Supplementary Material

Toll-Like Receptor 8 Agonist GS-9688 Induces Sustained Efficacy in the Woodchuck Model of Chronic Hepatitis B

Stephane Daffis, Scott Balsitis, Jason Chamberlain, Jim Zheng, Rex Santos, William Rowe, Dhivya Ramakrishnan, Divya Pattabiraman, Sandra Spurlock, Ruth Chu, Don Kang, Michael Mish, Ricardo Ramirez, Li Li, Bei Li, Sarina Ma, Magdeleine Hung, Christian Voitenleitner, Changsuek Yon, Manasa Suresh, Stephan Menne, Paul Cote, William E. Delaney IV, Richard Mackman and Simon P. Fletcher

Table of contents

Supplementary Methods

Investigational drugs

GS-9688 (selgantolimod), a small molecule agonist of TLR8, and GS-9620 (vesatolimod), a small molecule agonist of TLR7, were manufactured by Gilead Sciences, Inc. (Foster City, CA) (26). The small molecule tool agonists of TLR7 (GS-986) and TLR8 were also manufactured by Gilead Sciences, Inc. (Foster City, CA) (27).

PBMC analysis

Peripheral blood mononuclear cells (PBMCs) were isolated from human buffy coats or woodchuck heparinized whole blood using standard Ficoll density gradient separation. PBMCs were seeded at 2.0×10^6 cells per well in a flat-bottomed 12-well plate (RNA-Seq) or at 1.0×10^6 cells per well in a flat-bottomed 96-well plate (cytokine secretion) in RPMI 1640 medium containing 10% fetal bovine serum (FBS). PBMCs were stimulated with TLR agonists or DMSO at 37°C in a humidified 5% CO_2 incubator for 4-24 hours. Cell supernatants were then collected and stored in aliquots at -80°C. For woodchuck PBMCs, IL-12p40 levels were measured as described below. For human PBMCs, CXCL13 levels were measured by luminex assay (ThermoFisher Scientific, Waltham, MA) on a Luminex 200™ instrument accordingly to the manufacturer's instructions. For the RNA-Seq experiments, PBMCs were treated with DMSO, 2.4 nM TLR7 agonist (GS-986) or 625 nM tool TLR8 agonist. These compound concentrations were selected because (i) they represent the peak response in human PBMCs, and (ii) HEK293 reporter and PBMC cytokine assays indicated that the potency of TLR7 and TLR8 agonists was comparable in

human and woodchuck immune cells (data not shown). Of note, the tool TLR7 and TLR8 agonists have a comparable cytokine profile to GS-9620 and GS-9688, respectively, in human PBMCs (data not shown).

Woodchuck TLR8 cloning and sequencing

Woodchuck TLR8 was cloned using the same approach that was previously used to clone woodchuck TLR7 (10). Briefly, total RNA was isolated from woodchuck PBMCs using RNeasy extraction kit (Qiagen, Valencia, CA) and cDNA was synthesized from total RNA using SuperScript III First Strand Synthesis System (Life Technologies, Carlsbad, CA) according to manufacturer's instructions. Woodchuck TLR8 sequences were identified by a PCR-based strategy using primers specific for highly conserved regions of human TLR8 (accession number AY0358296.1) and mouse TLR8 (accession number AY035890.1). The PCR products were sequenced and used to design woodchuck-specific primers. The 5'- and 3'-ends of woodchuck TLR8 were identified using 5' and 3' rapid amplification of cDNA ends (RACE) Systems kits, respectively, according to the manufacturer's instructions (Life Technologies, Carlsbad, CA). The full coding sequence of woodchuck TLR8 was then synthetized and cloned into the expression vector pUNO3 (InvivoGen, San Diego, CA) to generate the expression construct pUNO3-wTLR8 (GenScript, Piscataway, NJ). Of note, the woodchuck TLR8 nucleotide sequence was subsequently confirmed by comparison with the full-length coding sequence obtained from the woodchuck transcriptome assembly (10). The complete wTLR8 sequence has been deposited into NCBI (accession number MN593722).

Sequence alignments

Alignment of the woodchuck TLR8 sequence with the TLR8 sequences from human (*homo sapiens*, accession number AAZ95441.1), cynomolgus monkey (*macaca fascicularis*, accession number BAG55068.1), cow (*bos taurus*, accession number ABQ52584.1), pig (*sus scrofa*, accession number NP_999352.1), horse (*equus caballus*, accession number ABM87942.1), cat (*felis catus*, accession number ABS28967.1), mouse (*mus musculus*, accession number AAK62677.1) and rat (*rattus norvegicus*, accession number ABM92444.1) was performed using the Clustal Omega software (http://www.ebi.ac.uk /Tools/msa/clustalo/).

