interest to use this co-culture system (as well as trying 3D systems as discussed in the introduction) without GM/M-CSF to determine if monocyte able to differentiate only with hepatocytes secretion, and which phenotype is obtained. Of note, as recommended by Murray and colleagues, we confirmed most of our results using *ex-vivo* stimulated liver ResMΦ, suggesting that even if not perfect, our model is of relevance here.

1. HBV modulates liver ResMΦ secretive phenotype

First, using liver biopsies from CHB patients, we correlated the presence of HBc with an increase in CD163 MΦ anti-inflammatory marks, suggesting that patients infected with HBV had a more tolerogenic phenotype (Faure-Dupuy, Delphin, Aillot, Dimier, Lebossé, Fresquet, Parent, Sebastian Matter, et al. 2019). Then, using two models to mimic liver ResMΦ and InfResMΦ, namely MDMs and PLMs, we demonstrated that HBV-D is capable of reducing the pro-inflammatory secretions of the prototypic antiviral cytokine IL-1 β , as well as increasing the ones of IL-10 (Faure-Dupuy, Delphin, Aillot, Dimier, Lebossé, Fresquet, Parent, Sebastian Matter, et al. 2019). Hence, HBV seems capable of modulating ResMΦs (PLMs as a model) which are directly at the infection site (i.e in close contact with the infected hepatocytes), as well as decreasing the pro-inflammatory state of InfResMΦ (M1-MDM model) or increasing the activation of anti-inflammatory MΦ (M2-MDMs). Altogether, this suggest that even if HBV infection would be detected, leading to the recruitment of infiltrating monocytes, they might not be able to ensure their antiviral role once on site. As we only performed analysis with *ex-vivo* purified cells, *in vivo* experiments should be performed to further investigate and confirm this hypothesis.

In PLMs and M2-MDMs, which are of a more anti-inflammatory phenotype, only 16h or 3h were needed, respectively, to observe the aforementioned modulations, suggesting an easy-to-implement mechanism. However, modulations of M1-MDMs required HBV presence during their 6 days differentiation process. In CBI patients, HBV particles, and especially HBsAg, are in large quantities in the circulation, in most patient' phases, thus, the recruited monocytes are surely in contact with the virus before reaching the liver. If these experiments showed us that HBV is able to induce a tolerogenic phenotype, it also suggests that its clearance from the circulation could enable a recovery of the recruited monocytes inflammatory state. Indeed, M1-MDMs incubated with HBV during their 3h activation process (i.e after a complete differentiation with GM-CSF for six days) were not impacted. This may suggest that M1-MDMs correctly differentiated (i.e without HBsAg in the circulation) are in a too high pro-inflammatory state for HBV to have an impact. This hypothesis is further demonstrated by the fact that we did not recapitulated our observations using THP-1 (data not shown), that have a higher basal level of pro-inflammatory activation. Hence, the NFkB-driven activation of the immune compartment within the liver observed in patients (see introduction) might be too important for HBV to counteract. At steady state, HBV, contrary to HDV, is not a strong inducer of IFN or the NFkB pathway (Wieland et al. 2004; Fletcher et al. 2012; Lebossé et al. 2017). Thus, an interesting hypothesis is that HBV could only be equipped to deal with low-level inflammation, which is in line with the silent infection observed for decades (i.e no sign of liver inflammation or disease) before patients enter phase 2, associated with ALT elevation, immune activation and subsequent liver disease. Interestingly, the exact triggers for a patient to enter phase 2 are not known to date, due to difficulties to detect such patients before the onset of the disease start (i.e phase 2/3 at least). It should be of interest to further investigate such mechanisms and thus understand how the immune compartment is activated/inhibited more precisely.

2. Mechanism of HBV-induced modulations

a. Which viral component is impacting MΦ?

Our observations from the study we published in J Hepatol were mainly phenotypic and we decided to get more insight into the exact mechanism of HBV-mediated impact on MΦ.

Several experimental evidences led us to conclude that blood-circulating forms of HBV are responsible for the HBV-induced modulations of MΦ. First, HBV does not replicate in MΦ, most probably as these cells lack the expression of transcription factors essential for HBV RNAs synthesis (Faure-Dupuy, Delphin, Aillot, Dimier, Lebossé, Fresquet, Parent, Sebastian Matter, et al. 2019; Y. Zheng, Li, and Ou 2004, 4). However, we observed HBc in close contact with liver ResMΦ in patient' IHCs, suggesting that a non-replicative interaction between the two is not impossible (i.e maybe HBc is interacting with liver ResMΦ surface proteins or is internalized). Second, incubation with AAV-HBV (i.e to deliver the HBV genome into the cells without HBsAg and HBeAg) did not impact the IL-6, IL-1β secretion by M1-MDMs (Faure-Dupuy, Delphin, Aillot, Dimier, Lebossé, Fresquet, Parent, Sebastian Matter, et al. 2019). Of note, no neo-synthesis of HBeAg or HBsAg was observed in M1-MDMs transduced with AAV-HBV since expression of the transgenes are under the control of a hepato-specific promoter. However, it means that the genome alone, in a different envelope, is not capable of inducing the observed modulations. Third, M1-MDMs incubated with an UV inactivated HBV virus (i.e HBV replication incompetent) led to the same reduction in the levels of secreted IL-6 and IL-1β, as observed before (Faure-Dupuy, Delphin, Aillot, Dimier, Lebossé, Fresquet, Parent, Sebastian Matter, et al. 2019). Finally, as HepAD38 supernatant concentration could contain non-HBV related molecules, we concentrated HepG2 supernatant (the parental cell line) and did not observe any differences in cytokine secretion upon incubation of such inoculum, compared with untreated cells.

The same experiment should and will be performed regarding HDV virions. Contrary to HBV, HDV is capable of replicating within other cell types (without secretion) when NTCP recognition is bypassed (i.e transfection of viral genome) (Polo, Lim, et al. 1995; Polo, Jeng, et al. 1995). As MΦ are professional phagocytes, capable of engulfing a large variability of pathogens, HDV could enter through a non-specific pathway to deliver its viral genome. However, such entry would require several escape mechanisms amongst which phagolysosome degradation that were, to date, not documented regarding HDV.

So, the molecule responsible for the observed modulation should be a protein present in the viral inocula.

One copy of HBV-Pol is contained within each Dane Particle (Nassal 2008) and HBx RNAs were detected in viral inocula from HepAD38 cells and patient serums (Niu et al. 2017), suggesting that these proteins, that were both described as modulator of the immune system (Wei et al. 2010; D. Liu et al. 2014; H. Wang and Ryu 2010), could potentially be responsible for the HBV-induced modulation of MΦ. However, the low concentrations of these two proteins; compared to large amount of HBeAg and HBsAg (Volker Bruss 2007) in the blood of infected patients (or viral inocula produced *in vitro*), favor the hypothesis of a modulation mediated by HBeAg or HBsAg that are already known to be involved in immunomodulatory processes on MΦ (Zannetti et al. 2016; Y. Wang et al. 2019; Gruffaz et al. 2013; M. Jiang et al. 2014; X. Yu et al. 2017). To search for the viral agent responsible for the observed modulations, most studies incubate their

cell of interest with recombinant protein (i.e rHBsAg, rHBeAg/HBc). However, recombinant proteins are often far from the protein observed *in vivo*. As a reminder, they are often produced in yeast or bacteria (i.e contaminant can activate MΦ) or are dissolved in DMSO, a well-known immune modulator (Kelly et al. 1994). Besides, the conformation can differ between physiological and recombinant protein, as for instance, HBc recombinant can be found in monomer, dimer or naked capsid (C.-L. Zheng et al. 2019). Finally, HBsAg is a well-known lipid coated protein, and this composition could differ from its physiological form in non-hepatocyte cells (hepatocytes have a highly specific composition putatively not recapitulated in other models (Vanlandschoot et al. 2007). Thus, to circumvent all these issues, we set up a co-culture model in transwell (i.e no cell-to-cell contact) between M1-MDMs and HepAD38 cells, treated with tetracycline and/or siRNA to clear the supernatant from its viral protein. In addition, we also set up a similar co-culture system with Huh7 integrated or not with HBsAg alone (not the full HBV genome). Using these models, we demonstrated that HBsAg production alone is capable of inducing the previously observed modulations, and loss of HBsAg rescued this phenotype.

Even if this hypothesis is the most relevant one, due to the amount of HBsAg present in the supernatant compared with other viral components, it is worth discussing other putative ones.

First, we observed similar results in co-culture of M1-MDMs with HepAD38 treated or not with tetracycline (Tet). The Tet-OFF condition led to HBeAg and HBV DNA drastic decrease in the supernatant, suggesting that these are indeed not related to the modulations observed. However, HBx RNA is still produced in Tet-off HepAD38 (Ladner et al. 1997). Even if only low levels of HBx RNA were observed in HepAD38 supernatant, the hypothesis of HBx RNA transfer within MΦ is unlikely, but cannot be excluded.

Exosomes are 30-150 nm diameter vesicles secreted by almost all cell types, containing various components (lipids, nucleic acids, proteins) which reflect the cell's status (S. Li et al. 2019). They are also capable of shielding the internalized components from cytoplasmic or extracellular (trafficking in fluids) degradation, acting as important cargos of cellular communication. In viral infection, where the cells are most often entirely hijacked by the pathogen toward their establishment and replication, exosomal components are profoundly disturbed. Hence, internalization of both (i) viral nucleic acid for propagation, such as HIV and HCV RNA, or (ii) anti-inflammatory inducers increased by viruses, have been observed (S. Li et al. 2019). Concerning HBV, its nucleic acids and proteins were shown internalized in patient' exosomes and supernatant from HepG2 infected cells (S. Li et al. 2019; Y. Yang et al. 2017). The HBx mRNA and protein were also found in exosomes extracted from the supernatant of HBV transfected Huh7 (Kapoor et al. 2017). Thus, the hypothesis of HBx transfer into liver ResMΦ by exosomes should be investigated. However, exosomes also contain HBsAg and were associated with CHB patient' NK cell dysfunction (Y. Yang et al. 2017) through blockade of degranulation and antiviral cytokines production. Also, monocytes treated with EVs from HepAD38 have increased expression of PD-L1 (Kakizaki et al. 2018). While the authors conclude of an EV-driven mechanism because EVs from HepAD38 +/- TET had the same effects, one should not forget that tetracycline treatment do not inhibit HBsAg and HBx production, thus further characterization of the inocula are required to this intention. Thus, our hypothesis of an HBsAg-driven modulation stands true in the light of an exosome-mediated regulation of liver ResMΦ. On the contrary, EV extracted and IP-depleted from HBsAg were also capable of inducing a reduction in IL-6 production by PBMC, through this time, transport of proteosomal catabolic

molecules to the immune cells (Jia et al. 2017). Other components could be transported, such as the exosome-mediated transfer of ISG activated following HCV infection, shown to be responsible for the control of the infection (Giugliano et al. 2015). While this should not be possible for HBV, as it does not trigger the IFN pathway, it should be investigated for HDV as it does. Relative to HDV, there is, to date, only one short article. Jung and colleagues recently observed an activation of PBMC and MDMs (M-CSF) following exposure of EV derived from HDV infected cells, compared with non-infected ones, as seen with increased levels of TNF α and IL-6 secretions (Jung et al., n.d.). As expected, they were able to detect HDV RNAs in their extracted EVs.

Another mechanism of interest is miRNA modulations of the immune compartment, as previously presented in the introduction. Interestingly, both HBsAg subviral particles and exosomes are able to transport them (Novellino et al. 2012; Y. Shi et al. 2021; Enomoto et al. 2017). Production of more than 100 EV-associated miRNA is shifted by HBV infection in PHH (Enomoto et al. 2017), leading, amongst other mechanisms, to the inhibition of IL-21 production in T cells. In 11 CHB patients, subviral particles were associated with liver specific miRNA, amongst which immune regulatory miR-106b and miR-223 (Novellino et al. 2012).

Thus, even if, in regard to the literature and the previous discussion, it is most probable that HBsAg is responsible for the aforementioned observations, some confirmations are still required. To this intention, GW4869 or Manumycin A, amongst other exosome inhibitors (Catalano and O'Driscoll, n.d.) will be tested in the lab. However, as exosomes and HBV shares the same secretory pathway, and some inhibitors (GW4869) are associated with anti-inflammatory polarization, thorough controls of the experiments will be required to correctly decipher this mechanism (HBV secretion control, characterization of the supernatant, quantification of cytokine secreted). Besides, miRNA modulation has never been investigated in our setting and is, of course, required.

b. How HBsAg interact with M Φ ?

From the first study, we knew that HBV modulations should not involve endocytosis, as Cytochalasin D treatment did not rescue the observed phenotype (Faure-Dupuy, Delphin, Aillot, Dimier, Lebossé, Fresquet, Parent, Sebastian Matter, et al. 2019). However, endocytosis is not the sole way to internalize pathogens for the professional phagocytosis that are liver ResM Φ . Thus, confirmation using other inhibitors, such as Latrunculin A or hyaluronate, are required to conclude further.

TLR4 pathway inhibition was already documented by incubation of THP-1 with a recombinant HBsAg (Q. Wang et al. 2013; M. Jiang et al. 2014; Zannetti et al. 2016; Cheng et al. 2005). In most cases, it was associated with a decrease in MAPK/NF κ B pathways (Q. Wang et al. 2013; M. Jiang et al. 2014) or NF κ B alone (Cheng et al. 2005), without further mechanistic insights. TLR4-L associated IL-6 production (but not IL-10) of *ex-vivo* stimulated PBMCs from CBI patients was significantly decreased in HBsAg high compared with HBsAg low patients (M. Jiang et al. 2014). Finally, Zannetti and colleagues efficiently found HBV internalized within THP-1 cells using a specific AlexaFluor HBV virions and confocal microscopy, suggesting that the virus is indeed capable of entering M Φ (Zannetti et al. 2016). In this study, HBsAg was again correlated with a

decrease in TLR activation, which led to AIM2 inflammasome blockade. They observed a reduction of IRF7 mRNA and protein, leading to an abrogation of IRF7-mediated AIM2 promoter activation and subsequent inflammasome inhibition (Zannetti et al. 2016). This latter study was of high interest to us as we observed a decrease of IL-1 β secretion upon AIM2-specific ligand activation in HBV incubated M1-MDMs and PLMs (Faure-Dupuy, Delphin, Aillot, Dimier, Lebossé, Fresquet, Parent, Sebastian Matter, et al. 2019). However, no AIM2 or IRF7 mRNA modulations were observed in our hands, most probably due to the strong differences existing between THP1 and MDMs/PLMs. However, we observed a decrease of IL-1 β , IL-12 and TNF α mRNA in both PLMs and M1-MDMs upon HBV incubation (Faure-Dupuy, Delphin, Aillot, Dimier, Lebossé, Fresquet, Parent, Sebastian Matter, et al. 2019), suggesting that HBsAg is modulating, at least in part, TLRs signaling and the subsequent NF κ B pathway activation of antiviral cytokines.

Interestingly, M1-MDMs exposed to HBV-D had increased expression, at the mRNA and protein levels of inhibitory receptor (Supplementary results, Figure 3), namely PD1/PDL1 axis and Tim3/Gal9. This is consistent with the already published literature describing an immunosuppressive myeloid compartment within the liver of CBI patients, especially the increase in MDSCs. However, we did not perform FACS analysis with our samples yet and it is thus still unclear if we obtained M-MDSCs (CD11b⁺CD14⁺HLA-DR^{-/lo}CD15⁻S100a9^{high}) or not (Gabrilovich 2017). Besides, M-MDSCs recruitment is associated with a low but consistent stimulation of the myeloid compartment (Condamine, Mastio, and Gabrilovich 2015b). However, no cytokine secretions were detected upon HBV incubation alone (i.e without LPS stimulation) in our experiment, either after 16h incubation in PLMs, or 6 days with MDMs (data not shown). Thus, it is unclear, to date, if we are indeed in the presence of *bona fide* M-MDSCs. However, as clear increase in inhibitory receptors is observed, it is most probable that this immunosuppressive phenotype could impact lymphocyte activation status, as previously observed, and suggested by us (Jin et al. 2010; D. Cao et al. 2013; Y. Tian et al. 2016; Haijun Li et al. 2018; Nebbia et al. 2012; Hang Li et al. 2012a). Indeed, even if increased IL10 secretive levels has no direct impact on HBV establishment within hepatocytes (Faure-Dupuy, Delphin, Aillot, Dimier, Lebossé, Fresquet, Parent, Sebastian Matter, et al. 2019), it impacted effector functions of T lymphocytes (as seen with TNF α secretion decrease), suggesting that it could participate in the exhaustion observed in CBI patients. Of note, IL-10 production by liver ResM Φ was already associated with downregulation of adaptive immune responses as well *in vivo* and using more read-out than us (L. Xu et al. 2014; A. Huang et al. 2014; Kong et al. 2014).

