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14. ABSTRACT

More than 400 million people worldwide are chronically infected with the hepatitis B virus (HBV). Estimates of the chronic hepatitis B (CHB) prevalence in the US range from 850,000 to 2.2 million people. CHB often progresses to cirrhosis, liver failure, and hepatocellular carcinoma (HCC). The existing antiviral drugs for treatment of HBV are not curative and require life-long treatment, which results in viral resistance. Therefore, new therapeutic approaches for the treatment of chronic HBV are sorely needed for this high prevalent disease. The main goal of this project is to improve immunogenicity and efficacy of the candidate immunotherapeutic vector CARG-301 using virus-like vesicle (VLV) platform for delivery of 3 HBV antigens and suppression of immune checkpoint PD-L1. After testing the efficacy of the CARG-301-based immunotherapy in a preclinical model of CHB, we will develop the cGMP manufacturing process and initiate IND-enabling studies to prepare CARG-301 for clinical trials. If our approach is successful, a treatment regimen of CARG-301 could overcome immune tolerance in CHB subjects with high viral load and antigenemia, prevent disease progression, and would improve quality of life of CHB patients while having a profound clinical and healthcare implications.

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1. INTRODUCTION

More than 400 million people worldwide are chronically infected with the hepatitis B virus (HBV). Estimates of the chronic hepatitis B (CHB) prevalence in the US range from 850,000 to 2.2 million people. CHB often progresses to cirrhosis, liver failure, and hepatocellular carcinoma (HCC). The existing antiviral drugs for treatment of HBV are not curative and require life-long treatment, which results in viral resistance. Therefore, new therapeutic approaches for the treatment of chronic HBV are sorely needed for this high prevalent disease. The main goal of this project is to improve immunogenicity and efficacy of the candidate immunotherapeutic vector CARG-301 using virus-like vesicle (VLV) platform for delivery of 3 HBV antigens and suppression of immune checkpoint PD-L1. After testing the efficacy of the CARG-301-based immunotherapy in a preclinical model of CHB, we will develop the cGMP manufacturing process and initiate IND-enabling studies to prepare CARG-301 for clinical trials. If our approach is successful, a treatment regimen of CARG-301 could overcome immune tolerance in CHB subjects with high viral load and antigenemia, prevent disease progression, and would improve quality of life of CHB patients while having a profound clinical and healthcare implications.

2. KEYWORDS

Immunotherapy, hepatitis B virus, therapeutic vaccine, combination therapy, immune checkpoint inhibition, PD-1, PD-L2, shRNA

3. ACCOMPLISHMENTS

The specific aims of the project are as follows:

1. Characterize the immunogenicity of CARG-301 modified constructs in normal mice.
2. Evaluate vaccine efficacy in mouse model of chronic HBV infection.
3. To develop CARG-301 manufacturing process and assess its PK/PD and toxicology.

HIGHLIGHTS

- **CARG-301 constructs modified with either a secretion signal alone or PD-L1 shRNA alone or in combination thereof are expressed and secreted efficiently. Research grade material of CARG-301 variants can be scaled-up and produced at high titers.**
- **Immunogenicity screening of CARG-301 variants in mice indicates that addition of the secretion signal to the core antigen or the addition of PD-L1 shRNA to CARG-301 tends to increase the frequency of the HBV-specific effector T cells.**
- **We demonstrate that PD-L1 shRNA cloned into VLV vector is functional *in vitro* and *in vivo***
- **The overall immunogenicity of middle S antigen (MHBs) in the CARG-301 construct is noticeably lower when compared to the immunogenicity of reference CARG-201 suggesting that expression of MHBs in polycistronic unit is position-dependent.**
- **CARG-301 (*MHBs*-Pol-HBc) was redesigned and engineered as CARG-301v2 (Pol-HBc-*MHBs*). The latter configuration yields improved MHBs expression ~2-fold better than the prototype CARG-301**
- **We used a reference and analogous construct (CARG-201) to establish AAV-HBV mouse model for testing the efficacy of CARG-301v2 variants.**

UNDER SPECIFIC AIM #1

Task 1: Optimize modified CARG-301 vectors for expression and inhibition studies

- Monitor expression and secretion of proteins by Western blot
- Measure inhibition of PD-L1 by shRNA using BHK-21/PD-L1 and cancer cells.

Milestone #1: Secretion is optimized – **100% achieved**

Milestone #2: Maximal inhibition by shRNA – **80% achieved**

Task 2: Examine single immunization of modified constructs

- Secure Yale IACUC and ACURO approval processes
- Scale-up and production of research grade VLV

- Immunize mice (C57BL/6) and assay for T-cells by flow cytometry
- Compare antibody production in immunized mice

Milestone #1:
(a) Yale IACUC and DoD ACURO Approvals: Yale IACUC protocol 2022-20444 was approved by Yale IACUC on 03/23/2022 and by the DoD ACURO on 08/23/2022– **100% achieved**
(b) Yale IRB and DoD Office of Human Research Oversight (OHRO) Approvals: Yale IRB protocol 2000035313 was approved by Yale Human Research Protection Program Institutional Review Board on 05/26/2023 and by the DoD OHRO on 06/21/2023– **100% achieved**

Milestone #2: Aggregate T-cell response toward all HBV antigens – **100% achieved**

Milestone #3: Serum abs specific for all antigens – **20%**

UNDER SPECIFIC AIM #2
<u>Task 1. Evaluate if modifications of CARG-301 improve efficacy</u>
- AMC IACUC and ACURO approval processes
- Scale-up and production of research grade VLV
- Establish HBV persistent C57BL/6 mice
- Prime and boost immunization of mice with modified constructs
- Analyze HBV-specific biological responses
- Evaluate intrahepatic genome replication
- Monitor HBV protein expression and antigenemia
- Assess liver injury and inflammation

Milestone #1: Albany Medical College IACUC approval for animal studies was obtained on March 17, 2022. ACURO approval for animal studies on September 12, 2022 – **100% achieved.**

Milestone #2a: Establishment of AAV-HBV persistent in mice – **12.5% achieved.**

Milestone #2b: Utilize model to achieve several logs reduction in serum HBsAg levels in >80% of the mice (**In 2nd Year**).

