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PhD THESIS SUMMARY

Production and characterization of chimeric subviral particles for the development of a novel vaccine against Hepatitis B

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Study aim

Globally, over 290 million people are chronically infected with Hepatitis B Virus (HBV). Annually, chronic infections lead to more than 800.000 deaths due to liver-associated complications such as cirrhosis and hepatocellular carcinoma. Current antiviral therapies have significantly attenuated the impact of HBV infections, but they cannot completely remove the virus from infected hepatocytes. Therefore, vaccination remains the most effective method of controlling the spread of HBV. The currently available vaccine is based on the HBV S envelope protein produced in yeast and has led to a significant decrease in the rate of HBV infection. However, the countries with the highest infection prevalence are often less developed from an economic standpoint and lack the infrastructure necessary for cold-storage and transport of the vaccine. Moreover, 5-10% of vaccinated individuals do not develop protective antibody titers and recent studies have shown a loss of protective antibody titers at 10-15 years post-vaccination in responsive individuals. Another issue is the emergence of „vaccine escape" mutant viruses which cannot be recognized by the anti-S antibodies induced by the current vaccine, which indicates the need for alternative, more immunogenic vaccines.

As such, in this study, we aimed to develop a novel concept for the production of highly immunogenic HBV antigens, as well as their expression in both mammalian cells and plants, as a low-cost alternative for the production of vaccine antigens. The S protein which is used in the currently available vaccine has a particular property, namely that it can self-assemble into subvrial particles (SVPs) which are immunogenic but not infectious. This property was used to obtain chimeric S/preS1 antigens by inserting immunogenic epitopes from the preS1 domain of the L envelope protein, which is involved in the interaction between the virus and its receptor, into the antigenic loop of the S protein.

Besides the expression in mammalian cells, we also wanted to investigate the possible use of plants as an antigen expression system. A major disadvantage of glycoprotein production in plants, such as *N. benthamiana*, is the difference in glycosylation patterns between mammalian cells and plants, which can influence their antigenic properties. Moreover, these plant-specific glycans can be highly immunogenic and cause allergic reactions in humans. The S envelope protein has a single glycosylation site at Asn146, which is situated within the antigenic region

and is physiologically occupied in only 50% of cases, being involved in immune response modulation.

Therefore, the second objective of this study was the expression of the chimeric antigens in a novel *N. benthamiana* line with a „humanized" N-glycosylation pattern via CRISPR/Cas9, a process which involves the knockout of β-1,2-xylose and α-1,3-fucose (*N. benthamiana* FX-KO) and analysis of the impact of N-glycosylation on the antigenicity of evaluated chimeric proteins. We also aimed to evaluate the immunological properties of the chimeric antigen expressed in WT and FX-KO *N. benthamiana* via mice immunization and analyze the neutralization capacity of the obtained antisera against WT and „vaccine escape" HBV.

Chapter 1. Introduction

HBV is an enveloped DNA virus with a partially double stranded genome which is a member of the *Hepadnaviridae* family of the *Orthohepadonavirus* genus of viruses. The viral particles, with a diameter of 42 nm and also known as Dane particles, are infectious particles with a lipid envelope which consists of the three viral envelope proteins, small (S), medium (M) and large (L), which envelop an icoshaedral capsid with a 30 nm diameter containing the viral genome (Figure 1). The viral genome is represented by a partially double-stranded relaxed circular DNA (rcDNA) of approximately 3.2 kilobases (kb) with an incomplete (+) strand and a complete (-) strand. The genome encodes seven viral proteins: (1) viral polymerase, (2) capsid (core) protein and the HBe antigen, (3) HBx protein) and (4) three envelope proteins, which are synthesized using three alternative start codons (Figure 1).

Besides the infectious viral particles, HBV induces the secretion of subviral particles (SVPs) which do not contain the viral genome and are non-infectious. Some studies show that these can be secreted up to 10.000 fold more than infectious virions, which is considered to be a viral immune escape mechanism (Hu and Liu, 2017).
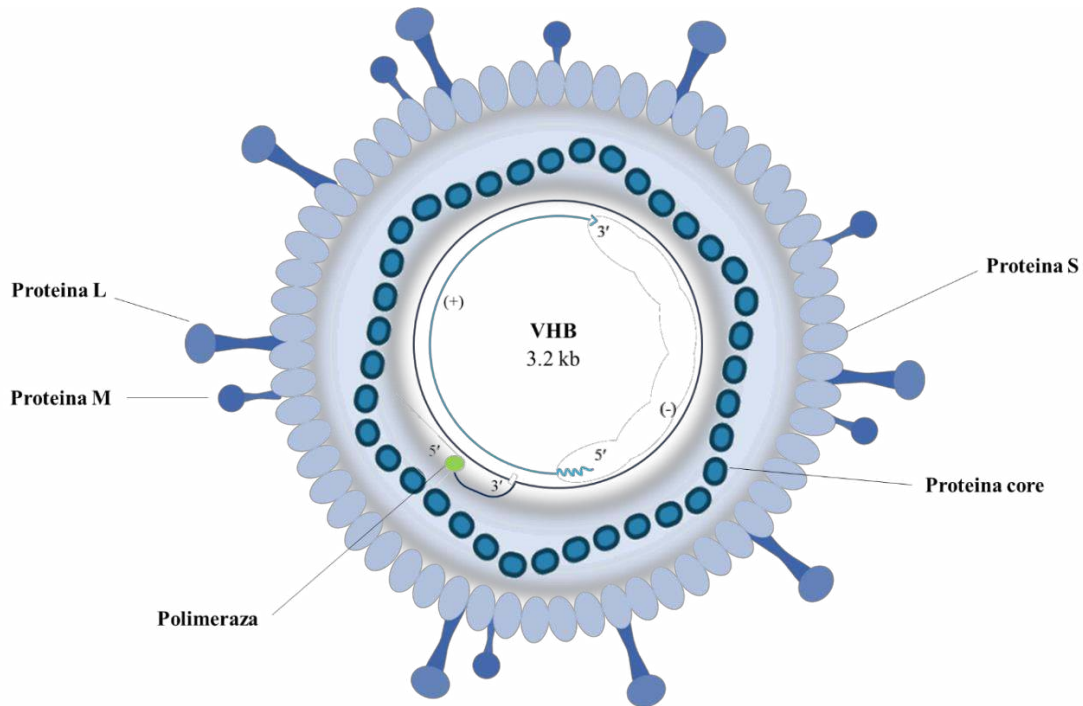
Figure 1. Schematic representation of HBV structure. HBV is an enveloped DNA virus. Its envelope is formed from a lipid bilayer containing the viral envelope proteins (S, M, L). The viral genome consists of a partially double-stranded DNA surrounded by a viral capsid consisting of core protein dimers. The viral polymerase is covalently attached to the 5′ end of the (-) strand.