Of special interest, Tim3 is an inhibitory receptor capable of modulating M Φ phenotype, and is especially known for its modulation of the TLR4 pathway (Ocaña-Guzman, Vázquez-Bolaños, and Sada-Ovalle 2018; X. Yang et al. 2013).

Nowadays, four Tim-3 ligands have been identified, galectin-9, phosphatidylserine (PS) residues, HMGB1 and CEACAM-1. In infectious diseases, Tim-3 is mainly associated with immune inhibition as it is closely correlated with the establishment of CD8⁺T cell exhaustion in HIV (Jones et al. 2008), LCMV (Jin et al. 2010), Friend Virus (Takamura et al. 2010), Hepatitis C (Golden-Mason et al. 2009) and B Viruses (Yuan Liu et al. 2016). Moreover, even if often associated with PD-1 expression, recent evidences suggest that resistance to antiPD-1/PD-L1 treatments in patients is correlated with increased Tim-3 expression (Shayan et al. 2017) and thus combination therapy of both receptors inhibitors is actually on trial with promising results (He et al. 2018). Hence, the role of Tim-3 in infectious diseases and cancer has gained in

importance in the past few years but, unfortunately, little is known on its molecular pathway and mode of action.

Studies suggest that, depending on which ligand and/or Tim-3 level of expression, this pathway either can activate or inhibits M Φ pro-inflammatory phenotype (Ocaña-Guzman, Torre-Bouscoulet, and Sada-Ovalle 2016). Besides, HBsAg contain PS residues (Meyer et al. 1999). To investigate the putative involvement of Tim3, we blocked it using neutralizing antibodies on M1-MDMs, with inconclusive results (data not shown). This was most probably due to Fc antibody recognition by MDMs that led to pro-inflammatory over-activation without proper Tim-3 inhibition, as observed with IgG control comparison (data not shown). Unfortunately, to date, no chemical inhibitors of Tim-3 are known, other than antibodies.

Other receptors involved in M Φ anti-inflammatory polarization are Tyro3, Axl and MerTK (TAM-R) (Cook et al. 2013; Foley 2013), and were found associated positively with HBV persistence (M.-T. Huang et al. 2015). TAM-Rs's ligand are Pros1 and Gas6, both needs to bind PS residues before binding to their receptors, residues that are found in numerous viral particles, amongst which HBsAg (Ghosh Roy 2020). Their activation is inducing IFN response and TLR pathway inhibition through both direct (Axl association with IFNAR) and indirect (subsequent activation of SOCS1/3) mechanisms (Ghosh Roy 2020). Unfortunately, our screening at the mRNA level detected no differences in the expression of TAM-Rs and their ligands (data not shown), their protein expression is however still to be investigated.

Of note, even if we focus here on TLR4 induced pathway, it is worth noticing that we also observed TLR3-L induction in PLMs inhibited by HBV (Gruffaz et al. 2013). This is not mutually exclusive to our previous hypothesis as TAM-R are broadly inhibiting TLRs pathway and Tim-3 blockade led to TLR3-L signaling recovery in *ex-vivo* incubated PBMCs (Ghosh Roy 2020)

Altogether, our hypothesis mainly involves an association of HBsAg with inhibitory receptors, which is still to date to be confirmed. We believe that it may not be solely HBsAg itself, but it's lipid coat, may also triggers the observed changed in M Φ s. To this intention, and as HBsAg is the main driver of immunosuppression, for most liver immune cells (van der Aa et al. 2016; Vincent et al. 2011a; Martinet et al. 2012; Fang et al. 2015; Pal et al. 2019a; W. Zhang et al. 2019; Wherry and Kurachi 2015), there is a need for glycoproteomic and lipid analysis of HBsAg from different genotypes, HBV-related disease phases, and HBV-HDV co-infected patients. Indeed, the differences observed in between genotypes or disease phases might be attributed to different quality and/or composition of HBsAg, modulating its attachment on M Φ , something which, to date has not been investigated.

In a broader view and as the liver control most of the metabolic changes happening in our body, hepatotropic viruses are associated with general changes in lipid, fatty acids and protein biogenesis and consumption. HBV is not an exception, as observed in several metabolomic analysis of patient' sera (Gavilanes, Gonzalez-Ros, and Peterson 1982; E.-S. Park et al. 2014; Arain et al. 2018; T. Wu et al. 2017). In general, an enhanced fatty acid synthesis and lipogenesis is observed, but also an increase fatty acid oxidation and lipolysis (J. Zhang et al. 2021; Y.-X. Shi, Huang, and Yang 2016). All in all, the virus is driving metabolic modulations to ensure sufficient production of material and energy for its replication in hepatocytes. Thus, the consequent strong differences in the membrane lipid composition should impact the composition of HBsAg lipid coat as well.

To this intention, metabolomic studies on liver ResM Φ are still lacking, but required, due to the increased literature intertwining polarization changes with specificities in oxygen consumption and metabolite production (Galván-Peña and O'Neill 2014; C. H. Patel et al. 2019; Viola et al. 2019). In this work, we observed a decrease of HIF1 α at the mRNA level in both PLMs and M1-MDMs (Faure-Dupuy, Delphin, Aillot, Dimier, Lebossé, Fresquet, Parent, Sebastian Matter, et al. 2019). HIF1 α is a well-known marker of broken Krebs cycle and subsequent succinate accumulation, associated with a pro-inflammatory M Φ profile (Galván-Peña and O'Neill 2014). In addition, most of the work conducted on MDSCs and HBV suggest that the high content of Arginine-I present in these suppressive cells is associated with L-Arginine deprivation in the liver environment and subsequent T cell exhaustion (L-Arginine is a key metabolite for T cell) (Dorhoi et al. 2019; Fang et al. 2015; F. Yang et al. 2019; Nebbia et al. 2012). Thus, it is highly probable that liver ResM Φ are metabolically switched by the infection. Fortunately, a new trends in cancer therapy consist in repurposing already approved anti-metabolic drugs, (with the goal to target the anti-inflammatory environment) (Varghese et al. 2021, 839; Wicker et al. 2021, 839), it is time to perform the same for antiviral therapy. Of note, a screen of different metabolic modulators is currently ongoing in the lab in both infected hepatocytes and M Φ .

3. HBV genotypes, HDV and macrophages

As a follow-up of our first study, we investigated the impact of different HBV genotypes on M Φ phenotype, namely HBV-B and HBV-C, in addition to the already used HBV-D. Interestingly, HBV-C was found especially efficient in reducing pro-inflammatory cytokines production whereas HBV-B led to increased anti-inflammatory IL-10 secretions. If we take a look at viral inocula composition (Supplementary results – Figure 1) for each of these genotypes, we observe that HBV-B has especially high levels of HBsAg and HBeAg compared to HBV-C and HBV-D, whereas the two latter were similar, at least in their ratio in between secreted parameters. As we normalized our inocula on HBV DNA and not antigenemia, HBV-B incubated MDMs had more HBsAg and HBeAg than HBV-D and-C. However, it is my belief that the observed modulations cannot be attributed only with an increase in viral protein levels, as we showed a dose-dependent response in M1 and M2-MDMs exposed to HBV total inocula (Faure-Dupuy, Delphin, Aillot, Dimier, Lebossé, Fresquet, Parent, Sebastian Matter, et al. 2019). Thus, an increase such as the one observed in PLMs by HBV-B (i.e 20-fold more IL-10 compared with control) should be accompanied by at least a modest but significant decrease in IL-1 β secretion, which is not the case here. In addition to the already mentioned modulations of HBsAg coating as an explanation of HBV modulation, another interesting hypothesis would be the changes observed in the ratio between S/M and L-HBsAg (Peiffer et al. 2018; Helene Norder et al. 2004). Indeed, as the proportions differs, the conformation should as well, and putative differences in receptor binding could arise. Besides, HBV-E is the sole representant of the ayw4 serotype, with a unique deletion in PreS1, supposedly leading to a larger M-HBsAg (Anna Kramvis 2014). Thus, modulation of HBsAg sequence and/or assembly could also be of importance here.

Due to difficulties and low quantities of the produced HBV genotypes, incubation of HBV-B with M1-MDMs was not performed yet. It is however, one of the important perspectives of this work, as well as trying other HBV, but also HDV, genotypes.

Huh7-HBsAg-HDV+ cells are producing HDV-I enveloped in HBsAg from HBV-D, however it would be interesting to envelope HDV in different HBsAg genotype and investigate if we recapitulate the observed inhibition of MDMs antiviral secretion. In addition, what we observe may only

consist in HBsAg effect and the fact that there is HDV within do not change anything, as co-culture with Huh7-HBsAg+ led to similar phenotype.

HDV is able to modify components of the liver environment which may be involved in M Φ pro-inflammatory activation, such as IFN activation (Alfaiate et al. 2016) and the previously observed boosting of HBV epitope presentation (Tham et al. 2020). However, more immune-relevant models should be used to investigate such issues (i.e co-culture with infected PHH). On the other hand, a recent study on 50 mono and co-infected patients, suggest that HBsAg evolution is impacted by HDV presence in a way favoring immune escape (Sajjad et al. 2021)(i.e mutation leading to less binding on MHC-I and-II, generation of new T cell epitopes). Thus, it is of high interest to really decipher the specific modulations that can be attributed specifically to HDV. To this intention, virions from HBV or HBV/HDV infected patients could be extracted to be incubated with MDMs or PLMs (from non-infected patients).

It should thus be of interest to better characterize the immune profile of HBV and HDV patients, depending on their immune status, but also, their genotype. Immunomodulatory treatments are the new trend in HBV/HDV drug development (Schmidt et al. 2021; Erken et al. 2018; Xiaoning Wu et al. 2018; Korolowicz et al. 2016; Janssen et al. 2018; El-Khoueiry et al. 2017; Buchmann et al. 2013; Dembek, Protzer, and Roggendorf 2018; Meng, Chen, and Lu 2020), and most experiments are conducted using HBV-D and HDV-I genotype, when it is known that there is a genotype-dependent response to the first immunomodulatory treatment used, the pegIFN α (Anna Kramvis 2014; Xianyu et al. 2018; Sugauchi et al. 2003; Sonneveld et al. 2012). Indeed, low (HDV, HBV-C and HBV-D) or good (HBV-B) pegIFN α response is observed, whereas such correlation was not made for NAs (Anna Kramvis 2014; Xianyu et al. 2018; Sugauchi et al. 2003; Sonneveld et al. 2012; Niro et al. 2006). Thus, gaining in understanding and characterization of the genotype-dependent immune responses (not only of M Φ , but as a general mechanism) could enable us to better apprehend these resistance phenomena, an important task, especially when taking into account that HDV, HBV-C and-D are also one of the most aggressive HCC inducers.

To date, very few studies investigated the differences in immune status from pan-genotypic patients, most of them being related to the genotype present within the laboratory region. To this end, an international effort must be undertaken.

4. *In vivo* analysis of the myeloid compartment?

As previously discussed, the most convenient animal model to work on HBV-related immunity is the AAV-HBV injected C57Bl6 mice. Studies have already been conducted on this model to characterize its immune adaptive compartment (Dion et al. 2013), which is, as in patients, exhausted, but nothing has been done to date on the innate myeloid one. However, people are largely conducting studies on the different myeloid cells' population in this model, without the need to compare it to non-AAV-HBV injected mice. Without an appropriate characterization, it is impossible to correctly appreciate if what we observe consist in an increase in pro-inflammatory marks from basal level, or just a return to baseline. Besides, to date, we observed an increase in anti-inflammatory marks *in vitro* and in patients' biopsies, but we do not know if the AAV-HBV model behave likely.

Hence, characterization of AAV-HBV mice compartment is to date, essential. This is why we and collaborators are currently performing it in the lab, using FACS, IHCs and RNA extraction as a read-out (data not shown – not all read-out performed/analyzed).

One really exciting perspective to this work would be to implement new and more precise technologies, such as transcriptomic, to characterize better the myeloid compartment present within the liver, and even investigate different timelines. In a collaboration between Florent Ginhoux's and Matteo Iannacone's lab, using these techniques, two distinct liver ResM Φ populations were observed in HBV Transgenic mice, KC1 and KC2 (De Simone et al. 2021). Interestingly, KC2 were being especially potent in breaking CD8T cell exhaustion through antigen presentation, upon IL2 treatment. It should thus be of interest to investigate their presence in AAVHBV injected mice, and possibly in HBV/HDV coinfection settings as HDV was recently demonstrated as being an inducer of HBV presentation on MHC (Tham et al. 2020). It is however mostly preliminary results and it is thus unclear if these KC1 and KC2 are differentially expressed in the infected liver, and how they participate in (i) the immunopathology and (ii) the CD8+T cell exhaustion at steady state (i.e without IL-2 treatment).

5. IL-1 β treatment leads to viral RNA decrease

Using CM from MDMs, we demonstrated that HBV-induced modulation on M Φ was blocking the antiviral effect on its establishment (Faure-Dupuy, Delphin, Aillot, Dimier, Lebossé, Fresquet, Parent, Sebastian Matter, et al. 2019), suggesting that HBV modulations could be required by the virus for its survival. We decided to focus on IL-1 β cytokine for the rest of the study as we previously observed its highly potent impact on HBV infection (Isorce et al. 2016) and it was present in doses sufficient for HBV inhibition in CMs.

a. IL-1 β is able to inhibit HBV establishment in hepatocytes

In our first study, we observed that 1 ng/mL of IL-1 β pre-treatment (one day prior the infection) was able to decrease HBV establishment (as seen with cccDNA expression) by up to 75% without any cellular cytotoxicity. Interestingly, the treatment was associated with NTCP downregulation in dHepaRG cells, but not in PHH, suggesting that even if HBV entry could be impacted, it is not the sole antiviral mechanism mediated by the cytokine. Watashi and colleagues observed a similar decrease of HBV establishment, with IL-1 β being the most efficient antiviral cytokine, amongst a screening of 37 immune mediators (Watashi et al. 2013). Interestingly, they observed an increase of the Cytidine Deaminase (AID) after 16h of IL-1 β treatment, and were able to induce HBV DNA hypermutation in HepG2 over-expressing AID cells, suggesting a role for AID in HBV establishment inhibition. However, we did not observe such increase in AID at the mRNA level upon IL-1 β treatment in infected dHepaRG and PHH (data not shown), suggesting that the observed effect might be independent of such protein, in our models.

Even if not tested, to date, on HDV establishment, IL-1R activation might be of interest in co-infection as well. Indeed, bulevirtide, the new HDV antiviral drug, acts by blocking virion entry within hepatocytes and thus precluding viral propagation (Kang and Syed 2020). Preliminary results of phase III suggest a decrease in overall HDV infection in patients (to be confirmed at the end of phase III), implying that entry inhibitors or NTCP expression modulators, which are poorly efficient *in vivo* against HBV, could be of interest in co-infected patients.

b. IL-1 β inhibit already established HBV and HDV infection in hepatocytes

We observed a similar dose-dependent decrease of HBV-A to-E and HDV RNA upon IL-1 β treatment. First, it is crucial to reproduce these experiments in a pan-genotypic manner (HBV-F to J) and to confirm our preliminary results on HBV-A and HBV-B. Such analysis of different HBV genotypes drug sensitivity has never been performed to date but seem crucial as already established differences in IFN response has been observed in different patient's cohorts (Y. Guo et al. 2019; Erhardt et al. 2005; Sonneveld et al. 2012). However, this could suggest that NF κ B inducers are more potent in broadly decreasing HBV/HDV viral RNA than IFN.

We observed a shorter half-life of HBV RNA upon IL-1 β treatment in dHepaRG cells treated with triptolide, suggesting that an IL-1R downstream protein is capable of degrading HBV RNA. However, another action on transcriptional inhibition cannot be ruled out only with this experiment. To investigate further this hypothesis, run-on could be performed to see if nascent HBV RNA transcription are also decreased by the treatment, or not. Nonetheless, as HBV RNA is produced at low levels in dHepaRG, long BrdU exposition is required to detect nascent RNA (2hours) and it is thus impossible to segregate transcriptional inhibition from degradation using this technic (at least in our hands, to date). A similar run-on approach is feasible for HDV as well with the same limitation. A specific effort for setting up an appropriate run-on experiment is currently ongoing in the lab.

