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Optimisation d'un vaccin bivalent VHB-VHC par son association avec l'apolipoprotéine E et par immunisations séquentielles avec des particules arborant des protéines d'enveloppe de VHC de divers génotypes

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RÉSUMÉ

L'hépatite C, affectant 71 millions de porteurs chroniques, constitue un réel problème de santé publique. Bien que l'introduction des agents antiviraux à action directe ait amélioré le traitement de cette maladie, ces médicaments ne protègent pas d'une réinfection, ni de la progression vers une maladie sévère du foie. L'Organisation Mondiale de la Santé a pour objectif d'éradiquer l'hépatite virale à l'horizon 2030, mais des études prospectives ont montré que, si les mesures n'évoluent pas, cela ne sera possible que dans quelques pays. Par conséquent, le développement d'un vaccin prophylactique contre le virus de l'hépatite C (VHC) reste une priorité, au même titre que l'application des initiatives pour le dépistage et le traitement.

Dans cette optique, notre équipe de recherche a développé un concept de vaccination original basé sur les protéines d'enveloppe entières (E1 ou E2) d'un VHC de génotype 1a, préalablement fusionnées à la protéine d'enveloppe S du virus de l'hépatite B (VHB) et qui se co-assemblent en particules sous-virales d'enveloppe non infectieuses, hautement immunogènes et secrétées, ressemblant au vaccin contre le VHB. Ces particules chimères ont induit de fortes réponses immunitaires humorales chez le lapin, ainsi que la production d'anticorps capables de neutraliser le VHC *in vitro*.

L'objectif de ce projet était d'améliorer la façon de présenter nos immunogènes, afin d'optimiser selon deux approches les propriétés cross-neutralisantes des anticorps induits par la vaccination. Dans un premier temps, nous avons tenu compte de l'association des protéines d'enveloppe du VHC avec l'apolipoprotéine E (apoE), une molécule impliquée dans la modulation de la sensibilité à la neutralisation du VHC par les anticorps. Nous avons donc développé des particules chimères arborant l'apoE en vue de mimer au mieux l'interface apoE-protéines d'enveloppe en surface du VHC. Nous avons ainsi pu montrer que la présence de l'apoE dans la genèse de nos particules vaccinales améliore la conformation de la protéine E1 et les capacités neutralisantes des anticorps anti-E2. Dans un second temps, nous avons produit des particules vaccinales portant des protéines d'enveloppe E2 de différents génotypes (1, 3 et 4) et immunisé des lapins par des injections séquentielles afin d'améliorer la maturation de l'affinité des lymphocytes B. Dans l'attente de l'analyse des réponses humorales (en cours de réalisation au moment de la finalisation de ce manuscrit), nous présentons la production et la caractérisation de ces particules, ainsi que le protocole d'immunisation.

Mots clés : vaccination prophylactique, vaccin bivalent VHB-VHC, anticorps neutralisants, apolipoprotéine E, maturation de l'affinité des lymphocytes B

ABSTRACT

With 71 million chronic carriers, hepatitis C is a major threat to public health. Although the introduction of direct-acting antivirals has improved the treatment of the hepatitis C virus (HCV) infection, they do not protect from reinfection or the progression to severe liver diseases. The World Health Organization aims to eradicate viral hepatitis by 2030, but recent prospective studies have shown that this would only be possible in few countries if the current measures do not evolve. Therefore, the development of a prophylactic HCV vaccine is still a priority, along with the implementation of screening and treatment initiatives.

In line with this, our group has developed an original vaccination concept based on full-length genotype 1a HCV envelope proteins (E1 or E2) fused to the heterologous hepatitis B virus (HBV) S envelope protein. They self-assemble into highly immunogenic, non-infectious and secreted subviral envelope particles (SVPs), resembling the HBV vaccine. Immunization of rabbits with the chimeric particles has shown to induce strong humoral responses directed against both viruses, as well as antibodies capable to neutralize HCV *in vitro*. However, neutralization of genetically-distant genotypes remains a challenge.

The aim of this project was to improve the way immunogens are presented for the generation of neutralizing humoral responses against HCV by two approaches. Firstly, we took into consideration the association of HCV with the apolipoprotein E (apoE), a molecule involved in the modulation of sensitivity to antibody-mediated HCV neutralization. Therefore, we produced chimeric HBV-HCV SVPs bearing apoE on their surface to mimic precisely the interactions in the apoE-HCV envelope proteins interface. We found that the association of apoE improved the conformation of the E1 protein and the neutralizing capacity of the anti-E2 antibodies. Secondly, we produced SVPs bearing HCV E2 protein of genotype 1, 3 or 4 and we immunized rabbits sequentially to improve the affinity maturation process of B cells. Here we present the production and characterization of SVPs, as well as the immunization protocol, pending the analysis of the humoral and neutralizing responses, still in progress at the time this manuscript was finalized.

Keywords: prophylactic vaccination, HBV-HCV bivalent vaccine, neutralizing antibodies, apolipoprotein E, B lymphocyte affinity maturation

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ABBREVIATIONS

A

aa	amino acid
AIDS	acquired immunodeficiency syndrome
Alb	albumin
ALT	alanine transferase
APC	antigen-presenting cell
apoA	apolipoprotein A
apoB	apolipoprotein B
apoC	apolipoprotein C
apoE	apolipoprotein E
AR	antigenic region
AS	antigenic site

B

BCR	B cell receptor
β-gal	beta-galactosidase
BHK-21	baby hamster kidney-21 (cells)
bNab	broad neutralizing antibody

C

CD	cluster of differentiation
CD81bl	cluster of differentiation 81 binding loop
cDNA	complementary DNA
CHO	Chinese hamster ovary (cells)
CIDEB	cell-death inducing DFFA-like effector B
cLD	cytosolic lipid droplet
CLDN1	claudin-1
co-IP	co-immunoprecipitation
COPII	coat protein complex II
covid	coronavirus disease
cryo-EM	cryogenic electron microscopy
CSR	class-switch recombination

D

DAA	direct-acting antiviral

DC	dendritic cell
DMSO	dimethyl sulfoxide
ds	double-stranded

E

EASL	European Association for the Study of the Liver
EDTA	ethylenediaminetetraacetic acid
EGFR	endothelial growth factor receptor
eIF2-α	eukaryotic initiation factor 2-alpha
ELISA	enzyme-linked immunosorbent assay
ELISpot	enzyme-linked immunospot
EM	electron microscopy
ER	endoplasmic reticulum
ESCRT	endosomal sorting complexes required for transport

F

Fab	fragment antigen-binding
FDA	Food and Drug Administration
FDC	follicular dendritic cell
FFU	focus-forming unit
FP	fusion peptide

G

GBD	global burden of disease
GBV-B	George Baker virus-B
GC	germinal center
GFP (or gfp)	green fluorescent protein
GT	genotype

H

HBV	hepatitis B virus
HCC	hepatocellular carcinoma
HCV	hepatitis C virus
HCVcc	hepatitis C virus in cell culture
HCVpp	hepatitis C virus pseudoparticles
HDL	high-density lipoprotein
HEK293T	human embryonic kidney 293T (cells)
HIV	human immunodeficiency virus
hph	hygromycin B phosphotransferase
HRP	horseradish peroxidase

HSPG	heparan sulfate proteoglycan
HVR	hypervariable region

I

IFN	interferon
Ig	immunoglobulin
igVR	intergenotypic variable region
IL	interleukin
IL-10R	interleukin-10 receptor
IL28B	interleukin-28B gene
IRES	internal ribosome entry site
IRF	interferon regulatory factor
ISG	interferon-stimulated gene
IVDU	intravenous drug user

J

JAK	Janus kinase

K

KC	Kupffer cell

L

LD	lipid droplet
LDL	low-density lipoprotein
LDLR	low-density lipoprotein receptor
luLD	luminal lipid droplet
LVP	lipoviral particle

M

mAb	monoclonal antibody
MAVS	mitochondrial antiviral signaling
MBL	mannose-binding lectin
mDC	myeloid dendritic cell
MHC	major histocompatibility complex
MIIC	major histocompatibility complex class II compartment
miR-122	microRNA-122
MPL	monophosphoryl lipid A
mRNA	messenger RNA
MSM	men who have sex with men
MTP	microsomal transfer protein

MW	membranous web

N

Nab	neutralizing antibody
NANBH	non-A non-B hepatitis
NAP1L1	nucleosome assembly protein 1-like 1
nE1	envelope protein E1 N-terminal domain
NFκB	nuclear factor-kappa B
NK	natural killer
NMR	nuclear magnetic resonance
NS	non-structural
NTPase	nucleoside triphosphatase

O

OCLN	occludin
OD	optical density
OPD	o-phenylenediamine dihydrochloride
ORF	open reading frame

P

pAb	polyclonal antibody
pac	puromycin N-acetyltransferase
PAMP	pathogen-associated molecular pattern
PCR	polymerase chain reaction
PD1	programmed cell death protein 1
PDB	Protein Data Bank
pDC	plasmocytoid dendritic cell
pegIFN	pegylated interferon
PKR	protein kinase R
PRR	pattern recognition receptor

R

RAS	resistance-associated substitution
RdRp	RNA-dependent RNA polymerase
RIG-I	retinoic acid-inducible gene I

S

S	hepatitis B small envelope protein
SARS-CoV-2	severe acute respiratory syndrome coronavirus 2
SCID	severe combined immunodeficiency disorder

SDS-PAGE	sodium-dodecyl sulfate-polyacrylamide gel electrophoresis
sE2	soluble HCV envelope protein E2
SFV	Semliki forest virus
SHBsAg	small envelope protein hepatitis B surface antigen
SHM	somatic hypermutations
SOCS3	suppressor of cytokine signaling 3
SR-BI	scavenger receptor-class B type I
ss	single-stranded
STAT1	signal transducer and activator of transcription 1
SVP	subviral particle
SVR	sustained virologic response

T

TAP	transporter associated with antigen processing
TBS	Tris-buffered saline
TBK1	TANK-binding kinase 1
TCR	T-cell receptor
TEM	transmission electron microscopy
Tfh	T follicular helper
TLR	toll-like receptor
TMD	transmembrane domain
TNE	Tris-NaCl-EDTA
TRIF	Toll–interleukin-1 receptor domain–containing adaptor inducing interferon-β

U

uPA	urokinase plasminogen activator
UTR	untranslated region

V

V_H	heavy chain variable segment
VLDL	very low-density lipoprotein
VLDLR	very low-density lipoprotein receptor
VLP	virus-like particle

W

WHO	World Health Organization
WT	wild-type

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Part I

INTRODUCTION

1

HEPATITIS C

1.1 EPIDEMIOLOGY OF HCV INFECTION

Hepatitis C is a liver disease caused by hepatitis C virus (HCV). In 2015, the World Health Organization (WHO) estimated that 71 million people were living with HCV infection globally, defining this disease as a major problem of public health. Moreover, 1,75 million new HCV infections are estimated to occur every year, while 400 000 deaths are caused by the HCV chronic infection annually. In fact, the number of deaths due to viral hepatitis (caused by hepatitis B virus (HBV) and HCV) continues to increase, in contrast to deaths due to other diseases such as tuberculosis, malaria and the acquired immunodeficiency syndrome (AIDS), caused by the human immunodeficiency virus (HIV) (Figure 1). Thus, the WHO aims to eliminate viral hepatitis by 2030 through the reduction of incidence by 90% and mortality by 65% (World Health Organization, 2017).

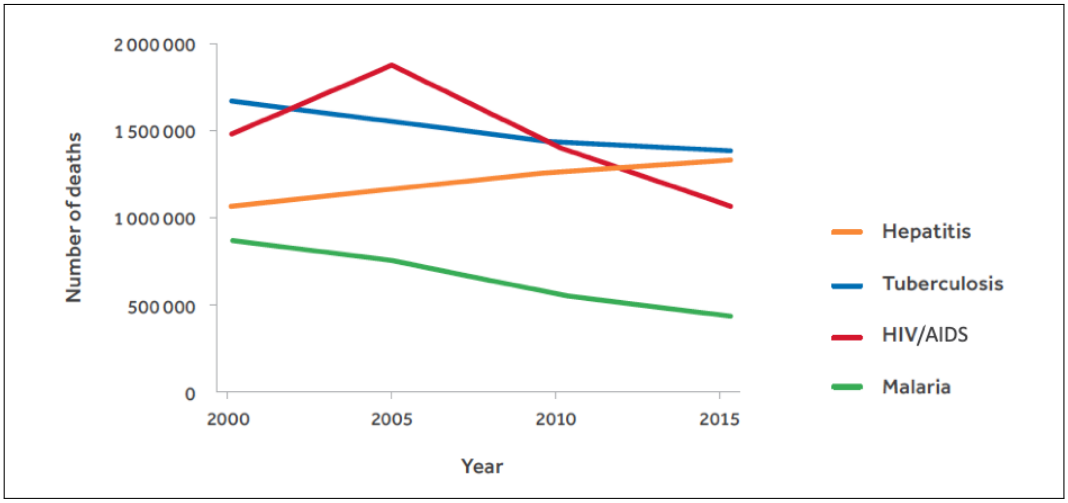

Figure 1: Comparison of the global annual mortality of viral hepatitis, tuberculosis, AIDS and malaria. Adapted from the global hepatitis report 2017 (World Health Organization, 2017).

Recent studies have shown that reaching these goals globally would be difficult and that they could only be achieved in a few countries or after 2030. Hill and colleagues evaluated the net cure of HCV by considering the number of cures, deaths and new infections in one year for 91 countries and estimated that with the current global net annual change, elimination of HCV is not feasible (Hill, Nath, and Simmons, 2017). Another study from Heffernan and collaborators predicted,

through a mathematical model with data from 190 countries, diverse scenarios in regard to HCV elimination. They found that the WHO's goals could only be reached shortly after 2030 if measures for the prevention, screening and treatment of HCV were implemented. However, the cost of these strategies can attain tens of billions of US dollars by 2030 (Heffernan et al., 2019). Razavi and colleagues analyzed the progress in the eradication of HCV in 45 high income countries and concluded that only 9 of them would be able to eliminate HCV by 2030 (Razavi et al., 2020). In conclusion, these reports suggest that current strategies need to be improved in order to achieve the targets for reduction in incidence and mortality established by the WHO.

For the screening and monitoring of HCV-infected patients, tools are extensive and currently available. Serological tests that detect the presence of anti-HCV antibodies in serum from patients after an exposure to this virus are mainly based on rapid diagnostic tests and enzyme-linked immunoassays [reviewed in (Peeling et al., 2017)]. From a comprehensive analysis, Gower and colleagues estimated the global serological prevalence at 1,6%, which means that around 115 million people have been diagnosed with a late, cleared or chronic infection (Gower et al., 2014). Concerning the diagnosis of active infections, the detection of HCV RNA is performed by methods based on real-time polymerase chain reaction (PCR) from which results are highly specific and sensitive. An alternative tool to evaluate active infections is the detection of the HCV core antigen (Chevaliez and Pawlotsky, 2018). Albeit less sensitive, it is also cheaper and easier to perform than the PCR technique. In fact, studies have shown that the HCV core antigen concentration correlates with HCV RNA levels when monitoring HCV-infected patients (Pérez-García et al., 2019; Tilborg et al., 2018). HCV genotype and subtype can be determined through sequencing and phylogenetic analysis, which is sometimes crucial to implement the best therapy and to identify resistance-associated substitutions (RAS) when the treatment fails to resolve the HCV infection (Manns et al., 2017).

Despite the availability of these diagnostic tools, the main obstacle in the screening of HCV-infected patients is the lack of symptoms, at least during the first years of the chronic infection, and consequently, the non-awareness of the disease. In 2017, the WHO reported that around 20% of patients knew their status, and out of them, only 7% to 13% were treated (Figure 2) (World Health Organization, 2017). Screening strategies, which are a key point to identify and to break the chains of transmission, differ from each country. In the case of France, the proportion of people chronically infected to HCV unaware of their condition has decreased from 43% to 25% in 10 years (Brouard et al., 2015). Meanwhile in England, since 2014, the dried blood spot testing was implemented for the detection of HCV in prison inmates, a high risk population, resulting in the improvement of screen-

ing rates (21% to 46%) (Mohamed et al., 2020). However, the access to testing in low- and middle-income countries, with around 75% of the total of people living with HCV, still remains an obstacle (World Health Organization, 2021). HCV prevention and screening programs are considered highly cost-effective strategies, because they not only help in the reduction of incidence and mortality, but also look after the national economies (Pedrana et al., 2018; Tordrup et al., 2019).

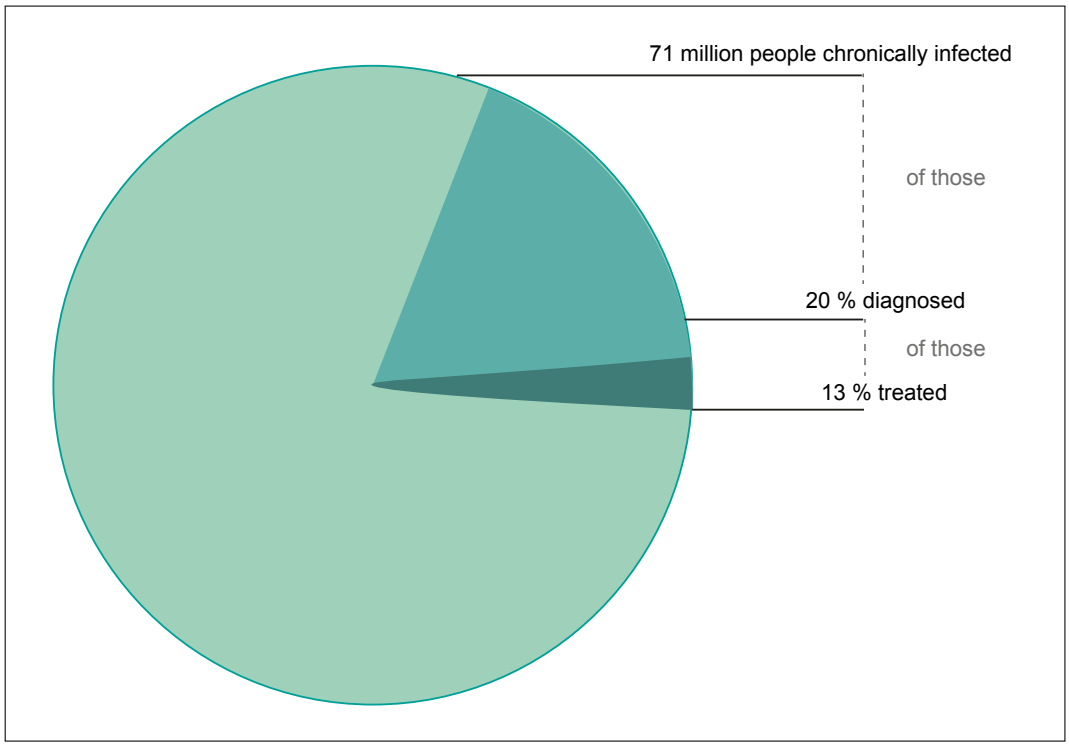

Figure 2: Global coverage of HCV screening and treatment. The 71 million people chronically infected with HCV represent the 100%, out of which only 20% of patients are diagnosed. Out of these patients who are diagnosed, only 13% are treated. The data were obtained from the global hepatitis report 2017 (World Health Organization, 2017).

Because transmission of HCV occurs through blood-to-blood contact, some population groups are at high risk to get infected. The intravenous drug users (IVDUs) are one of the major groups, mainly as a consequence of the needle sharing practice. Today, prevention programs for the distribution of sterile syringes or needles and opioid substitution treatments help to reduce the risk of HCV transmission (Platt et al., 2017). However, IVDUs, accounting for 8% of the global burden of the disease, are hard to target, due to their living conditions and to their difficult access to healthcare (Degenhardt et al., 2017; World Health Organization, 2017). A study from 2017 showed that 52,3% of IVDUs had been exposed to HCV (presence of anti-HCV antibodies) and 21,7% of them were under 25 years (Degenhardt et al., 2017). Regarding pregnant women, mother-to-child transmission of HCV can occur during pregnancy. Pregnant women infected with HCV

can give birth to HCV-infected children in 5,8% of cases with HCV-mono-infection and 11,8% of HIV-HCV co-infection cases (Benova et al., 2014).

Co-infection of HCV with other viruses such as HIV and HBV is frequent because they share similar routes of transmission. Men having sex with men (MSM) without protection are as well at high risk to get infected with HCV. A meta-analysis of HIV-HCV co-infection estimated a burden of 2,3 million people co-infected, which represents a global prevalence of 6,2% (Platt et al., 2016). In addition, other studies reported that HIV infection in MSM can increase 4 to 6 times the risk to contract HCV, compared to HIV-negative MSM (Platt et al., 2016; Yaphe et al., 2012). In HBV-HCV co-infection both viruses infect hepatocytes and therefore, this co-infection has been associated with an increased risk to develop fibrosis and decompensated cirrhosis in patients with chronic hepatitis (Pol et al., 2017; Yan et al., 2016). In addition, treatment of both viruses together is complex and, in some cases, HCV treatment can lead to HBV reactivation (Calvaruso et al., 2018; Collins et al., 2015; Liu et al., 2018).

1.2 HCV GENETIC DIVERSITY

HCV has been classified into eight different genotypes and more than 67 subtypes, with an intergenotypic and intragenotypic variation of 30% and 15%, respectively (Hedskog et al., 2019; Simmonds et al., 1994; Smith et al., 2014). HCV genotypes are distributed across the world (Figure 3), but the most represented ones are genotype 1 (44%), 3 (25%) and 4 (15%) (The Polaris Observatory HCV Collaborators, 2017).

In France, around 58% of HCV infections are from genotype 1a, which is also predominant in other European countries (40-99% prevalence), followed by genotype 3 (1-40% prevalence) (Alberti et al., 2016). Prevalence of genotype 2 is higher in Asia and West Africa (Coppola et al., 2019). The region with the highest prevalence of HCV genotype 3 (around 70% of total infected) is Southern Asia. This genotype has been associated with a highest prevalence of steatosis (lipids accumulation within hepatocytes) and a higher risk for developing hepatocellular carcinoma (HCC) (Bochud et al., 2009; Hourioux et al., 2007; Mihm et al., 1997; Nkontchou et al., 2011; Probst et al., 2011). In Egypt, the country with the highest HCV prevalence in the world (15%), 93,1% of the HCV infections are from genotype 4 (Petruzziello et al., 2016; The Polaris Observatory HCV Collaborators, 2017). Genotype 5 is mostly found in Southern and Eastern sub-Saharan Africa (80%), and only one subtype has been identified. The first case of genotype 5 infection was reported in South Africa, which today has a high proportion (54%) of infections due to this genotype (Asselah et al., 2018). Genotype 6 is mainly found in the southeast of Asia, in countries such as Laos and Cambodia, in which 96%

and 56% of cases, respectively, are from this genotype (Asrani et al., 2019). Concerning genotype 7, only few cases of patients in Canada, but originally from Central Africa, have been identified (Murphy et al., 2015). In 2018, the genotype 8 was identified in 4 HCV-infected patients from India, as a novel HCV genotype with one subtype (8a, homology among them >90%) (Borgia et al., 2018) (Figure 4).

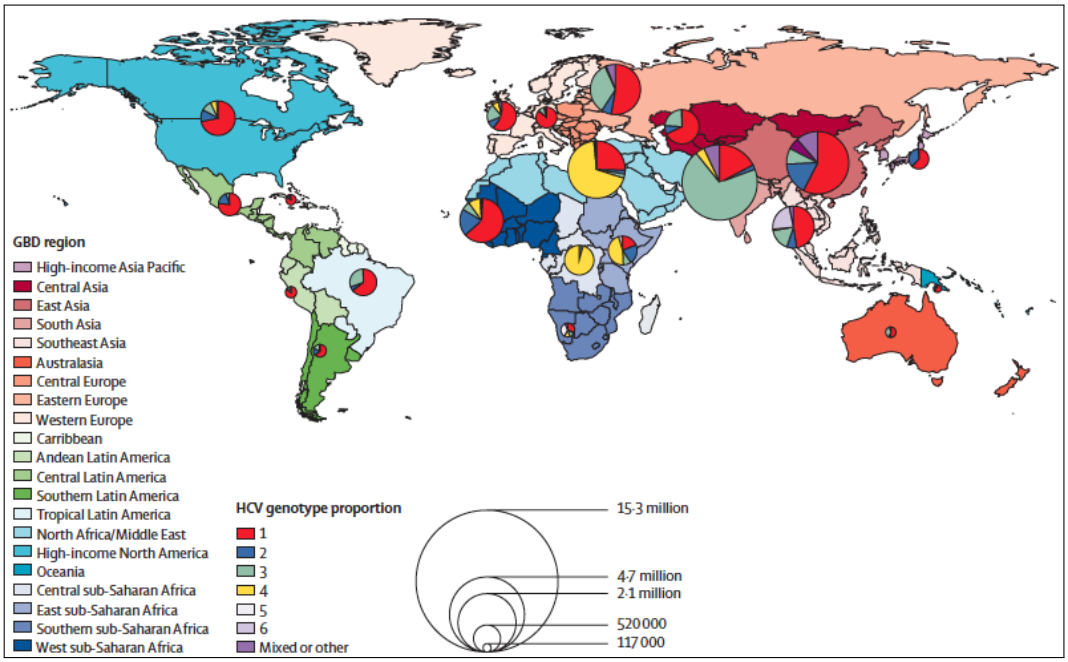

Figure 3: Global distribution of HCV genotypes. Countries are classified into global burden of disease (GBD) regions of different colors. The distributions of genotypes per GBD region are represented in a pie chart. (The Polaris Observatory HCV Collaborators, 2017)

Besides genotypes and subtypes, HCV has viral quasispecies that differ by 1 to 3% (Bukh, 2016; Farci, Bukh, and Purcell, 1997; Martell et al., 1992). The genetic variability of HCV is mainly due to the high viral replication level of HCV and to the high-error-prone rate of its RNA-dependent RNA polymerase (RdRp) (10^{-3} errors/site) (Powdrill et al., 2011). Because of this lack of proofreading mechanism, the viral population found in the host is composed of closely related HCV virions (quasispecies) that can escape from neutralizing antibodies (Farci et al., 1994; Shimizu et al., 1994). Several studies have suggested that the progression of the acute disease may be related with an increased genetic diversity generated by the immune pressure leading to viral persistence, while the reduction in this diversity may be associated with clearance (Dowd et al., 2009; Farci, 2000; Laskus et al., 2004). However, other studies have suggested that a more homogeneous viral population might lead to the progression of HCV infection, which might be related to a failure in the immune response (Izopet et al., 2000; Sullivan et al., 2007).

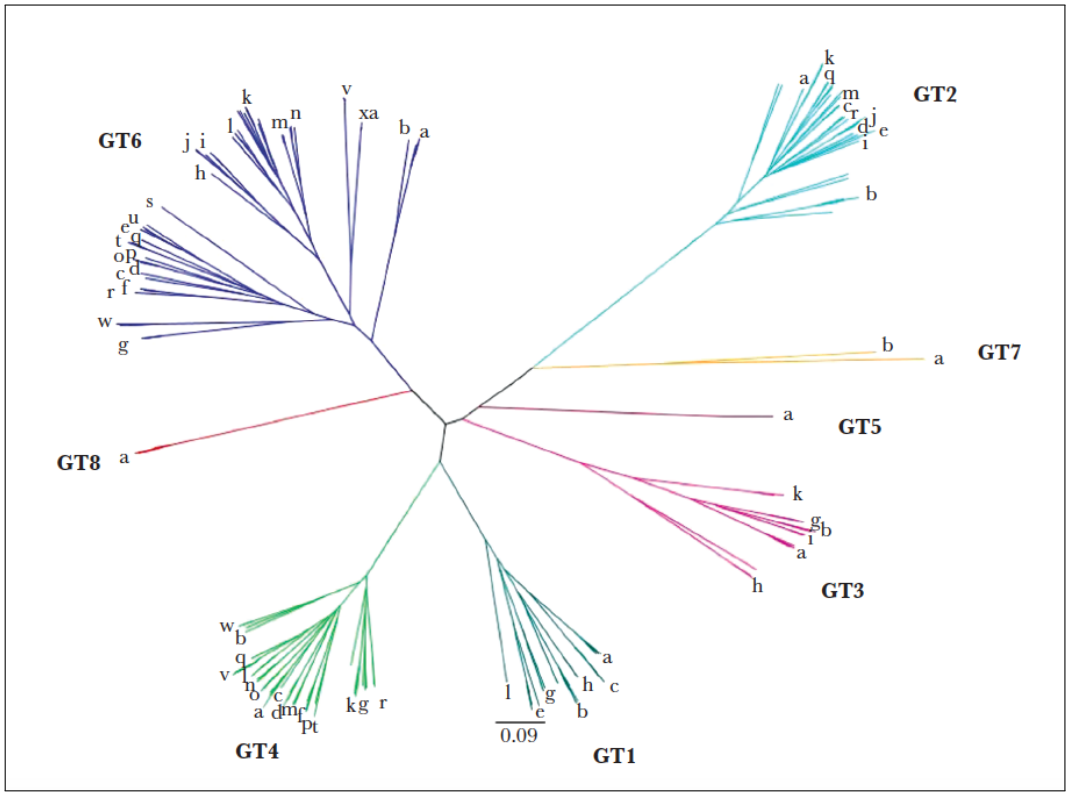

Figure 4: Phylogenetic tree of HCV genotypes 1 to 8. This tree was proposed based on the analysis of the full-length open reading frame of the genotypes (GT) 1-7 HCV reference sequences and the 4 sequences of the recently identified genotype 8 HCV. (Borgia et al., 2018)

1.3 ACUTE AND CHRONIC HEPATITIS C

The progression of hepatitis C can lead to an acute disease that may be spontaneously resolved or to a chronic condition. The acute phase of the infection lasts until 6 months, in which the majority of patients are asymptomatic or have mild non-specific symptoms (Manns et al., 2017). HCV RNA can be detected from day 2 to 14 after exposure, while HCV seroconversion occurs from day 20 to 120 (Glynn et al., 2005). Following a plateau phase in the detection of HCV RNA, at the end of which the levels of alanine aminotransferase (ALT) raise, there could be either the spontaneous clearance of the infection (observed in around 25% of patients) or the development of a chronic disease (Figure 5) (Hajarizadeh, Grebely, and Dore, 2013; Osburn et al., 2010). Resolution of HCV occurs thanks to specific immune responses, while evolution to a chronic infection happens in spite of a weak but not-lasting immune response (Thimme, 2021). Moreover, studies have shown that spontaneous clearance is favored by a genetic polymorphism near the *IFNL3* gene, formerly known as interleukin-28 gene (*IL28B*), which codes for the type III interferon (IFN) (Shi et al., 2012; Thomas et al., 2009).

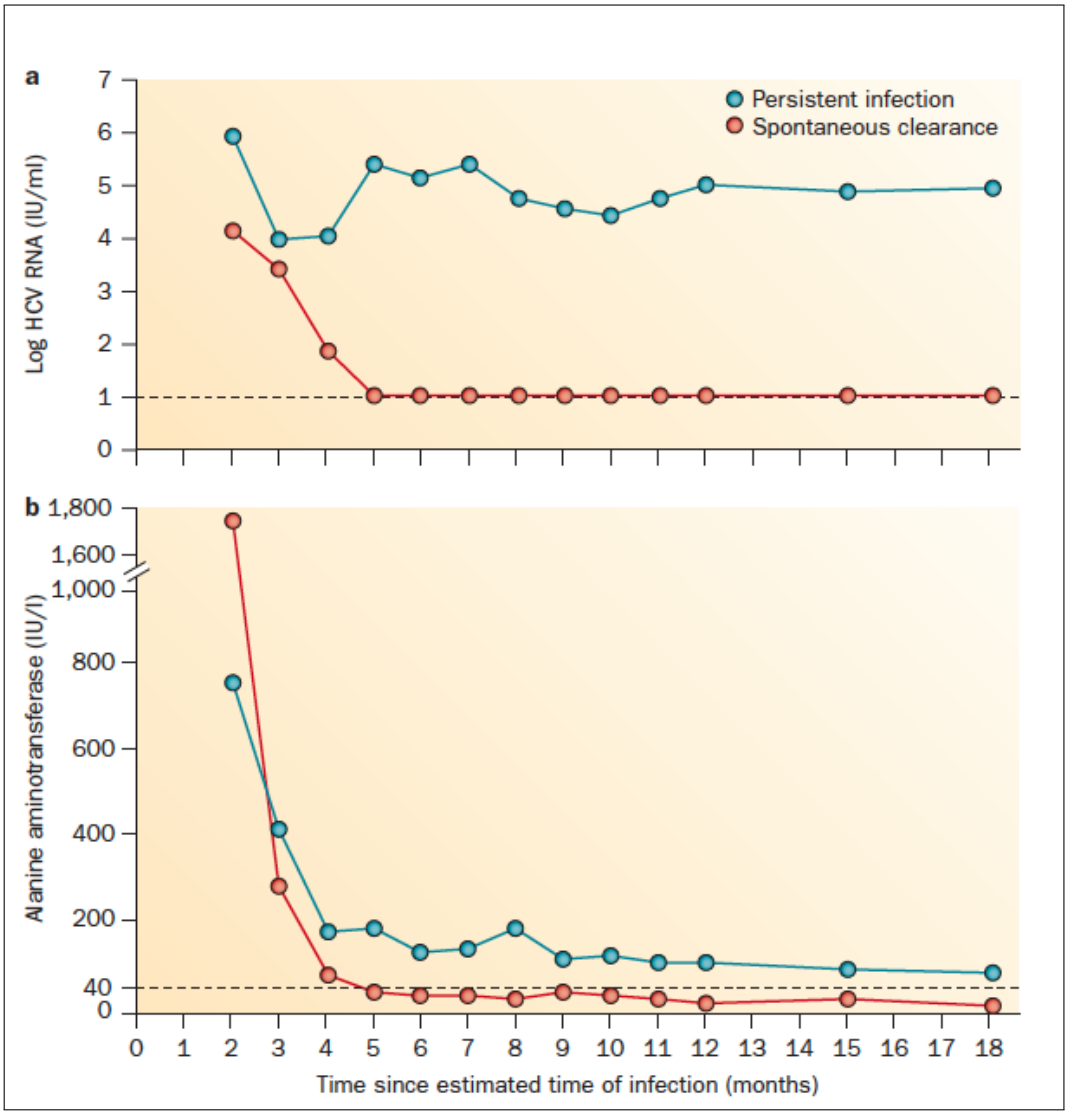

Figure 5: Dynamics of the HCV RNA levels and alanine aminotransferase in the case of spontaneous clearance or persistent HCV infection. a. HCV RNA levels decline in the spontaneous resolution of the infection, while in the case of persistency these levels are high and detectable. **b**. Alanine aminotransferase levels decline in both outcomes of the infection, but remain detectable in persistent infections. (Hajarizadeh, Grebely, and Dore, 2013)

Acute HCV infection becomes persistent in around 75-80% of the cases (Figure 6). Moreover, between 10 and 20% of patients with chronic hepatitis will develop a severe liver disease, such as cirrhosis and HCC (Westbrook and Dusheiko, 2014). Infection with HCV first leads to inflammation, damage and fibrogenesis of the liver causing scarring of the tissue, which can later drive to liver cirrhosis (15-25% of the cases). Cirrhosis is an advanced stage of liver fibrosis with a distorted vasculature of the liver (Schuppan and Afdhal, 2008), and between 1 and 7% of patients with liver cirrhosis may develop HCC (Hoshida et al., 2014). HCC is the fourth leading cause of death by cancer, the sixth most frequently

diagnosed cancer and the most frequent liver cancer (Bray et al., 2018). It was observed that 80% of patients with HCC also present HBV or HCV infection (El-Serag, 2012). Some risk factors that promote the development of HCC are the sex (male), the age (>75 years old), the presence of comorbidities (steatosis, insulin resistance), the alcohol abuse, co-infections and infection with genotype 3 HCV [reviewed in (McGlynn, Petrick, and El-Serag, 2021)].

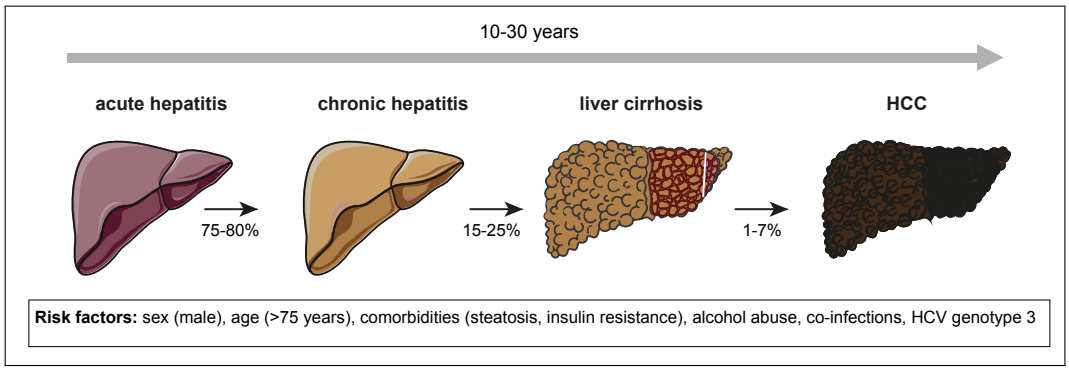

Figure 6: Natural history of hepatitis C. HCV infects hepatocytes and causes acute hepatitis that is mostly asymptomatic and lasts between 0 and 24 weeks. More than 70% of the cases will evolve into a chronic hepatitis disease within 10 to 30 years. Because of HCV, there is inflammation and scarring of the liver tissue causing fibrosis that, in 15 to 25% of the cases, can progress into liver cirrhosis. Patients with cirrhosis can develop a decompensated form of cirrhosis or HCC, both severe liver diseases. The data were obtained from (Heim, 2013; Hoshida et al., 2014; McGlynn, Petrick, and El-Serag, 2021; Westbrook and Dusheiko, 2014).

Nowadays, effective drugs to clear HCV are available, but chronic hepatitis can progress to HCC even after successful treatment. Currently available therapies (direct-acting antivirals (DAAs) or interferon-based) seem to reduce the risk of developing HCC, but the annual HCC incidence in "cured" patients is still of 0,1-2% depending if patients present liver cirrhosis (El-Serag et al., 2016; Ioannou, Green, and Berry, 2018; Kanwal et al., 2017). Although several studies suggested that active HCC in patients may reduce the efficacy of treatment (Ji et al., 2019; Prenner et al., 2017; Soria et al., 2017), DAAs-based therapies have been reported to improve the survival of these patients by reducing the risk of hepatic decompensation (Cabibbo et al., 2019; Dang et al., 2020).

1.4 THE ERA OF DIRECT-ACTING ANTIVIRALS

The first therapies for HCV-infected patients were based on the recombinant IFN alpha (IFNα), and later improved by combining it with ribavirin, a nucleoside analogue with antiviral activity. This combination led to a sustained virologic response (SVR) rate of around 38% (McHutchison et al., 1998). The SVR was defined as the resolution of the infection (absence of HCV RNA) at least 12 weeks

after the end of the treatment (Jacobson et al., 2012; Wedemeyer et al., 2012). Later, the IFNα was pegylated (peg-IFNα) to increase its stability and to reduce the frequency of administration in patients for whom treatment was hard. This modification improved the SVR rate of treated patients to around 50% (Lindsay et al., 2001; Zeuzem et al., 2000). In 2011, effective drugs called DAAs were developed and approved by the Food and Drug Administration (FDA). The first accepted drugs were telaprevir and boceprevir; when administrated in combination with peg-IFNα and ribavirin, they led to a SVR rate of 75% in patients (Bacon et al., 2011; Jacobson et al., 2011). It was not until 2014 that DAAs were approved to be used alone in IFN-free treatments (Pawlotsky et al., 2014).

The targets of DAAs are the non-structural (NS) proteins 3, 5A and 5B (NS3, NS5A and NS5B) of HCV (Table 1) and patients treated within DAAs-based therapies can reach SVR rates above 95% (Pawlotsky et al., 2020). Currently, five regimens have been approved for its use in Europe: sofosbuvir, sofosbuvir/velpatasvir, sofosbuvir/velpatasvir/voxilaprevir, glecaprevir/pribrentasvir and grazoprevir/elbasvir. The administration of these DAA regimens should be done according to the European Association for the Study of the Liver (EASL) recommendations (Table 2) considering the genotype and subtype, the status of the cirrhosis (if advanced, proteases inhibitors are contraindicated) and the prior exposure to a treatment (including the IFN-based therapies). Ideally, when patients come from a geographical zone where HCV subtypes (1l, 4r, 3b, 3g, 6u, 6v) mainly resistant to NS5A inhibitors have been identified, HCV genotype and subtype should be determined before therapy (Childs et al., 2019; Fourati et al., 2019; Pawlotsky et al., 2020).

The high genetic diversity of HCV makes this virus difficult to clear. The lack of proofreading of the HCV RdRp generates heterogeneous populations of viral quasispecies. If these mutations are found in the target sequences of DAAs, they are called RAS because they reduce the susceptibility of viral quasispecies to treatment. Most of RAS have been identified in the NS5A sequence and if they have a prevalence of at least 15%, they are considered clinically relevant (Pawlotsky, 2016). Basically, the administration of DAAs selects positively resistant variants (with RAS) that lead to the failure of the treatment (Pawlotsky, 2016; Sarrazin et al., 2016). The RAS can also appear after exposure to DAAs-based therapies and their prevalence might depend on the HCV subtype (Welzel et al., 2017; Zeuzem et al., 2017).

Access to treatment remains very limited, with an estimated administration to 7-13% of the diagnosed HCV chronic carriers worldwide (Edlin, 2016; World Health Organization, 2017). This lack of accessibility is mainly caused by the unaffordable prices of DAAs. In high income countries, such as the USA, in which people with no medical insurance are incapable to pay for DAAs-based therapies,

the reimbursement of DAAs is frequently denied by insurances if there is no evidence of liver cirrhosis (Gowda et al., 2018; Trooskin, Reynolds, and Kostman, 2015). However, the prices of DAAs have lowered because of several international initiatives. In some high-income countries (France, Netherlands and Australia), national programs were created to absorb part of treatment costs and to ensure the access to effective therapies (Cooke et al., 2019). Furthermore, pharmaceutical companies have signed agreements to manufacture generic versions of their drugs in low- and middle-income countries, which have allowed the reduction of prices and thus the accessibility to treatment (Cooke et al., 2019). For instance, thanks to these actions in low- and middle-income countries, the price of a 12-week sofosbuvir treatment dropped from US$84000 to US$900. This generic sofosbuvir has been shown to be as efficient to treat HCV as the branded sofosbuvir (Hill et al., 2016; Hill et al., 2017; University of Washington, 2019).

The access to treatment based on DAAs in the USA remains also restricted for drug users (Barua et al., 2015). If HCV-infected IVDUs were treated with effective therapies, the burden of this disease could be truly reduced cost-effectively (Barbosa et al., 2019; Martin et al., 2016). Drug users can achieve high cure rates as other patients do, but the problem is that reinfection rates are elevated because of risk behaviors (Boglione et al., 2017; Midgard et al., 2016), and unfortunately, DAAs-based therapies do not protect from reinfection. Simmons and colleagues evaluated through a meta-analysis the risk of reinfection post-treatment for high-risk groups. The risk of reinfection for IVDUs and prison inmates, as well as HCV/HIV-coinfected patients was calculated of 10,67% and 15,02%, respectively, highlighting the importance of controlling the epidemics through prevention campaigns (Simmons et al., 2016a). Overall, a prophylactic HCV vaccine would be an efficient tool to control the transmission of this virus in high-risk populations and to respond to the current problems with DAAs-based therapies (reinfection, failure due to resistance, HBV reactivation) (Echevarria et al., 2019; Stone et al., 2016).

Table 1: Classification of direct-acting antivirals by mode of action. The direct-acting antivirals (DAAs) are classified in non-structural (NS) 3 protease inhibitors, NS5A inhibitors and NS5B RNA-dependent RNA polymerase inhibitors. The data were obtained from (Asselah et al., 2018).

Mode of action	DAA
NS3 protease inhibitors "-previr"	Glecaprevir Grazoprevir Paritaprevir Simeprevir Voxilaprevir
NS5A inhibitors "-asvir"	Daclatasvir Elbasvir Ledipasvir Ombitasvir Pribrentasvir Velpatasvir
NS5B RNA-dependent RNA polymerase inhibitors "-buvir"	Sofosbuvir (nucleoside) Dasabuvir (non-nucleoside)

Table 2: Recommendations of therapies for adult patients (>18 years) according to their HCV genotype and subtype, to their cirrhosis status and to their prior exposure to treatment (Pawlotsky et al., 2020).

Type of treatment	Genotype	Cirrhosis status	Prior treatment experience	Sofosbuvir/ velpatasvir	Glecaprevir/ pibrentasvir	Sofosbuvir/ velpatasvir/ voxilaprevir	Grazoprevir/ elbasvir
Genotype/subtype determination-based treatment	Genotype 1a, 1b, 2, 4, 5 and 6	No cirrhosis	Treatment-naïve	12 weeks	8 weeks	No	12 weeks (genotype 1b only)
			Treatment-experienced				
		Compensated (Child-Pugh A) cirrhosis)	Treatment-naïve				
			Treatment-experienced		12 weeks		
	Genotype 3	No cirrhosis	Treatment-naïve	12 weeks	8 weeks	No	No
			Treatment-experienced		12 weeks		No
		Compensated (Child-Pugh A) cirrhosis)	Treatment-naïve	12 weeks with weight-based ribavirin[a]	8-12 weeks[b]	12 weeks[a]	No
			Treatment-experienced		16 weeks		No
	S ubtype 1l, 4r, 3b, 3g, 6u, 6v or any other subtype naturally harbouring one or several NS5A RASs[c]	No cirrhosis	Treatment-naïve	Unknown	Unknown	12 weeks	No
			Treatment-experienced				
		Compensated (Child-Pugh A) cirrhosis)	Treatment-naïve				
			Treatment-experienced				

2

HEPATITIS C VIRUS

2.1 THE DISCOVERY OF HCV

In 1975, Feinstone and colleagues described for the first time HCV as the non-A non-B hepatitis (NANBH) virus in patients with transfusion-associated hepatitis (Feinstone et al., 1975). But it was not until 1989 that Houghton and collaborators isolated a cDNA clone derived from this infectious agent by screening of cDNA libraries from NANBH-infected chimpanzees, using NANBH-patients plasma (Figure 7) (Choo et al., 1989). Later on, an assay to detect anti-HCV antibodies was developed, allowing the screening of patients, as well as blood donors and recipients (Kuo et al., 1989). In 1997, HCV was conclusively defined alone as the causative agent of hepatitis C, establishing the basis for the future research on this virus (Kolykhalov, 1997). The scientists who led to the discovery of HCV were awarded the Nobel Prize in Physiology or Medicine 2020.

2.2 VIRAL LIFE CYCLE

HCV is a small, enveloped, positive-sense single-stranded (ss) RNA virus classified as the type member of the *Hepacivirus* genus of the *Flaviviridae* family. HCV virions circulate in the bloodstream of infected patients as lipoviral particles (LVPs) because they associate to host low-density lipoproteins (LDL) and very-low-density lipoproteins (VLDL). The size of these LVPs ranges between 50 and 80 nm within a low buoyant density of <1,03 and 1,10 g/mL (André et al., 2002; Bradley et al., 1991; Catanese et al., 2013; Gastaminza et al., 2010; Kaito et al., 1994; Maillard et al., 2001; Nielsen et al., 2006; Piver et al., 2017). The HCV LVPs are formed of a nucleocapsid that contains the viral genome, surrounded by an endoplasmic reticulum (ER)-derived viral envelope in which HCV envelope glycoproteins E1 and E2 are anchored (Figure 8).

HCV is a hepatotropic virus that infects primarily hepatocytes thanks to the recognition and interaction with specific cellular receptors and molecules. HCV first binds to the cell surface through the lipoproteins (such as apolipoprotein E (apoE)) that interact by low-affinity binding with the syndecan-1, syndecan-2 and syndecan-4 heparan sulfate proteoglycans (HSPG) (Barth et al., 2003; Fan et al., 2017; Jiang et al., 2013; Lefèvre et al., 2014; Shi, Jiang, and Luo, 2013), the scavenger receptor-class B type I (SR-BI) (Catanese et al., 2007; Dao Thi et al., 2012;

Maillard et al., 2006; Voisset et al., 2005), the LDL receptor (LDLR) (Agnello et al., 1999; Monazahian et al., 1999) and the VLDL receptor (VLDLR) on host cells (Ujino et al., 2016). Then, the exposition of viral envelope proteins allows HCV to interact, through the E2 glycoprotein, with the cellular receptor SR-BI (Bartosch et al., 2003; Scarselli et al., 2002). This association helps the E2 protein bind the cluster of differentiation (CD) 81 (Pileri et al., 1998; Zeisel et al., 2007), which activates signaling pathways through tyrosine kinase receptors such as the epidermal growth factor receptor (EGFR), and promotes actin rearrangements by the activation of Rho GTPases (Baktash et al., 2018; Brazzoli et al., 2008; Lupberger et al., 2011). After attachment, these signals stimulate the formation of the claudin-1 (CLDN1)-CD81 co-receptor complex, essential for the internalization of HCV (Evans et al., 2007; Harris et al., 2010).

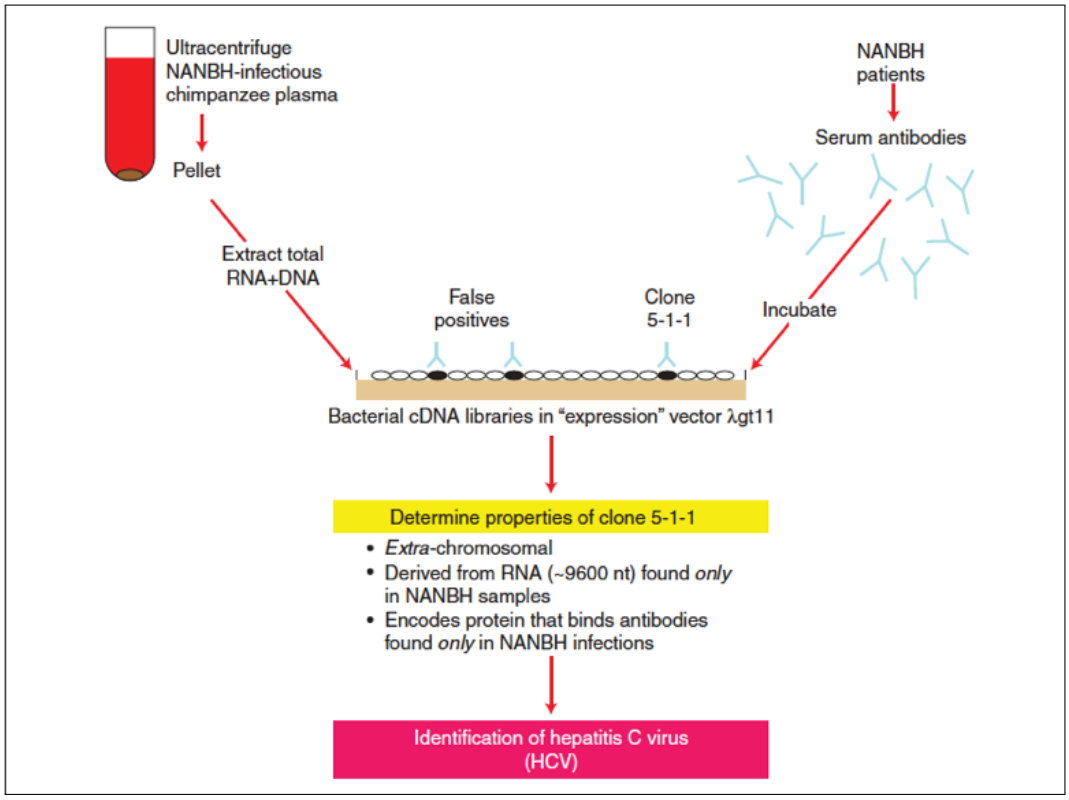

Figure 7: Protocol for the first isolation of HCV performed by Choo and colleagues. Libraries of bacterial complementary DNA (cDNA) from non-A non-B hepatitis (NANBH)-infected chimpanzees in the expression vector λgt11 were screened by using plasma from NANBH-infected patients. The clone 5-1-1 (one of the five positive clones) was identified as NANBH origin with specific properties that led to the identification of HCV. (Choo et al., 1989; Houghton, 2019)

Besides the cell-free transmission, infection of hepatocytes can also happen in a cell-to-cell way. This type of transmission is not well described, but studies have shown that it depends on post-binding receptors such as the tight junction proteins CLDN1 and occludin (OCLN), together with apoE (Fan et al., 2017; Gon-

DAR et al., 2015; HUEGING et al., 2014). The OCLN, as the CLDN1, is a tight junction protein (found in the basolateral membrane) also implicated in the entry process, at a post-binding step. In fact, this molecule, along with the CD81, determines the tropism of HCV for human cells (PLOSS et al., 2009). The cell-to-cell transmission route is a mechanism for the virus to escape from neutralizing antibodies and to resist against DAAs-based treatment (BRIMACOMBE et al., 2011; TIMPE et al., 2008; XIAO et al., 2014).

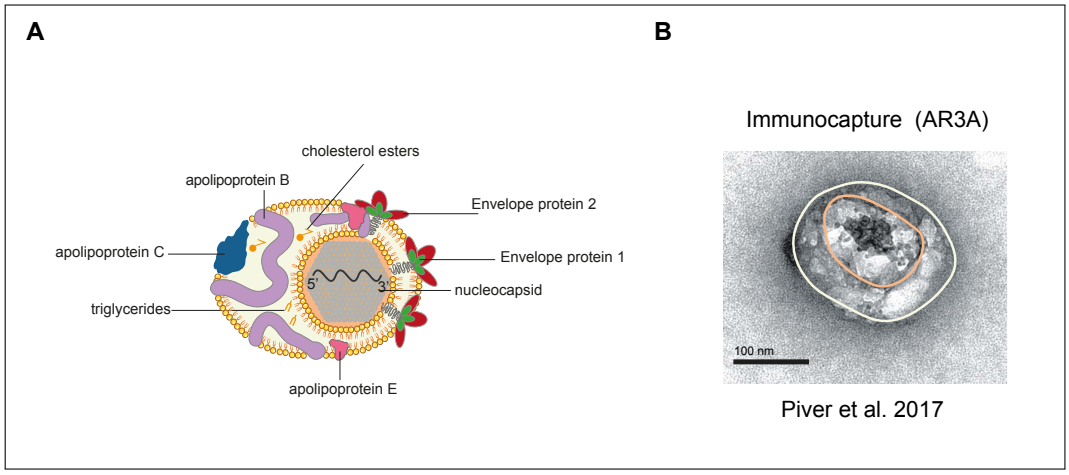

Figure 8: Modeling of the HCV lipoviral particle. A. Putative HCV lipoviral particle (LVP). HCV is formed with a nucleocapsid containing the positive single-stranded RNA viral genome, enclosed by an endoplasmic reticulum (ER)-derived membrane, in which the envelope proteins E1 and E2 are anchored. All this is closely associated to apolipoproteins (B, C and E) and neutral lipids (cholesterol esters and triglycerides). **B**. Electron micrograph of LVP from HCV-infected serum, captured with a monoclonal antibody (AR3A) targeting a conformational-dependent epitope of the E2 protein (PIVER et al., 2017). The yellow circle surrounds the LVP, while the orange one surrounds the nucleocapsid, as colored in panel A. Lipids are enclosed in the zone between the yellow and the orange rings.

Internalization of HCV occurs via a clathrin-mediated and dynamin-dependent endocytosis, through vesicles in which a pH-dependent fusion takes place (BLANCHARD et al., 2006; HAID, PIETSCHMANN, and PÉCHEUR, 2009). The fusion of viral and cellular membranes occurs thanks to the presence of a fusion protein. In the case of HCV, the E1 glycoprotein seems to have this role, but this mechanism is still not completely elucidated and the contribution of both envelope proteins to this step might be possible (DOUAM et al., 2014; GARRY and DASH, 2003; PERIN et al., 2016; TONG et al., 2017).

Following the uncoating, the particle is released to the cytosol and the viral RNA is liberated. The HCV genome is a (+) ssRNA molecule of 9,6 kb length containing one open reading frame (ORF) flanked by two untranslated regions (UTRs), 5'UTR and 3'UTR (FRIEBE et al., 2001). The 5'UTR is a highly conserved region that contains a type III internal ribosome entry site (IRES), which allows a cap-independent translation of the positive-strand RNA (serving as messenger

RNA) (Tsukiyama-Kohara et al., 1992; Wang, Sarnow, and Siddiqui, 1993). The IRES interacts with the ribosomal 40S unit, as well as other factors that modulate HCV translation (Fuchs et al., 2015; Kieft et al., 2001). For instance, two copies of the microRNA-122 (miR-122), a host molecule specific to the liver, bind to the 5'UTR (Jopling, 2005; Lagos-Quintana et al., 2002). The miR-122 was reported to stimulate the HCV RNA replication (Chang et al., 2008; Masaki et al., 2015), to enhance the translation of HCV proteins (Henke et al., 2008; Schult et al., 2018) and to protect genomic RNA from degradation (Machlin, Sarnow, and Sagan, 2011; Sedano and Sarnow, 2014). Moreover, the miR-122 circulating in HBV- or HCV-infected patient sera has been reported as a marker of chronic hepatitis infection (Cermelli et al., 2011; Waidmann et al., 2012).

A single polyprotein precursor of 3000 amino acids (aa) is directly translated at the ER membrane. Cellular and viral proteases cleave the polyprotein to generate ten products: three structural (core, E1 and E2 glycoproteins) and seven NS viral proteins (p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B) [reviewed in (Moradpour and Penin, 2013)]. Cleavage of core, E1, E2 proteins and the p7/NS2 junction is mediated by the ER signal peptidase and signal peptide peptidase from the host, while the cleavage between other NS proteins happens through the NS2 and NS3/4A viral proteases (Figure 9).

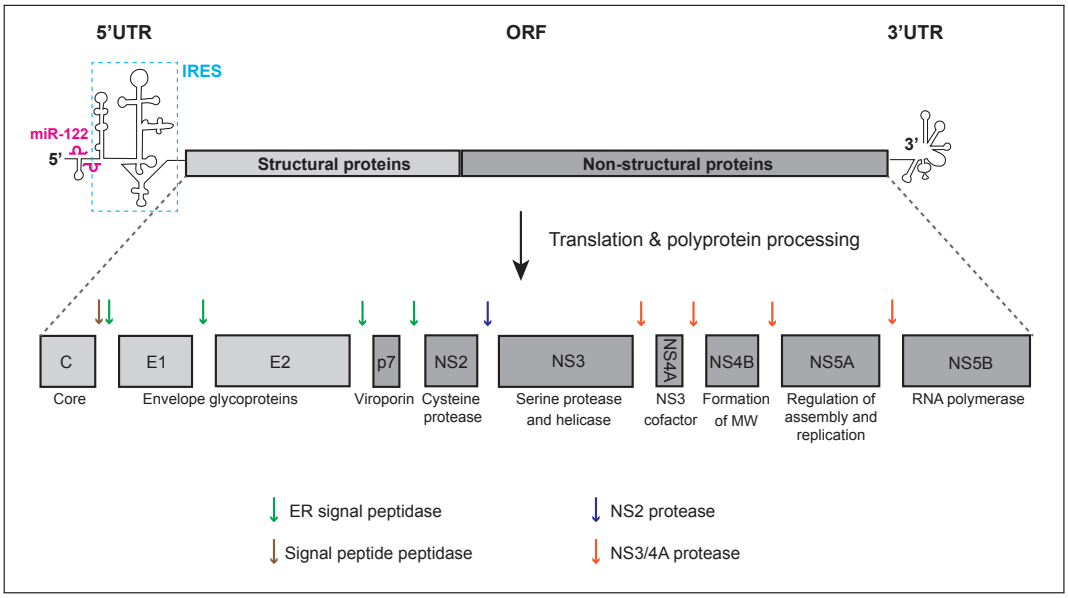

Figure 9: HCV genome organization and polyprotein processing. HCV possesses a positive single-stranded RNA genome of 9,6 kb that contains one open reading frame (ORF) flanked by untranslated regions (UTRs): 5'UTR and 3'UTR. Two copies of the microRNA-122 (miR-122) (in pink) from the host bind to the 5'UTR and stimulate the HCV genome replication and translation of proteins. The ORF codes for one polyprotein via an internal ribosome entry site (IRES)-mediated (indicated in blue) translation. The polyprotein precursor is processed by cellular (green and brown arrows) and viral (purple and orange arrows) proteases to obtain 3 structural proteins and 7 non-structural proteins. MW: membranous webs. The data were obtained from (Scheel and Rice, 2013).

The core is a 21-kDa protein that forms the viral capsid. The immature core protein (23 kDa) is composed of 2 domains and a signal sequence. The N-terminal domain binds the RNA and is necessary for the capsid formation (Majeau et al., 2004), the C-terminal domain containing two amphipathic helices allows the interaction with the ER-membrane and the lipid droplets (LDs) (Barba et al., 1997; Boulant et al., 2006) and the signal sequence serves for the translocation of the E1 protein (Santolini, Migliaccio, and La Monica, 1994). The immature core protein is released from the protein precursor by the ER-signal peptidase, and then the signal sequence at the C-terminal is cleaved by the signal peptide peptidase, obtaining a mature core protein of 21-kDa (McLauchlan, 2002; Santolini, Migliaccio, and La Monica, 1994). This processing of the core protein has been shown to be necessary for viral propagation (Okamoto et al., 2008). To form the viral capsid, the core protein assembles in dimers by disulfide bonds and then into oligomers (Boulant et al., 2005; Ivanyi-Nagy et al., 2006; Kushima, Wakita, and Hijikata, 2010). The accumulation and interaction of the core with the LDs seem to be crucial for viral assembly (Miyanari et al., 2007; Shavinskaya et al., 2007).

The envelope glycoproteins E1 and E2 are type I transmembrane proteins cleaved from the protein precursor by the ER signal peptidase. The N-terminal ectodomains are translocated into the ER lumen and the C-terminal transmembrane domains (TMDs) retain the proteins in the ER membranes (Cocquerel et al., 2002; Deleersnyder et al., 1997). The E1 and E2 proteins can interact with each other and form large covalent complexes stabilized through disulfide bonds on the surface of virions or non-covalent heterodimers intracellularly (Vieyres et al., 2010). These proteins will be described in detail in section 2.4.

The p7 protein is a small transmembrane protein of 7 kDa that belongs to the family of viroporins and shows a "hairpin-like" conformation (Cook et al., 2013; Foster et al., 2014; Montserret et al., 2010; OuYang et al., 2013). The p7 protein cleavage from the E2/p7 and the p7/NS2 junctions is mediated by ER signal peptidase (Lin et al., 1994). This protein has ion channel activity that has been shown to protect virions from low-pH conditions allowing the production of infectious virions (Wozniak et al., 2010) and to participate in the assembly and release of infectious virions (Steinmann et al., 2007). The interactions of the p7 protein with structural and non-structural proteins have been evaluated. For instance, it was reported that the p7 protein interacts with NS2 through their TMDs (Popescu et al., 2011). The interaction p7-NS2 seemed to modulate redistribution of the core from LDs to the ER (Boson et al., 2011; Gentzsch et al., 2013). Moreover, a recent study showed that the timing of the cleavage at the E2/p7 junction modulates the assembly and secretion of infectious virions (Denolly et al., 2017).

The NS2 is a membrane-associated cysteine protease of 23 kDa that liberates NS3 from the NS2/NS3 junction (Grakoui et al., 1993; Hijikata et al., 1993;

Lorenz et al., 2006). Independently from the protease activity of the NS2 protein, the interactions between the NS2-p7, NS2-NS5A and NS2-E2 proteins have been reported to promote the assembly of virions (Jirasko et al., 2010; Jones et al., 2007; Popescu et al., 2011; Stapleford and Lindenbach, 2011).

The NS3 is a 70-kDa protein with serine protease activity (N-terminal) that cleaves itself from the NS4A (its cofactor), and also at the junction between NS4A and NS4B. The NS4A associates with NS3 to form the NS3/4A complex that liberates the NS4B, NS5A and NS5B proteins (Bartenschlager et al., 1995; Brass et al., 2008; Tomei et al., 1993). When NS3 is cleaved from NS2, a hydrophobic conserved surface of NS3 promotes hyperphosphorylation of NS5A, and therefore, viral replication (Isken et al., 2015). The NS3 protein also possesses an NTPase/helicase activity at its C-terminal domain, which unfolds double-stranded (ds) RNA or RNA with complex secondary structure (Kim et al., 1995). This activity has shown to be essential for the RNA replication (Lam and Frick, 2006).