All three envelope proteins contain the S domain (226 amino acids) at the C-terminal end, with the M and L proteins being extended at the N-terminal with the preS2 domain (55 amino acids) or both preS2 and preS1 domain (108/119 amino acids depending on genotype), respectively. The S domain consists of four transmembrane domains and an antigenic loop displayed on the surface of viral/subviral particles which contains the „a" antigenic determinant, which is the target of neutralizing antibodies (Carman et al., 1990). The conformation of this determinant is extremely important for antibody recognition and is stabilized by disulfide bonds. The S domain also contains a glycosylation site at position Asn146 which is normally occupied in only 50% of synthesized proteins and plays a role in immune response modulation. An important property of the S protein is its ability to self-assemble into immunogenic subviral particles which are secreted in excess compared to virions. The preS1 domain of the L protein contains the major infectivity determinants, as the 2-48 amino acids in the N-terminal region of

the preS1 domain are crucial for the interaction between the virus and its entry receptor, namely NTCP (Sankhyan et al., 2016).

The HBV life cycle begins with the attachment of viral particles to heparan sulphate proteoglycans located on the membrane of hepatocytes, which favors the steric interaction between the L protein preS1 domain and the NTCP receptor located on the basolateral membrane of hepatocytes (Yan et al., 2012). Recently the epidermal growth factor receptor (EGFR) was shown to be a co-factor involved in HBV entry by forming a complex with HBV and NTCP and mediating viral entry (Iwamoto et al., 2019). The virus is then internalized via clathrin- (Herrscher et al., 2020; Huang et al., 2012) and caveolin-dependent (Macovei et al., 2010) endocytosis. The nucleocapid is then transported to the nucleus via microtubules where the capsid is disassembled, releasing the rcDNA and core protein subunits into the hepatocyte nucleus. In the nucleus, the rcDNA goes through a series of biochemical reactions mediated by host-cell factors to be converted to covalently closed circular DNA (cccDNA), which will serve as a template for viral replication (Nassal, 2015). Once formed, the cccDNA will be organized into viral minichromosomes which are extremely stable and will act as a reservoir for virus persistence in the cell (Allweiss and Dandri, 2017). The cccDNA is then used as a template to transcribe the 4 viral mRNAs (3.5 kb, 2.4 kb, 2.1 kb and 0.7 kb), with the help of host RNA polymerase II. The transcripts are then translocated to the cytosol where translation and protein synthesis will take place. Afterwards, viral capsid formation will be initiated via assembly of core proteins and packaging of pregnomic RNA (pgRNA) and viral polymerase. Inside the capsid, the polymerase will initiate synthesis of the two viral DNA strands, leading to the formation of rcDNA (Tong and Revill, 2016). Newly formed nucleocapsids will follow two paths: some will be transported back to the nucleus to maintain constant cccDNA loads while most nucleocapsids will be enveloped and secreted. For the formation of the viral envelope, the cytoplasmatic domain of the preS1 and S envelope proteins located on the membrane of multivesicular bodies (MVBs) will interact with the nucleocapsid and initiate the sorting of envelope proteins into a highly packaged formation, thereby facilitating the internal budding process (Watanabe et al., 2007). These are then sorted into Endosomal Sorting Complex Required for Transport (ESCRT) and secreted. Besides virions, SVPs consisting mostly of S protein and without the viral genome as well as naked nucleocapsids and HBe antigen will also be secreted.

Currently available therapies for treatment of chronic HBV infections are interferon α (IFN-α) and nucleotide/nucleoside analogues (NUCs). IFN-α directly inhibits viral DNA replication by indirectly regulating host immune responses (Allweiss and Dandri, 2017; Dill et al., 2014). IFN-α treatment leads to a decrease in viremia and an increase in anti-HBs and -HBe antibody titers but is often associated with systemic secondary effects and contraindications. NUCs are the most frequently used anti-HBV therapy and function by blocking reverse transcriptase activity, thereby inhibiting viral replication. Although treatment with these medications can significantly decrease viral replication, it does not completely eliminate the virus from hepatocytes, thereby requiring lifelong administration. Moreover, long-term treatment can lead to the selection of treatment-resistant mutants (Lok et al., 2016). Newer generations of antiviral treatments currently in development focus on finding novel ways of removing cccDNA from hepatocytes, thereby leading to a complete cure. These can be divided into two categories: (1) compounds that directly interfere with the viral life cycle and (2) immunomodulators that target reactivation of the immune response (Sandmann and Cornberg, 2021) with promising results in preliminary clinical trials.

The first vaccine against HBV was developed in 1982 and was based on S-protein SVPs purified from the inactivated sera of HBV carriers. This vaccine was shown to be highly effective but its production was difficult and costly. To address these disadvantages, the next generation of vaccines, which were approved in 1986, was based on a recombinant S-protein produced and purified in yeast (*Saccharomyces cerevisiae*) (Kim, 2009). This vaccine is still in use today and has led to a significant release in the global number of HBV infections, inducing protective antibody titers in over 90% of recipients (Rubin et al., 2014). However, high costs and the 3-dose intramuscular vaccination schedule, as well as the need for cold transport and storage represents an economic barrier for vaccination programs in less developed countries, which often also have the highest prevalence of HBV infections (Gerlich, 2017).

Although the efficacy of the currently available vaccines is very well characterized, the longevity of the induced immune response has recently been questioned. Studies have shown that those vaccinated at birth can lose their protective antibody titers at 10-15 years post-vaccination. Moreover, 5-10% of the vaccinated population does not develop a sufficient immune response, due to a multitude of factors such as age, gender, obesity level, smoker status, the presence of other chronic diseases such as diabetes or renal diseases or a compromised

immune system due to human immunodeficiency virus (HIV) infection. Furthermore, even patients who develop protective antibodies post-vaccination may be susceptible to infection with HBV variants that contain mutations within the major antigenic determinant of the S envelope protein which inhibits virus recognition by vaccine-induced anti-S antibodies (Bian et al., 2013; Lai et al., 2012).

As such, it is necessary to develop novel more immunogenic HBV antigens to induce an immune response in non-responders and mitigate the threat posed by „vaccine escape" HBV variants. Therefore, the thrid generation of HBV vaccines also contain the L and M envelope proteins. Recently, the American Food and Drug Administration (FDA) has approved the use of a novel HBV vaccine containing all three envelope proteins and produced in mammalian cells for use in adults, which was shown to induce protective antibody titers even in non-responders to the current vaccine (Vesikari et al., 2021a; Vesikari et al., 2021b).

The L envelope protein is of particular interest for the development of more immunogenic antigens, as the N-terminal region of its preS1 domain is involved in binding to the HBV entry receptor NTCP. Moreover, a recent study has shown that the L protein can induce neutralizing antibodies against HBV "vaccine escape" variants, which are not recognized by antibodies induced by S-based vaccines (Washizaki et al., 2022). A major disadvantage of vaccines containing only the L protein is that this protein doesn't self-assemble into SVPs, thereby inducing a weaker immune response, as the antigenic epitopes are not properly displayed to the immune system (Bian et al., 2017). An alternative strategy is the development of chimeric S/preS1 antigens, which take advantage of the ability of the S protein of self-assembly into SVPs, thereby ensuring proper exposure of immunogenic epitopes from the preS1 region. This type of strategy was used by our group to develop the chimeric S/preS1[21-47] antigen, which contained the 21-47 amino acid region from the preS1 domain of the L protein (genotype D) inserted between amino acids 126/127 of the S antigenic loop (Dobrica et al., 2017) which was previously shown to accommodate foreign epitopes. This antigen was shown to induce better humoral and cellular immune responses when compared to vaccination with the S protein in mice, thereby indicating that this is a promising strategy for the development of alternative vaccines.