<u>**Accomplished Goals**</u>
Major activities of Year 1 focused on **Specific Aim 1** (Task 1 and Task 2) all in preparation for Task 3. These preparatory activities included submission and receiving the approvals of the animal protocol for assessment of the immunogenicity of CARG-301 in mice and the protocol for the minimal risk study in human peripheral blood mononuclear cells (PBMC) (please see Key Information with approval dates above). We also ensured that the PD-L1 shRNA cloned into CARG-301 did not compromise the vector's ability to down regulate PD-L1 *in vitro*. However, it still remained within the realm of possibility that when delivered in vivo, shRNA modified-CARG-301 may fail to downregulate endogenous PD-L1 in mice. We demonstrate that using a tumor model that VLV-shRNA can inhibit tumor regression when delivered by intra-tumoral injection indicating that it is eminently feasible to retain function of shRNA *in vivo*.

The initial comparative study was carried out to assess modified and original CARG-301 immunogenicity in immunologically naïve C57BL/6J mice. This study was complemented by an assessment of the HBV antigen expression in hamster BHK-21 and human HEK 293T cells. Human MHC class I HLA.A2 transgenic mice were procured to initiate breeding to produce enough animals for additional immunogenicity studies. Furthermore, quotes for the PBMC, peptide, and antibody orders were received; the procurement and validation of the reagents is in progress. An initial run with a reference VLV and positive control peptides and subsequent analyses of cytokine production has been carried out.

The second major activity of Year 1 was focused on initiating the establishment of the mouse model needed for efficacy testing in **Specific Aim 2** (Task 1 and Task 2). These preparatory activities included submission and receiving the approvals of the animal protocol needed for assessment of the efficacy of CARG-301 in mice at Albany Medical College. We also obtained from commercial sources, serotype 8 of the Adeno-associated virus (AAV) encoding a 1.3-mer HBV genome prepared by SignaGen Laboratories (Frederick, MD). HBV replication was established in male C57BL/6 mice with transduction via intravenous injection of 1×10^{11} genome copies of AAV-HBV.

Specific objectives of the activities included the comparison of immunogenicity of the original CARG-301 (expressing HBV middle S antigen or MHBs; catalytically dead HBV polymerase antigen, and non-secreted core antigen) with modified CARG-301 variants (which included a secretion signal for the HBV core antigen, PD-L1 shRNA and their combination) versus the reference VLV CARG-201 (expressing HBV MHBs and HBc antigens only). The objective was to carry out the immunogenicity study in immunologically naïve C57BL/6J mice and mice expressing human HLA.A2 MHC class transgene, and *in vitro* in human PBMC. Another objective was to establish an *in vivo* AAV-HBV mouse model system for testing the efficacy of the CARG-301 variants in order to select and prioritize the top performer for preclinical development. To achieve this goal, we used a reference and analogous construct (CARG-201) to establish the system.

Significant results and key outcomes: CARG-301 constructs modified with either a secretion signal or PD-L1 shRNA or in combination are expressed and secreted efficiently. Research grade material of CARG-301 variants can be scaled-up and produced at high titers**.** We have demonstrated that the shRNA cloned into a VLV vector can downregulate expression of a stably transfected PD-L1 in BHK21 cells. The ability of shRNA to regress the growth of PD-L1-rich tumor indicates that shRNA is functional in vivo.

Immunogenicity screening of CARG-301 variants in mice indicates that addition of the secretion signal to the core antigen or the addition of PD-L1 shRNA to CARG-301 tends to increase the frequency of the HBV-specific effector T cells. The initial study in C57BL/6 mice with the original and modified CARG-301 variants suggests that addition of the secretion signal to the core antigen or PD-L1 shRNA tends to increase the frequency of the HBV-specific effector T cells measured by quantification of the IFNγ and TNF-positive T cells with flow cytometry upon restimulation with peptides derived from HBV S, Core and Polymerase antigens. However, the overall immunogenicity of the CARG-301 for the middle S antigen was noticeably lower when compared to the immunogenicity of CARG-201. Modifications to the VLV vector design in CARG-301 required rearrangement of the payload antigen order: middle S antigen (MHBs) was moved from the 3' end location of the VLV mRNA into the position immediately downstream of the SFVnsp1-4 to accommodate a second subgenomic polycistronic RNA expressing polymerase (Pol), core (HBc) and VSV-G. We suspected that these rearrangement effects which were further compounded by the addition of the secretion signal to core antigen or PD-L1 shRNA expression cassette, may have resulted in less-than-optimal expression of the MHBs antigen. Although the initial immunoblotting analyses of the antigen expression did not reveal the difference, follow up studies with more accurate densitometry analyses and normalization to VSV-G validated our concerns. Similar changes to the design of the reference VLV CARG-201 to accommodate PD-L1 shRNA and/or the secretion signal for core antigen resulted in reduction of the modified CARG-201 immunogenicity in naïve mice (data not shown) and efficacy in the AAV-HBV model (reported by Dr. Robek, our collaborator at Albany Medical College). Based on these findings, we redesigned the CARG-301 vector to place the MHBs antigen at the 3' end of the VLV RNA to restore and improve the expression levels of this key antigen. We have since constructed an optimal CARG-301 which we dub version 2 (CARG-301v2) in which MHBs expression is 2-fold better than the CARG-301 prototype. The optimized CARG-301 vector variants have already been validated for antigen expression, and we plan to compare their immunogenicity in naïve mice, side by side with the original CARG-301 and CARG-201. The best construct will be taken into further immunogenicity studies in HLA-A2 transgene mice and with human PBMC.

Other achievements: In preparations for the studies with CARG-301 in human PBMC, we refined the flow cytometry and ELISPOT protocols for downstream analyses of cytokine production after consultations with investigators at CTL Immunospot, who have extensive experience with immunogenicity studies during clinical trials. The protocol refines should improve our chances of success when we initiate the immunogenicity testing of the optimized CARG-301v2.