The NS4B is a highly hydrophobic transmembrane protein of 27 kDa cleaved from the polyprotein precursor by the NS3/4A complex. The NS4B protein is composed of an N-terminal domain, a core of 4 or 5 TMDs (if translocation of the N-terminal domain) and a C-terminal domain. The N-terminal and C-terminal domains are found in the cytosolic side, but the former can translocate to the ER lumen (Lundin et al., 2003), and the second might be involved in the formation of the replication complex (Gouttenoire et al., 2009). The NS4B protein induces membrane rearrangements derived from the ER, called membranous web, that serve as platform for the viral replication (Egger et al., 2002; Gosert et al., 2003). The main type of vesicles formed in the membranous matrix are the double membrane vesicles, which require the action of several NS proteins (Ferraris, Blanchard, and Roingeard, 2010; Romero-Brey et al., 2012). These formations seem to be triggered by the self-interaction and oligomerization of the NS4B protein (Gouttenoire et al., 2010; Paul et al., 2011).

The NS5A is a 56-58-kDa phosphoprotein organized in 3 domains (Tellinghuisen et al., 2004). The domain I is involved in the RNA binding (Huang et al., 2005; Hwang et al., 2010), while residues from the domain II are crucial for the replication of the HCV genome (Tellinghuisen et al., 2008). Moreover, the domain III of NS5A participates in the interaction with the core protein (Appel et al., 2008; Masaki et al., 2008). This protein has also shown to be involved in the formation of membranous webs and the binding with LDs (Miyanari et al., 2007). NS5A can be phosphorylated in many serine and threonine residues (Tanji et al., 1995), which impacts the RNA replication, virion assembly and production of infectious virions (Evans, Rice, and Goff, 2004; Eyre et al., 2016; Masaki et al., 2014; Tellinghuisen, Foss, and Treadaway, 2008). Finally, this protein was shown to interact with host molecules such as the apoE (Benga et al., 2010; Cun, Jiang, and

Luo, 2010; Jiang and Luo, 2009) and the phosphatidylinositol 4-kinase IIIα (Reiss et al., 2011).

The NS5B is a 68-kDa enzyme responsible for the RNA replication and one of the main targets of DAAs (Behrens, Tomei, and De Francesco, 1996). Observations from crystallography studies showed that NS5B has the classical right hand conformation of RNA polymerases, comprising domains resembling fingers, thumb and palm (Ago et al., 1999; Bressanelli et al., 1999). This enzyme has been shown to synthesize RNA at the 3′ terminus via a *de novo* mechanism (Luo et al., 2000; Zhong et al., 2000) and, as it has been stated before, this enzyme has low fidelity, leading to the generation of the viral quasispecies (Powdrill et al., 2011). The RdRp uses the (+) ssRNA as template to synthesize a negative strand of RNA, and then to produce new positive strands that will be used as templates for protein translation, for RNA replication or for packaging in nascent virions. The RNA replication has been reported to take place in double-membrane vesicles derived from the ER (Ferraris, Blanchard, and Roingeard, 2010; Paul et al., 2013; Romero-Brey et al., 2012) or in membrane fragments from the Golgi apparatus (Hansen et al., 2017), with properties similar to detergent-resistant membranes (Shi et al., 2003).

For the assembly of viral particles, HCV hijacks the VLDL biosynthesis pathway (Gastaminza et al., 2008; Huang et al., 2007). During the assembly, the core protein homodimerizes and seems to accumulate on the cytosolic LDs (cLDs) (Barba et al., 1997; Boulant, Targett-Adams, and McLauchlan, 2007), containing neutral lipids such as triglycerides and cholesterol esters surrounded by a phospholipid monolayer [reviewed in (Olzmann and Carvalho, 2019)]. The core-NS5A interaction helps to transfer the RNA from the replication to the assembly site (Miyanari et al., 2007). The NS2 and p7 proteins interact and recruit the NS3/4A complex, which also allows the recruitment of the viral core to the assembly site (Counihan, Rawlinson, and Lindenbach, 2011; Phan et al., 2009). For instance, an assembly model has proposed that the E1E2p7NS2 complex accumulates in the ER membrane, and the p7 protein has a central role by modulating the NS2-NS5A interaction and the transfer of the E1 and E2 proteins to the assembly site (Denolly et al., 2017).

Nascent HCV viral particles fuse to pre-VLDL ones in the ER lumen to form mature HCV LVPs (Bartenschlager et al., 2011). These pre-VLDL particles are synthesized thanks to the interaction of the apoB100 (non-exchangeable protein) and the microsomal transfer protein (MTP). Neutral lipids are transferred to apoB100, and so the pre-VLDL is released in the ER lumen where other lipid components such as exchangeable apolipoproteins (E and C) and triglycerides are incorporated [reviewed in (Tiwari and Siddiqi, 2012)]. The maturation of the HCV LVPs continues in the subsequent compartments at which lipid components can

be integrated, and envelope proteins also maturate by the formation of disulfide bonds and the acquisition of glycans at the N-glycosylation sites. Transport of virions has been suggested to occur through coat protein complex II (COPII)-derived vesicles and Golgi compartments, following the pathway of the VLDL biogenesis (Syed et al., 2017). Budding of HCV particles may occur through the endosomal sorting complexes required for transport (ESCRT), but this step still needs to be completely elucidated (Ariumi et al., 2011; Corless et al., 2010; Tamai et al., 2012).

There is increasing evidence that lipidation of HCV may also or exclusively take place in an extracellular milieu. This means that virions might be lipidated outside the cells and the association of HCV with the low-density molecules may occur through non-covalent interactions [reviewed in (Cosset et al., 2020)]. In fact, recent studies have reported that maturation of hepatitis C virus in cell culture (HCVcc) happens after secretion thanks to the presence of extracellular serum factors (Bankwitz et al., 2017; Denolly et al., 2019). The Figure 10 summarizes a proposed complete infectious cycle of HCV.

2.3 ROLES OF THE APOLIPOPROTEIN E IN THE HCV LIFE CYCLE

As described above, there is a tight relationship between HCV and the host lipid metabolism. Although the mechanism of LVP formation is still not fully elucidated, HCV particles associated with lipoproteins in the serum of HCV-infected patients have been characterized. However, the *in vitro* culture of LVPs from donors has resulted ineffective. The purification and analysis of these particles have led to the identification of apolipoproteins: apoA-I, apoB, apoC (I-III) and apoE (André et al., 2002; Diaz et al., 2006; Mancone et al., 2011; Meunier et al., 2008a). Moreover, it has been shown that these apolipoproteins are exposed on the surface of virions by the capture of LVPs from infected patients and on the surface of HCVcc by detection with specific antibodies (Boyer et al., 2014; Catanese et al., 2013; Gastaminza et al., 2010; Merz et al., 2011; Piver et al., 2017). The apoA, apoC and apoE are exchangeable proteins that can dissociate from one particle and re-associate to another, playing redundant roles (Fukuhara et al., 2014; Zhang, Qiao, and Luo, 2018). ApoE, in particular, has been reported to play important roles in the HCV life cycle.

The apoE is a 34-kDa protein (299 aa) synthesized in several organs (liver, brain, spleen and kidney), while the peripheral apoE is mainly produced by the hepatocytes (Elshourbagy et al., 1984). ApoE can be found in two states as a lipid-free or a lipid-bound protein. Some of the roles attributed to apoE are the mediation of the clearance of lipoproteins rich in triglycerides (high density lipoprotein (HDL), LDL, VLDL, chylomicrons) and the stabilization of lipoproteins [reviewed in (Phillips, 2014)]. The human *APOE* gene on chromosome 19 encodes

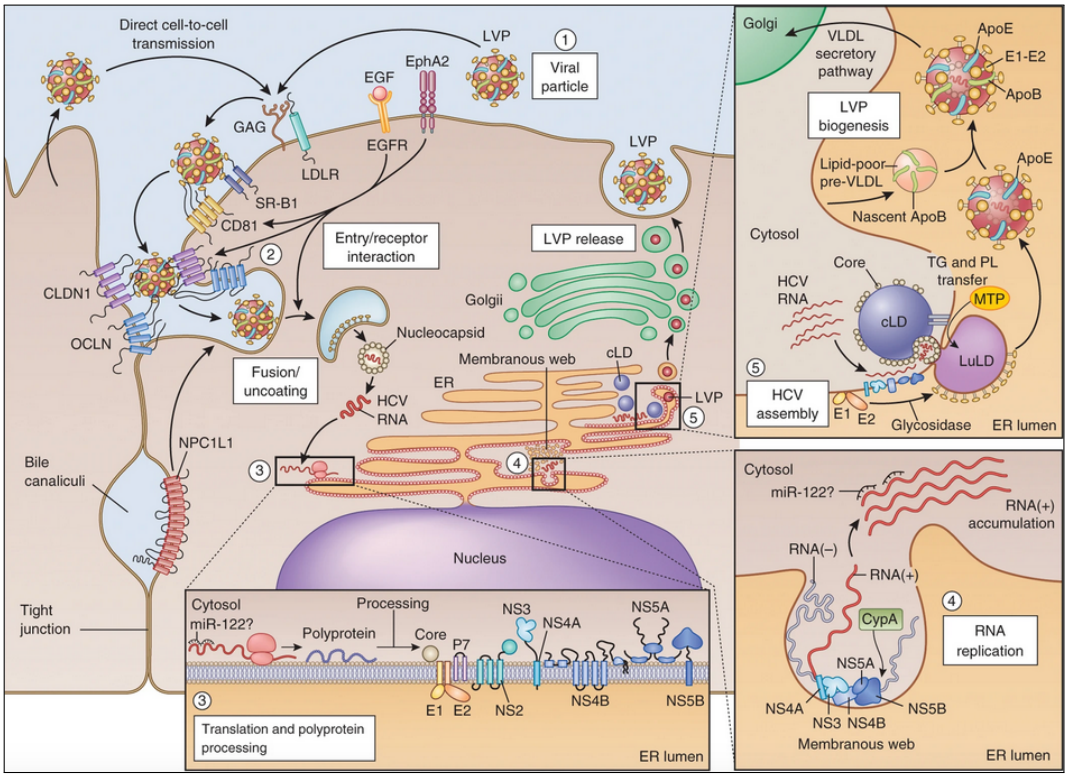

Figure 10: HCV life cycle. 1. HCV LVPs enter the cell through the recognition of cellular receptors such as the low-density lipoprotein receptor (LDLR), the scavenger receptor-class B type I (SR-BI) and the cluster of differentiation (CD) 81. **2**. HCV can also spread through cell-to-cell transmission by the tight junction proteins claudin-1 (CLDN1) and occludin (OCLN). HCV is internalized through clathrin-mediated and dynamin-dependent endocytosis, during which the virus is uncoated and RNA genome is liberated in the cytosol. **3**. The polyprotein precursor is translated and processed into structural (core and envelope proteins E1 and E2) and non-structural proteins (p7, NS2, NS3, NS4A, NS4B, NS5A and NS5B) associated to the endoplasmic reticulum (ER)-derived membranes. **4**. HCV RNA genome replication takes place in membranous webs within the replication complex (NS3/4A, NS4B, NS5A and NS5B). **5**. The core is transferred to cytosolic lipid droplets (cLDs) to form the nucleocapsid and the RNA is transferred by the NS5A. Nascent viral particles containing E1 and E2 on their surface fuse to luminal LDs (LuLDs) and to prevery-LDL (pre-VLDL). LVPs transit and mature following the VLDL secretory pathway and may be released through the endosomal sorting complex. Adapted from (Scheel and Rice, 2013).

for 3 isoforms: apoE2, apoE3 and apoE4, which differ in two amino acid changes at positions 112 and 158 of arginine or cysteine. These changes in residues were shown to define their binding affinity to receptors (Ruiz et al. 2005; Weisgraber et al. 1982). ApoE3 is the most frequent isoform in humans, present in around 70% of population, followed by apoE4 (20%) and apoE2 (10%) (MAHLEY, 1988). The isoform apoE4 has been identified as a major risk factor for the development of Alzheimer's disease (CORDER et al., 1993). On the contrary, the isoform apoE2 seems to have a protective effect against this disease and improve the longevity (GENIN et al., 2011; SHINOHARA et al., 2020). Because of the low receptor binding affinity of the apoE2 isoform, it has been associated with the type III hyperlipoproteinemia in 5 to 10% of the cases (DE BEER et al., 2002; MAHLEY, HUANG, and RALL, 1999). In fact, the impact of the three isoforms has been studied in HCV-infected patients. The isoform apoE3 was found to be associated to a persistent HCV infection (PRICE et al., 2006), while the isoform apoE4 to protect the liver from damage (MUELLER et al., 2016; WOZNIAK et al., 2002).

ApoE is composed of two domains (FIGURE 11): an N-terminal domain (aa 1-167) and a C-terminal domain (aa 206-299), linked by a hinge region (aa 168-205). The human apoE is post-translationally glycosylated via the acquisition of O-linked glycans at threonine 194, serine 290, serine 296 and serine 289, in a tissue-specific way, and sialylated via the addition of sialic acid. However, apoE has been observed to be N-glycosylated in mice [reviewed in (KOCKX, TRAINI, and KRITHARIDES, 2018)]. The Nuclear Magnetic Resonance (NMR) and X-ray structures of the lipid-free state of apoE revealed that the N-terminal domain is formed by 4 amphipathic α-helices disposed in the shape of a bundle (CHEN, LI, and WANG, 2011; DONG et al., 1994; WILSON et al., 1991). This domain is responsible for the binding to lipoprotein receptors (aa 136-150) such as the LDLR and VLDLR (HATTERS, PETERS-LIBEU, and WEISGRABER, 2006). The structure of the C-terminal domain was first defined as an α-helical region, in which the main site for the binding of lipids (aa 244-272) was identified (HATTERS, PETERS-LIBEU, and WEISGRABER, 2006; WANG et al., 1996). The structure of the full-length apoE3 has been resolved and it showed that the C-terminal domain contains 3 α-helices and many disordered regions that shield the receptor-binding site in the N-terminal domain (CHEN, LI, and WANG, 2011). A recent study proposed that the disordered regions in this domain may rearrange in such a way that the receptor- and the lipid-binding sites could be exposed (FRIEDEN, WANG, and HO, 2017). This proposition is supported by the fact that apoE has a higher affinity to the receptor when it is in a lipid-bound state (HATTERS, PETERS-LIBEU, and WEISGRABER, 2006).

ApoE is found on the surface of HCV LVPs and it has been shown to play a role in the attachment of HCV to the hepatocytes. This step occurs in part through the recognition of cellular receptors syndecan-1,-2 and-4 HSPG (JIANG et al., 2012,

2013; LEFÈVRE et al., 2014; SHI, JIANG, and LUO, 2013), LDLR (OWEN et al., 2009), SR-BI (DAO THI et al., 2012) and VLDLR (UJINO et al., 2016; YAMAMOTO et al., 2016) by apoE in HCV LVPs. It has been reported that specific antibodies directed against apoE could inhibit the attachment of the HCV LVPs. However, no inhibition could be observed once the virus had entered the cell (JIANG et al., 2012, 2013; OWEN et al., 2009). Moreover, Chang and collaborators showed that the amount of apoE on the surface of virions correlated with their infectivity (CHANG et al., 2007). These results were supported by studies showing that the activity of the lipases on HCV LVPs could reduce their infectivity and increase the buoyant density of particles (MAILLARD et al., 2011; SHIMIZU et al., 2010). Furthermore, Hishiki and colleagues reported that the infectivity of HCV was isoform-dependent by showing that the association of HCV with the apoE2 (compared to apoE3) led to the decrease of the infectivity (HISHIKI et al., 2010).

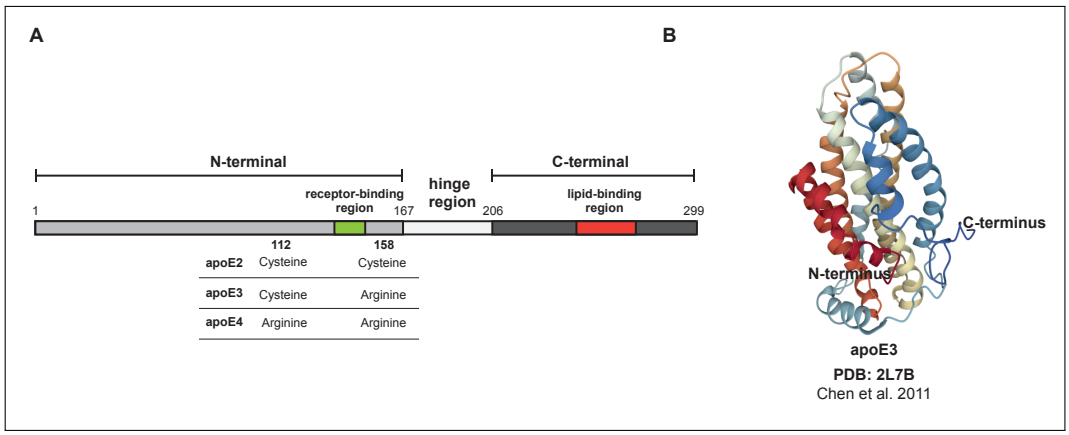

Figure 11: Structure of the apolipoprotein E. A. Representation of the linear structure of the apolipoprotein E (apoE). The apoE is a 299-aa length protein composed by two domains (N-terminal and C-terminal), linked by a hinge. In the N-terminal, there is the receptor-binding region (basic residues 136-150) and in the C-terminal the lipid-binding region (hydrophobic residues 244-272). Three isoforms have been identified: apoE2, apoE3 and apoE4, differing in changes at two positions: 112 and 158 (cysteine or arginine). **B**. Nuclear magnetic resonance structure of the full length apoE3 (PDB: 2L7B) by (CHEN, LI, and WANG, 2011). This protein has a bundle structure with an N-terminal domain formed by 4 α-helices, the C-terminal composed by 3 α-helices and some disordered regions, and the hinge with 2 α-helices.

ApoE is also required for the assembly and production of HCV. In a study proposed by Jiang and Luo, the inhibition of apoE in hepatoma cells led to the reduction in virion production, which was restored by the ectopic expression of apoE; while the knockdown of the apoB expression did not impair the virion production (JIANG and LUO, 2009). In addition, other groups reported that the expression of apoE in non-hepatic (Vero, HeLa and 293T cells) allowed the completion of the entire HCV life cycle, including the production of HCV pseudoparticles (HCVpp) (DA COSTA et al., 2012; HUEGING et al., 2014; MURAYAMA et al., 2016).

However, the use of apoE was evaluated in a comprehensive study for the production of HCVcc and HCVpp from genotypes 1-7, in HEK293T and Huh7.5 cells, respectively (Weller et al., 2017). The usage of apoE was shown to be strain- and cell-dependent, with JFH1-derived chimeras assembling differently. Furthermore, Fukuhara and colleagues reported that the impairment of the production of virions, due to the apoE knock-down, could be rescued by the ectopic expression of other apolipoproteins such as apoA1. They thus suggested that the amphipathic α-helices constituting these apolipoproteins were the crucial element for the production of HCV (Fukuhara et al., 2014). Once the virions are assembled, apoE seems to participate as well in the maturation of LVPs. Maturation has been described to occur intracellularly and/or extracellularly. Several studies have found that apoE is exchanged between LVPs and lipoproteins after release of the virus, which increases their infectivity (Bankwitz et al., 2017; Yang et al., 2016).

Another feature of apoE within the assembly of HCV is its capacity to interact with several viral proteins. First, it was reported that apoE could interact with the NS5A protein, implying apoE participates in early steps of the assembly (Benga et al., 2010; Cun, Jiang, and Luo, 2010). The region in apoE involved in this interaction was proposed to be the C-terminal domain, which contains the lipid-binding site (Cun, Jiang, and Luo, 2010). In fact, a recent report proposed that the interaction between apoE and NS5A might be mediated by the cell-death-inducing DFFA-like effector B (CIDEB) (Cai et al., 2016). The CIDEB is a molecule involved in the lipid metabolism and found to participate in the HCV entry (Wu et al., 2014). The inhibition of CIDEB abolished the interaction between apoE and NS5A, suggesting that the complex CIBED-NS5A may orchestrate the association of mature HCV in the ER lumen with pre-VLDL, through apoE (Cai et al., 2016).

Besides NS5A, apoE has been shown to interact with the HCV envelope glycoproteins (Boyer et al., 2014; Lee et al., 2014; Mazumdar et al., 2011). First, Mazumdar and colleagues showed that the E1 protein, but no E2, could interact specifically with apoE. The region of E1 responsible for this interaction was evaluated with synthetic peptides by enzyme-linked immunosorbent assay (ELISA). They found that binding determinants were in the N-terminal domain of the E1 protein, disposed in a specific conformation (Mazumdar et al., 2011). Then, Boyer and collaborators reported an interaction between apoE and the E1E2 complex that was confirmed by co-immunoprecipitation and microscopy experiments. However, they only detected the E2 protein from the E1E2 complex to be involved in this interaction (Boyer et al., 2014). In parallel, Lee and collaborators demonstrated that the association of the E1E2 heterodimer with apoE was mainly due to the E2 protein, and more specifically to the TMD of the E2 protein. They also showed that under high stringency conditions, apoE still coprecipitated with E2, suggesting the formation of a strong interaction between these two proteins (Lee et al.,

2014). Furthermore, by evaluating the composition of LVPs, it was shown that the amount of apoE molecules was much higher than the amount of E2 protein (Merz et al., 2011). Catanese and colleagues confirmed that apoE molecules significantly outnumbered the E2 protein (Catanese et al., 2013).

The association of HCV with apoE has also been related to the viral escape from the immune system. One mechanism of evasion is the cell-to-cell transmission, which could lead to a persistent disease. Hueging and colleagues showed that the absence of apoE in 293T/miR-122 cells prevented the transmission by cell-to-cell contact (Hueging et al., 2014). Moreover, Gondar and collaborators demonstrated that apoE was responsible for the spread of HCV from donor to acceptor hepatoma cells (Gondar et al., 2015). Another mechanism of evasion is the modulation of the sensitivity to neutralizing antibodies (Nabs). Bankwitz and colleagues reported that the incorporation of apoE into HCV particles (derived from primary human hepatocytes or hepatoma cells) enhanced the HCV infection, while the depletion of apoE abrogated that enhancement (Bankwitz et al., 2017). Fauvelle and collaborators further suggested that apoE may shield the E2 protein, allowing viruses with great amounts of apoE to escape from the antibody-mediated neutralization. The depletion of apoE from HCV particles led to the exposure of the antigenic domains B and C of E2, known to induce Nabs (Fauvelle et al., 2016a). Regarding the resistance to therapy due to apoE, a study showed that high serum apoE levels and thus high levels of LVPs were related to a reduced effectiveness of the peg-IFNα treatment (Sheridan et al., 2012).

Interestingly, the concentration of apoE in patient sera was estimated to be at least 100-fold more abundant than in cellular supernatants (Bankwitz et al., 2017). Current *in vitro* models (HCVcc and HCVpp) do not allow a complete lipidation of particles as observed in the LVPs isolated from patients. Therefore, new cellular models that improve the expression of the VLDL biosynthesis pathway or the mode of lipidation are needed to better understand the roles of molecules, such as apoE, in the viral life cycle (section 3.2).

2.4 HCV ENVELOPE GLYCOPROTEINS: STRUCTURE AND FUNCTIONS

The envelope glycoproteins E1 and E2 are found on the surface of HCV and therefore constitute one of the main targets of the host immune system. In fact, Nabs targeting these proteins have been isolated from infected patients, which makes them fundamental elements in the development of a prophylactic HCV vaccine. However, the precise characterization of their structures and roles on the viral life cycle remains under study. In this manuscript, E1 and E2 proteins will be numbered according to the HCV polyprotein and to the system proposed by Kuiken

and colleagues, unless indicated otherwise, in which they use the full-genome sequence of the isolate H77 (Genbank: AF009606) (KUIKEN et al., 2006).

2.4.1 *Envelope glycoprotein E1*

The E1 protein is a transmembrane protein of 190 aa (residues 192 to 383) with an N-terminal ectodomain and a C-terminal TMD (of 30 aa), which retains the E1 protein anchored to the ER membrane. The characterization through NMR of the TMD in membrane-mimicking environments (i.e. SDS micelles) suggested that the E1 TMD may have an helical conformation (ZAZRIN, SHAKED, and CHILL, 2014). The N-terminal domain contains 5 N-glycosylation sites: 4 of them (at positions 196, 209, 234 and 305) are conserved among genotypes, while one site at position 250 is specific to genotypes 1b and 6 (FIGURE 12) (MEUNIER et al., 1999). Glycosylation plays an important role in the correct folding of the protein (GOFFARD et al., 2005), but also, in the case of glycans in the E2 protein, in the evasion of the host immune response. Mutations or removal of N-glycosylation sites have been shown to increase the immunogenicity of the E1 protein (FOURNILLIER et al., 2001; REN et al., 2016). Another element in the stabilization of the envelope proteins is the disulfide bonds. The E1 protein possesses 8 cysteines entirely conserved among genotypes and mutations at these sites have been reported to reduce the efficiency of virus assembly and the stability of virions (WAHID et al., 2013). However, the matching of cysteines to form disulfide bonds within E1 and the heterodimer E1E2, remains elusive (EL OMARI et al., 2014; FREEDMAN et al., 2017).

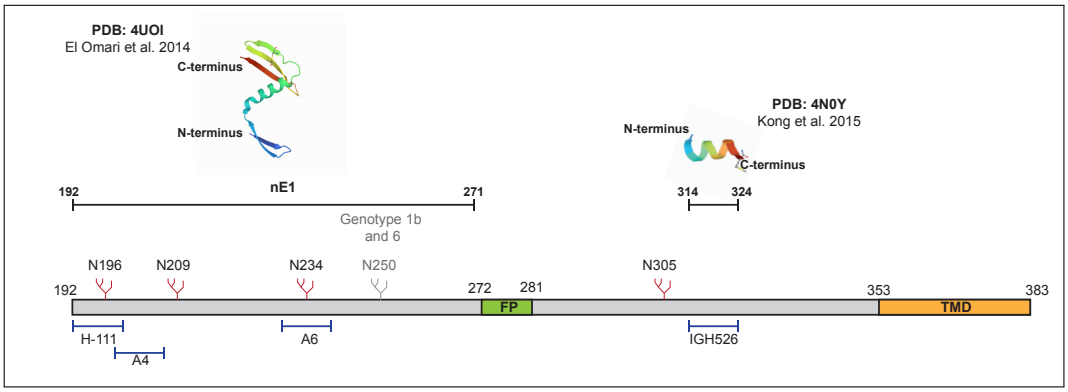

Figure 12: Structure of the HCV envelope E1 protein. The E1 protein contains 190 aa (160 aa for the ectodomain and 30 aa for the TMD), 5 N-glycosylation sites (in red) (N250 is exclusive from genotype 1b and 6, in gray) and a putative fusion peptide (FP). The crystal structure of the N-terminal domain of E1 (nE1) was determined (PDB: 4UOI), as well as the region 314-324 by co-crystallization with the human antibody IGH526 (PDB: 4N0Y). Antibodies binding sites: aa 192-202 for the human monoclonal antibody (mAb) H-111, aa 197-207 for the mouse mAb A4, aa 230-239 for the mouse mAb A6 and aa 313-324 for the human mAb IGH526.

The actual entire crystal structure of E1 is still unknown because this protein aggregates in the absence of the E2 protein (Cocquerel et al., 2000; Michalak et al., 1997; Patel, Patel, and McLauchlan, 2001). Therefore, only the N-terminal domain of a genotype 1 (H77 strain) E1 protein (nE1) has been crystallized in low-pH conditions and therefore, may represent a post attachment conformation (Figure 12) (El Omari et al., 2014). This study showed that nE1 dimers did not resemble the classical fusion proteins from flaviviruses, and they were rather small and structurally similar to the phosphatidylcholine transfer protein (El Omari et al., 2014). The authors suggested that this structure seems to bind another protein, which could be the apolipoproteins E and B previously proposed by Mazumdar and colleagues (Mazumdar et al., 2011). Another feature of the E1 protein is the ability to form trimeric structures, through the GxxxG motif at its TMD, within the E1E2 heterodimer conformation (Falson et al., 2015). The interaction between the TMDs of both envelope proteins was previously characterized and the residues involved in heterodimerization were as well GxxxG motifs in the TMDs of envelope proteins (Ciczora et al., 2007; Op De Beeck et al., 2000).

Despite the lack of a complete solved structure of the E1 protein, several roles in the viral life cycle have been attributed to this protein, for instance in the interaction with cellular receptors. Douam and collaborators reported that the binding of the E1E2 heterodimer to the cellular receptors, such as CLDN1, was modulated by the E1 protein while the E2 protein alone could not bind such receptor (Douam et al., 2014). Hopcraft and Evans also reported the interaction of the E1E2 heterodimer with CLDN1 through the E1 protein (Hopcraft and Evans, 2015). They showed that one single point mutation in the E1 protein allowed the virus to enter the CLDN1-knock-out cells using CLDN6 instead of CLDN1, a feature previously described in HCV (Haid et al., 2014; Meertens et al., 2008). Another group reported not only that the E1 protein could shift the HCV tropism for the CLDN1 receptor, but also that this protein might participate in the assembly of viral particles (Haddad et al., 2017). Apart from CLDN1, the CD36, a molecule from the family of the scavenger receptors, has been identified to interact with E1 in a cellular context (Cheng et al., 2016).

The E1 protein as well has been suggested to participate in the fusion step, in spite of the structural differences with the fusion proteins characterized from other viruses from the same family (Li and Modis, 2014). The putative fusion peptide (FP) region (aa 272-281) from the E1 protein has been described to be involved in the fusion process (Garry and Dash, 2003; Lavillette et al., 2007). Several groups have studied the role of the E1 protein by inducing mutations in the highly conserved region of the FP (Douam et al., 2014; Lombana et al., 2016; Perin et al., 2016; Tong et al., 2017). In fact, it was recently predicted, by a co-evolution analysis, that the fusion process depends on the modifications of the E1 and E2

proteins conformation, specifically, on the back layer domain of E2 (Douam et al., 2018).

The E1 protein has been described as less immunogenic than the E2 protein because Nabs mostly target the latter. Nevertheless, immunogenic regions of the E1 protein have been identified: at the N-terminal between aa 192-207 (Dubuisson et al., 1994; Keck et al., 2004), aa 230-239 (Mesalam et al., 2018), and the conserved region between aa 313-328 (Kong et al., 2015; Meunier et al., 2008b). One antibody targeting the first region is the human monoclonal antibody (mAb) H-111 (linear epitope aa 192-202) that was isolated from a genotype 1b HCV-infected patient with high titers of anti E1 antibodies. However, the H-111 mAb did not show a strong neutralizing activity (evaluated in the HCVpp system) (Keck et al., 2004). Other antibodies, the epitopes of which are found at the N-terminal, were also isolated, but they did not show any neutralizing activity: mouse mAb A4 (linear epitope aa 197-207) (Dubuisson et al., 1994) and the human mAb A6 (linear epitope aa 230-239) (Mesalam et al., 2018). Moreover, 2 mAbs (IGH505 and IGH526), targeting the discontinuous epitopes near the C-terminal of E1 and derived from the same donor, were able to neutralize HCVpp and HCVcc (genotypes 1a and 2a) (Meunier et al., 2008b). In fact, the structure of this region (aa 313-328) was solved by X-ray crystallography bound by the mAb IGH526, which epitope was found to be discontinuous within mostly E1 of the E1E2 heterodimer (Figure 12) (Kong et al., 2015).

2.4.2 *Envelope glycoprotein E2*

The E2 protein is composed of around 365 aa, 30 aa of which correspond to the TMD (residues 384 to 746 from the polyprotein). The E2 protein is organized in the following order (N-terminal→C-terminal) (Figure 13): the hypervariable region (HVR) 1 (aa 384-410), the front layer (aa 421-460), the HVR2 (aa 461-485), the CD81 binding loop (CD81bl) (aa 518-542) flanked by the β-sandwich (aa 486-570), the intergenotypic variable region (igVR) (aa 571-580), the back layer (aa 597-645), the stem region (aa 661-718) and the TMD (aa 718-736) (Gopal et al., 2017; Tzarum, Wilson, and Law, 2018). This protein is highly glycosylated with 11 N-linked glycans at positions 417, 423, 430, 448, 476, 532, 540, 556, 576, 623 and 645 (Goffard and Dubuisson, 2003). Moreover, 18 cysteines have been described to form 9 disulfide bonds that contribute to the structure and functions of the E1E2 heterodimer (Krey et al., 2010; McCaffrey et al., 2012).

The structure of the E2 ectodomain has been solved by several groups. Two of them followed a similar strategy to obtain the structure of the core of the E2 protein (Figure 13). Kong and colleagues solved the structure of a genotype 1a E2 core (aa 412 to 645) in complex to the fragment antigen binding (Fab) of the bro-

adly Nab (bNab) AR3C (Kong et al., 2013). They observed a well-defined globular structure of the E2 core with a central Immunoglobulin (Ig)-like β-sandwich (aa 492-566) flanked by a front (aa 424-459) and a back layer (aa 597-645), and some disordered regions. They also identified most of the glycans of the E2 protein, except for N417, N448, N476 and N576, suggesting that these glycans may mask neutralization epitopes (Kong et al., 2013). Khan and collaborators also solved the structure of a genotype 2a E2 core protein, but in complex with a non-neutralizing Fab 2A12. They observed the same globular structure and as well some glycans in the E2 core structure: N540, N556, N623 and N645 (Khan et al., 2014). Both studies identified conserved cysteines for the formation of disulfide bonds, but lacked the HVRs. More recently, Flyak and colleagues reported the co-crystallization of structures of genotype 1a and 1b E2 ectodomains (aa 384-643) with two bNabs isolated from patients who cleared the infection spontaneously, HEPC3 and HEPC74 (Flyak et al., 2018). Although both antibodies targeted the same region as the bNab AR3C, the binding site acquired different conformations, suggesting a somewhat flexibility in the structure of the E2 ectodomain (Flyak et al., 2018). Two additional studies reported the co-crystallization of the E2 ectodomain with bNabs, which confirmed the flexibility of the front layer region, and the need to study the genetic origin of the bNabs (Flyak et al., 2020; Tzarum et al., 2019).

Three HVRs have been identified in the ectodomain of the E2 protein: the HVR1, the HVR2 and the igVR, which correspond to 25% of the protein sequence (Hijikata et al., 1991; Krey et al., 2010; Weiner et al., 1991). The variability in these regions is due to the low-fidelity RNA polymerase (previously described) and the pressure of the immune system (Farci, 2000). The HVR1 is a 27-aa long sequence located at the N-terminus, that can differ up to 80% between genotypes and subtypes. HVR1 variants have been detected in the acute and chronic phases of the infection (Enomoto et al., 1993; Kurosaki et al., 1993; Sakamoto et al., 1994), as well as the antibodies against those variants (Kato et al., 1993; Mondelli et al., 1999). The antibodies induced by the HVR1 region have been described to be neutralizing and non neutralizing (Farci et al., 1996; Hsu et al., 2003; Vieyres, Dubuisson, and Patel, 2011). The binding of the little or no neutralizing antibodies to the HVR1 could lead to the blocking of conserved neighbor sites targeted by bNabs such as the epitope I (Keck et al., 2016b). In fact, the deletion of the HVR1 has been shown to increase the susceptibility to neutralization by Nabs in virions, suggesting a shielding effect of antigenic sites in the E2 protein by the HVR1 (Prentoe et al., 2016). Concerning the HVR2 and the igVR, they are not the targets of the Nabs, but they are structurally linked and contribute to the modulation of the accessibility to the CD81 binding site (Alhammad et al., 2015b; Farci et al., 1996). In fact, the deletion of at least one of these variable regions seems to have an impact on the E1E2 heterodimer formation (McCaffrey et al., 2011).

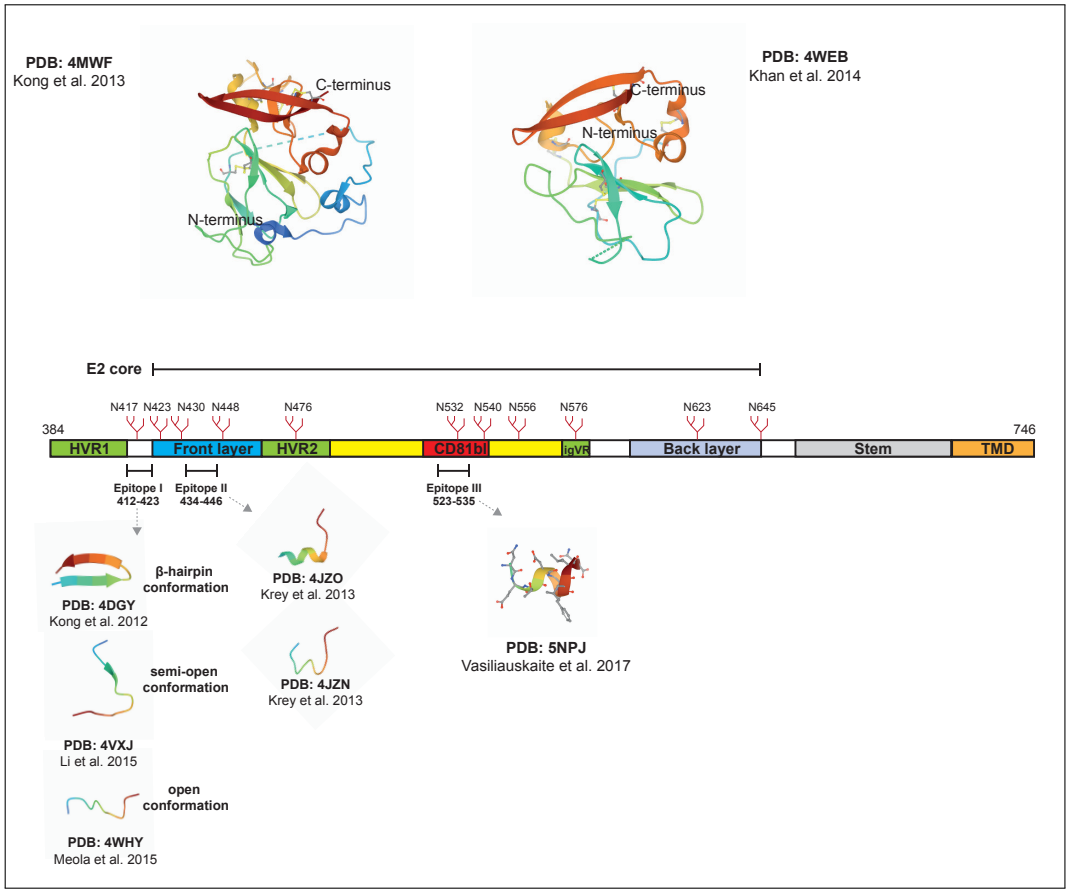

Figure 13: Structure of the HCV envelope E2 protein. The E2 protein contains 360 aa (30 aa for the TMD) in which 3 hyper variable regions (HVR1, HVR2 and igVR), a front layer, a back layer and a CD81-binding loop (CD81bl) and 11 N-glycosylation sites (in red above the linear structure) are represented. The crystal structure of the E2 core (aa 421-645) was obtained by two groups (PDB: 4MWF and 4WEB). Three epitopes for Nabs were identified (I, II and III). The epitope I can adopt 3 conformations: β-hairpin (PDB: 4DGY), semi-open (PDB: 4XVJ) and open (PDB: 4WHY). The epitope II was co-crystallized with the human monoclonal antibodies (mAbs) HC84.27 (PDB: 4JZO) and HC84.1 (PDB: 4JZN). The crystal structure of the epitope III, inside the CD81bl, (PDB: 5NPJ) was also obtained by co-crystallization with the mAb DAO5.

As described previously, the E2 protein participates actively in the entry step of the viral life cycle by interacting with the SR-BI (Bartosch et al., 2003; Scarselli et al., 2002) and the CD81 receptors (Pileri et al., 1998). The HVR1 of E2 was proposed to modulate the interaction of HCV with the SR-BI (Bartosch et al., 2005; Scarselli et al., 2002). The deletion or even mutations of the HVR1 region in E2 has been reported to enhance the binding of virions with the CD81 receptor (Bankwitz et al., 2010, 2014; Roccasecca et al., 2003) or the LDLR (Prentoe et al., 2014). Of note, the SR-BI is a HDL receptor that participates in the selective transport of the cholesterol (Acton et al., 1996). Therefore, the SR-BI can also interact with the HCV LVPs through the lipoproteins (Catanese et al., 2007; Dao Thi et al., 2012; Dreux et al., 2009; Maillard et al., 2006; Voisset et al., 2005), suggesting a complex relationship between HCV E2 (HVR1), the SR-BI and the lipoproteins linked to LVPs that is still not completely understood.

In the linear organization of the E2 protein, following the HVR1 there is the front layer that consists of a β-strand and a short α-helix (Kong et al., 2013). This region overlaps with epitopes of Nabs: the end of the epitope I (aa 412-423), known as antigenic site (AS) 412, and the entire epitope II (aa 434-446), known as AS434 (Zhang et al., 2007). These epitopes are conserved among genotypes. The epitope I is a conserved region with 2 N-glycosylation sites (N417 and N423). Antibodies directed to this site are only elicited in 2,5 to 15% of the patients (Tarr et al., 2007, 2012). This epitope is characterized by its flexibility and the possibility to adopt 3 different conformations: the β-hairpin, the open and the semi-open conformations (Figure 13) (Meola et al., 2015). The β-hairpin conformation is stabilized by hydrogen bonds and side chains with hydrophobic interactions (Kong et al., 2012b). Antibodies targeting this conformation of the epitope I are well characterized: the mouse mAb AP33 (Kong et al., 2012b; Owsianka et al., 2001; Potter et al., 2012; Tarr et al., 2006), the human mAb HCV1 (Broering et al., 2009; Kong et al., 2012a) and the mouse mAb Mab24 (Alhammad et al., 2015b). The HCV1 and AP33 have a broadly neutralizing activity and were shown to protect from challenge after passive immunization in chimpanzees (Morin et al., 2012) or mice with a humanized liver (Desombere et al., 2016), respectively. However, the escape from neutralization has been reported for the 3 antibodies (HCV1, AP33 and Mab24) due to glycan shifting (N417 to N415) at the antigenic site (Gu et al., 2018; Pantua et al., 2013). The semi-open conformation was described through the crystallization of the epitope I in complex with the human mAb HC33.1, an antibody isolated from a donor infected with HCV (Keck et al., 2013). This antibody was shown to neutralize HCV regardless of mutations and glycan shift that prevent neutralization with AP33 and HCV1 (Keck et al., 2014; Li et al., 2015). Moreover, other NAbs from the same panel (H33.4 and H33.8) targeting the semi-open conformation were inhibited by the binding of anti-HVR1 antibodies, due to the proximity of the HVR1

(Keck et al., 2016b). The open conformation has been characterized thanks to the rat mAb 3/11 whose binding to epitope was shown to be sensitive to denaturation (Flint et al., 1999; Meola et al., 2015; Tarr et al., 2006). This conformation was observed on the surface of virions during the entry, but was not as predominant as the β-hairpin conformation, contributing to the evasion of humoral responses (Meola et al., 2015).

Regarding the epitope II, it is composed of a 1,5 α-helix turn (aa 437-442) followed by an extended segment (aa 443-446) and flanked by two glycosylation sites N430 and N448. The N448 site has been reported to reduce sensitivity to neutralization and to be involved in the reduction of the HCV infectivity *in vitro* (Helle et al., 2010). Tarr and colleagues reported that antibodies targeting the epitope II showed a better neutralization of HCV when used in combination with antibodies against epitope I (Tarr et al., 2012). In contrast, Zhang and collaborators observed that non-Nabs binding the epitope II prevented the neutralization by Nabs targeting epitope I (Zhang et al., 2007). In addition, to evaluate the escape to neutralization by antibodies targeting the epitope II, Keck and colleagues used a set of 9 human mAbs, designated HC84, that were able to neutralize HCVcc and HCVpp. The culture of HCVcc with these antibodies resulted in no viral escape, in contrast with antibodies derived from epitope I (Keck et al., 2012). Then, Krey and collaborators crystalized the epitope II of a genotype 1a E2 protein in complex with two of the human mAbs HC84 and, by analyzing the epitope sequence, they found that residues 435, 436, 439 and 443 were highly conserved, which may be determinant for the binding and the broad neutralizing activity of these antibodies (Krey et al., 2013).

The epitope III (aa 523-535) is inside the CD81bl site. This conserved region of the E2 protein has been shown to be highly flexible, as it can be recognized by antibodies using different angles (Kong et al., 2016). In fact, Vasiliauskaite and colleagues designed a peptide from the Ig-like β-sandwich of the CD81bl that, in complex with the anti-E2 mAb DAO5, adopted an α-helix conformation, showing that the CD81bl may fold in at least two conformations. They also showed that CD81bl of the E2 proteins on the surface of HCVpp may be recognized by mAbs binding the different CD81bl conformations (Vasiliauskaite et al., 2017). Moreover, it has been suggested that the binding of this CD81bl site may require the recognition of amino acids in distant regions, such as the front and back layers (Gopal et al., 2017; Owsianka et al., 2006). For instance, mutation of residues in the back layer, through alanine scanning mutagenesis, altered the folding of the E1E2 heterodimer and thus its reactivity to mAbs (Gopal et al., 2017).

In addition to the previously described epitopes (I-III) within the structure of the E2 protein, some groups have also defined antigenic regions (AR1-5) (Giang et al., 2012; Law et al., 2008) and antigenic domains (A-E) (Keck et al., 2004, 2008,

2011, 2012, 2013; PIERCE et al., 2016). The three nomenclatures overlap in residues, but the domains and regions, in comparison with the epitopes, are defined in a conformational basis (FIGURE 14).

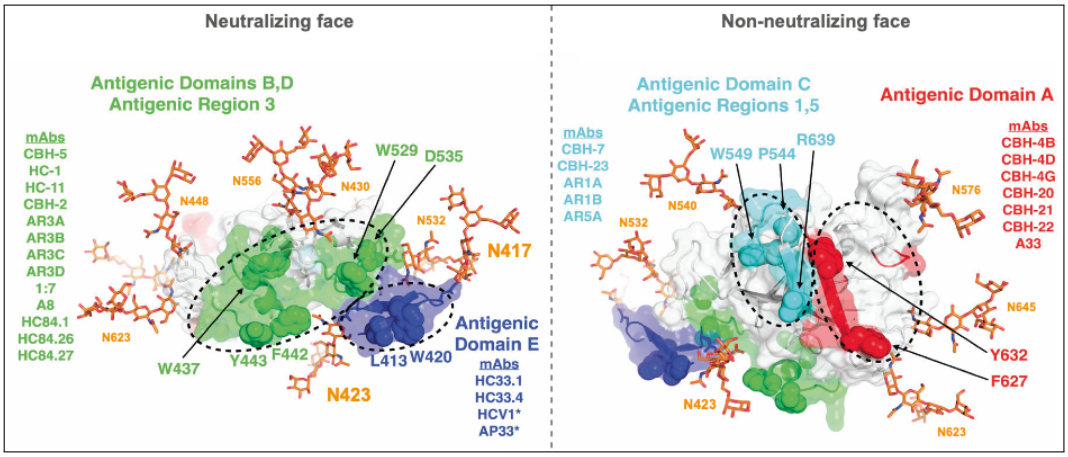

Figure 14: Representation of the antigenic domains and regions on the surface of the HCV E2 protein. The E2 protein has been described to have a neutralizing and a non-neutralizing face. Four binding sites and the representative antibodies are identified in green (antigenic region 3), purple (antigenic domain E), blue (antigenic regions 1, 5 and antigenic domain C) and red (antigenic domain A). The key residues for the binding of different antibodies are indicated in the figure, as well as the glycans N417-N645. Adapted from (FAUVELLE et al., 2016b).

Regarding the ARs, Nabs derived from these regions have been isolated from chronically infected patients (GIANG et al., 2012; LAW et al., 2008). Residues comprised in these regions are described in TABLE 3. Law and colleagues showed that complete antibodies recognizing the AR1 (proximal to the CD81-binding site) were not able to neutralize HCV, while Fabs from those antibodies did. Moreover, the AR2 region appears to be exposed on the surface of E2, as the antibody AR2A can neutralize HCV. They also showed that the AR3 is a highly conserved region capable of generating bNabs that block the E2-CD81 binding (LAW et al., 2008). In fact, this conformational region, comprising the CD81bl and the front layer, is called the neutralizing face of E2 (TZARUM, WILSON, and LAW, 2018). However, a recent study showed that a rare mutation in E1 may induce resistance to the bNab AR3A (VELÁZQUEZ-MOCTEZUMA et al., 2018). The AR4 and AR5 are adjacent regions that comprise residues from the E1E2 heterodimer interface. Antibodies targeting this region require a correct conformation of the E1E2 complex, suggesting that mutations at the aa 201-209, 657-659 and 692 may disrupt the conformation of the AR4 and AR5 (GIANG et al., 2012).

Concerning the nomenclature of domains (TABLE 4), the antigenic domain A induces non-neutralizing antibodies, maybe because of the proximity to the domain C (KECK et al., 2005). The antigenic domain B contains conserved epitopes that participate in the binding between the E2 protein and the CD81 receptor.

Domain-B-derived antibodies isolated from infected patients have shown strong neutralizing potential (Keck et al., 2008), even if virions with specific mutations have been shown to escape from them, such as the human mAbs CBH-2 and HC-11 (Keck et al., 2011). Likewise, epitopes in the domain D take part in the binding with the CD81 (Keck et al., 2012). In fact, the antigenic domains B and D overlap with the AR3 region. The antigenic domain E corresponds to the epitope I (or AS412) from which the human mAbs AP33, HCV-1 and HC33.1 have been isolated (Keck et al., 2014). The domain C overlaps with the AR5, but antibodies isolated from this domain do not bind to the E1E2 heterodimer (Keck et al., 2004).

Table 3: Characteristics of the defined antigenic regions of the E2 protein and the representative antibodies. The data were obtained from (Giang et al., 2012; Keck et al., 2018; Law et al., 2008).

Antigenic regions	Type of epitope	Residues	Antibodies	Neutralization?
AR1	Discontinuous	495, 519, 544, 545, 547, 548, 549 and 632	AR1A, AR1B	NO
AR2	Discontinuous	597-645 (back layer)	AR2A	YES
AR3	Discontinuous	396–424, 436–447 and 523–540	AR3A-D	YES
AR4	Discontinuous	E1E2 heterodimer with conserved residue 698	AR4A	YES
AR5	Discontinuous	E1E2 heterodimer with conserved residue 639	AR5A	YES

Table 4: Characteristics of the defined antigenic domains of the E2 protein and the representative antibodies. The data were obtained from (Fuerst et al., 2018; Keck et al., 2004, 2008, 2011, 2012, 2013).

Antigenic domains	Type of epitope	Residues	Antibodies	Neutralization?
A	Discontinuous	581-584, 627-633	CBH-20, CBH-21, CBH-22, CBH-4D	NO
B	Discontinuous	431-439, 529-535	CBH-5, CBH-2, HC-1, HC-11	YES
C	Discontinuous	544-549 and 523–540	CBH-7, CBH-23	YES
D	Discontinuous	420-428, 441-443, 616	HC84.24, HC84.26	YES
E	Continuous	412-423	AP33, HCV1	YES

Another relevant structural characteristic of E2 is the presence of 11 N-glycosylation sites, which are asparagine residues highly conserved. These glycans have been shown to play a role in the folding of the envelope proteins (GOFFARD et al., 2005) and the interaction with hepatocytes through lectin receptors (CHEN et al., 2014b; HELLE et al., 2010). However, the solved structure of the E2 ectodomain have shown that glycans also mask conserved epitopes such as the CD81-binding site (FIGURE 15) (KONG et al., 2013). Therefore, it has been suggested that in putative vaccines based on the E2 protein, the modification of the glycan patterns may increase the immunogenicity of that protein (LI et al., 2016, 2017).

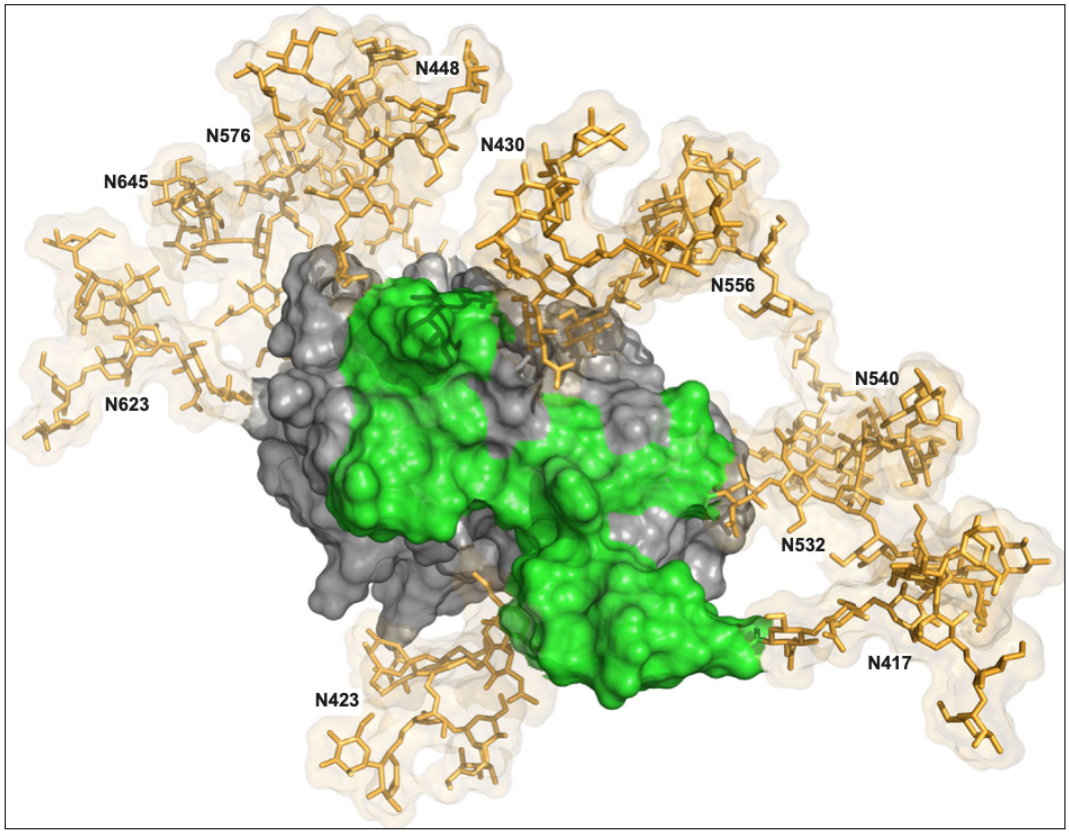

Figure 15: Representation of the N-glycans on the HCV envelope E2 protein. The E2 neutralizing epitopes (in green) are surrounded by N-glycosylations (golden sticks) that constitute a glycan shield. Ten of the eleven glycans are represented on this figure: N417, N423, N430, N448, N532, N540, N556, N576, N623 and N645. Adapted from (LAVIE, HANOULLE, and DUBUISSON, 2018).

3

CHALLENGES IN THE DEVELOPMENT OF AN HCV VACCINE

The introduction of effective therapies to treat HCV infection has indeed reduced the burden of this disease. However, as it was described in Chapter 1, these drugs do not protect from reinfection, nor do they fully prevent from the development of a severe liver disease, such as decompensated cirrhosis or HCC. In fact, rare subtypes of HCV have been recently identified as resistant to the DAA-based therapies. The development of an effective vaccine against this virus remains a priority for controlling the spread of HCV. However, the extreme genetic diversity of this virus reflected on the envelope proteins, the main targets of Nabs, represents a challenge in the development of vaccines. In addition, HCV can escape from the immune system through different escape routes, for instance via its interaction with apoE.

3.1 IMMUNE RESPONSES AGAINST HCV

Hepatitis C can be resolved in around 25% of the cases implying that an active immune response may be capable to control the infection (Micallef, Kaldor, and Dore, 2006; Osburn et al., 2010). In the case of reinfection after clearance, protective T and B cell responses controlled and reduced the magnitude of the viremia in chimpanzees (Lanford et al., 2004; Prince et al., 2005) and in humans 80% of the time (Osburn et al., 2010; Page et al., 2009). However, contradictory results have been obtained regarding the efficiency of this protection against different genotypes responsible from the primary infection (Islam et al., 2017; Prince et al., 2005). These differences in results may be due to factors associated with spontaneous clearance such as the sex (female) and gene polymorphisms (*IFNL4* genotype) (Sacks-Davis et al., 2015). In any case, the immunity-mediated early resolution of the disease is an important lead for the development of a vaccine against HCV.

3.1.1 *Innate immunity*

HCV enters the cell and the host pattern recognition receptors (PRRs) recognize the pathogen-associated molecular patterns (PAMPs), such as the poly-U/UC motif in the 3'UTR of the HCV RNA genome and its replication intermediate dsRNA that are bound by the retinoic acid-inducible gene-I (RIG-I) (Saito et al.,

2008; Schnell et al., 2012). It has also been shown that the toll-like receptors (TLRs) participate in the recognition of those HCV PAMPs (dsRNA or motifs in the ssRNA) (Lai et al., 2011; Wang et al., 2009; Zhang et al., 2009). These interactions activate signaling cascades that promote the production of IFN type I (IFNα and IFNβ) and III (interleukin (IL)-29 and IL-28A and B), as well as the activation of IFN-stimulated genes (ISGs) to prevent viral replication (Levy, Marié, and Durbin, 2011; Park et al., 2012). The first line of defense against HCV is the secretion of IFN by infected cells and by cells from the immune system. Furthermore, the mannose-binding lectin (MBL) receptor, a soluble PRR, has been reported to interact with the HCV envelope proteins and to activate the complement pathway (Brown et al., 2010). In fact, a low concentration of MBL has been suggested to be related to the spontaneous clearance, while an enhanced MBL activity has been suggested to promote the development of liver fibrosis (Brown et al., 2007; Saeed et al., 2013; Zhang et al., 2020).

The innate immune response during HCV infection can be avoided by viral proteins through the blocking the IFN production (Figure 16). The HCV core protein has been shown to induce the expression of the suppressor of cytokine signaling-3 (SOCS3). This inhibits the phosphorylation of the signal transducer and activator of transcription 1 (STAT1), which is important in the Janus kinase (JAK) signaling pathways for the transcription of ISGs in hepatocytes (Bode et al., 2003; Lin et al., 2006). The NS5A protein has been reported to suppress the STAT1 activity by direct interaction and phosphorylation of this protein, and therefore, to inhibit the production of IFN (Lan et al., 2007). This NS protein has also been shown to modulate the activity of the protein kinase R (PKR), but this mechanism remains controversial (Gale, Korth, and Katze, 1998; Podevin et al., 2001). The PKR is auto-phosphorylated in the presence of dsRNA, which blocks the synthesis of IFN by phosphorylation and inhibition of the eukaryotic initiation factor 2-α (eIF2-α) (Balachandran et al., 2000). The HCV IRES, found in the 5'UTR of the genome, has also been reported to bind the PKR and either activate or inhibit its activity (Toroney et al., 2010). The NS5A protein also evades the innate immune responses by targeting the nucleosome assembly protein 1-like 1 (NAP1L1) for degradation, which downregulates some ISGs (Çevik et al., 2017). Concerning the evasion through the NS3/4A complex, it has been shown to block the RIG-I pathway by cleavage of the Riplet protein, an E3 ubiquitin ligase that activates RIG-I (Vazquez, Tan, and Horner, 2019) and by the cleavage of the mitochondrial antiviral signaling (MAVS) protein, suppressing the production of IFNβ (Li et al., 2005b). The NS3/4A complex has also been reported to inhibit the IFN regulatory factor 3 (IRF) 3 activation pathway by proteolysis of the Toll-IL-1 receptor domain containing adaptor inducing IFNβ (TRIF) (Li et al., 2005a) and of the TANK-binding kinase 1 (TBK1) (Foy et al., 2005).

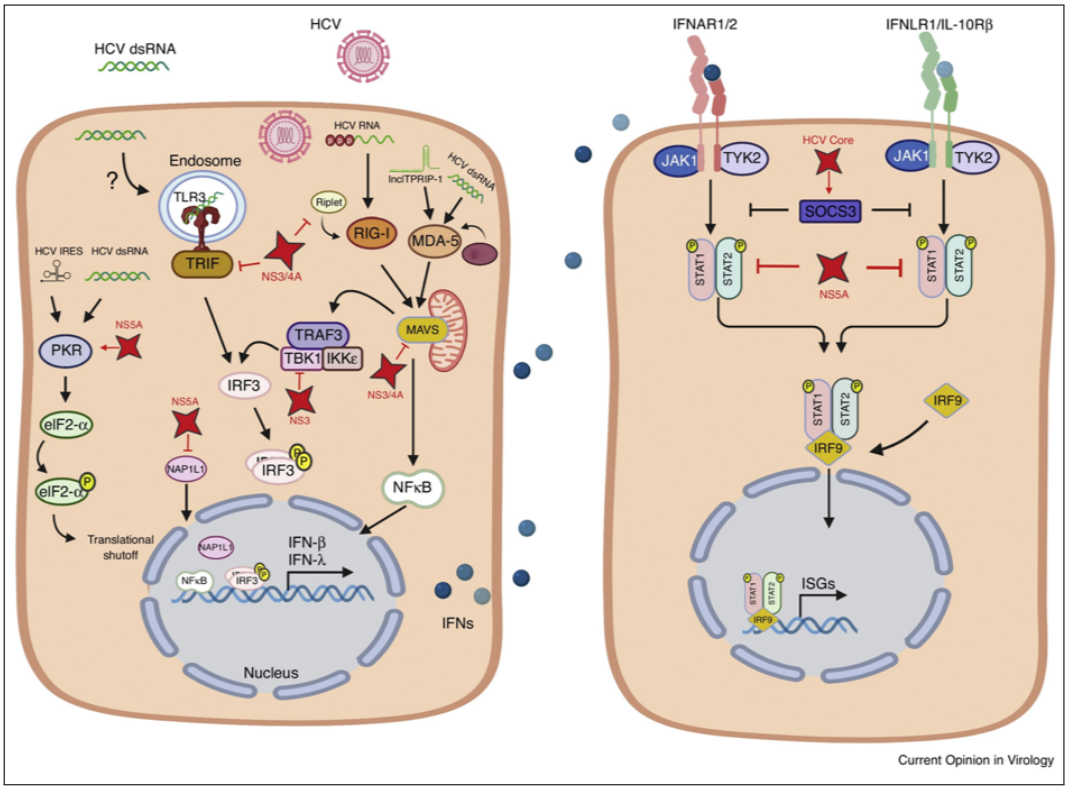

Figure 16: Inhibition of the cascades for the production of interferon and activation of interferon stimulated genes by HCV proteins. When HCV enters the cell (left panel), double-stranded (ds) RNA is sensed by toll-like receptor 3 (TLR3) and retinoic acid-inducible gene-I (RIG-I). The dsRNA and the HCV internal ribosomal entry site (IRES) activate the protein kinase R (PKR) that by phosphorylation of the eukaryotic initiation factor 2-α (eIF2-α) blocks the production of interferon (IFN). The NS5A also activates the PKR, but inhibits the nucleosome assembly protein 1-like 1 (NAP1L1), downregulating IFN production. The TLR3 signaling pathway can be blocked by the NS3/4A complex by proteolysis of the Toll-IL-1 receptor domain containing adaptor inducing IFNβ (TRIF) and the TANK-binding kinase 1 (TBK1). The RIG-I pathway can also be inhibited by the same complex by the cleavage of the Riplet ligase and the mitochondrial antiviral signaling (MAVS) protein. The IFN secreted is sensed by IFN receptors (right panel) that induce Janus kinase (JAK)-STAT signaling pathways. The HCV core protein activates the suppressor of cytokine signaling-3 (SOCS3) and inhibits phosphorylation of signal transducer and activator of transcription 1 (STAT1). The NS5A protein also blocks the activation of IFN stimulated genes (ISGs) by inhibiting STAT1. NFκB: nuclear factor-kappa B, IRF9: interferon regulatory factor 9, IL-10R: interleukin-10 receptor. (Stuart, Salinas, and Grakoui, 2021)

Among the immune cells involved in the innate responses against HCV are the natural killer (NK) cells, the Kupffer cells (KCs) and the dendritic cells (DCs). The NK cells have a cytolytic activity (perforins and granzyme B) and direct antiviral effect through the secretion of IFNγ (IFN type II) that has been shown to downregulate CLDN1 and CD81 receptors (Wei et al., 2009). The KCs are liver-resident macrophages that phagocyte extracellular components from infected cells (exosomes, apoptotic bodies) and produce pro-inflammatory cytokines. These mo-

lecules will attract other immune cells, and induce the death of hepatocytes, tissue inflammation and liver fibrosis [reviewed in (Boltjes et al., 2014)]. Although macrophages are known to play a role as antigen presenting cells (APC), KCs ineffectively induce the activation of T cells in HCV infection (You et al., 2008). The DCs can be divided in two subsets: the myeloid (mDCs) and plasmacytoid DCs (pDCs). The pDCs produce mainly the IFNα and other cytokines (Doyle et al., 2019), while the mDCs are in charge of the antigen presentation to T cells and so they trigger the adaptive immune response (Dolganiuc et al., 2008; Kadowaki et al., 2001). However, it has been reported that levels of mDCs and pDCs decreased in persistent infection and levels of IL-10 and IL-12 were altered, which may impair the T cell reactivity (Della Bella et al., 2007; Kanto et al., 2004).