Plants as an alternative system for HBV antigen production

In the past few decades, plants have been used as green factories for the synthesis of different biopharmaceutical compounds, proteins with important uses in medicine but also vaccines destined for use in both animals and humans. Plants are eukaryotes and, therefore, allow post-translational modification of expressed proteins, which is extremely important for the production of vaccines in plants, as complex modifications such as plant-specific N- and O-glycosylation can affect the immunogenicity of recombinant antigens produced in plants when compared to their mammalian cell-produced counterparts. Moreover, studies have shown that plants can process complex transmembrane viral proteins and sustain their oligomerization into virus-like particles (VLPs) with improved immunogenicity. Additionally, plant components which are often associated with VLPs tend to function as adjuvants, thereby improving immunogenicity. Another advantage of the production of such vaccines in plants is the low cost and time required for production, which makes scale-up much easier, as well as the low risk of contamination due to the lack of human or mammalian pathogens.

Heterologous protein expression in plants can be both transient and stable, with the genes encoding the protein of interest being introduced in the nucleus or the chloroplast, respectively. Genes are most frequently inserted via transfection with *Agrobacterium tumefaciens*. Viral vectors can also be used to introduce genes coding for foreign proteins in the plant genome. Considering the complexity of HBs antigen folding, its expression in plants (*Nicotiana tabacum*) for the first time as well as confirmation of its assembly into SVPs is considered an extremely important step in molecular farming. Moreover, immunization with plant-produced S antigen was shown to induce an HBV-specific immune response in mice B and T lymphocytes, thereby proving the viability of plants as a production system for HBV antigens. Subsequently, the S protein was produced in various plant species with varying success (Pantazica et al., 2021). However, no plant-produced HBV vaccine has been developed to date, although the high potential of such a platform has been illustrated by the recent approval of a plant-produced vaccine against COVID-19 in Canada for use in adults. This is the first plant-produced vaccine authorized for human use globally (National Advisory Committee on Immunization, 2022).

N-glycosylation is one of the most highly conserved post-translational modification pathways in eukaryotes, playing a crucial role in protein function and properties. Although the

N-glycosylation maturation steps are similar between plant and mammalian cells, plants synthesize N-glycans which contain β-1,2-xylose and α-1,3-fucose and lack sialic acid and α-1,6-fucose, which are specific for mammalian cells (Schoberer and Strasser, 2018). Additionally, studies have shown that plant-specific N-glycans can be immunogenic or cause allergic reactions in mammals, suggesting that long-term administration of such proteins may pose a risk to human health. As such, significant efforts have been made to „humanize" plant N-glycosylation patterns. Recently, the CRISPR/Cas9 technology was used to generate *N. benthamiana* strains lacking β-1,2-xylosyltransferase and α-1,3-fucosyltransferase activity (FX-KO) (Jansing et al., 2019). These plants were then used for the production of human monoclonal antibodies which were shown to lack plant-specific N-glycans and have similar activity to antibodies produced in mammalian cells (Frigerio et al., 2022; Jansing et al., 2019). However, no studies have investigated the use of FX-KO *N. benthamiana* plants for recombinant protein expression and the effect of N-glycan „humanization" on antigen immunogenicity.

HBV antigens such as the S protein and S-derived chimeric proteins are an ideal model to investigate the role of N-glycosylation on antigenicity, as these proteins have only one glycosylation site located within the antigenic domain, and studies have shown that it plays an important role in immune response modulation and escape (Dobrica et al., 2020). Therefore, any modification of this glycan could influence the antigenic properties of the produced protein.

Proposed antigens

Our group has previously developed a novel strategy for chimeric antigen construction which takes advantage of the property of the S envelope protein of self-assembling into SVPs. Thus, the 21-47 amino acid region of the preS1 domain was inserted within the antigenic loop of the S protein to form the S/preS1$^{21-47}$ chimeric antigen, which was then expressed in both mammalian cells and plants (Dobrica et al., 2017). Although this antigen was shown to induce a stronger immune response compared to the S antigen, issues such as its low expression and secretion would hinder its large-scale production.

Thus, this study aimed to optimise the chimeric antigen design by inserting partially overlapping regions of the preS1 domain extended towards the N-terminus when compared to the previously inserted region, in order to obtain a chimeric antigen with superior molecular and immunological properties (Figure 2). Therefore, using molecular biology techniques, the 10-36,

16-42 and 16-47 amino acid region from the N-terminus of the preS1 domain (genotype D) were inserted between amino acids 126/127 of the S antigenic loop or between amino acids 126-132 in the case of the 16-47 aminoacid insert to compensate its increased length.
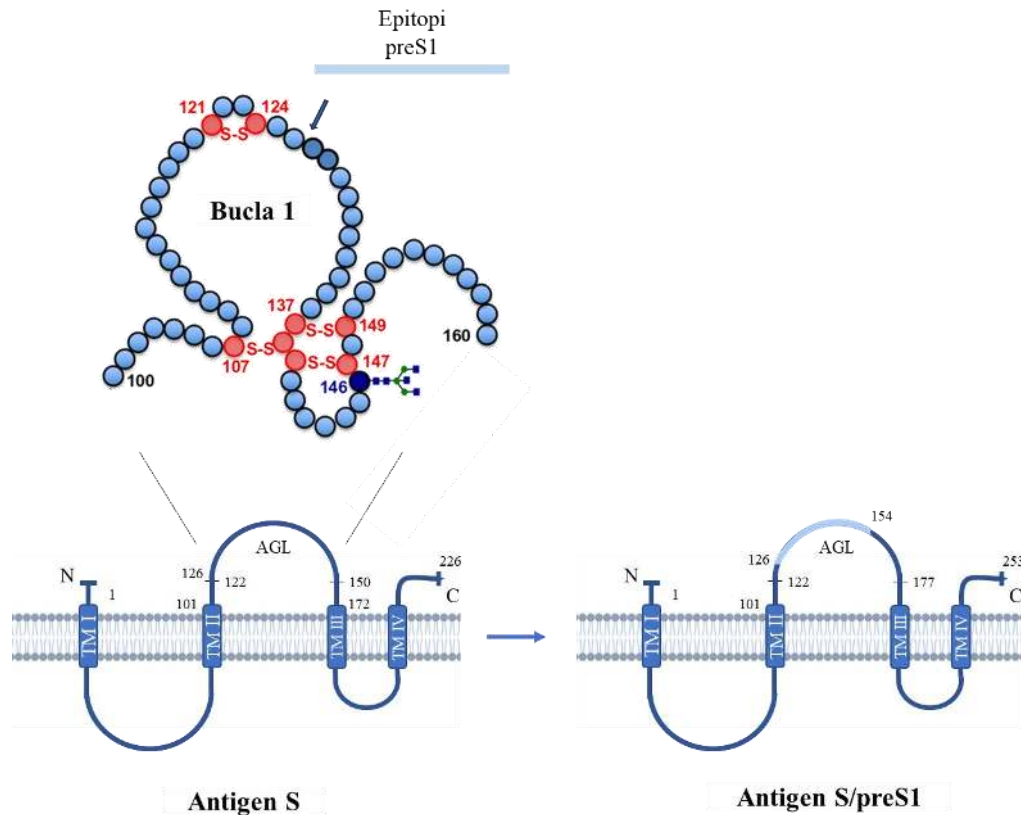
Figure 2. Schematic representation of the S/preS1 chimeric antigen design

Epitopes from the N-terminal region of the L protein preS1 domain were inserted between amino acids 126/127 of the S protein antigenic loop (AGL) or between amino acids 126-132 in the case of the $S\Delta^{127-131}/preS1^{16-47}$ antigen to accomodate for its length.