Before initiating the studies on modified CARG-301, a pilot study was performed to assess the impact of the planned CARG-301 modifications (addition of antigen secretion signals, expression of PD-L1 shRNA) on the immunogenicity and efficacy of a reference construct, CARG-201. Mice were transduced with AAV-HBV to generate animals that persistently replicated HBV and were subsequently prime-boost immunized with vectors expressing HBV antigens with or without the secretion signals, shRNA, or both. These studies revealed two key findings**.** ***First***, as expected, we found that adding a secretion signal to the HBV core protein significantly enhanced the antibody response to this antigen (**Fig. 1).** Note that in this competitive ELISA, lower O.D. values indicate higher antibody responses. ***Second***, unexpectedly, this study also revealed an influence of the protein-coding sequence position in the vector on the platform's efficacy (not shown). This information combined with the immunogenicity data have been employed to design an improved version of CARG-301 vectors dubbed CARG-301v2 to further enhance HBV antigen expression.

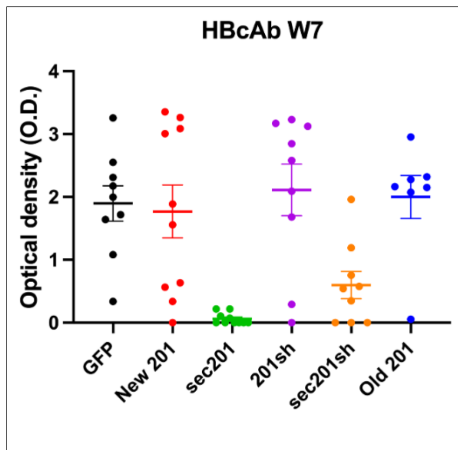

Figure 1. Competitive ELISA for the determination of total anti-HBc abs. In the designed ELISA system two specific antibodies were used, HBc-HRP and the other vaccine-induced HBc-antibody (present in serum) so that competition occurred between the two antibodies for the same antigen. Sera to be tested were added to these wells and incubated at 37°C and then washed. If antibodies are present, antigen-antibody reaction occurred and there is no antigen left for the HRP labeled Anti-HBc antibodies. These antibodies remained free upon addition and are washed off. Substrate was added but there was no enzyme to act on it, therefore, positive result showed no color or low color change. In other words, low positive samples showed low color when negative samples showed high color change (high absorbance at OD_{450} nm).

To better inform future study design and eventual CARG-301v2 clinical development, we also initiated an experiment to determine the impact of booster immunization on CARG-301 efficacy. Following transduction with AAV-HBV to create persistent HBV infection, mice were immunized with CARG-301 encoding the Indiana (IND) serotype VSV-glycoprotein (G). Four weeks later, the mice were boosted with CARG-301 encoding the New Jersey (NJ) VSV-G serotype (heterologous boost), CARG-301^{IND} (homologous boost), or a similar vector backbone engineered to express GFP (no boost). A group of control animals received both prime and boost with the GFP vector. Although this experiment is still in progress, and the final outcome remains to be determined, an interim analysis of an early time point suggests that heterologous prime-boost has superior anti-HBV efficacy compared to no boost or homologous prime-boost (not shown).

CARG-301 VLV optimization for expression and secretion

We have generated CARG-301 secreting all three antigens of HBV (secCARG-301). We have also engineered CARG-301 to incorporate shRNA alone (CARG-301.sh) or both secretion signals and shRNA (secCARG-301.sh). In order for CARG-301 constructs to induce immune responses that clear persistent HBV replication initiated in the mouse liver by adeno-associated virus (AAV) delivery requires, we deployed VLV vectors encoding VSV G proteins of different viral serotypes (VSV G^{IND} and VSV G^{NJ}) that will be used in sequential immunizations without being neutralized by antibody to VSV G (**Fig. 2**).

These-serotype switching, heterologous boosting agents have distinct biochemical properties and processing requirements (**Table 1**). BHK-21 cells were infected with CARG-301 VLV series separately at a multiplicity of

infection of 1. Cells were collected after 18 h, lysed, and evaluated in a Western blot using HBV preS2, anti-2A peptide, polymerase and anti-VSV-G (IND) antibodies.