As we cannot rule out this hypothesis to date, it is important to discuss the putative role of IL-1 β treatment on transcriptional inhibition.

First, several articles point out the presence of NF κ B binding site on viral genes, such as HIV-1, SV40 and several herpesviruses (Santoro, Rossi, and Amici 2003) but also HBV (Kwon and Rho 2002; Y.-C. Lin, Hsu, and Ting 2009) and HDV (observed in the lab). A total of four NF κ B binding sites were found in HBV adw subtype, on the nt (i) 207-216, (ii) 1408-1417, (iii) 1478-1486 and (iv) 2444-2452. HBc functions as a positive regulator of NF κ B subunit binding, at least on the 1408-1417 site (not shown for the others), leading to transcriptional activation. However, NF κ B dimers binding leads to different transcriptional impact depending on how the subunits are associated and which dimers it is composed of. Thus, one hypothesis would be a change in NF κ B dimers composition, or a change of NF κ B binding site on HBV DNA following IL-1 β treatment. This should be addressed by performing ChIP experiments of RelA/RelB/p65/p50 to see if HBV DNA is differentially bound to such subunits depending on IL-1 β treatment. Besides, a region of 43 amino acid in N-Term of p65 was able to bind and subsequently inhibit the Specificity Protein 1 (SP1) responsible for HBV transcriptional activation, suggesting that p65 association with the HBV DNA is not necessarily positive for the virus. HDV NF κ B binding site should and will be investigated in a similar manner (R-IP experiments), especially considering that nothing was published to date on their relevance in HDV replication and/or inhibition.

c. IL-1R mediated viral RNA degradation

One recently identified mechanism of action of HBV RNA degradation is to target their special mixed tails, as RG7834 does (Han et al. 2018). TENT4A/B enzymes role was for long described as inducers of exosome-mediated degradation of noncoding RNA through oligoadenosines addition at their 3'end (D et al. 2020). Interestingly, Sun and colleagues described that

TENT4A/B presence on HBV mRNA enable maintenance of its tail length and subsequent stabilization of the transcript, a process which is, to date, not shared with other viruses (D et al. 2020). Thus, RG7834, by binding and sequestering the TENT4A/B complex, block mixed tail establishment, leading to shortened HBV RNA which are more sensitive to exonucleases. Thus, RG7834, is a good control for specific HBV RNA degradation. As similar profile were observed in RG7834 and IL-1 β treated cells following triptolide treatment, we wondered if the IL-1R downstream pathway could induce a similar reduction of HBV RNA length. To this intention, we performed Northern Blot on total RNA extracted from cells treated with both drugs, or not. We successfully observed the shortening of HBV RNA in RG7834 treated cells, but not IL-1 β , suggesting that its degradation process was independent of RNA length modulations (Supplementary Results).

Another stabilizer of HBV RNA is the La autoantigen protein (Heise, Guidotti, and Chisari 2001). By binding to the stem-loop structure of HBV RNA (nt 1275-1291), the La protein acts as a chaperone which hinder sterically the RNAses access to HBV RNA cleavage site. Interestingly, cleavage product of the La protein were observed following IFN α and TNF α treatment and the p26 form associated with viral decrease (Heise, Guidotti, and Chisari 1999), thus IL-1R mediated cleavage of La autoantigen should be investigated further. Besides, La protein is also binding and stabilizing RNA viruses, as seen with HCV (Spångberg, Wiklund, and Schwartz, n.d.), so its binding to HDV RNA could be possible, especially as it contains a lot of loop and bulges. It is worth noticing that more recent work associated the La autoantigen with translational activation of poliovirus, HCV, ECMV, IAV and HIV (Izumi et al. 2004, 20). Whether it is the La autoantigen that stop binding to HBV RNA, or another destabilizing mechanism yet unknown, a second agent must be at play to fully degrade viral RNA, the endo/exo nucleases.

Most of the literature related to IL-1R pathway induced RNA degradation is associated with the activation of MCPIP-1 (regnase-1 or ZC3H12A) (M. Li et al. 2020; R.-J. Lin et al. 2014; 2013), as previously discussed in the introduction. It was actually first described as the negative regulatory feedback loop of IL-1R pathway (Skalniak et al. 2009), responsible for the appropriate return to homeostasis after NF κ B stimulation. This endonuclease specifically degrades IL-1 β , IL-6, IL-12 and some miRNA transcripts, most probably by binding to a stem-loop region (not clear to date) (Jura, Skalniak, and Koj 2012). Recently, MCPIP-1 degradation of HBV RNA following IL-1 β treatment was observed, with an association (R-IP experiment) between HBV RNA stem loop region and the RNase part of the endonuclease (M. Li et al. 2020, 1). Unfortunately, we did not recapitulate these observations (Supplementary results). One hypothesis is that the aforementioned study mainly used transfection of HBV RNA in MCPIP1 over-expressing cell lines or *in tubo* experiments. Hence, it is possible that the association and subsequent degradation of HBV RNA by MCPIP1 is possible, but not relevant in a more physiological context (i.e infection, immune-relevant models such as PHH and dHepaRG cells).

Other exonucleases were described as responsible for RNA degradation, such as the exosome complex, recruited by the Zinc-finger Antiviral Protein (ZAP or ZC3HAV1) (Mao et al. 2013). However most antiviral agents known to degrade HBV (or HDV) are IFN-induced proteins, and their induction by the NF κ B pathway is still to be proved.

To this intention, a large screening by siRNA of 20 putative endo/exonucleases targeting HBV and HDV RNA following NF κ B-stimulation is currently ongoing in the lab.

IL-1 β is highly interesting as its antiviral activity is targeting both the establishment and maintenance of HBV and HDV infection. Thus, by both degrading viral RNA in hepatocytes and avoid re-infection, IL-1R agonists could be of high interest in the development of new efficient antiviral drugs for both HBV and HDV infected patients. Besides, IL-1 β seems to possess a multi-genotypic effect, which is not the case of IFN.

6. How to treat patients undergoing HBV/HDV infections.

a. IL-1 β treatment for *in vivo* therapy

IL1B treatment at too high concentration or upon too many treatment leads to hepatocyte dedifferentiation (i.e decrease ApoB secretion and HNF4a production) (Study 1 and (Isorce et al. 2016)) and is associated *in vivo* with various side effects already mentioned (Veltri and Smith 1996). However, in our hands, no cytotoxicity or de-differentiation was observed in pre- or post-treatment with IL1B (100 pg/mL and one treatment) (Isorce et al. 2016; Faure-Dupuy, Delphin, Aillot, Dimier, Lebossé, Fresquet, Parent, Sebastian Matter, et al. 2019), suggesting, that IL-1 β act on viral RNA independently of these two parameters. However, such concentration may not be sufficient enough *in vivo* and several treatments might be required, leading to toxicity. It is first worth noticing that dedifferentiation *in vivo*, if punctual, does not come with strong liver impairment, it is a transient process happening after, for instance, alcohol poisoning (Do et al. 2013). If hepatocytes dedifferentiation leads to HBV loss (Schulze-Bergkamen et al. 2003; Y. Zheng, Li, and Ou 2004), it is not the case for HDV (J. Chang et al. 2008). Besides, the subsequent hepatocyte proliferation could enhance HDV propagation through cell-to-cell spreading (Giersch et al. 2019). Hence, a careful estimation of IL-1 β antiviral effect *in vivo* is required. One idea, as mentioned earlier, would be the use of immunocytokines (Neri and Sondel 2016) or AcTAKines (Van Den Eeckhout et al. 2020) to avoid side effects. Nonetheless, the sole use of IL-1 β might not be sufficient to restore the exhausted immune compartment present within the infected liver, and thus more broader strategies, targeting TLRs or metabolites, seems more relevant. Besides, the TLR/IL-1R downstream pathway on hepatocytes is highly similar and both were found crucial in restoring the appropriate CD8+T cell response in a model of hydrodynamic injection (Ma et al. 2017).

b. Relevance of restoring the myeloid compartment for clearance of HBV/HDV

In a mice model of mother-to-child transmission, HBeAg positivity in the mother was associated with HBV establishment in the progeny (Y. Tian et al. 2016). It was correlated with the induction of M Φ tolerance (M2-like phenotype) and subsequent CD8+T cell exhaustion. Total reversion upon chlodronate treatment highlighted the strong impact that tolerogenic M Φ can have on the outcome of HBV infection (Y. Tian et al. 2016). Besides, as mentioned, CBI is associated with MDSCs recruitment which are also associated with suppressive CTL functions by IL-10 secretion, IR-L expression and Arginase-I deprivation, amongst other mechanisms (A. Huang et al. 2014; Pallett et al. 2015b; F. Yang et al. 2019).

It has been established that the presence of myeloid cell aggregates following TLR9-L mediated activation led to HBV-specific T cell expansions associated with viral clearance, in several mice models (termed intrahepatic myeloid-cell aggregates for T cell expansions (iMATEs)) (L.-R. Huang et al. 2013; Kosinska et al. 2019). These aggregates are lost in CCR2-/- mice (conditional depletion by DTR), and a more profound analysis of iMATEs myeloid cells markers proved that they were mainly formed by infiltrating monocytes activated towards a pro-inflammatory phenotype (Huang et al., 2013).

These studies suggest that an appropriate recruitment of inflammatory M Φ phenotype is crucial for specific CTL expansion and activation, leading to subsequent HBV clearance.

c. Restoring the immune compartment, TLRs agonists

As previously exposed, the use of immune modulators, alone or in combination, has gained an increased interest in the past few years, especially TLR-Ls. However, not all TLRs are expressed on hepatocytes (Faure-Dupuy et al. 2018) and have thus no direct effect on HBV/HDV infection, which may explain their poor efficiency in clinical trials (Janssen et al. 2018). Besides, recent reports suggest that TLR8 agonisation in *ex vivo* stimulated control and patients PBMCs led to an efficient reduction of some immunoregulatory subsets (CD4+T cells and MDSCs), accompanied however with hepatocytes lysis and increase in inhibitory receptors (Gal9 and PD-L1) (i.e no restoration of CD8+T cell exhaustion) (Amin et al. 2020). As cellular apoptosis is one of the driver of viro-induced HCC, it is crucial to avoid such type of viral clearance (Peeridogaheh et al. 2018).

We in the lab, recently performed a screening of diverse TLR-L (Lucifora et al., 2018) and sorted TLR2-L and TLR3-L has being the most interesting in reducing HBV infection in hepatocytes. The two receptors are expressed on hepatocytes and immune cells (Faure-Dupuy et al. 2018), being able to induce antiviral cytokinic production in both, and subsequent reduction in HBV viral parameters, without rebound, suggesting that cccDNA is impacted (Lucifora et al., 2018). Interestingly, even if observed by some as decreased in PBMCs and KC of HBV-infected patients (Y.-W. Huang et al. 2013b; Visvanathan et al. 2007b), experiments from the lab suggest that only low-doses are sufficient enough to ensure appropriate downstream pathway activation and a positive feedback loop, at least for TLR2-L, leading to increase in the receptor's expression in hepatocytes (Manon Desmares PhD thesis).

However, when tested by systemic injection in a liver humanized mice model infected with HBV, results were disappointing (Lucifora et al., 2018). One hypothesis could be the lack of adaptive immunity in this model (thus biased immunity), hindering the efficiency of these ligands. In addition, systemic injection of an immunomodulator is surely not the best strategy, as it may not be addressed specifically to the liver. Even if no cytotoxicity was observed in mice, agonisation of TLR2 systemically could lead to the activation of all circulating cells expressing the receptor (i.e professional cells, like monocytes, but also endothelial cells), and is thus sub-optimal. To this intention, we collaborated with Bernard Verrier's team that developed Poly(Lactic Acid) Nanoparticles coated with TLR2-L (PAM3CSK4), as previously discussed (Lamrayah et al. 2019). The use of NanoP-TLR2-L enabled rapid (few minutes) and specific localization/delivery to the liver of the agonist. This led to a strong decrease of all HBV parameters, which was not observed with TLR2-L alone. Besides, we observed *in vitro* and *in vivo* induction of pro- over anti-inflammatory marks, which suggest an appropriate activation of the immune compartment. Ex-vivo incubated PLMs and LMNCs exposed to NanoP-TLR2-L were secreting IL-1 β in amount that we showed sufficient to reduce HBV and HDV infection (between 50 to 400 pg/mL, depending on NanoP-TLR2-L concentration). Interestingly, IL10 secretions,

even if not clearly induced, are not abrogated. This is of high importance as IL10 is a key guardian of liver homeostasis and its total inhibition could lead to uncontrolled pro-inflammatory activation. In AAV-HBV injected mice, NanoP-TLR2-L treatment led to the preferential increase of iNOS, IL-6 and IL-1 β over Arg-1 and CD206, when compared with untreated and TLR2-L treated cells, suggesting that, indeed NanoP-TLR2-L are highly potent in inducing pro-inflammatory marks within the liver. However, confirmations at the protein level are required to further conclude on the myeloid compartment activation within these mice. IHC staining of NanoP-TLR2-L treated mice are ongoing and preliminary results suggest the presence of iMATEs-like complexes within the liver ([Huang et al., 2013](#)) which, if confirmed, would suggest the efficiency of the treatment in restoring an appropriate CTL response (data not shown). Complementary analysis by FACS of the different immune populations and their specific phenotype will also be performed.

Unfortunately, the levels of HBV replication markers in mice of this cohort, as mentioned, was very low (only 10⁵ VGE/mL of viremia, when at least 10⁷ is required) and at that time, we only monitored the mice weight to address putative cytotoxicity. Even if mice behaved normally, had no septic choc and organs had similar size in between groups (i.e suggest no over-inflammation), more precise read-out are required, such as ALT level (for liver injury). In addition, we observed *in vitro* an impact of NanoP alone at high doses on LMNC and PLM secretion phenotype. Thus, even if *in vitro* comparison between NanoP and NanoP-TLR2-L demonstrated that NanoP alone are not capable of decreasing HBV infection in dHepaRG (Manon Desmares, PhD thesis), its *in vivo* impact on the immune compartment is still to be investigated.

Since then, efforts have been successfully undertaken to increase or infectious rate in this model, and a new cohort will soon be launched to address all the previous comments.

d. The use of immunomodulatory treatment in an exhausted compartment

Another point, which should be addressed upon immunomodulatory drugs trials in CHB or CHD patients, should be the immune exhaustion observed. As demonstrated in this thesis, and also by others ((Fang et al. 2015; Z. Chen et al. 2008; Müller and Zielinski 1990; Zannetti et al. 2016; M. Jiang et al. 2014) non exhaustive list), the liver immune micro-environment is hindered by HBV presence, mostly associated with HBsAg. Hence, the use of TLR-L, metabolites, or other immune activators may be precluded by the unresponsiveness of immune cells. To this intention, combination therapy should be tested, especially using NAPs or siRNA which were recently showed capable of decreasing HBsAg circulating amount in patients (Vaillant 2019; Man-Fung Yuen et al. 2019). Indeed, NAPs-induced HBsAg reduction was associated with a return to the appropriate immune control of HBV and HDV, when used in combination with IFN α (Vaillant 2019). However, IFN is not well-tolerated by patients, especially HBV-HDV co-infected patients which are often unresponsive ([Rizzetto, 2016](#)). Thus, one possibility would be the use of HBsAg reducing agent in combination with new innovative immunomodulators, such as the ones discussed earlier (immunometabolites, TLR-L) (Erken et al. 2018; Schmidt et al. 2021; Amin et al. 2020; Janssen et al. 2018; Korolowicz et al. 2016; Xiaoning Wu et al. 2018). In addition, a correct evaluation of immune subsets differences in HBV vs HBV/HDV patients are lacking to ensure correct repurposal of drugs efficient on mono-infection. Indeed, as HDV is a well-known inducer of hepatocyte IFN response (Alfaiate et al. 2016), the liver environment and subsequent response to immune inducers may differ.

Altogether, this work provided new evidences of HBV-mediated modulations of the M Φ compartment, as well as hinting the interest of NF κ B inducers in combinations therapy for both HBV and HDV clearance in patients. As discussed, the perspectives of this work are numerous and should require more international collaborations.