Chapter 2. MATERIALS AND METHODS

The proposed HBV antigens were expressed in HEK293T cells and in either wild type (WT) or FX-KO *N. benthamiana* plants. The antigens were then structurally and molecularly characterized by determining their ability to fold and form dimers, N-glycosylation, expression and secretion levels as well as antigenicity. The candidate with the best properties was then produced at a larger scale, purified and used for immunological studies in BALB/c mice. The

humoral immune response was then analyzed by quantifying IgG titers while the cellular immune response was determined by measuring cytokine secretion from stimulated splenocytes. The obtained antibodies were then characterized by determining their interaction with various viral epitopes and their ability to neutralize WT and „vaccine escape" HBV infection was investigated in an *in vitro* infection system using HepG2^{hNTCP} cells.

Chapter 3. RESULTS

Construction of the novel HBV chimeric antigens was based on a previously used strategy (Dobrica et al., 2017), involving the insertion of the 21-47 amino acid region of the L protein preS1 domain (genotype D) within the antigenic loop of the S protein between amino acids 126/127. Therefore, after aligning the preS1 sequences from the most prevalent HBV isolates, regions 10-36, 16-42 and 16-47 (genotype D) were chosen, which were partially overlapping the S/preS1$^{21-47}$ chimera but extended towards the N-terminus. As one of the constructs had an insert length of more than 27 amino acids (construct S/preS1$^{16-47}$), another construct was made in which amino acids 127-131 from the S protein were deleted to compensate for the increased length of the preS1 insert. The antigens were obtained via site-directed mutagenesis using PCR and specific primers.

The molecular and structural characterization of the novel chimeric antigens was done via transient transfection in HEK293T cells. Expression levels were analyzed via western blot with anti-preS1 antibodies and the results showed that the S/preS1$^{16-42}$ antigen has the highest expression level, even when compared to S/preS1$^{21-47}$ (Figure 3A, B). Insertion of a longer sequence (32 amino acids) from the preS1 region resulted in a decreased expression level, as can be seen for the S/preS1$^{16-47}$ and S$\Delta^{127-131}$/preS1$^{16-47}$ antigen confirming that this situs cannot accommodate large inserts without compromising protein integrity. Moreover, insertion of the same large epitope in a shorter antigenic loop was shown to be detrimental to the expression of the S$\Delta^{127-131}$/preS1$^{16-47}$ antigen, suggesting that this deletion could affect protein folding and, therefore, protein stability. This analysis also showed the successful post-translational modification of the antigens through the presence of bands corresponding to the glycosylated protein and protein dimers.

Next, the secretion level of the novel chimeric proteins was compared to that of the S/preS1$^{21-47}$ antigen via ELISA. We found a significantly higher accumulation of S/preS1$^{16-42}$

antigen compared to the other chimeric proteins tested (Figure 3C). As this difference in secretion level could be influenced by modifications within the antigenic domain of the S protein, thereby inhibiting recognition by conformation-dependent antibodies, pulse-chase and immunoprecipitation experiments were used to determine the influence of the inserted sequences on the structure, synthesis and secretion of the chimeric antigens. We showed that the S/preS1$^{16-42}$ antigen is recognized by both anti-S and anti-preS1 antibodies and has the highest expression level of the tested antigens. These data show that the 16-42 sequence is well tolerated by the antigenic loop and is properly displayed for recognition by anti-preS1 antibodies without affecting the integrity of the S antigenic domain (Figure 3D).

Thus, the S/preS1$^{16-42}$ antigen was selected for immunological studies and produced at a large scale. The antigen was then purified based on its ability to self-assemble into SVPs by ultracentrifugation on a sucrose gradient and then size-exclusion chromatography using the CaptoCore 400 resin. The purified antigens were then quantified and used for immunization in BALB/c mice via intramuscular injection. Analysis of the humoral and cellular immune response showed that the S/preS1$^{16-42}$ antigen has improved immunological properties when compared to the S antigen. Moreover, characterization of antibodies from immunized mice antisera showed that the chimeric antigen induces both anti-S and anti-preS1 antibodies, demonstrating that both epitopes are properly displayed on the surface of chimeric SVPs.
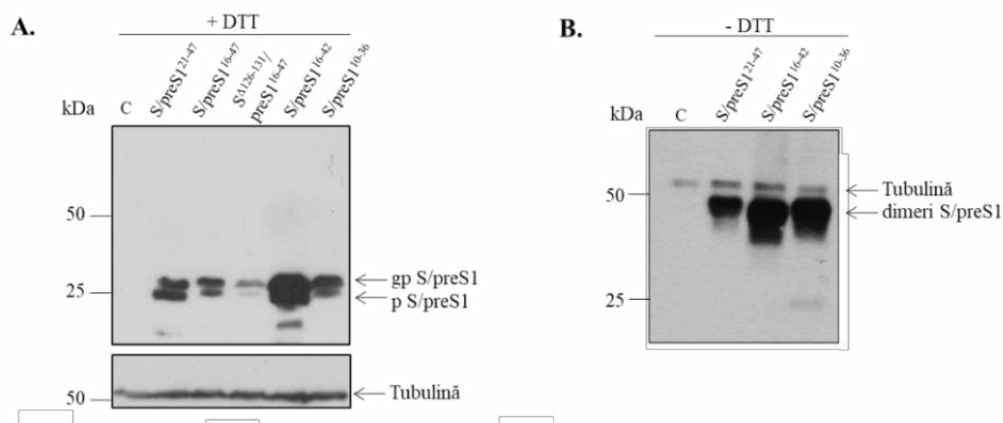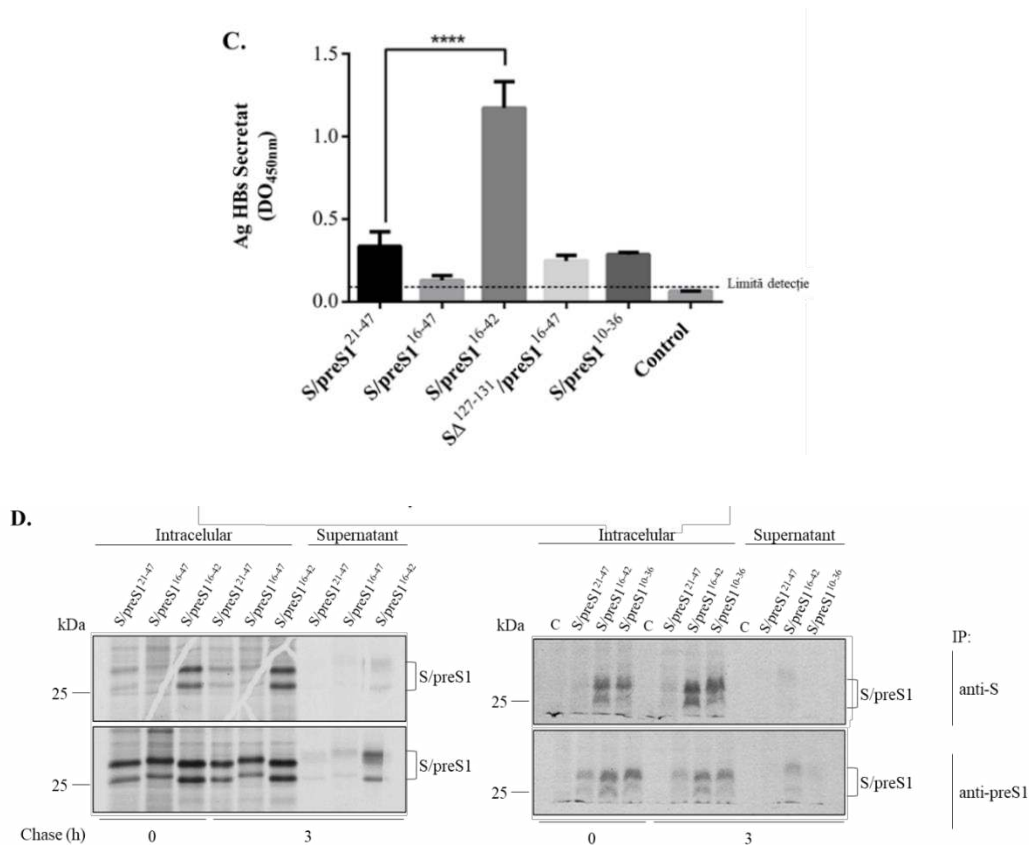
Figure 3. Expression and characterization of novel chimeric HBV antigens in mammalian cells. HEK293T cells were transfected with pCi plasmids encoding the indicated antigens or empty pCi vector as a control (C). Lysates were then analyzed via western blot under reducing (+DTT) **(A)** or nonreducing (-DTT) **(B)** conditions. Anti-preS1 antibodies were used to detect glycosylated (gp) and nonglycosylated (p) S/preS1, as well as S/preS1 dimers. **(C)** Extracellular media was collected at 72 h post-transfection and HBs antigen secretion was quantified via ELISA by measuring optical density at 450 nm. **(D)** At 24 h post-transfection, cells were marked with ^{35}S for 30 minutes and then chased for 3 h. Lysates and supernatants were then immunoprecipitated with anti-S and anti-preS1 antibodies, separated via SDS-PAGE and then visualized via autoradiography. Image adapted from (Pantazica et al., 2022).