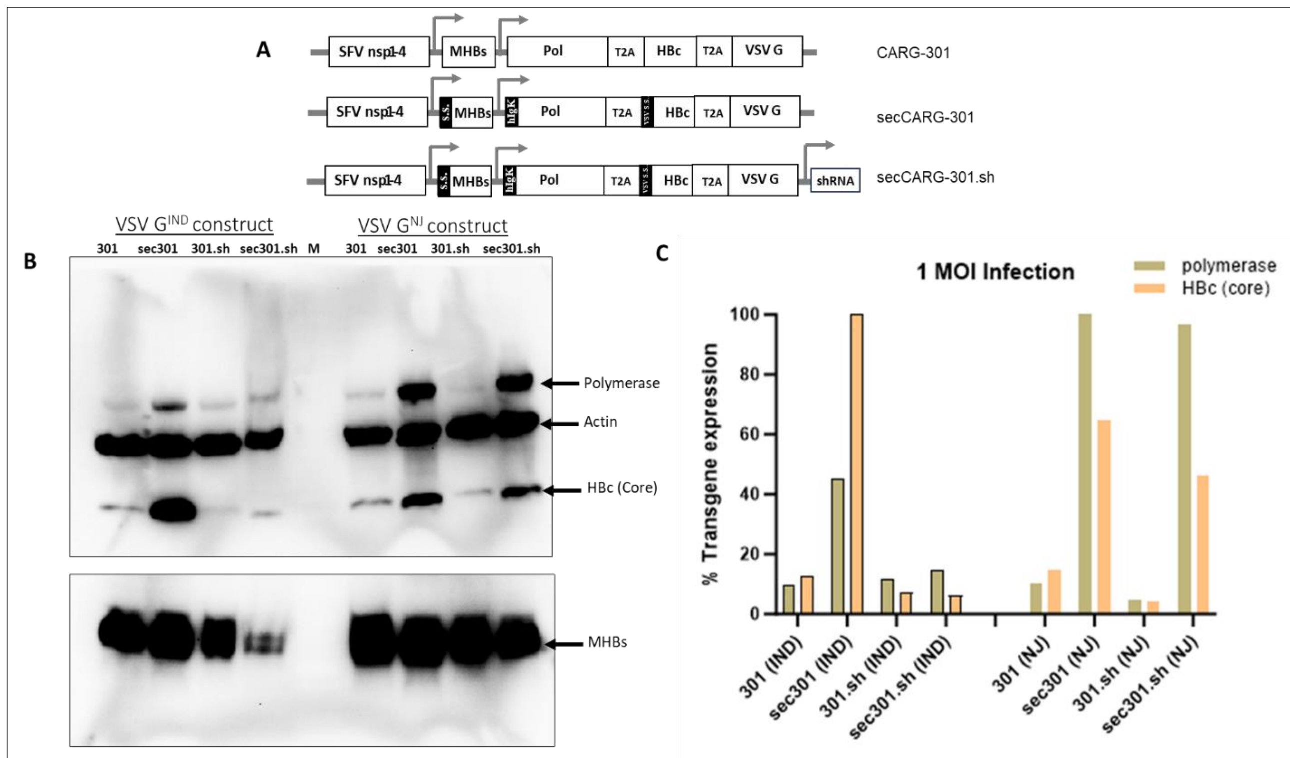

Figure 2. Expression and secretion of VLV-based recombinant multivalent HBV vaccines. (***A***) Schematic depiction of CARG-301 constructs. The four non-structural proteins of the Semliki Forest virus (SFV) replicase are designated (nsp1-4). HBV polymerase (Pol) is deleted of its terminal protein from the four structural domains comprising the enzyme. At its N-terminus is fused the human IgK signal sequence. The middle surface antigen (MHBs) and the core antigen (HBc) have the native and heterologous VSV G signal sequence fused to their amino termini respectively. Expression of Pol and core was linked to the downstream gene by a piconavirus, Thosea asigna virus 2A (T2A) skipping sites. ***(B)*** Expression of HBV genes as assayed by western blot analysis in BHK21 in cell lysate using anti-preS2 antibody, anti-peptide 2A antibody, and anti-VSV-G (Indiana serotype) antibody. (***C)*** Densitometric scanning of WB in (*B*) by normalization to β-actin gene expression. HBc, HBcAg; 301=CARG-301; sec301=secCARG-301; 301sh=CARG-301sh; sec301sh=secCARG-301shs; ss=signal sequence; MOI=multiplicity of infection. VSV G=vesicular stomatitis virus envelop glycoprotein. IND=Indiana serotype; NJ=New Jersey serotype. VSV G=vesicular stomatitis virus envelop glycoprotein.

Table 1: VLV Titers of Modified CARG-301 Constructs after Transfection

Construct	Titer (PFU/mL)
CARG-301^{IND}	1.00 x 10^6
CARG-301^{NJ}	9.50 x 10^6
CARG-301sh^{IND}	2.33 x 10^6
CARG-301sh^{NJ}	1.18 x 10^6
secCARG-301^{IND}	1.30 x 10^6
secCARG-301^{NJ}	2.80 x 10^6
secCARG-301sh^{IND}	2.08 x 10^6
secCARG-301sh^{NJ}	1.50 x 10^6

These-serotype switching, heterologous boosting agents have distinct biochemical properties and processing requirements (**Table 1**). BHK-21 cells were infected with CARG-301 VLV series separately at a multiplicity of infection of 1. Cells were collected after 18 h, lysed, and evaluated in a Western blot using HBV preS2, anti-2A peptide, polymerase and anti-VSV-G (IND) antibodies. HBV preS2 antibody detects an epitope corresponding to amino acids 132-137 of the middle surface antigen of HBV. Anti-2A peptide antibody detects the peptide remaining at the carboxy-terminal end of proteins following ribosome skipping (cleavage) at T2A sequences. VSV-G (IND) antibody is specific to the Indiana serotype of the glycoprotein and therefore detects VSV-G only from CARG-301-VLV-infected lysates, but not from CARG- 201-1-VLV-infected lysates. Therefore, we can produce CARG-201 VLVs with a consistent yield and antigen expression.

As seen in **Table 1**, we have completed the generation of CARG-301 secreting all three antigens (*sec*CARG-301). We have also engineered CARG-301 to incorporate shRNA alone (CARG-301.sh) or both secretion signals and shRNA (*sec*CARG-301.shNA). We will now test the immunogenicity of these constructs and prioritize them for efficacy studies in a chronic mouse model of HBV infection. The availability of constructs in both serotypes will allow us to employ a prime boost regimen if necessary. It is quite apparent that addition of the secretion signal or shRNA did not compromise VLV recovery.

Production of Research grade VLVs. We have achieved pilot scale production methods to produce viral titers of VLVs up to 10^9 PFU per mL, removing a major hurdle that has thwarted the further development of similar products (**Table 2**). For the production of VLV stocks, BHK-21 cells were cultured in DMEM supplemented with 5% fetal bovine serum (FBS). VLVs were produced by transfecting BHK-21 cells with the VLV plasmid DNA followed by collection of the master VLV stock. Propagation of the working stocks was performed by a single passage of the master stock in BHK-21 cells cultured in Opti-MEM™ Reduced Serum Medium (ThermoFisher, Waltham, MA,). Working stocks were concentrated using MacroSep® Advance 100K MWCO (Pall Laboratories, Port Washington, NY) and titrated using plaque assay in BHK-21 cells. Low MOI infections are critical for achieving high titer VLVs. Our QC tests of VLVs include Identity (SDS-PAGE, Western blot, RT-qPCR); Potency (Plaque assay, IFA).

Table 2: VLV Titers of Modified CARG-301 Constructs after Scale-up Production

Construct	Titer (PFU/mL)
CARG-301^{IND}	2.33 x 10^9
CARG-301^{NJ}	6.80 x 10^9
CARG-301sh^{NJ}	1.50 x 10^9
secCARG-301^{NJ}	2.00x 10^9
secCARG-301sh^{NJ}	1.68 x 10^9

Monitor PDL-1 inhibition by shRNA in normal and cancer cells.
In order to examine whether shRNA can inhibit PD-L1 expression *in vitro* we conducted the following experiment. MC38 cells were stimulated with 20 ng/ml mouse IFN-γ and at the same time infected with multiplicity of infection of 2 (2 x MOI) of the indicated VLV. IFN-γ is utilized to mimic *in vivo* cell signaling that induces PD-L1 expression and the response to IFN-γ will highlight the ability of the MC38 tumor cell line to mount an anti-immunity response to immune cell signaling. The PD-L1 expression levels with IFN-γ will was normalized to β-actin. The data show that there was a three-fold reduction in PD-L1 expression as compared to the control (**Fig 3**). In order to test whether shRNA can affect tumor growth¸ we tested VLV-shRNA construct in the MC38 model of syngeneic colon cancer in mice. Intra-tumoral injection of VLV-shRNA to subcutaneously grafted and established MC38 tumors markedly exhibited partial tumor growth inhibition that was statistically significant from the VLV-GFP used as a vector control (Figure **3c**). These results indicate that the PD-L1 shRNA payload can suppress tumor growth.