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« Qu'importe où nous allons, honnêtement. Je ne le cache pas. De moins en moins. Qu'importe ce qu'il y a au bout. Ce qui vaut, ce qui restera n'est pas le nombre de cols de haute altitude que nous passerons vivants. N'est pas l'emplacement où nous finirons par planter notre oriflamme, au milieu d'un champ de neige ou au sommet d'un dernier pic dont on ne pourra plus jamais redescendre. N'est plus de savoir combien de kilomètres en amont du drapeau de nos parents nous nous écroulerons ! Je m'en fiche ! Ce qui restera est une certaine qualité d'amitié, architecturée par l'estime. Et brodée des quelques rires, des quelques éclats de courage ou de génie qu'on aura su s'offrir les uns aux autres. Pour tout ça, les filles et les gars, je vous dis merci. Merci. »

Appendices

1. Oral presentation

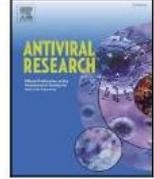
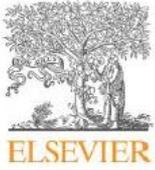
- × Annual meeting of the ANRS AC42 - February 2020, Paris, France.
- × BMIC Doctoral school days - November 2019, Lyon, France.

2. Poster presentation

- × 33rd Conference of the EMDS - September 2019, Marseille, France.

3. Publications

- × Current knowledge on Hepatitis Delta Virus replication
Julie Lucifora et Marion Delphin.
Antiviral Research, 2020.
- × How to get away with liver innate immunity? A viruses' tale.
Marion Delphin, Manon Desmares, Svenja Schuehle, Mathias Heikenwalder,
David Durantel, Suzanne Faure-Dupuy.
Liver International, 2021.
- × Hepatitis B virus-induced modulation of liver macrophage function
promotes hepatocyte infection
Suzanne Faure-Dupuy*, Marion Delphin*, Ludovic Aillot, Laura Dimier, Fanny
Lebossé, Judith Fresquet, Romain Parent, Michel Rivoire, Nathalie Bendriss-
Vermare, Angela Lam, Klaus Klumpp, Fabien Zoulim, David Durantel and Julie
Lucifora.
Journal of Hepatology, 2019.



Invited Review

Current knowledge on Hepatitis Delta Virus replication

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ABSTRACT

Hepatitis B Virus (HBV) that infects liver parenchymal cells is responsible for severe liver diseases and co-infection with Hepatitis Delta Virus (HDV) leads to the most aggressive form of viral hepatitis. Even though being different for their viral genome (relaxed circular partially double stranded DNA for HBV and circular RNA for HDV), HBV and HDV are both maintained as episomes in the nucleus of infected cells and use the cellular machinery for the transcription of their viral RNAs. We propose here an update on the current knowledge on HDV replication cycle that may eventually help to identify new antiviral targets.

1. HDV, a satellite virus

In the late 70s, the study of Hepatitis B Virus (HBV) infected patients experiencing extremely severe hepatitis lead to the discovery of a small virus named Hepatitis Delta Virus (HDV) (Rizzetto et al., 1977). This virus was later shown to be a defective satellite virus that uses the envelope of HBV (Fig. 1 and Fig. 2) to egress from and to re-enter into hepatocytes (Rizzetto et al., 1980). Apart from those steps, HDV intracellular RNA replication is independent of HBV. Hence, HDV RNAs can be detected for at least 6 weeks in the liver of experimentally infected HuHep mice in the absence of HBV (Giersch et al., 2014). Contrary to HBV, HDV was shown to be able to replicate in different tissues/cell types after transfection of a plasmid containing HDV genomes (head-to-tail dimers or trimers) used to launch the infection cycle (Polo, Jeng et al., 1995; Polo, Lim et al., 1995). Moreover, HDV-like viruses were recently identified in birds (Wille et al., 2018), snakes (Hetzl et al., 2019) but also fish, amphibians and invertebrates without association to any partner hepadnavirus (Chang et al., 2019). These recent data challenge the hypothesis of HDV solely originated from an escaped human gene (Salehi-Ashtiani et al., 2006) and suggest that HDV might be able to use other helper viruses to egress. Results of a series of *in vitro* experiments also showed that HDV ribonucleoprotein (RNP) can be packaged with envelopes from several non-HBV related viruses such as hepacivirus, flavivirus and vesiculovirus (Perez-Vargas et al., 2019), broaching the question of HDV also being transmitted by other viruses in humans (Fig. 3).

2. Organization of HDV genome in virion and intracellular replicative intermediates

Besides, the HBV envelope (or potentially other viral envelope), HDV virions contain a RNP composed of (1) a circular single-stranded negative RNA genome (HDV-G) of almost 1,7 kb (Wang et al., 1986), presenting an unbranched “quasi” double-stranded conformation, and of (2) both isoform of the viral proteins called S-HDAg and L-HDAg (Fig. 1). More precisely, HDV RNA forms an extended quasi-dsRNA containing numerous internal loops and bulges. Its association with HDAg would depend more on its secondary structure than on its primary sequence (Griffin et al., 2014). HDAg indeed binds as multimers (probably octamers (Zuccola et al., 1998; Cornillez-Ty and Lazinski, 2003; Alves et al., 2010)) to unbranched quasi-double-stranded HDV RNA segments (with length requirement of about 311 nt) to form nuclease-resistant complexes (Defenbaugh et al., 2009). Depending on the method used, it was reported that 30 to 200 molecules of HDAg would be bound to one molecule of HDV RNA (Ryu et al., 1993; Gudima et al., 2002; Defenbaugh et al., 2009) and therefore between 3 and 25 HDAg octamers per full-length HDV RNA. S-HDAg and L-HDAg, that can exist as either homomultimers or as heteromultimeric structures (Xia and Lai, 1992), have been found in equal quantity in circulating HDV virions (Bergmann and Gerin, 1986; Bonino et al., 1986).

HDV RNA replicative intermediates called anti-genomes (HDV-AG) are present in the nucleus of infected cells but not in circulating HDV virions (Chen et al., 1986). HDV-AG is fully complementary to HDV-G and bears the ORF for HDAg proteins expression. S-HDAg (195 amino acids) and L-HDAg (214 amino acids *i.e.* containing an extension of 19 amino acids compared to S-HDAg), originate from of a common 0,8 kb

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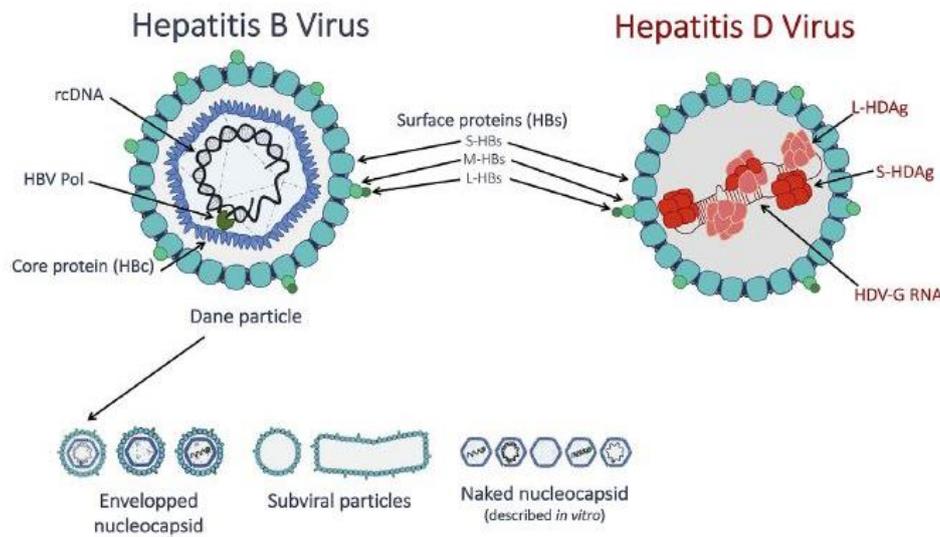


Fig. 1. Schematic representation of HBV and HDV particles. Left, infectious HBV virion (Dane particle) (upper) and non-infectious HBV particles, including enveloped capsid containing immature DNA/RNA, subviral particles (sphere and filament), and naked nucleocapsids (lower). Right, infectious HDV, of which envelope is identical to that of infectious HBV.

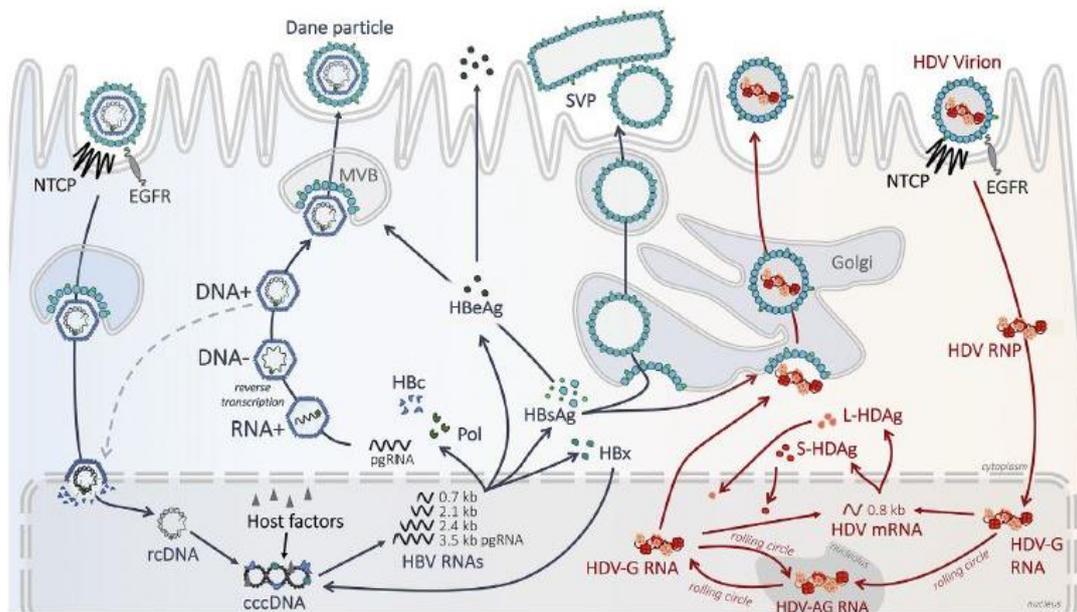


Fig. 2. Schematic overview of HBV and HDV life cycle. (HBV, left part) After entry through NTCP and EGFR, the HBV nucleocapsid is translocated to the nucleus where rcDNA is converted into cccDNA. The latter associate with viral and host factors to be used as a template for HBV RNAs synthesis. The pregenomic (pg) RNA and the viral polymerase (Pol) are encapsidated by multimerization of the core protein (HBc). After reverse transcription of pgRNA into rcDNA, mature nucleocapsids are enveloped and the infectious viral particles, named Dane particles, are secreted. Subviral particles (SVPs) forming spheres and filaments are assembled and secreted independently through the Golgi apparatus. **(HDV, right part)** Once in the cells, the HDV RNP translocates to the nucleus where the incoming genome (genomic HDV RNA; HDV-G) serves as template for the synthesis of the HDV mRNAs, allowing the synthesis of S-HDAg. HDV-AG (anti-genomic HDV RNA) and HDV-G and are synthesized from one another by a rolling-circle replication strategy. L-HDAg is produced later after editing of HDV-AG and subsequent synthesis of edited HDV mRNAs. Its association to HDV-G allow the nuclear export of new RNP and HDV particles assembly occur through the interaction of L-HDAg and HBsAg.

mRNA that is edited in the case of L-HDAg (Luo et al., 1990). Despite being both bound to HDV-G and having almost identical sequences, S-HDAg and L-HDAg have very distinct roles in the viral life cycle. S-HDAg is essential for HDV mRNA transcription and replication (Kuo et al., 1989; Glenn et al., 1990; Glenn and White, 1991; Harichandran et al., 2019), whereas L-HDAg is indispensable for virion assembly (Chang et al., 1991; Lee et al., 2001) but would represses HDV RNA synthesis (Chao et al., 1990; Glenn and White, 1991; Xia and Lai, 1992) (Fig. 4). More precisely, HDV-G RNA synthesis can be inhibited by low amount of L-HDAg whereas the synthesis of HDV-AG and HDV mRNA were found to be inhibited only when L-HDAg are in vast excess over S-HDAg (Modahl and Lai, 2000). According to the “black sheep” model proposed, L-HDAg would “poison” the homo-oligomeric S-HDAg

multimers bound to HDV-AG, thereby potentially disrupting the positive effect of S-HDAg on HDV-G RNA synthesis (Xia and Lai, 1992). Conformational differences between S-HDAg and L-HDAg induced by prenylation would be responsible for the differences in their trans-activating and trans-dominant inhibitory biological activities (Hwang and Lai, 1994).

3. Molecular aspects of genome replication and transcription – replicative cycle

A schematic overview of the HDV life cycle is presented in Fig. 2. As it uses HBV envelope proteins, HDV virions enter into the cells via the same mechanisms. Briefly, it is mediated by an attachment to heparan

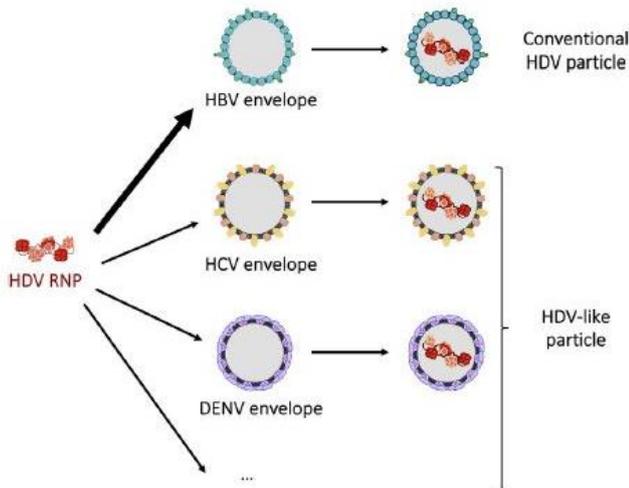


Fig. 3. Schematic representation of conventional and possible alternative HDV particles. HDV is a defective satellite virus that uses the envelope of HBV to egress from the hepatocytes. Recent data suggested that HDV might be able to use other helper viruses such as hepatitis C Virus (HCV) or Dengue virus (DENV).

sulfate proteoglycans at the surface of the hepatocytes followed by specific interactions of the virus with the Na⁺-taurocholate co-transporting polypeptide (NTCP) and the epidermal growth factor receptor (EGFR) (Urban et al., 2014; Iwamoto et al., 2019). Once in the cells, the HDV RNP translocates to the nucleus probably because of the NLS sequences present in S-HDAg (Chou et al., 1998; Tavanez et al., 2002). In the nucleus of infected cells, the incoming genomes (HDV-G) serve as template for the synthesis of the antigenomic sense HDV mRNAs containing poly(A) tails in 3' and cap structures in 5' (Hsieh et al., 1990; Modahl and Lai, 1998; Gudima et al., 2000) (Fig. 4). The presence of S-HDAg is mandatory to start HDV mRNAs transcription from circular HDV RNAs but only low amounts are actually required (Harichandran et al., 2019). These data strongly suggest that the quantity of S-HDAg brought by the incoming RNP is probably sufficient to initiate transcription from the HDV-G. Of note, the quantity of L-HDAg brought by the incoming RNP (equal amount as S-HDAg (Bergmann and Gerin, 1986; Bonino et al., 1986)) should not be sufficient to inhibit HDV mRNA synthesis as already mentioned above (Modahl and Lai, 2000). HDV-G also serve as template for HDV-AG synthesis by a rolling-circle replication strategy, as described for viroids (Branch and Robertson, 1984). HDV-AG RNAs in turn become the template for the rolling-circle amplification thereby yielding more genomic HDV-G (Fig. 4). Small self-cleaving RNA sequences (i.e. ribozyme) identified in HDV-G and HDV-AG (Sharmeen et al., 1988) are responsible for the cleavage of multimeric RNA molecules that arise

during the rolling circle amplification (Kuo et al., 1988; Jeng, Daniel et al., 1996) and represent the only enzymatic activity reported for HDV. Neither S-HDAg nor L-HDAg are required for HDV RNAs cleavage, but their presence enhances it (Lazinski and Taylor, 1994; Jeng, Su et al., 1996). The mechanisms behind the circularization and ligation of HDV RNAs remain elusive and may involve ribozyme self-ligation activity (Sharmeen et al., 1989) or cellular(s) factor(s) (Reid and Lazinski, 2000).