To determine whether antibodies generated via immunization with the S and S/preS1[16-42] antigen can protect against HBV infection we analyzed their neutralization capacity in an *in vitro* infection system. We found that antibodies resulting from immunization with both antigens

were able to specifically inhibit HBV infection when compared to the control group (Figure 4A). Moreover, antisera from the S/preS1[16-42] group had higher neutralization capacity when compared to the S group but the difference was not statistically significant. To determine the neutralization capacity of the obtained antisera against vaccine-escape mutants we introduced the mutation with the highest clinical prevalence, namely G145R, via site-directed mutagenesis (Bian et al., 2013). The obtained HBV-S[G145R] particles were produced in Huh7 cells, purified and characterized regarding their secretion, infectivity and ability to be recognized by conformation-dependent anti-S antibodies. Finally, we tested the neutralization capacity of the obtained antisera against this virus. Our data showed that antisera obtained from S/preS1[16-42] immunized mice significantly inhibited HBV-S[G145R] infection, at levels similar to those obtained for Myrcludex B (Figure 4B). In the case of sera obtained from mice immunized with the S protein we observed a more heterogenous response but without significant neutralization capacity against HBV-S[G145R] when compared to the control group.
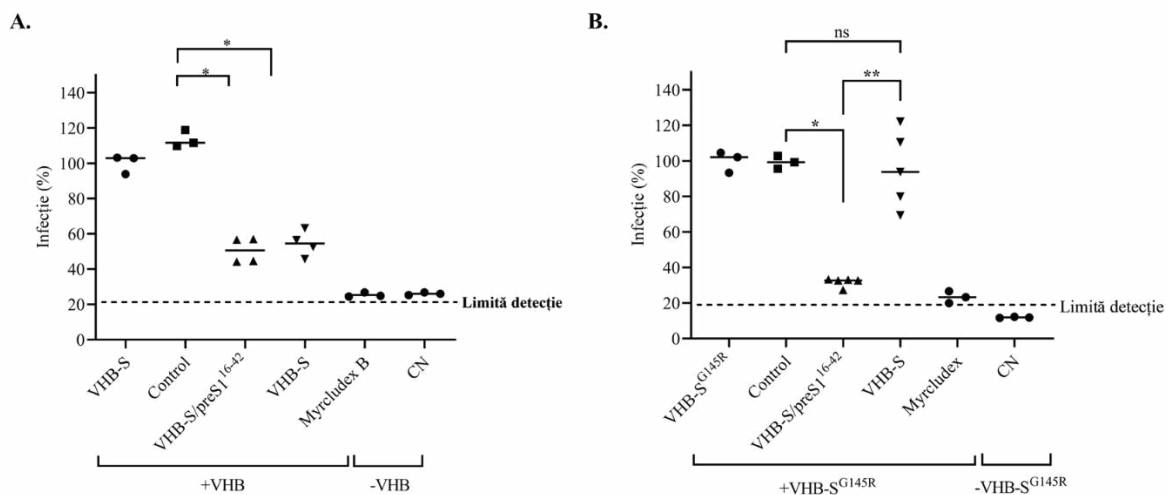
Figure 4. Neutralization capacity of S- and S/preS1[16-42]-induced antibodies against wild type and vaccine escape HBV. Pooled pre-immune sera or sera from mice immunized with S, S/preS1[16-42] or background proteins (control) were diluted and preincubated with **(A)** HBV or **(B)** HBV-S[G145R] (100 GEq/cell). HepG2[hNTCP] cells were incubated with the HBV-sera inoculum or uninfected as a negative control (CN). Myrcludex B-incubated cells for 3 h prior to infection were used as control for specific HBV inhibition. Extracellular media collected at day 11 post-infection were utilized to quantify HBe antigen levels via ELISA.

Data are represented as percentage of HBV infection in the presence of post-immune sera from infection values obtained in the presence of pre-immune sera at the same dilution (n=4). Values in the presence of Myrcludex B and in the negative control represent percentage of infection from HBV-only samples (n=4) (*, p < 0,05). Image adapted from (Pantazica et al., 2022).

In conclusion, data show that combining relevant S and preS1 epitopes in a single molecule is a promising strategy to improve the immune response and, therefore, the efficiency of HBV vaccination in non-responders to the current vaccine. Moreover, this strategy permits the development of a highly efficient vaccine against vaccine escape HBV variants. However, production in mammalian cells is costly. Therefore, our next step was to investigate the possibility of alternative production systems such as plants. Additionally, we wanted to investigate the possibility of producing this antigen in plants with „humanized" N-glycosylation to determine its effect on antigenicity and ability to activate the immune system and induce neutralizing antibodies.