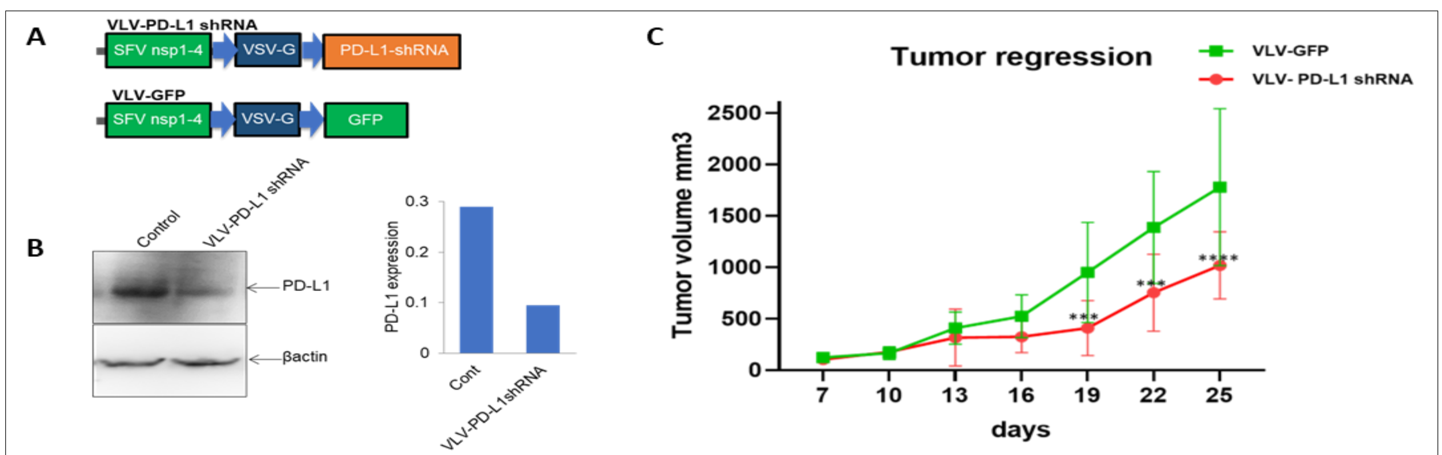

Figure 3. Armed VLV RNA replicon platform promotes tumor regression. A. Design of VLV constructs comprising SFV 1-4 non-structural protein (nsp) and VSV-G structural glycoprotein. Transgenes encoding GFP and shRNAs against PD-L1 were cloned into VLV vector backbone to generate respective payload VLV constructs. B: BHK-21 cells that stably express

PD-L1 were infected with 1 MOI VLV PD-L1 shRNA. Cells were lysed and blotted against anti-PD-L1 and anti-β-actin antibodies. Relative expression of PD-L1 compared to β-actin was quantified in the right panel. **C**: 5 x 10^5 MC38 cells were grafted subcutaneously to the flank of C57BL/6J mice. At indicated dates, 5 x 10^7 pfu indicated VLV agents were injected intratumorally. Tumor sizes were measured every three days, and average tumor sizes of each group were shown. Error bars denote S.E.M. ** p<0.01, **** p<0.0001.

BHK21 cells stably expressing PDL1 transgene were infected with PDL1 shRNA VLV at 1 MOI infection. Cells collected by scraping and analyzed by western blot probed with anti-PD-L1 (Bio X cell # BE0101), and anti-β-actin (Santa Cruz Biotechnology) antibodies. PD-L1 band densities were analyzed and normalized with actin loading control. In addition to the PD-L1 stably transfected BHK21 cells, we also employed a cancer cell line (MC38) which are known to express endogenously abundant levels of PD-L1. To examine the *in vitro* PD-L1 downregulation by VLV-PDL1-shRNA construct, MC38 cells were stimulated with 20 ng/ml mouse IFN-γ and at the same time infected with two MOI of the indicated VLVs. After 26 hours of stimulation/infection, cells were lifted and stained for PD-L1 (cat#124308, BioLegend), and analyzed for the percentage of PD-L1-positive and -negative cells by flow cytometry. For data analysis, we used GraphPad Prism software, version 9 (GraphPad Software, San Diego, CA). BHK21 cells stably expressing PDL1 transgene were infected with PDL1 shRNA VLV at 1 MOI infection. Cells collected by scraping and analyzed by western blot probed with anti-PD-L1 (Bio X cell # BE0101), and anti-β-actin (Santa Cruz Biotechnology) antibodies. PD-L1 band densities were analyzed and normalized with actin loading control.

Now that we have ascertained that shRNA is functional in vivo, we next tested whether CARG-301 modified with shRNA could inhibit PD-L1 *in vitro*. It is clear that shRNA expressed on VLV or in combination CARG-301 can inhibit PD-L1 expression (**Fig 4**).