The initiation site for synthesis of the HDV mRNAs may differ from the initiation point for synthesis of HDV-AG RNAs. This was suggested by the fact that the loss of HDV-AG accumulation induced by mutations in the region of the putative RNA promoter for HDAg could be reversed by small HDAg provided *in trans* (Wu et al., 1997). Results from primer extension assays obtained later also supported the same hypothesis (Modahl and Lai, 1998). Strong evidences support the hypothesis of HDV mRNA synthesis carried out by the DNA-dependent RNA polymerase II (Pol-II) (MacNaughton et al., 1991; Filipovska and Konarska, 2000; Modahl et al., 2000; Li et al., 2006). HDV would therefore be able to direct a DNA dependent cellular enzyme to an RNA template (Lehmann et al., 2007). If the quasi double stranded structure of HDV-G probably facilitates this hijacking, it is important to note that mammalian RNA-dependent mRNA amplification was probably underestimated until now and several studies recently showed that it could be a general physiological phenomenon (Rits et al., 2019; Volloch et al., 2019; Jain et al., 2020). A promoter region for HDV mRNA synthesis has been described in a region close to the end of the HDV-G RNA rod (Beard et al., 1996; Abraham and Pelchat, 2008). The start site of transcription would rather depend on the secondary structure of the HDV-G RNA than on its primary sequence (Filipovska and Konarska, 2000). Moreover, Pol-II was shown to recognize terminal stem-loop domains at the two poles of HDV-G (Greco-Stewart et al., 2007) where it forms, along with general transcription factors, pre-initiation complexes similar to those observed on canonical DNA promoters (Abraham and Pelchat, 2008) (Fig. 5). The main proteins of the nuclear paraspeckles (PSF, p54nrb and PSP1) would be associated to HDV RNA and involved in their accumulation (Beeharry et al., 2018). Even though debated and not clearly demonstrated, the synthesis of HDV-AG RNAs, that was reported to occur in the nucleolus, might be regulated by different mechanisms (Tseng et al., 2008) possibly involving Pol I-like polymerases (Modahl et al., 2000; Macnaughton et al., 2002; Li et al., 2006; Huang, Chen et al., 2008). On one hand, the involvement of Pol-I, which generally leads to transcripts that are not polyadenylated, would explain why the polyadenylation signal remains silent during the synthesis of HDV-AG RNAs by rolling circle amplification. On the other hand, nuclear run-on assay demonstrated that synthesis of HDV-AG could be inhibited by low concentrations of amanitin (specific for Pol-II transcription) (Chang et al., 2008) and HDV-G RNAs but also HDV-AG RNAs were detected after immunoprecipitation of Pol-II (Abeywickrama-Samarakoon et al., 2020). HDV-G RNA synthesis would be carried out by Pol-II and occur in PML body-associated structure

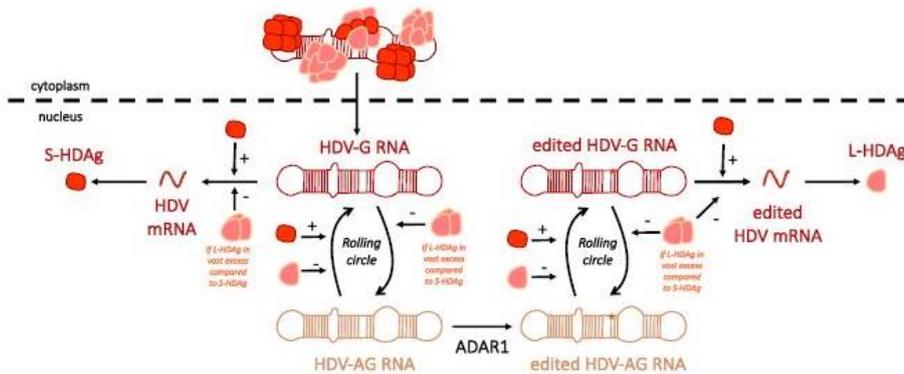


Fig. 4. Schematic representation of HDV RNAs synthesis. Genomic HDV RNA (HDV-G) serves as template for the synthesis of the HDV mRNAs and subsequent synthesis of S-HDAg. HDV-AG (anti-genomic HDV RNA) and HDV-G and are synthesized from one another by a rolling-circle replication strategy. Edition of a stop codon in HDV-AG by ADAR1 will give rise to edited HDV-G, edited HDV mRNAs and L-HDAg synthesis. The presence of S-HDAg is mandatory to start HDV mRNAs transcription from circular HDV RNAs. HDV-G RNA synthesis can be inhibited by low amount of L-HDAg whereas the synthesis of HDV-AG and HDV mRNA were found to be inhibited only when L-HDAg are in vast excess over S-HDAg.

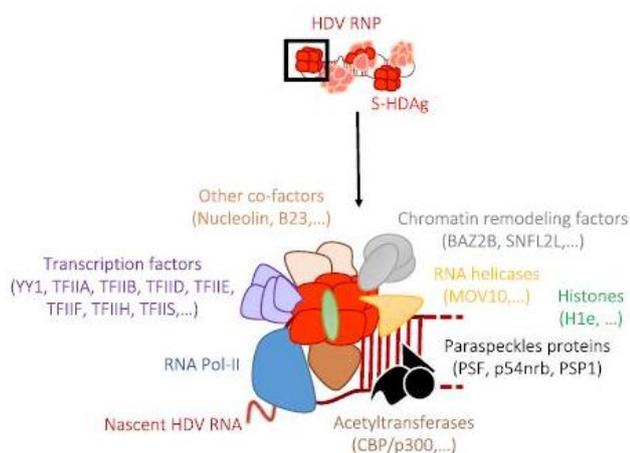


Fig. 5. Schematic overview of cellular factors possibly involved in the initiation of HDV RNA synthesis through their interaction with S-HDAg and/or HDV RNAs. Several lines of evidence suggest that S-HDAg would mimics histones and recruit cellular factors involved in the initiation of transcription to promote HDV RNAs synthesis. S-HDAg binds to HDV RNA as octamers and was shown to interact with the linker histone H1e, different subunit of Pol-II, transcription factors such as YY1, activating co-factors such CBP, p300, B23 and nucleolin, RNA helicase such as MOV10 as well as chromatin remodeling factors such as BAZ2B. The main proteins of the nuclear paraspeckles (PSF, p54nrb and PSP1) would be associated to HDV RNA and involved in their accumulation.

(Modahl et al., 2000; Macnaughton et al., 2002; Li et al., 2006; Huang, Chen et al., 2008).

Several lines of evidence suggest that S-HDAg would mimics histones and recruit cellular factors to promote HDV RNAs synthesis (Fig. 5). As histones on DNA (Kobayashi and Kurumizaka, 2019), S-HDAg binds to HDV RNA as octamers (Zuccola et al., 1998; Cornillez-Ty and Lazinski, 2003; Alves et al., 2010). S-HDAg (and not L-HDAg) interacts with the linker histone H1e and HDV replication is inhibited by deletion mutants of histone H1e (Lee and Sheu, 2008), thus emphasizing a role for this host factor in HDV life cycle. Of note, we confirmed the interaction of HDAG with several histones including histone H1e in HBV/HDV co-infected primary human hepatocyte (Lucifora et al., personal communication). S-HDAg interacts also with different subunit of Pol-II (Haussecker et al., 2008; Cao et al., 2009) and undergoes several post-translational modifications such as phosphorylation (Hong and Chen, 2010), acetylation (Mu et al., 2004; Huang, Mai et al., 2008), sumoylation (Tseng et al., 2010) and methylation (Li et al., 2004) that would be critical for its positive effect on the synthesis of HDV-G RNA and HDV mRNA synthesis but not on HDV-AG synthesis (Tseng et al., 2008). For instance, the phosphorylation status of the serine 177 of S-HDAg would be important for its interaction with the active phosphorylated form of Pol-II (Hong and Chen, 2010) whereas the acetylation of S-HDAg at the lysine 72 by the acetyltransferase p300 and the methylation at the arginine 13 by the arginine methyltransferase (PRMT1) would be important for S-HDAg nuclear localization and the enhancement of HDV-G RNA and HDV mRNA synthesis (Li et al., 2004; Mu et al., 2004; Huang, Mai et al., 2008; Tseng et al., 2008). S-HDAg has also been proposed to interact with transcription factors such as YY1 as well as with activating co-factors such CBP, p300 (Huang, Mai et al., 2008), B23 (Huang et al., 2001) and nucleolin (Lee et al., 1998) that together may facilitate the recruitment of Pol-II (Huang, Mai et al., 2008). MOV10, an RNA helicase, identified as a co-factor for RNA dependent RNA polymerase in plants, was also shown to interact with HDAG and would associate to small-capped antigenomic HDV RNAs to promote HDV RNAs synthesis (Haussecker et al., 2008). Very recently, a short linear interacting motif was found in S-HDAg (similar to the one present in histone H3) that allow the binding of S-HDAg to the BAZ2B-

associated remodeling factor (BRF). Importantly, RNA immunoprecipitation assays confirmed the binding of BRF remodelers to the HDV RNP in HDV-infected human hepatocytes suggesting (Abeywickrama-Samarakoon et al., 2020) again that S-HDAg would be essential to recruit different factors involved in the initiation of transcription. Many other cellular proteins interacting with S-HDAg (Cao et al., 2009) are probably involved in HDV RNA synthesis and further studies are required to get a full picture on these mechanisms. Besides its possible involvement in the recruitment of the transcription machinery, S-HDAg would also promote RNA elongation by removing inhibitory elongation factors (Yamaguchi et al., 2001). Even though, both mRNA transcription and HDV-AG RNA synthesis take place on the same template (HDV-G), they require different post-translational modifications of HDAG (Tseng et al., 2008) and as mentioned above are probably carried out by different transcription machinery (Modahl et al., 2000; Macnaughton et al., 2002; Li et al., 2006; Huang, Chen et al., 2008). It was recently suggested that, in the case of HDV-AG, S-HDAg would be more important for posttranscriptional events such as stabilization of the HDV-AG RNAs (to avoid nuclease activity) or promotion of ribozyme activity than directly for synthesis (Harichandran et al., 2019).

4. From HDV RNAs editing to virion production – morphogenetic cycle

If S-HDAg is produced early after infection by transcription of HDV mRNAs from the incoming HDV-G within the HDV RNP, L-HDAg is produced later after editing of the amber termination codon on HDV-AG (Luo et al., 1990) (Fig. 4). Using loss and gain of function approaches, the adenosine deaminase ADAR1 has been shown to be responsible for this editing. Whether only the non-inducible form (ADAR1-S) or also the interferon inducible form (ADAR1-L) is implicated in this editing is debated (Wong and Lazinski, 2002; Hartwig et al., 2006). As the structure adjacent to the amber site is suboptimal for editing and interaction with HDAG reduces the access to ADAR1 (Sato et al., 2004; Hsu et al., 2019), the expression of L-HDAg is delayed. This delayed expression allows a tuned progress from replicative to morphogenetic phases of the viral life cycle. The additional 19 amino acids present in L-HDAg contain a nuclear export signal (NES) (Lee et al., 2001) and a prenylation site (Glenn et al., 1992; Lee et al., 1994) that are required for viral assembly. The cellular L-HDAg NES-Interacting protein (NESI), identified by yeast two hybrid assays (Wang et al., 2005), was shown to be a nuclear membrane protein interacting with lamin A/C and nucleosporin thereby facilitating L-HDAg nuclear export (Huang et al., 2013). In parallel, L-HDAg forms a complex with cellular export receptor TAP (also called NXF1) and the adaptor protein Aly that would also be essential for HDV assembly (Huang et al., 2016). The isoprenylation site of L-HDAg is essential for its interaction with HBsAg (Hwang and Lai, 1993). The sequences and amount of HBsAg influence the assembly, release (Shih et al., 2008) as well as infectivity of HDV particles (Freitas, Abe et al., 2014). Trafficking of neo-formed HDV RNPs would occur through the *trans*-Golgi network and be clathrin-mediated (Huang, Chang et al. 2007, 2009; Wang et al., 2009).

5. Effect of HDV on its host

Although under diagnosed and underestimated because of the lack of standardized assays (Le Gal, Bricler et al., 2016), HBV/HDV co-infection is thought to be the most prevalent worldwide (compared to other co-viral infections). Indeed, at least 25 million people are estimated to be chronically co-infected with HBV and HDV (Wedemeyer and Negro, 2018). Two recent meta-analyses proposed two to three times more co-infection worldwide (Chen et al., 2018; Miao et al., 2019) but methods used in this study are still debated (Stockdale et al., 2018). HDV/HBV co-infection is also the most aggressive chronic form of viral hepatitis (Fu et al., 2016). In HBV positive patients with

fulminant hepatitis (a disease with an 80% rate of mortality within 2–10 days after infection), 33%–39% were tested positive for HDV (Smedile et al., 1982; Govindarajan et al., 1984). An accelerated progression towards fibrosis/cirrhosis followed by clinical liver decompensation and related death have also been described as major complications for chronically HDV/HBV infected patients (Buti et al., 2011). The risk of cirrhosis has been evaluated to be 23%, 41% and 77% after respectively 10, 20 and 30 years of co-infection (Yurdaydin et al., 2010). Studies reported at least a three-time higher risk of hepatocellular carcinoma (HCC) development in chronically HDV/HBV co-infected patients compared to HBV mono-infected ones (Fattovich et al., 2000; Ji et al., 2012; Kushner et al., 2015; Alfaiate et al., 2020). Moreover, HDV was identified as the main comorbidity factor in pluri-infected HIV patients in the national HIV Swiss cohort (Beguelin et al., 2017).

Nine to ten HBV genotypes (A to H; J has been recently added to the list but few full-length sequences are available) and eight HDV genotype (I–VIII) with distinct geographical distributions have been described (Littlejohn et al., 2016). A clear genotype-specific activity of HDV in supporting HDV RNA replication has been established by reporting the inability of HDV genotype III to support replication of HDV genotype I for instance (Casey and Gerin, 1998). The few existing and relevant clinical studies showed that viral genotypes (and some subtypes) highly influence the diseases outcome. Epidemiological data indicate that some HBV genotypes associate with a higher risk of chronic infection and/or evolution toward HCC. For instance, HBV genotype A in Japan is associated with a higher frequency of evolution to chronicity compared to other genotypes. In Europe, genotype A predominates over genotype D among HBV carriers (Lin and Kao, 2015). Similarly, it is well documented that some HBV genotypes are more prone to induce HCC (e.g. genotype C compared with genotype B in Asia; genotype A over other genotypes in Japan, genotype F1b in Alaska (Hayashi et al., 2018)). Moreover, HBV genotype C and HDV genotype I would be associated with lower remission rates and more aggressive outcomes as compared to HBV genotype B and HDV genotype II and IV respectively (Su et al., 2006). HBV genotypes may also influence HDV infection and replication since it was reported that HDV viral load is lower in patients co-infected with HBV genotype A compared to those co-infected with HBV genotype D or F and co-infection with HDV is more frequent in HBV genotype F infected patients (Kiesslich et al., 2009). Of note, since the viral genotypes have different geographical distributions, it is not excluded that environmental factors may also influence viruses replication and diseases outcome. Most of the studies performed *in vitro* or in animal models used HBV genotype D and HDV genotype I, and none reported a systematic comparison between the different viral genotypes.

In a majority of human HBV/HDV co-infection cases as well as in different animal models including chimpanzees, WHV-infected woodchucks and liver-humanized mice, HDV suppressed HBV replication (Rizzetto et al., 1980; Hadziyannis et al., 1985; Lee et al., 1987; Negro et al., 1989; Pastore et al., 1990; Colombo et al., 1991; Sagnelli et al., 1991; Arribas et al., 2005; Pollicino et al., 2011; Lutgehetmann et al., 2012). A recent cross-sectional study in a small cohort of 109 HBV/HDV infected patients reported HDV dominance in 75% of the cases, HBV dominance in 7% and no dominance for the rest of the cases. Interestingly, HDV dominance was associated with down-regulations of some interleukins, chemokines and cytokines as well as with a delayed in response to treatment with Pegylated interferon alpha (Peg-IFN- α) (Lutterkort et al., 2018). Of note, longitudinal analyses of HBV/HDV co-infected patients showed different profiles of viral dominance over time, emphasizing the complexity of the interactions between the two viruses and their host (Schaper et al., 2010). Limited knowledge has been generated to understand how HDV would interfere with HBV. It probably involves mechanisms independent from the adaptive immune system since it is also observed in isolated hepatocytes infected by both viruses (Alfaiate et al., 2016) and upon overexpression of HDV in

hepatoma cell lines (Wu et al., 1991). Using overexpression system, it has also been suggested that both forms of HDV might inhibit HBV replication by repressing the HBV enhancer activity (Williams et al., 2009). Moreover, the levels of HBV RNAs but not of cccDNA were decreased in the presence of HDV in non-transformed and immune-competent hepatocytes infected *in vitro*, suggesting an HDV-induced inhibition of HBV RNAs transcription or stability (Alfaiate et al., 2016). This is supported by the fact that besides their association to HDV RNAs, HDV proteins were shown to be able to associate to some cellular mRNA (Chen et al., 2019). Of note, infectious HDV particles can be produced in HBV non-replicating cells producing HBsAg from integrated HBV DNA (Freitas, Cunha et al., 2014) suggesting that chronic HDV infection could persist even though HBV replication would be suppressed highlighting the need to develop specific treatments for HDV infections.