The possibility of producing the novel chimeric antigen in plants such as *N. benthamiana* was investigated using the standard wild-type line as well as a novel CRISPR/Cas9-edited line with knockout of 2 β-1,2-xylosyltransferase genes and four α-1,3-fucosyltransferase genes (Jansing et al., 2019) (Figure 5A). HBV-S/preS1[16-42] antigen expression was first tested in WT plants via transient transformation with the corresponding plasmid. Data showed that both glycosylated (gp) and nonglycosylated (p) forms of the chimeric antigen were expressed in WT plants. Similarly, the FX-KO line was also able to sustain HBV-S/preS1[16-42] antigen expression (Figure 5B, C). After quantifying the antigen obtained from both WT and FX-KO plants we found that the average expression level of the chimeric antigen was 200 µg/g fresh weight. This is significantly higher than previously obtained HBsAg levels via agroinfiltration in different plant hosts (Pniewski, 2013) and similar to those obtained with the help of expression systems using viral plant vectors (Huang et al., 2008). Moreover, antigen separation via sucrose gradient showed that both plant systems permit assembly into SVPs.
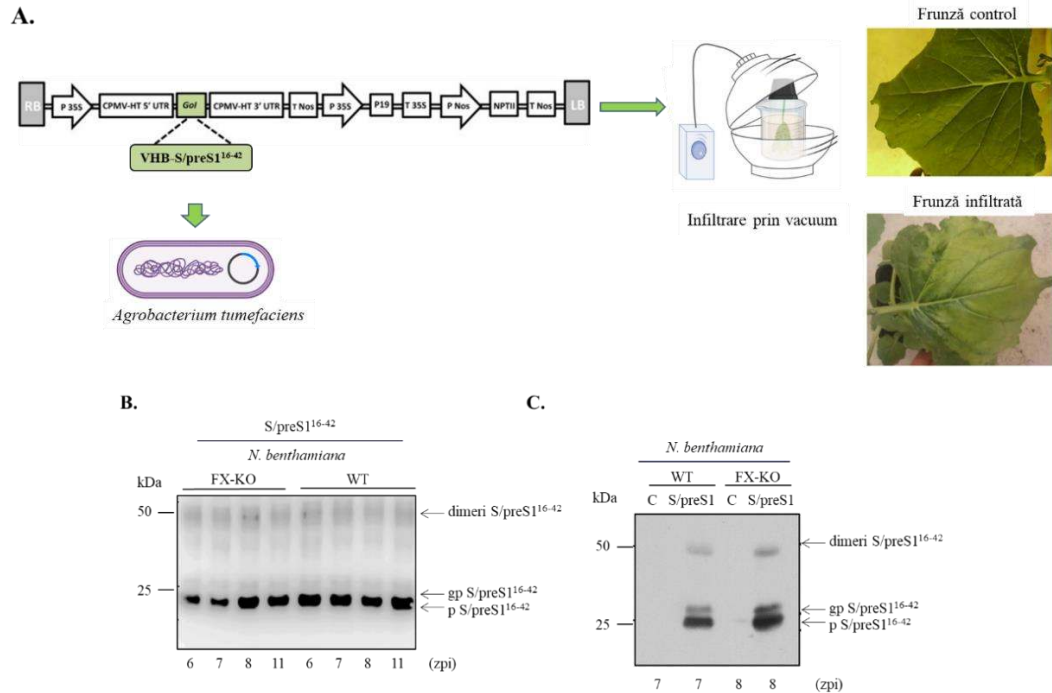
Figure 5. HBV-S/preS1$^{16-42}$ antigen expression in *N. benthamiana*. **(A)** Schematic representation of HBV antigen production protocol in *N. benthamiana*. **(B)** HBV-S/preS1$^{16-42}$ expressing WT and FX-KO *N. benthamiana* leaves were collected at the indicated times, lysed and analyzed via western blot using anti-preS1 antibodies. **(C)** Lysates of WT (7 dpi) FX-KO (8 dpi) or control *N. benthamiana* leaves expressing an irrelevant protein (7 dpi) were analyzed via western blot using anti-preS1 antibodies. Image adapted from (Pantazica et al., 2023).

For purification, lysates of WT and FX-KO *N. benthamiana* leaves expressing the HBV-S/preS1$^{16-42}$ were initially concentrated via ultracentrifugation on a 20% sucrose bed followed by sucrose gradient separation. Positive fractions were pooled, dialyzed against PBS and incubated with 1% activated charcoal followed by gel-filtration using the CaptoCore 400. Eluted positive fractions were concentrated and analyzed via SDS-PAGE followed by Coomassie Blue staining or western blot. Quantification of antigen levels and total protein contents in purified samples revealed approximately 80% purity.

HBV-S/preS1$^{16\text{-}42}$ monomers purified from WT and FX-KO N. benthamiana were extracted from gels and treated with PNGase A to release N-glycans, followed by analysis via UPLC-FLD/ESI-TOF MS. The data showed a significant difference in N-glycosylation patterns between the two systems. The mass spectra for the WT N. benthamiana-produced antigen showed a predominant signal (~63% of all glycans) representing the plant-specific N2H4FX glycan, which contains β-1,2-xylose and α-1,3-fucose. On the other hand, the N4H5 structure without β-1,2-xylose and α-1,3-fucose was the most prevalent glycan in FX-KO N. benthamiana-produced antigen samples (~55%). Other complex N-glycans which are mammalian cell-specific were also more abundant in these samples. These data describe the first detailed N-glycan analysis of an HBV antigen produced in plant cells and indicate that S/preS1$^{16\text{-}42}$ particles are transported along the secretory pathway to the trans-Golgi network where the N-linked glycans are processed to complex structures by specific glycosyltransferases.