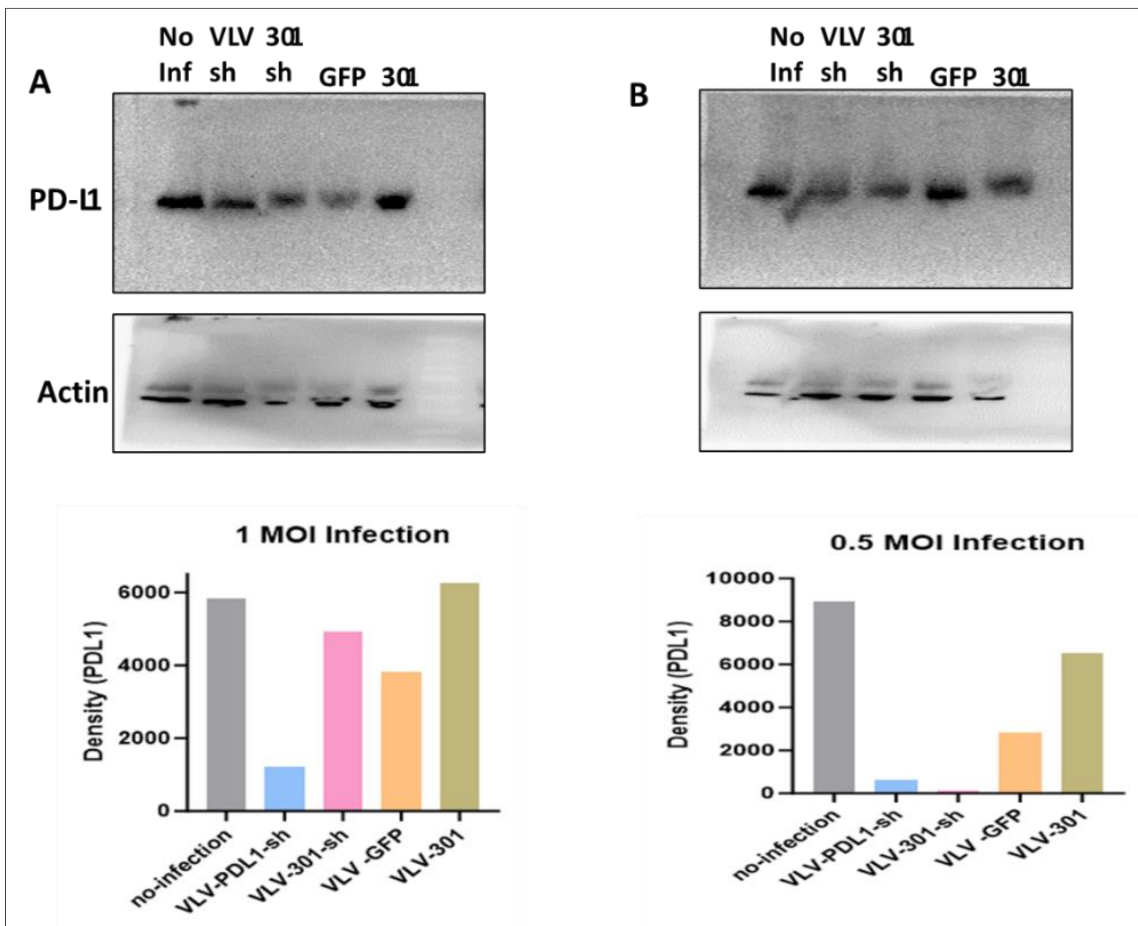

Figure 4. CARG-301 modified with shRNA inhibits PD-L1 expression in stably transfected BHK21 cells. Multiple PDL-1 specific shRNAs were inserted 3' of VSV G glycoprotein. A mouse cDNA clone of PDL-1, CD274/B7-H1/PD-L1 ORF (Sino biological Inc), was cloned into the mammalian expression vector, pCMV3-Flag-mCD274. BHK21 cells were transfected and hygromycin-resistant clones were selected and amplified. PDL1 expression in stable cells was analyzed by western blot with anti-PDL1 antibodies. VLVs produced by transfecting BHK21 cells using VLV-shRNA and CARG-

301shRNA VLVs were produced and tittered, 1 MOI (A) or 0.5 MOI (B) was used to infect BHK21:PDL1 stable cell line. Lysates were prepared by collecting cells after 32 hours. PDL1 downregulation was analyzed by western blots and compared with non-infected cells lysate. Band intensity quantified by GelQuant.NET software (BiochemLabSolutions.com) normalized to β-actin.

Redesign of CARG-301 to maximize expression of MHBs

The genome of alphaviruses such as SFV is translated into polyproteins that are processed into a viral replicase that produces both negative and positive strands. In infected cells, negative strand synthesis is short-lived and occurs only early, whereas positive strand synthesis is stable and occurs both early and late. The nsp1 functions in the initiation of transcription; nsp3 acts to form initial transcription complexes; and nsp2 and nsp4 first recognize positive strands as templates and then make negative strands the preferred templates. The premature termination of VLV genome leads to a shorter minus-RNA that can decrease MHBs expression cloned next to the first sub-genomic promoter in CARG-301. These shorter minus-RNA templates possibly generate lesser MHBs sub-genomic plus-RNAs and by produce lesser MHBs. Our goal is not to compromise MHBs expression and is vital to decrease HBs antigen levels in HBV chronic disease models. Therefore, in order to maintain optimal MHBs expression and to generate better efficacy, we cloned MHBs downstream of the VSV G gene under a separate sub-genomic promoter and named this newer version "CARG-301 version 2" or CARG-301v2 (**Fig. 6**). We see improved MHBs expression with this new design (**Fig. 7).**

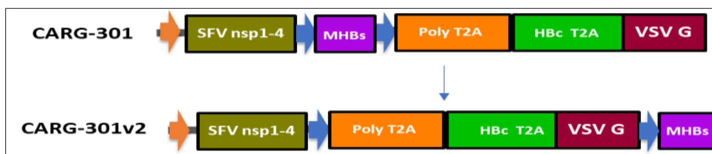

Figure 5: Scheme of a re-design of CARG-301 to maximize expression of MHBs.

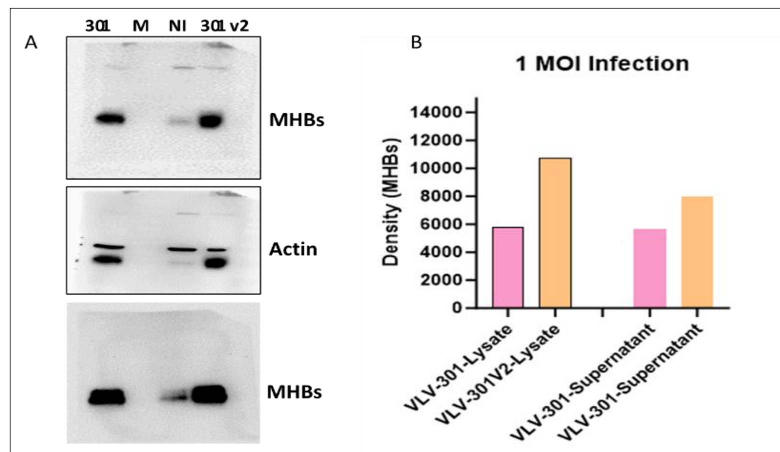

Figure 6: Re-designed CARG-301v2 secretes more efficiently than CARG-301. (A) Expression of HBV MHBs as assayed by western blot in BHK21 was compared in cell lysate (top panel) and culture supernatants (bottom panel). Actin protein is detected in the cell lysate (middle panel) but not in the cell supernatants. (B) Densitometric scanning of WB in (by normalization to β-actin gene expression.