The current knowledge, raised from both experimental and clinical studies, suggests that HBV is a weak inducer of innate responses and has evolved strategies to evade innate immune sensing (Wieland et al., 2004; Dunn et al., 2009; Luangsay et al., 2015), whereas HDV has been shown to activate the IFN pathway via MDA5 (Williams et al., 2009; Giersch et al., 2014; Alfaiate et al., 2016; Zhang et al., 2018). It has been suggested that both HBV and HDV could inhibit the JAK/STAT signaling pathway and hence the response to exogenous IFN (Christen et al., 2007; Pugnale et al., 2009). Moreover, the implications of the interplay between both viruses and hepatocyte innate immunity on pathogenesis remain to be understood. Despite being recognized for its antiviral effects on numerous viruses such as HBV, HDV or HIV for instance (Doyle et al., 2015; Isorce et al., 2016), the prolonged activation of the IFN pathway in chronic viral diseases may also promote pathogenesis. Indeed, IFN- α can have inhibitory effect on cell-mediated immunity through the production of immunosuppressive cytokines such as IL-10 and up-regulation of ligands for inhibitory receptors such as PDL1 that may contribute further to the loss of HIV-immune control (see (Doyle et al., 2015) for a review). In the case of HBV, it has been observed that IFN- α treatment increased the number of NK cells (Micco et al., 2013) thereby inducing killing of HBV-specific CD8 T cells by TRAIL-dependent mechanisms (Peppas et al., 2013). It is thus tempting to hypothesize that activation of the type-I IFN pathway by HDV may promote such mechanisms and thereby increase HBV pathogenesis in co-infected patients. HDV proteins were reported to alter autophagy process to promote its genome replication (Khabir et al., 2019), to cause oxidative stress (Williams et al., 2012) as well as modulating the TGF- β (Choi et al., 2007) and NF- κ B (Williams et al., 2012) signaling pathways. However, as already mentioned above, most of the studies performed on the interplay between HDV and cellular processes have been performed in artificial overexpressing systems, some even using non-hepatocytes cell lines. Numerous interactions between HDV proteins and cellular proteins have been reported (for review see (Goodrum and Pelchat, 2018)) but most of them need to be confirmed in infectious systems. Combining RNAi loss-of-function and small molecule screens in HDV-infected HuH7-NTCP, the team of Thomas Baumert recently identified the hypoxia-inducible factor (HIF)-signaling and insulin-resistance pathways as well as glycosaminoglycan biosynthesis and the pyrimidine metabolism as essential for HDV replication (Verrier et al., 2019). More precisely, using HDV primary human hepatocytes, they identified the carbamoyl-phosphatesynthetase 2, aspartate transcarbamylase and dihydroorotase (cad) enzyme and estrogen receptor alpha as key host factors for HDV life cycle (Verrier et al., 2019).

6. Conclusions

The current knowledge of the HDV structure and life cycle that we summarized above was mostly raised in the 90's from *in vitro* assays using elegant biochemical analyses but rather artificial models (i.e. overexpression of HDV proteins or RNAs in non-hepatocytic cell lines) because infection systems were not available at that time. Very few data

have been confirmed using more relevant systems. Studies are still required to uncover the different aspects of HDV life cycle as well as interactions with HBV and their host. This is mandatory to develop antiviral strategies efficient against HDV as illustrated with several host-targeting agents being currently clinically evaluated (Deterding and Wedemeyer, 2019).

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How to get away with liver innate immunity? A viruses' tale

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- Figures preparation: MD

Abstract:

In their never-ending quest towards persistence within their host, hepatitis viruses have developed numerous ways to counteract the liver innate immunity. This review highlights the different and common mechanisms employed by hepatitis viruses to (i) establish in the liver (passive entry or active evasion from immune recognition) and (ii) actively inhibit the innate immune response (i.e. modulation of pattern recognition receptor expression and/or signalling pathways, modulation of interferon response, and modulation of immune cells count or phenotype).

Key points:

- Hepatitis viruses subvert the immune system towards efficient internalization and replication;
- Hepatitis viruses can escape recognition by efficiently modulating antibody and complement recognition as well as inhibition of MHC presentation;
- Inhibition of PRR/IFN downstream signaling pathways prevent the production of cytokines and interferon;
- Hepatitis viruses modify the inflammatory balance towards the establishment of a tolerogenic environment;
- Innate immune modulators should be used as stand-alone or in combination with existing treatments to ensure potent virus elimination.

1. Introduction

The liver is constantly exposed to a large variety of pathogens (i.e. viruses, bacteria, and parasites) that can primarily infect the host through four different pathways, including (i) contaminated fluids, (ii) infected mosquito bites, (iii) contaminated food, and (iv) aerosols. This review will be focused on hepatitis viruses that replicate in the liver. Bacterial and parasite infections of the liver have been discussed elsewhere ^{1,2}.

Hepatotropic viruses infect approximately 540 million people per year, causing around 1.4 million deaths (WHO reports) (Figure 1). While they account for only 65.7% of all liver infections per year, viruses infecting through contaminated fluids represent 92% of related deaths (WHO reports). Once the infection is established, the hepatitis B (HBV), C (HCV), and Delta (HDV) viruses can lead to acute hepatitis (AH), fibrosis, cirrhosis, and/or hepatocellular carcinoma (HCC) ³. A vaccine targeting both HBV and its satellite virus, HDV, is available. However, patients still suffer from chronic hepatitis B (CHB) and thus need to be treated lifelong with nucleos(t)ide analogues (NA), pegylated interferon alpha (peg-IFN α), or a combination; however, these treatments lead to a functional cure in less than 10% of patients, highlighting the need for the development of new therapeutic approaches ³. Co-infection with HDV, diagnosed in around 5% of CHB patients ^{3,4}, accelerates the pathogenesis with a low response rate to peg-IFN α . Importantly, Bulevirtide, a viral entry inhibitor, has recently received a conditional authorisation to treat HDV-infected patients (under the name Hepcludex or Myrcludex B). Regarding HCV, new direct antiviral agents (DAAs) can cure the infection in chronically infected patients ³. The human cytomegalovirus (HCMV) can also infect the liver but only as a secondary site and therefore will not be further discussed in this review. Two viruses infect the liver through an enteric contamination (Figure 1): Hepatitis A virus (HAV) and Hepatitis E virus (HEV). They represent 24.9% of all liver infections and 4% of related deaths (WHO). HAV and HEV infections can be prevented by a vaccine and might cause AH. HEV has also been reported to cause chronic infections in immunocompromised patients ⁵.

Finally, four of these hepatotropic viruses infect their host via mosquito bites (Figure 1), namely Dengue virus (DENV), West Nile virus (WNV), Yellow Fever virus (YFV), and Zika virus (ZIKV). They represent 9.3% of liver infection, mostly attributed to DENV, and 4% of related deaths. However, due to the lack of information on the effect of these four viruses on innate immune

responses in the host, only Hepatitis viruses (i.e. HAV, HBV, HCV, HDV, and HEV) will further be discussed in this review.

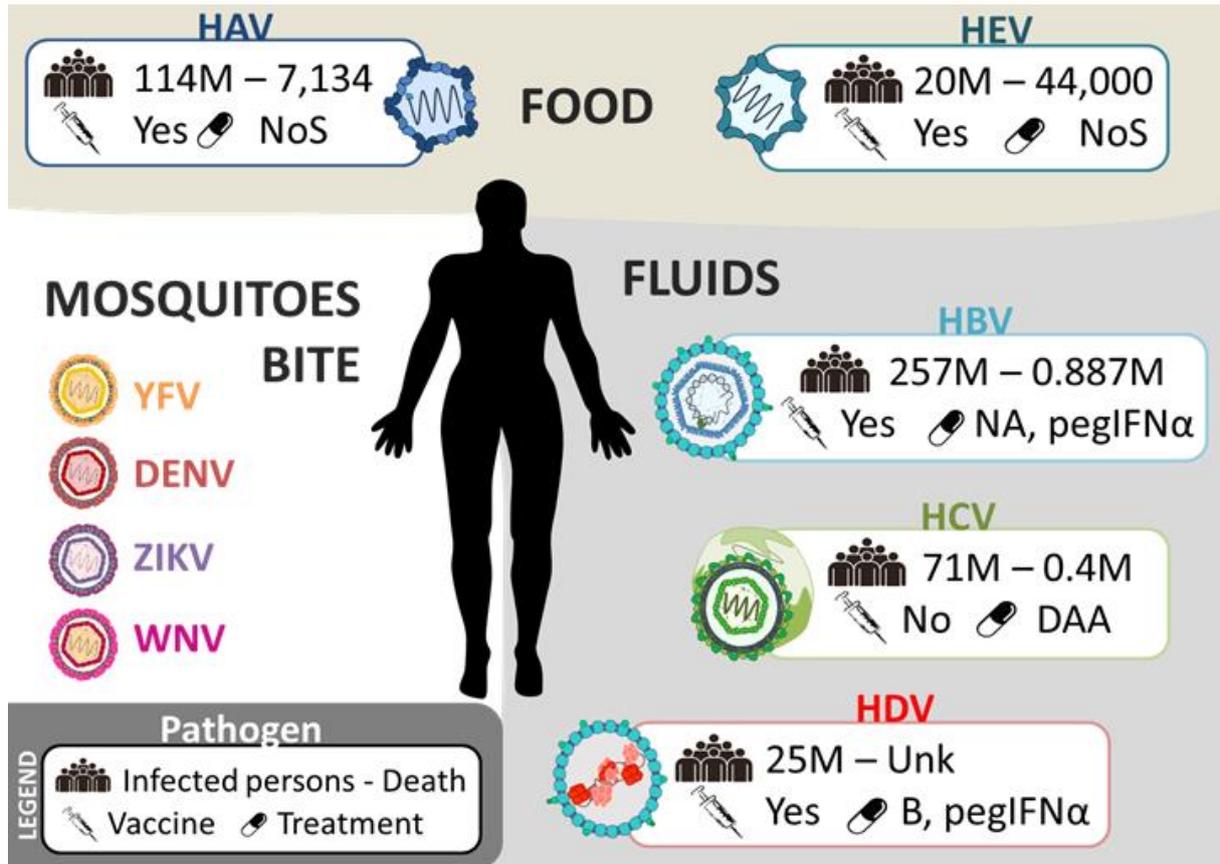


Figure 1: Liver viruses' infection route, epidemiology, and treatments.

Liver viruses are displayed depending on their entry route: fluids (sexual, blood transfusion, unsanitary drug use), food (ingestion), and mosquito's bite. Number of infected persons and the subsequent death toll, vaccine and treatment availability. NoS: Nothing Specific; NA: Nucleos(t)ides Analogues; Unk: Unknown.

Intriguingly, the liver, which is exposed to high concentrations of food-derived antigens, is described as tolerogenic⁶. Often seen as a secondary lymphoid organ, all cells are equipped to recognize and alert the host of a pathogen invasion⁷. This dichotomy is mostly attributed to the high threshold of stimulation that liver cells require to be activated as compared to other organs⁸. Consequently, it represents a perfect shelter for pathogens capable of avoiding recognition and/or for which the infection occurs with poor immune activation.

A healthy liver is composed of (i) parenchymal cells, the hepatocytes (70% of liver cells; participate in liver immune response), (ii) three liver-specific non-parenchymal cells (NPCs): the Liver Sinusoidal Endothelial Cells (LSECs) (20%; compose the sinusoidal wall), the Hepatic Stellate cells (HSCs) (5-8% when quiescent; liver fibroblasts), and Kupffer Cells (KCs) (4%; resident macrophages), and (iii) liver-unspecific NPCs, found in the circulation (monocytes)

and/or infiltrating the liver: Dendritic Cells (DCs) and lymphocytes ⁹. Concerning the latter, some subsets display innate functions, as the Natural Killer T (NKT) cells and Mucosal-associated invariant T (MAIT) cells which are equipped with antigen recognition capacities ¹⁰. MAIT cells can be activated by cytokines during virus infections ¹¹. Moreover, innate lymphoid cells (ILCs), despite lacking antigen-specific receptors, can mediate immune responses and regulate tissue homeostasis and inflammation. Natural Killer cells and type 1 innate lymphoid cells are the most abundant ILCs in the liver ¹⁰.

The innate immune system senses a broad range of Pathogen Associated Molecular Patterns (PAMPs), as well as Danger Associated Molecular Patterns (DAMPs; related to dysregulation of cellular functions). These conserved motifs are recognized by Pattern Recognition Receptors (PRRs), of which the Toll-like receptors (TLR) are the largest family ¹². Several PRRs are expressed by liver resident cells, allowing a broad sensing ^{7,13}. PRR activation results in the degradation of the microorganism, followed by presentation on the Major Histocompatibility Complex II (MHC-II) molecule ¹⁴. Altogether, such sensing mechanisms enable activation of various downstream immune pathways (nuclear factor-kappa B (NF- κ B), Akt, JAK-STAT, or interferon regulatory factor (IRF) pathways), which, in turn, lead to the production of effectors (cytokines or IFN) responsible for the appropriate orchestration of the immune response ¹⁵⁻¹⁷. These immune mediators can stimulate the respective pathways in an autocrine and paracrine manner, thereby increasing the activation of both professional and non-professional immune cells.

Thus, to establish and maintain infection within the liver, evasion from these mechanisms is required by pathogens.

2. How to establish infection in the liver: a virus' perspective

a. The Trojan Horse strategy

Such as ancient Greeks, two hepatitis viruses have developed means to enter cells without being detected, thereby eluding/delaying the activation of an immune response. To establish infection, different problems might arise: (i) access to the liver, (ii) access to permissive cells from the blood circulation, and (iii) efficient penetration into cells.

Pathogens mainly reach the liver through blood circulation. Thus, accessing parenchymal cells from the sinusoid, i.e. crossing the endothelium formed by LSECs, is often the first barrier encountered. LSECs are equipped with unique fenestration allowing nutrients, and most viruses, to pass through. It is worth mentioning that, upon specific liver pathologies such as fibrosis, fenestrae are lost, which could thereby prevent several viruses from accessing permissive cells ¹⁸. Consequently, these pathogens would need a “plan B” to ensure potent infection. Duck-HBV and HCV therefore use transcytosis through LSECs and KCs. While Duck-HBV seems to be scavenged by LSECs in a non-specific manner ¹⁹, HCV uses a receptor-mediated mechanism. Indeed, HCV-E2 interacts with DC-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) and liver/lymph node-specific intercellular adhesion molecule-3-grabbing integrin (L-SIGN) on KCs and LSECs, respectively ²⁰⁻²². However, it is still unclear if this process serves only to address HCV particles to LSECs fenestrae (passive entry) or if HCV hijacks this lectin pathway towards its translocation into the space of Disse (active entry). Of note, HIV-gp120 (Human Immunodeficiency Virus; which despite not being a liver-specific virus, has been evidence in vitro and in vivo to infect liver cells) has also been shown to interact with DC-SIGN. However, it is still unknown if this interaction is relevant for viral transcytosis in LSECs and/or KCs, and the in vivo relevance of these mechanisms remains unclear ^{23,24}. Whether it is an active or passive mechanism, transcytosis also offers the advantage of shielding the virus from recognition by intracellular PRRs. Finally, once it has reached the hepatocytes, HCV uses the association of its envelope proteins with high/very low/low density lipoproteins (HDLs/VLDLs/LDLs) to enhance receptor-mediated cellular entry or to enter by membrane fusion ²⁵.