3.1.2 *Adaptive immunity*

After the induction of innate immune responses, specific T cells are activated thanks to the professional immune cells for the antigen presentation, such as the DCs (Figure 17). These cells typically present the extracellular antigens through the major histocompatibility complex (MHC) class II. Antigens are internalized (endocytosis, pinocytosis or phagocytosis), and partially degraded into peptides of ~30 aa that are loaded to the MHC class II, at the MHC class II compartment (MIIC), and transported to the surface of the APCs. With this type of presentation, DCs activate the CD4+ T cells (Levin et al., 1993). In contrast, the CD8+ T cells require a more complex mechanism that is the cross-presentation, in which APCs present exogenous antigens through their MHC class I, usually presenting self-antigens and expressed by most of the nucleated cells. Antigens are internalized and processed in specialized compartments into peptides with low degradation and an improved epitope preservation. These peptides are loaded and presented with the MHC class I thanks to the activity of ER-resident proteins and further trafficking through ER-Golgi intermediate compartments [reviewed in (Gros and Amigorena, 2019; Kotsias, Cebrian, and Alloatti, 2019)].

Furthermore, the presentation of lipid-derived antigens has been reported to occur through the CD1, which could lead to the activation of a type of CD1-restricted T cells [reviewed in (Cohen, Garg, and Brenner, 2009)]. Interestingly, it has been shown that the apoE mediates this type of antigen presentation in order to develop responses against lipids, suggesting an immunomodulatory role (Van Den Elzen et al., 2005).

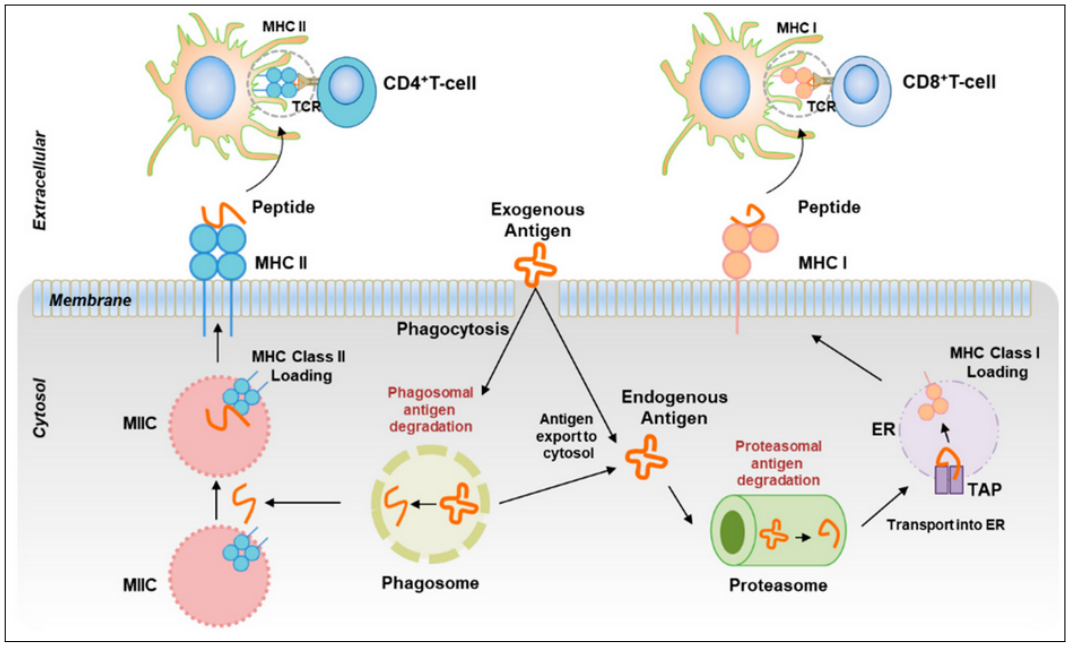

Figure 17: The MHC class II classic antigen presentation and the MHC class I cross-presentation pathways to activate T cells. The CD4+ T cells are activated through the MHC class II presentation pathway. Antigens are internalized and processed in the phagosome to generate peptides. Peptides are loaded in the MHC class II molecules in the MHC class II compartment (MIIC) and exported to the surface of the cell. The CD8+ T cells are primed through the co-presentation pathway. The exogenous antigen is endocytosed and processed by the proteasome. The generated peptides are transported to the endoplasmic reticulum (ER) through the transporter associated with antigen processing (TAP), loaded in the MHC class I and exported to the surface of the cell. TCR: T cell receptor. (Kumar et al., 2019)

3.1.2.1 *Cellular responses*

The CD8+ and CD4+ T cell responses are detected between 8 and 12 weeks after the infection, while HCV RNA can be detected from week 1-2 (Figure 18) (Major et al., 2004; Thimme et al., 2002). The CD8+ T cells are the initial set of cells responsible for the control of the infection (Jo et al., 2009), while the CD4+ T cells help maintaining the CD8+ T cell and the humoral responses (Raziorrouh et al., 2011; Spaan et al., 2015; Thimme et al., 2001). The CD8+ T cells are activated through the cross-presentation route, which may explain the delay of the response compared to the viremia (Shin et al., 2011). Effector CD8+ T cells have a cytolytic activity, but also produce cytokines (IFNγ) with antiviral effect. After clearance, these cells can differentiate into memory T cells (Kaech et al., 2002). Spontaneous clearance has been related to a strong CD8+ T cell response and the presence of CD4+ T cells (Osburn et al., 2010). Studies with chimpanzees showed that after clearance, the control of the reinfection was associated to both cellular responses (Grakoui et al., 2003; Shoukry et al., 2003). The subsets of CD4+ T cells associated with clearance are the T-helper 1 and T follicular helper (Tfh). The latter cells are responsible for

the activation of B cells for the generation of antibodies, but they disappear with the progression of the disease (Cobb et al., 2018; Raziorrouh et al., 2016).

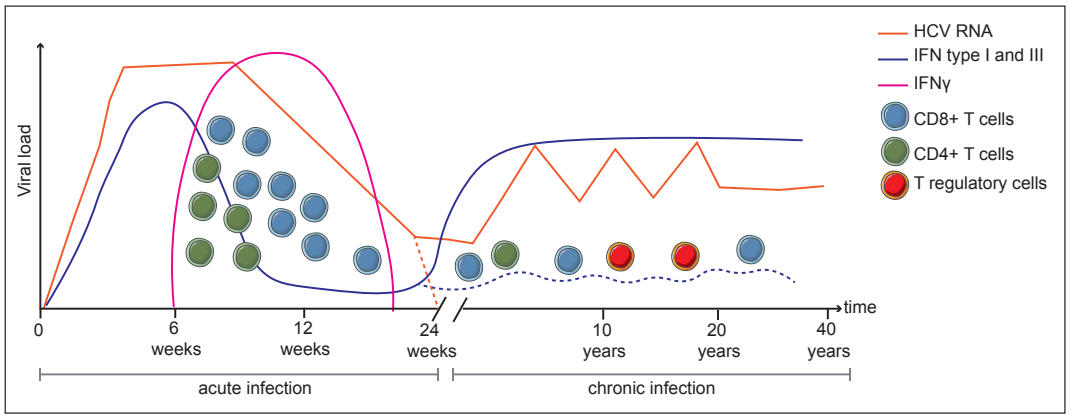

Figure 18: Evolution of the innate and T cell responses with the course of the HCV infection. In the early infection, the viral load (red line) increases rapidly within days after infection, along with the innate immune response through the secretion of interferon (IFN) type I and III (violet line). CD4+ (green cells) and CD8+ (blue cells) T cells proliferate. IFNγ (pink line) is produced by the CD8+ effector T cells. The replication of HCV decreases (dashed red line) at the end of the acute phase and the virus can be completely cleared by some patients. In the chronic infection, there is no production of IFNγ, but secretion of IFN type I and III can be induced in some patients. CD8+ T cells are impaired and less abundant, while CD4+ T cells tend to disappear in chronic infection. The CD4+ T regulatory cells proliferate in chronic infection and they may inhibit CD8+ T cells. Adapted from (Heim and Thimme, 2014).

Chronic hepatitis C is characterized by the exhaustion of T cells and the viral escape (Thimme, 2021). Exhaustion means that the functions of the T cells are impaired, which can be caused by the upregulation of inhibitory receptors such as the programmed cell death 1 (PD1) and downregulation of CD127 (Chen et al., 2020a; Radziewicz et al., 2007). Exhaustion may also be triggered by the constant interaction with the antigen (Bucks et al., 2009; Mueller and Ahmed, 2009). Moreover, the high error rate of HCV RNA polymerase promotes the generation of escape mutants, which leads to a broad repertoire T cells (Cox et al., 2005; Neumann-Haefelin et al., 2008; Puig et al., 2006). Escape mutations affecting CD8+ T cells can be present inside or in the flanking regions of epitopes of HCV NS proteins, which may impact their processing by the proteasome (Kimura et al., 2005; Schatz et al., 2008; Walker et al., 2016), the binding of processed peptides to the MHC class I (Erickson et al., 2001) or the recognition of these peptides by the T-cell receptor (TCR) (Dazert et al., 2009; Timm et al., 2004). As for the CD4+ T cells, mutations have also been identified in epitopes of the NS proteins (Lucas et al., 2007; Schulze zur Wiesch et al., 2012). However, these mutations have been described as nonsignificant compared to those specific to the CD8+ T cells (Fuller et al., 2010). Furthermore, the subset of CD4+ T regulatory cells has been reported to be implicated in the chronicity of the disease because of their role on the inhibi-

tion of CD8+ T cells (Boettler et al., 2005; Cabrera et al., 2004; Claassen et al., 2010).

DAA-based therapies seem to have a positive impact in the T cell-based immunity. In fact, it has been reported the partial reversion of exhausted CD8+ T cells and the restorage of the population of CD4+ T cells (Burchill et al., 2015; Meissner et al., 2017). However, a recent transcriptional study showed that the elimination of the antigen after a long exposition leads to an impaired set of memory CD8+ T cells, suggesting that chronic HCV leaves a molecular scar despite the cure with DAAs (Hensel et al., 2021).

3.1.2.2 *Humoral responses*

The humoral responses are triggered when the B cells encounter and bind the antigen through their B-cell receptor (BCR). The B cells migrate to the lymph nodes and establish the germinal centers (GCs) where a network of follicular DCs (FDCs) is also created. FDCs represent a constant supply of intact antigen for the B and Tfh cells (in different zones). The B cells proliferate and the size of the GC grows, forming two zones: the dark (mainly populated by closely packed B cells) and the light (mainly populated by Tfh cells). In the dark zone, B cells undergo somatic hypermutations (SHM) into the Ig variable region genes to improve their affinity to the antigen in a process called affinity maturation (Figure 19). Then, the Tfh cells promote the selection of the high-affinity B cells that present the antigen via their MHC class II in the light zone. A high affinity of the BCR for the antigen is translated into an improved antigen captured by the MHC and a high density of peptide-MHC on the surface of B cells. The B cells can undergo several rounds of mutations by re-entering the dark zone after interaction with Tfh cells, resulting in the selection of high affinity mutants. In the light zone, the change of the isotype of Ig in B cells can take place through a process called class-switch recombination (CSR). Therefore, upon interaction with Tfh cells, B cells can either re-enter the dark zone or differentiate into long-lived plasma cells that secrete large amounts of antibodies (IgM→IgG) or into memory B cells [reviewed in (De Silva and Klein, 2015)]. In the case of re-exposure, the memory B cells are activated and can undergo again the affinity maturation process or directly differentiate into long-lived plasma B cells to secrete high titers of IgG [reviewed in (Akkaya, Kwak, and Pierce, 2020)].

In acute HCV infection, the Tfh cells have been shown to participate in the affinity maturation process of B cells and to be associated with an early expansion of E2-specific B cells (Salinas et al., 2021). However, little is known about B cells in the context of chronic infections. The spontaneous clearance of HCV has been related to the early appearance of a humoral response (Kinchen et al., 2018; Osburn et al., 2014; Pestka et al., 2007; Walker et al., 2019). In fact, these primary neu-

tralizing antibodies leading to clearance have been associated with the presence of circulating Tfh cells (Zhang et al., 2019). In chronic infection, B cells have been reported to be perturbated by HCV because the E2 protein may interact with the CD81 receptor also found in the BCR complex. This association might lead to the aberrant proliferation of lymphocytes, as well as to the development of lymphoma (Cacoub et al., 2003; Ng et al., 2014; Quinn et al., 2001) with an annual incidence of 660,8 new cases per 100 000 patients (Monti et al., 2005).

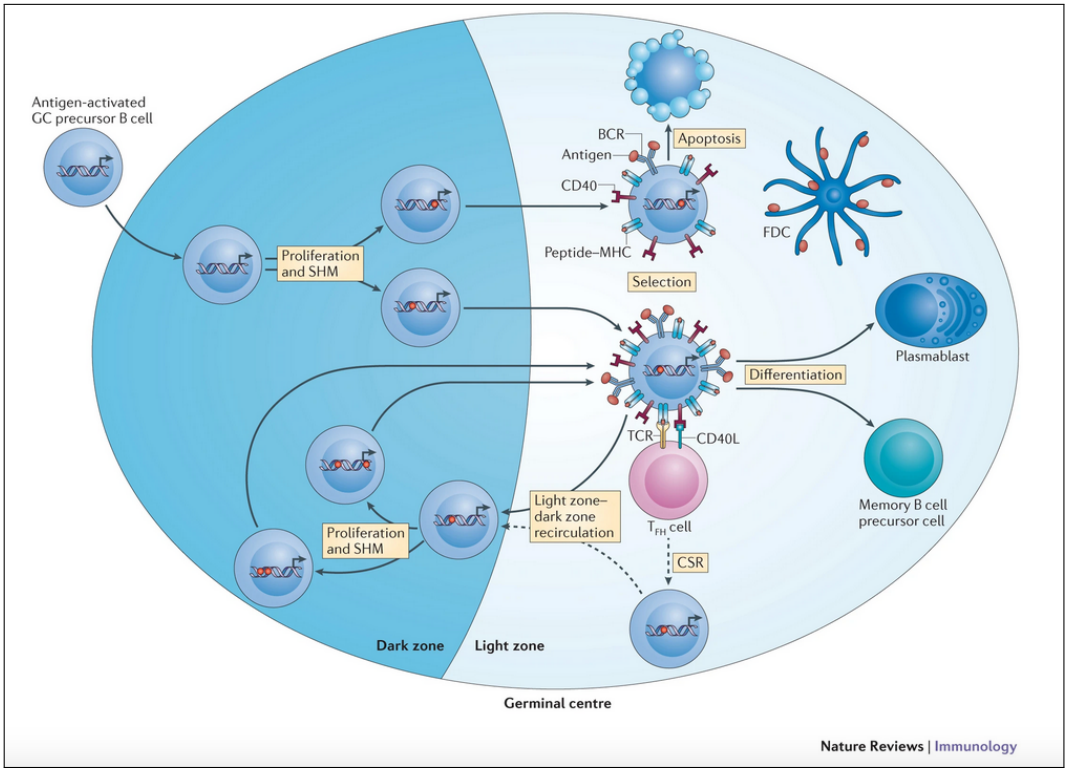

Figure 19: Affinity maturation process of B cells upon encounter with the antigen. B cells enter the lymph node and establish germinal centers (GCs) where they proliferate and undergo somatic hypermutations (SHM) on the immunoglobulin (Ig) variable genes (red dots). B cells transit to the light zone of the GC and interact with T follicular helper (Tfh) cells that select B cells in terms of their binding affinity to the antigen. B cells either differentiate into plasmablast (plasma B cells), memory B cells or re-enter the dark zone to proliferate and undergo SHM. B cells that show low binding affinity to the antigen in the light zone undergo apoptosis. The follicular dendritic cells (FDCs) are a source of antigen for Tfh and B cells. In the light zones, B cells can also experience a class-switch recombination (CSR), which means a change of the Ig isotype (i.e. from IgM to IgG), and then go back to the dark zone. (De Silva and Klein, 2015)

Antibodies in HCV infection have been reported to appear from week 5 to 8 (Glynn et al., 2005; Major et al., 2004). Antibodies isolated from patients who spontaneously cleared the infection have been characterized (Bailey et al., 2017; Flyak et al., 2018; Kinchen et al., 2019; Merat et al., 2016). Some antibodies from spontaneous clearance and chronic infections have been defined as bNabs that have been reported to protect from challenge with homologous and heterologous

HCV strains (Giang et al., 2012; Keck et al., 2016a; Law et al., 2008; Morin et al., 2012) or even abolish the infection (De Jong et al., 2014). The bNabs isolated from patients who cleared HCV spontaneously have been found to bind specific epitopes on the neutralizing face of the E2 protein (Bailey et al., 2017; Merat et al., 2016). The same epitopes were identified for antibodies isolated from chronically infected patients (Giang et al., 2012; Law et al., 2008). Therefore, the main difference between antibodies from patients who cleared the infection and those chronically infected might be the timing of the appearance of the humoral response (Pestka et al., 2007).

Brasher and colleagues analyzed the plasma of a large cohort of patients in early infection and found that antibodies with broad neutralizing activity were preferentially directed towards the AR2, AR3, AR4 regions and the domain D, suggesting that these sites should be considered for the design of immunogens for an HCV B-cell vaccine (Brasher et al., 2020). Besides, a recent complete comparative study characterized the immune response induced by a prototype vaccine based on recombinant E1E2 proteins (Chen et al., 2020b). They immunized nonhuman primates and mice with the same immunogen used in the clinical trial NCT00500747, in order to compare the responses between the human and the animal models. They found that most of the antibodies elicited by the immunogen targeted weak or non-neutralizing epitopes, such as the HVR1, in a similar way in all models. However, antibodies elicited in nonhuman primates and directed against the AR3 region were capable to cross-neutralize HCVcc and HCVpp from different genotypes. These bNabs were encoded by a set of genes from the heavy chain variable region (V_H) similar (90% homology) to the V_H1-69 lineage in humans (Chen et al., 2020b). The bNabs targeting the neutralizing face of E2 have been characterized and found to be mainly derived from the V_H1-69 gene, with only few SHM, and thus suggesting a rapid lineage development (Bailey et al., 2017; Flyak et al., 2018; Tzarum et al., 2019). The V_H1-69 lineage has also been identified for antibodies involved in the clearance of other viruses such as HIV (Breden et al., 2011; Huang et al., 2004) and influenza viruses (Sui et al., 2009; Throsby et al., 2008). Therefore, it has been suggested that B-cell vaccines should be based on the antigens found to elicit this kind of antibodies.

In any case, several mechanisms of resistance to Nabs have been described, such as the generation of mutations inside and outside the epitopes of antibodies. Bailey and collaborators found that the substitutions I538V, Q546L and T563V in genotype 1 E2 proteins (from a library of 19 sequences) conferred resistance to neutralization by the modification of the folding of the protein, even if they did not corresponded to binding residues for Nabs (Bailey et al., 2015). Other studies support these results by showing that distant mutations can affect the binding of antibodies (Prentoe et al., 2016; Rodrigo et al., 2017; Wasilewski,

Ray, and Bailey, 2016). As described in Chapter 2, the change in conformation of epitope I and the CD81bl can as well induce a resistance to Nabs (Meola et al., 2015; Vasiliauskaite et al., 2017). Furthermore, the glycan shielding of the HCV envelope proteins and the association to apoE have been reported to induce resistance to Nabs (Bankwitz et al., 2017; Fauvelle et al., 2016a; Helle et al., 2010; Pantua et al., 2013; Prentoe et al., 2019). Finally, another resistance mechanism described is the one mediated by the IFN-induced transmembrane proteins that were reported to drive the escape from Nabs in acute and chronic HCV infections (Wrensch et al., 2019).

With the recent failure of the clinical trial NCT0143635 based on the immunization with a T-cell vaccine (based on the induction of cellular responses) (Page et al., 2021), the future vaccine candidates should as well consider the generation of humoral responses through the presentation of previously described epitopes associated to bNabs (Chen et al., 2020b, 2021; Kinchen et al., 2018).

3.2 IN VIVO AND IN VITRO MODELS TO EVALUATE HCV VACCINES

Another challenge in the development of an HCV vaccine is the lack of appropriate animal models to evaluate vaccine candidates in pre-clinical stages. During the first years after the discovery of HCV, the chimpanzees (*Pan troglodytes*) were the nonhuman model of choice. Because of their genetic relatedness to humans, they can be infected with this virus. Although this animal model played an important role in the understanding of the acute and chronic HCV infections, experimentations within this model are expensive and raise ethical concerns (Bukh, 2004). In Europe, the use of chimpanzees is no longer allowed and legally regulated by the directive 2010/63/EU (Shelton-Davenport and Kahn, 2011). Today, vaccine candidates are mainly evaluated in small animals such as guinea pigs, rabbits or mice (for early stages of development) and nonhuman primates such as Rhesus macaques (for advanced stages). However, none of these models are susceptible to HCV infection. Therefore, the quality of antibodies generated through immunization of animals with vaccine candidates must be evaluated *in vitro*.

To overcome this challenge, small animal models permissive to HCV infection have been characterized or developed (summarized on Figure 20). The treeshrews (*Tupaia belangeri*) are small animals genetically related to chimpanzees that are susceptible to HCV infection and the development of an acute hepatitis (Xu et al., 2007). Additionally, rodent models able to be infected by HCV have been established and classified in: immunocompromised human liver xenograft, immunocompetent xenograft and genetically-modified mouse models (Burm et al., 2018). The Alb-uPA-SCID mice are immunocompromised rodents presenting a severe combined immunodeficiency disorder (SCID) and expressing the urokinase-

type plasminogen activator (uPA) transgene under the control of the albumin (Alb) promoter (MEULEMAN, VANLANDSCHOOT, and LEROUX-ROELS, 2003; MEULEMAN et al., 2005) that have been humanized through the transplantation of human hepatocytes (MERCER et al., 2001; TATENO et al., 2015; VANWOLLEGHEM et al., 2010). These mice have been shown to support robust HCV infection from different genotypes (BUKH et al., 2010). The dually engrafted models have also been established, for instance the double-humanized (liver and immune system) mice that have shown to be susceptible to HCV infection and generate immune responses (STRICK-MARCHAND et al., 2015; WASHBURN et al., 2011; WILSON et al., 2014). Genetically modified mouse models have also been developed, such as the Rosa26-Fluc mouse that supports low levels of HCV replication (DORNER et al., 2013) and the ICR-C/OTg mouse, in which HCV can establish a persistent infection but not HCC (CHEN et al., 2014a). However, not all these mouse models can be used for the evaluation of vaccines, because of the limited immune response or the difficulty to challenge after immunization. The use of viruses homolog to HCV (Hepaciviruses) is also an alternative of HCV-permissive animal models. That is the case of the rodent hepacivirus (BILLERBECK et al., 2017), the equine hepacivirus (PFAENDER et al., 2014), and the nonhuman primate hepacivirus George Barker virus-B (GBV-B) (BUKH et al., 2008). Compared to the immunocompetent mouse models, these viruses share features with HCV that may allow the evaluation of the efficiency of HCV vaccine candidates in natural hosts (cellular and humoral responses).

Concerning the *in vitro* evaluation of the neutralizing properties of antibodies induced by immunization, the most common systems are the HCVpp and the HCVcc (FIGURE 20). After the discovery of the virus, the establishment of a culture model of HCV from patient sera resulted inefficient. The first HCV *in vitro* system was the HCV replicon, which consists in one or two promoters, one selection gene, the NS proteins and the minimal elements (i.e. IRES) to ensure self-replication of the HCV genome. This construct is transfected in a hepatoma cell line (Huh7 cells), and cells containing the replicon are selected under antibiotic pressure. This system enabled the evaluation of the first DAAs, but it does not generate virions, due to the absence of the HCV structural proteins [reviewed in (LOHMANN and BARTENSCHLAGER, 2014)].

The HCVpp are based on a retroviral core (containing a reporter gene such as the luciferase) surrounded by a membrane in which the HCV envelope proteins E1 and E2 are anchored. These particles are produced in human embryonic kidney 293T (HEK293T) cells by co-transfection of three plasmids: one containing the gag-pol sequence (retroviral structure), another encoding the HCV envelope proteins and other with the reporter gene (luciferase or GFP). The HCVpp model allows to study the entry step (not dependent on lipid components) by infection of hepatoma cells with pseudotyped particles. When HCVpp enters the cell, the reporter

gene is released and it can be quantified (Bartosch, Dubuisson, and Cosset, 2003; Hsu et al., 2003).

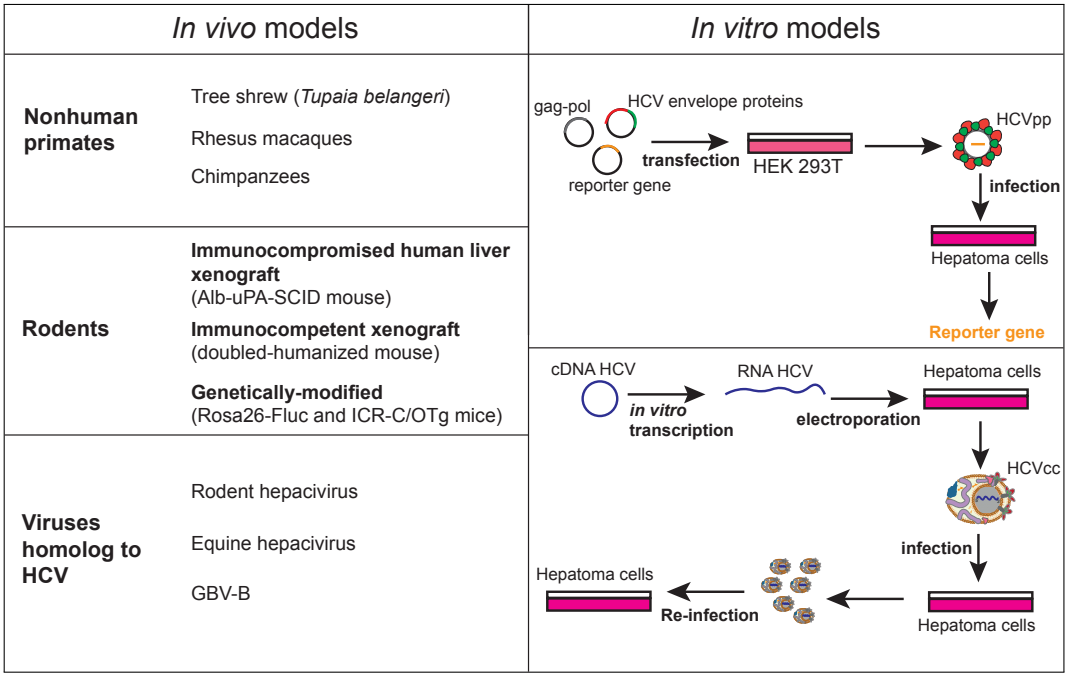

Figure 20: *In vivo* and *in vitro* models used for the study of the HCV infection. The *in vivo* models (left panel) discussed can be classified in three main categories: nonhuman primates, rodents and viruses homolog to HCV. Two main *in vitro* models (right panel) are currently used to evaluate the neutralizing properties of antibodies: the HCV pseudoparticles (HCVpp) and the HCV in cell culture (HCVcc). The HCVpp are generated by transfection of human embryonic kidney 293T (HEK293T) cells with a plasmid containing the gag-pol genes, a second one containing the reporter gene and a third one carrying the HCV envelope proteins. HCVpp harboring HCV envelope proteins on their surface are collected in the supernatant of cells and can be used to infect hepatoma cells. The HCVcc are generated by electroporation of hepatoma cells with the HCV RNA genome. HCVcc are collected in the supernatant of cells and can be used to infect hepatoma cells that will produce more HCVcc, and so on.

The HCVcc is currently the most relevant model for the evaluation of Nabs. It is based on the full-length genome from a genotype 2a HCV (JFH1) isolated from a japanese patient with fulminant hepatitis (Kato et al., 2001, 2003). The JFH1 genome was used as backbone for the development of chimeric viruses containing the sequences for the structural proteins, p7 and the complete or partial NS2 from one genotype, while the NS3 to NS5B belong to the JFH1 strain (Gottwein et al., 2007, 2009; Scheel et al., 2011; Wakita et al., 2005). The HCVcc particles are composed of the RNA genome surrounded by a nucleocapsid and a membrane-based envelope bearing the E1 and E2 proteins, as well as low-density molecules to form a LVP. In contrast to the HCVcc, the HCVpp, because of their production in HEK293T cells, are not associated to lipids and envelope proteins form noncovalent heterodimers (Flint et al., 2004; Op De Beeck et al., 2004). Both systems allow

the evaluation of Nabs and the results from these systems correlate, but a higher sensitivity has been attributed to HCVpp (Urbanowicz et al., 2016; Wasilewski, Ray, and Bailey, 2016). The HCVcc particles better represent the actual circulating LVPs than HCVpp, and have been shown to be infectious in humanized mice (Dorner et al., 2013).

Nevertheless, systems better mimicking the interaction of HCV with the lipid metabolism are currently under development, which may help to improve the knowledge on the particle formation and the way we evaluate the outcomes of immunization with vaccine candidates (Cochard et al., 2021; Denolly et al., 2019). Cochard and colleagues developed a culture system of differentiated Huh7.5 cells (using dimethyl sulfoxide (DMSO)) under hypoxia (1% O_2) in order to mimic the low-oxygen conditions of the liver. They observed an increased accumulation of LDs on these cells compared to those under standard culture conditions (no DMSO and 21% O_2), suggesting a modification of the cell lipid metabolism. When they infected differentiated hypoxic cells with genotype 2a HCVcc, HCV LVPs produced by these cells showed a lipidation and conformation similar to those of particles from HCV-infected patients (Cochard et al., 2021). Denolly and collaborators proposed a culture system of Huh7.5 cells supplemented with human serum, which allowed the production of HCVcc with similar characteristics to HCV LVPs from patients. These results suggest that maturation of HCV occurs extracellularly potentially because of serum factors present in the human serum and not in the fetal calf serum commonly used for cell culture (Denolly et al., 2019).

3.3 CURRENT PROTOTYPE VACCINES AGAINST HCV

The term *vaccine* appeared in 1798, when Edward Jenner inoculated an 8 year-old child with cowpox (mild form of smallpox) from hands lesions, later inducing protection from the challenge with smallpox. This practice was commonly applied earlier but through the inoculation with the actual infectious agent, sometimes leading to the death of patients (Smith, 2011). However, it was not until 1880 that the development of multiple vaccines began with Louis Pasteur and his inactivated rabies vaccine (Plotkin and Plotkin, 2011).

A vaccine is composed of a representative antigen from the pathogen (protein, polysaccharide) or the pathogen itself (live-attenuated or inactivated) that will induce protection in the case of a future exposure to the infectious agent. Today, a wide range of vaccine technologies are available: live-attenuated pathogens, inactivated pathogens, virus-like particle (VLP), subunits, viral-vectored, toxoids, polysaccharides and nucleic acid vaccines [reviewed in (Pollard and Bijker, 2021)]. To achieve better responses from vaccines, antigens are often combined with adjuvants to modulate their immunogenicity. The adjuvants comprise a wide diversity

of compounds (aluminum-based, lipid-based, emulsions and polymeric particles) that boost the innate immune response by interacting with the PRRs of cells like DCs and macrophages (Shah, Hassett, and Brito, 2017). Therefore, the use of adjuvants may be an important element in the development of a vaccine in order to induce a long-lasting adaptive immunity (Bonam et al., 2017).

The mechanism by which vaccines trigger the immune responses is similar to the one explained above for HCV. Briefly, the antigen is injected and processed by the APCs such as the DCs that will present it to the CD4+ and CD8+ T cells. The CD8+ T cells differentiate into effector and memory cells. B cells will proliferate and maturate with the support of CD4+ T cells, so they develop humoral responses of high affinity through the affinity maturation process. B cells will differentiate into plasma B cells (produce specific antibodies against the antigen) and memory B cells (proliferate and mediate the immune memory) (Figure 21) [reviewed in (Pollard and Bijker, 2021)].

Historically, live-attenuated and inactivated pathogens have been the most recognized technologies in the development of vaccines, such as those against poliovirus, smallpox, yellow fever, influenza, hepatitis A, varicella and measles viruses (Karch and Burkhard, 2016). In the case of HCV, an inactivated HCV vaccine has been proposed thanks to the development of the HCVcc culture system. The inactivated vaccine developed by Wakita and colleagues was based on a genotype 2a chimeric replication-deficient HCVcc (Akazawa et al., 2013; Murayama et al., 2007). These particles were produced in large-scale Huh7.5.1 cell cultures (supplemented with 2% fetal bovine serum), and purified over a sucrose cushion in ultracentrifugation with a recovery rate of 15% (Yokokawa et al., 2018). Immunization of BALB/c mice and nonhuman primates (marmosets *Callithrix jacchus*) with inactivated HCVcc induced cellular responses and specific antibodies against HCV structural proteins that cross-neutralize HCVcc containing the structural region of genotypes 1a, 1b, 2a and 3a isolates (Yokokawa et al., 2018). One advantage of this vaccine against a recombinant envelope protein is that lower doses of inactivated HCVcc were required for the induction of antibodies, in comparison with vaccines based on recombinant proteins (Akazawa et al., 2013). Moreover, antibodies induced in human liver chimeric uPa-SCID mice by immunization with this vaccine protected them from HCV challenge with the low viral doses (10^3 RNA copies) (Akazawa et al., 2013). However, these HCVcc displayed heterogeneous densities because of the association of lipids and apolipoproteins due to the presence of animal-derived serum. Therefore, Mathiesen and collabotarors reported the production of genotypes 1-6 HCVcc in a serum-free medium. These particles showed an homogeneous density profile, but maintaining their association to apoE, and similar biological characteristics to HCVcc, such as binding to CD81 and SR-BI receptors (Mathiesen et al., 2014). Furthermore, they recently published a proof-of-concept of the

purification process of UV-inactivated genotype 1a and 5a HCVcc-based vaccines consisting in initial clarification and concentration steps followed by steric exclusion chromatography and chromatography based on sulfated cellulose membrane adsorbers (heparin-mimicking for HCV binding) (LOTHERT et al., 2020). However, there is an increasing number of rationally-designed vaccines using only specific epitopes from the pathogen, which are safer than the live-attenuated or inactivated vaccines.

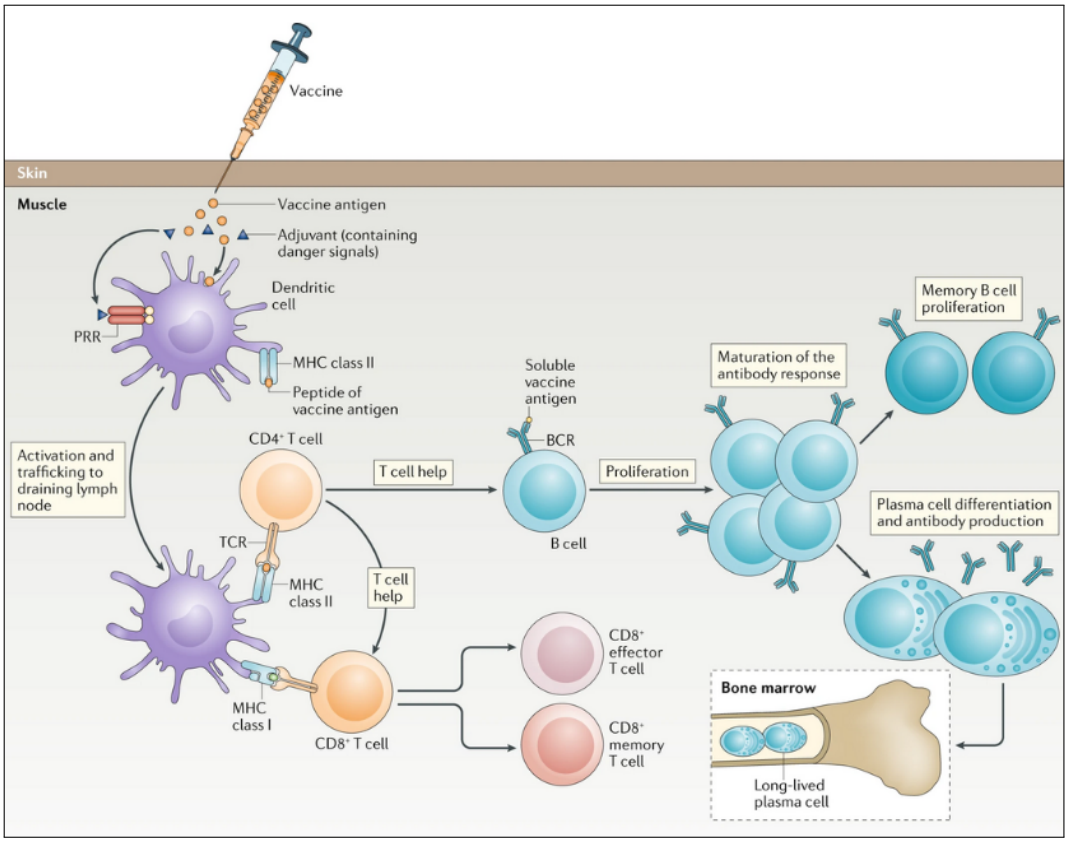

Figure 21: Generation of the immune responses by vaccination. The vaccine, composed of an antigen and an adjuvant, can be injected intramuscularly and dendritic cells will capture and internalize the antigen. The adjuvant can be bind by the pattern recognition receptors (PRRs), which trigger the innate immune response. Dendritic cells will present the antigen through the MHC class II to CD4+ T cells that bind it by the T cell receptor (TCR). CD8+ T cells will be activated by the cross-presentation of the antigen (MHC class I) to become effector and memory cells. Activated CD4+ T cells participate in the selection of high affinity B cells that can differentiate in memory B cells and long-lived plasma B cells. (POLLARD and BIJKER, 2021)

3.3.1 *HCV T-cell vaccine candidate*

The vaccine against HCV that has reached the most advanced phase (1/2) in clinical trials in humans is a T-cell vaccine. The vaccination strategy consists in a prime-boost with two different viral vectors expressing the genotype 1b (BK strain) HCV

NS3-5B proteins. This vaccine was first evaluated in chimpanzees using human adenovirus constructs that induced strong cellular responses and protected the 80% of animals from challenge with HCV (Folgori et al., 2006). The clinical trial phase 1 (NCT01070407) was performed to evaluate the safety of the prime-boost vaccination with two adenoviral vectors: the adenoviral vector 6 and the chimpanzee adenovirus 3 (ChAd3-NSmut). Barnes and colleagues found that the vaccine was well tolerated with no severe adverse effects, and led to the generation of cellular responses. However, they observed that the ChAd3-NSmut was a better priming vector (Barnes et al., 2012). A second clinical trial phase 1 (NCT01296451) was performed to evaluate the safety and the outcomes of a different viral vector, the modified vaccinia Ankara (MVA-NSmut). The use of this vector as a boost resulted in increased and better sustained CD4+T cell responses overtime (Swadling et al., 2014). Finally, a randomized clinical trial phase 1/2 (NCT01436357) with 548 participants (IVDUs), 274 of them following the prime-boost (ChAd3-NSmut-MVA-NSmut) regimen, resulted in the induction of T cell responses but did not prevent the development of chronic infection in comparison to the placebo group (274 participants) (Page et al., 2021). These results suggest the need of the induction of B cell responses in the prevention of the disease progression.

3.3.2 *HCV B-cell vaccine candidates*

As discussed by Page and colleagues, their T-cell vaccine did not express the HCV envelope glycoproteins, which may be necessary to promote a complete protection (Page et al., 2021). In fact, most of the current available vaccines for other pathogens are based on the development of Nabs (Ringel et al., 2018). In the case of HCV, the E1 and E2 proteins are the main targets of these antibodies, as described in the Chapter 2. Therefore, several groups around the world have used these proteins as immunogens for the development of their vaccine candidates (Table 5). However, the only B-cell vaccine based on these proteins that has been evaluated in a clinical trial is the recombinant HCV E1/E2/MF59 vaccine from the ex-Chiron Corporation. This vaccine is based on genotype 1a E1 and E2 proteins purified from mammalian cell cultures. Immunization of chimpanzees with this vaccine induced strong humoral responses that protected them from challenge with HCV. In animals with low antibody titers, acute infection occurred but it was retarded and resolved (Choo et al., 1994). Then, they evaluated different vaccine constructs (E2, E1E2 and E1E2p7) produced in Chinese hamster ovary (CHO) cells by immunization of rodents. The antibodies generated in this study were able to cross-neutralize genotypes 1a, 1b HCVpp and HCVcc and less efficiently the genotype 2a. They also found that E1E2 proteins were more immunogenic than the E2 protein alone (Houghton, 2011; Stamataki et al., 2007). Meunier and collea-

gues showed that the antibodies induced in chimpanzees of the study of Choo and colleagues could cross-neutralize HCVpp and HCVcc particles, but there was no correlation between the neutralization results in both animal models (Meunier et al., 2011).

The recombinant E1E2 protein adjuvanted with MF59 was assessed in the clinical trial phase 1 (NCT00500747) to evaluate their safety and to determine the ideal dose in humans. They found that participants immunized with their vaccine developed antibodies against E1E2 proteins and also observed proliferation of the T helper cells (Frey et al., 2010). The elicited antibodies were able to neutralize the homologous HCVpp and HCVcc, but the antibodies from half of the samples were reactive against the HVR1 of the E2 protein (Law et al., 2013; Ray et al., 2010; Stamataki et al., 2011). In fact, only around 50% of participants developed antibodies capable to cross-neutralize HCVcc harboring HCV envelope protein genotypes from various genotypes (1a, 1b, 2a, 4a, 5a and 6a better than 2b, 3a and 7a) (Law et al., 2013). Because of the high number of participants eliciting antibodies against the HVR1, Law and collaborators deleted this site from the recombinant E1E2 protein and evaluated the impact by vaccination in mice. They found that the deletion of this region did not improve the cross-neutralizing properties of the induced antibodies compared to the wild-type (WT) E1E2 proteins (Law et al., 2018).

Several other vaccine candidates based on recombinant HCV envelope proteins have been developed. Krapchev and colleagues proposed genotypes 1a, 1b and 2a HCV E1E2 heterodimers tagged with a Flag (E1E2-flag). These particles showed a correct folding, but antibodies induced by immunization of C57BL/6 mice had low neutralizing capacities (HCVcc 2a<1a) (Krapchev et al., 2018). Recently, a proof-of-concept for a native-like soluble E1E2 glycoprotein, with a leucine zipper scaffold that replaced the TMDs, was published. They showed that bNabs isolated from patients could recognize epitopes as in the original E1E2 heterodimer and that these new particles could induce antibodies capable to neutralize genotype 1a HCVpp and HCVcc (Guest et al., 2021).

The VLPs are proteins that self-assemble to generate empty structures physically resembling a virus. Because of their geometry, epitopes displayed across the surface of the VLPs have shown to strongly stimulate B cell responses (Zabel, Kündig, and Bachmann, 2013). In fact, these structures have been used for the development of HCV vaccines containing envelope proteins. Garrone and colleagues reported the generation of retroviral VLPs pseudotyped with E1 and/or E2 proteins in HEK293T cells. They immunized macaques with these particles and found that antibodies directed against E1 were difficult to achieve, but elicited antibodies that could cross-neutralize HCVpp (genotypes 1a, 1b, 2a, 2b, 4c) (Garrone et al., 2011). A group from the University of Melbourne developed HCV

VLPs containing structural proteins (core, E1 and E2) from a genotype 1a HCV, resembling HCV virions, which were produced in Huh7 cells. These particles induced specific CD8+ T cells and humoral responses able to neutralize genotype 1a HCVpp in BALB/c mice (Earnest-Silveira et al., 2016). Moreover, they proposed a quadrivalent vaccination strategy using HCV VLPs carrying structural proteins from genotypes 1a, 1b, 2a and 3a in mice that developed specific humoral and cellular responses (Christiansen et al., 2018). The same quadrivalent vaccine was evaluated in Landrace pigs, and the generated antibodies were able to neutralize genotype 2a and 3a HCVcc (Christiansen et al., 2019).

Nevertheless, as the major epitopes eliciting bNabs have been identified in the E2 protein, many vaccine candidates are based on this protein alone as a subunit vaccine. A group from the Institut Pasteur of Shanghai developed a genotype 1b TMD-truncated soluble E2 (sE2) vaccine. First, they compared the production yield of sE2 produced in *Drosophila* S2 and HEK293T cells and obtained at least 50 times more protein per liter with the S2 cells. Because of the different production systems (mammalian and insect cells), the glycosylation patterns generated by S2 cells on the sE2 protein were less complex than those on the one produced in HEK293T. After immunization of immunocompetent mice, they found that the sE2 produced in insect cells, was more immunogenic, which might be explained by an improved flexibility of the protein due to the less complex glycosylation pattern associated to S2 cells. Therefore, antibodies induced by the sE2 from insect cells cross-neutralize genotypes 1-7 HCVcc. Li and collaborators also deleted the HVR1 from E2 protein and they did not observe any difference for this sE2 protein in terms of immunogenicity (Li et al., 2016). This group pursued the validation of their sE2 vaccine with different adjuvants by performing immunizations of nonhuman primates (Rhesus macaques). This vaccine induced better T cell and neutralizing humoral responses with a combination of Alhydrogel 2% (aluminum-based) and monophosphoryl lipid (MPL) adjuvants, approved by the FDA. The cellular responses induced with this combination were memory-type with the production of IFNγ. Regarding the humoral responses, the cross-neutralizing potential was lower than the one observed in the first study with mice, which may be due to the dose or the animal model (Li et al., 2017). In a different study, they evaluated the sE2 protein from different genotypes (1b, 1a and 3a) as a cocktail (mixed in equal proportions) to improve the cross-neutralizing properties of induced antibodies. They found that the trivalent vaccine elicited a better cross-neutralizing response for some HCVcc than the monovalent sE2 vaccine (Wang et al., 2019). Recently, they produced the sE2 coupled to ferritin that self-assembles into nanoparticles, similar to VLPs. The conformation of sE2 was improved in the ferritin platform compared to sE2 alone, which enhanced its immunogenicity and the cross-neutralizing potential of elicited antibodies (Yan et al., 2020).

Another vaccine candidate based on the E2 protein alone has been developed from the group led by the Prof. Heidi Drummer in Australia. They produced a monomeric genotype 1a E2 protein deleted of its 3 variable regions (Δ123) that generated broad neutralizing responses (genotypes 1-7 HCVcc) by immunization of guinea pigs. They suggested that deletion of the variable regions may have allowed the exposition of epitopes occluded in the native structure (Vietheer et al., 2017). A recent report described the modification of this vaccine candidate by sequential reduction and oxidation that induced the formation of disulfide bonds, which led to multimeric particles. Despite the improvement in the production yield of the multimeric forms compared to the monomeric ones, bNabs showed reduced reactivity to the E2 protein in the multimeric forms. In fact, the neutralizing capacity of antibodies elicited with these particles in guinea pigs was only enhanced for genotype 1a HCVpp (Center et al., 2020).

Furthermore, Tarr and colleagues reported a prototype vaccine based on the consensus sequence of the core E2 (ΔHVR1 and ΔC-terminus), from 720 strains of genotype 1 HCV, produced in *Drosophila* S2 cells. Guinea pigs were immunized with this protein and the induced antibodies recognized mostly linear epitopes capable to neutralize exclusively HCVpp harboring envelope proteins of genotype 1a strains (Tarr et al., 2018). In line with this, a group from The Scripps research institute presented a rationally-designed genotype 1a and 6a E2 core constructs (regions inducing non-Nabs were deleted) associated to nanoparticles. These E2 core nanoparticles were produced with a high yield and high purity in mammalian cell cultures. Immunization of mice with nanoparticles, individually, led to antibodies with better neutralizing properties than soluble E2 cores. However, the mix of genotypes 1a and 6a particles resulted in lower antibody titers and not better neutralizing potential than the individual strategy (He et al., 2020).

Some research groups have as well developed vaccination strategies based on peptides from HCV proteins. That is the case of Dawood and colleagues who synthesized 6 peptides from genotype 4a HCV E1, E2, NS4B, NS5A and NS5B proteins. These peptides were chosen because of their high conserved residues among genotypes and their relationship with the induction of strong B and T cell responses associated to spontaneous clearance. Immunized BALB/c mice developed cellular and humoral responses (neutralization of genotype 2a and 4a HCVcc) in a dose-dependent manner (Dawood et al., 2019). Another group also proposed a vaccination based on peptides (with high sequence divergency) from the HVR1 region from patients. Immunization of mice with a mix of both peptides resulted in a better strategy than each peptide individually, and led to the cross-neutralization of HCVpp: genotypes 1a, 1b and 6a were better neutralized than genotypes 2a, 3a and 5a (Mosa et al., 2020).

Several groups have also focused their efforts on the development of vaccine candidates based on the hepatitis B surface antigen (HBsAg), which can self-assemble into VLPs. For example, our research group has developed an original vaccination concept based on the genotype 1a full-length HCV envelope proteins (E1 and E2) fused to the heterologous HBV small (S) envelope protein. These chimeric HBV-HCV proteins, co-expressed with the WT S envelope protein in mammalian cells, self-assemble into highly immunogenic and secreted subviral envelope particles (SVPs). Immunization with chimeric HBV-HCV SVPs has been reported to induce humoral responses against HBV and HCV in New Zealand rabbits, as well as antibodies capable to neutralize genotypes 1a, 1b, 2a, 3a and 4a HCVpp and HCVcc (Beaumont et al., 2013, 2016, 2020). Further details on this vaccine candidate will be discussed in section 4.2. Czarnorta and collaborators proposed a vaccine candidate in which the hydrophilic loops of the SHBsAg were substituted by the epitopes I, II or III of the genotype 1a HCV E2 protein. These chimeric particles were produced in *Leishmania tarentolae*, an expression system with a glycosylation pattern similar to mammalian cells (Czarnota et al., 2016, 2020). Antibodies targeting epitope I (aa 412-425), purified from immunized mouse sera, induced better cross-neutralization of HCVcc (genotypes 1a, 1b, 2a, 2b, 4a and 5a), when compared to antibodies against epitopes II and III (Czarnota et al., 2020). A very similar strategy was proposed by Wei and colleagues, who inserted epitopes from genotypes 1a, 1b and 2a HCV E2 proteins (including the HVR1) in the external hydrophilic loop of the SHBsAg. These particles were used individually or as a pool to immunize BALB/c mice. The highest titer of antibodies was obtained from immunization with the pool of VLPs bearing different epitopes, as well as the best cross-neutralizing potential (genotypes 1a, 1b and 2a HCVcc and HCVpp) (Wei et al., 2018).

Table 5: Current HCV B-cell vaccine candidates under development. The HCV vaccine candidates are classified by the approach: the E1 and E2 proteins, the E2 protein alone, peptides from HCV envelope proteins and the HBV small surface antigen (SHBsAg) bearing the complete or partial HCV envelope proteins.

Approach	Vaccine candidate	Genotype	Expression system	Models tested	Humoral responses	Cellular responses	References
E1 and E2 proteins	Recombinant E1E2/MF59	1a	CHO cells	Chimpanzees Healthy humans	Yes, antibodies anti-HVR1 and neutralization HCVcc genotypes 1a, 1b, 2a, 2b, 3a, 4a, 5a, 6a and 7a	Yes	Choo et al. 1994, Ray et al. 2010, Frey et al. 2010, Meunier et al. 2011, Stamataki et al. 2011, Law et al. 2013
	E1E2-flag/IFA adjuvant	1a, 1b, 2a	HEK293T cells	C57BL/6 mice	Yes, neutralization HCVcc genotype 1a and 2a	Not analyzed	Krapchev et al. 2018
	Soluble native-like E1E2	1a	Expi293F cells	CD-1 mice	Yes, neutralization HCVpp and HCVcc genotype 1a	Not analyzed	Guest et al. 2021
	MLV VLP E1E2/ polyphosphazene PCPP-R adjuvant	1a	HEK293T cells	BALB/c and C57BL/6J mice Cynomolgus macaques (*Macaca fascicularis*)	Yes, neutralization HCVpp genotype 1a, 1b, 2a, 2b and 4c	Yes	Garrone et al. 2011
	HCV VLP core, E1 and E2/alum	1a, 1b, 2a, 3a	Huh7 cells	BALB/c mice Landrace pigs	Yes, neutralization HCVcc 1a, 2a and 3a	Yes	Earnest-Silveira et al. 2016, Christiansen et al. 2018; 2019
E2 protein	Soluble E2/Ferritin/Alhydrogel+MPL	1a, 1b, 3a	*Drosophila* S2 cells	BALB/c mice Rhesus macaques	Yes, neutralization HCVcc 7 genotypes	Yes	Li et al. 2016; 2017, Wang et al. 2019, Yan et al. 2020
	E2 Δ123 variable regions/AddaVax™	1a	FS293F cells	Albino Dunkin Hartley guinea pigs	Yes, neutralization HCVcc 7 genotypes	Not analyzed	Vietheer et al. 2017, Center et al. 2020
	Consensus core E2 ΔHVR1 ΔC-term	1a (720 strains)	*Drosophila* S2 cells	Guinea pigs	Yes, neutralization HCVpp genotype 1a	Not analyzed	Tarr et al. 2018
	E2 core nanoparticles /AddaVax™	1a, 6a	HEK 293F and ExpiCHO cells	BALB/c mice	Yes, neutralization HCVpp genotypes 1a, 2a, 5a and 6a	Not analyzed	He et al. 2020
HCV protein peptides	Multiepitope peptide vaccine (E1, E2, NS4B, NS5A and NS5B)	4a	Synthesis by the 9-fluorenylmethoxy carbonyl method	BALB/c mice	Yes, neutralization HCVcc genotypes 2a and 4a	Yes	Dawood et al. 2019
	Bivalent HCV peptide (HVR1) vaccine/ Freunds complete or incomplete adjuvant	1a	Synthesis using Fmoc chemistry	BALB/c mice	Yes, neutralization HCVpp genotypes 1a, 1b, 2a, 3a, 4a, 5a, 6a	Not analyzed	Mosa et al. 2020
SHBsAg and HCV envelope proteins	Bivalent chimeric HBV/HCV vaccine/AddaVax™	1a, 3a, 4a	CHO cells	New Zealand rabbits	Yes, neutralization HCVpp and HCVcc genotypes 1a, 1b, 2a, 3a and 4a	Not analyzed	Beaumont et al. 2013; 2015; 2016; 2020
	HBV VLPs carrying HCV E2 protein epitopes/AddaVax™	1a	*Leishmania tarentolae*	BALB/c mice	Yes, neutralization HCVcc genotypes 1a, 1b, 2a, 2b, 4a and 5a	Not analyzed	Czarnota et al. 2016; 2020
	Chimeric HBV S antigen VLPs presenting HCV neutralization epitopes/AddaVax™	1a, 1b, 2a	HEK293T cells	BALB/c mice	Yes, neutralization HCVcc and HCVpp genotypes 1a, 1b and 2a	Not analyzed	Wei et al. 2018

4

A BIVALENT HBV-HCV VACCINE CANDIDATE

4.1 THE CASE OF THE HBV VACCINE

In 2015, the WHO estimated 257 million HBV chronic carriers worldwide, most of them in Africa and Asia-Pacific. This burden is mainly due to the asymptomatic nature of the disease, which has led to undiagnosed (9%) and untreated (8%) patients. This disease is transmitted through sexual and blood-to-blood contact, and one of the main routes is the mother-to-child transmission (World Health Organization, 2017). In fact, 90% of children infected with HBV by this type of transmission may develop a chronic infection (Beasley, 2009), which can be prevented by early immunization (Chen et al., 2012). An effective vaccine to prevent the infection with HBV has been introduced in 1980, which has decreased the global incidence of HBV in children by 3%. However, most of the HBV-infected people were born before the introduction of the vaccine, and belong to the current group of chronic carriers (World Health Organization, 2017).

HBV is an enveloped partially double-stranded, relaxed-circular DNA virus that belongs to the *Hepadnaviridae* family. Its 3,2 kb genome (within 4 ORF) encodes the core protein, the e-antigen, the X protein, the polymerase and the 3 envelope proteins (small, middle and large). Eight genotypes (A-H) and more than 30 sub-genotypes of HBV have been identified, with an intergenotypic and intragenotypic variation of more than 8% and 4 to 8%, respectively [reviewed in (Sunbul, 2014)]. Circulating HBV particles have been observed through electron microscopy (EM) as spheres with a diameter of 42 nm, carrying the HBsAg (Dane, Cameron, and Briggs, 1970). The infected cells produce envelope proteins in excess (10^4-10^6 fold) that self-assemble into noninfectious, immunogenic SVPs carrying as well the HBsAg. These SVPs have been found to be spherical and filament particles of 17-25 nm-diameter (Cao et al., 2019; Dane, Cameron, and Briggs, 1970; Short et al., 2009). The structures of these particles have been observed through cryo-EM. Short and colleagues analyzed the HBsAg filaments and found that these particles possess spikes on their surface. They also verified the composition of filaments and found that the HBV envelope proteins were distributed in 1:1:4 (large:middle:small) ratio (Heermann et al., 1984; Short et al., 2009). Recently, Cao and collaborators analyzed the spherical HBsAg, isolated from chronic carriers, and observed a spike-like structure observed on their periphery similar to the filaments (Figure 22) (Cao et al., 2019). Because of the different compo-

sition of filaments and spherical particles (only a small amount of large protein) (Heermann et al., 1984), it has been suggested that they do no traffic through the same compartments. Filaments presumably traffic through the multivesicular bodies and the ESCRT pathway (Jiang et al., 2016), as virions do for budding, while spherical particles are transported through the ER-Golgi complex (Huovila, Eder, and Fuller, 1992; Patzer et al., 1986). However, Patient and collaborators showed that S proteins assemble as branched filaments in the ER lumen. These filaments are packaged in ER-derived vesicles to the ER-Golgi intermediate compartment where they are unpacked and spontaneously convert into spherical particles (Patient et al., 2007).

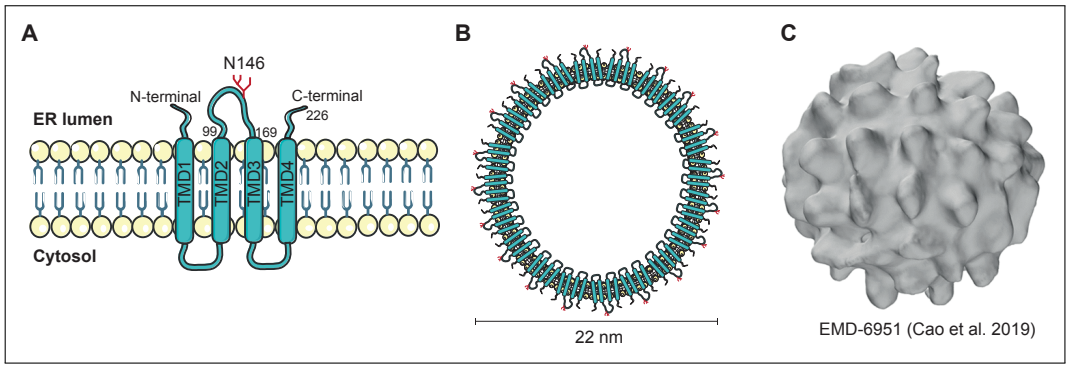

Figure 22: Representations of the hepatitis B surface antigen. A. The HBV small (S) envelope protein is composed of 4 transmembrane domains (TMD1, TMD2, TMD3 and TMD4) that generate one endoplasmic reticulum (ER)-luminal and two cytosolic loops. The luminal loop contains an N-glycosylation site at the residue 146. **B**. The S proteins can self-assemble, by creating dimers (48-60 subunits), into 22-nm diameter spherical particles. **C**. In the 3D reconstruction of the complete HBsAg (Cao et al., 2019), particles showed a spike-like surface explained by the presence of the luminal or antigenic loop of the S protein.

The 3 envelope proteins of HBV, which all carry the HBsAg, have in common the 226-aa domain that corresponds to the S envelope protein or the SHBsAg. It has been shown that the S protein alone can form particles of 22-nm diameter with an octahedral shape and around 48-60 subunits (dimers of S protein) (Gavilanes, Gonzalez-Ros, and Peterson, 1982; Gilbert et al., 2005). The S protein is inserted in the ER membrane and is composed of 4 TMDs (TMD1, TMD2, TMD3 and TMD4), two cytosolic loops and one loop directed to the ER lumen (Figure 22) (Eble, Lingappa, and Ganem, 1990; Eble et al., 1987; Liu et al., 2007). The S protein possesses one N-glycosylation site (N146), leading to two forms: the unglycosylated and the glycosylated S protein of 24 kDa and 27 kDa, respectively. The immunogenic region, also called "a" determinant, is found in the luminal loop, which will be exposed on the surface of the SVPs once particles are secreted from cells (Julithe, Abou-Jaoude, and Sureau, 2014). The "a" determinant along with two pairs of sub-determinants d/y and w/r define the HBsAg subtype [reviewed

in (Weber, 2005)]. The formation of disulfide bonds between S proteins seems to be an important element for the assembly of the particles, which has been proposed to begin with the homodimerization of the S protein (Wunderlich and Bruss, 1996). Suffner and colleagues identified the elements needed for this self-assembly of the SHBsAg: the first cytosolic loop, the TMD2 and the luminal loop (Suffner et al., 2018).

Due to the biophysical and chemical properties of the HBsAg, it was proposed as a potential HBV vaccine. In 1975, it was first reported that the purified HBsAg protected chimpanzees against HBV challenge (Prince et al., 1985). Furthermore, researchers from the University of Tours immunized for the first time humans with a formalin-inactivated HBsAg (purified by chromatography) from plasma of blood-donors (Maupas et al., 1976). Followed that, a placebo-controlled, randomized and double-blind trial of an HBV vaccine derived from plasma was performed and resulted in the generation of antibodies against the HBsAg for more than 95% of participants (Szmuness et al., 1981). However, plasma-derived vaccines have limitations such as the availability of donors and the possibility of contamination with other recently identified agents.

As an alternative for the use of plasma as the source of HBsAg, this antigen was produced in mammalian (CHO) or yeast (*Saccharomyces cerevisiae*) cells by using the recombinant DNA technology. These particles were synthesized in large amounts and they shared similar characteristics to those purified from human plasma such as the size (22-nm diameter) and density (1,2 g/mL), although SVPs were not secreted nor glycosylated in the yeast model (Miyanohara et al., 1983; Valenzuela et al., 1982). In a pre-clinical study in chimpanzees, the pharmaceutical company Merck evaluated this yeast-derived vaccine and showed that the antibodies induced by vaccination protected chimpanzees from challenge (McAleer et al., 1984). They also determined that the HBsAg subtype *adw* could induce protection against other subtypes (*adr* and *ayw*) (McAleer et al., 1984). Because of the successful induction of protection against HBV by these vaccines, the WHO recommended its implementation in national vaccination programs (World Health Organization, 1992). In 2015, the coverage of a 3-dose vaccine had reached 84%, but coverage of birth dose is still of 39% (World Health Organization, 2017).

Today, several HBV vaccines are used around the world, but the first two recombinant vaccines derived from yeast approved by the FDA were Engerix-B® (GlaxoSmithKline) and Recombivax HB® (Merck), both prepared with aluminum-based adjuvants [reviewed in (Zhao, Zhou, and Zhou, 2020)]. These vaccines are normally administered in a 3-dose schedule, but a 4th dose may be needed if individuals do not have enough anti-HBsAg antibodies (Coates et al., 2001). These vaccines have been reported to induce rates of protection above 90% that can be

easily achieved at young age and also to protect newborns from HBsAg-positive mothers (Lee et al., 2006). One example of a successful implementation of the HBV vaccine is that of Taiwan's mass vaccination program. Before vaccination, most of the chronic HBV carriers were infected at an early age, with 40% of the born HBV-infected children developing a chronic disease. Ten years after the beginning of the program, the coverage of the vaccine was of around 90%, which led to a decline in the seroprevalence from 9,8 to 1,3% (Chen et al., 1996) and a significant decrease in the incidence of HCC in children (Chang et al., 1997). This reduction in the incidence was confirmed by a follow-up study 20 years later, making this vaccine the first one to prevent cancer (Chang et al., 2009).

