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Supplemental Information

Virus-like Vesicles Expressing Multiple Antigens for Immunotherapy of Chronic Hepatitis B

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Supplemental Information

Transparent Methods

Plasmids and cells for VLV production. Plasmids for generation of empty vector VLV and the VLV expressing MHBs (MT2A) have been previously described (Reynolds et al., 2015; Rose et al., 2014). We custom synthesized DNA fragments encoding HBV Pol and HBcAg with 2A peptides and cloned them into the MT2A plasmid using conventional cloning and/or Gibson assembly at SbfI and PacI restriction sites. We used BHK-21 cells for generation of VLV master stocks by Lipofectamine-mediated transfection and production of VLV working stocks by infection. We maintained BHK-21 cells in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum (FBS), 50 U/ml of penicillin, and 2 mM L-glutamine and switched them to Opti-MEM I reduced serum medium for VLV production for 48 to 72 h after infection. After preclearing of the conditioned medium by centrifugation at 600g for 10 min, we concentrated working stocks by ultrafiltration using MacroSep® Advance 100 MWCO (Pall Laboratory) or Amicon Ultra 100K MWCO (EMD Millipore) centrifugal filter units. To quantify VLV titers, we used plaque assay or indirect immunofluorescence for VSV-G in BHK-21 as previously described (Reynolds et al., 2015; Rose et al., 2014).

Indirect immunofluorescence microscopy and flow cytometry. We seeded BHK-21 cells on coverslips one day prior to infection with VLVs at MOI=1 plaque forming unit (PFU)/cell. We fixed cells with 3% paraformaldehyde at 20 h post infection, washed with PBS containing 10 mM glycine (PBS-glycine), permeabilized with 0.1% Triton X-100 in PBS-glycine, and incubated with a 1:200 dilution of mouse monoclonal antibodies for MHBS (preS2, clone S 26),

HBV Pol (clone 2C8) from Santa Cruz Biotechnology, VSV-G (clones I1 and I1-4), or rabbit polyclonal for HBcAg (DAKO). After extensive washes, we stained the cells with 1:500 dilution of goat anti-mouse AlexaFluor® 488 IgG or goat anti-rabbit AlexaFluor® 594 (ThermoFisher). We mounted washed coverslips on slides using ProLong Gold antifade reagent with DAPI (ThemorFisher) and imaged with Leica DMIRB microscope with 20×/0.75 objective, DC350FX camera (Leica) and ImagePro software or Nikon Eclipse 80i epifluorescence microscope with Retiga 2000R camera and NIS Elements software.

To validate expression of antigens by flow cytometry, we seeded BHK-21 cells in 12-well or 6-well tissue culture plates one day prior to infection with VLVs at MOI=1 plaque forming unit (PFU)/cell. We harvested cells at 20 h post infection with TrypLE Express (ThermoFisher), washed in FACS buffer (3% FBS in PBS supplemented with 0.1% sodium azide), treated with fixation and permeabilization buffer (ThermoFisher), and incubated with the PreS2, Pol, HBcAg and VSV-G antibodies listed above followed by rat monoclonal anti-mouse IgG1 conjugated to PE-Cy7 (BioLegend), goat anti-mouse IgG2a conjugated to AlexaFluor® 647 and goat anti-rabbit conjugated to AlexaFluor® 488 (ThermoFisher) antibodies. We acquired a minimum of 10,000 cells satisfying live cell forward and side scatter parameters using LSR II flow cytometer at the Cell Sorter Core Facility (Yale University School of Medicine) and analyzed the data with FlowJo software (version 7.6.5).

Immunoblotting. We seeded BHK-21 cells in 100-mm tissue culture plates one day prior to infection with VLVs at MOI=1 plaque forming unit (PFU)/cell. We prepared protein lysates by scraping the cells in cold lysis buffer containing 1% Igepal (Sigma) and Complete™ protease inhibitor cocktail and preclearing by centrifugation at 14,000 g for 10 min at 4°C. We separated

proteins by SDS-PAGE in 4%–15% precast gradient gels (Biorad), transferred them onto nitrocellulose membranes, blocked, and incubated with rabbit polyclonal anti-2A-peptide (EMD Millipore), anti-VSV-G or anti-actin (SantaCruz) antibodies and HRP-conjugated or near infrared fluorescently labeled secondary antibodies. We scanned and analyzed immunoblots using ChemiDoc (Bio-Rad) or Odyssey imaging system (LICOR).

In vivo studies (immunogenicity studies and models of HBV challenge and chronic infection). All studies with animals were designed and carried out according to the recommendations in the Guide for the Care and Use of Laboratory Animals of the U. S. National Institutes of Health, Eighth Edition. IACUCs of University of Connecticut Health Center, Yale University, Albany Medical Center and Southern Research approved the studies carried out at the corresponding institutions. For immunogenicity studies, we used 6-8 week-old C57BL/6J male mice (Jackson Laboratory) at Yale University and CaroGen laboratory at UCHC TIP facility. We immunized animals with 10^8 PFU/mouse in 1 ml of PBS via i.p. route and collected spleens from euthanized mice at day 7 post immunization. Control mice received equal volume of PBS at the time of immunization. We prepared single cell suspension of splenocytes by ACK lysis buffer, counted cells and stained with directly conjugated antibodies for T cell markers (CD4-Pacific Blue clone GK1.5, CD8-AlexaFluor® 488 clone 53-6.7, CD62L-PE clone MEL-14, CD44-AlexaFluor® 647 clone IM7) in the presence of FcBlock (BD Biosciences). We also stimulated 10^6 splenocytes per well with custom synthesized HBV peptides (Genscript) in the presence of 1X Brefeldin A (BioLegend) for 6 to 12 h prior to fixable viability staining eFluor-780 (ThermoFisher) and surface staining with CD4-Pacific Blue, CD8-AlexaFluor® 488 (BioLegend). After extensive washes and treatment with fixation and permeabilization buffer