To determine the impact of host-specific N-glycosylation on the antigenicity of HBV-S/preS1$^{16\text{-}42}$, we analyzed its binding capacity against a panel of commercial conformation-dependent anti-S antibodies via ELISA (Figure 6). To obtain more robust data, the comparison was made between mammalian cell-derived antigen and antigen produced in WT or FX-KO *N. benthamiana*. As we expected, the most efficient binding was observed in the case of the mammalian cell-produced antigen. The antigen produced in WT *N. benthamiana* has a lower binding affinity against all tested antibodies. However, an improvement in binding efficiency was observed for the antigen produced in FX-KO *N. benthamiana*, approaching those obtained for HEK293T-produced antigen in some cases. Therefore, although the exact viral epitopes recognized by these antibodies are unknown, our data suggest that the N-glycan structure plays an important role in this process.
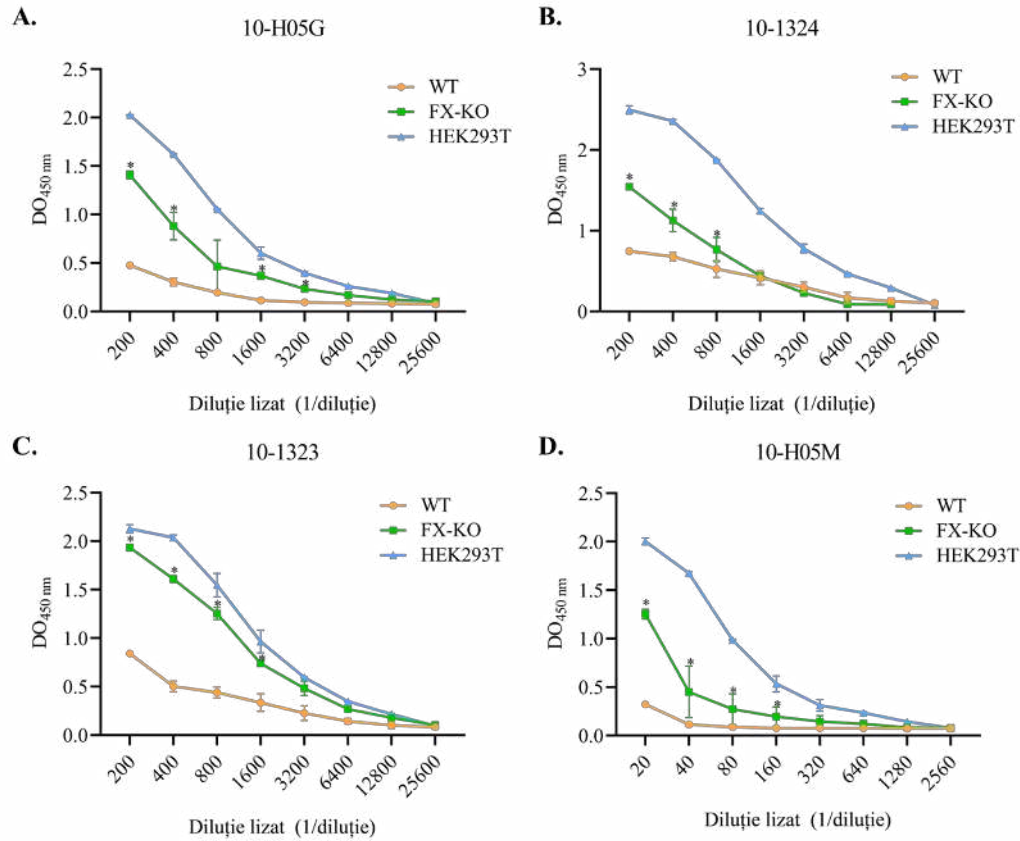
Figure 6. Antigenicity of the S/preS1$^{16-42}$ antigen produced in WT and FX-KO _N. benthamiana_. (A-D) A panel of conformation-dependent, monoclonal anti-S antibodies were used to coat 96-well plates before incubation with serial dilutions of lysates of WT and FX-KO _N. benthamiana_ leaves or HEK293T cells expressing the HBV S/preS1$^{16-42}$ antigen. The plates were incubated with a secondary rabbit anti-S antibody followed by an HRP-conjugated anti-rabbit antibody and the corresponding substrate. Antigen binding is shown as optical density values measured at 450 nm. Data are represented as means ± SD (n=4). Statistical analysis was performed by using the Mann-Whitney U test for each dilution. *, $p < 0.05$ (WT versus FX-KO).Image adapted from (Pantazica et al., 2023).

Considering the improved antigenic properties of the FX-KO plant-produced antigen, we next assessed its immunogenicity compared to its WT pant-produced counterpart _in vivo_. As such, groups of 7 Balb/c mice were intramuscularly injected with 3 doses at 14-day intervals of 25 µg of antigen in the presence of AddaVax, a squalene-based oil-in-water adjuvant. Studies have shown that this class of adjuvants protects the antigen from premature elimination and

activates both the humoral and cellular immune response (O'Hagan et al., 2021). We found that both antigens induced a significant IgG titer, with higher titers being observed in the case of FX-KO immunized mice. Moreover, analysis of the IgG1 and IgG2a subclasses, which are markers of humoral and cellular immunity, respectively, confirmed the superior immunogenicity of the FX-KO *N. benthamiana-* produced HBV antigen.

Analysis of the cellular immune response in HBV-stimulated splenocytes from immunized mice showed that immunization with the HBV antigen produced in FX-KO plants induced the strongest immune response, with the highest level of IFN-γ and IL-5 secretion when compared to its WT counterpart. Moreover, IFN-γ and IL-5 secretion from splenocytes stimulated with PBS was extremely low (almost undetectable), further confirming that the immune response is highly specific towards HBV.

Finally, we investigated the neutralization capacity of the antibodies induced by immunization with WT and FX-KO *N. benthamiana*-produced HBV-S/preS1$^{16-42}$ against HBV infection *in vitro* (Figure 7). WT and vaccine escape mutant HBV (HBV-S^{G145R}) were incubated with diluted pre-immune and day 49 sera from mice immunized with only adjuvant or the antigen produced in WT or vaccine escape HBV. The inoculum was then used to infect HepG2^{hNTCP} cells (100 Geq/cell). The infection was then quantified by determining HBeAg secretion via ELISA and normalized against levels obtained in the presence of pre-immune sera for each group. The data showed that the sera from mice immunized with the HBV-S/preS1$^{16-42}$ antigen, regardless of the production system, induced a strong neutralization of both WT (Figure 7A) and HBV-S^{G145R} (Figure 7B) compared to the control group, which is consistent with the previous results of neutralization assays of the same antigen produced in mammalian cells. However, immunization with HBV-S/preS1$^{16-42}$ produced in FX-KO *N. benthamiana* led to the production of antibodies with higher neutralizing capacity against both WT and vaccine escape HBV compared to the WT plant-produced antigen. These results suggest that better presentation of relevant epitopes, with neutralizing activity against HBV, to the immune system, as is the case for the FX-KO plant-produced antigen, contributes to the immunogenicity observed in this study.
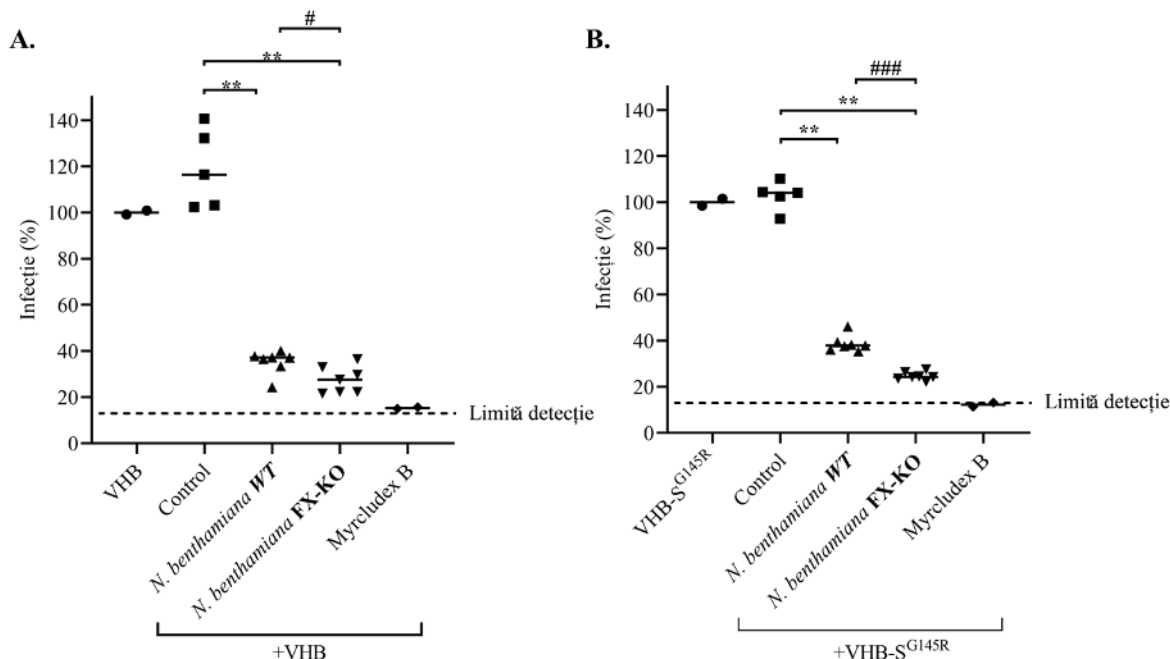
Figure 7. WT- and vaccine escape mutant HBV neutralization activity of WT and FX-KO *N. benthamiana*-derived S/preS1$^{16-42}$ antisera. Sera from mice immunized with S/preS1$^{16-42}$ antigen produced in WT or FX-KO *N. benthamiana* or only adjuvant (Control) were diluted and incubated with WT **(A)** or -S^{G145R} **(B)** HBV (GEq/cell). HepG2^{hNTCP} cells were then incubated with this inoculum or only HBV. Cells preincubated with Myrcludex B were used as a control for specific HBV inhibition. Infection levels were quantified by determining HBeAg secretion levels via ELISA. Data are presented as percentage of HBV infection in the presence of immune sera from infection percentages in the presence of pre-immune sera at the same dilution. Comparisons between antigen and control group (**, p < 0,01) and between WT and FX-KO groups (#, p < 0,05; ###, p < 0,001) are presented.