4.2 PREVIOUS STUDIES ON OUR BIVALENT HBV-HCV VACCINE CANDIDATE

The generation of HBV-HCV chimeric SVPs was done by taking advantage of the auto-assemble capacity of the HBV S protein. Regarding the HCV envelope proteins, it has been previously shown that full-length HCV envelope proteins (E1 and E2) are retained in the ER because of their TMDs, but the deletion of those domains impairs the immunogenicity of these proteins (Flint et al., 2000; Forns et al., 2000). Therefore, Patient and colleagues replaced the TMD1 of the HBV S protein (subtype *adw*) with the TMD of the full-length E1 or E2 proteins (genotype 1a, Genbank: AF529293) to obtain chimeric E1-S and E2-S proteins. In order to evaluate the spontaneous assembly of chimeric SVPs, they used Semliki Forest virus (SFV)-derived vectors as expression system (Patient et al., 2007, 2009). The SFV is an alphavirus from which a commonly used vector is derived and is able to self-replicate inducing high expression levels of the foreign gene of interest by transfection of cells [reviewed in (Piver et al., 2005)].

Baby hamster kidney-21 (BHK-21) cells were transfected with SFV-derived vectors (pSFV) containing the sequence of the S, the E1-S or the E2-S proteins. They found that S proteins were able to self-assemble into SVPs of 22-nm diameter, detected in western blotting at 24 and 27 kDa. However, no particles were observed in cells transfected with pSFV-E1-S or pSFV-E2-S, even if E1-S and E2-S proteins were detected by western blotting at 54 and 85 kDa, respectively. In contrast, co-transfection of the SFV-derived vector carrying the sequence of the WT S protein and those bearing either E1-S or E2-S proteins led to the formation of chimeric HBV-HCV SVPs. Then, CHO cell lines stably expressing proteins of interest were established by transduction of cells with recombinant lentiviruses containing the sequences of the S (pHR′^{hph}-S hph: hygromycin phosphotransferase), E1-S or E2-S (pHR′^{gfp}-E1-S or -E2-S gfp: green fluorescent protein) proteins. Resistant clones to hygromycin (CHO-S) and expressing the gfp (CHO-S+E1-S and CHO-S+E2-S) were selected and expanded. These CHO clones allowed the secretion of chimeric

SVPs (S, S+E1-S and S+E2-S) with the ectodomains of the HCV envelope proteins exposed on their surface (FIGURE 23) (PATIENT et al., 2009). These results set the basis for the development of our vaccine candidates. Of note, although the CHO cells were initially preferred for the development of these vaccine candidates, we believe that they can also be produced in yeast as the current HBV vaccine. Indeed, since the absence of glycosylation (or partial glycosylation) of the HBV S protein produced in yeast cells does not impair its immunogenicity, it could also be the case for the HCV envelope proteins.

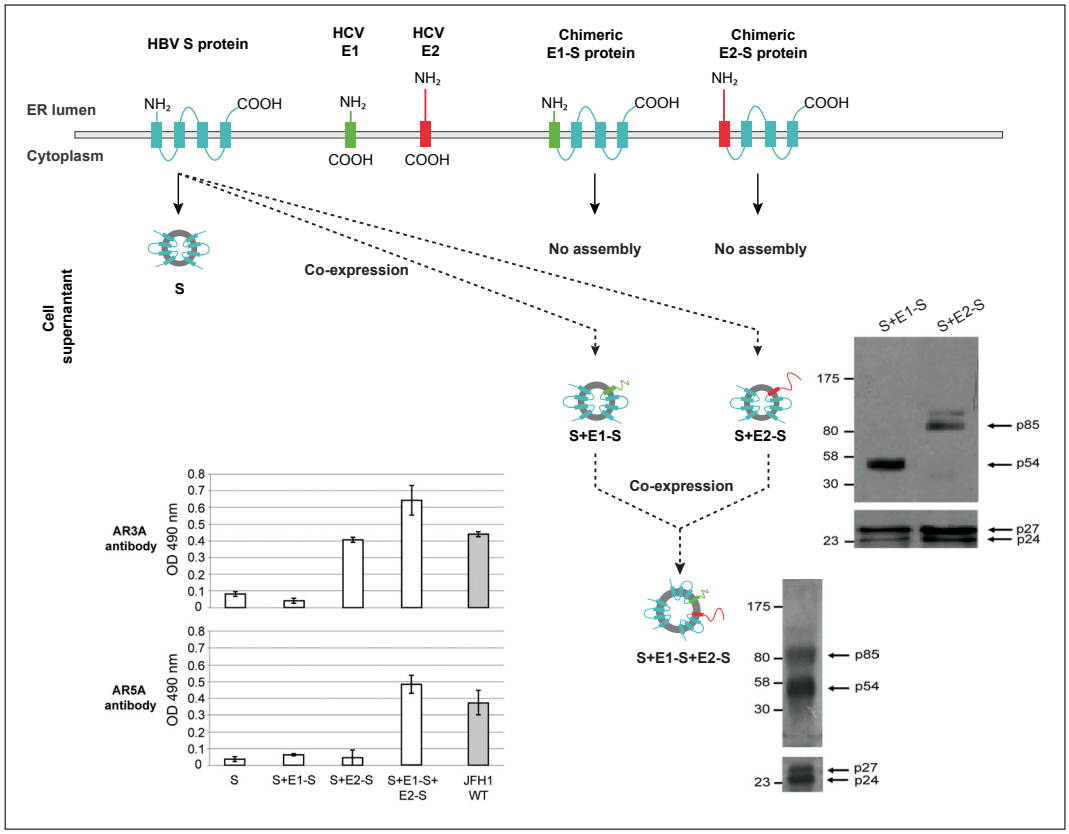

Figure 23: Formation of chimeric HBV-HCV subviral envelope particles. The transmembrane domain (TMD) 1 from the HBV small (S) envelope protein was replaced with the TMD of HCV E1 or E2 proteins. Co-expression of the S protein with chimeric E1-S and/or E2-S proteins led to the secretion of chimeric HBV-HCV subviral particles (SVPs) from Chinese hamster ovary (CHO) cells, while expression of chimeric E1-S or E2-S particles alone resulted in no assembly into particles. Proteins of interest were detected by western blotting (S: p24/p27, E1-S: p54 and E2-S: p85). The folding of HCV envelope proteins on the surface of SVPs was evaluated by enzyme-linked immunosorbent assay (ELISA) with monoclonal antibodies AR3A and AR5A recognizing conformational epitopes on the E2 and E1E2 proteins, respectively. Adapted from (BEAUMONT et al., 2013).

After that, Beaumont and colleagues reported the first immunization protocol with chimeric HBV-HCV particles, previously described, and one more containing both envelope proteins (S+E1-S+E2-S). Chimeric HBV-HCV SVPs were produced in CHO cells cultured in mass and purified following basic methods for

the purification of HBV SVPs. The folding of the HCV envelope proteins was verified by ELISA with mAbs AR3A and AR5A recognizing conformation-dependent epitopes of the E2 protein or the E1E2 heterodimer, respectively. Both antibodies detected proteins of interest, but with a higher specificity in the S+E1-S+E2-S particles (Figure 23). New Zealand rabbits were immunized with 15 µg of adjuvanted (AddaVax™) SVPs (S+E1-S, S+E2-S or S+E1-S+E2-S) or with 15 µg of Engerix-B® (HBV vaccine) or with adjuvant alone at days 0, 14 and 28. More than 90% of the rabbits immunized with SVPs or Engerix-B® developed antibodies against the HBsAg, measured with the chemiluminescence microparticle immunoassay (ARCHITECT system), suggesting a protection from the HBV. The antibodies elicited against E1E2 proteins were measured by an "in-house" ELISA. Despite similar levels of antibodies, those induced by immunization with S+E2-S particles had a better cross-neutralizing potential (genotypes 1a, 1b, 2a and 3a HCVpp and HCVcc) than those elicited by immunization with S+E1-S particles (Figure 24). S+E1-S and S+E2-S particles elicited high levels of anti-E1E2 antibodies individually, but when HCV envelope proteins were presented in the same particle, the neutralizing potential of the antibodies was not improved. These results suggested that the heterodimers formed on the surface of SVPs may mask epitopes responsible for the induction of antibodies (Beaumont et al., 2013).

Then, they evaluated if previous immunization against HBV could interfere with the production of antibodies against the HCV E2 protein. Therefore, New Zealand rabbits were immunized with 4 doses of 15 µg Engerix-B® (days 0, 14, 28 and 42) and 42 days later, with 4 doses of 15 µg S+E2-S particles (days 84, 98, 112, 126). The presence of anti-S antibodies did not impair the generation of antibodies against the E2 protein in the small animal model. Although these results would need to be confirmed in humans, they are encouraging for the development of our bivalent HBV-HCV vaccine and of special interest because, as stated before, around 85% of the world population has already received at least 3 doses of one of the HBV vaccines (Beaumont and Roingeard, 2015).

The next study consisted in the evaluation of the neutralizing properties of antibodies generated by the immunization of rabbits with a mixture of S+E1-S and S+E2-S particles. First, sera from rabbits immunized with S+E1-S or S+E2-S particles (1st immunization protocol) were characterized for specific anti-E1 or anti-E2 responses. They observed that antibodies generated by immunization with S+E1-S+E2-S particles were indeed specific against E1 and E2, but titers were lower than those of antibodies elicited with individual S+E1-S or S+E2-S particles. When mixing one serum from each immunization group (S+E1-S and S+E2-S), they found that the neutralizing potential improved, highlighting the additive properties of these anti-E1 and anti-E2 antibodies (Figure 25) (Beaumont et al., 2016).

After that, they designed an immunization protocol to compare the use of a mixture of S+E1-S and S+E2-S particles, the sequential injections of S+E1-S particles (2 doses), followed by S+E2-S particles (2 doses) and inversely. They found that immunization with the mixture of particles induced stronger humoral responses against E1 and E2 proteins than those observed with S+E1-S+E2-S particles and the sequential immunizations. The neutralizing potential of antibodies induced by immunization with the mixture of particles was also significantly increased against genotypes 1a, 1b, 2a and 4a HCVcc, compared to the neutralizing potential of antibodies elicited by S+E1-S+E2-S-immunization (TABLE 6). Therefore, vaccination with a mixture of particles presenting both HCV envelope proteins separately seems to be the best strategy for the induction of antibodies with cross-neutralizing activity (BEAUMONT et al., 2016).

In the most recent publication related to our vaccine candidate (reproduced in Appendix A), we reported the evaluation of a mixture of chimeric HBV-HCV particles bearing HCV envelope proteins from the three most prevalent genotypes (1, 3 and 4). The aim of this study was to increase the cross-neutralizing properties of antibodies elicited by immunization with our vaccine. Chimeric HBV-HCV particles were generated using HCV envelope proteins of genotypes 3a and 4a isolated from HCV-infected patients. The immunization protocol was designed with the following groups: a) S+E2-S particles harboring genotype 1a HCV E2 proteins ($E2^{1as}$), b) a cocktail of S+E2-S particles presenting genotype 1a, 3a and 4a HCV E2 proteins ($E2^{1a+3a+4a}S$), c) a cocktail of S+E1-S and S+E2-S particles harboring genotype 1a, 3a and 4a HCV E1 or E2 proteins ($E1^{1a+3a+4a}S/E2^{1a+3a+4a}S$), d) a cocktail of S+E1-S and S+E2-S particles bearing genotype 4a HCV E1 or E2 proteins ($E1^{4a}S/E2^{4a}S$) and e) S particles or adjuvant alone (BEAUMONT et al., 2020). The group of rabbits immunized with $E2^{1a+3a+4a}S$ particles elicited antibodies with significantly improved neutralization capacities against genotypes 3a and 4a HCVcc, compared to the group immunized with $E2^{1a}S$ particles. However, the cocktail $E1^{1a+3a+4a}S/E2^{1a+3a+4a}S$ did not lead to better results (Figure S3 in Appendix A, p. 219 and p. 223). This may be due to the presentation of many different immunogens to the immune system, which may result in the frustration of the affinity maturation process of B cells. Another outcome of this study was the improved neutralizing properties of antibodies induced by $E1^{4a}S/E2^{4a}S$ particles against the HCVcc from the same genotype suggesting that the use of mixtures of antigens to target endemic HCV genotypes, such as genotype 4 in Egypt, may be an interesting vaccination strategy (BEAUMONT et al., 2020).

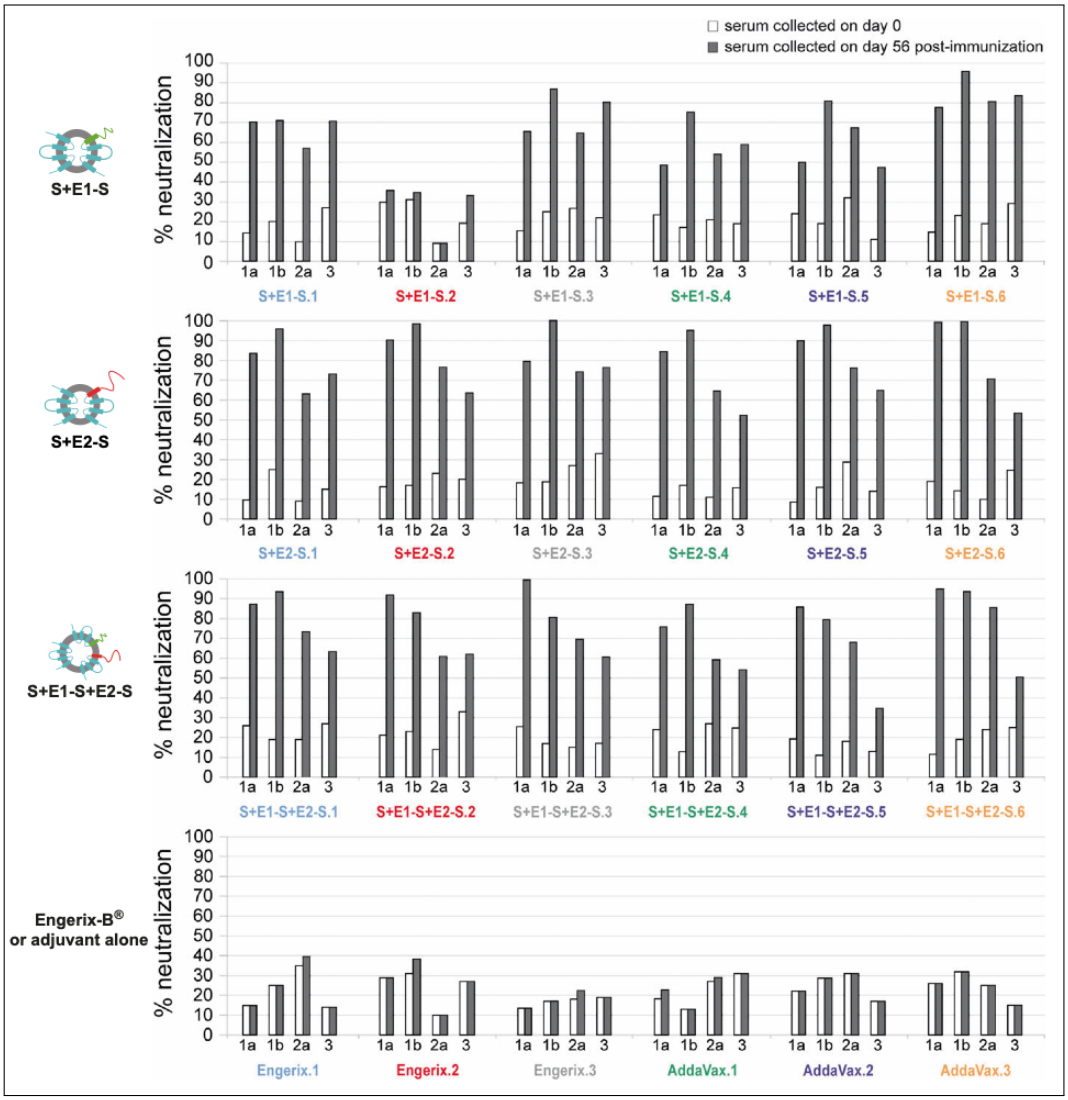

Figure 24: Cross-neutralizing properties of antibodies induced by immunization with chimeric HBV-HCV particles. Rabbit sera from day 56 and day 0 (1:5 dilution) were incubated genotypes 1a (H77/JFH1), 1b (J4/JFH1), 2a (JFH1) and 3 (S52/JFH1) HCV in cell culture (HCVcc) (100 focus-forming units (FFUs)) for 1 hour at 37°C. Then, the mix serum-virus was incubated in Huh7.5 cells for 6 hours at 37°C. 48 hours after incubation, the infection levels were determined by a FFU staining assay. (Beaumont et al., 2013)

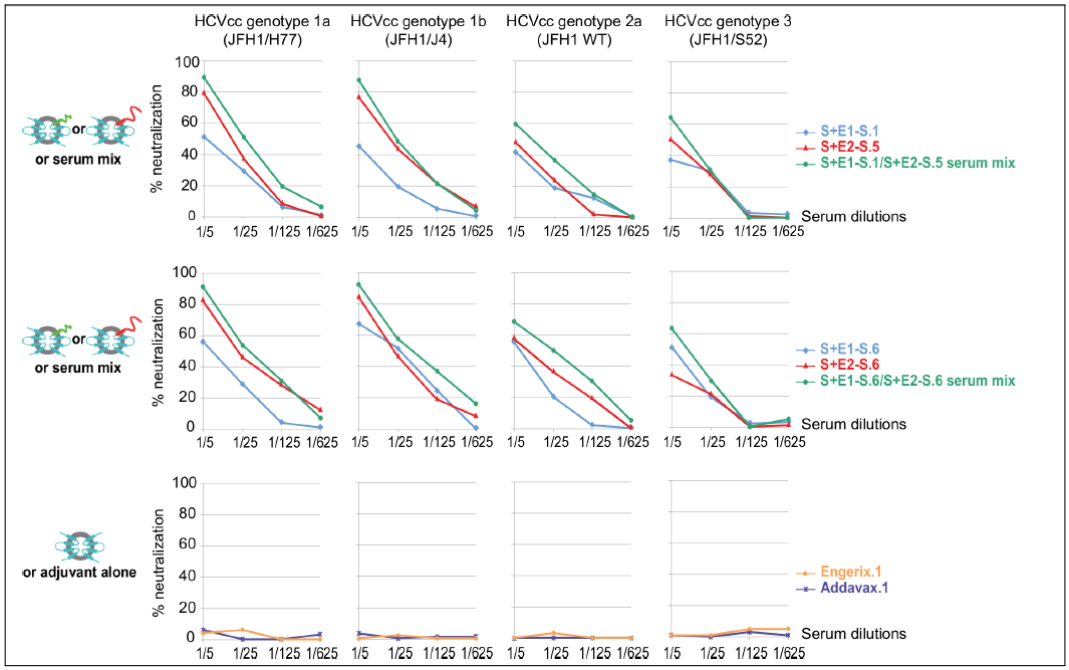

Figure 25: Cross-neutralizing properties of two pairs of mixtures prepared with equal amounts of each serum (anti-E1 and anti-E2 sera). Sera with the best cross-neutralizing properties, analyzed in (BEAUMONT et al., 2013), were selected, as well as one of each control (Engerix-B® and AddaVax™). Mixtures or individual serum were incubated (1/5, 1/25, 1/125 or 1/625 dilutions) with genotype 1a (H77/JFH1), 1b (J4/JFH1), 2a (JFH1) or 3a (S52/JFH1) HCV in cell culture (HCVcc) (100 focus-forming units (FFUs)) for 1 hour at 37°C. Then, the mix serum-virus was incubated in Huh7.5 cells for 6 hours at 37°C. 48 hours after incubation, the infection levels were determined by a FFU staining assay. The percentage (%) of neutralization was determined by subtracting the % of neutralization obtained with the pre-immune serum (day 0) from the one obtained with the post-immune serum (day 56) from the same rabbit. (BEAUMONT et al., 2016)

Table 6: Comparison of the neutralizing properties of antibodies induced by different vaccination strategies against genotypes 1a, 1b, 2a, 3 and 4 HCV in cell culture (HCVcc) (BEAUMONT et al., 2016).

	HCVcc genotype neutralization (% median)				
	1a	1b	2a	3	4
Mix of S+E1-S & S+E2-S (particles bearing E1 and E2 separately)	68.75	70.16	49.40	46.71	52.39
S+E1-S+E2-S (particles with E1E2 heterodimers)	56.25 *	59.26 *	37.30 **	40.85 (NS)	32.98 **
(S+E1-S then S+E2-S sequential immunization)	42.12 **	34.39 **	25.14 **	12.50 **	14.96 **
(S+E2-S then S+E1-S) sequential immunization	56.85 *	48.55 **	34.10 **	32.95 **	14.57 **
Engerix or AddaVax alone	0 **	0.29 **	0.58 **	0 **	0 **

Nonparametric Mann-Whitney tests (statistical analysis performed by comparing the median percentage neutralization obtained by immunization with the mixture of particles bearing E1 and E2 separately, with that obtained by other vaccination strategies)
(NS) non-significant
(*) $p<0.05$
(**) $p<0.01$

Part II

OBJECTIVES

The success of an effective vaccine candidate relies on its capacity to induce strong immune responses against the pathogen of interest. In the case of the development of an HCV vaccine, both cellular and humoral immune responses are needed for the resolution of the infection. In fact, the recent failure of an HCV T-cell vaccine in preventing a chronic disease has indirectly proved that humoral responses are important in the development of a protective immunity. Antibodies directed against HCV have been extensively evaluated in the several phases of the disease. Epitopes identified as neutralizing have also been described in the HCV envelope proteins, mainly in the E2 protein. Thus, an HCV B-cell vaccine must at least contain the latter. Nevertheless, HCV presents high genetic variability, mostly concerning the E2 protein, which possesses 3 variable regions. Therefore, the antibodies elicited by a vaccine need to be of broad-neutralizing nature. Furthermore, several mechanisms developed by HCV to escape the immune system have been described, such as the high level of glycosylation of the envelope proteins, which may also alter the binding of antibodies to proteins.

The vaccine candidate presented here is of VLP type and is based on the HBV S protein that self-assembles into secreted and highly antigenic particles of 22-nm diameter. When co-expressing the WT HBV S and chimeric HBV-HCV proteins (E1-S and E2-S) in CHO cells, particles with the HCV envelope proteins exposed on their surface are secreted in the cells supernatants. The conformation of envelope proteins has been verified through the use of antibodies recognizing discontinuous epitopes only accessible in the native conformation of the protein. However, we hypothesize that the precise conformation of HCV envelope proteins may be more easily achieved by considering and integrating their interactants as observed in the viral context. In order to better simulate these protein-protein interactions at the surface of the virus, it would be particularly interesting to consider that HCV associates with molecules from the host lipid metabolism.

This association of HCV with lipids has been related to the escape from neutralization by antibodies (Bankwitz et al., 2017; Fauvelle et al., 2016a). One of the host molecules associated with HCV is the apoE, which has not only been reported to be involved in the attachment of HCV by interacting with cell receptors, but also in the production of infectious virions. Moreover, studies on the association of apoE with HCV have reported contradictory results concerning which HCV envelope protein is involved in this interaction. One group has shown that apoE interacts specifically with E1, but not with E2 (Mazumdar et al., 2011), while others have reported that the interaction of the E1E2 heterodimer with apoE occurs mainly through the E2 protein (Boyer et al., 2014; Lee et al., 2014). These results regarding the association of apoE with HCV envelope proteins were the first indication to postulate that the conformation of E1 and E2 proteins might be also dependent on interactions with apoE.

In addition to these studies, Fauvelle and colleagues showed that depletion of apoE from producer cells resulted in virions more susceptible to neutralization, while their capacity to escape was restored by ectopic expression of apoE. Moreover, they found that mutations at the aa 447 (phenylalanine→alanine/leucine) of the E2 protein (strain H77c) impaired the association of apoE, as mutants were poorly neutralized and captured with anti-apoE antibodies. They showed that this alteration was not due to the amount of apoE incorporated on the particle, but to a change in the conformation of the apoE-E2 complex. Therefore, the modulation of antibody-mediated neutralization sensitivity may be dependent on the amount of incorporated apoE and the apoE-E2 conformation (Fauvelle et al., 2016a). These results suggested that apoE play indeed a role on the conformation of E2 protein and it should be considered for the development of an HCV vaccine.

The induction of neutralizing antibodies not only depends on the exposition of the precise epitopes, but also on the immunization strategy. The production of high-affinity antibodies directed against a specific antigen occurs through the affinity maturation of B cells. Briefly, B cells recognize the free antigen through their BCR and they migrate to lymph nodes to establish GCs where they undergo SHM on their receptor to improve their affinity to a specific epitope. During this process, B cells interact with the FDCs that present antigens, so the BCR can internalize it and present peptides through their MHC class II. B cells present the antigen at their surface and compete to interact with Tfh cells that will select positively (if strong binding of the MHC-peptide antigen) and differentiate B cells into plasma or memory cells. Most of the B cells experience several rounds of mutations and selection to produce antibodies of higher affinity [reviewed in (De Silva and Klein, 2015)]. Therefore, immunization strategies to correctly induce the affinity maturation process of B cells are necessary to elicit bNabs.

Like HCV, HIV has high genetic diversity and therefore, the development of a pan-genotypic HIV vaccine remains a challenge. The presentation of multiple variants of the antigen at the same time (cocktail) was shown to result in the frustration of the affinity maturation process (Childs, Baskerville, and Cobey, 2015). However, recent computational and experimental studies on the affinity maturation process of B cells have reported that a sequential immunization strategy with variants of the HIV-derived antigen would be the solution to generate antibodies with high affinity to those variants (Mohan et al., 2018; Sprenger et al., 2020; Wang et al., 2015). This strategy has also been evaluated for vaccines against dengue and influenza viruses (Hou et al., 2020; Liu et al., 2019; Zhou et al., 2017). The presentation of one antigen at the time allows the generation of specific antibodies to conserved epitopes in the variants presented through sequential immunizations. In fact, our recent study regarding the use of a cocktail led to the improvement on the neutralizing potential of the induced humoral responses, when only vari-

ants of the E2 protein were used. However, when the variants of the E1 protein were added to this cocktail, the humoral responses were impaired, suggesting a possible frustration of the B cell affinity maturation (Beaumont et al., 2020).

Therefore, the aim of this project was to improve the way HCV envelope proteins were presented at the surface of SVPs to induce the generation of antibodies with better cross-neutralizing properties against HCV by 2 approaches (Figure 26): 1) the improvement of the actual envelope protein conformation by mimicking the interactions at the interface HCV envelope proteins-apoE on the surface of our SVPs as presented on virions; and 2) the implementation of a sequential vaccination strategy by immunizing with chimeric HBV-HCV particles presenting HCV E2 proteins of different genotypes (1, 3 or 4), one at a time, to improve the affinity maturation process of B cells.

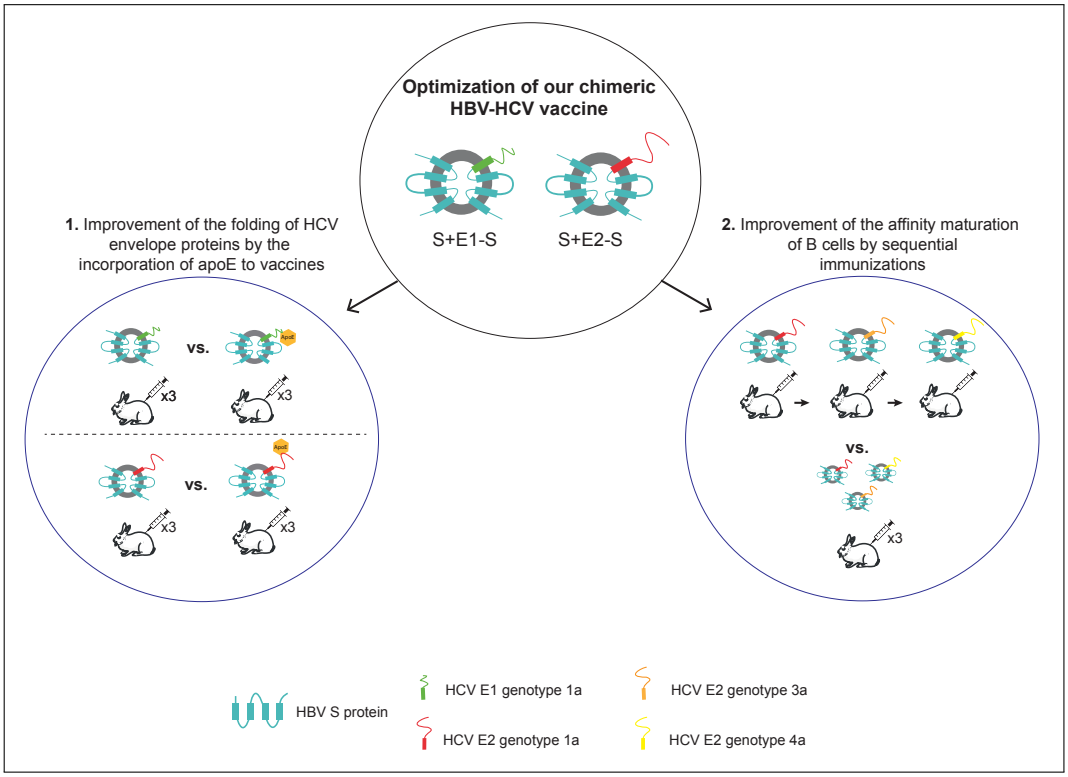

Figure 26: Approaches for the optimization of the bivalent HBV-HCV vaccine. 1. The incorporation of apoE into chimeric HBV-HCV subviral particles (SVPs) to improve the folding of HCV envelope proteins (E1 or E2) present on the surface of SVPs. **2**. The vaccination of rabbits by sequential immunization with particles bearing HCV envelopes from different genotypes (1a, 3a and 4a) to improve the affinity maturation of B cells.

Part III

RESULTS

1

STUDY 1. INCORPORATION OF APOLIPOPROTEIN E INTO CHIMERIC HBV-HCV SUBVIRAL PARTICLES

The first approach considered to optimize our vaccine candidate and explored as part of my thesis project was the evaluation of the impact of the co-presentation of apoE on the folding and immunogenicity of the HCV envelope proteins (E1 and E2). Both, apoE and HCV E1 or E2 proteins were presented on the surface of chimeric HBV-HCV particles, in order to precisely mimic the interactions at the surface of HCV. The findings from study, presented below, were summarized in the publication (reproduced in Appendix B):

Gomez-Escobar E, Burlaud-Gaillard J, Visdeloup C, Ribeiro E Silva A, Coutant P, Roingeard P, Beaumont E. Incorporation of apolipoprotein E into HBV-HCV subviral envelope particles: implications for an improved hepatitis vaccine strategy.
Under review in *Scientific Reports*.

1.1 ANALYSIS OF THE INTERACTIONS BETWEEN APOE AND HCV ENVELOPE PROTEINS

First, we evaluated the interactions between apoE and the HCV envelope proteins through co-immunoprecipitation (co-IP) experiments. We used BHK-21 cells to express proteins of interest, and then, captured the immune complexes with a goat anti-apoE polyclonal antibody (pAb) (AB947) or with non-specific goat IgG (isotype control). We found that apoE could interact specifically with the HCV envelope proteins (E1 and E2) (Figure 27) and with the chimeric HBV-HCV envelope proteins (E1-S and E2-S) (Figure 28). We reported for the first time the interactions between apoE and both HCV E1 and E2 proteins, in contrast with previous studies alleging for only one of them.

1.2 CHARACTERIZATION OF PURIFIED CHIMERIC HBV-HCV SVPS BEARING APOE

CHO cell clones stably expressing proteins of interest (S, E1-S, E2-S and apoE) and allowing the formation of chimeric HBV-HCV particles bearing apoE were generated. SVPs with and without apoE (S, S+apoE, S+E1-S, S+E1-S+apoE, S+E2-S and S+E2-S+apoE) were purified following classical methods for the purification

of HBV SVPs. Vaccine particles were then characterized by western blotting, ELISA and transmission EM (TEM). We observed the following:

- ApoE was detected on the surface of vaccine particles of interest by an anti-apoE in ELISA and TEM experiments, even in particles containing only the WT HBV S protein (FIGURE 29). These results suggest a direct interaction between apoE and the HCV envelope protein (previously verified by co-IP experiments), as well as between apoE and the HBV S protein.
- ApoE was incorporated on vaccine particles (verified by western blotting: FIGURE 29A), but in different amounts (S+E2-S+apoE>S+E1-S+apoE>S+apoE). The difference in the amount of apoE detected may be due in part to the cumulation of protein-protein interactions (S-apoE, E1-apoE or E2-apoE) and to the possible multiple binding sites to apoE in the E2 protein.
- The incorporation of chimeric E1-S or E2-S proteins on SVPs seemed to be modulated by the presence of apoE in the particle formation. We analyzed the changes in protein quantity by calculating the densitometry of the E1-S and E2-S bands for the western blot in FIGURE 29A with the ImageJ software. We found that there was 1,5-fold more E1-S protein on particles bearing apoE than in those that did not, while the opposite was observed for the E2-S protein: 3,4-fold less protein in particles carrying apoE.
- Specific mAbs against E1 (A4 and H-111) and E2 (AP33 and AR3A) proteins were able to recognize linear and conformation-dependent conserved epitopes on the exposed HCV envelope proteins (FIGURE 30A), suggesting that these proteins were presented in a native-like conformation. However, the detection signals of E1-S protein were better in particles bearing apoE, while the opposite was observed for E2-S protein.
- To better analyze the implication of apoE on the conformation of HCV envelope proteins, we compared the optical densities (ODs) obtained with each mAb (FIGURE 30A) to the relative densities of E1-S or E2-S bands from FIGURE 29A previously calculated (S+E1-S=1 and S+E1-S+apoE=1,5; S+E2-S=3,4 and S+E2-S+apoE=1). The ratios obtained for each antibody and SVP (FIGURE 30B) indicated that by normalizing the amount of envelope proteins, mAbs displayed a better binding to E1-S or E2-S proteins, which may imply a better conformation of these proteins in particles co-presenting apoE.

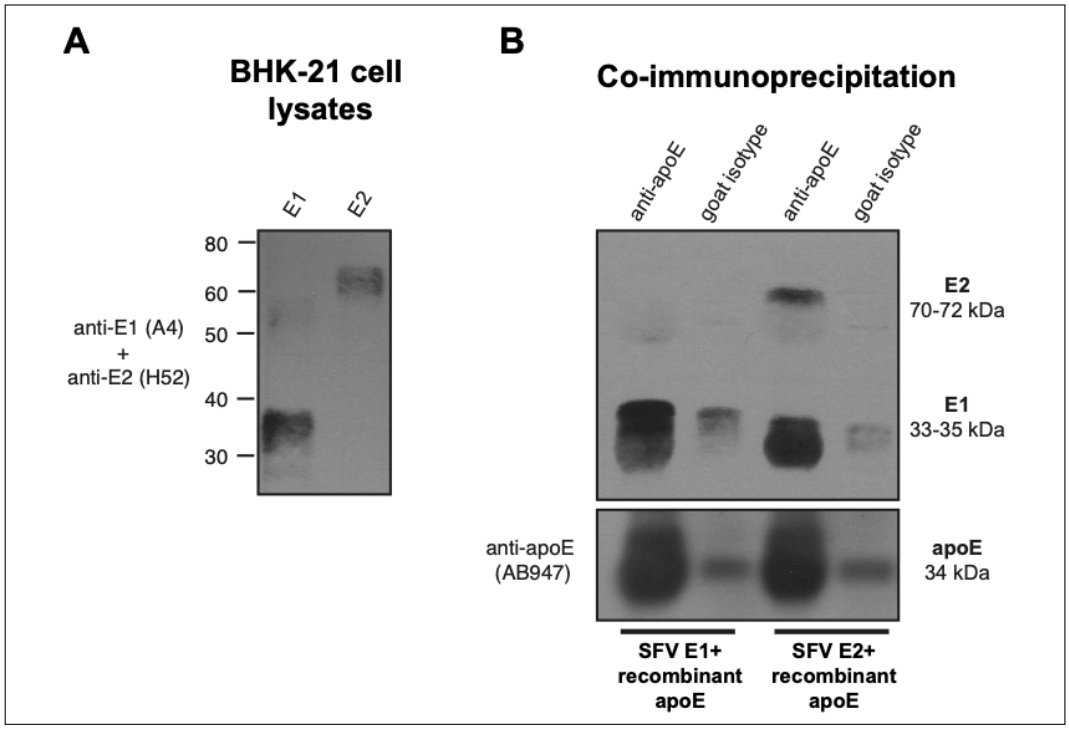

Figure 27: Evaluation of the interaction between apoE and HCV envelope proteins in co-immunoprecipitation assays. A. Western-blot analysis of baby hamster kidney-21 (BHK-21) cell lysates expressing HCV envelope proteins E1 or E2, detected with mouse anti-E1/anti-E2 monoclonal antibodies (mAbs) (A4 and H52). **B**. Western-blot analysis of immune complexes from co-immunoprecipitation with a goat anti-apoE polyclonal antibody (pAb) (AB947) or a goat isotype. Proteins were detected with anti-E1/anti-E2 mAbs (A4 and H52) and anti-apoE pAb (AB947).

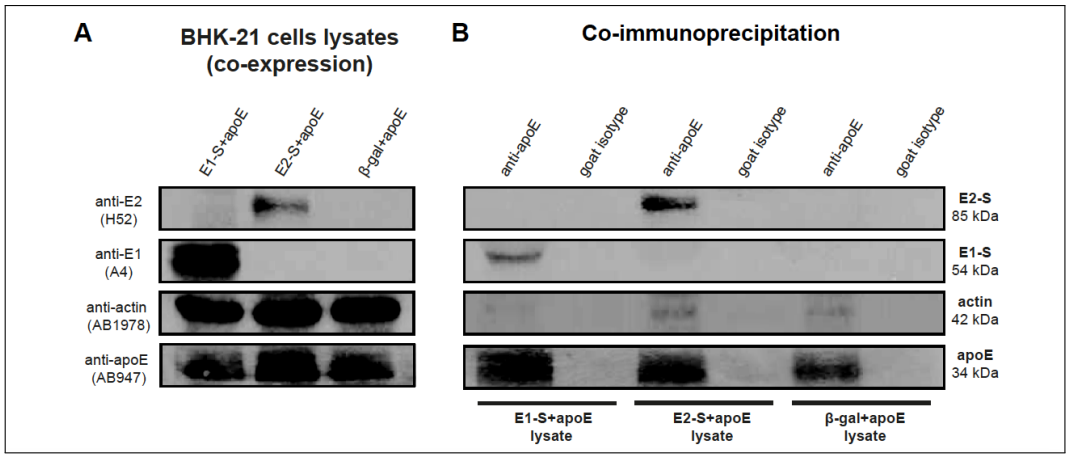

Figure 28: Evaluation of the interaction between apoE and HCV envelope glycoproteins fused to HBV S envelope protein (E1-S or E2-S) in co-immunoprecipitation assays. A. Western-blot analysis of baby hamster kidney-21 (BHK-21) cell lysates co-expressing chimeric HBV-HCV E1-S or E2-S proteins, and apoE, detected with the mouse anti-E1 monoclonal antibody (mAb) (A4), mouse anti-E2 mAb (H52), goat anti-apoE polyclonal antibody (pAb) (AB947) and mouse anti-actin mAb (AB1978). **B**. Western-blot analysis of immune complexes from co-immunoprecipitation assays of BHK-21 cell lysates of interest with anti-apoE pAb (AB947) or with non-specific goat IgG (isotype control). Analyses were performed with the same antibodies as in panel A.

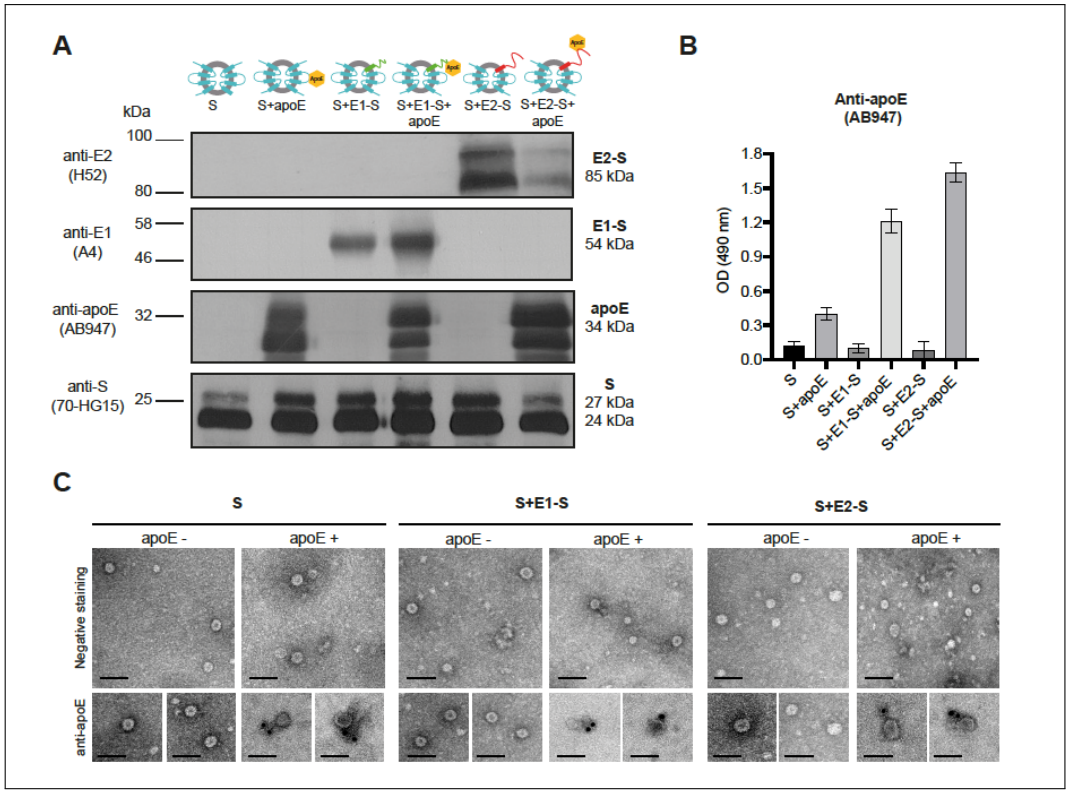

Figure 29: Characterization of the incorporation and presentation of apoE in purified HBV-HCV subviral particles. A. Western-blot analysis of subviral particles (75 µg/mL of HBV small surface antigen (SHBsAg)) and detection of proteins of interest with the mouse anti-E1 monoclonal antibody (mAb) (A4), mouse anti-E2 mAb (H52), goat anti-S polyclonal antibody (pAb) (70-HG15) and goat anti-apoE pAb (AB947). **B**. Detection of apoE on the surface of particles by enzyme-linked immunosorbent assay (ELISA). Subviral particles (25 µg SHBsAg) were captured with lectin, and apoE was detected with the anti-apoE (AB947) antibody. The experiment was performed once in triplicate, and the results are expressed as mean optical density (OD) (490 nm) values ± standard deviation. **C**. Observation of subviral particles by TEM. Particles were analyzed by negative staining with 2% uranyl acetate solution (upper panels) and by the same method after immunogold staining with an anti-apoE pAb (AB947) (lower panels). Scale bars: 50 nm (in upper and lower panels).

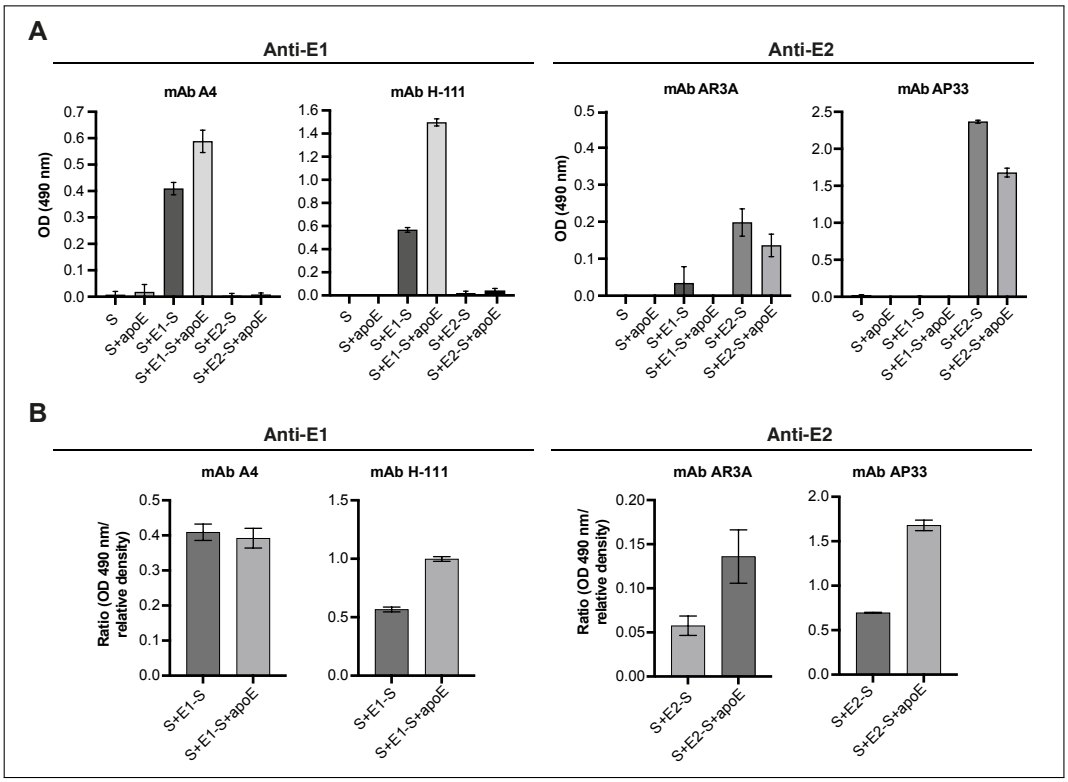

Figure 30: Analysis of the incorporation and presentation of E1-S and E2-S proteins in purified chimeric HBV-HCV subviral particles. A. Detection of HCV E1 or E2 proteins on the surface of subviral particles (SVPs) by enzyme-linked immunosorbent assay (ELISA). Subviral particles (25 µg of HBV small surface antigen(SHBsAg)) were captured with a goat anti-S polyclonal antibody (pAb) (70-HG15), and proteins were detected on the surface of the particles with a mouse anti-E1 monoclonal antibody (mAb) (A4), a human anti-E1 mAb (H-111), a human anti-E2 mAb (AR3A) or a mouse anti-E2 mAb (AP33) antibodies. Experiments were performed once in triplicate and results are expressed as the mean optical density (OD) (490 nm) values ± standard deviation. **B**. The levels of chimeric HBV-HCV E1-S or E2-S proteins detected were normalized according to the amount of each protein incorporated into the subviral particles. The results are expressed here as the ratio of OD (490 nm) values obtained by ELISA to the relative density of bands for the same proteins incorporated subviral particles calculated by densitometry (S+E1-S=1, S+E1-S+apoE=1,5, S+E2-S=3,4 and S+E2-S+apoE=1).

1.3 ANALYSIS OF HUMORAL RESPONSES

Groups of 8 female New Zealand rabbits were immunized at days 0, 14 and 28, with 3 doses of 15 µg of adjuvanted (AddaVax™) SVPs of interest and control groups of 4 rabbits were immunized with 15 µg of S, S+apoE or adjuvant alone. Serum samples were taken at days 0,12, 26, 42 and 56 for the analysis of humoral responses (FIGURE 31). Anti-S antibodies were analyzed through a chemiluminescent microparticle immunoassay (ARCHITECT system, Abbott Diagnostics) (FIGURE 32). They were detected for all the rabbits immunized with SVPs and a slight reduction in the antibody titer was observed for rabbits vaccinated with particles bearing apoE. However, no significant difference was obtained by day 56 when compared by pairs using the non-parametric Mann-Whitney test: S vs. S+apoE (p=0,1143), S+E1-S vs. S+E1-S+apoE (p=0,6454) and S+E2-S vs. S+E2-S+apoE (p=0,4418) (FIGURE 33).

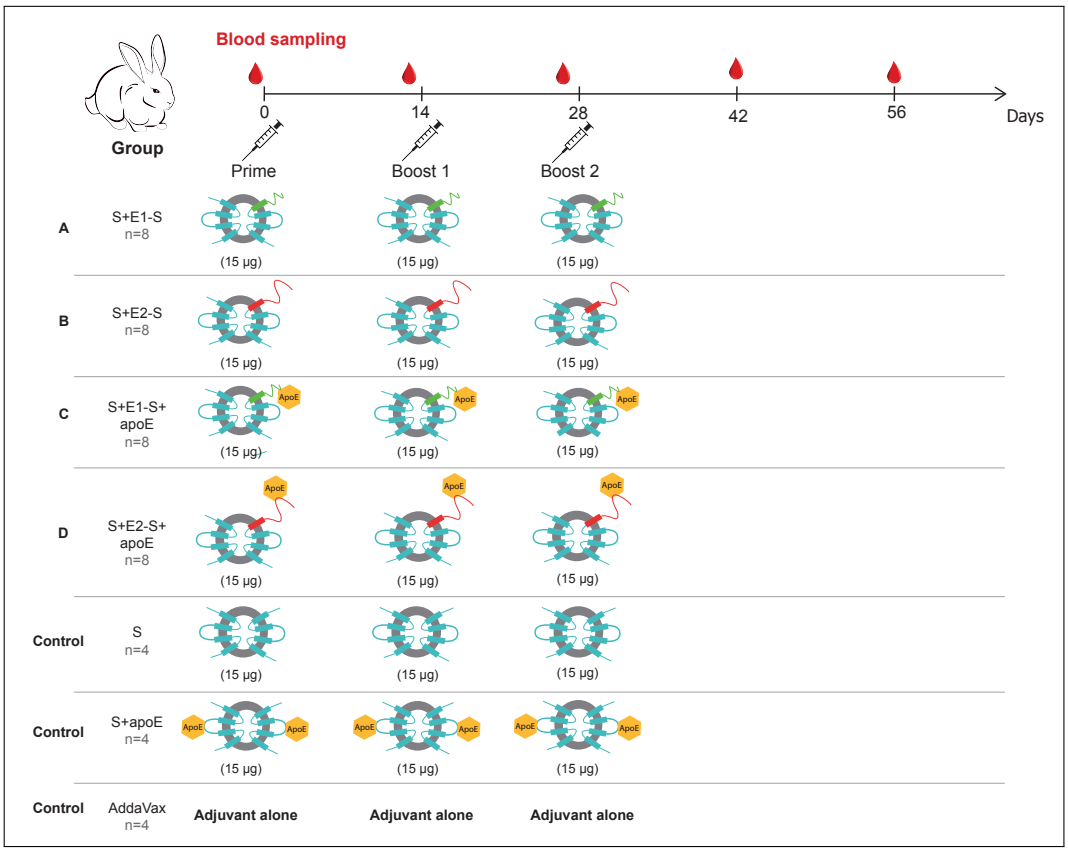

Figure 31: Immunization protocol of New Zealand rabbits with subviral particles bearing apoE or not. Groups of 8 rabbits were immunized on days 0, 14 and 28 with 15 µg of adjuvanted (AddaVax™) S+E1-S, S+E2-S, S+E1-S+apoE or S+E2-S+apoE particles, while control groups of 4 rabbits were immunized with 15 µg of adjuvanted (AddaVax™) S or S+apoE particles or AddaVax™ adjuvant alone. Blood samples were taken at days 0, 12, 26, 42 and 56 for the analysis of humoral responses.

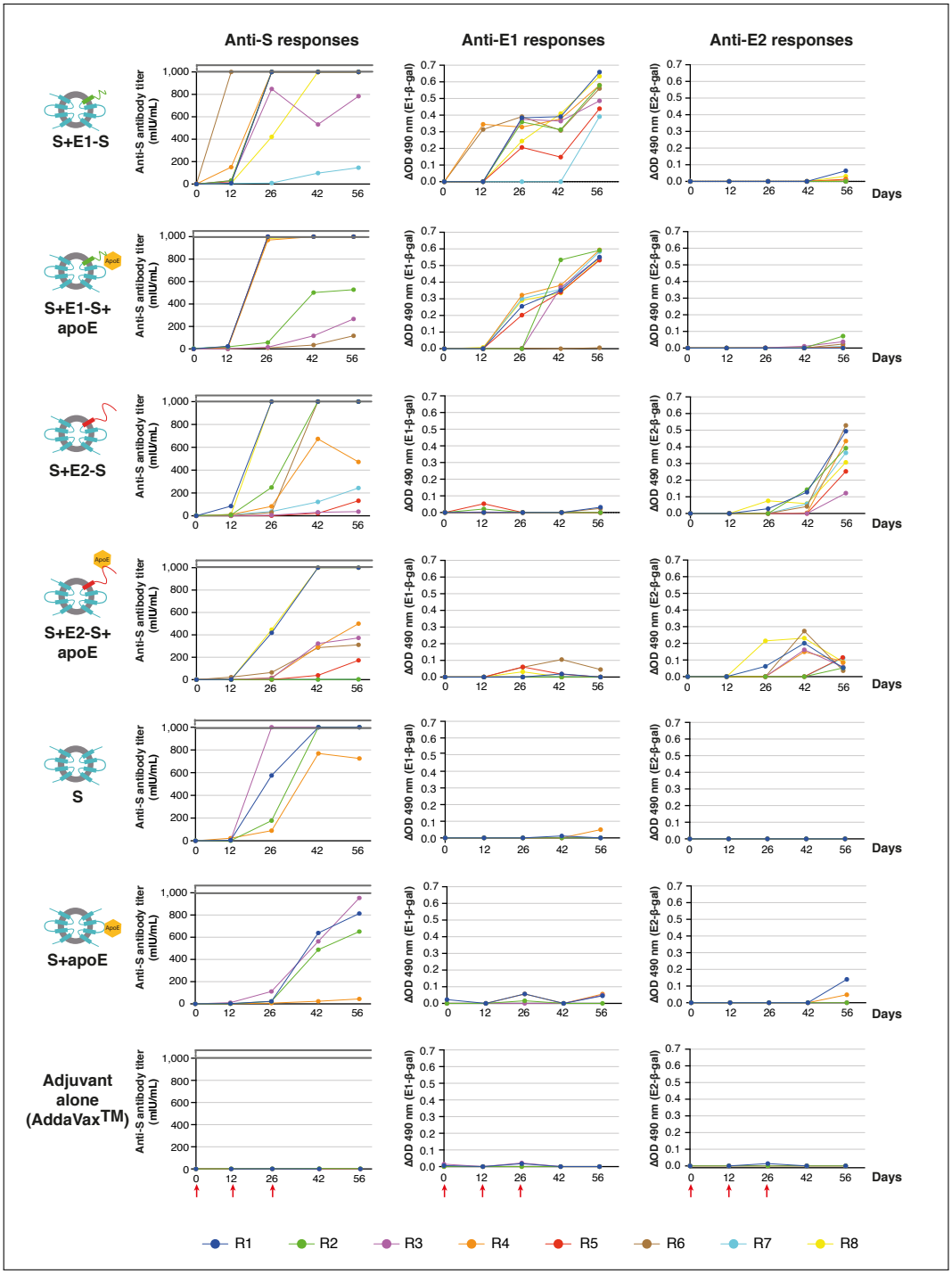

Figure 32: Anti-S and anti-E1/-E2 responses in rabbits immunized with HBV-HCV subviral particles bearing or not apoE, prepared with the AddaVax™ adjuvant. Specific anti-S and anti-E1/-E2 responses were evaluated in rabbit sera with a chemiluminescent microparticle immunoassay (ARCHITECT system) and with "in-house" enzyme-linked immunosorbent assays (ELISAs), respectively. Sera from rabbits immunized with S, S+apoE and AddaVax™ adjuvant alone were used as controls. Results for anti-E1/anti-E2 responses are expressed as the difference (Δ) in optical density (OD) (490 nm) (anti-E1 or anti-E2 – β-galactosidase), whereas anti-S titers are expressed in mIU/mL. Red arrows indicate the time at which immunizations occurred (days 0, 14 and 28).

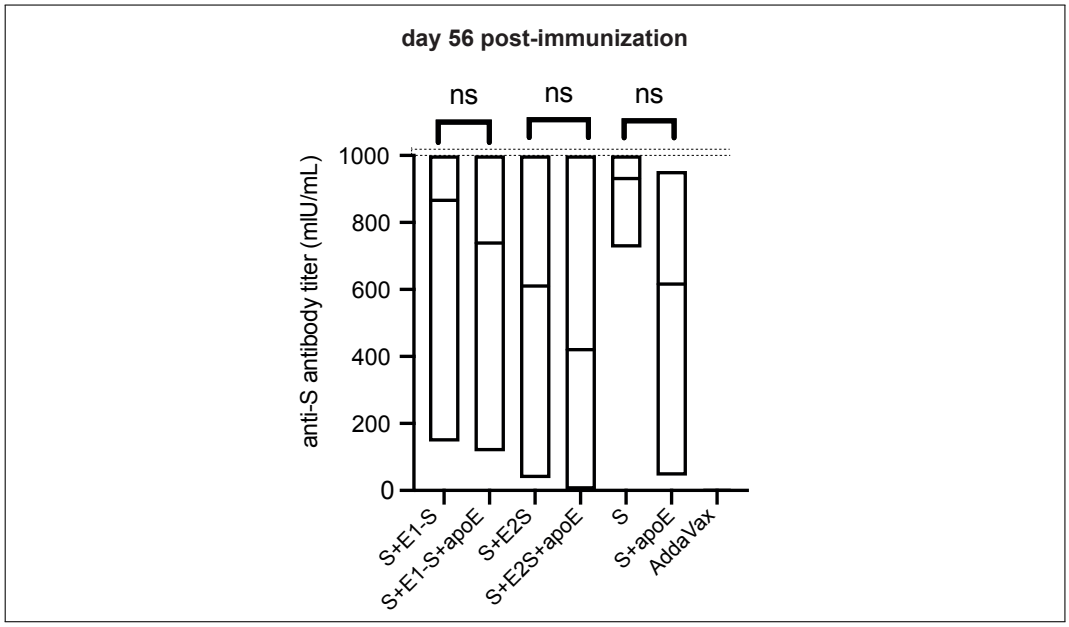

Figure 33: Anti-S responses at day 56 post-immunization induced in New Zealand rabbits. Antibody titers against the HBV S protein induced by immunization with chimeric subviral particles bearing or not apoE were evaluated with chemiluminescent microparticle immunoassay (ARCHITECT system) and were compared using the Mann-Whitney nonparametric test ($p<0,05$). ns=nonsignificant. The gray dashed lines represent the upper detection limit of 1000 mIU/mL of the ARCHITECT system.

Anti-E1/-E2 responses were evaluated through "in-house" ELISAs based on the use of BHK-21 cell lysates expressing the genotype 1a proteins E1 or E2 as solid phase of the test (Figure 32). We first observed that despite the higher amount of E1-S protein incorporated in particles bearing apoE, anti-E1 antibody titers were similar for groups immunized with S+E1-S or S+E1-S+apoE particles. However, the levels of these antibodies elicited with S+E1-S+apoE particles were more homogeneous for all rabbits than those induced with S+E1-S particles, which was maybe due to the improved conformation of E1 protein by apoE. Concerning the anti-E2 responses, higher levels of antibodies were elicited by immunization with S+E2-S particles than with the S+E2-S+apoE ones by day 56. This is not surprising in regard to the very low amount of E2-S protein incorporated in those SVPs. However, antibodies elicited by S+E2-S+apoE particles showed a peak at day 42 (above titers from S+E2-S group) but dropped by day 56.

Antibodies directed against apoE were also evaluated through "in-house" ELISAs using a purified human apoE (isoform E3, MyBioSource) or BHK-21 cell lysates expressing apoE as solid phase (Figure 34). In both experiments, we observed a basal level of anti-apoE antibodies before immunization. After immunization, levels of antibodies directed against apoE increased (in different proportions) for all of the rabbits immunized with SVPs bearing or not apoE. We believed that this could be due to the presence of cell- or serum-derived remnants in our pre-

parations that may trigger the observed responses. This is supported by the fact that rabbits immunized with adjuvant only showed no or very little increase in the apoE antibody titers.

1.4 ANALYSIS OF THE NEUTRALIZING PROPERTIES OF ANTIBODIES INDUCED BY IMMUNIZATION WITH SVPS OF INTEREST AGAINST HCVCC

The neutralizing potential of antibodies was evaluated using a genotype 1a (H77 isolate) and 2a (WT JFH1) HCVcc. The mean % of neutralization for both genotypes with antibodies elicited by immunization with S+E1-S+apoE particles were slightly higher (61,09% and 58,08%) than those from antibodies induced by immunization with S+E1-S particles (48,17% and 47,10%). In addition, the % of neutralization of the genotype 1a HCVcc with sera from S+E1-S+apoE-immunized rabbits were more homogeneous (standard deviation of 9,53% versus 22,10%) than that of the rabbits immunized with S+E1-S particles, as observed for the antibody titers. Concerning the anti-E2 antibodies elicited with particles bearing apoE, the mean % of neutralization of both genotypes were lower (28,23% and 40,06%) than those from antibodies induced by immunization with S+E2-S particles (59,49% and 48,60%) (FIGURE 35A). These results were not surprising in regard to the low antibody titers at day 56 for rabbits immunized with S+E2-S+apoE particles.

Because of the disappointing % of neutralization of the group S+E2-S+apoE, and to better analyze these results, we investigated the neutralizing potential related to the anti-E2 antibody titers for the best 4 rabbits (% of neutralization>median) from groups immunized with S+E2-S and S+E2-S+apoE SVPs. When we normalized the anti-E2 antibody titers for these rabbits at day 56, the neutralizing potential of antibodies induced by immunization with particles bearing apoE appeared to be significantly better against genotypes 1a and 2a HCVcc than for those induced with S+E2-S particles (FIGURE 35B). We hypothesize that the very low % of neutralization was in fact due to the low quantity of antibodies used to neutralize the HCVcc and not to their quality. Therefore, the estimation of ratios related to the antibody titers allowed us to better evaluate the neutralization potential of these antibodies.

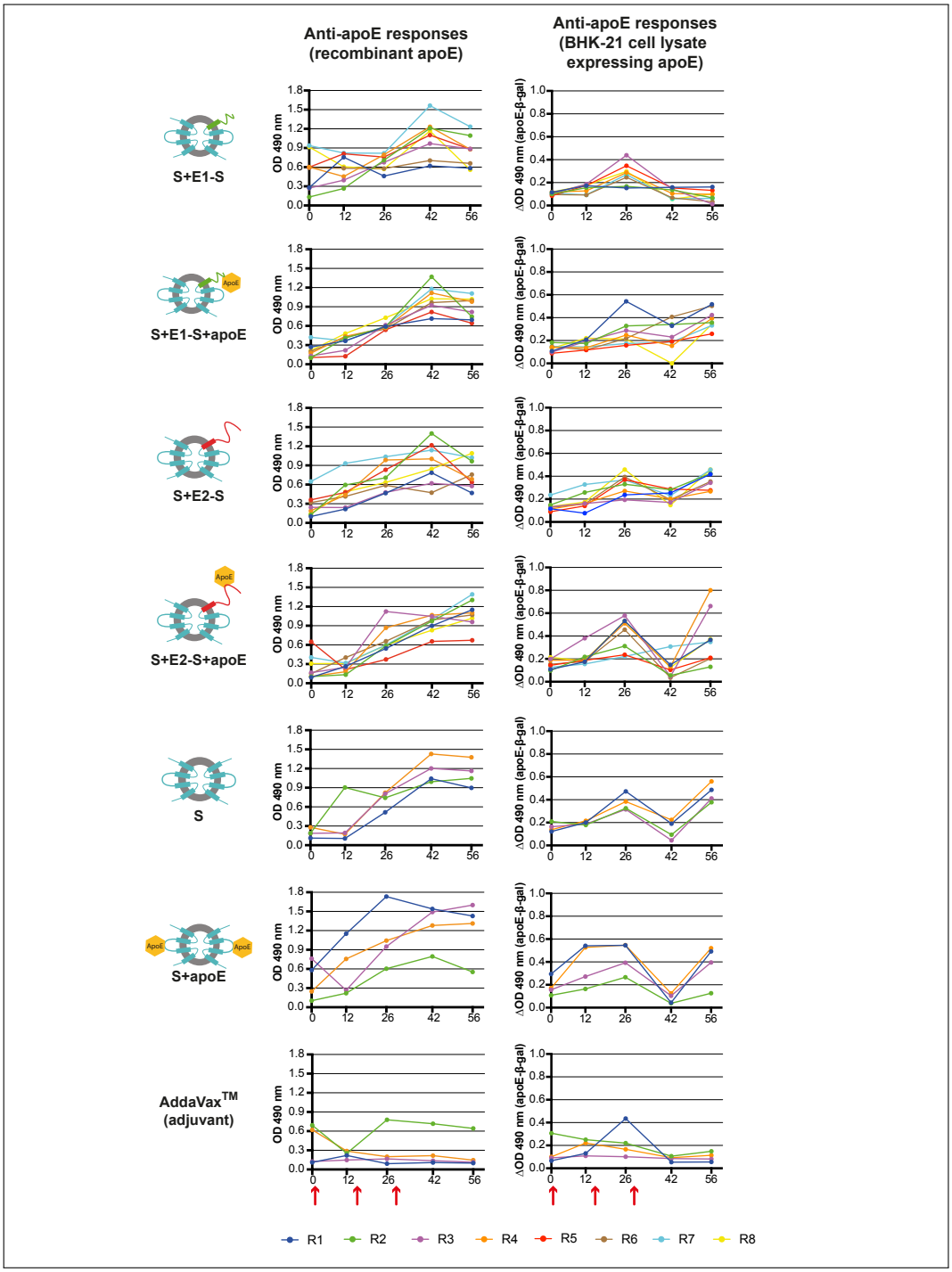

Figure 34: Anti-apoE responses in rabbits immunized with HBV-HCV subviral particles bearing or not apoE, prepared with AddaVax™ adjuvant. Antibodies against apoE were evaluated through "in-house" enzyme-linked immunosorbent assays (ELISAs) using the purified human apoE (isoform E3) or baby hamster kidney-21 (BHK-21) cell lysate expressing the human apoE or β-galactosidase (β-gal) (control) as solid phase. Sera from rabbits were incubated in 96-well plates previously coated with the solid phase and then, detected by a goat $F(ab')_2$ anti-rabbit IgG (H+L) antibody. Results for anti-apoE responses using the purified human apoE are expressed as the OD 490 nm, whereas those using BHK-21 cell lysates are expressed as the difference (Δ) in optical density (OD) 490 nm (anti-apoE – β-gal). Red arrows indicate the time at which immunizations occurred (days 0, 14 and 28).