CARG301.shv2 inhibits PDL1 expression

We compared PD-L1 downregulation using newly generated CARG-301shv2 constructs with previously generated CARG-301sh construct. The differences between these two constructs are the positions of shRNA and MHBs gene sequences in the VLV vector (**Fig. 7**). By modifying MHBs position close to 3'UTR, we measured increased MHBs expression (**Fig 8**). We know that genes cloned away from 3'UTR are expressed less efficiently compared to the gene cloned next to 3'UTR. This is possibly due to premature termination of the VLV genome negative RNA strand during its replication. MHBs expression is very important to decrease HBs antigen levels in chronic HBV patients. To maintain optimal MHBs expression from the VLV vector we switched shRNA and MHBs positions in vector. To check PD-L1 down-regulation in these newer version VLV constructs, (CARG301shv2) we used them to infect PDL1 stably expressing BHK21 cells at 1 MOI infection. Lysates prepared with RIPA buffer and ran on 4-15% SDS-PAGE gel. We observed less PD-L1 downregulation in newly generated CARG-301.sh V2 constructs compared to CARG-301.sh. We also see a difference in PDL1 downregulation between 2xsh and 3xsh RNA constructs. This lower than the expected PD-L1 downregulation

may be due to shRNA insert cloned away from 3'UTR compared to previously generated CARG301sh. However, when compared with control VLV-GFP, we observe PD-L1 downregulation in all shRNA constructs (**Fig 8**). We will further optimize infection conditions, particularly at low multiplicity of infection conditions, and will compare for PD-L1 decrease. In addition, we will also further optimize designs by increasing the number of shRNA to get a better than or comparable PD-L1 response as we observed with prototypic CARG301sh.

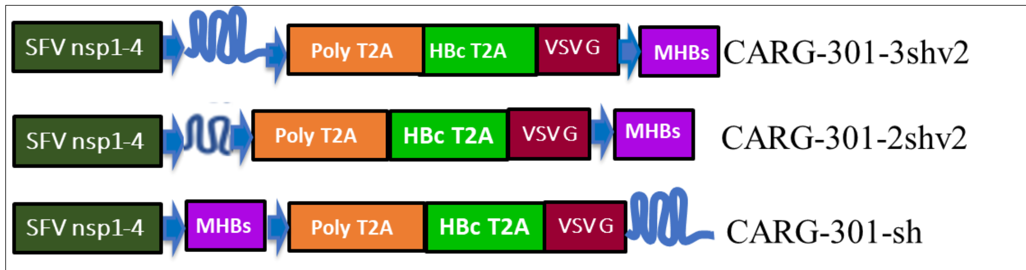

Figure 7: Scheme of a re-design of CARG-301shRNA based constructs to maximize inhibition of PD-L1

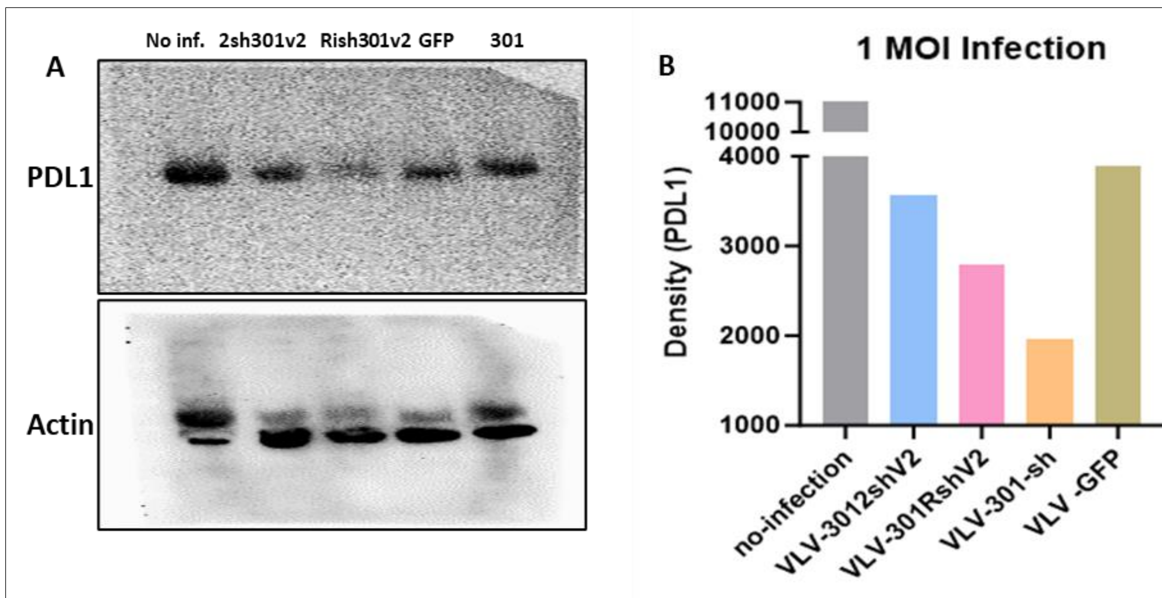

Figure 8: CARG-301shv2 inhibits PD-L1 expression in stably transfected BHK21 cells. (A) Lane 1 is un-infected control, lane 2 is CARG301.2sh (2xshRNA), lane 3 is Rish301v2 [CARG301.3sh (3 shRNA)], lane 4 is GFP control and lane 5 is CARG301.sh. (B) Blots probed for PDL1 and actin and quantified expression using BIO-RAD Quantity-one software.

What opportunities for training and professional development has the project provided?
Nothing to Report.

How were the results disseminated to communities of interest?
Nothing to Report.