Chapter 4. DISCUSSION AND CONCLUSIONS

This study presents an optimization of the design of chimeric HBV S/preS1 antigens, with the production of 4 new constructs. These consist of partially overlapping sequences from the antigenic domain of the preS1 region which were inserted into the antigenic loop of the S protein to enable exposure of these epitopes on the surface of SVPs. The constructs were expressed in mammalian cells and characterized. We selected a novel antigen candidate, HBV-S/preS1$^{16-42}$,

which had significantly improved expression and secretion levels compared to all tested antigens (Dobrica et al., 2017). Immunological analysis showed that the novel chimeric antigen (produced in HEK293T cells) induced a stronger immune response compared to the S protein, while the resulting antibodies were capable of neutralizing both WT and vaccine escape (-S^{G145R}) HBV infection, suggesting that the novel antigen is a promising candidate of alternative vaccines against HBV.

Considering the advantages associated with production of antigens for vaccine development in plants, the novel antigen candidate was proposed for production in *N. benthamiana*. Studies have shown that plant N-glycosylation can affect the properties of recombinant proteins produced in plants. Therefore, this study represents the first analysis of the effect of plant N-glycosylation on the molecular, antigenic and immunological properties of proteins destined for vaccine development. We showed that „humanization" of N-glycosylation improved the recognition of the S/preS1$^{16-42}$ antigen by conformation-dependent anti-S antibodies, suggesting that the N-glycan plays a role in masking epitopes from the immune system. Moreover, the immunological analysis showed that the S/preS1$^{16-42}$ antigen produced in *N. benthamiana* with „humanized" N-glycosylation induced a significantly improved immune response and antibodies with increased neutralizing activity against WT and vaccine-escape mutant HBV. These data show for the first time the importance of N-glycosylation patterns in regard to antigen immunogenicity and illustrated the potential of FX-KO *N. benthamiana* as a production platform for HBV antigens.

Moreover, we showed that immunization with the chimeric S/preS1$^{16-42}$ antigen induces both anti-S and anti-preS1 antibodies, demonstrating that this strategy assures echimolar display of both epitopes on the surface of formed SVPs. However, it remains unclear whether the improved neutralization capacity of the resulting antibodies after immunization with the chimeric antigen is due to the combination of both anti-S and anti-preS1 antibodies or only the anti-preS1 antibodies. As such, in future studies we aim to determine whether depletion of anti-preS1 antibodies from the antisera affects the neutralization capacity and investigate whether introduction of the preS1 epitope into the S-protein structure affects the neutralizing activity of the resulting anti-S antibodies. This issue is of particular importance as the presence of anti-S antibodies is crucial for the neutralization of other HBV genotypes, as most sequence differences between genotypes are in the preS1 region. As such, a second future direction is

testing the neutralization capacity of the antibodies induced by the S/preS1$^{16\text{-}42}$ antigen against other HBV genotypes to confirm whether it could be a good candidate for a pan-genotypic vaccine.

Furthermore, another line of research of this study is the production of the S/preS1$^{16\text{-}42}$ antigen in low-cost alternative production systems, such as microalgae. As such, during the 1SEE project, we obtained preliminary data which confirm the possibility of expressing the chimeric HBV-S/preS1$^{16\text{-}42}$ antigen in the microalga *Porphyridium purpureum*, as well as confirming that it can assemble into subviral particles. The large-scale production of the chimeric antigen in microalgae, analysis of its glycosylation profile, as well as its antigenic and immunological properties, are future goals in order to further optimize production systems for this antigen.

Considering all the properties of the S/preS1$^{16\text{-}42}$ antigen described in this study, which make it a promising candidate for alternative vaccine development, a next step would be initiation of more advanced preclinical studies. These can include genetically modified infectable mouse models, in so-called „challenge" experiments, which involve complete immunization of mice followed by infection with HBV to determine the protective capacity of the vaccine. Other preclinical studies would involve testing the safety profile and efficacy of the vaccine in primate models, with a similar immune system to humans. If the results of all these studies are favorable, the final step would be initiation of clinical studies involving patients, in order to approve the vaccine for human use.

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1. **Pantazica, A.-M.**, van Eerde, A., Dobrica, M.-O., Caras, I., Ionescu, I., Costache, A., Tucureanu, C., Steen, H., Lazar, C., Heldal, I., Haugslien, S., Onu, A., Stavaru, C., Branza-Nichita, N. and Liu Clarke, J., The "humanized" N-glycosylation pathway in CRISPR/Cas9-edited *Nicotiana benthamiana* significantly enhances the immunogenicity of a S/preS1 Hepatitis B Virus antigen and the virus-neutralizing antibody response in vaccinated mice. *Plant Biotechnol J*. **2023**, doi: 10.1111/pbi.14028, **IF 13.263, Q1, AIS 2.17**

2. **Pantazica A-M**, Dobrica M-O, Lazar C, Scurtu C, Tucureanu C, Caras I, Ionescu I, Costache A, Onu A, Clarke JL, Stavaru C and Branza-Nichita N., Efficient cellular and humoral immune response and production of virus-neutralizing antibodies by the Hepatitis B Virus S/preS1[16-42] antigen. *Front. Immunol.*, **2022**, 13:941243, doi: 10.3389/fimmu.2022.941243.**IF 8.787, Q1, AIS 1.943**

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