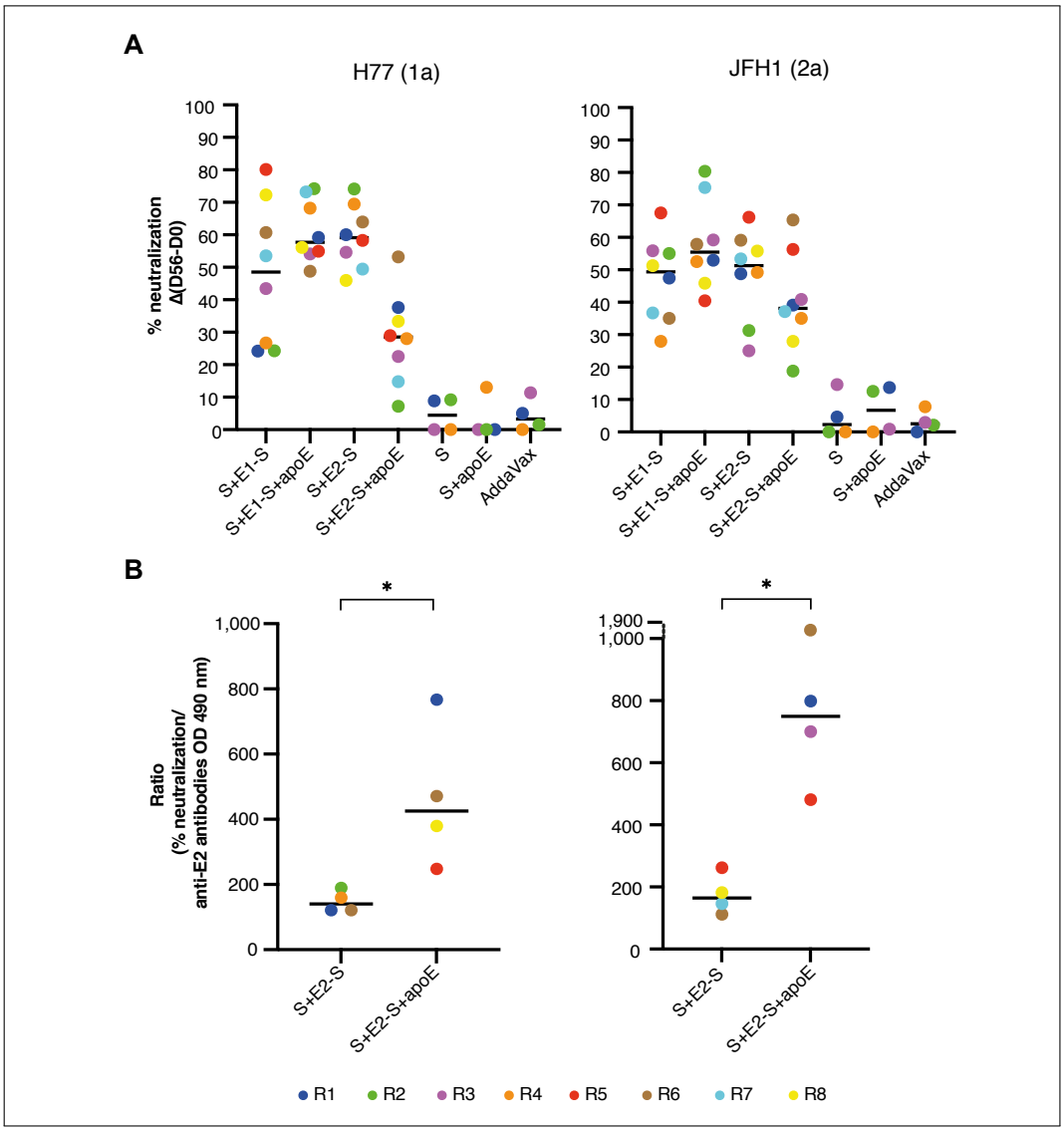

Figure 35: Neutralizing potential of antibodies induced by the immunization of rabbits with purified HBV-HCV subviral particles with and without apoE. A. % neutralization of genotype 1a and 2a HCV in cell culture (HCVcc). Rabbit serum samples (collected on days 0 and 56), at a 1:5 dilution, were incubated with HCVcc harboring HCV envelope proteins from genotype 1a (H77 isolate) and 2a (wild-type JFH1), which were then used to infect Huh7.5 cells. Infection levels were determined 48 hours post-infection, in a focus-forming unit staining assay. The % neutralization was determined by subtracting the % neutralization obtained with the pre-immune serum (day 0) from that obtained with the post-immune serum (day 56) from the same rabbit. The assay was performed once in triplicate, and the results are expressed as mean values. Each colored point represents the % neutralization value calculated for a serum sample from an individual immunized rabbit, whereas the horizontal line represents the median value. **B**. Analysis of the neutralizing capacity of anti-E2 antibodies for the best four rabbits (% neutralization > the median) of each group (S+E2-S versus S+E2-S+apoE), related to the antibody levels, evaluated by enzyme-linked immunosorbent assay (ELISA) on day 56. Results are expressed here as the ratio of the change in % neutralization between days 0 and 56 (Δ(D56-D0)) to the OD (490 nm) of anti-E2 antibodies obtained by ELISA on day 56. The groups were compared using the non-parametric Mann-Whitney U test. (*) p<0,05

2

STUDY 2. SEQUENTIAL IMMUNIZATION WITH CHIMERIC HBV-HCV SUBVIRAL PARTICLES BEARING HCV ENVELOPE PROTEINS FROM VARIOUS GENOTYPES

The second approach for the optimization of our vaccine candidate consisted in the immunization of rabbits with particles bearing HCV E2 proteins from genotypes 1a, 3a and 4a, in a sequential schedule and the comparison of the cross-neutralizing properties of these antibodies with the ones elicited by immunization with a cocktail from the same particles. Here I present the results from the production, purification and characterization of the vaccine particles, as well as the preliminary analysis of the anti-S responses.

2.1 MATERIALS AND METHODS

2.1.1 *Production and purification of chimeric HBV-HCV particles bearing HCV E2 envelope protein genotypes 1a, 3a and 4a*

CHO cell clones stably expressing chimeric HBV-HCV proteins were generated as described previously (Beaumont et al., 2020; Patient et al., 2009). Briefly, CHO cells were transduced with recombinant lentiviruses carrying the sequence of the HBV S protein and the hph gene (pHR'^{hph}-S). A clone resistant to hygromycin (CHO-S) and expressing the S protein was selected and expanded. Subsequently, the CHO-S clone was transduced with lentiviruses carrying either the chimeric E2 genotype 1a (E2^{1a})S protein and the gfp gene (pHR'^{gfp}-E2-S), or chimeric E2 genotype 3a or 4a (E2^{3a} or E2^{4a})S proteins and the puromycin N-acetyltransferase (pac) gene (pHR'^{pac}-E2^{3a}S or pHR'^{pac}-E2^{4a}S). A GFP-positive clone (CHO-S+E2^{1a}S) and puromycin-resistant clones (CHO-S+E2^{3a}S and CHO-S+E2^{4a}S) were selected and expanded. Selected clones were cultured with the Advanced DMEM/F12 medium supplemented with a low-concentration serum (2,5% fetal calf serum) in HYPERflask® vessels (1720 cm² culture surface). Supernatants from each culture were harvested every 10 to 12 days.

Chimeric HBV-HCV SVPs (S+E2^{1a}S, S+E2^{3a}S, S+E2^{4a}S) were purified from cell supernatants as described elsewhere (Beaumont et al., 2013; Patient et al., 2009). Briefly, total proteins were precipitated by incubation overnight at 4°C with a 45% solution of ammonium sulfate (pH 7,5). The precipitated proteins were recovered by centrifugation and dialyzed against Tris-NaCl-EDTA (TNE) buffer (10

mM Tris-HCl [pH 7,5]/100 mM NaCl/1 mM EDTA) overnight at 4°C. The SVPs were purified by successive isopycnic ultracentrifugations on cesium chloride gradients with a density of 1,22 g/cm^3. SHBsAg-positive fractions of gradients were identified in an "in-house" ELISA. To do this, particles were captured with a goat pAb directed against the HBV S protein (70-HG15) on 96-well plates. Then, particles were detected by an anti-S mAb (clone H25B10) that was subsequently recognized by a horse radish peroxidase (HRP)-conjugated secondary anti-mouse mAb anti-mouse IgG1 (H+L) (clone Sb77e). Fractions containing the HBV S protein were pooled and dialyzed against TNE buffer overnight at 4°C. Purified vaccine particles were concentrated with the Amicon Ultra-15 100k device (Millipore) and their final SHBsAg concentrations were determined with the same "in-house" ELISA, with a sequentially diluted recombinant SHBsAg subtype *adw* as the standard (R86872; Meridian Life Science). SVPs purity was calculated as the ratio of SHBsAg concentration to total protein concentration assessed by determining the absorbance measured at 280 nm with a NanoDrop spectrophotometer.

2.1.2 *Characterization of chimeric HBV-HCV subviral particles S+E2^{1a}S, S+E2^{3a}S and S+E2^{4a}S*

The presence of proteins of interest was evaluated by western blotting and ELISA. For western blotting, particle samples containing equal amounts of SHBsAg (50 µg/mL) were prepared in reducing sample buffer (1X Laemmli buffer supplemented with 25% β-mercaptoethanol) and boiled for 5 minutes. They were then subjected to sodium-dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in 12% acrylamide gels and the resulting bands were electroblotted onto a nitrocellulose membrane. Non-specific sites were blocked by incubation with 2% non-fat dry milk in 1X Tris-buffered saline (TBS) solution for 1 hour at room temperature. Membranes were incubated overnight at 4°C with a goat pAb directed against S protein (70-HG15), a mouse anti-E2 mAb (clone AP33) or a goat anti-S pAb (R247). The membranes were then washed with 0.3% Tween® in 1X TBS solution and incubated with horseradish peroxidase-conjugated secondary antibodies for 1 hour at room temperature. Membranes were washed again with 0.3% Tween® buffer and then developed by enhanced chemiluminescence.

For ELISA, particles with equal amounts of SHBsAg (5 µg) were captured with lectin (*Galanthus nivalis*) (2 µg/well) on 96-well plates. Primary antibodies detecting the HCV E2 protein were added: the mouse anti-E2 mAbs (clones AP33 and H52) or the human anti-E2 mAb (clone AR3A). Then, HRP-conjugated secondary antibodies rat anti-mouse IgG1 (H+L) (clone Sb77e) or mouse anti-human IgG Fc (clone JDC10) were incubated. HRP substrate (O-phenylenediamine dihydrochloride (OPD)) was incubated to generate a yellow-orange product. The reaction was

stopped with 50 µL of H_2SO_4 2N and the plate was read at 490 nm and 630 nm, the reference wavelength.

2.1.3 *Immunization of New Zealand rabbits with chimeric HBV-HCV S+E2^{1a}S, S+E2^{3a}S, S+E2^{4a}S particles*

Groups of 8 female New Zealand rabbits were immunized with 15 µg of SVPs prepared with AddaVax™ (oil-in-water nanoemulsion, an MF-59-like adjuvant) at days 0, 14 and 28 as follows: a) S+E2^{1a}S particles b) a cocktail of S+E2^{1a}S, S+E2^{3a}S and S+E2^{4a}S particles (5 µg of each one) or c) S+E2^{1a}S, S+E2^{3a}S or S+E2^{4a}S particles administered sequentially. Control groups were composed of 4 rabbits immunized with either S particles or AddaVax™ adjuvant alone. Blood sampling was done at days 0, 12, 26, 42 and 56 to analyze humoral responses. Immunizations and sampling were performed by Agro-Bio (La Ferté Saint-Aubin, France), a company accredited by the French authorities for the performance of protocols on laboratory animals in accordance with European Directive 2010/63/EU. The protocol was approved by Agro-Bio Ethics Committee C2EA-99 (French Ministry of Research, agreement B45-146-01).

2.1.4 *Analysis of humoral responses (**ongoing**)*

Antibodies against S protein were analyzed with a chemiluminescent microparticle immunoassay using the ALINITY system (Abbott Diagnostics). Anti-E2$^{1a/3a/4a}$ will be evaluated through an "in-house" ELISA previously described (Beaumont et al., 2013, 2016, 2020). Briefly, BHK-21 cells will be transfected by electroporation with SFV-derived vectors: pSFV3-β-gal (control) or pSFV-E2 (genotypes 1a, 3a or 4a). Cell lysates (E2^{1a},E2^{3a} and E2^{4a}) will be mixed in equal protein amounts and used as solid phase for all the ELISAs. For "in-house" ELISAs, 2 µg/well of cell lysates (β-gal or mix of E2 proteins) will be captured on 96-well plates by lectin (*Galanthus nivalis*), previously saturated by incubation with a blocking solution (10% nonfat dry milk + 5% sheep serum-TBS 1X) for 4 hours at 37°C. A second blocking of non-specific binding sites will be performed after incubation of lysates under the same conditions. Then, 100 µL of serum from immunized rabbits (day 0, 12, 26, 42 or 56) will be added to the plates and incubated for 1 hour at 37°C. Secondary HRP-conjugated goat F(ab')$_2$ anti-rabbit IgG (H+L) antibody will be added and incubated for 1 hour at 37°C. After that, the OPD (an HRP substrate) will be added and the reaction will be stopped after 15 minutes with 2 N H_2SO_4. Absorbance will be read at 490 nm and the OD obtained in β-gal wells will be subtracted from the OD obtained for wells with E2 proteins lysates.

2.1.5 *Analysis of the neutralizing properties of antibodies against genotypes 1a, 3a and 4a HCVcc (**ongoing**)*

The neutralizing properties of antibodies will be evaluated *in vitro* with the HCVcc model. HCVcc harboring HCV envelope proteins from genotype 1a (H77 isolate), 3a (S52 isolate) and 4a (ED43 isolate) were previously generated by the transfection of hepatoma Huh7.5 cells with the full genomic HCV RNA, transcribed *in vitro*, as previously described (Gottwein et al., 2009; Kato et al., 2003). Viral infectivity was determined in an FFU staining assay described elsewhere (Beaumont et al., 2013). Briefly, 10 000 cells/well on a 96-well plate were infected with the HCVcc at different dilutions and incubated for 6 hours at 37°C, under a humidified atmosphere containing 5% CO_2. The inoculum was removed and replaced by fresh medium. Cells were incubated again at 37°C and the infection was stopped 48 hours later. FFUs were stained with a colorimetric enzyme immunoassay.

For neutralization assays, 50 µL of inoculum, corresponding to 100 FFUs, will be mixed with 25 µL of a 1:5 dilution of rabbit serum (day 0 or 56) and incubated for 1 hour at 37°C. We will then infect 10 000 Huh7.5 cells with 75 µL of the virus-serum mixture for 6 hours at 37°C. The next steps of the protocol will be the same as for the infectivity test, described above. The percentage of neutralization will be determined by subtracting the percentage of neutralization obtained with the pre-immune serum (day 0) from that obtained with the post-immune serum (day 56) from the same rabbit.

2.2 RESULTS

2.2.1 *Characterization of subviral particles*

Particles were produced and purified with a mean purity of 15%. The presence of proteins of interest was first evaluated by western blotting. We observed the two forms (unglycosylated and glycosylated) of HBV S proteins at 24 and 27 kDa, respectively, using the anti-S pAb (70-HG15) in all the SVP preparations (Figure 36A). The incorporation of chimeric E2-S proteins was evaluated with the mAb AP33 (Figure 36B), but it could only detect $E2^{1a}S$ and $E2^{4a}S$, not the $E2^{3a}S$, which may be due to a sequence divergence ($E2^{3a}S$ homology of 74% and 67,1% with $E2^{1a}S$ and $E2^{4a}S$, respectively) previously evaluated (Figure S2 in Appendix A, p. 219 and p. 222). Therefore, we used another anti-S antibody pAb (R247) to attempt the detection of the chimeric $E2^{3a}S$ protein (85 kDa) through the recognition of the S domains, which was difficult to determine with the pAb 70-HG15 because of the background noise. The western blotting with the pAb R247 allowed the observation of a band corresponding to the size of the E2-S protein in SVPs

S+E2^{1a}S and S+E2^{3a}S SVPs, but not in S+E2^{4a}S SVPs (FIGURE 37), which was already verified by other antibodies.

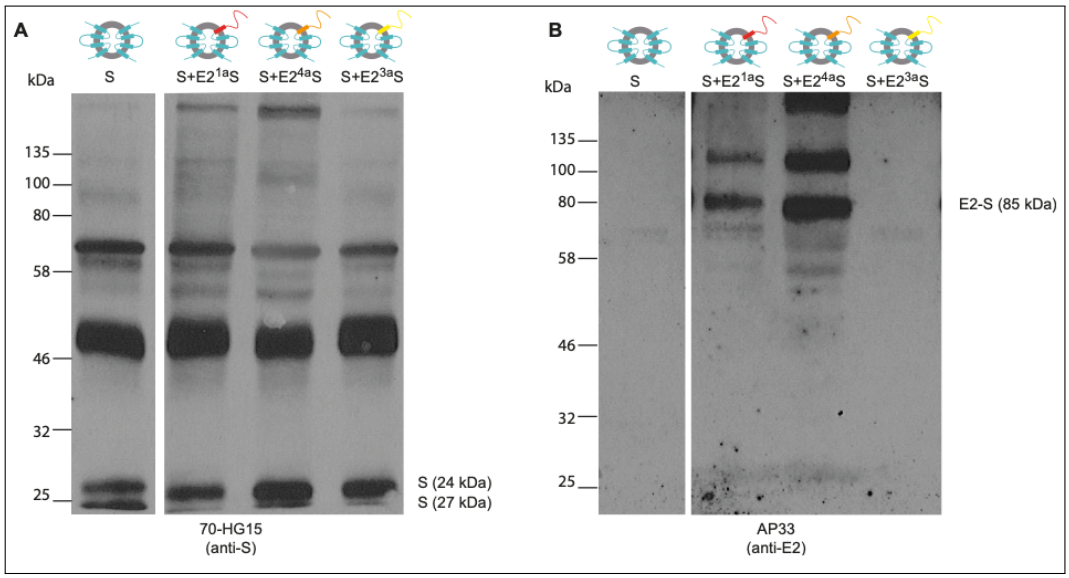

Figure 36: Western blotting of chimeric HBV-HCV subviral particles for the detection of proteins of interest. A. The HBV S protein was detected by the goat anti-S polyclonal antibody (70-HG15) at 24 and 27 kDa (unglycosylated and glycosylated forms) for all the particles. **B**. The chimeric E2-S proteins (85 kDa) were detected by the mouse anti-E2 monoclonal antibody (clone AP33) at 85 kDa for the S+E2^{1a}S and S+E2^{4a}S subviral particles.

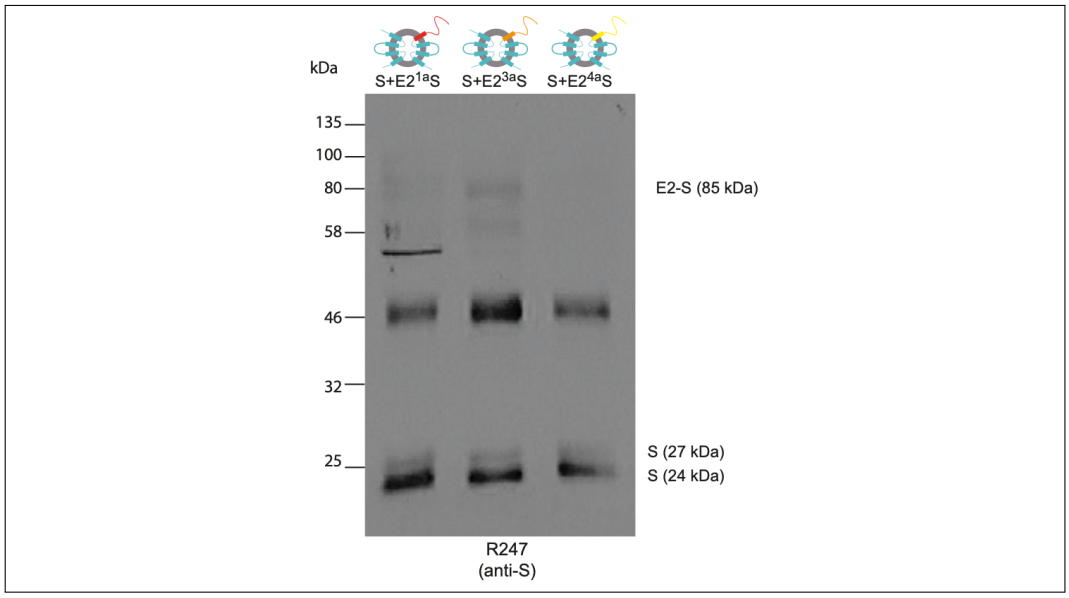

Figure 37: Western blotting of chimeric HBV-HCV subviral particles for the detection of the HBV S protein. The two forms (unglycosylated and glycosylated) of the HBV S proteins were detected (24 and 27 kDa) in all the particles, while the chimeric E2-S protein (85 kDa) was only detected for S+E2^{1a}S and S+E2^{3a}S, by the rabbit anti-S polyclonal antibody R247. We observe a band (~58 kDa) that do not correspond to the proteins of interest that might be due to a problem in the revelation in X ray films.

To validate the presence of E2-S proteins on particles, we performed ELISA experiments using different antibodies that bind to the HCV E2 protein (FIGURE 38). The mAb AR3A, recognizing a conformational epitope on the E2 protein, was able to bind the E2 protein in the 3 different SVPs, but with different affinities. In addition, we used mAbs H52 and AP33 which bind the linear epitopes aa 644-655 and aa 412-423, respectively, on the HCV E2 protein. The mAb AP33 presented similar results in ELISA as in the western blotting (FIGURE 36B): no recognition of the chimeric E2^{3a}S protein. While the mAb H52, the epitope of which is near the C-terminus of the HCV E2 protein, showed a good binding to the E2^{1a}S, followed by E2^{3a}S, but no detection of the E2^{4a}S protein. Therefore, we could validate the presence of all proteins of interest despite the high variability of the HCV E2 proteins.

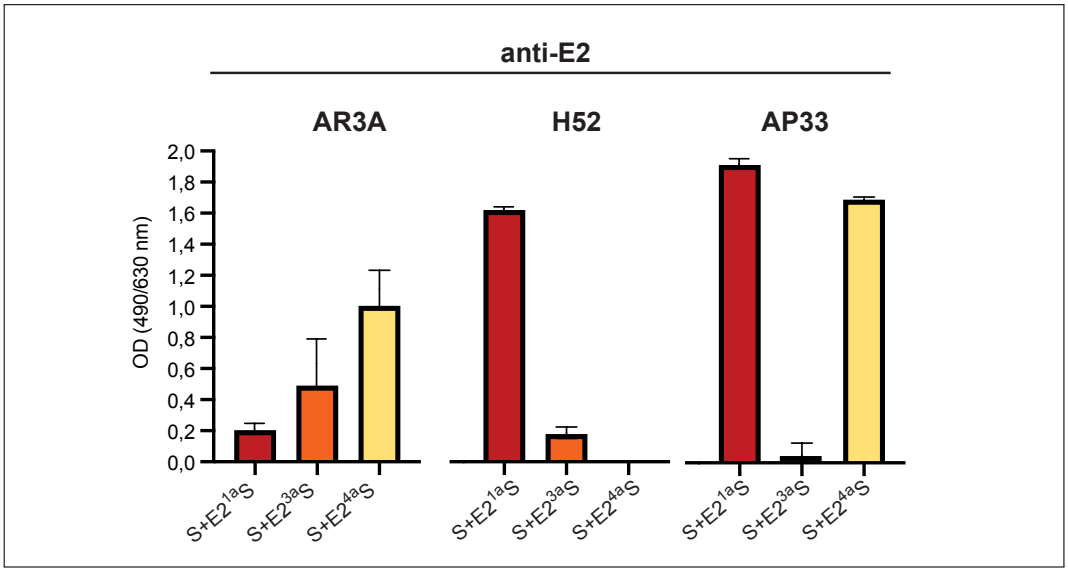

Figure 38: Detection of genotype 1a, 3a and 4a HCV envelope E2 protein on the surface of chimeric HBV-HCV particles. Subviral particles (5 µg of SHBsAg) were captured with lectin on 96-well plates. Then, primary antibodies against E2 protein were added: the human anti-E2 monoclonal antibody (mAb) AR3A recognizing a conformation-dependent epitope and the mouse anti-E2 mAbs (clone AP33 and H52) recognizing linear epitopes. Secondary antibodies anti-mouse IgG1 (H+L) (clone Sb77e) or anti-human IgG Fc (clone JDC10) were incubated. The HRP substrate (O-phenylenediamine dihydrochloride) was added to generate a yellow-orange product measured at 490 nm and a reference 630 nm, to obtain the optical density (OD) 490/630 nm.

Once particles were characterized, they were prepared with AddaVax™ in a 1:1 (vol:vol) ratio for the immunization of rabbits. The immunization protocol was executed as illustrated on FIGURE 39. All serum samples have been collected, and the evaluation of the humoral responses and the neutralizing properties of antibodies is currently ongoing.

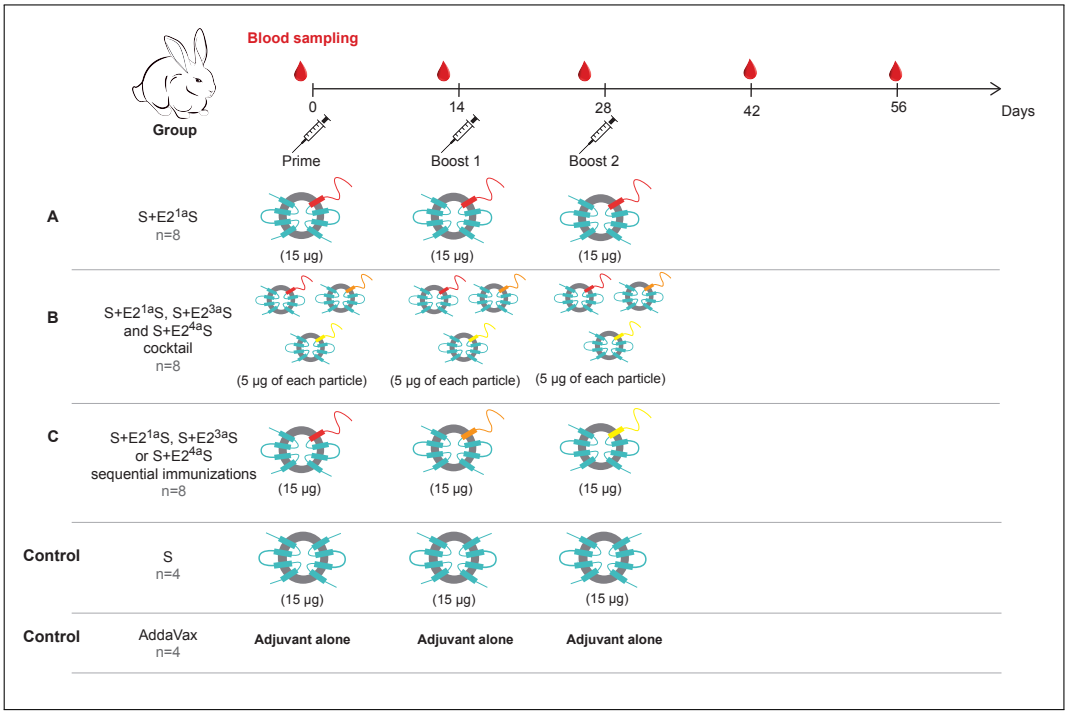

Figure 39: Immunization protocol of New Zealand rabbits with chimeric HBV-HCV particles bearing HCV E2 envelope protein from different genotypes (1a, 3a and 4a). Groups of 8 female New Zealand rabbits were immunized at days 0, 14 and 28 with 15 µg of adjuvanted (AddaVax™) S+E2^{1a}S or a cocktail of S+E2$^{1a+3a+4a}$S (5 µg each particle) or S+E2^{1a}S→S+E2^{3a}S→S+E2^{4a}S administered sequentially. Samples were obtained at days 0, 12, 26, 42 and 56 for the evaluation of humoral responses.

2.2.2 *Analysis of humoral responses induced by immunization of rabbits with SVPs of interest*

Anti-S responses from the serum samples (days 0, 12, 26 and 42) were already evaluated (FIGURE 40). We observed that antibodies against the HBV S protein were developed for all the rabbits immunized with SVPs. Anti-S titers from the 3 test groups (S+E2^{1a}S, cocktail of S+E2$^{1a+3a+4a}$S and sequential injections of S+E2$^{1a\rightarrow3a\rightarrow4a}$S) at day 42 were compared using the non-parametric Kruskal-Wallis test ($p<0,05$) and found to be not significatively different ($p=0,1759$). We expect that the anti-S responses will continue to increase by day 56, and some of the rabbits will reach the 1000 mIU/mL, as observed in other immunization protocols. If the evaluation of humoral responses is concluded in time, results will be presented during the thesis defense.

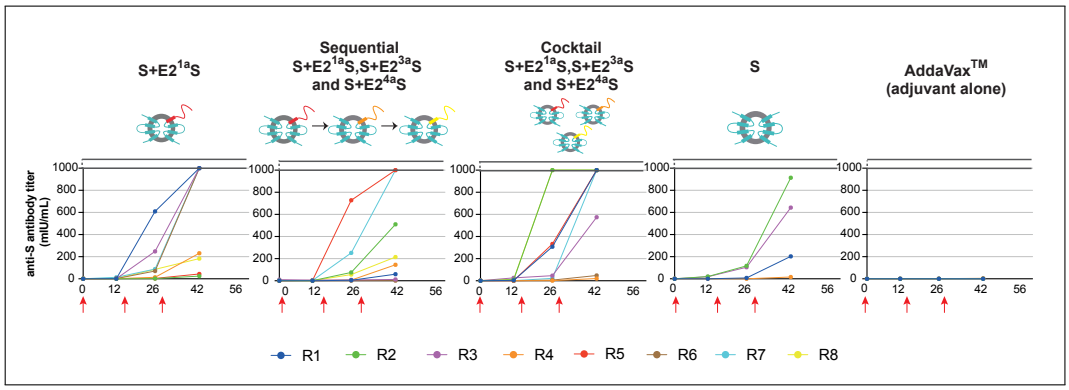

Figure 40: Preliminary results on the analysis of humoral responses. Anti-S antibodies of serum samples (days 0, 12, 26 and 42) from rabbits immunized with S+E2^{1a}S particles, S+E2$^{1a\rightarrow3a\rightarrow4a}$S sequentially administered, a cocktail of S+E2$^{1a+3a+4a}$S particles, S particles or adjuvant (AddaVax™) were analyzed with a chemiluminescent microparticle immunoassay using the ALINITY system (Abbott Diagnostics). Red arrows indicate the time at which immunizations occurred (days 0, 14 and 28).

Part IV

DISCUSSION

An HCV vaccine is needed to control the burden of hepatitis C. Although the DAAs are very effective to cure HCV infection, HCV can leave a molecular scar and liver diseases can continue to progress even after treatment in some patients. The high genetic variability of HCV and its viral escape mechanisms make the development of an HCV vaccine a difficult task. We have previously reported the generation of chimeric HBV-HCV particles that induce in the animal model antibodies able to neutralize efficiently HCVpp and HCVcc from closely related genotypes. Therefore, we aimed to increase the neutralization potential of these antibodies and to increase their cross-neutralizing properties against genetically distant genotypes. During the conduct of this project, we first explored the association of HCV with apoE, which was previously suggested to be important for the generation of an HCV vaccine because of its association to the envelope proteins and its impact on the neutralization sensitivity modulation (Fauvelle et al., 2016a; Wrensch et al., 2018).

Indeed, both HCV envelope proteins have been proposed to associate with apoE (Boyer et al., 2014; Lee et al., 2014; Mazumdar et al., 2011). We showed that both HCV envelope proteins could interact with apoE, not only by co-IP experiments but also by producing chimeric HBV-HCV SVPs carrying apoE on their surface. However, the amount of apoE detected on the surface of particles differed for each type of SVPs. Particles presenting the HBV S and the E2-S proteins incorporated more apoE, followed by S+E1-S+apoE and S+apoE particles. In addition to the apoE-HCV envelope proteins interactions, we observed that SVPs made with the S protein alone also incorporated and exposed apoE on their surface, suggesting a potential interaction of apoE with the HBV S protein. This finding is in agreement with the recent results regarding the implication of apoE on the production of infectious HBV (Qiao and Luo, 2019). Therefore, we first hypothesized that the cumulative interactions of apoE with S and E2 in the particle formation process might provoke the capture of an increased amount of apoE into S+E2-S+apoE particles. Regarding the S+E1-S+apoE particles, we observed little difference in the amount of apoE incorporated in these SVPs compared to S+apoE particles, despite the difference of apoE observed in CHO cell lysates (Appendix C). This observation suggests that the E2 protein might contain more binding sites to apoE than the E1 protein, resulting in a higher amount of apoE incorporated. This is further supported by studies detecting higher amounts apoE than E2 proteins on the viral surface (Catanese et al., 2013; Merz et al., 2011). In addition, the interaction apoE-E1 might be less strong than the apoE-E2 one, which was shown before to be resistant to stringency implying a tight association (Lee et al., 2014).

In the E1 protein, Mazumdar and colleagues suggested that the N-terminal ectodomain of this protein oriented in a specific conformation might be involved in the interaction with apoE (Mazumdar et al., 2011). For the interaction of apoE

with the E2 protein, Fauvelle and collaborators proposed that the region surrounding the aa 447 in E2 might be responsible for the interaction with apoE. Because they also showed that depletion of apoE uncovered domains B and C of E2, which are composed of discontinuous epitopes near the CD81 binding region, we hypothesize that there are in fact more than one binding site in E2 to apoE (Fauvelle et al., 2016a). Structurally, the binding of apoE to the E2 protein may change the conformation of E2, allowing the presentation of sites naturally exposed on the surface of HCV. Lee and colleagues also attempted to find the region of E2 involved in this interaction and found that the deletion of the TMD of the E2 protein abrogated the apoE-E2 association (Lee et al., 2014).

Concerning the region(s) of apoE involved in the interaction with envelope proteins, they have not been determined yet. However, for the association of apoE with the NS5A protein Cun and colleagues found that the region comprised between aa 205-280 at the C-terminal domain of apoE was indispensable. This region in apoE has been previously identified for the binding with lipoproteins (Cun, Jiang, and Luo, 2010; Wang et al., 1996). Moreover, Lee and collaborators showed that the interaction apoE-E2 was a protein-protein interaction, and not a lipid-mediated one (Lee et al., 2014). In the case of apoE-HCV envelope protein association, it could be possible that the same region may be involved in order to leave the N-terminal domain free for the interaction with the cellular receptors at the entry step of the viral cycle.

The co-expression of apoE altered the amount of the chimeric HBV-HCV envelope proteins incorporated into the SVPs. The S+E1-S+apoE SVPs contained 1,5-fold more E1-S protein than the S+E1-S particles, while the S+E2-S+apoE SVPs incorporated 3,4-fold less E2-S protein than particles without apoE. These HCV envelope proteins were found on the surface of SVPs and characterized by using mAbs targeting different epitopes on these proteins. For instance, we used the human mAb H-111 and the mouse mAb A4 that target overlapping regions between the aa 192-207 at the N-terminal domain of the E1 protein (Dubuisson et al., 1994; Keck et al., 2004). The recognition of E1 in HBsAg-normalized particles by both antibodies was better in SVPs containing apoE. In fact, the greater difference was observed with the H-111 mAb (>2-fold), one of the few HCV-neutralizing antibodies targeting E1. The normalization of ODs related to the amount of E1 protein showed that this protein in particles containing apoE was better or equally recognized by the H-111 or the A4 mAbs, respectively. These observations suggest that the conformation of E1 protein may be improved by its association with apoE.

The detection of the E2 protein on the surface of HBsAg-normalized particles by the mouse AP33 and the human AR3A mAbs was inferior for SVPs bearing apoE (~1,5-fold with both antibodies). When the ODs were related to a normalized amount of E2 protein, the tendencies were inverted. Results with the mAb AP33

suggest that in SVPs bearing apoE, the β-hairpin conformation of the epitope I of E2 may be better recognized than in S+E2-S particles (Kong et al., 2012b). The AR3A is a bNab that binds a discontinuous epitope on the antigenic domains B and D of the E2 protein (Law et al., 2008). Because the domains B and C of E2 have been proposed to be involved in the interaction with apoE, the association of this protein may interfere with the binding of the mAb AR3A. However, in particles bearing apoE, these domains of E2 were better recognized by the mAb AR3A than in those without apoE. We thus believe that the interaction apoE-S in S+E2-S+apoE particles may also change the way apoE associates with E2, allowing the binding of the conformation-dependent mAb in spite of the low amount of E2 protein.

Despite the higher amount of E1 protein in S+E1-S+apoE than in S+E1-S particles quantified by densitometry, the particles bearing apoE did not elicit greater antibody titers than those that do not. However, the antibody levels induced against E1, detected by ELISA after immunization with S+E1-S+apoE SVPs were remarkably more homogeneous than those induced with S+E1-S particles. We suppose that the homogeneity in the anti-E1 responses may be due to the estimated improved conformation of the E1 protein in the presence of apoE. The interaction apoE-E1 might stabilize the conformation of E1 protein, and thus increase the recognition of epitopes by B cells that will generate specific high affinity antibodies. An immunomodulatory role has been attributed to apoE, as it participates in the presentation of lipid antigens to a specific subset of T cells that promote the activation of B cells (Allan et al., 2009; Barral et al., 2008; Van Den Elzen et al., 2005). Therefore, we cannot exclude that apoE might have a role in the modulation of the humoral responses.

Concerning the titers of antibodies elicited against E2 by S+E2-S+apoE particles, they were well inferior than those induced by S+E2-S SVPs. This result is not surprising, considering the 3,4-fold lesser amount of E2 protein on particles carrying apoE. However, when we compared the antibody titers at days 26 and 42, some rabbits immunized with S+E2-S+apoE particles showed titers above those from the S+E2-S group. Unfortunately, antibodies from S+E2-S+apoE-immunized rabbits dropped immediately by day 56, for unknown reasons. We hypothesize that the earlier responses may be related to the immunomodulatory role of apoE, and the drop of titers may be due to the low amount of the E2 antigen presented, leading to non-sustained antibody responses.

The *in vitro* evaluation of the neutralizing potential of anti-E1/-E2 antibodies was done using the HCVcc model. First, we performed neutralization assays with HCVcc harboring HCV envelope proteins from a genotype 1a isolate (H77/JFH1), the same genotype as the HCV envelope proteins presented in the SVPs. As observed for the anti-E1 responses, the neutralization of HCVcc with antibodies elicited

with S+E1-S+apoE were more homogeneous, but also slightly higher (not significant) than with antibodies from rabbits immunized with S+E1-S particles.

The analysis of the neutralization of HCV with antibodies elicited with S+E2-S+apoE or S+E2-S particles was more complex because by day 56, anti-E2 titers were very low for the S+E2-S+apoE group. The assay was performed for both groups under the same conditions and the results that we obtained were not a surprise: the % of neutralization of HCV for the rabbits immunized with particles bearing apoE was significantly lower compared to those immunized with S+E2-S SVPs. However, we should consider that the number of antibodies incubated to neutralize HCV was not the same. In other flavivirus models, a number of 30 antibodies has been predicted to be the minimal quantity for neutralization of one virion [reviewed in (Pierson and Diamond, 2015)]. In our study, it is likely that insufficient antibodies in sera from rabbits immunized with S+E2-S+apoE particles to neutralize HCVcc may be the reason of the inferior % of neutralization.

To evaluate the actual neutralizing potential of antibodies, we selected the best neutralizing sera and we calculated the % of neutralization to a normalized antibody titer. By doing this, we observed an inversion of the tendency: the neutralizing potential of antibodies elicited by S+E2-S+apoE particles was significantly higher than that of antibodies induced by S+E2-S particles. These results suggest that the anti-E2 responses from vaccination with particles bearing apoE may mediate an improvement of HCV neutralization. The same results were observed for the neutralization of a genotype 2a HCV isolate (WT JFH1): slight improve in the neutralization by antibodies from S+E1-S+apoE-immunized rabbits, significantly lower neutralization by antibodies from S+E2-S+apoE-immunized rabbits and increased neutralizing potential for S+E2-S+apoE-induced antibodies when the % of neutralization was calculated regarding antibody titers. Therefore, the impact of apoE could be transposable for at least these 2 HCV genotypes.

The main inconvenient of our vaccine particles bearing the E2 protein and apoE was the low amount of incorporated E2 protein. This problem needs to be addressed to balance the amount of both proteins (apoE and E2) on SVPs and generate enough amounts of anti-E2 antibodies. In fact, when we generated the CHO clones expressing S+E2-S+apoE SVPs, we verified the expression of all proteins of interest by western blotting (Appendix C). The results showed an equivalent E2 protein expression in CHO-S+E2-S and CHO-S+E2-S+apoE clones. Therefore, we believe that the increased incorporation of apoE in particles bearing the S and E2 proteins may occur during the particle formation process and might be triggered by apoE-S interaction and the presumably multiple binding sites implicated in the apoE-E2 association.

When we realized the poor antibody responses induced by S+E2-S+apoE particles, we developed a CHO cell clone stably expressing both envelope proteins

and apoE to produce S+E1-S+E2-S+apoE SVPs. The amount of envelope proteins exposed on the surface of SVPs was verified by ELISA, and specifically for the E2 protein it was compared to that observed for the S+E2-S+apoE particles (Appendix D). We observed that the E2 protein was recognized 1,7-fold greater in S+E1-S+E2-S+apoE than in S+E2-S+apoE particles by the mAb AP33, and the detection of E2 protein was equivalent to the SVPs without apoE. The humoral responses induced by immunization with particles bearing both HCV envelope proteins with or without apoE are currently under study and we expect to obtain complementary results on the impact of apoE.

Besides the anti-E1/-E2 antibodies, we measured the antibodies against apoE. The human apoE (isoform E3) exposed at the surface of the vaccine particles has 74,8% homology with the apoE found in rabbits (Appendix E), we thus expect the development of some antibodies. We observed a basal level of anti-apoE antibodies detected in all rabbits before immunization, but the anti-apoE titers increased in rabbits immunized by SVPs bearing apoE or not, indicating the presence of cell or serum remnants that may have biased our results. The lack of neutralization from antibodies induced by the S+apoE particles suggests that if they were antibodies directed against apoE, their presence did not seem to influence the HCV neutralization.

The second approach of this project was the sequential immunization of rabbits with SVPs bearing HCV envelope E2 proteins from genotypes 1a, 3a or 4a. The particles were produced, purified and characterized by using multiple antibodies recognizing the S and the E2 proteins. The main difficulty was the recognition of the E2 proteins from different HCV genotypes, for which several antibodies were used. The AR3A mAb, recognizing a conformational-dependent epitope, allowed us to verify the presence of the E2 protein on the surface of the S+E2^{1a}S, S+E2^{3a}S and S+E2^{4a}S, but within different affinities. We believe that these differences are partly due to the quantity of E2 incorporated on the particles, of which we cannot be certain because not all the E2 proteins from the different SVPs could be observed in the same western blotting experiment. Additionally, it has been suggested that the conformation of the E2 protein on the surface of virions shows some flexibility, specifically on its CD81bl, which is comprised in the AR3A epitope, explaining the different ODs obtained with the mAb AR3A (Vasiliauskaite et al., 2017). Concerning the mAb AP33, it binds the epitope I that is conserved among genotypes but cases of viral escape have been reported. In fact, the E2^{3a}S protein presents two substitutions in two residues at the N-terminus of the epitope (aa 412-423 QL**I**N**T**NGSWHIN) (I414V and T416A) not observed in the E2^{1a}S or the E2^{4a}S (Figure S2 in Appendix A, p. 219 and p. 222). These mutations have been reported to reduce the binding of antibodies recognizing the β-hairpin conformation of the epitope I (Aleman et al., 2018; Alhammad et

al., 2015a,b), which may explain the lack of detection of our genotype 3a HCV E2 protein. Regarding the epitope of mAb H52 (aa 644-655 **E**HR**LQA**ACNWTR), several mutations were also observed for the $E2^{3a}S$ (L647F and Q648T) and the $E2^{4a}S$ (E644K, L647M and A649P) proteins, not previously documented as escape mutations, but that may explain the differences in the binding of mAb H52 to the proteins.

The first analyses of the anti-S responses have shown no significant differences between the rabbits immunized with different regimens by day 42, as expected. The control groups also responded as expected, rabbits immunized with the S particles developed antibodies against the HBsAg, while those immunized with adjuvant alone showed no antibodies. To determine the impact of the sequential immunization strategy, we plan to complete the analyses of the humoral responses induced against our vaccine particles.

Part V

CONCLUSIONS AND PERSPECTIVES

The HCV envelope proteins are the main targets of Nabs and thus, essential elements for the induction of humoral responses to control HCV infection. Vaccine candidates against HCV should present at least the HCV E2 protein, containing most of the neutralizing epitopes of the virus, in a native-like conformation to elicit bNabs by immunization. However, HCV can escape the immune system by several mechanisms such as the association with lipids. We considered this association by producing and purifying chimeric HBV-HCV SVPs that carry apoE, which has been shown to interact with HCV envelope proteins and modulate the sensitivity to antibody-mediated neutralization. The presence of apoE on the surface of SVPs had an impact on the folding of the HCV envelope proteins presented, as well as on the neutralizing potential of the antibodies against the HCV E2 protein. We concluded that apoE could probably improve vaccine strategies based on the HCV envelope proteins. However, one limitation concerning our SVP vaccine strategy is the low level of incorporation of the E2 protein in the SVPs when apoE is co-expressed in CHO cells. The reasons for this low incorporation are not known but could be related to a defect of incorporation of the chimeric protein E2-S into the SVP due to its strong interaction with apoE. Fortunately, we have since produced SVPs that incorporate both E1-S and E2-S chimeric proteins and the level of incorporation of the E2-S is better in this case. Therefore, we expect the level of anti-E2 antibodies to be higher with this vaccination strategy. We also plan to evaluate the neutralization potential of the antibodies induced by immunization with particles bearing apoE towards HCV produced in culture systems allowing a similar lipidation to that observed in LVPs found in the bloodstream of HCV-infected patients. This will be done with highly infectious native-like hepatitis C virus particles produced by a model of differentiated cells in prolonged hypoxia, recently established in our laboratory.

In this project we also attempted to improve the affinity maturation of B cells by sequential immunization of rabbits with HCV E2 proteins from genotype 1a, 3 and 4 isolates, chosen because they are the most prevalent in the world. The main difficulty on the execution of this approach was that particles bearing the genotype 3 E2 protein were hard to characterize because of the high degree of genetic variation at epitopes of the antibodies used for this purpose. The immunization through a sequential approach has been shown to generate broad neutralizing humoral responses against other viruses such as HIV, influenza and dengue viruses (Hou et al., 2020; Liu et al., 2019; Sprenger et al., 2020; Wang et al., 2015). In the case of our strategy, the E2 envelope proteins used for this protocol shared around 70% of the residues and were injected in equal amounts as follows: genotype 1a→genotype 3a→genotype 4a. We expect to complete the analysis of the humoral responses induced by this strategy and to compare the results from those

obtained in our previous study that simply used a mixture of these SVPs bearing the different E2 proteins.

The vaccine candidate proposed here has been exclusively evaluated for the induction of neutralizing antibodies. However, the clearance of the HCV has been associated to the induction of both immune responses, humoral and cellular (Osburn et al., 2010, 2014). Therefore, it would be interesting to evaluate as well the induction of cellular responses after immunizations of small animals with our vaccine particles. This could done by techniques such as the enzyme-linked immunospot (ELISpot) for the detection of cytokine-secreting cells. The presence of cellular responses after immunization would be an added value for our bivalent HBV-HCV vaccine.

HCV envelope proteins are highly glycosylated and the N-glycans have shown to play a role in the escape from the immune system (Helle et al., 2010; Pantua et al., 2013). Moreover, a group from the Shanghai Pasteur Institute showed that the production of their sE2 vaccine candidate in insect cells led to a high production yield of proteins with better immunogenicity and ability to induce cross-neutralizing antibodies, in comparison to the sE2 produced in mammalian cells (Li et al., 2016). The hypothesis put forward by the authors was a potential increased flexibility of the protein due to the less complex glycosylation pattern. Therefore, to evaluate the impact of the different glycosylation patterns on the immunogenicity of our chimeric HBV-HCV vaccine particles, the use of different production systems such as yeast and insect cells is currently under study by our group. Concerning the yeast system, in addition to the modified glycosylation pattern from mammalian cells, it presents other several advantages for our vaccine candidate such as the established large-scale industrial processes, the high production yields and the reduced production costs verified for the production of many HBV vaccines.

Nevertheless, CHO cells are used for the production of most of the recombinant proteins and monoclonal antibodies approved for therapeutic use in humans (Fischer, Handrick, and Otte, 2015; Kim, Kim, and Lee, 2012). Today, our CHO cell clones producing the chimeric HBV-HCV vaccine particles are grown in adherent cultures with media containing animal serum. For the production of our particles in large-scale, CHO cells would need to be adapted to a serum-free medium and ideally to be grown in suspension in bioreactors (Moleirinho et al., 2019). These conditions may also allow the development of a more efficient purification process based on chromatography techniques that would improve the recovery and purity of the subviral particles (Zhao et al., 2019).

To sum up, potential successful vaccines need at least to achieve effective immune responses in animal models and to be produced in systems to obtain adequate purities and production yields to be eventually evaluated in humans. The

holy grail in the development of the HCV vaccine might be the induction of bNabs for the protection from infection and from the development of chronic hepatitis. The ongoing covid-19 pandemic outbreak has led to the rapid development and approval of vaccines, derived from nucleic acid and viral-vectored technologies, which have shown to protect at least partially from the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Therefore, if a prospect HCV vaccine can induce at least partial protection for the high-risk populations and from endemic genotypes in high prevalent regions, it would be a good start in the road of HCV eradication.

Part VI

RÉSUMÉ DE LA THÈSE EN FRANÇAIS

1 INTRODUCTION

1.1 L'HÉPATITE C : ÉPIDÉMIOLOGIE, DIAGNOSTIC ET TRAITEMENT

L'hépatite C, qui affecte 71 millions de porteurs chroniques du virus, constitue un réel problème de santé publique. La mortalité due aux hépatites virales continue d'augmenter chaque année, contrairement à celles relatives aux autres maladies infectieuses. C'est pour cette raison que l'Organisation Mondiale de la Santé (OMS) s'est donné pour objectif d'éradiquer l'hépatite virale à l'horizon 2030, en réduisant de 90 % l'incidence et de 60 % la mortalité associées (World Health Organization, 2017). Malheureusement, des études récentes ont montré que si les mesures actuelles n'évoluent pas, les objectifs d'éradication ne seront atteints que dans quelques pays. Ces études ont aussi montré la nécessité de mettre en place davantage d'initiatives pour le diagnostic et le traitement de cette maladie, tout en soulignant l'importance de les associer avec un vaccin contre le VHC (Heffernan et al., 2019; Hill, Nath, and Simmons, 2017; Razavi et al., 2020).

Le diagnostic est une étape essentielle pour l'élimination de l'hépatite virale. L'hépatite C chronique étant une maladie souvent asymptomatique, seuls 20 % des malades sont diagnostiqués et 13 % de ces individus diagnostiqués sont traités (World Health Organization, 2017). Les infections dues au VHC sont initialement détectées par des tests sérologiques basés sur des réactions immuno-enzymatiques qui permettent d'identifier les anticorps spécifiquement dirigés contre ce virus, signe d'une infection ancienne ou en cours. Dans un deuxième temps, l'identification d'une infection chronique en cours est réalisée par la quantification de l'ARN du VHC à l'aide de méthodes basées sur la réaction en chaine de la polymérase (Peeling et al., 2017). La détection de l'antigène de capside du VHC permet aussi un diagnostic moins onéreux des infections actives (Pérez-García et al., 2019; Tilborg et al., 2018). Des programmes nationaux pour le diagnostic des populations à risque ont été créés dans certains pays développés (Brouard et al., 2015; Mohamed et al., 2020).

Les principales populations considérées à risque sont les usagers de drogues injectables, les prisonniers, ainsi que les hommes ayant des relations sexuelles avec des hommes, car la transmission du VHC se fait par contact sanguin (Degenhardt et al., 2017). Les femmes enceintes co-infectées par le virus de l'immunodéficience humaine (VIH) et le VHC ont aussi un risque accru de donner naissance à des enfants infectés par le VHC, par rapport aux mères mono-infectées par le VHC

(Benova et al., 2014). Les co-infections par le VHC et d'autres virus comme le virus de l'hépatite B (VHB) augmentent le risque de développer une maladie chronique du foie (Pol et al., 2017; Yan et al., 2016).

L'hépatite C peut se manifester comme une infection aiguë qui peut évoluer soit vers une guérison, soit vers une infection chronique. L'hépatite aiguë est asymptomatique ou engendre dans la plupart des cas peu de symptômes (Manns et al., 2017). L'ARN viral peut être détecté quelques jours après le contact avec le virus, tandis que les anticorps anti-VHC n'apparaissent qu'à partir de la 4e semaine (Glynn et al., 2005). Dans 25 % des cas environ, les patients résolvent l'infection de façon spontanée. Par contre, plus de 70 % des patients développent une hépatite chronique (Westbrook and Dusheiko, 2014). L'hépatite chronique est associée à une fibrose du foie qui peut évoluer en cirrhose et carcinome hépatocellulaire. Certains facteurs de risque de développer un cancer du foie ont été identifiés : l'âge (>75 ans), le sexe (homme), la consommation d'alcool, les co-infections (VHB, VIH), les comorbidités (diabète, stéatose) et l'infection par des VHC de génotype 3 (McGlynn, Petrick, and El-Serag, 2021).

Dans le but de traiter l'hépatite C de façon efficace, des thérapies basées sur l'utilisation de l'interféron (IFN) et des agents antiviraux à action directe (AADs) ont été développées (Lindsay et al., 2001; McHutchison et al., 1998; Pawlotsky et al., 2014; Zeuzem et al., 2000). Les cibles des AADs sont les protéines non structurales (NS) du VHC NS3/4A, NS5A et NS5B. Ces médicaments ont conduit, chez plus de 90 % des patients, à une réponse virologique soutenue, 12 semaines après la fin du traitement (Pawlotsky et al., 2020). Un défi à relever par ces antiviraux est la haute diversité génétique du VHC.

Cette variabilité génétique est due au taux élevé de réplication du virus et à son ARN polymérase ARN-dépendante qui peut générer jusqu'à 10^{-3} erreurs par site et qui n'est pas pourvue d'une activité correctrice (Powdrill et al., 2011). C'est pour cette raison que 8 génotypes et plus de 67 sous-types du VHC ont été identifiés dans le monde (Hedskog et al., 2019; Simmonds et al., 1994; Smith et al., 2014). Le génotype 1 est le plus représenté, avec 44 % des infections, suivi par le génotype 3 (25 %) et le génotype 4 (15 %) (The Polaris Observatory HCV Collaborators, 2017). En plus des génotypes et des sous-types, des quasi-espèces du VHC, qui varient de 1 à 3 %, ont également été identifiées au sein d'un même patient (Bukh, 2016; Farci, Bukh, and Purcell, 1997; Martell et al., 1992). L'administration des AADs a été préconisée par l'Association européenne pour l'étude du foie (EASL) avec la recommandation d'identifier les sous-types du VHC par analyse phylogénétique chez les patients provenant des régions où des sous-types résistants aux antiviraux ont été préalablement repérés (Childs et al., 2019; Fourati et al., 2019; Pawlotsky et al., 2020).

En revanche, les AADs ne protègent ni d'une réinfection par le VHC, ni de la progression vers une maladie sévère du foie (Simmons et al., 2016b). En outre, le prix de ces médicaments dans les pays à faibles revenus demeure élevé, malgré les efforts déployés pour développer des médicaments génériques ou pour réduire leur coût à travers les programmes nationaux (Cooke et al., 2019). Par conséquent, le développement d'un vaccin pourrait être un outil pour surmonter les obstacles aux traitements actuels contre le VHC.

1.2 LE VIRUS DE L'HÉPATITE C

Le VHC est formé d'une nucléocapside qui contient une molécule d'ARN de polarité positive de 9,6 kb, enveloppée par une membrane dérivée du réticulum endoplasmique (RE) dans laquelle les protéines d'enveloppe (E1 et E2) sont ancrées. Le tout est associé à des lipides et lipoprotéines de faibles et très faibles densités (LDL et VLDL) issus de l'hôte et circule dans le sang sous la forme de particules lipovirales (LVPs). Ces particules ont une taille de 50 à 80 nm et une densité de 1,03-1,10 g/mL (André et al., 2002; Catanese et al., 2013; Gastaminza et al., 2010; Piver et al., 2017).

Le VHC s'attache à la surface des hépatocytes via des molécules, comme l'apolipoprotéine E (apoE), qui interagissent avec les récepteurs cellulaires comme le récepteur scavenger classe B type I (SR-BI), le récepteur des lipoprotéines de basse densité (LDLR) et le récepteur des lipoprotéines de très basse densité (VLDLR) (Agnello et al., 1999; Ujino et al., 2016; Voisset et al., 2005). Ensuite, la protéine d'enveloppe E2 entre en contact avec les récepteurs CD81 (cluster de différentiation 81) et claudine-1 (CLDN1) qui déclenchent l'internalisation du virus (Lupberger et al., 2011; Pileri et al., 1998; Zeisel et al., 2007). Le virus est internalisé par endocytose clathrine- et pH-dépendante. L'acidification au sein de la vacuole d'endocytose cause la fusion des membranes virale et endosomale, et une décapsidation concomitante (Blanchard et al., 2006; Haid, Pietschmann, and Pécheur, 2009). L'ARN génomique est alors libéré dans le cytoplasme et sert immédiatement d'ARN messager (ARNm) pour la traduction des protéines virales qui est initiée grâce au site d'entrée interne du ribosome (IRES) (Tsukiyama-Kohara et al., 1992; Wang, Sarnow, and Siddiqui, 1993). Le VHC possède un seul cadre ouvert de lecture qui génère une seule polyprotéine qui s'insère dans la membrane du RE. Elle est clivée par des protéases cellulaires (signal peptidase et signal peptide peptidase) et virales pour obtenir 3 protéines structurales (protéine de capside, E1 et E2) et 7 protéines non structurales (p7, NS2, NS3, NS4A, NS4B, NS5A et NS5B) (Moradpour and Penin, 2013).

Les protéines d'enveloppe E1 et E2 sont des protéines avec un domaine transmembranaire (TMD) C-terminal qui traverse la membrane du RE et un ec-

todomaine N-terminal orienté vers la lumière du RE (Cocquerel et al., 2002; Deleersnyder et al., 1997). Ces protéines forment des complexes à la surface des virions par la formation de ponts disulfures (Vieyres et al., 2010). La protéine E1 possède 190 acides aminés (aa) et 4 ou 5 sites de N-glycosylation (Meunier et al., 1999). Seule la structure du domaine N-terminal de cette protéine a été obtenue, car elle forme des agrégats en l'absence de la protéine E2 (El Omari et al., 2014). En outre, il a été montré que la protéine E1 forme des trimères à la surface des virions et participe à l'entrée et la fusion virales (Douam et al., 2014; Falson et al., 2015; Lavillette et al., 2007). Bien que cette protéine soit moins immunogénique que la protéine d'enveloppe E2, des anticorps ciblant certaines régions de cette protéine ont toutefois pu être isolés (Dubuisson et al., 1994; Keck et al., 2004; Kong et al., 2015; Meunier et al., 2008b).

La protéine d'enveloppe E2 est la mieux caractérisée en termes de structure et de sites antigéniques. Elle est formée de 365 aa et comporte 11 sites de N-glycosylation (Goffard and Dubuisson, 2003). De nombreuses structures de la protéine E2 ont été cristallisées et couplées à différents anticorps, isolés à partir de patients infectés par le VHC, permettant ainsi l'identification des caractéristiques structurales et de flexibilité de cette protéine (Flyak et al., 2018, 2020; Khan et al., 2014; Kong et al., 2013). La protéine E2 comporte aussi les régions hypervariables 1 et 2 (HVR1 et HVR2) et la région variable intergénotypique (igVR), correspondant à 25 % de la protéine (Hijikata et al., 1991; Weiner et al., 1991). La région HVR1 se trouve dans la partie N-terminale et peut induire la production d'anticorps ayant parfois des propriétés neutralisantes faibles (Farci et al., 1996; Hsu et al., 2003; Vieyres, Dubuisson, and Patel, 2011). Par contre, des études ont montré que cette région pouvait également masquer d'autres épitopes importants de par sa position et par l'interaction des anticorps avec cette région, bloquant ainsi l'accès aux anticorps neutralisants (Keck et al., 2016b; Prentoe et al., 2016). Trois épitopes (I-III), 5 régions (AR1-5) et 5 domaines (A-D) antigéniques ont été identifiés au sein de la protéine E2 (Giang et al., 2012; Keck et al., 2008, 2012, 2014; Kong et al., 2013; Law et al., 2008; Li et al., 2015; Meola et al., 2015; Vasiliauskaite et al., 2017). Les anticorps ciblant les domaines et régions antigéniques sont dépendants de la conformation de la protéine E2. L'étude de ces anticorps a permis d'identifier la face neutralisante de cette protéine, qui comprend la région antigénique 3 (AR3), les domaines antigéniques B et D, ainsi que les épitopes II et III.

La réplication de l'ARN viral a lieu dans des réseaux membranaires dérivés du RE avec la participation des protéines NS3/4A, NS4B, NS5A et NS5B (Ferraris, Blanchard, and Roingeard, 2010; Romero-Brey et al., 2012). L'ARN polymérase ARN-dépendante (NS5B) synthétise un brin d'ARN de polarité négative qui sert à son tour de matrice pour la synthèse de nouveaux brins d'ARN de polarité positive, qui constituent alors des génomes viraux ou des ARNm pouvant être

traduits en protéines virales. L'assemblage semble commencer par l'accumulation des protéines de capside à la surface des gouttelettes lipidiques, et se poursuit par l'association de l'ARN génomique avec la protéine de capside qui semble être engagée par la protéine NS5A (Barba et al., 1997; Miyanari et al., 2007). Les particules présentant des protéines d'enveloppe à leur surface seraient alors formées dans la lumière du RE où elles fusionneraient avec des particules lipidiques (Bartenschlager et al., 2011). Les LVPs seraient ensuite transportées à l'extérieur de la cellule en transitant dans des compartiments dérivés de l'appareil de Golgi utilisés pour la synthèse des VLDL (Syed et al., 2017).