Hence, HBV and HCV are capable of hiding from the immune system, as well as use host proteins, towards their efficient internalization into permissive cells.

b. Catch me if you can: how liver viruses escape recognition

i. Extracellular escape: complement pathway and pathogenic evasion

The complement pathway enables efficient recognition of circulating pathogens resulting in the production of inflammatory cytokines, recruitment of phagocytes, and lysis of the recognized

pathogen; mechanisms that viruses need to avoid for their efficient viral infection and spreading²⁶.

Evasion from complement-dependent antibody-recognition can be achieved by several mechanisms: (i) coating of viral particles by host proteins; (ii) formation of quasi-species by mutation; (iii) viral decoy. HCV has been suggested to avoid antibody recognition by association of envelope proteins with host lipoproteins and glycans²⁵. Similarly, HAV and HEV are secreted in a quasi-enveloped form (eHAV and eHEV) that hide hyper-immunogenic capsid/core components in a host-membrane cloak²⁷. Finally, HCV and HBV structural proteins and genetic material (DNA and RNA) are found in patients' exosomes^{28,29}. These extravesicular vesicles offers a unique hiding place as they are composed of cellular membrane – thus appearing as self – and they can enter other cells by fusion of the exosome with the cell membrane. This mechanism was also observed in other types of infections (e.g. bacterial, fungal, parasitic), highlighting a redundant mechanism employed by pathogens to avoid immune activation and easily reach and infect targeted cells. Moreover, HCV - but also other RNA viruses from the *Flaviviridae* family (DENV, ZIKV, YFV, and WNV)- and HDV hinder epitope recognition and subsequent antigen presentation by MHC molecules by selecting emerging mutations in the most targeted epitopes, often surface proteins, which generates a constant need for novel epitope-specific antibodies in patients^{30,31}. To this end, the composition and localization of glycans attached to viral envelope proteins, such as HCV-E2, can also be modulated³². The formation of quasi-species, while it can hindered viral elimination by the complement pathway, also represent a major drawback in the development of an effective vaccine against viruses with rapid mutations' emergence.

In addition, during HBV infection, non-infectious subviral particles (only containing HBV-HBsAg embedded in a lipid membrane) are secreted in excess (10⁴ to 10⁵ more than infectious particles) and serves as decoy against the immune system^{33,34}. Indeed, the excess of subviral particles secreted will allow the infectious particles, that are also covered by HBV-HBsAg embedded in a lipid membrane, to hide in plain sight. Consequently, entry inhibitors or antibodies-mediated elimination of HBV requires to deliver an important excess of molecules to insure both viral and subviral particles targeting.

Finally, HCV has developed active mechanisms to evade complement pathways by recruiting the host factor CD59 (membrane inhibitor of reactive lysis) to serve as inhibitor^{34,35}. Indeed,

CD59 is a glycosylphosphatidylinositol-anchored membrane protein that can inhibit the formation of the membrane attack complex in order to regulate complement activation ³⁶. Evidence from patients suggest incorporation of CD59 in HCV-virions ³⁴ (Figure 2, point#1).

Altogether, these different strategies enable HAV, HBV, HCV (and other flaviruses), and HEV to escape antibody- and complement-mediated death.

ii. Peek-a-Boo: intracellular hiding of viruses

Once they have entered permissive cells, viruses need to avoid intracellular immune recognition by PRRs.

HBV establishes a host-like form/state leading to the sustained presence of the virus within the liver and possible re-activation of the disease. HBV genome lays in the nucleus of infected cells as a chromosome-like structure, the cccDNA (covalently-closed circular DNA), thereby not constituting PAMPs. Thus, even if in some peculiar experimental conditions, cytosolic PRRs were able to recognize HBV genome ³⁷, only transient and weak or no innate immune responses are detected in hepatocytes following natural HBV infection ^{38,39}. Outside of the nucleus, the nucleocapsid shields HBV from PRR recognition ⁴⁰. As a consequence, drugs destabilizing HBV nucleocapsid can restore immune detection by DNA sensors, and have shown promising results *in vivo* and *in vitro* ⁴¹.

HCV can also evade cytosolic recognition and ensure local concentration of replication-relevant factors by modulating host membranes and generating a new compartment (i.e. double membrane vesicles of 150 nm diameter) ⁴². Altogether, these strategies decrease the amount of PAMPs available for recognition.

3. Liver viruses strike back: active inhibition of innate immune sensing

a. Targeting professional immune sensors

i. PRRs

PRR sensing and activation induce NF- κ B and IFN pathways (discussed in part 3.b.), which are essential to initiate pathogen clearance. Therefore, viruses have evolved strategies to dampen PRR expression/activity.

TLR9, TLR3, and TLR2 expression are downregulated in PBMCs, liver macrophages, and/or hepatocytes from chronic HBV-HBeAg+ patients⁴³⁻⁴⁵ (Figure 2, point#2). Also, HBV-HBsAg protein, by binding to CD14 (TLR4 co-receptor), is able to decrease TLR4 signalling both *in vitro* and *in vivo*^{46,47} (Figure 2, point#2). However, TLR downregulation could not be recapitulated *ex vivo* when using strong immune inducers, suggesting that inhibition by HBV can be overcome above a certain activation threshold^{48,49}. Although not clinically confirmed, HCV-NS3/4 targets Riplet, essential for activation of RIG-I, an intracellular PRR⁵⁰ (Figure 2, point#3).

Thus, among hepatitis viruses, HBV, which is the only virus which does not trigger immune responses upon primary infection, is also the only virus that has evolved towards efficient inhibition of PRR expression and/or activity.

ii. Targeting MHC presentation

Efficient pathogen recognition results in loading of intracellular- or extracellular-derived peptides onto MHC-I or MHC-II molecules, respectively. The liver is the only organ where presentation is not hindered by the endothelial barrier, allowing CD8+ T cells activation⁵¹.

CD8+ T cell exhaustion (i.e. decrease CD8+ T cell function) is a hallmark of CHB patients. Recently, this mechanism was, at least in part, attributed to hepatocyte priming^{52,53}. However, KC and hepatic DC priming led to T cell activation, putatively correlating with viral clearance^{52,53}. Altogether, this indicates a differential modulation and efficiency of MHC presentation in liver cells during HBV infection. However, the specific mechanism is still to be unraveled. In HBV-HDV co-infected patients, HDV, by inducing the IFN pathway, boosts HBV presentation⁵⁴. Even if not sufficient to reverse CD8+ T cell exhaustion, it is however beneficial for the clearance of HBV during engineered T cell therapy, highlighting the importance to activate the innate immune response in the design of new anti-viral therapies. On the other hand, MHC-I presentation is attenuated in HCV-infected cells treated with IFN, leading to decreased CD8+T cell effector functions⁵⁵ (Figure 2, point#4).

Altogether, HBV and HCV can circumvent activation of immune responses by (i) decreasing innate recognition and (ii) benefiting from tolerogenic environments or modulating MHC-mediated presentation.

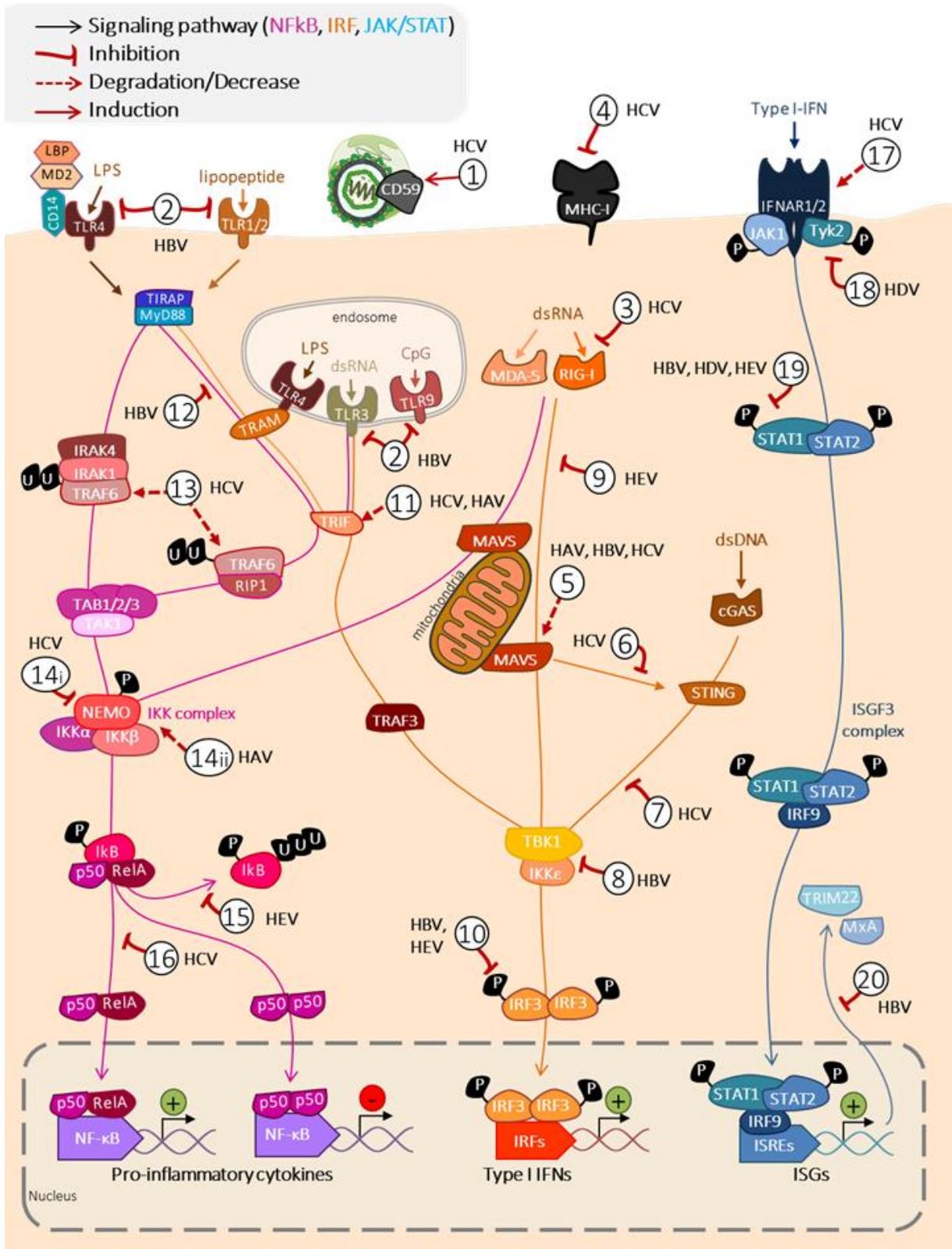


Figure 2: Effect of pathogens on intracellular immune signalling pathways.

1. Induction of CD59 and Factor H expression mediated by WNV, DENV, and ZIKV; 2. Inhibition of TLR1/2, TLR3 and TLR9 by HBV; 3. Inhibition of RIG-I by HCV; 4. Inhibition of MHC-I presentation by HCV; 5. (Si) Inhibition of MAVS interaction with mitochondria by HCMV or (Sii) MAVS degradation by HAV, HBV, and HCV; 6. Inhibition of MAVS-STING interaction by HCV; 7. Inhibition of STING-TBK1 interaction by HCV; 8. Inhibition of TBK1/IKKε interaction by HBV; 9. Inhibition of RIG-I/TBK1 interaction by HEV; 10. Inhibition of IRF3 phosphorylation by HEV; 11. Degradation of

TRIF by HCV and HAV;**12.**Inhibition of TRAM activation by HBV;**13.**Degradation of TRAF6 by HCV;**14.**(14i) Inhibition of NEMO activation by HCV and (14ii) IKK complex degradation by HAV;**15.**Inhibition of I κ B polyubiquitin-mediated proteasome addressing by HEV;**16.**Inhibition of NF- κ B subunits translocation to the nucleus by HCV;**17.**Decrease of IFNAR1 expression by HCV;**18.**Inhibition of Tyk2 phosphorylation by HDV;**19.**Inhibition of STAT1 phosphorylation by HBV, HDV, and HEV;**20.**Inhibition of TRIM22 and MxA expression by HBV. Light coloured arrows: signalling pathway (pink for NF- κ B, orange for IRF, and blue for JAK/STAT; red stopped arrows: inhibition; red dotted: degradation; red arrows: induction. cGAS: cyclic GMP-AMP synthase; TBK1: TANK-binding kinase, TRAM: TRIF-Related Adaptor Molecule.

b. Modulating intracellular innate immune pathways

i. PRRs' downstream signalling pathways

As mentioned, activation of PRRs triggers two major signalling pathways: IRF and NF- κ B. Their induction is controlled by key adaptor molecules, namely mitochondrial antiviral signalling protein (MAVS; RNA sensing), STING (DNA sensing), and MyD88 or TIR domain-containing adaptor inducing IFN β (TRIF; most TLR signalling).

Upon RNA virus recognition by RIG-I or MDA5, MAVS is activated resulting in induction of NF- κ B signalling and pro-inflammatory cytokines secretion. However, MAVS is impaired in its function by HAV, HCV, and HBV^{56,57}, leading to immune impairment (Figure 2, point#5i-5ii). MAVS cleavage and degradation is mediated by: (i) HCV-NS3/4A (serine protease), (ii) HAV-3ABC (cysteine protease)^{56,58,59}, and (iii) HBV-HBx (through Lys136 ubiquitination)⁵⁷. Of note, except for HCV, for which a cleavage by virion-encoded proteases was also observed *in vivo*, most of these studies were only performed *in vitro* (infection or overexpression of viral proteins). This phenotype could, however, explain the reduced pro-inflammatory cytokines observed in patients.

STING, a cytosolic DNA sensor, interacts independently with both MAVS and TBK1 (a protein at the crossroad of the NF- κ B and IRF signalling), leading to the activation of NF- κ B and IRF3/7 activation, and IFN and cytokines production. Interaction of STING with TBK1 is inhibited by HCV-NS4B⁶⁰ (Figure 2, point#6-7). Similarly, the interaction between TBK1/IKK γ and the DEAD box RNA helicase (DDX3; implicated in the induction of IFN-I) is abrogated by HBV-Pol⁶¹ (Figure 2, point#8) and the deubiquitination process, required for RIG-I/TBK1 activation, is reduced by the HEV-ORF1 papain-like cysteine protease (PCP) domains (Figure 2, point#9)⁶². Subsequently, the downstream IRF3/7 activation is prevented. In addition, IRF3/7 are directly inhibited *in vitro*

through blockade of poly(I:C)-mediated IRF3 phosphorylation or degradation of IRF7 mRNA as implemented by HEV-ORF1 X domain and HBV-HBsAg, respectively (Figure 2, point#10)^{62,63}.

In vitro, TLR3 and TLR4 signaling can be impaired by modulating the expression of their adaptor molecule TRIF. TRIF is cleaved and degraded by HCV-NS3/4A, HCV-NS4B, and HAV-3CD viral proteases^{64,65} (Figure 2, point#11). In addition, HBV and HCV are able to inhibit MyD88-dependent TLR signalling (i.e. most of the other members of the TLR family) through sequestration or degradation of the signalling molecules TRAM and TRAF6, thus preventing immune activation^{66,67} (Figure 2, point#12-13).

Further downstream, NF- κ B pathway signalling molecules are targeted in several liver infections. Whereas two NF- κ B signalling have been described (i.e. canonical pathway induced through NEMO and non-canonical induced pathway through NIK), mostly the canonical pathway has been shown to be targeted by hepatitis viruses. One of the major targets in the cascade is the IKK complex, formed of NEMO (also termed IKK γ), IKK α , and IKK β . Association of the linear ubiquitin chain assembly complex (LUBAC) with NEMO is essential for its appropriate activation, a process which is precluded by the competitive binding of HCV-NS3 to LUBAC⁶⁸ (Figure 2, point#14i). In addition, the IKK complex can be degraded through NEMO cleavage by HAV-3C at the Q304 residue⁶⁹ (Figure 2, point#14ii). Further downstream, the canonical pathway signals through the I κ B complex, composed of I κ B α and I κ B β . The ubiquitin-mediated addressing of the I κ B complex to the proteasome by the E3 ubiquitin ligase β -TRCP releases the NF- κ B complex, enabling its translocation into the nucleus. This process is prevented by the binding of HEV-pORF2 to the E3 ubiquitin ligase TRCP^{70,71} (Figure 2, point#15). Finally, the translocation of several NF- κ B subunits, especially RelA, to the nucleus is inhibited by HCV-core and HCV- polyprotein⁷² (Figure 2, point#16).