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

We will next complete the assembly and optimization of all CARG-301v2 variants in both NJ and IND serotypes for immunogenicity testing and for prime-boosting in efficacy studies. Concomitant with the mice experiments, we shall conduct immunogenicity studies using PBMCs from human subjects which is a more relevant model than a mouse model. In this way we will thus be able to confirm and complement our mouse immunogenicity data and to generate data that will allow us to select the top performers for *in vivo* efficacy studies in the AAV-HBV model we have established. In the unlikely event that these experiments fail to differentiate between the various promising constructs, we still have the option of going straight into the AAV-HBV mice since we have shown that the control CARG-301 as well as an analogous reference construct secCARG-201 are efficacious in AAV-HBV model. We are therefore confident that our experiments will enable the selection of a lead candidate for preclinical development, cGMP manufacturing and timely filing of IND by the end of the award.

4. IMPACT:

What was the impact on the development of the principal discipline(s) of the project?
Nothing to Report.

What was the impact on other disciplines?
Nothing to Report.

What was the impact on technology transfer?
Nothing to Report.

What was the impact on society beyond science and technology?
Nothing to Report.

5. CHANGES/PROBLEMS:

Nothing to Report

6. PRODUCTS: Nothing to Report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

CaroGen Site
Valerian Nakaar, Ph.D., Principal Investigator, (2.0 calendar months): No Change
Joseph Rininger, Ph.D., Co-Investigator, (1.0 calendar months): No Change
King Lee, Ph.D. RAC, Co-Investigation, (1.0 calendar months): No Change
Marie Krady, Ph.D. Sr Research Scientist (2.4 calendar months): No Change
Bhaskara Madina, Ph.D. Principal Research Scientist (3 calendar months): No Change

Yale University Site
John Rose, PhD, Principal Investigator (1.8 calendar months): No Change
Timur Yarovinsky, MD PhD, Co-Investigator (2.4 calendar months): No Change

Albany Medical College Site
Michael Robek, PhD, Principal Investigator (2.4 calendar months): No change

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

CHANGES IN ACTIVE SUPPORT

Robek, Michael D.

Current

W81XWH2210762 *(this award)* (Nakaar) 09/15/22 - 09/14/24 1.2 cal months / year
US Dept. of the Army total direct + indirect
Subaward from CaroGen Corporation
Combination of HBV immunotherapy with shRNA-mediated PD-1/PDL checkpoint inhibition for treatment of chronic HBV
The major goal of this subcontract is to test the hypothesis that the combined effects of shRNA-mediated PD-L1 inhibition and the improved secretion of the HBV antigens will confer on multivalent CARG-301 a superior therapeutic index.
Aim 1. Characterize the immunogenicity of CARG-301 modified constructs in normal mice.
Aim 2. Evaluate vaccine efficacy in a mouse model of chronic HBV infection.
Aim 3. To develop CARG-301 manufacturing process and assess its PK/PD and toxicology.
Primary Place of Performance: Albany Medical College, Albany, NY
Contact: Valerian Nakaar, CaroGen Corporation, vnakaar@carogencorp.com

R01 AI148354 (Robek) 05/05/20 - 04/30/25 3.0 cal months / year
NIH/NIAID total direct + indirect
Human mechanisms of virus persistence in an AAV-based mouse model of chronic HBV infection
The major goal of this project is to understand how mechanisms of immunological dysfunction contribute to HBV persistence using a mouse model of chronic infection.
Aim 1. Assess the inhibition of HBV-specific T cells by NK cells.
Aim 2. Define the role of regulatory cells, cytokines, and metabolic pathways.
Aim 3. Evaluate T cell inhibitory receptor function and exhaustion.
Primary Place of Performance: Albany Medical College, Albany, NY
Contact: Rajen Koshy, NIAID/NIH, rkoshy@niaid.nih.gov

R13 AI051522 (Robek) 08/01/22 - 07/31/23* 0.12 cal months / year
NIH/NIAID/NIAMS *competitive renewal
Upstate New York Immunology Conference pending total direct + indirect
The major goal of this proposal is to provide financial support for a regional immunology conference.
Aim 1. To provide a forum for members of the regional immunology community to present their work, exchange ideas, and develop collaborations.
Aim 2. To give students and postdoctoral fellows an opportunity to meet distinguished national leaders in the field, as well as other trainees and PIs from area institutions.
Aim 3. To give students and postdoctoral fellows an opportunity to present their work in a meeting format, and obtain appropriate feedback on their projects.
Aim 4. To increase local, national, and international awareness of immunology research being conducted in the region.
Primary Place of Performance: Albany Medical College, Albany, NY
Contact: Susan F. Cooper, NIH/NIAID

Scientific Advisory Board and Consulting Agreement 03/03/2016 - present 0.0 cal months / year

CaroGen Corporation, Farmington, CT Primary Place of Performance: Albany, NY Contact: Bijan Almassian, Carogen Corp.	+ stock options with unknown fair market value	
Institutional funds for research support and graduate student stipends Albany Medical College Primary Place of Performance: Albany Medical College, Albany, NY Contact: William Hammer, Albany Medical College, hammerw@amc.edu	annual (Est.)	0.0 cal months / year

In-Kind

Summary of In-Kind Contribution: Proprietary virus-like vesicle vectors provided by CaroGen Corporation (Farmington, CT) to be tested in active U.S. Dept. of the Army subcontract.
Status of Support: Active
Primary Place of Performance: Albany Medical College, Albany, NY
Project/Proposal Start and End Date (MM/YYYY) (if available): 09/2022 – 08/2024
Person Months (Calendar/Academic/Summer) per budget period: N/A
Estimated Dollar Value of In-Kind Information: estimate

Summary of In-Kind Contribution: Peptides provided by Gilead Sciences (Foster City, CA).
Status of Support: Active
Primary Place of Performance: Albany Medical College, Albany, NY
Project/Proposal Start and End Date (MM/YYYY) (if available): N/A
Person Months (Calendar/Academic/Summer) per budget period: N/A
Estimated Dollar Value of In-Kind Information: estimate

Changes in current support:

1. This award began on 09/15/2022 (added to current support)
2. NIH R13 AI051522 began on 8/1/2022 (added to current support)
3. NIH R01 AI124006 ended on 01/31/2023 (removed from current support)
4. CaroGen sponsored research agreement ended on 12/31/2022 (removed from current support)

Overlap

No scientific overlap exists between the application under consideration and any other current support.

What other organizations were involved as partners?
Nothing to Report.

8. SPECIAL REPORTING REQUIREMENTS
Nothing to Report

9. APPENDICES:
Nothing to Report