Bien que le VHC soit étroitement associé au métabolisme lipidique, le mécanisme de formation des LVPs reste peu connu. La purification de LVPs circulant chez les patients a permis d'identifier des apolipoprotéines associées au VHC : l'apoA, l'apoB, l'apoC et l'apoE. Ces apolipoprotéines sont exposées à la surface des LVPs et des particules de VHC produites en culture cellulaire (VHCcc) et peuvent être échangées d'une particule à l'autre (André et al., 2002; Diaz et al., 2006; Mancone et al., 2011; Meunier et al., 2008a). L'apoE participe à de nombreuses étapes du cycle du VHC. L'apoE circulante est une protéine de 34 kDa, synthétisée principalement par le foie, qui joue un rôle dans la clairance et la stabilisation des lipoprotéines (HDL, LDL, VLDL et chylomicrons) (Phillips, 2014). Des 3 isoformes d'apoE identifiées (apoE2, apoE3 et apoE4), l'apoE3 est celle qui est la plus présente chez l'homme (70 %), suivie par l'apoE4 (20 %) et l'apoE2 (10 %) (Mahley, 1988). Cette isoforme majoritaire a été associée au développement d'une hépatite C chronique, alors que l'apoE4 présente plutôt une fonction protectrice (Mueller et al., 2016; Price et al., 2006; Wozniak et al., 2002). L'apoE est composée de deux domaines : un domaine N-terminal responsable de l'association avec des récepteurs des lipoprotéines (LDLR et VLDLR) et un domaine C-terminal qui contient le site d'association aux lipides (Chen, Li, and Wang, 2011; Hatters, Peters-Libeu, and Weisgraber, 2006).

Dans le cycle viral, l'apoE participe à l'attachement du VHC à la cellule par interactions avec des récepteurs cellulaires (Dao Thi et al., 2012; Lefèvre et al., 2014; Owen et al., 2009). En effet, des anticorps ciblant cette protéine sont capables d'inhiber cette étape du cycle viral (Jiang et al., 2012, 2013; Owen et al., 2009). De plus, l'infectivité des particules a été corrélée avec la quantité d'apoE présente à la surface des LVPs et à l'isoforme d'apoE considérée (apoE3>apoE2) (Hishiki et al., 2010). L'apoE participe aussi à l'assemblage et la genèse des nouveaux virions. Des études ont montré que la production du VHC par des cellules hépatiques pouvait être bloquée par une inhibition de l'expression de l'apoE, et ensuite restaurée par l'ajout ectopique de cette protéine (Jiang and Luo, 2009). La production des virions peut aussi être restaurée par l'ajout d'autres apolipoprotéines formées par des hélices α amphipathiques (Fukuhara et al., 2014).

Il a été également montré que l'apoE pouvait interagir avec certaines protéines du VHC comme la protéine NS5A (Benga et al., 2010; Cun, Jiang, and Luo, 2010) et les protéines d'enveloppe E1 et E2 (Boyer et al., 2014; Lee et al., 2014; Mazumdar et al., 2011). Les résultats issus des études d'interactions avec E1 et E2 sont toutefois contradictoires. Une étude a montré que l'apoE interagissait uniquement avec la protéine E1 (Mazumdar et al., 2011), alors que deux autres études ont trouvé que E2 était la protéine responsable de l'interaction entre l'apoE et la protéine E1E2 (Boyer et al., 2014; Lee et al., 2014). De plus, en étudiant la composition des LVPs, il a été montré qu'il y avait plus de molécules d'apoE que de protéines d'enveloppe E2, ce qui peut indiquer l'existence de plusieurs sites d'interaction de l'apoE avec la protéine E2, et ce contrairement à ce qui a pu être observé pour la protéine E1 (Catanese et al., 2013; Merz et al., 2011).

L'apoE joue aussi un rôle majeur dans l'échappement viral à la réponse immune. Il a été montré que l'apoE pouvait favoriser la transmission du virus par contact cellule-cellule en agissant conjointement avec les protéines CLDN1 et occludine présentes sur les membranes basolatérales des hépatocytes, évitant alors son contact avec le milieu extracellulaire (Gondar et al., 2015; Hueging et al., 2014). L'apoE peut aussi moduler la sensibilité du VHC à la neutralisation par des anticorps en changeant la configuration de l'interaction apoE-E2, et ce de manière dépendante de la quantité d'apoE présente dans les LVPs (Bankwitz et al., 2017; Fauvelle et al., 2016a).

1.3 DÉVELOPPEMENT D'UN VACCIN CONTRE L'HÉPATITE C

Puisque l'hépatite C est résolutive dans environ 25 % des cas, il apparait alors évident que certaines réponses immunes sont capables de contrôler efficacement cette infection (Micallef, Kaldor, and Dore, 2006; Osburn et al., 2010). Des études menées chez le chimpanzé ont montré que, dans le cas de réinfections après clairance virale, des réponses cellulaires et humorales pouvaient réduire la virémie (Lanford et al., 2004; Prince et al., 2005). Ces résultats ont encouragé le développement de vaccins contre le VHC.

La première ligne de défense contre un pathogène est la réponse immunitaire innée. Quand le virus entre dans la cellule, des motifs localisés dans son ARN génomique simple brin et dans son intermédiaire de réplication ARN double brin sont reconnus par des récepteurs de reconnaissance de motifs moléculaires comme RIG-I (gène I inductible par l'acide rétinoïque) et TLR3 (récepteur de type Toll) (Saito et al., 2008; Wang et al., 2009). L'interaction entre les motifs et leurs récepteurs déclenche la synthèse d'IFN et l'activation des gènes stimulés par l'IFN (Levy, Marié, and Durbin, 2011). Malheureusement, le VHC peut échapper au système immunitaire inné grâce à ses protéines core, NS3/4A et NS5A qui in-

hibent les voies de production de l'IFN (Bode et al., 2003; Lan et al., 2007; Vazquez, Tan, and Horner, 2019). Les cellules du système immunitaire qui participent principalement à cette réponse sont les cellules *natural killer*, les cellules de Kupffer et les cellules dendritiques (CDs). Ces cellules participent à l'élimination du virus en produisant des cytokines à effet antiviral ou activatrices des cellules immunitaires (Boltjes et al., 2014; Doyle et al., 2019; Wei et al., 2009). Les CDs sont aussi des cellules présentatrices d'antigènes qui vont donc contribuer au déclenchement de la réponse immune adaptative (Dolganiuc et al., 2008; Kadowaki et al., 2001).

Les CDs présentent ainsi les antigènes via leurs complexes majeurs d'histocompatibilité (CMH) de classe II aux cellules T CD4+ (Levin et al., 1993). Tandis que les cellules T CD8+ sont activées par la voie de la cross-présentation dans laquelle les CDs présentent l'antigène via leurs CMH de classe I (Shin et al., 2011). Les réponses cellulaires (CD4+ et CD8+) sont détectées chez les patients entre 8 et 12 semaines après la contamination. Les cellules T CD8+, après activation, deviennent des cellules effectrices qui secrètent de l'IFNγ et des cellules T mémoires (Kaech et al., 2002). Les cellules T CD4+ aident à maintenir les réponses T CD8+ et contribuent à la sélection des cellules B de haute affinité (Raziorrouh et al., 2011; Spaan et al., 2015; Thimme et al., 2001). Les cellules T folliculaires helper (Tfh ; sous-type de cellules T CD4+) participent spécifiquement au processus de maturation de l'affinité des cellules B, et tendent à disparaître au cours de la maladie (Cobb et al., 2018; Raziorrouh et al., 2016).

Dans une maladie chronique, les réponses cellulaires semblent être épuisées, c'est-à-dire non fonctionnelles, à cause de : l'expression altérée des récepteurs au sein des cellules T qui inhibent leur activité (Chen et al., 2020a; Radziewicz et al., 2007) , l'interaction prolongée des cellules T avec le virus (Bucks et al., 2009; Mueller and Ahmed, 2009) et la génération de mutations dans les épitopes des cellules T (Cox et al., 2005; Puig et al., 2006). Après traitement avec les AADs, les réponses cellulaires semblent être rétablies (Burchill et al., 2015; Meissner et al., 2017), mais il semblerait toutefois que l'hépatite C chronique laisse une empreinte moléculaire au sein des cellules T CD8+, en dépit d'une résolution de l'infection (Hensel et al., 2021).

En parallèle avec les réponses cellulaires, les réponses humorales débutent lors de la rencontre entre les cellules B et l'antigène. Ces cellules migrent aux ganglions lymphatiques et établissent des centres germinatifs (CGs), où elles subissent des hypermutations somatiques importantes pour la génération d'immunoglobulines ayant une haute affinité pour l'antigène. Dans ce processus, les cellules Tfh aident à la sélection des cellules B qui peuvent ensuite entrer dans les CGs ou devenir des cellules B plasmatiques ou mémoires (De Silva and Klein, 2015). Les cellules B plasmatiques produisent des anticorps sur le long terme et résident dans la moelle osseuse, alors que les cellules B mémoires restent dans un état quiescent

jusqu'à ce qu'elles soient réactivées lors d'une réinfection (Akkaya, Kwak, and Pierce, 2020).

Les anticorps anti-VHC peuvent être détectés dès la cinquième semaine suivant le contage viral (Glynn et al., 2005). La clairance spontanée du virus a été corrélée à une détection précoce de la réponse humorale (Osburn et al., 2014). Comme décrit précédemment, la cible principale des anticorps neutralisants est la protéine d'enveloppe E2, et plus spécifiquement la face neutralisante de cette protéine. Des études menées avec des anticorps isolés à partir de porteurs chroniques du virus et de personnes ayant guéri spontanément ont montré que, dans les deux cas, les anticorps ciblent les mêmes épitopes (Bailey et al., 2017; Giang et al., 2012; Law et al., 2008; Merat et al., 2016), mais que leur vitesse d'apparition est différente (Pestka et al., 2007). En effet, les anticorps issus d'une clairance spontanée présentent moins de mutations somatiques (processus de maturation rapide) et sont dérivés du gène codant la partie variable de la chaine lourde V_H1-69 (Bailey et al., 2017; Flyak et al., 2018; Tzarum et al., 2019). La capacité d'un vaccin à induire une réponse humorale soutenue est devenue de grande importance, surtout depuis l'échec récemment reporté des essais cliniques de phase 1 et 2 menés avec le vaccin connu pour induire une forte immunité T (Page et al., 2021).

Afin de vérifier l'efficacité des candidats vaccins, des modèles animaux sont nécessaires avant une évaluation chez l'homme. Le chimpanzé a beaucoup été utilisé par le passé pour des études visant une meilleure compréhension de la maladie (Bukh, 2004). Aujourd'hui, pour des raisons éthiques, cette pratique est toutefois régulée par des directives internationales. Par conséquent, des modèles animaux de petite taille comme des souris humanisées pour le foie et le système immunitaire ou génétiquement modifiées ont été établis (Burm et al., 2018). Ces nouveaux modèles permettent une infection par le VHC, mais qui n'est pas optimale pour l'évaluation des vaccins, du fait du déficit immunitaire des animaux. Des modèles *in vitro* sont donc utilisés pour évaluer le pouvoir neutralisant des anticorps induits par immunisation. Deux systèmes ont été mis en place : les pseudoparticules du VHC (VHCpp) (Bartosch, Dubuisson, and Cosset, 2003) et les VHC en culture cellulaire (VHCcc) (Kato et al., 2003; Wakita et al., 2005). La morphologie des VHCcc ressemble le mieux à celle des LVPs trouvées chez les patients, mais avec un niveau de lipidation inférieur. De ce fait, de nouveaux systèmes de culture sont en cours de développement en vue d'une utilisation pour l'évaluation des réponses humorales dans de meilleures conditions (Cochard et al., 2021; Denolly et al., 2019).

En général, les vaccins se composent d'un antigène (le pathogène vivant ou inactivé, ou une partie du pathogène) et d'un adjuvant (des polysaccharides, des lipides ou de l'aluminium) qui aide à déclencher la réponse immunitaire innée. Pour le développement des vaccins, les techniques d'inactivation ou d'atténuation

des pathogènes sont largement utilisées, mais de nouvelles technologies, comme celle de l'ADN recombinant, ont permis le développement de vaccins basés sur des « virus-like particles » (VLPs), des sous-unités, des vecteurs viraux et des acides nucléiques (Pollard and Bijker, 2021).

Dans le cas de l'hépatite C, une grande variété de concepts vaccinaux a été proposée. Un vaccin basé sur l'usage d'un VHCcc de génotype 2a inactivé a induit des réponses cellulaires et humorales chez la souris et le ouistiti (Yokokawa et al., 2018). Des souris ayant un foie humanisé ont aussi été immunisées avec ces particules virales inactivées et ont développé des anticorps cross-neutralisants contre le VHC et les protégeant d'un challenge viral (Akazawa et al., 2013). En parallèle, un vaccin basé sur l'usage de vecteurs viraux et visant l'induction d'une réponse cellulaire a été évalué chez le chimpanzé et l'homme (Barnes et al., 2012; Folgori et al., 2006; Swadling et al., 2014). Ce vaccin a fait l'objet d'essais cliniques de phases 1 et 2, mais la vaccination n'a malheureusement pas empêché le développement d'une hépatite C chronique (Page et al., 2021).

Concernant les vaccins visant l'induction d'une réponse humorale, de nombreux prototypes ont été proposés ces dernières années. Le seul candidat vaccin qui a atteint la phase 1 des essais cliniques est basé sur l'usage des protéines d'enveloppe recombinantes E1E2 produites en cellules de mammifères (Frey et al., 2010; Law et al., 2013; Ray et al., 2010). Différentes espèces ont été immunisées avec ce vaccin et ont développé des réponses cellulaires et humorales spécifiques contre le VHC (Choo et al., 1994; Meunier et al., 2011; Stamataki et al., 2007). Par contre, les anticorps induits chez l'homme ciblaient la région HVR1 et n'étaient donc pas cross-neutralisants (Ray et al., 2010). En plus de ce vaccin, des VLPs élaborées à partir de la core du VHC ou d'un Gag rétroviral et décorées par les protéines d'enveloppe du VHC ont été évaluées chez l'animal (Christiansen et al., 2019; Garrone et al., 2011).

D'autres groupes de recherche ont conçu des concepts vaccinaux basés uniquement sur la protéine d'enveloppe E2, qui constitue la principale cible des anticorps neutralisants. C'est le cas d'une équipe de l'Institut Pasteur de Shanghai, qui a proposé un vaccin basé sur l'usage d'une protéine E2 soluble (sE2) produite en cellules d'insectes issues de *Drosophila melanogaster* (cellules S2) (Li et al., 2016). Ce système de production génère des protéines avec des motifs de glycosylation moins complexes que lors d'une synthèse en cellules de mammifères. Il semblerait que les motifs de glycosylation générés par les cellules S2 confèrent une flexibilité accrue à la protéine sE2, ce qui se traduit par une meilleure induction des anticorps cross-neutralisants (Li et al., 2016). Ce vaccin a aussi été évalué chez la souris et le macaque sous la forme d'un cocktail de protéines sE2 de différents génotypes (Li et al., 2017; Wang et al., 2019).

La protéine d'enveloppe E2 a subi des nombreuses modifications dans le but d'induire des réponses immunitaires optimales contre le VHC. Certains groupes de recherche l'ont délétée de sa région HVR1 (Tarr et al., 2018), voire de ses 3 régions hypervariables (Vietheer et al., 2017), d'autres l'ont également couplée à des nanoparticules (He et al., 2020; Yan et al., 2020), et enfin d'autres n'en ont utilisé que des peptides d'intérêt comme candidats vaccins (Dawood et al., 2019; Mosa et al., 2020). En général, les anticorps induits par ces vaccins neutralisent des VHC du même génotype que celui utilisé pour le vaccin, mais le défi à relever reste la cross-neutralisation des génotypes génétiquement distants.

Notre groupe de recherche a développé un concept vaccinal basé sur la capacité d'auto-assemblage de la protéine d'enveloppe S du VHB, actuellement utilisée comme vaccin contre ce virus (Beaumont et al., 2013, 2016, 2020). À la différence des autres vaccins développés contre le VHC en considérant cette protéine S, nous utilisons, dans notre approche, des protéines d'enveloppe du VHC complètes, et non pas uniquement des épitopes d'intérêt (Czarnota et al., 2020; Wei et al., 2018). L'intérêt de notre vaccin repose sur sa capacité à générer des réponses immunitaires contre ces deux virus hépatotropes et à être produit selon les procédés industriels déjà développés pour la production des vaccins contre le VHB.

1.4 LE CANDIDAT VACCIN BIVALENT CONTRE LE VHB ET LE VHC

L'hépatite B est une pathologie virale qui affecte 257 millions de porteurs chroniques, principalement en Afrique sub-saharienne et en Asie du sud. L'hépatite B est souvent asymptomatique et donc difficile à diagnostiquer dans les phases précoces de l'infection. Le mode de transmission dominant de la mère enceinte à l'enfant engendre une maladie chronique dans 80-90 % des cas (World Health Organization, 2017).

Le VHB est un virus enveloppé ayant un génome à ADN circulaire partiellement bicaténaire de 3,2 kb codant pour plusieurs protéines dont 3 protéines d'enveloppe. Des génotypes et sous-types du VHB ont aussi été identifiés, mais le VHB présente toutefois une variabilité moindre que le VHC (Sunbul, 2014). Le VHB circule sous la forme de particules, appelées particules de Dane, de 42 nm de diamètre et portant l'antigène de surface du VHB (AgHBs) (Dane, Cameron, and Briggs, 1970). Les cellules infectées par ce virus secrètent des protéines d'enveloppe en excès qui s'assemblent en particules non-infectieuses (filaments ou sphères) de 17-25 nm de diamètre, portant l'AgHBs (Cao et al., 2019; Dane, Cameron, and Briggs, 1970; Short et al., 2009). Ces structures ont été caractérisées et observées par microscopie électronique (ME) et cryo-ME (Cao et al., 2019; Short et al., 2009). Des 3 protéines d'enveloppe, la protéine S est la plus petite et ses domaines sont partagés par les 2 autres protéines (M et L). En effet, la protéine S

peut s'assembler en particules octaédriques contenant uniquement cette protéine (48-60 sous-unités) (Gavilanes, Gonzalez-Ros, and Peterson, 1982; Gilbert et al., 2005). La protéine S possède 4 TMDs (TMD 1-4) qui forment une boucle antigénique orientée vers la lumière du RE (exposée ensuite à la surface du virion ou de la particule d'excès d'enveloppe) et deux autres boucles du côté cytosolique (exposées vers l'intérieur du virion ou de la particule d'excès d'enveloppe) (Eble et al., 1987).

Les particules sous-virales (SVPs) d'enveloppe ont donc été proposées comme candidat vaccin contre le VHB. Elles ont d'abord été purifiées à partir de plasma de patients et utilisées pour immuniser des chimpanzés, puis des hommes volontaires sains, qui ont pour la plupart développé des anticorps contre le VHB (Maupas et al., 1976; Prince et al., 1985; Szmuness et al., 1981). Cependant, du fait du risque infectieux potentiel lié à l'utilisation de plasma de patients, ce vaccin a été progressivement remplacé par des vaccins utilisant la technologie de l'ADN recombinant, les particules sous-virales étant produites en cellules de mammifères et en levures (Miyanohara et al., 1983; Valenzuela et al., 1982). Même si ces particules produites possédaient des caractéristiques similaires à celles dérivées du plasma humain, elles n'étaient toutefois pas secrétées, ni glycosylées (ou peu glycosylées). Les anticorps induits par une vaccination avec ces particules chez des chimpanzés les ont protégés du challenge avec le VHB (McAleer et al., 1984). Après une évaluation chez l'homme ayant conduit à des résultats encourageants, l'OMS a recommandé d'inclure ce vaccin dans les programmes de vaccination des pays (World Health Organization, 1992). La campagne de vaccination menée à Taïwan est un exemple de l'efficacité de ce vaccin. Dix ans après le début de la campagne, 90 % de la population avait reçu 3 doses du vaccin et la séroprévalence du VHB avait diminué de 9,8 % à 1,3 % (Chang et al., 1997; Chen et al., 1996). Par ailleurs, la réduction de l'incidence du carcinome hépatocellulaire a été vérifiée 20 ans plus tard, faisant du vaccin contre l'hépatite B le premier vaccin anti-cancer (Chang et al., 2009).

Notre vaccin bivalent a été développé à partir de la protéine d'enveloppe S du VHB. Le TMD1 de la protéine S a été substitué par les TMD des protéines d'enveloppe (E1 ou E2) complètes du VHC afin qu'elles ne soient plus retenues dans le RE (Flint et al., 2000; Forns et al., 2000). L'assemblage de ces protéines chimères (E1-S et E2-S) a été évalué dans des cellules de mammifères, mais elles ne se sont révélées s'assembler qu'en présence de la protéine S sauvage (Patient et al., 2009). Afin de produire des particules de façon stable, des lignées cellulaires de mammifères ont été générées par transduction des cellules d'ovaires de hamster chinois (CHO) avec des lentivirus contenant les transgènes S, E1-S et/ou E2-S. Des clones de CHO exprimant de façon stable les protéines d'intérêt S, E1-S et/ou E2-S ont été sélectionnés et amplifiés pour une production en masse des particules S,

S+E1-S, S+E2-S et S+E1-S+E2-S (Beaumont et al., 2013; Patient et al., 2009). Avant l'immunisation d'animaux, la présence et l'exposition des protéines d'enveloppe du VHC à la surface des particules ont été vérifiées grâce à la reconnaissance d'épitopes conformationnels spécifiques de E2 ou du complexe E1E2 par des anticorps monoclonaux (Beaumont et al., 2013).

Des lapins ont été immunisés avec 15 µg d'immunogènes préalablement mélangés avec de l'adjuvant (AddaVax™). Après immunisation, plus de 90 % des lapins avaient généré des anticorps contre le VHB, évalués avec un test immunologique microparticulaire par chimiluminescence. Les anticorps induits par immunisation avec les particules S+E2-S, mesurés par un ELISA « maison », ont montré un meilleur pouvoir neutralisant que ceux induits par les particules S+E1-S. Par contre, les particules portant les deux protéines d'enveloppe n'ont pas induit la production d'anticorps présentant un meilleur pouvoir neutralisant que celui des anticorps induits par immunisation avec des particules S+E2-S (Beaumont et al., 2013).

Par la suite, il a été montré que l'immunisation avec un cocktail de particules S+E1-S et S+E2-S induisait de meilleures réponses humorales neutralisantes que l'immunisation avec des particules S+E1-S+E2-S (Beaumont et al., 2016). En revanche, la neutralisation des génotypes génétiquement distants du génotype 1a n'était pas optimale. Nous avons donc généré des particules chimères arborant des protéines d'enveloppe E1 et E2 de divers génotypes (1a, 3a et 4a). Les lapins immunisés avec un cocktail de particules S+E2-S de génotype 1a, 3a et 4a ont généré des anticorps ayant un pouvoir neutralisant significativement plus élevé à l'égard des génotypes 3a et 4a, en comparaison avec les anticorps induits par des particules S+E2-S de génotype 1a. En revanche, l'ajout des protéines E1 de génotype 1a, 3a et 4a au cocktail n'a pas amélioré la réponse, ce qui suggère une frustration potentielle de la maturation de l'affinité des lymphocytes B.

2

OBJECTIFS DU PROJET

La réussite d'un vaccin contre le VHC repose sur sa capacité à induire des réponses cellulaires et humorales soutenues. L'induction d'anticorps neutralisants est notamment jugée essentielle depuis l'échec des essais cliniques menés avec un candidat vaccin connu pour induire uniquement des réponses cellulaires. Les épitopes de ces anticorps se trouvent dans les protéines d'enveloppe du VHC, et plus particulièrement dans la protéine d'enveloppe E2. Le VHC présente une grande diversité génétique, surtout au sein de cette protéine d'enveloppe qui contient 3 régions présentant une haute variabilité de séquence.

Le vaccin proposé par notre équipe est basé sur la protéine d'enveloppe S du VHB et sur des protéines d'enveloppe du VHC complètes fusionnées à cette protéine S qui s'auto-assemblent et génèrent des particules chimères VHB-VHC secrétées par les cellules CHO de façon stable. Les protéines d'enveloppe du VHC ont été détectées à la surface de ces particules et leur conformation a été vérifiée à l'aide d'anticorps reconnaissant des épitopes spécifiques de la structure native des protéines. Par contre, il est possible que la conformation optimale de ces protéines ne puisse être atteinte que si elles sont présentées à la surface de nos particules avec les mêmes interactants trouvés à la surface du VHC, comme les molécules de faible densité dérivées de l'hôte.

Cette association avec l'apoE favorise notamment l'échappement du VHC au système immunitaire. Il a été montré que l'apoE participe à l'attachement du virus à la cellule et à la production des virions infectieux, mais aussi qu'elle interagit avec les protéines d'enveloppe du VHC (E1 ou/et E2) (Boyer et al., 2014; Lee et al., 2014; Mazumdar et al., 2011). Nous avons donc émis l'hypothèse que la conformation des protéines d'enveloppe à la surface du VHC puisse être dépendante de leur interaction avec l'apoE. Il a d'ailleurs été montré que la mutation d'un acide aminé de la protéine E2 modifie l'interaction avec l'apoE, rendant alors le VHC susceptible à la neutralisation par des anticorps anti-apoE. Cette susceptibilité à la neutralisation n'était pas due à une moindre quantité d'apoE incorporée aux virions, mais à un changement de la conformation de l'interaction apoE-E2 (Fauvelle et al., 2016a).

La génération d'anticorps neutralisants de qualité ne dépend pas uniquement de la bonne conformation des immunogènes, mais aussi de la stratégie de vaccination choisie. En effet, les anticorps spécifiques d'un pathogène sont produits par des cellules B qui subissent un processus de maturation de leur affi-

nité, comme précédemment décrit (De Silva and Klein, 2015). Une évaluation des stratégies d'immunisation mises en œuvre dans le cadre du développement d'un vaccin contre le VIH a montré que l'immunisation avec un cocktail de variantes d'un immunogène ne permettait pas une maturation correcte des cellules B responsables des réponses humorales, mais au contraire qu'elle pouvait engendrer une frustration de ces dernières (Childs, Baskerville, and Cobey, 2015). En revanche, il a été montré que des immunisations séquentielles avec ces variantes induisent efficacement la production d'anticorps avec une haute affinité (Mohan et al., 2018; Sprenger et al., 2020; Wang et al., 2015). Dans une étude précédente, nous avons observé que l'immunisation de lapins avec une mixture de protéines E2 de génotype 1a, 3a et 4a améliorait significativement le pouvoir neutralisant des anticorps induits. Par contre, l'ajout à cette mixture des protéines E1 de génotype 1a, 3a et 4a avait un effet négatif sur les réponses humorales. Ces résultats pouvaient suggérer une potentielle frustration de la maturation des lymphocytes B.

L'objectif de ce projet est donc d'améliorer la présentation des protéines d'enveloppe du VHC pour la génération de meilleures réponses neutralisantes selon deux approches : 1) l'incorporation de l'apoE dans nos particules sous-virales, afin de mimer au plus près les interactions à l'interface apoE-protéines d'enveloppe observée à la surface du VHC et 2) l'immunisation de lapins de façon séquentielle avec des particules chimères arborant des protéines d'enveloppe E2 de génotype 1a, 3a ou 4a afin d'améliorer la maturation de l'affinité des lymphocytes B.

3

RÉSULTATS

3.1 ÉTUDE 1. INCORPORATION DE L'APOLIPOPROTÉINE E DANS LES PARTICULES SOUS-VIRALES CHIMÈRES VHB-VHC

Nous avons tout d'abord évalué la co-exposition de l'apoE et des protéines d'enveloppe du VHC (E1 ou E2) à la surface de nos particules sous-virales afin de nous assurer de mimer au plus près les interactions survenant naturellement entre ces protéines au sein des virions. Les résultats principaux de cette approche ont été résumés dans la publication suivante, actuellement en révision dans la revue *Scientific Reports* (et reproduite dans l'Annexe B) :

Gomez-Escobar E, Burlaud-Gaillard J, Visdeloup C, Ribeiro E Silva A, Coutant P, Roingeard P, Beaumont E. Incorporation of apolipoprotein E into HBV-HCV subviral envelope particles : implications for an improved hepatitis vaccine strategy.

3.1.1 *Analyses des interactions entre l'apoE et les protéines d'enveloppe du VHC*

Nous avons évalué les interactions de l'apoE avec les protéines d'enveloppe du VHC (E1 et E2), mais aussi avec les protéines chimères E1-S et E2-S, par des techniques de co-immunoprécipitation. Nous avons pu montrer que l'apoE était capable d'interagir avec les deux protéines d'enveloppe natives et chimères, contrairement à ce qui avait été reporté dans la littérature par d'autres investigateurs.

3.1.2 *Caractérisation des particules sous-virales chimères VHB-VHC*

Les particules vaccinales arborant ou pas de l'apoE (S, S+apoE, S+E1-S, S+E1-S+apoE S+E2-S et S+E2-S+apoE) ont été produites par des clones de cellules CHO exprimant les protéines d'intérêt de façon stable et permettant la genèse de particules. Ensuite, nous les avons purifiées à partir des surnageants de culture par des méthodes traditionnelles de purification des particules sous-virales. Finalement, nous avons caractérisé les particules vaccinales par des techniques de western blot et de microscopie électronique en transmission (MET), mais aussi par des tests immuno-enzymatiques de type ELISA. Nous avons réalisé les observations suivantes :

- L'apoE a été détectée à la surface de toutes les particules censées arborer l'apoE, y compris à la surface des particules composées uniquement de la protéine d'enveloppe S du VHB, suggérant alors une interaction entre ces deux protéines.
- L'apoE a été incorporée dans les particules en proportions distinctes selon les protéines d'enveloppes co-exprimées : les particules contenant la protéine E2 étant celles les plus riches en apoE (S+E2-S+apoE> S+E1-S+apoE> S+apoE).
- La quantité de protéines chimères E1-S et E2-S paraît être modulée par la présence d'apoE lors de la formation des particules. Nous avons donc évalué la variation de la quantité de ces protéines en analysant la densitométrie des bandes correspondant à E1-S et E2-S révélées lors du western blot de caractérisation des particules vaccinales d'intérêt. La protéine E1-S a été incorporée 1,5 fois plus dans les particules arborant l'apoE, alors que la protéine E2-S l'a été 3,4 fois moins.
- Les protéines E1-S se sont également révélées être mieux reconnues à la surface des particules arborant l'apoE par les anticorps monoclonaux H-111 et A4. Le phénomène inverse a par contre été observé pour les protéines E2-S en utilisant les anticorps monoclonaux AP33 et AR3A.
- Afin de comprendre l'impact de la présence d'apoE sur la conformation des protéines d'enveloppe du VHC, nous avons calculé les ratios entre les densités optiques (DOs) obtenues avec chaque anticorps et les densités relatives aux bandes de western blot observées pour les protéines E1-S et E2-S (S+E1-S = 1 et S+E1-S+apoE = 1,5 ; S+E2-S = 3,4 et S+E2-S+apoE = 1), préalablement évaluées par densitométrie. Les ratios calculés indiquent une meilleure reconnaissance des protéines d'enveloppe par les anticorps quand l'apoE est co-présentée dans les particules.

3.1.3 *Analyse des réponses humorales*

Une fois que les particules ont été caractérisées, nous les avons mélangées avec de l'adjuvant (AddaVax™) (vol :vol) préalablement aux immunisations. Des groupes de 8 lapins ont été immunisés aux jours 0, 14 et 28 avec 15 µg de particules S+E1-S, S+E1-S+apoE, S+E2-S ou S+E2-S+apoE, alors que des groupes contrôles de 4 lapins ont été immunisés avec 15 µg de particules S ou S+apoE ou avec de l'adjuvant seul. Des prélèvements ont été effectués aux jours 0, 12, 26, 42 et 56 en vue de l'analyse des réponses humorales. Les réponses anticorps anti-S ont été évaluées par un test immunologique microparticulaire par chimiluminescence (système ARCHITECT). Ces analyses ont montré l'induction d'une réponse anticorps anti-S chez tous les lapins, toutefois légèrement plus faible chez les animaux immunisés avec des particules arborant l'apoE. Concernant les anticorps dirigés contre les protéines d'en-

veloppe E1 et E2, ils ont été évalués par des ELISAs « maison ». Les titres en anticorps anti-E1 semblaient similaires, malgré l'observation d'une quantité légèrement accrue d'E1 dans les particules S+E1-S+apoE, mais remarquablement plus homogènes que ceux induits par immunisation avec des particules sans apoE. Les titres en anticorps anti-E2 étaient plus importants au jour 56 post-immunisation pour les lapins immunisés avec les particules S+E2-S, mais pas aux jours 26 et 42. Une chute des titres en anticorps a effectivement été observée entre les jours 42 et 56 chez les lapins immunisés avec les particules arborant de l'apoE.

Nous avons aussi essayé d'évaluer la présence d'anticorps dirigés contre l'apoE par des ELISAs « maison » basés sur l'usage soit d'un lysat de cellules exprimant l'apoE, soit d'une apoE humaine purifiée à partir du plasma. Dans les deux cas, nous avons observé des niveaux de base en anticorps dirigés contre cette protéine qui évoluaient différemment pour chaque groupe de lapins immunisés. Il semblerait possible que des protéines cellulaires contaminant les lots de vaccins induisent une réponse non spécifique qui puisse être détectée par ces ELISAs. En effet, les lapins immunisés avec de l'adjuvant seul se sont révélés présenter des titres en anticorps plutôt stables.

3.1.4 *Analyse des propriétés neutralisantes des anticorps induits par immunisation avec les particules vaccinales*

Concernant les propriétés neutralisantes des anticorps générés, elles ont été évaluées à l'aide de VHCcc arborant des protéines d'enveloppe de génotype 1a (isolat H77) ou 2a (WT JFH1). Les anticorps induits avec les particules S+E1-S+apoE ont des % de neutralisation pour les deux génotypes plus élevés que ceux obtenus avec les anticorps générés par immunisation avec les particules S+E1-S. De plus, pour le génotype 1a, les % de neutralisation étaient plus homogènes pour les lapins immunisés avec des particules arborant l'apoE. En revanche, les % de neutralisation des deux génotypes en utilisant des sérums de lapins immunisés avec des particules S+E2-S+apoE, qui avaient montré de faibles titres en anticorps au jour 56, ont été bien moins bons que pour le groupe immunisé avec des particules sans apoE. Afin de mieux évaluer les résultats pour ces deux groupes, nous avons rapporté les % de neutralisation des 4 meilleurs répondeurs aux titres en anticorps obtenus au jour 56. En calculant ces ratios, nous avons remarqué que si l'on normalisait la quantité d'anticorps utilisée pour ces expériences, le pouvoir neutralisant des anticorps induits avec des particules contenant de l'apoE était significativement supérieur à celui des anticorps générés avec des particules S+E2-S.

3.2 ÉTUDE 2. L'IMMUNISATION SÉQUENTIELLE DE LAPINS AVEC DES PARTICULES ARBORANT DES PROTÉINES D'ENVELOPPE E2 DE DIVERS GÉNOTYPES

La seconde approche du projet portait sur l'amélioration de la maturation de l'affinité des lymphocytes B en procédant à des immunisations séquentielles avec des protéines E2 de génotype 1a, 3a et 4a. Les propriétés cross-neutralisantes des anticorps induits avec cette stratégie d'immunisation seront comparées avec celles des anticorps générés par des immunisations avec des cocktails de protéines E2 de génotype 1a, 3a et 4a.

3.2.1 *Matériels et méthodes*

3.2.1.1 *Production et purification des particules chimères VHB-VHC présentant des protéines d'enveloppe E2 de génotype 1a, 3a et 4a*

Les clones de cellules CHO ont été développés par transduction avec des lentivirus contenant les gènes codant pour la protéine S du VHB et l'enzyme hygromycine phosphotransferase conférant une résistance à l'hygromycine. Un clone résistant à cet antibiotique (CHO-S) a été sélectionné et amplifié. Ce clone a ensuite été transduit avec des lentivirus contenant soit la séquence codant pour la protéine chimère $E2^{1a}S$ (VHC de génotype 1a) et le gène codant la protéine fluorescente verte (GFP), soit les protéines chimères $E2^{3a}S$ (VHC de génotype 3a) ou $E2^{4a}S$ (VHC de génotype 4a) et le gène de résistance à la puromycine (enzyme puromycine N-acétyltransférase). Les clones exprimant la protéine GFP ou résistants à la puromycine ont été sélectionnés et amplifiés. Ils ont ensuite été cultivés dans un milieu adapté, supplémenté avec une faible concentration de sérum de veau fœtal, et dans des contenants de 1720 cm^2 de surface pour assurer une production en masse des particules vaccinales d'intérêt.

Les surnageants de culture ont été récoltés tous les 10 à 12 jours en vue d'une purification des particules. D'abord, les protéines totales ont été précipitées dans une solution de sulfate d'ammonium et récupérées par centrifugation. Les précipités de protéines ont été dialysés contre un tampon à pH neutre (Tris-NaCl-EDTA) à 4°C sous agitation. Ensuite, deux ultracentrifugations isopycniques en gradient continu de CsCl ont été effectuées. Les fractions récupérées ont été évaluées par des ELISAs « maison » capables de détecter l'AgHBs. Les fractions positives en AgHBs ont été regroupées, dialysées et concentrées. Les concentrations finales en particules ont été déterminées avec le même ELISA « maison » et en utilisant une gamme d'AgHBs purifié. La pureté de ces particules a été obtenue en divisant la concentration d'AgHBs par la concentration en protéines totales.

3.2.1.2 *Caractérisation des particules chimères VHB-VHC*

Nous avons caractérisé les particules purifiées (S+E2^{1a}S, S+E2^{3a}S et S+E2^{4a}S) par des techniques de western blot et d'ELISA. Pour le western blot, nous avons fait migrer les particules (concentration de 75 µg/mL d'AgHBs) dans des gels de polyacrylamide en présence de SDS. Les protéines ont été ensuite transférées sur des membranes de nitrocellulose et hybridées avec des anticorps polyclonaux dirigés contre la protéine S (R247 et 70-HG15) ou un anticorps monoclonal détectant la protéine E2 (AP33). Des anticorps secondaires couplés à l'enzyme peroxydase de raifort (HRP) ont été incubés et les protéines ont ensuite été révélées par une réaction de chimiluminescence. Pour les ELISAs, chaque particule (l'équivalent de 5 µg d'AgHBs) a été capturée dans des puits de plaques préalablement couverts par de la lectine et les protéines E2 ont été détectées par les anticorps monoclonaux AP33, H52 et AR3A.

3.2.1.3 *Immunisation des lapins avec des particules chimères S+E2^{1a}S, S+E2^{3a}S, S+E2^{4a}S*

Le protocole d'immunisation a été réalisé par la société Agro-Bio (La Ferté Saint-Aubin). Trois groupes de 8 lapins ont été immunisés avec 15 µg de particules mélangées préalablement avec l'adjuvant AddaVax™ (vol :vol) aux jours 0, 14 et 28 : a) S+E2^{1a}S, b) mixture de S+E2^{1a}S, S+E2^{3a}S et S+E2^{4a}S (5 µg de chaque particule) ou c) S+E2^{1a}S→S+E2^{3a}S→S+E2^{4a}S selon un schéma d'immunisations séquentielles. Deux groupes de 4 lapins ont aussi été immunisés soit avec 15 µg de particules S adjuvantées, soit avec le même volume d'adjuvant seul. Des prélèvements ont été effectués aux jours 0, 12, 26, 42 et 56 en vue d'une analyse des réponses humorales.

3.2.1.4 *Analyse des réponses humorales (**en cours**)*

Les analyses des réponses anti-S et anti-E2 sont en cours de réalisation. Les titres en anticorps anti-S observés aux jours 0,12, 26 et 42 ont déjà été évalués par des tests immunologiques microparticulaires par chimiluminescence (système ALINITY, Abbott Diagnostics). Les échantillons prélevés au jour 56 seront explorés par la même technique dans les jours à venir. Les anticorps anti-E2$^{1a/3a/4a}$ seront analysés par des ELISAs « maison » basés sur l'utilisation de lysats de cellules BHK-21 exprimant les protéines E2 de génotype 1a, 3a et 4a ou la β-galactosidase (β-gal) comme contrôle.

3.2.1.5 *Analyse des propriétés neutralisantes des anticorps vis-à-vis de VHCcc génotype 1a, 3a et 4a (**en cours**)*

Les VHCcc arborant les protéines d'enveloppe de VHC de génotype 1a (isolat H77), 3a (isolat S52) ou 4a (isolat ED43) ont été générés par transfection de cellules

Huh7.5 avec les génomes recombinants complets associés (H77/JFH1, S52/JFH1 et ED43/JFH1). L'infectivité de ces virus a été déterminée par une technique de détection des foyers infectieux (FFUs).

Les tests de neutralisation seront réalisés en utilisant 50 µL d'inoculum viral (correspondant à 100 FFUs) et 25 µL de sérum de lapin (dilution 1/5). Les mixtures virus-sérum seront incubées pendant 1 heure à 37°C, puis mises en contact avec des cellules Huh7.5 pendant 6 heures à 37°C. L'inoculum sera éliminé et remplacé par du milieu frais et les cellules seront ensuite incubées pour 48 heures supplémentaires. Les cellules seront alors lavées, et l'infectivité sera déterminée par le même test de détection des foyers infectieux. Le % de neutralisation sera calculé en déduisant le % de neutralisation au jour 0 de celui obtenu au jour 56.

3.2.2 *Résultats*

3.2.2.1 *Caractérisation des particules chimères S+E2^{1a}S, S+E2^{3a}S et S+E2^{4a}S*

Les particules ont été purifiées et présentent une pureté moyenne de 15 %. La présence des protéines d'intérêt (S, E2^{1a}S, E2^{3a}S, E2^{4a}S) a bien été mise en évidence par western blot en utilisant différents anticorps d'intérêt. Par contre, nous avons rencontré de grandes difficultés pour reconnaître la protéine E2^{3a}S avec l'anticorps AP33 dirigé contre la protéine E2. Cette dernière a toutefois été détectée par l'anticorps ciblant la protéine S du VHB. Avec les ELISAs, nous avons pu montrer que les protéines d'enveloppe E2 de divers génotypes étaient présentes à la surface des particules, et ce dans une conformation adéquate pour la reconnaissance par l'anticorps monoclonal AR3A détectant un épitope conformationnel. Les anticorps monoclonaux reconnaissant d'autres épitopes au sein de la protéine E2 ont également été testés. Tout comme observé précédemment en western blot, les anticorps AP33 et H52 n'ont pas su détecter respectivement les protéines E2^{3a}S et E2^{4a}S.

3.2.2.2 *Analyses des réponses humorales*

Nous avons analysé les réponses anticorps anti-S induites par immunisation avec les différentes particules dans des échantillons prélevés aux jours 0, 12, 26 et 42. Grâce à l'usage d'un test statistique non paramétrique (test de Mann-Whitney), nous avons comparé les médianes des titres en anticorps anti-S observés au jour 42 chez les groupes de lapins immunisés par les particules S+E2^{1a}S, par un cocktail de particules S+E2$^{1a+3a+4a}$S ou selon un schéma d'immunisations séquentielles avec les particules S+E2$^{1a\rightarrow 3a\rightarrow 4a}$S. Comme attendu, nous n'avons pas observé de différence significative entre les médianes de ces groupes au jour 42 du protocole.

4

DISCUSSION

L'objectif général de ce projet était d'améliorer le potentiel neutralisant des anticorps induits par notre vaccin bivalent VHB-VHC. Dans un premier temps, nous avons exploré l'association entre les protéines d'enveloppe du VHC et l'apoE, préalablement suggérée comme étant un élément important dans la génération d'un vaccin contre le VHC. Il a été précédemment montré que l'apoE pouvait interagir avec les protéines d'enveloppe du VHC (Boyer et al., 2014; Lee et al., 2014; Mazumdar et al., 2011). L'existence de ces interactions a effectivement été confirmée par nos expériences de co-IP et par notre capacité à produire des particules chimères VHB-VHC portant à leur surface les protéines d'enveloppe et l'apoE. La quantité d'apoE détectée n'était pas équivalente dans toutes les particules (S+E2-S+apoE>S+E1-S+apoE>S+apoE). Cette différence peut être due à l'existence de multiples interactions entre les protéines présentes dans les particules. L'interaction S-apoE a été indirectement dévoilée au travers de notre capacité à générer des particules S+apoE, ce phénomène étant pleinement en accord avec une étude récente montrant l'implication de l'apoE dans la production du VHB (Qiao and Luo, 2019). Les particules contenant la protéine E2 ont incorporé bien plus d'apoE que les particules S+E1-S+apoE, qui elles aussi en ont incorporé légèrement plus que les particules S+apoE, ceci soulignant la potentielle existence de multiples sites d'interaction au sein d'E2. Il a aussi été montré précédemment que l'interaction apoE-E2 était hautement résistante au traitement avec du SDS (Lee et al., 2014).

Certaines régions des protéines d'enveloppe ont été suggérées comme intervenant dans l'interaction avec l'apoE. Pour E1, le domaine N-terminal replié dans une conformation spécifique a été proposé (Mazumdar et al., 2011), alors que pour E2, la région environnant le résidu 447, mais aussi d'autres sites dans les domaines antigéniques B et C et le TMD ont été suggérés (Fauvelle et al., 2016a; Lee et al., 2014). En ce qui concerne l'apoE, la région d'interaction avec les protéines d'enveloppe n'a pas encore été identifiée. En revanche, le domaine C-terminal de l'apoE a été identifié comme impliqué dans son interaction avec la protéine NS5A (Cun, Jiang, and Luo, 2010).

La quantité de protéines d'enveloppe incorporée paraît être modifiée en fonction de la co-expression de l'apoE. Une quantité plus élevée de protéine chimère E1-S a été incorporée dans les particules contenant de l'apoE, alors qu'une faible quantité de la protéine chimère E2-S était incorporée dans les particules S+E2-S+apoE. Cette différence peut être expliquée par la présence de l'apoE et par son

interaction avec les protéines S, E1 et E2 lors de la formation des particules. Les protéines d'enveloppe ont également été détectées à la surface des particules en utilisant des anticorps monoclonaux (A4, H-111, AP33 et AR3A). La reconnaissance des protéines E1 et E2 s'est d'ailleurs révélée être meilleure pour les particules arborant l'apoE, suggérant une meilleure mise en conformation des protéines d'enveloppe du VHC en présence d'apoE.

Lors de l'immunisation des lapins avec ces particules, les titres en anticorps induits ont varié. Les titres en anticorps anti-E1 induits par immunisation avec des particules contenant ou pas de l'apoE étaient similaires, mais bien plus homogènes lors d'immunisations avec les particules S+E1-S+apoE. Les titres en anticorps anti-E2 étaient plus élevés aux jours 26 et 42 pour les lapins immunisés avec les particules S+E2-S+apoE, mais ont ensuite drastiquement chuté au jour 56. Cette différence peut être attribuée à la faible quantité d'E2 incorporée dans ces particules, mais aussi à un rôle potentiel de l'apoE dans la modulation des réponses humorales (Allan et al., 2009; Barral et al., 2008; Van Den Elzen et al., 2005).

Les % de neutralisation des VHCcc de génotype 1a et 2a obtenus avec les anticorps dirigés contre la protéine E1 ont été légèrement plus élevés pour ceux induits par immunisation avec des particules S+E1-S+apoE. À l'inverse, les anticorps induits par immunisation avec les particules S+E2-S+apoE ont conduit à de plus faibles % de neutralisation de ces VHCcc que ceux générés par immunisation avec les particules S+E2-S. Par contre, le titre en anticorps anti-E2 du groupe de lapins immunisés avec les particules S+E2-S+apoE au jour 56 était bien plus faible, ce qui ne permettait pas de comparer le potentiel neutralisant de ces anticorps. Pour cette raison, nous avons rapporté les % de neutralisation aux titres en anticorps, et dans ces nouvelles conditions, les potentiels neutralisants des anticorps induits par immunisation avec des particules arborant l'apoE se sont révélés être significativement plus élevés. Ceci peut suggérer que si la quantité d'E2 incorporée dans les particules avait été supérieure, les anticorps induits, qui semblent être de meilleure qualité, auraient pu mieux neutraliser le VHC *in vitro*.

Les anticorps anti-apoE ont aussi été évalués, mais des résultats peu concluants ont été obtenus, peut-être à cause des contaminants cellulaires ou des résidus de sérum de veau fœtal supplémentant le milieu de culture. En conclusion de cette approche, le plus gros inconvénient était la faible quantité d'E2-S incorporée dans les particules S+E2-S+apoE. Quand nous avons vérifié la quantité de la protéine E2-S présente dans les lysats des clones stables utilisés pour la production de particules, la protéine E2-S était produite à des niveaux équivalents par les deux clones CHO-S+E2-S et CHO-S+E2-S+apoE. Cela suggère donc que cette différence d'incorporation résulte du processus de formation des particules sous-virales dans les cellules CHO. Au vu de cette différence, nous avons développé des clones de

cellules CHO exprimant les protéines d'intérêt de manière à générer des particules S+E1-S+E2-S+apoE. En caractérisant ces particules, nous avons montré que les protéines d'enveloppe du VHC étaient aussi bien reconnues dans les particules arborant l'apoE que dans celles qui en sont dépourvues, et aussi que la protéine E2-S était mieux détectée que dans les particules S+E2-S+apoE. Nous espérons que les réponses humorales induites par immunisation avec ces nouvelles particules seront meilleures que celles observées avec les particules ayant uniquement la protéine E2 et l'apoE.

Concernant la deuxième approche, les particules arborant les protéines d'enveloppe E2 de génotype 1a, 3a et 4a ont été produites, purifiées et caractérisées. La caractérisation des protéines $E2^{3a}S$ a été un défi, car elles n'étaient pas reconnues par les anticorps utilisés. L'anticorps monoclonal AR3A, qui reconnaît spécifiquement un épitope conformationnel de E2, a été le seul capable de détecter l'ensemble des protéines issues des 3 génotypes du VHC. De plus, les protéines $E2^{3a}S$ et $E2^{4a}S$ présentaient des mutations au niveau d'acides aminés présents dans les épitopes linéaires des anticorps monoclonaux AP33 et H52, empêchant ainsi la détection de toutes les protéines. Les premières analyses des réponses anticorps anti-S ont montré des résultats similaires pour tous les groupes de lapins immunisés au jour 42.

5

CONCLUSIONS ET PERSPECTIVES

Les protéines d'enveloppe du VHC sont les cibles principales des anticorps neutralisants. De ce fait, les futurs développements de vaccins dirigés contre le VHC devraient au moins impliquer la protéine d'enveloppe E2, qui contient la plupart des épitopes de ces anticorps. Le VHC utilise divers mécanismes pour échapper au système immunitaire, tels que son association avec les lipides. Afin de considérer cela dans notre concept vaccinal, nous avons produit et purifié des particules chimères VHB-VHC arborant les protéines d'enveloppe E1 ou E2 et l'apoE. La présence de l'apoE a impacté la conformation des protéines d'enveloppe du VHC et le potentiel neutralisant des anticorps anti-E2 induits par immunisation. Au vu de ces résultats, il paraît légitime de penser que cette approche, qui considère l'interaction de l'apoE avec les protéines d'enveloppe du VHC, mais aussi le rôle de l'apoE dans la mise en conformation de ces immunogènes de choix, puisse être d'un grand intérêt pour tous les développements de vaccins basés sur les protéines d'enveloppe du VHC. Pour ce qui est de notre vaccin basé sur des SVPs VHB-VHC, l'incorporation de la protéine E2 est trop faible dans les SVPs contenant également de l'apoE. Il nous faudra donc remédier à ce problème. Celui-ci sera peut-être résolu du fait de l'obtention récente de SVPs présentant à la fois E1, E2 et l'apoE dans lesquelles nous avons montré un bien meilleur niveau d'incorporation de E2. Une autre perspective de cette étude serait l'évaluation du potentiel neutralisant des anticorps induits par immunisation avec des particules arborant l'apoE à l'égard de virus produits dans des systèmes générant une lipidation similaire aux LVPs circulant chez les patients.

Dans un second temps, nous avons essayé d'améliorer le processus de maturation de l'affinité des lymphocytes B en procédant à un schéma d'immunisations séquentielles chez le lapin avec des particules arborant des protéines d'enveloppe E2 issues d'isolats de VHC de différents génotypes (70 % d'homologie). La principale difficulté de cette approche était la caractérisation des particules générées, car les protéines E2 de génotype 3a et 4a présentaient des mutations dans les épitopes reconnus par les anticorps utilisés. L'immunisation séquentielle a préalablement été évaluée pour des candidats vaccins développés contre d'autres virus, tels que le VIH et les virus de la grippe et de la dengue (Hou et al., 2020; Liu et al., 2019; Sprenger et al., 2020; Wang et al., 2015). Le protocole d'immunisation des lapins est achevé et nous espérons terminer rapidement les analyses des réponses hu-

morales induites par la vaccination de manière à les comparer à celles de l'étude utilisant un cocktail de ces mêmes particules (Beaumont et al., 2020).

Le candidat vaccin proposé ici a été exclusivement évalué pour l'induction d'anticorps neutralisants. Toutefois, la clairance du VHC a été associée à l'induction de réponses à la fois humorales et cellulaires (Osburn et al., 2010, 2014). Par conséquent, il serait intéressant d'évaluer également l'induction des réponses cellulaires après l'immunisation de petits animaux avec nos particules vaccinales, ce qui pourrait être fait grâce à des techniques telles que l'ELISpot pour la détection de cellules sécrétrices de cytokines. La présence de réponses cellulaires après immunisation serait une valeur ajoutée pour notre vaccin bivalent VHB-VHC.

Les protéines d'enveloppe du VHC sont hautement glycosylées et ces N-glycanes jouent un rôle dans l'échappement du virus au système immunitaire (Helle et al., 2010; Pantua et al., 2013). De plus, un groupe de recherche a montré une amélioration de l'immunogénicité de son candidat vaccin (protéine E2 soluble) et des propriétés cross-neutralisantes des anticorps induits par cette protéine produite en cellules d'insectes, en comparaison avec cette même protéine produite en cellules de mammifères. Pour toutes ces raisons, notre équipe de recherche a récemment débuté une analyse comparative de l'immunogénicité de notre candidat vaccin produit à l'aide de différents systèmes de production (cellules de mammifères et d'insectes, levures) générant des processus de glycosylation très distincts. Au vu de l'existence de nombreuses chaînes de production en levures du vaccin développé contre le VHB et des coûts et rendements de production constatés, ce système présente des avantages compétitifs certains pour un futur développement industriel.

Les cellules CHO sont utilisées pour la production de la plupart des protéines recombinantes et anticorps monoclonaux à usages thérapeutiques chez l'être humain (Fischer, Handrick, and Otte, 2015; Kim, Kim, and Lee, 2012). Aujourd'hui, pour des raisons de logistique, nos vaccins sont produits dans ces cellules, cultivées de manière adhérente dans des milieux supplémentés par du sérum animal. Une adaptation des clones de cellules CHO à une culture en suspension dans des milieux dépourvus de sérum nous faciliterait la tâche quant à notre quête de production vaccinale à grande échelle (Moleirinho et al., 2019). Ces nouvelles conditions de culture pourraient également s'accompagner d'une amélioration des procédés de purification, qui pourraient notamment s'appuyer sur des techniques plus sophistiquées de chromatographie (Zhao et al., 2019).

Pour résumer, les candidats vaccins devraient être capables de générer des réponses immunitaires efficaces chez l'animal avant d'être produits dans des systèmes adaptés pour obtenir des bons rendements de production et des puretés suffisantes pour leur évaluation chez l'homme. Dans le cadre du développement des vaccins contre le VHC, le graal serait l'induction d'anticorps largement neutra-

lisants qui assureraient une protection globale efficace à l'encontre de l'ensemble des génotypes du VHC et qui préviendraient la progression de l'infection vers une maladie chronique du foie. L'actuelle pandémie de covid-19 a conduit au développement et à l'approbation rapides de nombreux vaccins qui se sont montrés assurer une protection au moins partielle à l'égard du SARS-CoV-2. Dans le cas du VHC, un vaccin capable d'induire une protection partielle pour les populations identifiées à haut risque et vivant dans les régions ayant les plus hauts taux de prévalence constituerait un atout important pour l'éradication du VHC.

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APPENDICES

A

PAPER 1

Mixing particles from various HCV genotypes increases the HBV-HCV vaccine ability to elicit broadly cross-neutralizing antibodies

Elodie Beaumont, Benoit Joël Clément, Vanessa Guérin, Lucie Chopin, Emmanuelle Roch, Elsa Gomez-Escobar, Philippe Roingeard

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Abstract

The development of a safe, effective and affordable prophylactic vaccine against hepatitis C virus (HCV) remains a medical priority. Hepatitis B-C subviral envelope particles, which could be produced by industrial procedures adapted from those established for the hepatitis B virus vaccine, appear promising for use for this purpose. The prototype HBV-HCV bivalent vaccine-bearing genotype 1a HCV envelopes can induce neutralizing antibodies against this genotype, but is less effective against other genotypes. We show here, in a small animal model, that the use of a set of vaccine particles harbouring envelopes from different HCV genotypes in various association strategies can induce broad neutralizing protection or an optimized protection against a particular genotype prevalent in a given region, such as genotype 4 in Egypt. This vaccine could help to control the hepatitis C epidemic worldwide.

KEYWORDS

HCV genetic variability, hepatitis B virus, hepatitis C virus, neutralizing antibodies, prophylactic vaccine, subviral envelope particles

1 | INTRODUCTION

The World Health Organization (WHO) has estimated that hepatitis C virus (HCV) infection could be eradicated by 2030 if the HCV epidemic worldwide decreases (ie the number of patients cured by direct-acting antivirals (DAAs) consistently and significantly exceeds the number of new infections) by at least 7% per year. However, recent studies have shown that the global annual net regression, estimated at only 0.4%, is far from meeting this objective.[1] The cost of DAAs, which has recently decreased considerably, is less of a problem than the screening logistics required to identify the 71 million chronic carriers of HCV worldwide that have yet to be treated with DAAs. Indeed, due to the absence of symptoms in most HCV-infected individuals, only a small fraction (probably around 5%) of these individuals are aware that they are infected. Chronic carriers of HCV that are not diagnosed may, therefore, transmit the virus to healthy people, and it is estimated that the world reservoir of HCV-infected individuals increases by two million newly infected subjects each year. This increase is not limited to low economic countries, as the Centers for Disease Control recently reported a near tripling of HCV incidence in the US over the last 5 years, to reach a 15-year high of 41,200 new cases in 2016.[2] A recent mathematical model evaluating the impact on hepatitis C eradication of scaling up prevention and treatment indicated that it would be extremely difficult

Abbreviations: DAAs, direct-acting antivirals; FFU, focus-forming unit; HBsAg, hepatitis B virus S envelope protein; HBV, hepatitis B virus; HCV, hepatitis C virus; HCVcc, hepatitis C virus in cell culture; OD, optical density; WHO, World Health Organization; β-gal, β-galactosidase.

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FIGURE 1 Immunization protocol design and analysis of the antibody responses induced by various vaccination strategies. (A) Groups of 12 rabbits were immunized three times subcutaneously with AddaVax-adjuvanted chimeric HBV-HCV envelope subviral particles harbouring a genotype 1a E2 envelope glycoprotein (15 μg) or with different cocktails consisting of equal amounts of chimeric particles harbouring genotype 1a, 3a and 4a HCV E1 or E2 envelope glycoproteins, on days 0, 14 and 28. Groups of six rabbits immunized with the AddaVax adjuvant alone or with Engerix, a commercially available HBV vaccine (15 μg) were used as negative and positive controls respectively. Serum samples were collected from rabbits at various time points (days 0, 14, 28, 42 and 56), for the characterization of antibody responses and evaluation of the neutralizing potential of the antibodies induced by vaccination. (B) Specific anti-E1/anti-E2 and anti-HBsAg responses were evaluated in rabbit sera with in-house ELISAs[8,9] and a routine immunoassay (Abbott Laboratories), respectively. Results are expressed as the difference in optical density (anti-E1- or anti-E2-β-Gal) and anti-HBsAg titre (mIU/ml) respectively.

to achieve this goal.[3] Improvements would be required in blood safety and infection control, together with the expansion or creation of services for intravenous drug users, and extensive screening and treatment, the cost of which has been estimated by the WHO to be at least US$12 billion.[4] Furthermore, although antivirals drugs are highly effective at controlling infection, they cannot prevent re-infection with HCV, a situation that is becoming increasingly common among drug users in industrialized countries.[5] Moreover, approximately 15% of subjects with chronic hepatitis C infection go on to develop cirrhosis or even liver cancer after eradication of the virus by DAAs.[6] For all these reasons, the development of a prophylactic anti-HCV vaccine remains an important objective.

An original vaccine based on full-length HCV E1 or E2 envelope glycoproteins fused to the heterologous hepatitis B virus (HBV) S envelope protein (HBsAg) has been developed.[7] These chimeric envelope proteins self-assemble into highly immunogenic, non-infectious and secreted subviral envelope particles resembling to the HBV vaccine and are able to elicit specific antibody responses against HCV and HBV envelope proteins.[8,9] More importantly, prototype vaccine particles bearing the E1 or E2 envelope proteins from a genotype 1a HCV isolate-induced antibodies capable of neutralizing heterologous genotype 1a and 1b HCV strains efficiently in vitro. However, these antibodies appeared to have a lower cross-neutralizing potential against viruses of genotypes 3 and 4, which are genetically more distant.[8,9] As genotypes 1, 3 and 4 are the most prevalent worldwide (83.8% if considered together) and the most common in several parts of the world, the objective of this study was to increase the broad neutralizing properties of antibodies induced by such HBV-HCV bivalent vaccines through the development of new subviral vaccine particles bearing envelope glycoproteins from genotype 3 and 4 HCV isolates.

2 | METHODS

2.1 | Production of the vaccine particles

Chimeric HBV (subtype *adw*) – HCV (genotype 1a) envelope particles bearing the full-length HCV E1 ($E1^{1a}S$ particles) or E2 proteins ($E2^{1a}S$ particles) were produced and purified as previously described.[8] Using the same strategy, extensively described elsewhere,[7,8] we designed, produced and purified new vaccine particles bearing full-length genotype 3a or 4a HCV E1 or E2 glycoproteins (envelope sequences provided in Figure S1 and S2) named $E1^{3a}S$, $E2^{3a}S$, $E1^{4a}S$ and $E2^{4a}S$.

2.2 | Immunization of New Zealand rabbits

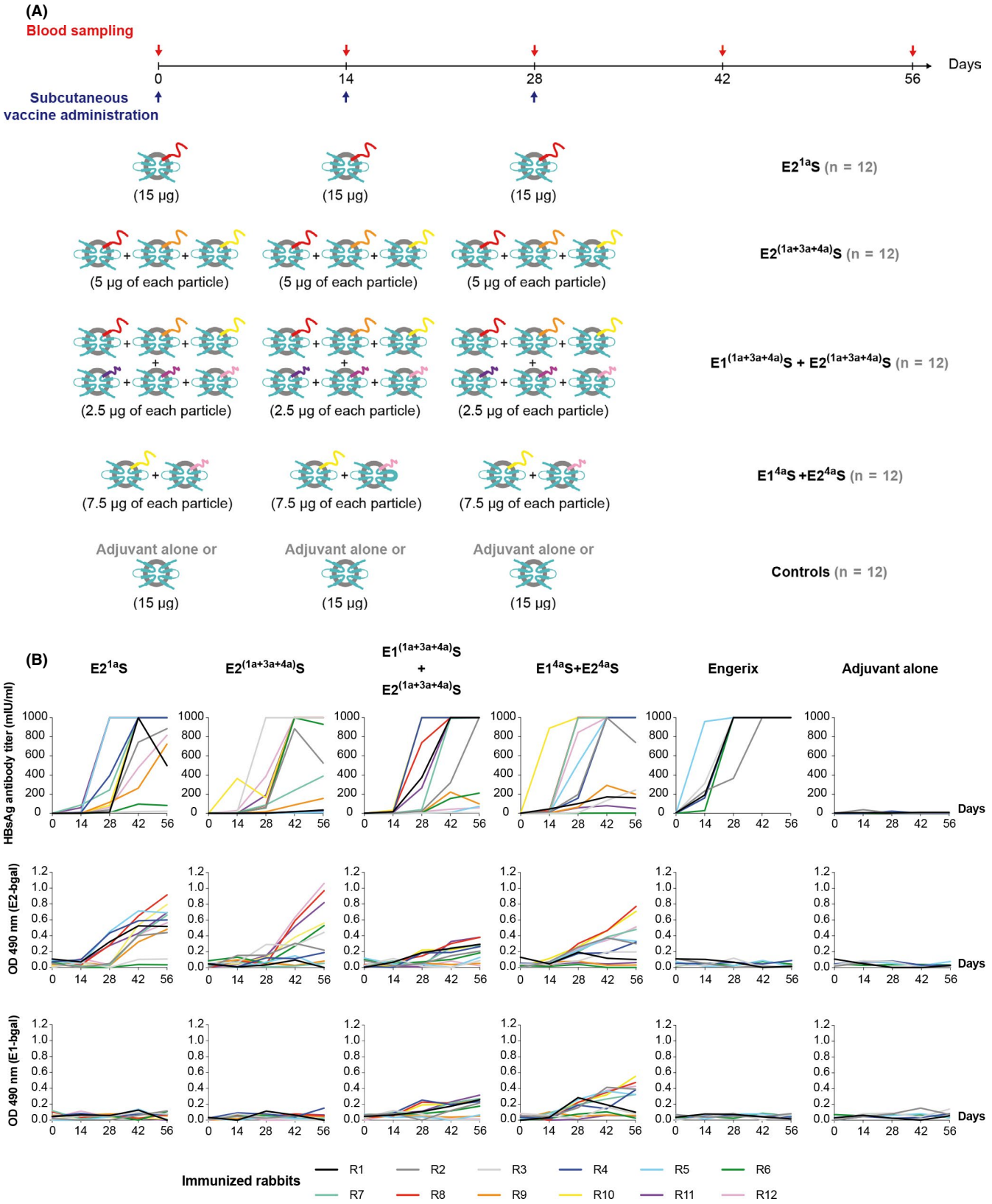
We immunized four groups of 12 rabbits subcutaneously on days 0, 14 and 28, with 15 μg of AddaVax-adjuvanted immunogens, quantified by HBsAg ELISA, as follows: (i) only $E2^{1a}S$ particles; (ii) equal amounts of $E2^{1a}S$, $E2^{3a}S$ and $E2^{4a}S$ particles (5 μg of each particle); (iii) equal amounts of $E1^{1a}S$, $E2^{1a}S$, $E1^{3a}S$, $E2^{3a}S$, $E1^{4a}S$ and $E2^{4a}S$ particles (2.5 μg of each particle) and (iv) or equal amounts of $E1^{4a}S$ and $E2^{4a}S$ particles (7.5 μg of each particle). Groups of six rabbits immunized with the AddaVax adjuvant alone or with Engerix, a commercially available HBV vaccine, were used as negative and positive controls, respectively. Serum samples were collected from rabbits at various time points (days 0, 14, 28, 42 and 56) for the characterization of antibody responses. Immunizations were performed by Agro-Bio (La Ferté-Saint-Aubin, France), a company accredited by the French authorities for the performance of protocols on laboratory animals in accordance with European Directive 2010/63/EU. The protocol was approved by the Agro-Bio Ethic Committee C2A-99 (French Ministry of Research, agreement B45-146-01).