(ThermoFisher), we stained cells with PE-conjugated anti-mouse IFNγ (clone XMG1.2) and AlexaFluor® 647-conjugated anti-mouse TNF (clone MP6-XT22). We acquired a minimum of 50,000 cells satisfying live cell forward and side scatter parameters using LSR II flow cytometer at the Cell Sorter Core Facility (Yale University School of Medicine) and analyzed the data with FlowJo software (version 7.6.5). The H-2^b peptides used for $CD8^+$ T cell stimulation were as follows: HBP-44 (NLNVSIPWTHKVGNF), HBP-396 (FAVPNLQSL), HBP-419 (VSAAFYHLPL), HBC-93 (MGLKFRQL), HBS-353 (VWLSVIWM), HBS-371 (ILSPFLPL). As a control peptide, we used H-2^d restricted peptide from HBV Pol (HYFQTRHYL). The threshold for cytokine positive $CD8^+$ T cells was manually set using similarly gated and stimulated $CD8^+$ T cells from spleens of non-immunized mice.

For acute challenge with HBV-AAV, we used 6-8 week-old CB6F1/J male mice (Jackson Laboratory) at Albany Medical College. We chose the CB6F1/J strain ($H2^{d/b}$ haplotype) over C57BL/6 strain ($H2^{b-}$ haplotype) for the HBV challenge because they generate a response to a dominant protective epitope of the surface antigen (HBS-191) restricted to $H2^d$ haplotype that facilitates HBV clearance. We immunized animals with 10^8 PFU/mouse via the i.p. route six weeks prior to delivery of 10^{11} genome copies of the AAV2/8 vector carrying 1.3 copies of the HBV genome (genotype D) via the i.v. route. Frequency of IFNγ-producing cells was measured by ELISPOT assay as previously described (Reynolds et al., 2017).

We established experimental chronic HBV replication by transduction of 6-8 week old C57BL/6J male mice in the facility of Southern Research (Birmingham, AL) following description of the model (Dion et al., 2013). After the HBV infection was confirmed by rising levels of serum HBsAg, we formed experimental groups with 10 animals per group based on normalization of

HBsAg levels at day 21 post infection such that each group had the same mean and standard-deviation of 70±29 ng/mL HBsAg. Therefore, each group had a very similar range of HBsAg levels just prior to administration of VLVs. We immunized mice with 3xT2A or empty vector VLV (10^8 PFU/mouse) via i.p. route at day 28 post infection. We continued monitoring HBsAg levels for additional 13 weeks. On days 65 and 84, we administered plasmid DNA adjuvanted with 20 μg/mouse monophosphoryl lipid A from *Salmonella minnesota* (Invivogen) via the i.m. route as follows: the group of mice immunized with 3XT2A received three separate plasmids expressing HBsAg, HBV Pol and HBcAg under the promoter region of the rhesus monkey EF1α gene in pVAC1 vector (Invivogen, 50 μg/mouse ea.); the group of mice immunized with empty vector VLV received empty pVAC1 vector (150 μg/mouse). We cloned HBsAg, HBcAg and Pol into the pVAC1 vector (Invivogen) and used EndoFree plasmid maxi kit (QIAGEN) to isolate DNA for the booster immunizations. To measure liver HBV RNA, we euthanized 5 mice per group to collect liver samples on day 70 and at the conclusion of the study on day 119. The numbers of IFNγ-producing $CD8^+$ T cells were determined similarly to the analyses of immunogenicity in the naïve mice, except that HBV Pol and HBsAg antigen peptides were pooled for each antigen separately and no peptide control was used for background determination.

Analytical assays for serum HBV antigens and liver HBV RNA. We collected blood samples from anesthetized mice weekly to measure serum HBsAg and HBeAg in the acute challenge experiment using ELISA kits from International Immunodiagnostics as previously described (Reynolds et al., 2015). We used HBsAg ELISA kit from XpressBio and the HBsAg standard from CellBioLabs to measure serum HBsAg in the chronic HBV infection model.

We collected liver samples from euthanized mice, snap-froze them in liquid nitrogen, and stored at -80^{o} C or on dry ice until processing for RNA isolation. We isolated total RNA using the RNeasy Mini kit (QIAGEN) and used High Capacity cDNA Reverse Transcription kit (Applied Biosystems) for cDNA synthesis. We used quantitative TaqMan^{TM} Fast Advanced Master Mix (Applied Biosystems) and TaqMan^{TM} Assay Mix containing previously described probe and specific primers for detection of HBV: probe, 6FAM- 5' CCT CTT CAT CCT GCT GCT ATG CCT CAT C 3'-MGBNFQ, forward primer 5'-GTG TCT GCG GCG TTT TAT CA-3', and reverse primer 5'-GAC AAA CGG GCA ACA TAC CTT-3', and mouse GAPDH as an endogenous loading control on a StepOnePlus real time PCR system (Applied Biosystems) using StepOne software v2.3 (Garson et al., 2005). We used the comparative ΔCT method to determine HBV RNA abundance relative to mouse GAPDH mRNA.

Statistical analyses. To determine the difference between the experimental groups, we used 1-way ANOVA for endpoint analyses (HBV RNA) or 2-way ANOVA for analyses over the time course (HBsAg) with Sidak's multiple comparison test. To perform all calculations we used GraphPad Prism software, version 7 (GraphPad Software, San Diego, CA).

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