HEK293 TLR assays

Human embryonic kidney 293 (HEK293) cells (#CRL-1573; ATCC®, Manassas, VA) were seeded at 20,000 cells per well in flat-bottomed 96 well plates in DMEM-GlutaMAX-I supplemented with 10% FBS. Eight hours post-seeding, the cells were transfected with 10 ng of a reporter vector pNiFty2-luc, consisting of the firefly luciferase gene under the control of the transcription factor NF-κB (InvivoGen, San Diego, CA), together with 10 ng of a vector expressing woodchuck TLR8 (pUNO3-wTLR8), woodchuck TLR7 (pUNO3-wTLR7), human TLR8 (pUNO3-hTLR08; InvivoGen, San Diego, CA) or human TLR7 (pUNO3-hTLR07; InvivoGen, San Diego, CA) using Lipofectamine 3000 (Life Technologies, Carlsbad, CA) according to the manufacturer's instructions. Sixteen hours post-transfection, the cells were stimulated with DMSO (control) or various concentrations of GS-9688 or vesatolimod for 16 hours. The cells were then lysed and luciferase activity (relative light units, RLU) was quantified using ONE-Glo™ Luciferase assay system

(Promega, Madison, WI) with a Victor3 Luminescent Device (PerkinElmer, Waltham, MA). Reporter gene induction was expressed as RLU fold-change relative to the DMSO control. The minimal effective concentration (MEC) was defined as the minimal concentration of compound that induced a 2-fold increase relative to control. MEC was calculated using GraphPad Prism version 7.03 (GraphPad Software, San Diego, CA).

Woodchuck studies

All animals used in these studies were eastern North American woodchucks (*Marmota monax*) that were born and reared in the animal facility of Northeastern Wildlife, Inc. (Harrison, ID). Animals were housed in floor pens containing cedar or aspen wood shavings, maintained under daily cycles of 12 hours of light and 12 hours of dark, and fed with Purina rabbit chow (16% crude protein [≥18% for pups and pregnant animals], 2% crude fat, ~15% crude fiber, vitamin A (4500 IU), 1% calcium and 0.5% phosphate). Animal were not fasted prior to treatment. GS-9688 (formulated in 2% ethanol, 40% polyethylene glycol 300, 58% water, pH=2) and vesatolimod (formulated in 0.001 N hydrochloric acid with 0.005% (w/v) propyl gallate) were diluted 1:1 in a liquid woodchuck diet (79002 Liquid Woodchuck Control Diet, Dyets, Inc., Bethlehem, PA) and administered in a dose volume of 1 mL/kg. Vehicle-treated woodchucks were administered base formulation diluted 1:1 in liquid diet.

Single dose GS-9688 and vesatolimod studies in uninfected woodchucks

Uninfected adult woodchucks were assigned to treatment groups based on gender and body weight. For each study, woodchucks (n=3-6 per dose group) were orally administered a

single dose of vehicle, GS-9688 or vesatolimod. For all studies, evaluations included clinical observations, clinical pathology (hematology, coagulation and clinical chemistry), pharmacokinetics (PK) and pharmacodynamics (PD). Normal lymphocyte and platelet count ranges were calculated from hematology data for n=42 healthy woodchucks and are expressed as the 95% confidence interval of the geometric mean.

Repeat dose GS-9688 and vesatolimod efficacy studies in WHV-infected woodchucks

Woodchucks were infected as neonates (3-5 days of age) by subcutaneous inoculation of 10^7 ge/mL woodchuck hepatitis virus (WHV, strain WH7P2A). The age of the woodchucks ranged from 12 to 18 months at the start of the study. Chronically infected animals had detectable serum WHV DNA, serum WHsAg and serum antibody to WHV core antigen (anti-WHc) and were negative for serum antibody to WHsAg (WHsAb). Woodchucks were assigned and stratified by gender and body weight, as well as by pretreatment (baseline) serum WHV DNA and WHsAg levels into treatment and vehicle groups. The woodchucks were also screened for hepatocellular carcinoma (HCC) by measuring serum levels of gamma-glutamyl transferase (GGT), an established biomarker of HCC in woodchucks (28), as well as by visual inspection of the liver during the pre-study biopsy performed prior to study initiation. Woodchucks with elevated serum GGT levels (>3 U/L) and/or observable liver tumors were either excluded from the study or were included in the vehicle group. The study designs are summarized in Supplementary Fig. 3, 7 and 14.