2.3 | Analysis of anti-HBsAg and anti-E1/E2 antibody responses

Anti-HBsAg and anti-E1/E2 assays were conducted and analysed as described in detail elsewhere.[8,9] Briefly, anti-HBsAg antibodies were quantified with the ARCHITECT system (Abbott Laboratories). Anti-E1 and anti-E2 responses were evaluated with in-house ELISAs based on the use of solid phases consisting of appropriate lysates (or mixtures of lysates) of baby hamster kidney (BHK-21) cells expressing the same HCV E1 or E2 proteins of genotypes 1a, 3a and 4a as were exposed at the surface of the vaccine particles, which were compared with control cells expressing β-galactosidase (β-gal). For each serum sample tested, the optical density (OD) value obtained for wells with capture by β-gal protein was subtracted from that obtained for wells with capture by E1 or E2 proteins.

2.4 | Neutralization assays with HCV in cell culture

HCV in cell culture (HCVcc) harbouring HCV envelope glycoproteins derived from genotype 1a (JFH1/H77), 1b (JFH1/J4), 2a (JFH1), 3a (JFH1/S52) and 4a (JFH1/ED43) isolates were used to assess and compare



the neutralizing potential of the anti-HCV envelope protein antibodies present in pre- and post-vaccination serum samples from rabbits. Virus stocks were generated by transfecting hepatoma Huh7.5 cells with in vitro transcribed full-genomic HCV RNA and viral infectivity was monitored using a focus-forming unit (FFU) staining assay, as described elsewhere.[8] FFU were counted under a microscope, supernatant infectivity titres were determined as the number of FFU/ml and titrated viral stocks were diluted to obtain 2,000 FFU/ml in growth medium.

A volume of 50 µl, corresponding to 100 FFU, was then incubated for 1 hour at 37 °C with 25 µl of five-fold serial dilutions of each rabbit serum collected on days 0 and 56. The virus/serum mixture was then used to infect 10,000 Huh7.5 cells plated the previous day on 96-well plates (Falcon) for 6 hours at 37°C. The virus-containing medium was then removed, replaced with fresh medium and the infected cells were incubated at 37°C. Infectivity was evaluated 48 hours after infection, as described above. The assay was performed once in triplicate and the results are expressed as mean values.

2.5 | Sequence analysis

The amino-acid sequences encoding the E1 or E2 envelope proteins presented at the surface of the HCVcc were downloaded from the GenBank database (deposited under the accession numbers: JF343780.1 (H77/JFH1), FJ230881.1 (J4/JFH1), AB047639.1 (JFH1), EU204645.1 (S52/JFH1) and MK600383.1 (ED43/JFH1)) and aligned with those encoding the E1 or E2 envelope proteins presented at the surface of the vaccine particles (Figure S1 and S2). Multiple sequence alignment was computed using ClustalW (BioEdit software) and edited manually.

2.6 | Statistical analysis

The observed differences in neutralizing antibody induction capacity between the vaccination strategies for a given genotype were analyzed statistically with nonparametric Mann-Whitney tests. These distribution comparison tests were conducted considering only the datasets obtained for the six rabbit sera presenting the most sustained anti-HBV and anti-HCV antibody responses and also containing the antibodies with the most significant cross-neutralizing properties. A p value < 0.05 was considered to be statistically significant.

3 | RESULTS

All HBV-HCV vaccine particles (whether used alone or in cocktails, Figure 1A) induced specific antibody responses against the HCV and HBV envelope proteins (Figure 1B). After the evaluation of the anti-HBsAg, anti-E1 and anti-E2 antibodies levels, the neutralizing properties of antibodies induced by the various vaccination strategies in immunized rabbits were investigated using HCVcc harbouring HCV envelope glycoproteins derived from different heterologous genotype 1a, 1b, 2a 3a and 4a strains (Figure S3). By paying particular attention to the neutralizing properties of the six rabbit sera with the strongest humoral responses to vaccination in each group (the rabbits with the highest levels of anti-HBsAg antibodies were also those with the highest levels of anti-E1 and anti-E2 antibodies), we demonstrated that immunization with a cocktail of E2S particles bearing HCV genotype 1a, 3a and 4a E2 envelope glycoproteins significantly increased the broad neutralizing properties of the antibodies induced (p-values = 0.026 and 0.005 for HCV genotype 3 and 4 neutralization, respectively, when a comparison is made with animals immunized with $E2^{1a}S$ particles only) (Figure 2). More importantly, we clearly show that the antibodies produced in response to vaccination with this cocktail were above all capable to neutralize infections with an HCVcc of genotype 2a harbouring an E2 envelope sequence not present in the vaccine cocktail (Figures 2 and S3). The strength of these broad cross-neutralizing responses was confirmed with additional neutralizing assays using serial dilutions of rabbit sera, showing that these broad neutralizing properties of the antibodies induced were also observed for high serum dilutions (Figure S4). The addition of particles bearing E1 glycoproteins to this cocktail of particles bearing E2 proteins from genotypes 1a, 3a and 4a clearly reduced the positive impact of this multi-genotypes strategy. By contrast and importantly, the use of a cocktail of $E2^{1a}S$ + $E2^{3a}S$ + $E2^{4a}S$ particles did not significantly decrease the ability of the vaccine to induce neutralizing antibodies against HCV genotypes 1a and 1b, relative to the prototype $E2^{1a}S$ vaccine. Nevertheless, a cocktail of only two types of particles, bearing HCV genotype 4 E1 and E2 glycoproteins separately, was identified as the best candidate vaccine for protection against this particular genotype. It clearly gave the best protection in comparisons of animals immunized with the $E1^{4a}S$ + $E2^{4a}S$ particle cocktail and animals immunized with the prototype $E2^{1a}S$ vaccine (p-value = 0.005, Figure 2). However, even though the median value for neutralization obtained in animals immunized with $E1^{4a}S$ + $E2^{4a}S$ particle cocktail was higher than that for animals immunized with the $E2^{1a}S$ + $E2^{3a}S$ + $E2^{4a}S$ particle cocktail (81.5% *vs* 70.5%, Figure 2), the difference was not significant, probably due to the small number of animal.

4 | DISCUSSION

DAAs have revolutionized the treatment of chronic HCV infection. Nevertheless, it has become clear that it will be very difficult to eradicate hepatitis C without a prophylactic vaccine against this virus.[10,11] Various vaccination strategies are currently being tested for HCV,[12-14] but the development of such vaccines is difficult, and their place in vaccination programs remains unclear. In this context, a bivalent HBV-HCV vaccine would be more likely to be adopted, as its main function would be to provide protection against hepatitis B, and any additional protection, even if only partial, against HCV would be a bonus. The relevance of this vaccine approach is reinforced by the capacity of this vaccine candidate to induce anti-HBsAg responses, although slightly lower than those induced by the commercially Engerix vaccine. It cannot be excluded that this slight decrease in HBsAg immunogenicity is due to the fusion of the envelope proteins, but this observation is probably related to the difference in purity existing between the vaccine prepared in an academic laboratory and the highly purified industrially produced Engerix vaccine. This approach would also strengthen the notion of the hepatitis B vaccine being the first anti-cancer vaccine, as it would result in a new vaccine providing protection against different virus-induced liver cancers.

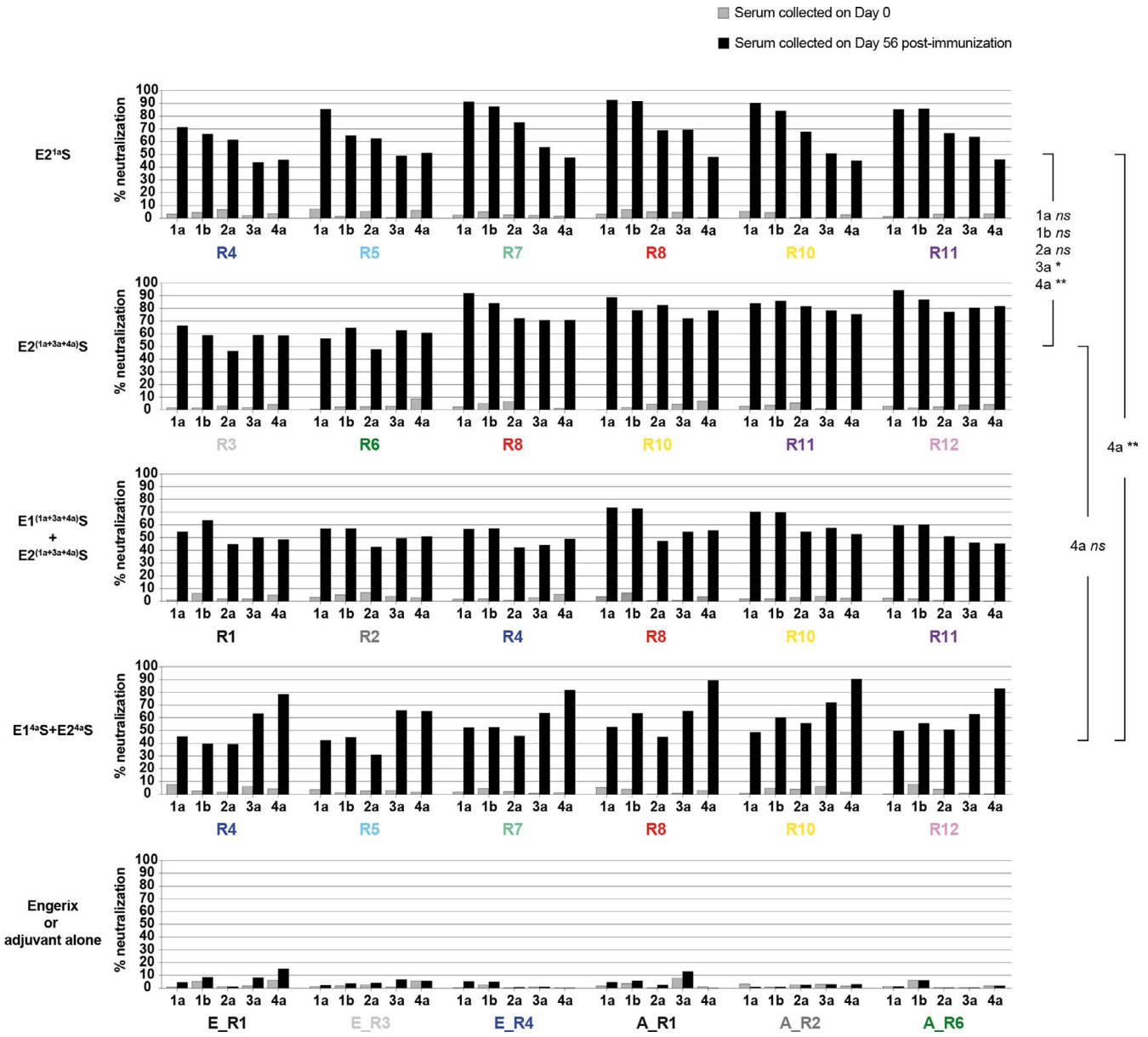


FIGURE 2 Neutralizing potential of antibodies induced by the various vaccination strategies. Cross-neutralizing properties against HCVcc of the anti-HCV antibodies induced by the various vaccination strategies evaluated on the six rabbit serum samples with the strongest humoral responses to vaccination in each group. Five-fold dilutions of rabbit serum samples collected on days 0 and 56 were first incubated with HCVcc harbouring HCV envelope glycoproteins derived from genotype 1a, 1b, 2a, 3a and 4a isolates for 1 hour at 37°C, which were then used to infect Huh7.5 cells for 6 hours. Infection levels were determined after 48 hours of incubation at 37°C, in a FFU staining assay[8]. FFU were counted under a microscope, and the percentage neutralization in the presence of the post-immune serum (Day 56) was compared with that in the presence of pre-immune serum (Day 0) from the same rabbit. The assay was performed once in triplicate, and the results are expressed as mean values. Statistical analyses were performed with nonparametric Mann-Whitney tests. * $p<0.05$; ** $p<0.005$; *ns* = nonsignificant.

Our previous immunization studies, performed with chimeric HBV-HCV envelope subviral particles, showed that particles containing $E2^{1a}$ proteins gave the best neutralizing coverage, but that particles containing $E1^{1a}$ proteins could also induce neutralizing antibodies against HCV genotype 1a and 1b, and, to a lesser extent, against other HCV genotypes.[8] In a second set of experiments, we showed that the most efficient strategy for inducing both anti-E1 and anti-E2 neutralizing antibodies was to mix subviral HBV-HCV envelope subviral particles containing $E1^{1a}$ proteins with particles containing $E2^{1a}$ proteins.[9] In the multi-genotype strategy presented here, the addition of a cocktail of particles bearing E1 proteins to the cocktail of particles bearing E2 proteins clearly decreased the positive impact of this multi-genotype approach, probably by reducing the amount of each immunogen in the dose of vaccine administered and imposing conflicting selection forces which frustrate the affinity maturation of the B

lymphocytes. However, if limited to a cocktail of multi-genotype particles containing only E2 proteins, this approach clearly yielded a much better broad cross-neutralizing response to the main HCV genotypes 1a, 1b, 2a, 3a and 4a. The strength of this broad cross-neutralizing response was greatly amplified by the fact that antibodies produced in response to vaccination with this cocktail were capable to neutralize HCV of genotype 2a, and this also for high serum dilutions. Nevertheless, a cocktail of particles bearing HCV E1 or E2 glycoproteins of a particular genotype separately would be the best strategy for protection against a given genotype (reference 9 for the genotype 1; this study for the genotype 4). These data suggest that a set of HBV-HCV envelope subviral vaccine particles bearing HCV envelope glycoproteins of different genotypes could be used, in various combination strategies, to induce a broad neutralizing protection against different HCV genotypes or to provide protection against a particular genotype being prevalent in a given region. This second approach would clearly be relevant for genotype 4 in Egypt, which has the highest prevalence of HCV infection in the world, where more than 90% of HCV-infected subjects are infected with genotype 4 viruses.[15]

In conclusion, the mixing of different envelope-based subviral particles can be used to optimize the bivalent HBV-HCV prophylactic vaccine, particularly for use in cases of potential exposure to different HCV genotypes. As the neutralizing breadth of anti-HCV antibodies was investigated in this study using a limited panel of historical important reference strains which are known for some to be highly sensitive to neutralization,[16,17] and as the replication-competent cell culture viruses have been described varied widely in terms of sensitivity to neutralization by monoclonal antibodies,[16,18,19] it will be important in future experiments to investigate these phenotypic differences. Further studies, including the immunization of humans, will, of course, also be required before definitive conclusions can be drawn. In addition, it will be interesting to conduct further studies to analyze precisely the genotype-specific or more conserved epitopes targeted by the antibodies induced by the different vaccination strategies. However, this vaccine would be simple to produce, as it could be processed by industrial procedures adapted from those established for the HBV vaccine, and it could potentially help to control the global hepatitis C epidemic.

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CONFLICT OF INTEREST

Philippe Roingeard is co-inventor of the patent "Fusion proteins and use thereof for preparing hepatitis C vaccines" (U.S. No. US008765143B2) that is currently exploited by the ViroCoVax company. Remaining authors declare that they have no conflict of interest.

AUTHOR CONTRIBUTIONS

E.B. and P.R. conceptualized and designed the experiments, analyzed the data and wrote the manuscript. E.B., B.J.C., V.G. and L.C. performed the experiments. E.R. and E.G.-E. provided technical support. All authors approved the final version of the manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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SUPPLEMENTAL FIGURE LEGENDS

FIGURE S1 Comparison of the amino-acid sequences of the E1 proteins harbored at the surface of the vaccine particles and the HCV in cell culture used to conduct the neutralization assays. (A) Multiple sequence alignment of the E1 proteins. Amino-acid sequences of the E1 proteins from genotype 1a, 3 and 4 viruses selected for the development of the vaccine particles (represented in blue) were aligned with the amino-acid sequences of the E1 proteins presented at the surface of the genotype 1a, 3 and 4 HCV in cell culture neutralized by the antibodies induced through vaccination (shown in black). Signal sequence responsible for translocation of the E1 ectodomain into the endoplasmic reticulum lumen is boxed in red. Amino-acid identities with the uppermost sequence (.) and substitutions are indicated. (B) Comparative analysis of the sequence homologies. The numbers indicate the similarities at the amino-acid level in percentage.

FIGURE S2 Comparison of the amino-acid sequences of the E2 proteins harbored at the surface of the vaccine particles and the HCV in cell culture used to conduct the neutralization assays. (A) Multiple sequence alignment of the E2 proteins. Amino-acid sequences of the E2 proteins from genotype 1a, 3 and 4 viruses selected for the development of the vaccine particles (represented in blue) are aligned with the amino-acid sequences of the E2 proteins presented at the surface of the genotype 1a, 3 and 4 HCV in cell culture neutralized by the antibodies induced through vaccination (shown in black). Signal sequence responsible for translocation of the E2 ectodomain into the endoplasmic reticulum lumen is boxed in red. Amino-acid identities with the uppermost sequence (.), gaps (-) and substitutions are indicated. (B) Comparative analysis of the sequence homologies. The numbers indicate the similarities at the amino-acid level in percentage.

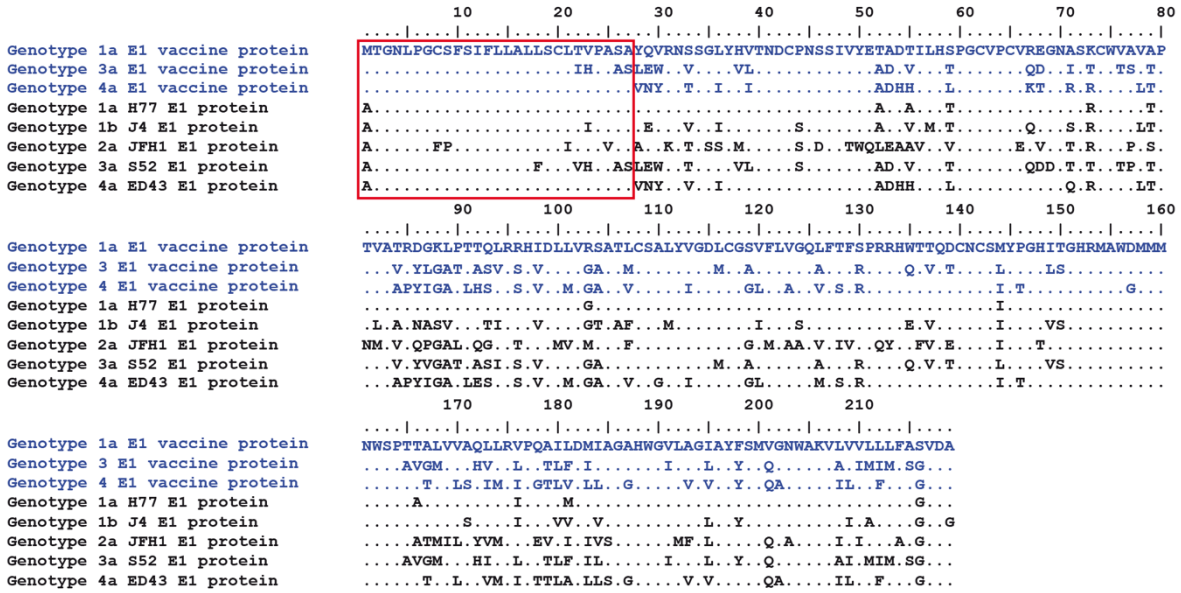
FIGURE S3 Neutralizing potential of antibodies induced by the various vaccination strategies in all immunized rabbits. Cross-neutralizing properties against HCVcc of the anti-HCV antibodies induced by the various vaccination strategies evaluated on all rabbit serum samples. Five-fold dilutions of rabbit serum samples collected on days 0 and 56 were first incubated with HCVcc harboring HCV envelope glycoproteins derived from genotype 1a, 1b, 2a, 3a and 4a isolates for 1 hour at 37°C, which were then used to infect Huh7.5 cells for 6 hours. Infection levels were determined after 48 hours of incubation at 37°C, in a FFU staining assay[8]. The percentage of neutralization was determined by subtracting the infectious

titer obtained with the pre-immune serum (day 0) from that obtained with the post-immune serum (day 56) from the same rabbit. The assay was performed once in triplicate, and the results are expressed as mean values. Each colored point represents the neutralization value observed for the serum sample from an individual immunized rabbit. The horizontal line represents the median value. The conservation of the color code between the main and supplementary figures allows a close correlation of these data with those resulting from the evaluation of the antibody responses.

FIGURE S4 Strengh of the broad cross-neutralizing antibody response. Evaluation of the cross-neutralizing properties against HCVcc of 5-fold serial serum dilutions of the six rabbits with the strongest humoral responses to vaccination in each group. Dilutions of rabbit sera collected on days 0 and 56 were first incubated with HCVcc harboring HCV envelope glycoproteins derived from genotype 1a, 1b, 2a, 3a and 4a isolates for 1 hour at 37°C, which were then used to infect Huh7.5 cells for 6 hours. Infection levels were determined after 48 hours of incubation at 37°C, in a FFU staining assay[8]. FFU were counted under a microscope, and the percentage of neutralization was determined by subtracting the infectious titer obtained with the pre-immune serum (D0) from that obtained with the post-immune serum (D56) from the same rabbit. The assay was performed once in triplicate, and the results are expressed as mean values.

Figure S1

A

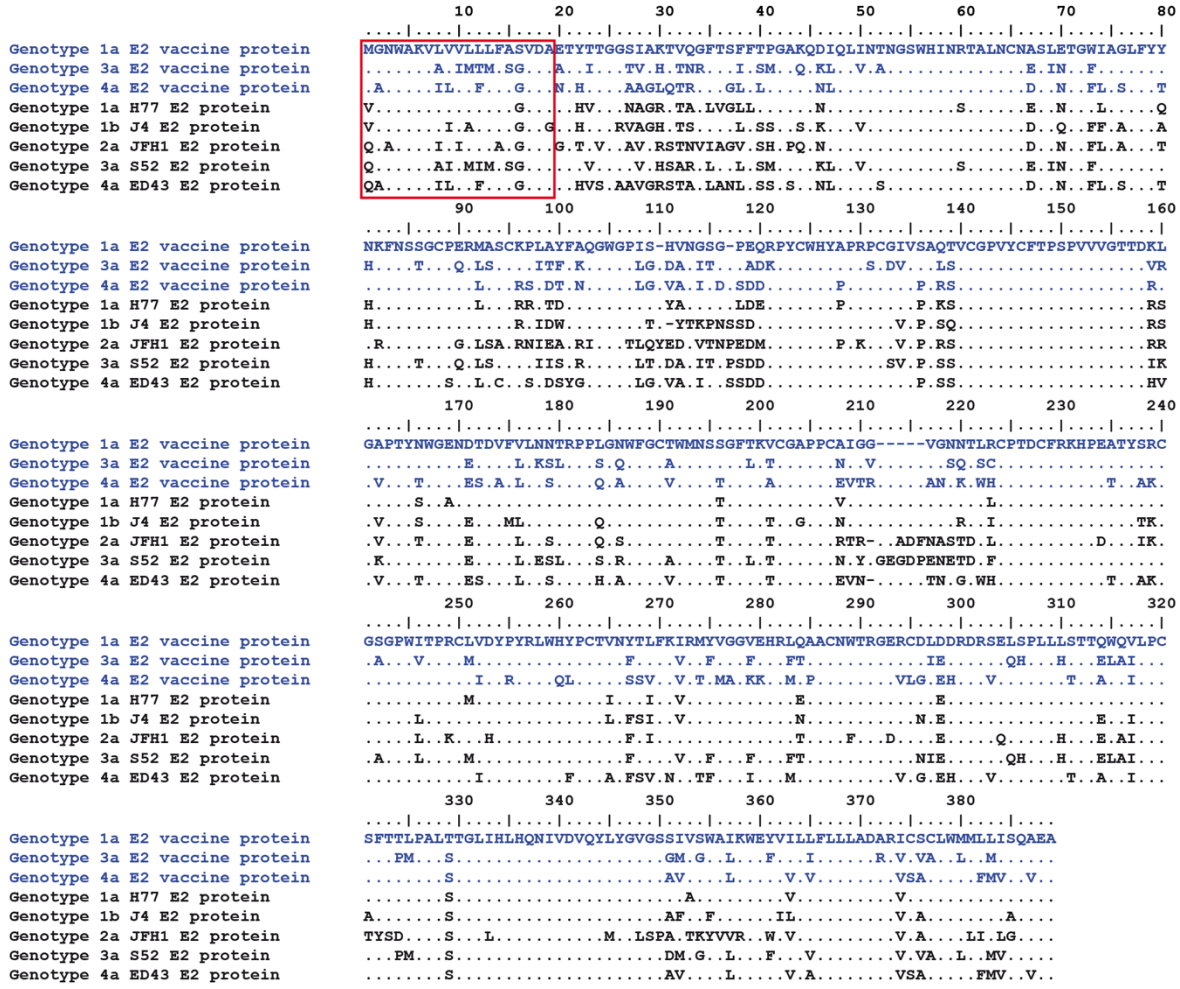


B

E1 amino acid sequence (% homology)	Genotype 1a vaccine candidate	Genotype 3a vaccine candidate	Genotype 4a vaccine candidate
Genotype 1a vaccine candidate		69.4	70.8
Genotype 3a vaccine candidate	69.4		67.6
Genotype 4a vaccine candidate	70.8	67.6	
Genotype 1a H77/JFH1 HCVcc	94.5	70.3	72.1
Genotype 1b J4/JFH1 HCVcc	78.1	68.5	69.9
Genotype 2a JFH1 HCVcc	63.5	54.8	58.9
Genotype 3a S52/JFH1 HCVcc	67.6	92.7	66.7
Genotype 4a ED43/JFH1 HCVcc	72.6	67.1	92.7

Figure S2

A



B

E2 amino acid sequence (% homology)	Genotype 1a vaccine candidate	Genotype 3a vaccine candidate	Genotype 4a vaccine candidate
Genotype 1a vaccine candidate		74	72.8
Genotype 3a vaccine candidate	74		67.1
Genotype 4a vaccine candidate	72.8	67.1	
Genotype 1a H77/JFH1 HCVcc	86.4	72	75.6
Genotype 1b J4/JFH1 HCVcc	79.2	71.5	74
Genotype 2a JFH1 HCVcc	67.1	65.8	67.4
Genotype 3a S52/JFH1 HCVcc	71.5	86.1	68.1
Genotype 4a ED43/JFH1 HCVcc	71.5	68.1	86.9

Figure S3

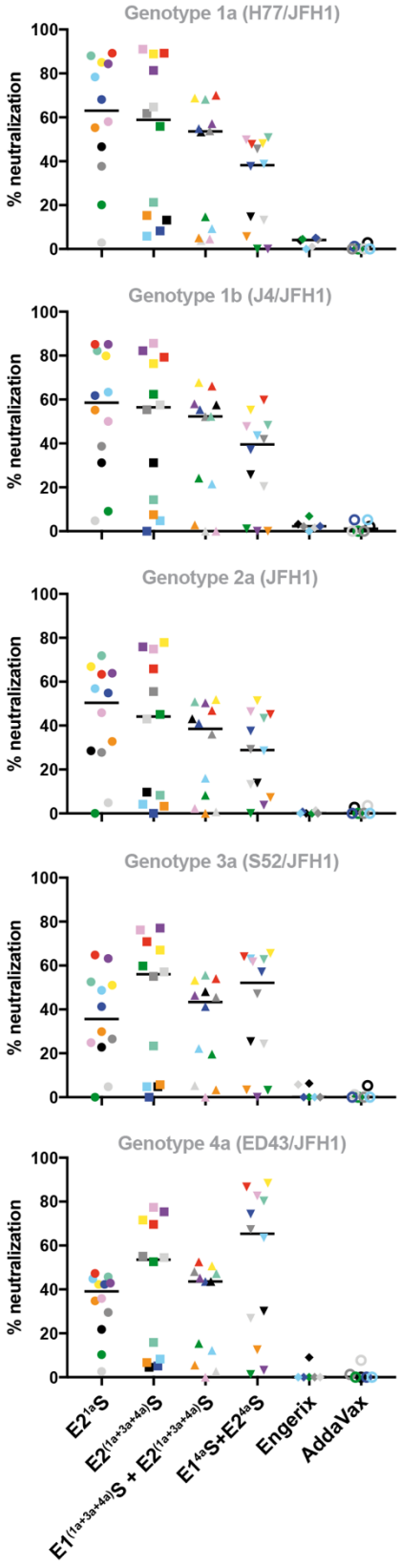
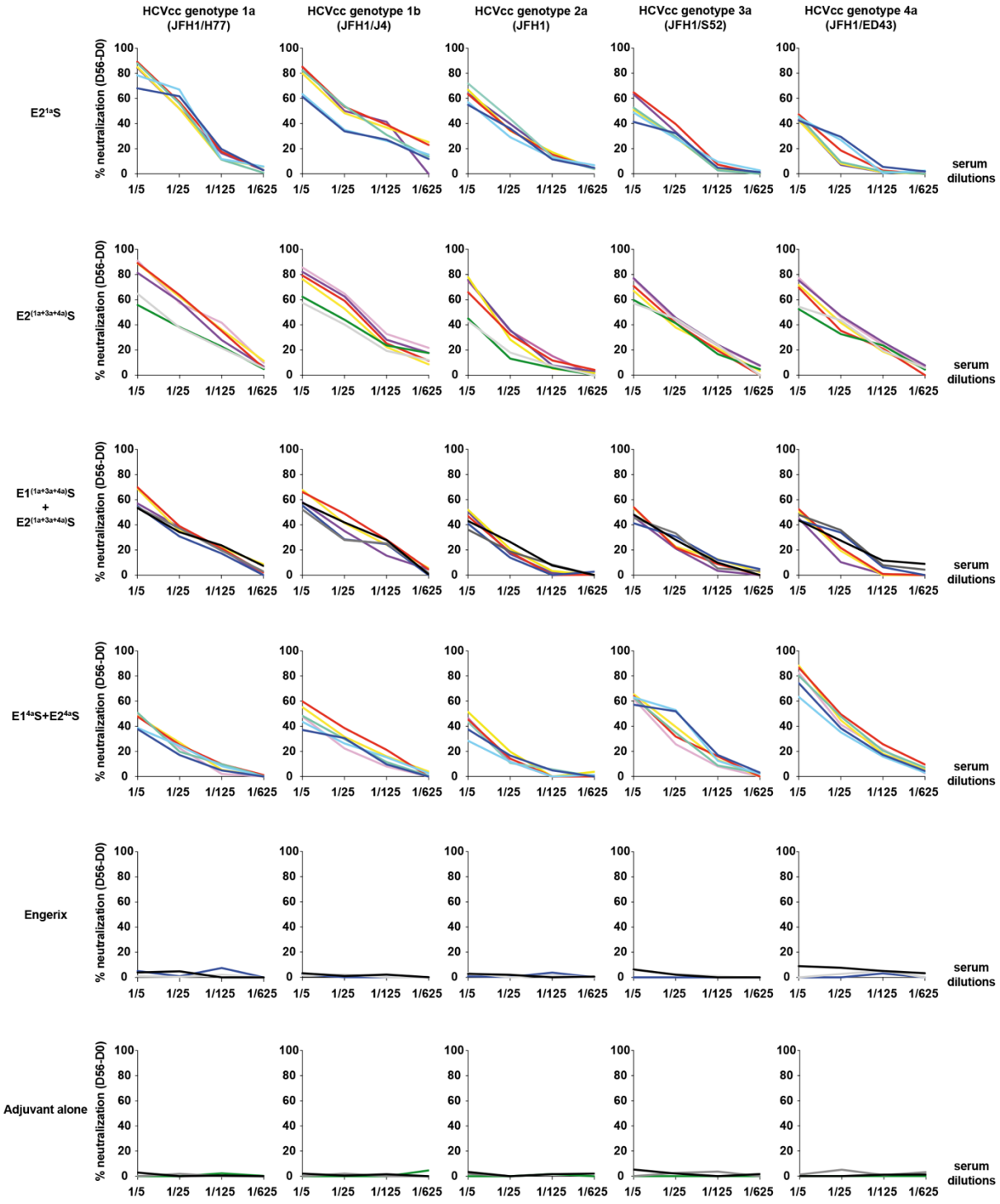

Genotype 1a (H77/JFH1)
Genotype 1b (J4/JFH1)
Genotype 2a (JFH1)
Genotype 3a (S52/JFH1)
Genotype 4a (ED43/JFH1)
% neutralization
100
80
60
40
20
0
E2^1aS
E2^(1a+3a+4a)S
E1^(1a+3a+4a)S + E2^(1a+3a+4a)S
E1^4aS+E2^4aS
Engerix
AddaVax

Figure S4


HCVcc genotype 1a (JFH1/H77)
HCVcc genotype 1b (JFH1/J4)
HCVcc genotype 2a (JFH1)
HCVcc genotype 3a (JFH1/S52)
HCVcc genotype 4a (JFH1/ED43)
E2^1aS
E2^(1a+3a+4a)S
E1^(1a+3a+4a)S + E2^(1a+3a+4a)S
E1^4aS+E2^4aS
Engerix
Adjuvant alone
% neutralization (D56-D0)
100
80
60
40
20
0
1/5
1/25
1/125
1/625
serum dilutions

B

PAPER 2

Incorporation of apolipoprotein E into HBV-HCV subviral envelope particles: implications for an improved hepatitis vaccine strategy

Elsa Gomez-Escobar, Julien Burlaud-Gaillard, Clara Visdeloup, Adeline Ribeiro E Silva, Pauline Coutant, Philippe Roingeard, Elodie Beaumont

Manuscript under revision in *Scientific Reports*

Incorporation of apolipoprotein E into HBV-HCV subviral envelope particles: implications for an improved hepatitis vaccine strategy

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ABBREVIATIONS

apoE	apolipoprotein E
β-gal	beta-galactosidase
BHK-21	baby hamster kidney-21 cells
CHO	Chinese hamster ovary cells
co-IP	co-immunoprecipitation
DAA	direct-acting antiviral
E1	HCV envelope glycoprotein 1
E1-S	chimeric HBV-HCV E1 protein
E2	HCV envelope glycoprotein 2
E2-S	chimeric HBV-HCV E2 protein
ELISA	enzyme-linked immunosorbent assay
FCS	fetal calf serum
FFU	focus-forming unit
HBsAg	hepatitis B surface antigen
HBV	hepatitis B virus
HCC	hepatocellular carcinoma
HCV	hepatitis C virus
HCVcc	hepatitis C virus in cell culture
LVP	lipoviral particle
mAb	monoclonal antibody
Nabs	neutralizing antibodies
OD	optical density
pAb	polyclonal antibody
PBS	phosphate-buffered saline

PCR	polymerase chain reaction
S	HBV small envelope protein
SHBsAg	small hepatitis B surface antigen
SD	standard deviation
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SFV	Semliki Forest virus
SVP	subviral particle
TBS	Tris-buffered saline
TEM	transmission electron microscopy
TNE	Tris-NaCl-EDTA
WHO	World Health Organization
WT	wild-type

ABSTRACT

Hepatitis C is a major threat to public health for which an effective treatment is available, but a prophylactic vaccine is still needed to control this disease. We designed a vaccine based on chimeric HBV-HCV envelope proteins forming subviral particles (SVPs) that induce neutralizing antibodies against HCV *in vitro*. Here, we aimed to increase the neutralizing potential of those antibodies, by using HBV-HCV SVPs bearing apolipoprotein E (apoE). These particles were produced by cultured stable mammalian cell clones, purified and characterized. We found that apoE was able to interact with both chimeric HBV-HCV (E1-S and E2-S) proteins, and with the wild-type HBV S protein. ApoE was also detected on the surface of purified SVPs and improved the folding of HCV envelope proteins, but its presence lowered the incorporation of E2-S protein. Immunization of New Zealand rabbits resulted in similar anti-S responses for all rabbits, whereas anti-E1/-E2 antibody titers varied according to

the presence or absence of apoE. Regarding the neutralizing potential of these anti-E1/-E2 antibodies, it was higher in rabbits immunized with apoE-bearing particles. In conclusion, the association of apoE with HCV envelope proteins may be a good strategy for improving HCV vaccines based on viral envelope proteins.

INTRODUCTION

About 71 million people worldwide are chronically infected with hepatitis C virus (HCV), a major cause of severe chronic liver disease that may progress to hepatocellular carcinoma (HCC).[1,2] Therapies based on the use of direct-acting antivirals (DAAs) are available and highly effective (with cure rates above 95%), but with some limitations. Most infected individuals are asymptomatic, so only a small percentage are diagnosed and, therefore, treated. The cost of DAAs, which has decreased in recent years, is less of a problem than the cost of the screening logistics required to identify chronic carriers of HCV worldwide that have yet to be treated.[3] Moreover, reinfection can occur, because these drugs have no protective effect, and they do not fully prevent the development of severe liver diseases, such as HCC, either. [4–6] DAAs can also fail to cure infections involving several distantly related genotypes of HCV. [7–9] The World Health Organization (WHO) aims to eliminate viral hepatitis as a public health threat by 2030. However, it was estimated in 2015 that two million new HCV infections occur annually, which is much greater than the number of people cured (843,000 individuals).[1] Recent studies have suggested that the WHO's goals are likely to be reached only locally in a few countries, not globally.[10,11] In light of this evidence, it will be difficult to control the spread of HCV without a prophylactic vaccine.

The spontaneous clearance of HCV has been observed in 15-25% of patients and is related to early cellular and humoral responses.[12–14] This early induction of neutralizing antibodies

(Nabs), together with a T-cell response, is the key to containing the infection and provides important leads for the development of a prophylactic vaccine. Most of the preventive vaccines against various pathogens currently available are based on the development of Nabs.[15] In the case of HCV, the envelope glycoproteins (E1 and E2) are the natural targets of these antibodies and have been widely used in various prototype vaccines.[16–20] One of these candidate vaccines, based on recombinant HCV E1 and E2 proteins, was shown to elicit high titers of anti-HCV envelope antibodies with cross-neutralizing properties, together with a T-cell response, in a human phase I clinical trial (NCT00500747).[16,21] However, the E1 and E2 glycoproteins are transmembrane proteins and are thus difficult to extract and purify in large-scale manufacturing processes. We have tried to overcome this problem by developing a prototype HCV vaccine consisting of full-length HCV E1 or E2 glycoproteins (from genotype 1a) fused to the heterologous hepatitis B virus (HBV) small envelope protein (S), also known as small hepatitis B surface antigen (SHBsAg), that self-assemble into highly immunogenic, noninfectious and secreted subviral particles (SVPs) resembling the HBV vaccine.[22] We have shown that these particles can induce strong specific antibody responses directed against the HCV and HBV envelope proteins in immunized rabbits, together with Nabs capable of neutralizing HCV infection *in vitro*.[23,24] Moreover, we have shown that the E1 and E2 proteins are more immunogenic when presented separately in the SVPs.[25] Finally, we recently reported that immunization with mixtures of SVPs presenting HCV envelopes of different genotypes improves the induction of cross-neutralizing antibodies against various genotypes of HCV.[26]

However, it is important to take the close interactions of HCV with host lipid metabolism into account when developing a vaccine against this virus. HCV associates with lipid components and circulates in the bloodstream of infected patients as lipoviral particles (LVPs), which help the virus to escape from Nabs.[27] Apolipoprotein E (apoE) is a component of HCV LVPs that

has been shown to play a key role in the modulation of Nab sensitivity.[28] ApoE is a polymorphic lipoprotein that not only interacts with HCV envelope glycoproteins, but also participates in various stages of the viral cycle: entry, assembly and the maturation of infectious virions.[29–32] Consequently, apoE seems to be an important element for ensuring the correct folding and presentation of HCV envelope proteins as on the viral surface. In this study, we aimed to improve our vaccine model by reproducing the interactions between HCV envelope glycoproteins and apoE at the surface of HBV-HCV SVPs, with the ultimate objective of increasing the neutralizing properties of the antibodies induced by immunization.

RESULTS

Effective interaction between apoE and chimeric HBV-HCV envelope proteins

The presence of apoE and chimeric E1-S or E2-S proteins in the baby hamster kidney-21 cell (BHK-21) lysates used for co-immunoprecipitation (co-IP) assays was evaluated by western blotting (Fig. 1a). The protein complexes co-immunoprecipitated and captured with anti-apoE antibodies coupled to G-sepharose beads were denatured and analyzed by the same technique. The proteins of interest were detected at the expected sizes, whereas no proteins were captured with a non-specific goat isotype (Fig. 1b). These results confirm the interactions between apoE and chimeric HBV-HCV proteins and the possibility of producing vaccine particles reproducing the interactions between these proteins.

Efficient incorporation and presentation of apoE on the surface of the vaccine particles

SVPs produced by Chinese hamster ovary cell (CHO) clones stably expressing proteins of interest were purified with a mean purity of 13%. The incorporation of proteins of interest into purified SVPs was first verified by western blotting (Fig. 2a). We found that apoE was efficiently incorporated into SVPs, even those consisting of the wild-type (WT) HBV S protein

alone. In particles containing the WT HBV S and E1-S or E2-S proteins, apoE clearly modulated the incorporation of chimeric HBV-HCV proteins. The amount of E1-S protein in apoE-containing SVPs was slightly higher than that in SVPs without apoE, whereas the opposite effect was observed for the incorporation of E2-S protein (Fig. 2a). Furthermore, the amount of apoE was always higher in particles bearing the E2-S protein than in those presenting the E1-S protein. These differences in incorporation were observed in several different purification batches. We subsequently checked that apoE was not only incorporated, but also exposed on the surface of the SVPs (Fig. 2b and 2c), by two different techniques. Enzyme-linked immunosorbent assays (ELISAs) (Fig. 2b) showed that the amount of apoE exposed on the different particles followed the same trend, E2-S>E1-S>S, as observed in the western blotting analysis (Fig. 2a). Finally, transmission electron microscopy (TEM) observations confirmed that apoE was present on the surface of SVPs and that particles had a morphology identical to that of WT HBV S particles (Fig. 2c).

Differential incorporation and presentation of chimeric HBV-HCV envelope proteins in the presence and absence of apoE

We characterized this modulation of the incorporation of chimeric HBV-HCV envelope proteins into SVPs in more detail, by studying the HCV envelope proteins presented on the surface of particles in ELISAs with different antibodies (Fig. 3a). The detection of E1-S, by two monoclonal antibodies (mAbs) (A4 and H-111), revealed this protein to be present in larger amounts on S+E1-S+apoE particles than on S+E1-S particles. Conversely, the levels of E2-S protein detected with two mAbs (AP33 and AR3A) were poor when apoE was copresented on the SVPs. We analyzed the effect of apoE on the folding of chimeric E1-S and E2-S proteins, by normalizing ELISA results against the amount of these proteins incorporated into SVPs. The western blots were analyzed by densitometry (Fig. 2a), and the relative densities of E1-S and

E2-S proteins were calculated in particles bearing apoE (S+E1-S+apoE and S+E2-S+apoE), in comparison to S+E1-S and S+E2-S, respectively. We found that there was 1.5 times more E1-S protein in apoE-containing particles than in S+E1-S SVPs, whereas the amount of E2-S protein in S+E2-S+apoE particles was lower, by a factor of 3.4, than that in S+E2-S particles. The optical density (OD)-to-relative density ratios (Fig. 3b) showed that higher levels of E1-S protein were detected on S+E1-S+apoE SVPs than on S+E1-S particles with the H-111 mAb, whereas this protein was detected at similar levels on both types of particle with the A4 mAb. The E2-S protein was much better recognized by the AP33 and AR3A mAbs (possessing a linear and a conformational epitope, respectively) when apoE was copresented on the SVPs. These results suggest that apoE improved the folding of both chimeric E1-S and E2-S proteins.

Immunogenicity of vaccine particles with and without apoE

Sera were collected from the immunized rabbits on days 0, 12, 26, 42 and 56, for the evaluation of humoral immune responses (Fig. 4). Anti-S responses were broadly similar for all groups of rabbits, but were slightly delayed in the groups immunized with apoE-bearing particles. The same phenomenon was observed when WT S vaccine particles were compared with S+apoE SVPs. The anti-E1 responses of rabbits immunized with S+E1-S+apoE SVPs tended to be slightly stronger, and remarkably more homogeneous (the kinetic curves of 7 of the 8 rabbits were similar) than those of rabbits immunized with S+E1-S particles. This observation can be explained by the observed improvements in E1-S protein folding when apoE was copresented, resulting in greater immunogenicity. The curves for the anti-E2 responses of rabbits immunized with S+E2-S+apoE SVPs showed peaks for anti-E2 antibody levels on day 42, followed by a rapid decrease in the response at day 56. These responses were clearly inferior on day 56 than those observed with S+E2-S particles, probably due to the much smaller amounts of E2-S protein observed in S+E2-S+apoE SVPs.

Neutralization properties of the antibodies induced by immunization with vaccine particles with and without apoE

Sera (collected on days 0 and 56) from rabbits immunized with SVPs containing or not containing apoE were evaluated in neutralization assays against hepatitis C virus in cell culture (HCVcc) harboring HCV envelope proteins from genotype 1a (isolate H77) (Fig. 5a). The percentage (%) neutralization achieved with serum from rabbits immunized with S+E1-S+apoE particles was higher than that achieved with serum from rabbits immunized with S+E1-S particles; the median and mean values for the S+E1-S+apoE group (57.68% and 61.09%) were higher than those for the S+E1-S group (48.53% and 48.17%). The data for the S+E1-S+apoE-immunized rabbits were more homogeneously distributed than those for the S+E1-S group (standard deviations of 9.53 and 22.10, respectively), confirming the phenomenon observed for the anti-E1 responses analyzed by ELISA (Fig. 4). Nevertheless, the % neutralization obtained following immunization with S+E2-S+apoE particles was lower (median 28.52% and mean 28.23%) than that achieved by immunization with S+E2-S SVPs (median 59.14% and mean 59.49%).

Given the much lower levels of anti-E2 antibodies detected on day 56 in rabbits immunized with S+E2-S+apoE particles (Fig. 4), and to optimize the evaluation of the neutralizing potential of these antibodies, we selected the best four rabbits and calculated the ratio of their % neutralization relative to the OD (490 nm) values obtained for anti-E2 responses by ELISA on day 56. The ratios (Fig. 5b) obtained for rabbits immunized with S+E2-S+apoE particles were higher than those for rabbits immunized with S+E2-S SVPs (median values of 342.2 and 154.7, respectively). The data for both groups were evaluated with the non-parametric Mann-Whitney *U* test and a significant difference (*p* value=0.0286) was observed, indicating that the

anti-E2 antibodies generated through the copresentation of apoE on chimeric HBV-HCV SVPs were of higher quality.

DISCUSSION

HCV-associated apoE has been shown to help the virus to avoid neutralization by antibodies isolated from chronically infected patients. Functional analyses with human mAbs showed that conformational epitopes of E2 protein were exposed after apoE depletion and that the level and conformation of virion-associated apoE affected the ability of the virus to escape neutralization by antibodies.[28] These important findings revealed a novel strategy contributing to ability of HCV to escape the immune system and establish chronic infection. We hypothesized that immunogens mimicking epitopes at the interface between HCV envelope proteins and apoE might generate a better neutralizing humoral immune response against HCV. The objective of this study was, therefore, to test this hypothesis using our bivalent HBV-HCV vaccine model.

We first showed, through co-IP experiments, that chimeric E1-S and E2-S proteins were able to interact with apoE, even in the context of fusion with the HBV S protein. The interaction between apoE and the E1-E2 heterodimer is well documented,[29–31] but conflicting results have been obtained in previous studies, with one study showing that the E1 protein was responsible for this interaction,[29] whereas two other studies implicated the E2 protein.[30,31] We found that both our chimeric HBV-HCV envelope proteins were able to interact with apoE, making it possible to investigate the incorporation of apoE into vaccine particles. We successfully produced HBV-HCV SVPs bearing apoE, the presence of which was confirmed by western blotting, ELISA and TEM experiments. Curiously, apoE was also incorporated into particles containing only the WT HBV S protein, suggesting a direct interaction between these two proteins. Indeed, this result is consistent with a recent study reporting that an association of

apoE with HBV is essential for virus production.[33] Nevertheless, this interaction raises the question of the protein domains involved in the association between apoE and the chimeric proteins. ELISA and western blotting experiments showed that larger amounts of apoE were incorporated into particles containing E1-S or E2-S proteins than into particles containing only the WT HBV S protein, suggesting a possible cumulative effect of these different interactions. To verify this hypothesis, we were able to demonstrate by co-IP experiments that native HCV envelope proteins (both E1 and E2) interact with apoE (see Supplementary Fig. S1 online; procedures are also described in the Supplementary Information file). Moreover, the vaccine particles bearing the chimeric E2-S protein incorporated the largest amounts of apoE, implying either stronger protein-protein interactions or an involvement of more than one protein domain in this interaction. This may reflect the reported higher stringency of the apoE-E2 interaction than of the apoE-E1 interaction.[31] In any case, despite the large amount of apoE incorporated into SVPs, particularly those carrying the E2 protein, TEM analyses showed that the particles generated had the same morphology as the original HBV vaccine.

We found that the incorporation of E1-S was not affected by the presence of apoE, whereas that of E2-S was strongly impaired in the presence of apoE. The reasons for this difference in incorporation are unknown, but may be related to the amount of apoE detected in these particles. This phenomenon was repeatedly observed in the preparation of several batches of purified SVPs. Indeed, these results are consistent with previous studies showing that, in a viral context, apoE is exposed at a higher copy number than the HCV E2 protein. [34,35] It is, therefore, possible that, during the SVP budding process, a strong interaction occurs between E2-S and apoE, or that the presence of a large number of apoE molecules interferes with the incorporation of the chimeric E2-S protein into particles, instead favoring the integration of WT S proteins. In any

case, more experiments are required to validate these hypotheses and to shed light on the process of particle formation.

Following normalization of the detection rates of chimeric E1-S and E2-S proteins on the surface of the SVPs, we found that apoE improved the folding of these proteins. We used mAbs directed against HCV envelope E1 and E2 proteins for the recognition of chimeric envelope proteins. The AR3A mAb was able to detect its conformational epitope in the HCV E2 protein, and this detection was improved by the incorporation of apoE into particles. The AP33 mAb, the epitope of which is located in the conserved region downstream (aa 412-423) from hypervariable region 1 of the E2 protein, also recognized the E2 protein more efficiently when apoE was present. Similar findings were obtained for the H-111 mAb, one of the few antibodies with neutralizing activity directed against the HCV E1 protein. The access of antibodies to these epitopes has already been described as a presumably important element in the development of an effective HCV vaccine.[36] Our findings therefore show that the HBV-HCV particles are a potentially good model for mimicking the apoE/HCV envelope-glycoprotein interface.

An analysis of the immune responses of rabbits vaccinated with SVPs showed that apoE had no statistically significant impact on the level of anti-S antibodies by day 56. However, anti-S responses were delayed in rabbits immunized with vaccine particles bearing apoE, possibly due to the immunomodulatory functions attributed to apoE.[37] Anti-E1 antibody responses were efficient with and without apoE, but were much more homogeneous for rabbits immunized with S+E1-S+apoE SVPs. A similar homogeneity was found for the % neutralization of HCVcc genotype 1a, with slightly higher median values for rabbits immunized with S+E1-S particles. We cannot rule out the possibility that this improvement in neutralization was due to the induction of anti-apoE antibodies in immunized animals. Unfortunately, we were unable to

establish an ELISA for the specific detection of such antibodies. Furthermore, this increase in % neutralization did not seem to be related to a neutralizing activity of potential anti-apoE antibodies, because animals immunized with S+apoE particles did not develop a neutralizing response against HCV. The results for the E1 protein are promising, as S+E1-S+apoE SVPs could be used in a mixture of vaccine particles. In a previous study, we evaluated this strategy with particles bearing the E1 and E2 proteins separately and found that anti-E1 and anti-E2 antibodies had additive neutralizing properties, suggesting that this approach would be relevant for inducing dual anti-E1 and anti-E2 responses.[25]

Unfortunately, the anti-E2 responses obtained in rabbits immunized with S+E2-S+apoE particles were rather disappointing when compared with those obtained for rabbits immunized with S+E2-S SVPs. However, these results are not surprising, as only very small amounts of E2-S protein were incorporated into the apoE-containing particles. The anti-E2 response displayed a small peak at day 42 (above the anti-E2 responses from the S+E2-S group at that time point), before drastically falling by day 56, whereas antibody levels continued to increase in animals vaccinated with S+E2-S SVPs. This phenomenon remains unexplained and requires further investigation. These results may be related to the complex roles of apoE in modulating the immune response.[37] The neutralizing responses in animals immunized with S+E2-S+apoE particles were disappointing, as for the anti-E2 antibody levels determined by ELISA, with a much lower % neutralization than for immunization with S+E2-S particles. Nevertheless, after the normalization of % neutralization based on antibody levels at day 56, the antibodies generated in the presence of apoE were found to have better neutralizing properties than those induced by S+E2-S SVPs. To evaluate the neutralizing potential of those antibodies against another HCV genotype, we performed neutralization assays of HCVcc genotype 2a (isolate JFH1) and we observed comparable results to the homologous neutralization (see

Supplementary Fig. S2). These results suggest that this approach is potentially interesting for vaccine strategies aiming to increase the neutralizing potential of anti-E2 antibodies. In the case of our bivalent HBV-HCV vaccine model, the problem of the low level of E2-S protein incorporation into apoE-containing particles will need to be resolved. We therefore now plan to modulate the expression of apoE in order to decrease the apoE/E2 ratio in the secreted SVPs.

In conclusion, this study shows that both chimeric HBV-HCV proteins were able to interact with apoE. The presentation of E1-S or E2-S proteins, in complex with apoE, resulted in the induction of a more efficient HCV-neutralizing humoral response than the presentation of these proteins without apoE. The results obtained with our HBV-HCV vaccine model could potentially be exploited for immunization with E1-containing SVPs. Unfortunately, apoE greatly decreased the incorporation of E2 protein into the vaccine particles. This problem will need to be addressed for the development of a similar strategy for immunization with particles bearing the E2 protein. In spite of that, these results demonstrate the potential benefits of incorporating apoE into vaccine particles for the presentation of HCV envelope proteins to the immune system, and this protein may be useful for other envelope-based vaccine approaches against HCV.

MATERIALS AND METHODS

The authors of this article certify that all experiments were performed in accordance with relevant guidelines and regulations, including the recommendations of the ARRIVE guideline.

Analysis of interactions between apoE and chimeric HBV-HCV envelope proteins through co-immunoprecipitation assays

Co-IP assays were performed on lysates of BHK-21 cells co-expressing apoE and chimeric HBV-HCV envelope proteins (E1-S or E2-S) (HBV subtype *adw* and HCV genotype 1a) or β-galactosidase (β-gal) from original Semliki Forest virus (SFV)-derived vectors. We ensured that all these proteins of interest were produced by cotransfecting cells with each of the previously described pSFV-E1-S, pSFV-E2-S and pSFV3-β-gal vectors[22] separately, and with a new pSFV1-apoE vector (β-gal+apoE, E1-S+apoE or E2-S+apoE). This new plasmid was generated by the same strategy as the other SFV-derived vectors.[22] Briefly, the gene encoding human apoE isoform E3 was amplified by polymerase chain reaction (PCR) from the pOTB7-apoE plasmid (kindly provided by Dr. Catherine Schuster)[38], with the Phusion® high-fidelity DNA polymerase (New England Biolabs) and primers flanked by *Bam*HI restriction sites (underlined), forward: 5'-ATAGGATCCATGAAGGTTCTGTGGGCTGC-3' and reverse: 5'-ATAGGATCCTTAGTGATTGTCGCTGGGCACAG-3'. The PCR product was inserted into the pGEM®-T vector (pGEM®-T Easy Vector System; Promega), from which it was excised with *Bam*HI enzyme (New England Biolabs) for insertion into the *Bam*HI restriction site of the pSFV1 vector (Invitrogen). All PCR products were verified by DNA sequencing. Recombinant SFV RNA synthesis, and the cotransfection and lysis of BHK-21 cells were performed as described elsewhere.[23]

For co-IP assays, the previously described cell lysates and either goat polyclonal (pAb) anti-apoE antibody (AB947; Millipore) or goat-isotype control (R&D Systems) were incubated overnight at 4°C with rec-Protein G-Sepharose® 4B conjugate beads (Invitrogen) in 1X phosphate-buffered saline (PBS; Gibco) solution. The beads were washed in 0.1% Triton X-100 (Sigma-Aldrich) in 1X PBS solution, and the immune complexes were analyzed by western blotting in denaturing conditions. For this purpose, samples containing equal amounts of total protein were prepared in reducing sample buffer (1X Laemmli buffer supplemented with 25%

β-mercaptoethanol (Sigma-Aldrich)) and boiled for 5 minutes. They were then subjected to sodium-dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in 12% acrylamide gels and the resulting bands were electroblotted onto a nitrocellulose membrane (Amersham Protran; GE Healthcare). Non-specific sites were blocked by incubation with 2% non-fat dry milk in 1X Tris-buffered saline (TBS; Euromedex) solution for 1 hour at room temperature. Membranes were incubated overnight at 4°C with a mouse mAb directed against E1 (clone A4; generously provided by Dr. Harry Greenberg)[39], a mouse anti-E2 mAb (clone H52; generously provided by Dr. Jean Dubuisson)[40], an anti-apoE pAb (AB947) or a mouse anti-actin mAb (AB1978, clone AC-15; Sigma-Aldrich). The membranes were then washed with 0.3% Tween® (Sigma-Aldrich) in 1X TBS solution and incubated with horseradish peroxidase-conjugated secondary antibodies for 1 hour at room temperature. Membranes were washed again with 0.3% Tween® buffer and then developed by enhanced chemiluminescence. BHK-21 cell lysates, used for co-IP assays, were also analyzed according to the same western blotting protocol.

Development of stable CHO clones co-expressing apoE and chimeric HBV-HCV envelope proteins

Stable clones of CHO cells co-expressing WT HBV S protein (HBV subtype *adw*) and chimeric HBV-HCV E1-S or E2-S envelope proteins (HCV genotype 1a) were generated by lentiviral transduction, as described elsewhere.[22] New clones co-expressing these proteins of interest and apoE were developed according to the same strategy. Briefly, CHO-S, CHO-S+E1-S and CHO-S+E2-S clones were stably transduced with a lentiviral expression vector encoding apoE and the blasticidin-S deaminase. Clones (CHO-S+apoE, CHO-S+E1-S+apoE and CHO-S+E2-S+apoE) resistant to the antibiotics hygromycin and blasticidin (InvivoGen) were amplified and protein levels were evaluated by western blotting, as described above, with anti-E1 mAb (A4), anti-E2 mAb (H52), anti-apoE pAb (AB947) and goat anti-S pAb (70-HG15; Fitzgerald).

Production and purification of vaccine particles

CHO clones (CHO-S, CHO-S+apoE, CHO-S+E1-S, CHO-S+E1-S+apoE, CHO-S+E2-S and CHO-S+E2-S+apoE) were cultured in DMEM/F12 medium (Gibco) supplemented with 10% heat-inactivated fetal calf serum (FCS; Eurobio Scientific), 100 U/mL penicillin and 100 µg/mL streptomycin (penicillin-streptomycin; Gibco) at 37°C, under a humidified atmosphere containing 5% CO_2. Clones were gradually adapted to a low-serum (2.5% FCS) Advanced DMEM/F12 medium (Gibco) appropriate for mass production. In some cases, depending on the clone of interest, the medium was supplemented with antibiotics (hygromycin and/or blasticidin). Each clone was cultured in HYPERFlask® cell culture vessels (Corning). Every 10 to 15 days, the supernatant was harvested and the culture vessels were fed with cells and fresh media.

SVPs (S, S+apoE, S+E1-S, S+E1-S+apoE, S+E2-S and S+E2-S+apoE) were purified from the supernatants of cell cultures as described elsewhere.[22,23] Briefly, total proteins were precipitated by incubation overnight at 4°C with ammonium sulfate ($(NH_4)_2SO_4$). The precipitated proteins were recovered by centrifugation and dialyzed against Tris-NaCl-EDTA (TNE) buffer (10 mM Tris-HCl [pH 7.5]/100 mM NaCl/1 mM EDTA) overnight at 4°C. The SVPs were purified by successive isopycnic ultracentrifugations on cesium chloride (CsCl) gradients. SHBsAg-positive fractions of gradients were identified in a previously described "in-house" ELISA[22], pooled and dialyzed against TNE buffer overnight at 4°C. Purified vaccine particles were concentrated with the Amicon Ultra-15 100k device (Millipore) and their final concentrations of SHBsAg were determined with the same "in-house" ELISA, with a sequentially diluted recombinant hepatitis B surface antigen (HBsAg) subtype *adw* (R86872; Meridian Life Science) as the standard. SVP purity was calculated as the ratio of SHBsAg concentration to

total protein concentration assessed by determining the absorbance measured at 280 nm with a NanoDrop spectrophotometer (DeNovix).

Analysis of protein incorporation into purified SVPs

The presence of proteins of interest (S, chimeric E1-S or E2-S proteins, and apoE) in purified SVPs was first evaluated by western blotting (with an equal concentration of 75 µg/mL SHBsAg), as described above, with the anti-E1 mAb (A4), anti-E2 mAb (H52), anti-apoE pAb (AB947) and anti-S pAb (70-HG15). Densitometry with ImageJ software (NIH) was performed to measure the intensity of the E1-S and E2-S bands on western blots (Fig. 2a). The relative densities of these protein bands were calculated in pairs (S+E1-S and S+E1-S+apoE; S+E2-S and S+E2-S+apoE) with the particles having the lowest intensities for the E1-S or E2-S bands used as the reference (relative density=1).

We evaluated the presence of apoE on the surface of particles by ELISA and TEM. For ELISA, SVPs (containing 25 µg SHBsAg) were captured by lectin (Sigma-Aldrich) in a 96-well plate (Immulon 2 HB; Thermo Fisher Scientific), and apoE was detected with a specific antibody (AB947). The assay was performed once in triplicate, and results are expressed as mean OD (490 nm) ± standard deviation (SD). For TEM, formvar/carbon-coated nickel grids were placed on drops of samples for five minutes, and were then rinsed twice with 1X PBS. Then, grids were incubated on a drop of 1% bovine serum albumin (Sigma-Aldrich)-PBS, and then on a drop of goat anti-apoE pAb (AB947) diluted 1:100 in 1X PBS, for one hour. The grids were washed six times with 1X PBS (5 minutes each), and then incubated for 1 hour on a drop of gold-conjugated (6 nm) donkey-anti-goat IgG (Aurion) diluted 1:30 in 1X PBS. Grids were washed again with 6 drops of 1X PBS, post-fixed in 1% glutaraldehyde and rinsed with three drops of distilled water. Negative staining was then performed with three consecutive contrast

steps, using 2% uranyl acetate (Agar Scientific), for analysis under a transmission electron microscope (JEOL 1011). Negative staining of the samples without labeling was also performed.