Altogether, inhibition of sensors (MAVS and STING), of adaptor molecules (TRIF and MyD88), or of NF- κ B pathway directly, highlight a suboptimal activation of immune responses upon hepatitis viruses' infection. Although confirmation in patients is missing, these inhibitory mechanisms of NF- κ B and IRF signalling pathways could contribute to the decreased levels of inflammatory mediators observed in infected patients.

ii. IFN receptor downstream signalling

IFN-I (IFN α and IFN β) and IFN-II (IFN γ) bind to their respective receptors, IFNAR and IFNGR, and activate downstream signalling. Subsequently, interferon-stimulated genes (ISGs) are induced, promoting anti-viral activity. It is worth noting that aside from the described inhibitory mechanisms, some viruses trigger IFN production through PRR recognition of their nucleic acids, such as HCV detection by RIG-I and HDV detection by MDA-5. However, some evidence suggests interference with the IFN pathway which could dampen further immune activation even after successful pathogen recognition.

Firstly, in hepatoma cells, expression of IFNAR or that activation of its signalling through the adaptor molecule Tyk2 is precluded by HCV and HDV, respectively ^{73,74} (Figure 2, point#17-18). Further downstream, activity of the transcription factors STAT1/2 (signal transducer and activator of transcription) is prevented by HBV-pol and HDV proteins *in vitro* ^{73,75} (Figure 2, point#19). HBV-mediated inhibition of STAT1/2 nuclear translocation was confirmed in CHB patients' liver biopsies. Although studies in HDV patients are lacking, the observed inhibition could partially account for the low efficacy of IFN α treatment in HDV patients ⁷⁵.

Moreover, some evidence points towards direct inhibition of ISG expression, leading to a decreased production of important antiviral effectors, such as the myxovirus resistance A (MxA), only observed in HBV-transfected Huh7, and tripartite motif 22 (TRIM22) protein, as seen in primary human hepatocytes and liver biopsies ^{76,77} (Figure 2, point#20). This double inhibition (i.e. of the signalling and of the effectors expression) could be an evolutionary mechanism to ensure control of the anti-viral immune responses during infection. Furthermore, *in vitro* studies demonstrated reduced IFN- β promoter activity caused by HCV-induced autophagy in host cells ⁷⁸.

In summary, IFN signalling, leading to the expression of ISGs, is crucial to control virus infection making it a privileged target of HBV, HCV, and HDV to evade the immune system and set a persistent infection.

c. Modulating innate immune cells

Hepatitis viruses are also capable of modulating the number of innate immune cells with anti-viral functions, through (i) an increase of suppressor immune cells, or (ii) an inhibition of proliferation/an induction of apoptosis.

The Myeloid-Derived Suppressor Cells (MDSCs), for instance, favour an immunosuppressive environment. They are found broadly recruited to the liver in HBV-infected patients^{79,80} as well as increased in the circulation of HCV-infected patients⁸¹ (Figure 3-A). In CHB patients, increase of MDSC has also be linked to decrease T cell activation^{79,80}. Of note, increased pro-inflammatory cells can also deteriorate pathogenesis as observed in CHB patients, showing correlations between ILC1s numbers and hepatic damage¹⁰ (Figure 3-A).

On the contrary, chronic HCV patients show lower hepatic NK cell numbers, following increased KLRG1 (a lymphocyte co-inhibitory receptor) expression⁸² (Figure 3-A). Innate-like invariant NKT cells are significantly reduced in CHB patients, and the residual iNKT cells show aberrant activation in response to its specific agonist⁸³ (Figure 3-A). Furthermore, MAIT cells are decreased in the blood of patients with chronic HBV, HCV, and HDV infection as well as in HCV/HIV co-infection⁸⁴ (Figure 3-A). In HBV patients, the number of livers MAIT cells inversely correlates with liver fibrosis and inflammation¹¹. Induction of MAIT apoptosis by these viruses, however, was not shown and the cell decrease was rather explained by a persistent immune activation during chronic infection.

In addition to the cell number, viruses develop strategies to interfere with immune cell polarization (i.e. pro-inflammatory vs. anti-inflammatory phenotypes). Indeed, increasing anti-inflammatory secretions while inhibiting pro-inflammatory secretions, offers a highly tolerogenic environment suitable for infection establishment and maintenance.

Cytokine secretion profile is a key aspect of immune cell phenotypes. Pro-inflammatory cytokines such as PBMC-derived IL-12 (*ex vivo* stimulation) and/or MAIT cell-derived IFN γ and TNF α are downregulated in chronic HBV and HCV patients^{84,85} (Figure 3-B, point#1,2). The reduction of other cytokines such as IL-1 β , TNF α , IL-6, or IFN-I is mostly described *in vitro* (e.g. human KCs), using virions or specific viral proteins, but could also contribute to the reduced pro-inflammatory responses observed in HBV and HCV patients^{8,86} (Figure 3-B, point#1,3).

On the other hand, anti-inflammatory cytokines (IL-10, TGF- β) and/or surface markers (CD163) are upregulated in patients with HBV^{8,85,87} (Figure 3-B. point#4-5) as well as in patient sera and *in vitro* models of HCV infection (Figure 3-B, point#4)⁸⁸.

Hepatitis viruses further dampen immune responses by modulation of surface receptors and their ligands affecting immune cell activation, inhibition, and apoptosis. Indeed, FasL upregulation is observed in HBV patients' KCs, favouring cell death⁸⁹ (Figure 3-B, point#6). Of

Figure 3: Modulation of immune cells count and phenotype by liver viruses.

Panel A. Modulation of immune cell count by liver pathogens. **Panel B.** Modulation of immune cell phenotype by liver pathogens. **1.** Downregulation of the secretion of IL-1 β , IL-6, IL-12, and TNF α by HBV in KCs; **2.** Downregulation of IFN- γ and TNF α secretion in MAIT cells by HBV and HCV; **3.** Inhibition of the TLR3-mediated IL-12 and IFN-I secretion by HCV in KCs; **4.** Upregulation of IL-10 and TGF- β secretion by HBV and HCV in KCs; **5.** Upregulation of CD163 by HBV on KCs; **6.** Upregulation of FasL by HBV on KCs; **7.** Upregulation of PD-1 and CTLA-4 by HBV and HCV; **8.** Downregulation of NKG2D by HBV, and HCV in NKs; **9.** Inhibition of NKp30 and NKp46 by HCV on NKs. Light coloured arrows: secretion pathways; red stopped arrows: inhibitions; red dotted arrows: downregulation; simple red arrows: upregulation.

note, similar observations are found with other viral infections, but have yet to be confirmed in patients ⁸⁶. More evident is the enhanced expression of inhibitory checkpoint proteins/receptors, impairing CD8+ T cell and NK cell activity, which was shown on livers from CHB patients (i.e. PD-1) and MAIT cells of chronic HBV and HCV patients (i.e. PD-1, CTLA-4), indicating an exhausted phenotype ⁹⁰⁻⁹² (Figure 3-B, point#7). NK cell activation is directly inhibited by downregulation of the activating receptor NKG2D in HCV ⁸⁸ and CHB patients ⁹³ (Figure 3-B, point#8). Besides, HCV also downregulates expression of the activating receptors NKp46 and NKp30 in vitro ⁹⁴ (Figure 3-B, point#9).

Finally, DC activation and migration towards lymphoid organs, which is important for induction of adaptive immune responses, is impaired in HCV infected patients ^{95,96}.

Therefore, HBV, HCV, and HDV can modulate the number, the phenotype, and the response of innate immune cells to create a tolerogenic environment, in which lymphocytes activation is strongly impaired. Consequently, these viruses can maintain a chronic infection for decades, leading to uncontrolled disease progression, such as HCC.

4. Towards the future of hepatitis viruses' treatment

A large spectrum of treatments already exists against hepatitis viruses. These treatments may target different steps of the pathogen life cycle as, viral entry (Myrcludex B against HBV and HDV...), replication (nucleos(t)ides analogues against various viruses...), and egress (Nucleic Acid Polymers against HBV and HCV). However, even though these treatments are efficient to control the infection, they are often not sufficient to fully eliminate it and/or come with strong side effects. Thus, development of new therapeutics is still needed.

A large effort has been made in the last decade to eliminate hepatitis infection and more especially chronic hepatic infection. Recently, a treatment leading to control and elimination of

Issue	Treatment	Pathogens		
		Validated Therapeutic	In clinical trial or in development	Author's suggestions
Evasion of antibody recognition	Neutralization	x	HCV bNABs	eHAV, eHEV, HBV, bNABs
Downregulation of the pro-inflammatory phenotype (PRR signaling, pro-inflammatory cytokines, IFN)	PRRs agonists	x	HBV TLR2 agonist	x
			x	HBV TLR3 agonist
			x	HBV TLR4 agonist
			HBV TLR7 agonist	x
			HBV TLR8 agonist	x
			HCV TLR9 agonist	HBV TLR9 agonist
	IFN or pro-inflammatory cytokines	HBV, HCV, HDV pegIFNα	x	HBV, HCV and HDV Pro-inflammatory cytokine
Others	x	HBV LTBR agonist	x	
Increase in anti-inflammatory phenotype	Anti-inflammatory cytokine release inhibition	x	x	HBV and HCV Anti-TGF-βR1 small-molecule kinase inhibitor
				HBV and HCV Anti-IL10
Increase in anti-inflammatory phenotype	Phenotype modulators	x	x	HBV and HCV CB-839 and/or other metabolic modulators
				HBV and HCV Anti-CSF-1R or CCR2 antibody
Modulating immune cell count	Blocking suppressive cells recruitment	x	x	HBV and HCV Gemcitabin, LXR agonist (MDSC depletion)

Issue	Treatment	Pathogens		
		Validated Therapeutic	In clinical trial or in development	Author's suggestions
Modulating immune cell count	Increase in pro-inflammatory cells	x	x	HCV NK cell therapy
				HCV DC therapy
Activatory/inhibitory receptors modulation	Inhibitor of inhibitory receptors	x	HBV and HCV Anti-PD-1/PD-L1	x
			x	HBV and HCV Other Inhibitory Receptors blockers (Tim-3, CTLA-4)
			x	HCV Anti-NKG2A

Table 1: Established, in development, or suggested treatments targeting innate immune responses modulated by liver pathogens.

HCV has been developed. Glecaprevir and pibrentasvir have shown great efficiency in reducing viral parameters, a phenotype that is long-lasting ⁹⁷. However, the cost of this treatment hinders its accessibility to all HCV infected patients. Similar efforts have been made to find a cure for CHB and HDV infected patients. Whereas several DAA types (e.g. Myrcludex B, capsid modulators...) ⁹⁸⁻¹⁰⁰ are currently under investigation for the treatment these patients, none of these treatments has shown a curative effect *in vivo* yet.

While efforts are pursued to develop specific DAAs to target these viruses, another axis of therapeutics has been pursued: restoring and/or modulating innate immune responses. Such treatments could be used as mono- or co-therapy of already existing molecules (i.e. DAAs). Indeed, first reducing viral pressure on the innate immune system with a DAA and then activating immune responses, might be the missing key towards hepatitis virus clearance, as suggested for HBV elimination ¹⁰¹. Notably, the specific immune pathways that are dysregulated by viruses should be consider in the development of therapeutics. Indeed, the immune pathways targeted by hepatitis viruses could an evolutionary mechanism to ensure viral survival, highlighting that activation of these immune pathways could eliminate the infection.

First in line, broadly neutralizing antibodies (bNAbs) are widely investigated, with both entry-inhibiting and neutralizing activities ¹⁰². bNAbs isolated from HCV-recovered patients showed efficient neutralization activity against HCV ^{103,104}. Thus, bNAbs should be envisaged for complement pathway escapes (i.e. eHAV, eHEV, and HBV) (Table 1). However, antibody-dependent enhancement (ADE) represents a major challenge in the development of bNAbs. Some viruses misuse “neutralizing” antibodies (with weak neutralizing activity and/or low concentrations in the blood) to infect the cell via an FcR-dependent mechanism ^{105,106}. ADE is a common phenomenon that has been described for a variety of pathogens and is currently investigated in SARS-CoV2 infection ^{107,108}.

Moreover, the fragile balance between pro- and anti-inflammatory responses is crucial for the elimination of pathogens. Several ways can be envisaged to counteract pathogens’ manipulation of the inflammatory response.

On one hand, increase of pro-inflammatory responses can be achieved through different means. Treatments with PRR agonists have been largely considered in the last decade as it can (i) induce the production of pro-inflammatory cytokines and other anti-pathogenic molecules, (ii) increase by a positive feedback loop the expression of the sensors itself, (iii) modify cell phenotype (anti- towards pro-inflammatory), and (iv) favour the recruitment of other immune cells. Nowadays, PRR agonists are mainly used as adjuvant in vaccines, but they are progressively considered as stand-alone agents. Several in vitro and in vivo testings of these agonists are currently ongoing in the context of hepatitis virus infections, and some are already being tested in clinical trials ^{109–118} (Table 1). Extension of these tests to pathogens that are known to dysregulate given pathway should be considered (e.g. HBV decreases TLR3 expression in patients, thus TLR3 agonist should be tested against HBV infection...) ¹¹⁹ (Table 1).

Also, the direct systemic administration of inflammatory mediators, such as peg-IFN α , has been used for decades as a treatment against viral infection (e.g. peg-IFN α against HBV and HDV...), but its limited efficacy (i.e. the low rate of viral elimination) does not make it a promising agent for hepatitis virus elimination. For decades, other inflammatory mediators have been tested for the treatment of hepatitis viruses but have shown limited efficacy or no additional benefit as compared to the already available therapies (e.g. IFN β against HBV...). Other pro-inflammatory mediators with more potent anti-viral effect could be envisaged (e.g. IL-1 β , lymphotoxin β receptor) ^{120,121}. However, quantity and timing of these treatments need to be tightly monitored as uncontrolled inflammation might lead to tremendous side effects, such as

cytokine storms ^{122,123}. Another issue to consider is the appropriate addressing to the liver and uptake of these proposed treatments, which remains, to date, a challenging issue. Indeed, intravenous administration is unreasonable for long-term treatment, especially when considering immune activating agents, while oral administration requires efficient crossing of the intestinal wall.

On the other hand, decreasing anti-inflammatory responses might be sufficient, or at least a first step towards the activation of a functional innate immune response. Direct inhibition of anti-inflammatory cytokines (i.e. TGF- β , IL-10) by either small molecules or neutralizing antibodies, has shown great results in the treatment of several cancers and should be envisaged for liver pathogens targeting these responses (e.g. HBV) ^{124–126} (Table 1). Additionally, several molecules have been used to target the anti-inflammatory phenotype often displayed by tumour associated macrophages (TAMs), as extensively described by others ¹²⁷. These modulators, which often target surface markers or key metabolic pathways (i.e. anti-CSF1R or anti-CCR2, and CB839, respectively), should be tested in the management of HBV and HCV infection, in which macrophages display a similar phenotype (Table 1). MDSCs, recruited in HBV and HCV infections, might also be therapeutic targets. Strategies, aiming at either depleting these cells or inhibiting their recruitment are currently developed, as suggested for some infections using Gemcitabin or LXR agonists, among others ¹²⁸. In parallel, increasing the recruitment of “positive” immune cells to the liver, particularly DCs, will help to implement potent immune responses and activate specific T cells. Increasing DC count and/or reverting DC inhibition has been studied in different pathologies and should be considered for HCV infections in which similar modulation is observed ¹²⁹ (Table 1). Similarly, modulation of NK cells count has shown promising results ¹³⁰.

Last but not least, modulation of inhibitor receptors has been used in the treatment of several cancers, and could/should be repurposed for the treatment of liver infections. Anti-PD-1/PD-L1 treatments have already been tested against HBV and HCV infection, in which they improved the activation of a functional immune response ^{131,132} (Table 1). Besides, blockade of other inhibitory receptors has shown encouraging results in HBV infection, alone or in combination with anti-PD-1/PD-L1, and thus should be considered as well ¹³³. All these strategies benefited from a combination therapy with a viremia-reducing DAA, proving the need and efficiency of co-therapy.

5. Conclusion

This review describes how hepatitis viruses have evolved a broad spectrum of mechanisms to evade innate immune responses and maintain their infection in the liver. Moreover, while we only discussed the effect of hepatitis viruses on liver's innate immune responses, common phenotypes/mechanisms could be found in other liver pathogen infections, namely other viruses, bacteria and parasites. Therapeutically targeting one or several of these mechanisms could enhance efficacy of existing treatments or lead to the development of new therapies to eliminate hepatitis viruses.

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