At the end of each study, woodchucks were euthanized and necropsied. Euthanasia was performed under deep general anesthesia via intracardiac administration of a solution

containing 390 mg pentobarbital sodium and 50 mg/mL phenytoin sodium. Woodchucks that developed HCC during the study were euthanized at the time of diagnosis (determined by in-life parameters and/or GGT levels) and confirmed by gross observation at necropsy. Woodchucks that were euthanized or died unexpectedly during the study were submitted to a complete necropsy performed in the laboratory facility of Northeastern Wildlife, Inc. Woodchucks were monitored at various time points before, during and after treatment for multiple parameters including safety, tolerability, PK, PD, and antiviral efficacy. Tolerability was assessed on-site via in-life parameters including daily visual observation of the woodchucks, as well as body weight and temperature. Clinical pathology was assessed by analyzing serum chemistry, hematology and coagulation parameters at Cornell University, Animal Health Diagnostic Center (Ithaca, NY). PK samples were analyzed at Gilead Sciences, Inc. (Foster City, CA) and Covance laboratories (Madison, WI). PD endpoints were analyzed at Gilead Sciences, Inc. and Covance laboratories (Redmond, WA). Serum and hepatic viral endpoints as well as the PBMC proliferative response to WHV peptides were analyzed at Georgetown University (Washington, DC).

Pharmacokinetics of GS-9688

Plasma samples were stored at -20°C until shipped to Gilead Sciences. Fifty µL of plasma was treated with 100 µL of acetonitrile containing an internal standard (an analog of GS-9688 at 100 nM), mixed and then centrifuged at 1000 x *g* for 20 minutes. Fifty µL of supernatant was then transferred to a 96-well plate and diluted with 150 µL of water. An aliquot of 20 µL of this solution was injected into the TSQ Quantum LC-MS/MS system (Thermo Finnigan, San Jose, CA). Non-compartmental pharmacokinetic analysis was

performed to estimate pharmacokinetic parameters using Phoenix (version 1.3). The lower limit of quantification (LLOQ) of the assay is 0.1 nM.

WHV parameters

Serum WHV DNA was quantified by two different methods depending on concentration: dot blot hybridization or real-time PCR assay, as described previously (29). The lower limit of detection (LLOD) for the PCR assay is approximately 3 x 10^2 WHV ge/mL. Serum WHsAg and anti-WHsAg antibodies (WHsAb) were measured by WHV-specific enzyme immunoassays (30). Flash-frozen wedge liver biopsies obtained by open surgery were used to measure intrahepatic WHV nucleic acid levels. Liver WHV RNA levels were measured quantitatively by Northern blot hybridization (31) and liver WHV DNA replicative intermediates (RI) and WHV cccDNA were quantitatively determined by Southern blot (32). Briefly, for WHV DNA RI quantitation, samples were extracted, digested with the restriction enzyme *Hind*III and assessed quantitatively against a standard curve of a linearized, full-length WHV DNA genome using agarose gel electrophoresis followed by Southern blot hybridization on nitrocellulose membranes with a ^{32}P-labeled fragment of purified WHV DNA. Data were expressed as pg WHV DNA RI/μg cellular nucleic acid. For WHV cccDNA quantitation, analysis was performed by Southern Blot by measuring the single band corresponding to the 3.2 kb genome-size DNA species. Data were expressed as pg WHV cccDNA/μg cellular nucleic acid. For WHV RNA quantitation, samples were extracted and assessed quantitatively against a standard curve of a linearized, full-length WHV DNA genome using formaldehyde agarose gel electrophoresis followed by Northern blot hybridization on nitrocellulose membranes with a ^{32}P-labeled fragment

of purified WHV DNA. Data were expressed as pg WHV RNA/µg cellular nucleic acid. Data were normalized by quantifying the levels of the housekeeping gene β-actin using stripped and re-hybridized membranes with a ^{32}P-probe specific for woodchuck β-actin. The LLOD is 20-200 ng/mL for serum WHsAg (20 ng/mL for the study summarized in Supplementary Fig. 3; 200 ng/mL for the study summarized in Supplementary Fig. 7), 100 U/mL for WHsAb and approximately 2 pg/µg cellular nucleic acid for WHV RNA, WHV DNA RI and WHV cccDNA. When WHV parameters were below the LLOD, the LLOD value was plotted. Antiviral response to GS-9688 treatment was defined as $\geq$1 $\log_{10}$ reduction in quantitative serum WHsAg at one or more time point during the dosing period.