The incorporation and folding of chimeric E1-S and E2-S proteins at the surface of the purified SVPs were also evaluated by an ELISA based on the capture of SVPs (25 µg SHBsAg) in 96-well plates (Nunc Immuno plates; Thermo Fisher Scientific) with the anti-S pAb (70-HG15), followed by detection with the anti-E1 mAb (A4), the human anti-E1 mAb (clone H-111; generously provided by Dr. Steven Foung)[41], the human anti-E2 mAb (AR3A; kindly provided by Dr. Mansun Law)[42] or the mouse anti-E2 mAb (clone AP33; Genentech)[43]. The assay was performed once in triplicate, and the results are expressed as mean OD values (490 nm) ± SD. Protein levels were normalized by calculating the ratios between the ODs (490 nm) obtained for each anti-E1 and anti-E2 antibody and the relative density of the E1-S and E2-S proteins, respectively, calculated as described above.

Immunization of female New Zealand rabbits

Vaccine doses were prepared with 15 µg of purified SVPs mixed in a 1:1 ratio (vol:vol) with AddaVax™ (InvivoGen), a squalene-based oil-in-water nanoemulsion. Female New Zealand rabbits were immunized by Agro-Bio (La Ferté Saint-Aubin), a company accredited by the French authorities for the performance of protocols on laboratory animals in accordance with European Directive 2010/63/EU. The protocol was approved by Agro-Bio Ethics Committee C2EA-99 (French Ministry of Research, agreement B45-146-01).

Groups of eight rabbits were immunized subcutaneously with particles of interest (S+E1-S, S+E1-S+apoE, S+E2-S or S+E2-S+apoE), and control groups of four rabbits were injected with

AddaVax™ adjuvant alone, or with S or with S+apoE particles on days 0, 14 and 28. Serum samples were collected from rabbits at various time points (days 0, 12, 26, 42 and 56) for the characterization of humoral responses.

Analysis of anti-S, anti-E1 and anti-E2 responses

Anti-S antibodies were quantified with the ARCHITECT system (Abbott Laboratories). Anti-E1 and anti-E2 responses were evaluated with an "in-house" ELISA based on the use of lysates of BHK-21 cells expressing HCV E1 or E2 proteins, versus β-gal (control), from SFV-derived vectors. Cell lysates containing β-gal, HCV E1 or E2 proteins were obtained as described elsewhere.[23,25] A detailed description of ELISAs can be found in the Supplementary Information file. The results for each sample are expressed as the difference between the mean OD (490 nm) for HCV E1 or E2 wells and the mean of OD (490 nm) for β-gal wells.

Neutralization of HCV in cell culture

The neutralizing properties of antibodies were evaluated *in vitro* with the HCVcc model. First, HCVcc harboring HCV envelope proteins from genotype 1a (isolate H77) [44] were generated by the transfection of hepatoma Huh7.5 cells (kindly provided by Prof. Charles Rice)[45] with the full genomic HCV RNA, transcribed *in vitro*, as previously described.[46] Viral infectivity was determined in a focus-forming units (FFU) staining assay described elsewhere.[23] In addition, we verified the presence of apoE in HCVcc used for neutralization assays (data not shown).

In neutralization assays, 50 µL of inoculum, corresponding to 100 FFUs, was mixed with 25 µL of a 1:5 dilution of rabbit serum (day 0 or 56) and incubated for 1 hour at 37°C. We then infected 10,000 Huh7.5 cells with 75 µL of the virus-serum mixture for 6 hours at 37°C, under a humidified atmosphere containing 5% CO_2. The inoculum was then removed and replaced

with fresh medium and the cells were incubated for a further 37°C. Infectivity was evaluated 48 hours post-infection, as described above. The % neutralization was determined by subtracting the infectious titer obtained with the pre-immune serum (day 0) from that obtained with the post-immune serum (day 56) from the same rabbit. The assay was performed once in triplicate, and the results are expressed as mean % neutralization values for each rabbit.

The neutralizing potential of the antibodies from the best four rabbits (% neutralization > median value) from the S+E2-S and S+E2-S+apoE groups was analyzed by calculating the ratio of % neutralization to the corresponding anti-E2 responses (OD 490 nm) at day 56. Results are expressed as ratios and the median value is shown.

Statistical analysis

Data analyses were conducted with Prism8 (GraphPad), and non-parametric Mann-Whitney *U* tests for group comparisons; p values<0.05 were considered significant.

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AUTHORS CONTRIBUTIONS

Conceptualization and design of the study: EB, EGE and PR. Experiments and procedures: EGE, EB, JBG, CV, ARS and PC. Analysis and interpretation of data: EGE, EB and PR. Drafting and writing of the article: EGE, EB and PR.

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Additional Information

COMPETING INTERESTS

PR is co-inventor of the patent "Novel fusion proteins and use thereof for preparing hepatitis C vaccines"; EGE, PR and EB are co-inventors of the patent "New immunogenic compositions and their use in the preparation of vaccines against hepatitis C and hepatitis B". The remaining authors declare no competing interests.

DATA AVAILABILITY

The datasets generated during the current study are available from the corresponding author on reasonable request.

FIGURE LEGENDS

Figure 1. Evaluation of the interaction between apoE and HCV envelope glycoproteins fused to HBV S envelope protein (E1-S or E2-S) in co-immunoprecipitation assays. (**a**) Western-blot analysis of BHK-21 cell lysates co-expressing chimeric HBV-HCV E1-S or E2-S proteins, and apoE with the anti-E1 mAb (A4), anti-E2 mAb (H52), anti-apoE pAb (AB947) and anti-actin mAb (AB1978). (**b**) Western-blot analysis of immune complexes from co-immunoprecipitation assays of BHK-21 cell lysates of interest with anti-apoE pAb (AB947) or with non-specific goat IgG (isotype control). Analyses were performed with the same antibodies as in panel a.

Figure 2. Characterization of the incorporation of apoE and its presentation in purified HBV-HCV subviral particles. (**a**) Western-blot analysis of subviral particles (75 µg/mL SHBsAg) and detection of proteins of interest with the anti-E1 mAb (A4), anti-E2 mAb (H52), anti-S pAb (70-HG15) and anti-apoE pAb (AB947). (**b**) Detection of apoE on the surface of particles

674 by ELISA. Subviral particles (25 µg SHBsAg) were captured with lectin, and apoE was
675 detected with the anti-apoE (AB947) antibody. The experiment was performed once in
676 triplicate, and the results are expressed as mean OD (490 nm) values ± SD. (**c**) Observation of
677 subviral particles by TEM. Particles were analyzed by negative staining with 2% uranyl acetate
678 solution (upper panels) and by the same method after immunogold staining with an anti-apoE
679 pAb (AB947) (lower panels). Scale bars: 50 nm (in upper and lower panels).
680
681 **Figure 3.** Analysis of the incorporation of E1-S and E2-S proteins and their presentation in
682 purified HBV-HCV subviral particles**.** (**a**) Detection of HCV E1 or E2 proteins on the surface
683 of SVPs by ELISA. Subviral particles (25 µg SHBsAg) were captured with an anti-S pAb (70-
684 HG15), and proteins were detected on the surface of the particles with anti-E1 (mAbs A4 and
685 H-111) or anti-E2 (mAbs AR3A and AP33) antibodies. Experiments were performed once in
686 triplicate and results are expressed as the mean OD (490 nm) values ± SD. (**b**) The levels of
687 chimeric HBV-HCV E1-S or E2-S proteins detected were normalized according to the amount
688 of each protein incorporated into the subviral particles. The results are expressed here as the
689 ratio of OD (490 nm) values obtained for the detection of chimeric HBV-HCV proteins by
690 ELISA to the relative density of bands for the same proteins in subviral particles (Fig. 2a) ±
691 SD.
692
693 **Figure 4.** Humoral immune responses in rabbits immunized with HBV-HCV subviral particles
694 with and without apoE presentation, with the AddaVax^{TM} adjuvant. Specific anti-E1/anti-E2
695 and anti-S responses were evaluated in rabbit sera with "in-house" ELISAs and a routine
696 immunoassay (ARCHITECT), respectively. Sera from rabbits immunized with S, S+apoE and
697 AddaVax^{TM} adjuvant alone were used as controls. Results for anti-E1/anti-E2 responses are
698 expressed as the difference (Δ) in OD (490 nm) (anti-E1 or anti-E2 – β-Gal), whereas anti-S

699 titers are expressed in mIU/mL. Red arrows indicate the time at which immunizations occurred
700 (days 0, 14 and 28).
701
702 **Figure 5.** Neutralizing potential of antibodies induced by the immunization of rabbits with
703 purified HBV-HCV subviral particles with and without apoE. (**a**) % neutralization of HCVcc
704 genotype 1a. Rabbit serum samples (collected on days 0 and 56), at a 1:5 dilution, were
705 incubated with HCVcc harboring HCV envelope proteins from genotype 1a (isolate H77),
706 which were then used to infect Huh7.5 cells. Infection levels were determined 48 hours post-
707 infection, in a focus-forming unit staining assay. The % neutralization was determined by
708 subtracting the infectious titer obtained with the pre-immune serum (day 0) from that obtained
709 with the post-immune serum (day 56) from the same rabbit. The assay was performed once in
710 triplicate, and the results are expressed as mean values. Each colored point represents the %
711 neutralization value calculated for a serum sample from an individual immunized rabbit,
712 whereas the horizontal line represents the median value. The color code established for the
713 antibody responses (Fig. 4), one color per rabbit, was conserved to simplify the correlation of
714 results from both experiments. **(b)** Analysis of the neutralizing capacity of anti-E2 antibodies
715 for the best four rabbits (% neutralization > the median) of each group (S+E2-S versus S+E2-
716 S+apoE), related to the antibody levels, evaluated by ELISA on day 56. Results are expressed
717 here as the ratio of the change in % neutralization between days 0 and 56 (Δ(D56-D0)) to the
718 OD (490 nm) of anti-E2 antibodies obtained by ELISA on day 56 (Fig.4). The groups were
719 compared in non-parametric Mann-Whitney *U* test. (*) p=0.0286

Figure 1

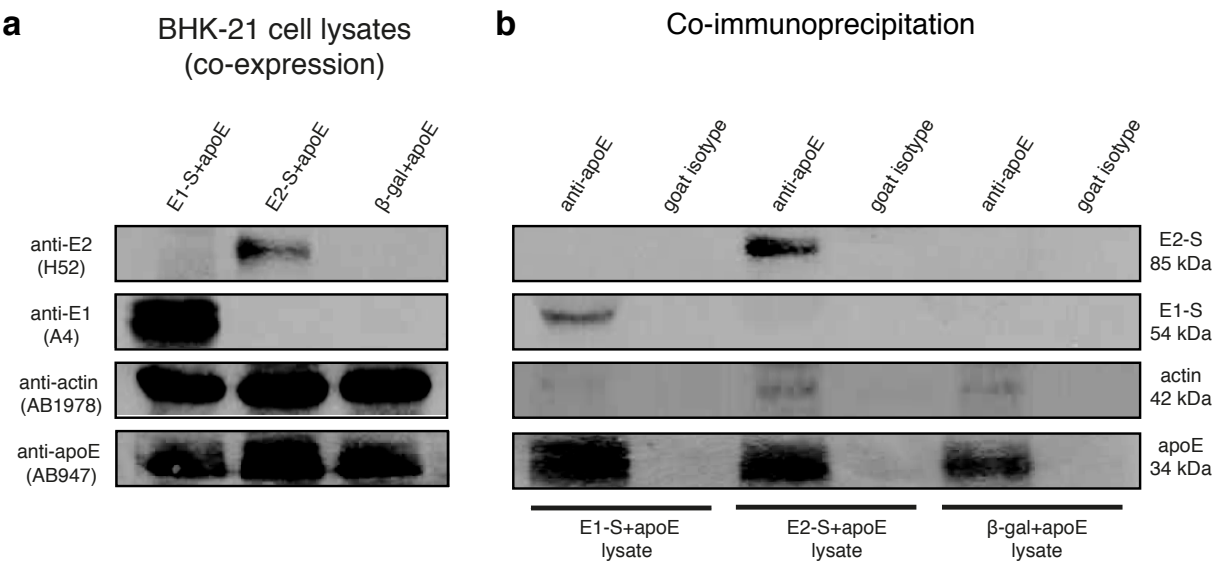

a
BHK-21 cell lysates
(co-expression)
E1-S+apoE
E2-S+apoE
β-gal+apoE
anti-E2
(H52)
anti-E1
(A4)
anti-actin
(AB1978)
anti-apoE
(AB947)
b
Co-immunoprecipitation
anti-apoE
goat isotype
anti-apoE
goat isotype
anti-apoE
goat isotype
E2-S
85 kDa
E1-S
54 kDa
actin
42 kDa
apoE
34 kDa
E1-S+apoE
lysate
E2-S+apoE
lysate
β-gal+apoE
lysate

Figure 2

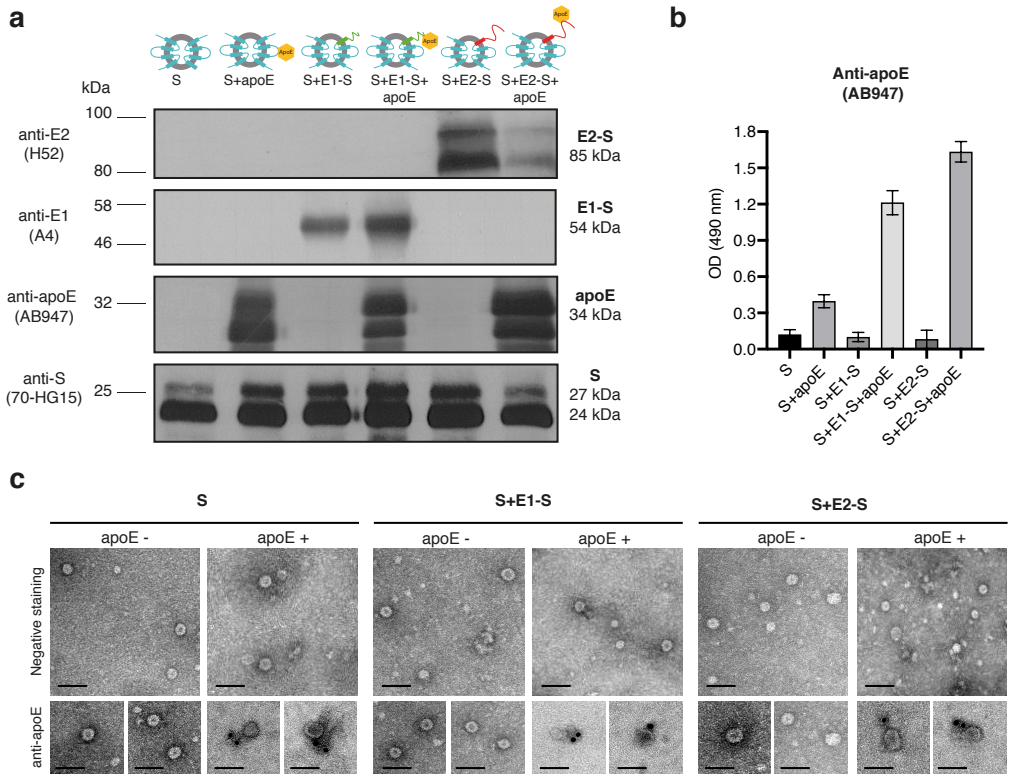

a
S
S+apoE
S+E1-S
S+E1-S+ apoE
S+E2-S
S+E2-S+ apoE
kDa
100
80
58
46
32
25
anti-E2 (H52)
anti-E1 (A4)
anti-apoE (AB947)
anti-S (70-HG15)
E2-S 85 kDa
E1-S 54 kDa
apoE 34 kDa
S 27 kDa 24 kDa
b
Anti-apoE (AB947)
OD (490 nm)
1.8
1.5
1.2
0.9
0.6
0.3
0.0
S
S+apoE
S+E1-S
S+E1-S+apoE
S+E2-S
S+E2-S+apoE
c
S
S+E1-S
S+E2-S
apoE -
apoE +
Negative staining
anti-apoE

Figure 3

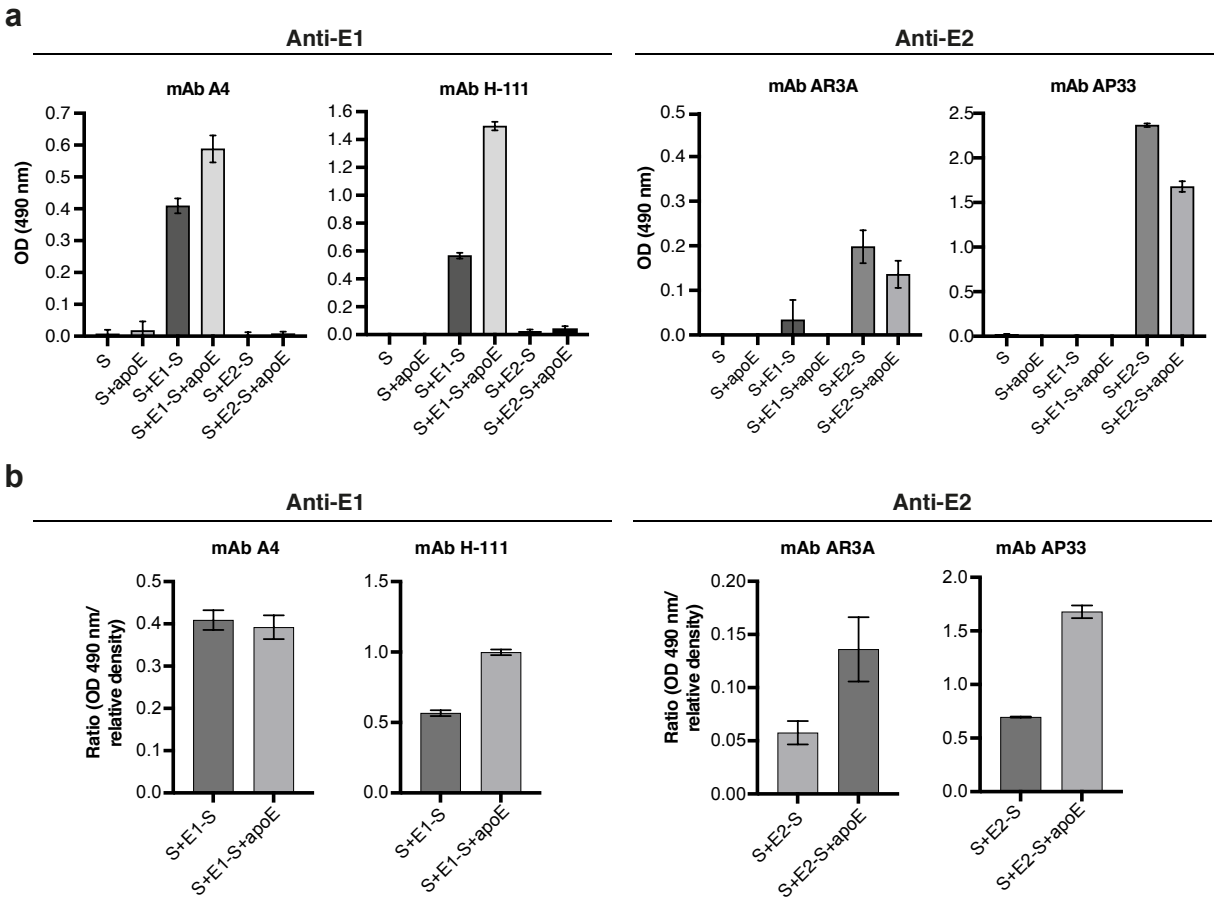

a
Anti-E1
Anti-E2
mAb A4
mAb H-111
mAb AR3A
mAb AP33
OD (490 nm)
S
S+apoE
S+E1-S
S+E1-S+apoE
S+E2-S
S+E2-S+apoE
b
Anti-E1
Anti-E2
mAb A4
mAb H-111
mAb AR3A
mAb AP33
Ratio (OD 490 nm/ relative density)
S+E1-S
S+E1-S+apoE
S+E2-S
S+E2-S+apoE

Figure 4

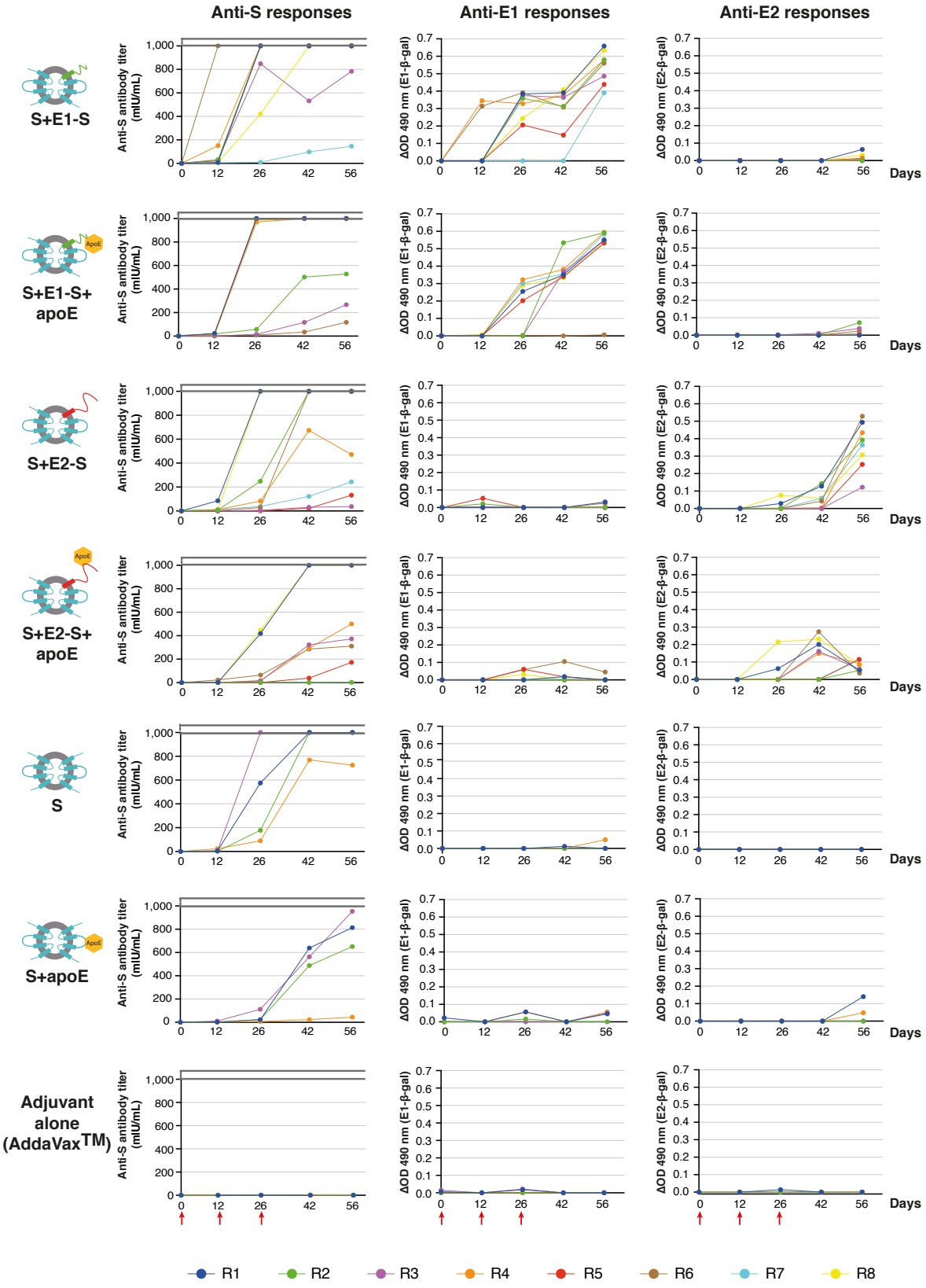
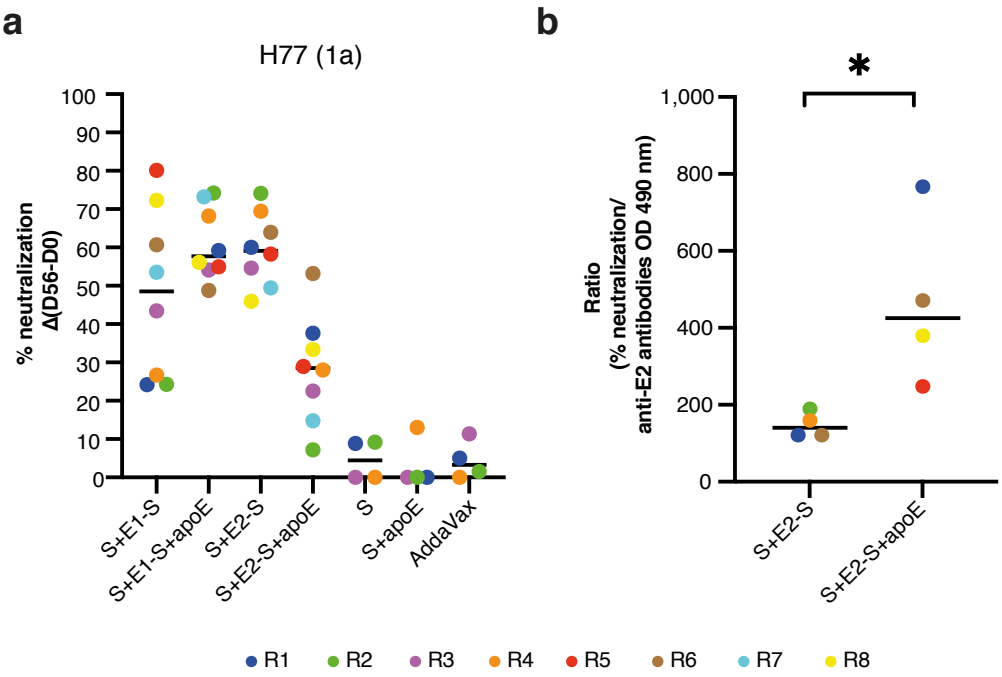

Anti-S responses
Anti-E1 responses
Anti-E2 responses
S+E1-S
S+E1-S+ apoE
S+E2-S
S+E2-S+ apoE
S
S+apoE
Adjuvant alone (AddaVax™)
Anti-S antibody titer (mIU/mL)
ΔOD 490 nm (E1-β-gal)
ΔOD 490 nm (E2-β-gal)
Days
R1
R2
R3
R4
R5
R6
R7
R8

Figure 5


a
H77 (1a)
% neutralization Δ(D56-D0)
100
90
80
70
60
50
40
30
20
10
0
S+E1-S
S+E1-S+apoE
S+E2-S
S+E2-S+apoE
S
S+apoE
AddaVax
b
*
Ratio (% neutralization/ anti-E2 antibodies OD 490 nm)
1,000
800
600
400
200
0
S+E2-S
S+E2-S+apoE
R1
R2
R3
R4
R5
R6
R7
R8

Incorporation of apolipoprotein E into HBV-HCV subviral envelope particles: implications for an improved hepatitis C vaccine strategy

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SUPPLEMENTARY MATERIALS AND METHODS

Analysis of anti-E1 and anti-E2 responses

ELISAs were performed in 96-well Immulon 2 HB plates coated by overnight incubation with 2 µg/well lectin from *Galanthus nivalis* (Sigma-Aldrich) at 4°C. Non-specific binding sites were saturated by incubation with a blocking solution (10% non-fat dry milk + 5% sheep serum-TBS 1X) for 4 hours at 37°C. We then added 2 µg/well cell lysate (ß-gal, HCV E1 or E2 proteins) and incubated overnight at 4°C. A second blocking of non-specific binding sites was then performed, as described above. We added 100 µL of serum from immunized rabbits (day 0, 12, 26, 42 or 56) to the plates and incubated for 1 hour at 37°C. Secondary HRP-conjugated goat $F(ab')_2$ anti-rabbit IgG (H+L) antibody (Southern Biotech) was added and the plates were incubated for 1 hour at 37°C. We then added O-phenylenediamine dihydrochloride (SIGMAFAST™ OPD; Sigma-Aldrich) as an HRP substrate. The reaction was stopped after 15 minutes by adding 2 N H_2SO_4, and absorbance was read at 490 nm.

Analysis of interactions between apoE and HCV envelope proteins through co-immunoprecipitation assays

Co-immunoprecipitation (co-IP) assays were performed on lysates of BHK-21 expressing HCV envelope proteins (E1 or E2) (genotype 1a) from original Semliki Forest virus (SFV)-derived vectors. First, we ensured that HCV envelope proteins were produced by transfecting cells with each of the previously described pSFV-E1 and pSFV-E2, [1] separately. Recombinant SFV RNA synthesis, and the transfection and lysis of BHK-21 cells were performed as described elsewhere. [2]

For co-IP assays, the previously described cell lysates were pre-incubated with a purified native human apoE (isoform E3; My BioSource). Then, the protein complexes, with either an anti-apoE goat polyclonal antibody (pAb) (AB947) or a goat-isotype control (R&D Systems), were incubated overnight at 4°C with rec-Protein G-Sepharose® 4B conjugate beads in 1X PBS. The beads were washed in 0.1% Triton X-100 in 1X PBS buffer, and the immune complexes were analyzed by western blotting in denaturing conditions (as described in the main manuscript) with mouse anti-E1/anti-E2 monoclonal antibodies (mAbs) (A4 and H52) and goat anti-apoE pAb (AB947). BHK-21 cell lysates, used for co-IP assays, were also analyzed according to the same western blotting protocol.

REFERENCES

1. Beaumont, E., Roch, E., Chopin, L. & Roingeard, P. Hepatitis C virus E1 and E2 Proteins used as separate immunogens induce neutralizing antibodies with additive properties. *PLoS One* **11**, e0151626 (2016).
2. Beaumont, E., Patient, R., Hourioux, C., Dimier-Poisson, I. & Roingeard, P. Chimeric

hepatitis B virus/hepatitis C virus envelope proteins elicit broadly neutralizing antibodies and constitute a potential bivalent prophylactic vaccine. *Hepatology* **57**, 1303–1313 (2013).

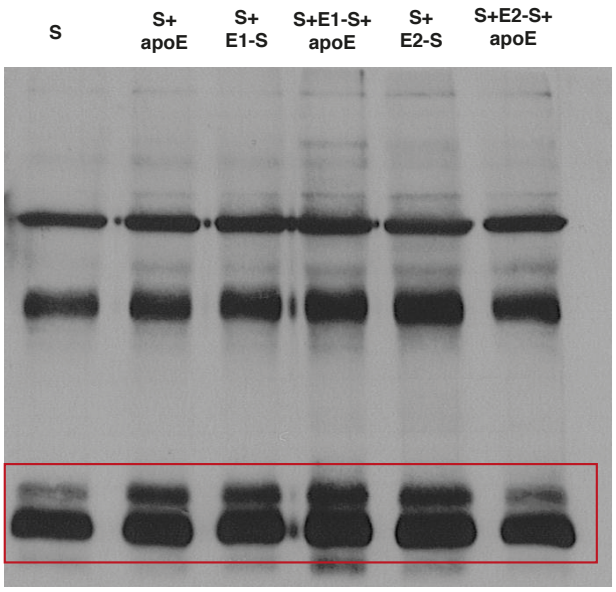
SUPPLEMENTARY FIGURE LEGENDS

Supplementary Figure S1. Evaluation of the interaction between apoE and HCV envelope proteins through co-immunoprecipitation assays. **(a)** Western-blot analysis of BHK-21 cell lysates expressing HCV E1 or E2 envelope proteins, detected with anti-E1/anti-E2 mAbs (A4 and H52). **(b)** Western-blot analysis of immune complexes from co-immunoprecipitation with anti-apoE (AB947) or goat isotype. Proteins were detected with anti-E1/anti-E2 mAbs (A4 and H52) and anti-apoE (AB947).

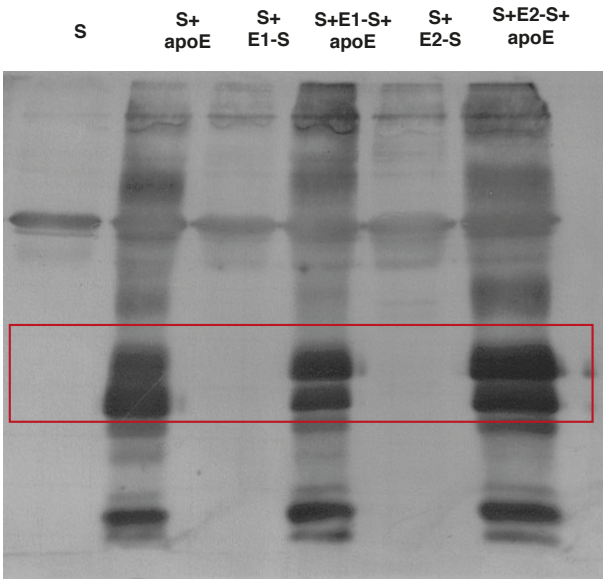
Supplementary Figure S2. Neutralizing potential of antibodies induced by the immunization of rabbits with purified HBV-HCV subviral particles with and without apoE, against HCV genotype 2a. (**a**) % neutralization of HCVcc genotype 2a. Rabbit serum samples (collected on days 0 and 56), with a 1:5 dilution, were incubated with HCVcc harboring HCV envelope proteins from genotype 2a (isolate JFH1), which were then used to infect Huh7.5 cells. Infection levels were determined 48 hours post-infection, in a focus-forming unit staining assay. The % neutralization was determined by subtracting the infectious titer obtained with the pre-immune serum (day 0) from that obtained with the post-immune serum (day 56) from the same rabbit. The assay was performed once in triplicate, and the results are expressed as mean values. Each colored point represents the % neutralization value calculated for a serum sample from an individual immunized rabbit, whereas the horizontal line represents the median value The color code established for the antibody responses (Fig. 4), one color per rabbit, was conserved to simplify the correlation of results from both experiments. **(b)** Analysis of the neutralizing

capacity of anti-E2 antibodies for the best four rabbits (% neutralization > the median) of each group (S+E2-S versus S+E2-S+apoE), in terms of antibody level, as evaluated by ELISA, on day 56. Results are expressed here as the ratio of the change in % neutralization between days 0 and 56 (Δ(D56-D0)) to the OD (490 nm) of anti-E2 antibodies obtained by ELISA on day 56 (Fig.4). The groups were compared in non-parametric Mann-Whitney *U* test. (*) $p<0.05$

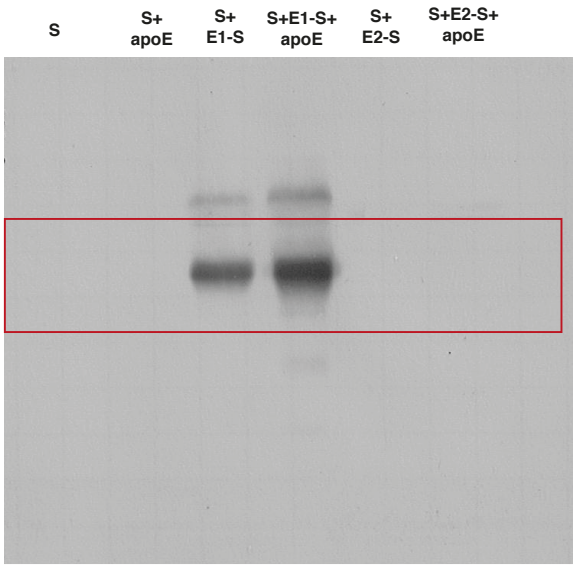
Full gel pictures Fig. 2a



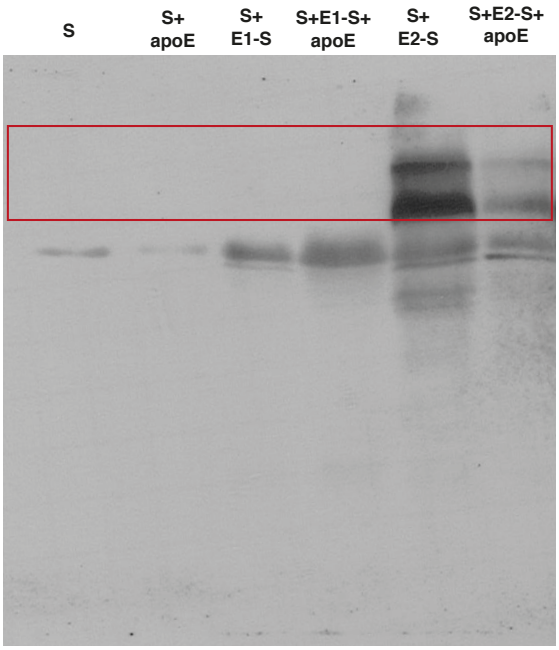
anti-S (70-HG15)



anti-apoE (AB947)



anti-E1 (A4)



anti-E2 (H52)

Figure S1

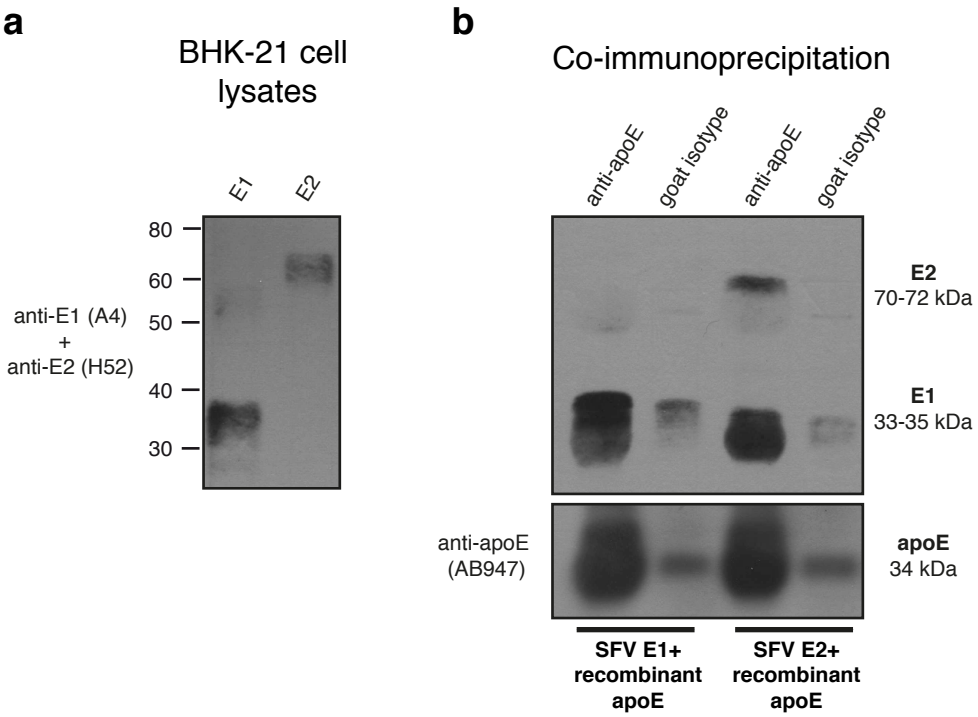
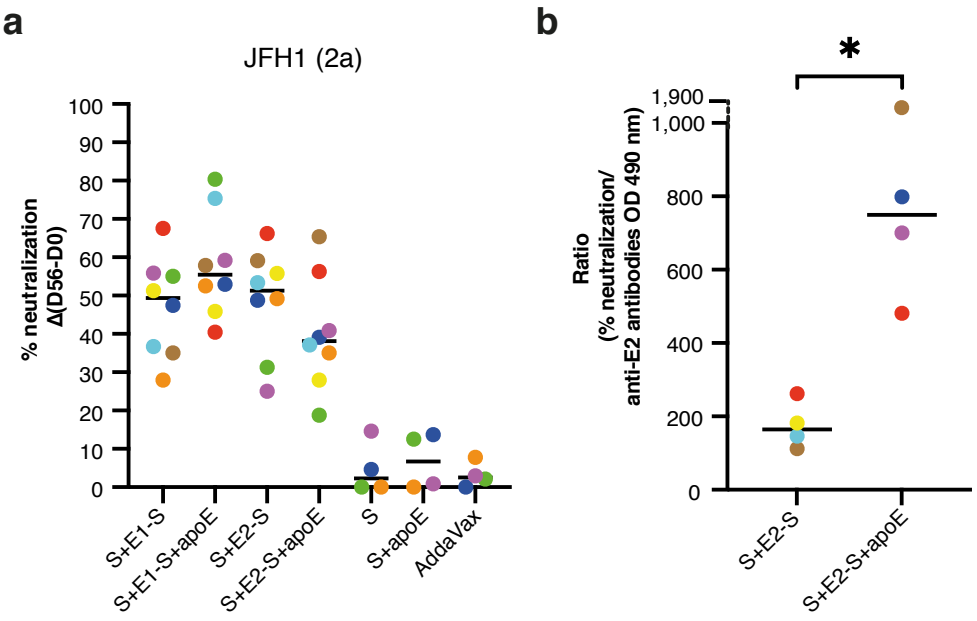

a
BHK-21 cell lysates
E1
E2
80
60
50
40
30
anti-E1 (A4)
+
anti-E2 (H52)
b
Co-immunoprecipitation
anti-apoE
goat isotype
anti-apoE
goat isotype
E2
70-72 kDa
E1
33-35 kDa
anti-apoE
(AB947)
apoE
34 kDa
SFV E1+ recombinant apoE
SFV E2+ recombinant apoE

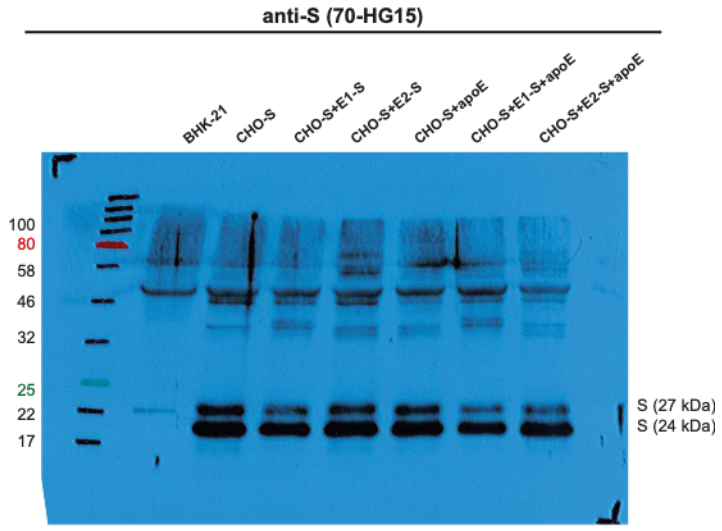
Figure S2

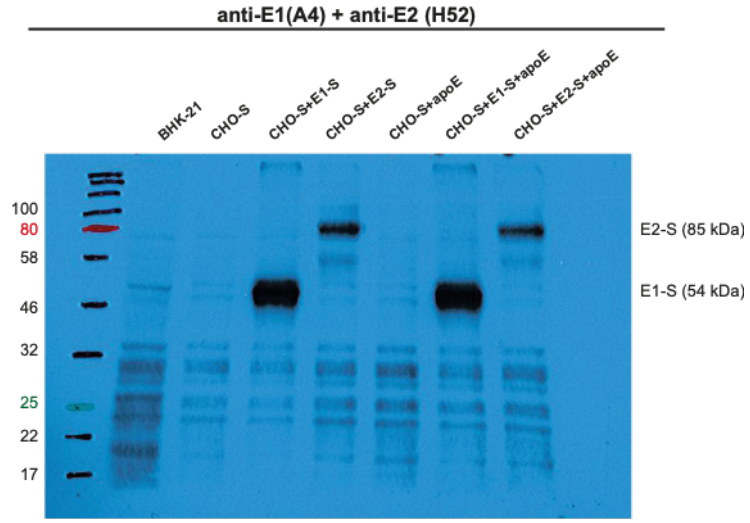

a
JFH1 (2a)
% neutralization Δ(D56-D0)
100
90
80
70
60
50
40
30
20
10
0
S+E1-S
S+E1-S+apoE
S+E2-S
S+E2-S+apoE
S
S+apoE
AddaVax
b
*
Ratio (% neutralization/ anti-E2 antibodies OD 490 nm)
1,900
1,000
800
600
400
200
0
S+E2-S
S+E2-S+apoE

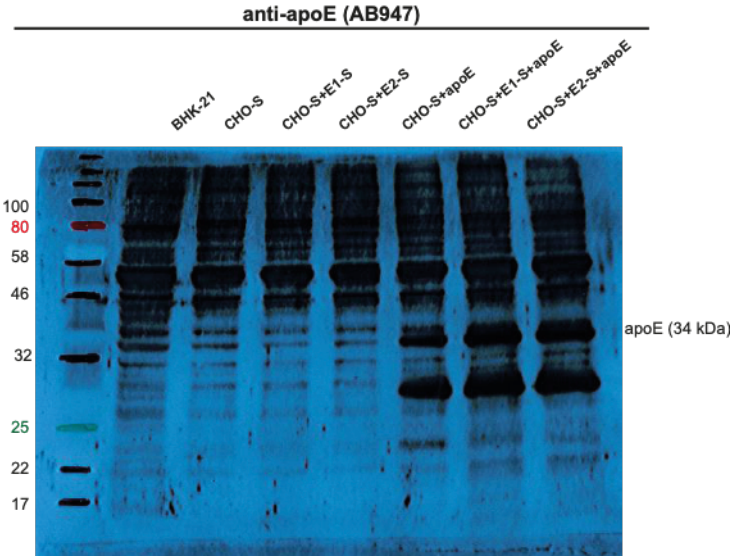
C

DETECTION OF PROTEINS OF INTEREST IN CHO CELL CLONES LYSATES

CHO clones expressing the proteins of interest (S, E1-S, E2-S and apoE) were lysed using a 1% Triton-PBS 1X buffer supplemented with proteases inhibitors. Samples containing the same amount of total protein were prepared with a reducing sample buffer (Laemmli 1X + 25% β-mercaptoethanol buffer). Samples were loaded to a 12% SDS-PAGE. Proteins were migrated and then electroblotted to a nitrocellulose membrane. Nonspecific sites were blocked with a 2% nonfat dry milk solution and then probed with primary antibodies: goat anti-S pAb (70-HG15), mouse anti-E1 and anti-E2 mAbs (A4 and H52) or goat anti-apoE pAb (AB947). Secondary HRP-coupled anti-mouse or anti-goat antibodies were then incubated. Signals were developed using an enhanced chemiluminescent technique. Proteins of interest were detected at their expected sizes: S (24 and 27 kDa), E1-S (54 kDa), E2-S (85 kDa) and apoE (34 kDa). BHK-21 cells lysate was used as negative control in these experiments.



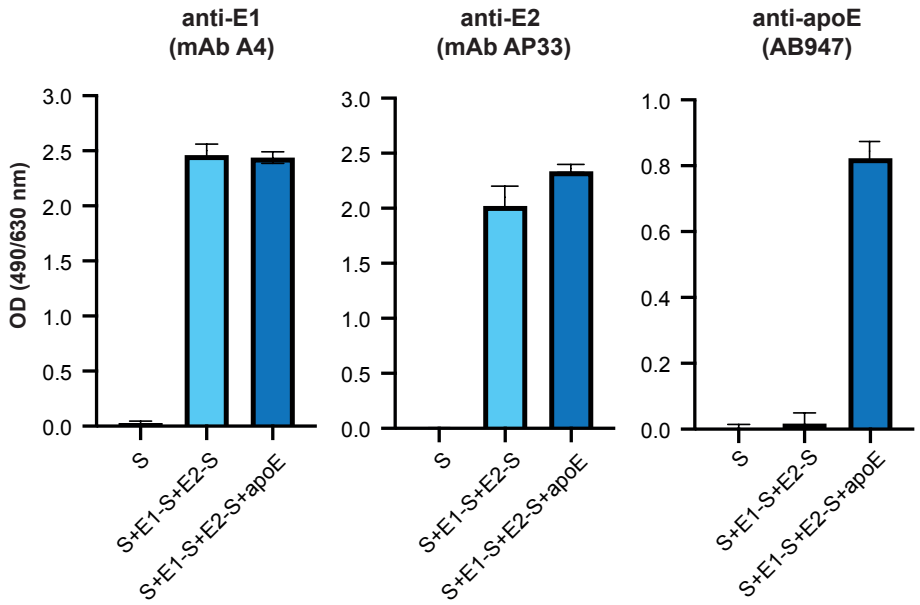

anti-E1(A4) + anti-E2 (H52)
BHK-21
CHO-S
CHO-S+E1-S
CHO-S+E2-S
CHO-S+apoE
CHO-S+E1-S+apoE
CHO-S+E2-S+apoE
100
80
58
46
32
25
22
17
E2-S (85 kDa)
E1-S (54 kDa)


anti-apoE (AB947)
BHK-21
CHO-S
CHO-S+E1-S
CHO-S+E2-S
CHO-S+apoE
CHO-S+E1-S+apoE
CHO-S+E2-S+apoE
100
80
58
46
32
25
22
17
apoE (34 kDa)

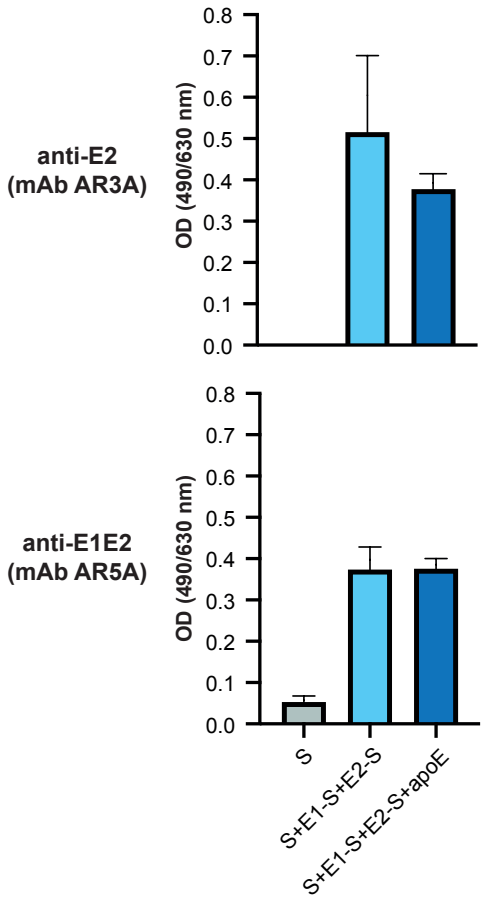
D

ANALYSIS OF CHIMERIC HBV-HCV PARTICLES BEARING HCV ENVELOPE PROTEINS (E1 AND E2) AND APOE

Particles bearing both HCV envelope proteins with or without apoE were produced by CHO clones stably expressing proteins of interest (S, E1-S, E2-S and apoE). S+E1-S+E2-S+apoE and S+E1-S+E2-S SVPs were purified with classical methods for the purification of HBV SVPs, previously described. We verified the presence of HCV envelope proteins and apoE on the surface of particles through ELISA assays, capturing particles with anti-S antibody or lectin. We observed that the levels of E1 and E2 proteins detected on the surface or particles were equivalent in particles bearing or not apoE, while apoE was detected only in S+E1-S+E2-S+apoE particles. Then, when using antibodies recognizing conformation-dependent epitopes, we observed slight differences in the detection between both types of particles. For the mAb AR3A (anti-E2), the epitope was better recognized in particles without apoE, while for the mAb AR5A (anti E1E2) similar ODs were obtained.

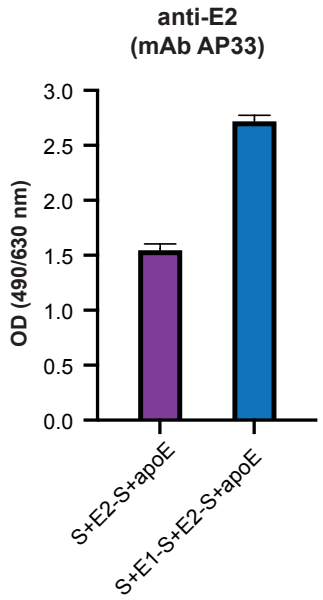


Detection of proteins of interest on the surface of particles. Samples (S, S+E1-S+E2-S and S+E1 S+E2 S+apoE) with an equal amount of SHBsAg were captured with a goat pAb anti-S (70-HG15) on 96-well plates. Proteins were then detected by a mouse mAb anti-E1 (clone A4), a mouse mAb anti-E2 (clone AP33) or a goat pAb anti apoE (AB947). Experiments were performed once in triplicate and results are expressed as the mean OD (490/630 nm).



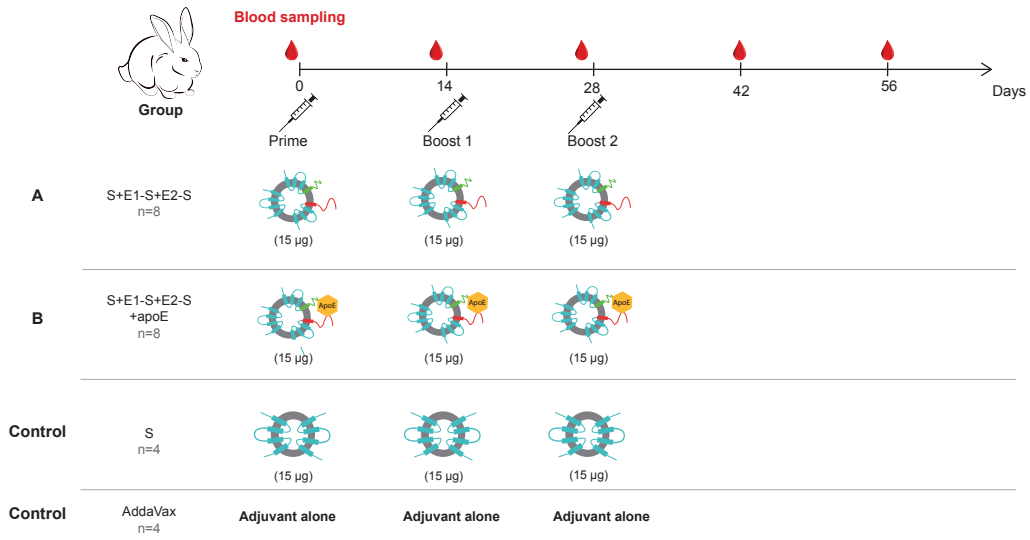
Detection of conformation-dependent epitopes of HCV envelope proteins on the surface of particles. Samples (S, S+E1-S+E2-S and S+E1 S+E2 S+apoE) with an equal amount of SHBsAg were captured with lectin on 96-well plates. Proteins were then detected by human mAbs anti-E2 (clone AR3A) or anti E1E2 (clone AR5A). Experiments were performed once in triplicate and results are expressed as the mean OD (490/630 nm).

Then, we wanted to compare the level of the chimeric E2-S protein exposed in the new S+E1-S+E2-S+apoE particles and in the S+E2-S+apoE ones. We performed the ELISA assay capturing the SVPs with the anti-S antibody 70-HG15 and using the same amount of HBsAg in the samples. We observed that the levels of E2-S presented in the S+E1-S+E2-S+apoE particles were 1,7-fold greater than in S+E2-S+apoE. These results suggest that immunization of rabbits with these new particles may elicit higher levels of anti-E2 antibodies generated in the presence of apoE.



Detection of HCV envelope E2 proteins on the surface of particles. Samples (S+E2-S+apoE and S+E1 S+E2 S+apoE) with an equal amount of SHBsAg were captured with an anti-S antibody (70HG-15) on 96-well plates. Proteins were then detected by a mouse mAb anti-E2 (clone AP33). The experiment was performed once in triplicate and results are expressed as the mean OD (490/630 nm).

Because of these encouraging results, we decided to perform an immunization protocol of New Zealand rabbits to evaluate the humoral responses induced with particles carrying both HCV envelope proteins and apoE. Two groups of 8 rabbits were immunized with 15 µg of adjuvanted SVPs S+E1-S+E2-S or S+E1-S+E2-S+apoE and two groups of 4 rabbits with 15 µg of adjuvanted S particles or adjuvant alone. Immunizations were done at day 0, 14 and 28, while blood samples at days 0, 12, 26, 42 and 56 to evaluate anti-E1/-E2 and anti-S responses.



Immunization protocol of New Zealand rabbits with chimeric HBV-HCV particles bearing HCV envelope proteins and apoE. Groups of 8 female New Zealand rabbits were immunized at days 0, 14 and 28 with 15 µg of adjuvanted (AddaVax™) S+E1-S+E2-S or S+E1-S+E2-S+apoE and control groups of 4 rabbits were immunized the same days with 15µg of adjuvanted S particles or adjuvant alone. Samples were obtained at days 0, 12, 26, 42 and 56 for the evaluation of humoral responses.

E

HUMAN AND RABBIT APOLIPOPROTEIN E SEQUENCES ALIGNMENTS

EMBOSS Matcher (https://www.bioinformatics.nl/cgi-bin/emboss/matcher)

Aligned sequences: 2

```
>human_apoE_UniProt_P02649
MKVLWAALLVTFLAGCQAKVEQAVETEPEPELRQQTEWQSGQRWELALGRFWDYLRWVQTLSEQVQEE
LLSSQVTQELRALMDETMKELKAYKSELEEQLTPVAEETRARLSKELQAAQARLGADMEDVCGRLVQY
RGEVQAMLGQSTEELRVRLASHLRKLRKRLLRDADDLQKRLAVYQAGAREGAERGLSAIRERLGPLVE
QGRVRAATVGSLAGQPLQERAQAWGERLRARMEEMGSRTRDRLDEVKEQVAEVRAKLEEQAQQIRLQA
EAFQARLKSWFEPLVEDMQRQWAGLVEKVQAAVGTSAAPVPSDNH
>rabbit_apoE_UniProt_P18287
MKVWWAVLAAAILAGCRAQTEQEVEVPEQARWKAGQPWELALGRFWDYLRWVQSLSDQVQEELLSSQV
TQELTMLMEETMKEVKAYKSELEEQLSPMAQEHRARLSKELQVAGALEADMEDVCNRLAQYRGEAQAM
LGQSTEELARAFSSHLRKLRKRLLRDAEDLQKRMAVYGAGAREGAERGVSAVRERLGSRLERGRLRVA
TVGTLAGRPLRERAQAWGERLRGHLEEVGSRARDRLNEVREQVEEVRVKVEEQAPQMRLQAEAFQARL
KSWFEPLVEDMQRQWAGLVEKLQAAMPSKAPAAAPIENQ
```

Length: 317
Identity: 237/317 (74,8%)
Similarity: 269/317 (84,9%)
Gaps: 8/317 (2,5%)

```
               10         20         30         40         50
 human MKVLWAALLVTFLAGCQAKVEQAVETEPEPELRQQTEWQSGQRWELALGR
rabbit ...W..V.AAAI....R.QT..E..V-..-----.AR.KA..P.......

               60         70         80         90        100
 human FWDYLRWVQTLSEQVQEELLSSQVTQELRALMDETMKELKAYKSELEEQL
rabbit .........S..D.............TM..E.....V...........

              110        120        130        140        150
 human TPVAEETRARLSKELQAAQARLGADMEDVCGRLVQYRGEVQAMLGQSTEE
rabbit S.M.Q.H.........V.G.-.E.......N..A.....A..........

              160        170        180        190        200
 human LRVRLASHLRKLRKRLLRDADDLQKRLAVYQAGAREGAERGLSAIRERLG
rabbit .ARAFS.............E.....M...G..........V..V.....

              210        220        230        240        250
 human PLVEQGRVRAATVGSLAGQPLQERAQAWGERLRARMEEMGSRTRDRLDEV
rabbit SRL.R..L.V....T...R..R...........GHL..V...A....N..

              260        270        280        290        300
 human KEQVAEVRAKLEEQAQQIRLQAEAFQARLKSWFEPLVEDMQRQWAGLVEK
rabbit R...E...V.V....P.M..............................

               310
 human VQAAVGTSA-APVPSDN
rabbit L...MPSK.P.AA.IE.
```

Elsa GOMEZ ESCOBAR

Optimisation d'un vaccin bivalent VHB-VHC par son association avec l'apolipoprotéine E et par immunisations séquentielles avec des particules arborant des protéines d'enveloppe de VHC de divers génotypes

Résumé

L'hépatite C, affectant 71 millions de porteurs chroniques, constitue un réel problème de santé publique. Bien que l'introduction des agents antiviraux à action directe ait amélioré le traitement de cette maladie, ces médicaments ne protègent pas d'une réinfection, ni de la progression vers une maladie sévère du foie. L'Organisation Mondiale de la Santé a pour objectif d'éradiquer l'hépatite virale à l'horizon 2030, mais des études prospectives ont montré que, si les mesures n'évoluent pas, cela ne sera possible que dans quelques pays. Par conséquent, le développement d'un vaccin prophylactique contre le virus de l'hépatite C (VHC) reste une priorité, au même titre que l'application des initiatives pour le dépistage et le traitement.

Dans cette optique, notre équipe de recherche a développé un concept de vaccination original basé sur les protéines d'enveloppe entières (E1 ou E2) d'un VHC de génotype 1a, préalablement fusionnées à la protéine d'enveloppe S du virus de l'hépatite B (VHB) et qui se co-assemblent en particules sous-virales d'enveloppe non infectieuses, hautement immunogènes et secrétées, ressemblant au vaccin contre le VHB. Ces particules chimères ont induit de fortes réponses immunitaires humorales chez le lapin, ainsi que la production d'anticorps capables de neutraliser le VHC in vitro.

L'objectif de ce projet était d'améliorer la façon de présenter nos immunogènes, afin d'optimiser selon deux approches les propriétés cross-neutralisantes des anticorps induits par la vaccination. Dans un premier temps, nous avons tenu compte de l'association des protéines d'enveloppe du VHC avec l'apolipoprotéine E (apoE), une molécule impliquée dans la modulation de la sensibilité à la neutralisation du VHC par les anticorps. Nous avons donc développé des particules chimères arborant l'apoE en vue de mimer au mieux l'interface apoE-protéines d'enveloppe en surface du VHC. Nous avons ainsi pu montrer que la présence de l'apoE dans la genèse de nos particules vaccinales améliore la conformation de la protéine E1 et les capacités neutralisantes des anticorps anti-E2. Dans un second temps, nous avons produit des particules vaccinales portant des protéines d'enveloppe E2 de différents génotypes (1, 3 et 4) et immunisé des lapins par des injections séquentielles afin d'améliorer la maturation de l'affinité des lymphocytes B. Dans l'attente de l'analyse des réponses humorales (en cours de réalisation au moment de la finalisation de ce manuscrit), nous présentons la production et la caractérisation de ces particules, ainsi que le protocole d'immunisation.

Mots clés : vaccination prophylactique, vaccin bivalent VHB-VHC, anticorps neutralisants, apolipoprotéine E, maturation de l'affinité des lymphocytes B

Abstract

With 71 million chronic carriers, hepatitis C is a major threat to public health. Although the introduction of direct-acting antivirals has improved the treatment of the hepatitis C virus (HCV) infection, they do not protect from reinfection or the progression to severe liver diseases. The World Health Organization aims to eradicate viral hepatitis by 2030, but recent prospective studies have shown that this would only be possible in few countries if the current measures do not evolve. Therefore, the development of a prophylactic HCV vaccine is still a priority, along with the implementation of screening and treatment initiatives. In line with this, our group has developed an original vaccination concept based on full-length genotype 1a HCV envelope proteins (E1 or E2) fused to the heterologous hepatitis B virus (HBV) S envelope protein. They self-assemble into highly immunogenic, non-infectious and secreted subviral envelope particles (SVPs), resembling the HBV vaccine. Immunization of rabbits with the chimeric particles has shown to induce strong humoral responses directed against both viruses, as well as antibodies capable to neutralize HCV in vitro. However, neutralization of genetically-distant genotypes remains a challenge.

The aim of this project was to improve the way immunogens are presented for the generation of neutralizing humoral responses against HCV by two approaches. Firstly, we took into consideration the association of HCV with the apolipoprotein E (apoE), a molecule involved in the modulation of sensitivity to antibody-mediated HCV neutralization. Therefore, we produced chimeric HBV-HCV SVPs bearing apoE on their surface to mimic precisely the interactions in the apoE-HCV envelope proteins interface. We found that the association of apoE improved the conformation of the E1 protein and the neutralizing capacity of the anti-E2 antibodies. Secondly, we produced SVPs bearing HCV E2 protein of genotype 1, 3 or 4 and we immunized rabbits sequentially to improve the affinity maturation process of B cells. Here we present the production and characterization of SVPs, as well as the immunization protocol, pending the analysis of the humoral and neutralizing responses, still in progress at the time this manuscript was finalized.

Keywords: prophylactic vaccination, HBV-HCV bivalent vaccine, neutralizing antibodies, apolipoprotein E, B lymphocyte affinity maturation