Quantitative RT-PCR

Whole blood (2.5 mL) was collected in PAXgene® tubes, maintained at room temperature for at least 2 hours and then stored at -80°C until processing. Total RNA was extracted by Covance Laboratories (Redmond, WA) using the PAXgene 96 Blood RNA extraction kit (PreAnalytiX, Switzerland) according to the manufacturer's instructions. Quantitative RT-PCR (qRT-PCR) was performed with the TaqMan Fast Virus 1-Step Master Mix (Applied Biosytems, Carlsbad, CA) using custom primers and probes (Life Technologies, Carlsbad, CA) for woodchuck MARCO (forward primer 5'-GGA CAG AAA GGG ACA AAA GGA GAA T-3', reverse primer 5'-CGA CCC GGT TCT TCC CAT TT-3' and probe 5'-ATG CCT GGG ATT CCT G-3'), woodchuck ISG15 (assay order ID #AI89L6B) and woodchuck β-actin (forward primer 5'-ACA GCC GAG CGG GAA A -3', reverse primer 5'-GCA ACG TAG CAC AGC TTC TC -3' and probe 5'-TCG TGC GTG ACA TCA A-3'). Woodchuck β-actin mRNA expression was used to normalize target gene expression.

RNA-Seq analysis

Isolation of total cellular RNA and RNA-Seq was conducted by Expression Analysis (Durham, NC) as described previously (10). Sequence count normalization, differential gene expression analysis, false discovery rate (FDR) calculation, gene set enrichment analysis (GSEA) and Ingenuity® Pathway Analysis (IPA; Qiagen, Redwood City, CA) were performed as described previously (10, 24). All differentially expressed genes (DEGs) had an absolute fold change >2 and FDR <0.05. Cell type enrichment analysis was performed using the xCell method for 39 immune cell types (33). The xCell z-score is a normalized score which is expressed relative to the mean of overall enrichment of each cell type. For the data presented in Fig. 7B-D, fold-change was calculated by dividing the expression level in each animal (transcripts per million) by the geometric mean of the expression level for the uninfected animals.

Woodchuck PBMC proliferation assay

PBMCs were assessed for their proliferation capacity in response to treatment using an ex vivo proliferation assay, as previously described (34). Briefly, woodchuck PBMCs were isolated from whole blood samples using standard Ficoll gradient centrifugation. PBMCs were then cultured in 96-well opaque plates (Greiner; MilliporeSigma, St. Louis, MO) in the presence of 10 µg/mL WHV peptides (dissolved in 0.2% DMSO) which covered the entire WHV core, WHsAg or WHx protein. DMSO alone was used as negative control. After 5 days in culture, PBMC proliferation was determined using a standard viability assay (CellTiter-Glo) according to manufacturer's instructions (Promega, Madison, WI). Luminescence was subsequently measured using a Centro LB 960 Luminometer (Berthold Technologies, Oak Ridge, TN). The luminescence signal of triplicate cultures was

averaged and expressed as a proliferation index, defined as luminescence (WHV peptide)/ luminescence (DMSO). A proliferation index of ≥ 2.0 was considered a positive response.

Expression, purification and analytical characterization of woodchuck cytokines

Complete coding sequences for the woodchuck IFN-γ, IL-12A (IL-12p35) and IL-12B (IL-12p40) genes were obtained from the woodchuck transcriptome assembly (10). All genes were codon optimized for expression. The woodchuck IFN-$\gamma_{1\text{-}166}$ gene (full-length) was cloned into pcDNA3.1+ using In-Fusion® HD cloning kit (TaKaRa Bio, Mountain View, CA) to generate a wIFN-γ - murine IgG2a Fc expression construct (wIFN-γ-mG2aFc). A woodchuck IL-12B$_{1\text{-}327}$ (full-length) and IL-12A$_{24\text{-}223}$ (mature sequence without leader sequence) gene fusion separated by a G4S linker was cloned into the pSECTAG-A vector to generate a wIL-12B-$(G4S)_3$-wIL-12A expression construct. The woodchuck IFN-α5A$_{1\text{-}190}$ gene (full length; accession number AF338274.1) followed by an 8HIS-FLAG epitope tag was cloned into pcDNA3.1+ to generate a wIFN-α5-HIS-FLAG expression construct.

The expression vectors were transfected into Expi293™ cells using ExpiFectamine™ according to the manufacturer's protocol (Life Technologies Carlsbad, CA). Clarified supernatant was harvested 4 -5 days post-transfection for protein purification. Culture media containing wIFN-γ-mG2aFc was supplemented with 2 mM EDTA and cOmplete™ protease inhibitor tablets immediately after harvest and loaded onto a MabSelect SuRe (MSS) column pre-equilibrated with 20 mM Tris pH 7.4, 2 mM EDTA, 150 mM NaCl and 1 mM NaN_3 pH 7.5 (buffer A). The MSS column was washed with buffer A for 10 column volumes (CV) before elution with 100 mM sodium acetate pH 3.7 for 5 CV. The eluted

pool was dialyzed overnight into 1 x PBS at 4°C. The dialyzed sample was supplemented with 2 mM $CaCl_2$, then Factor Xa was added at a 1:50 (w:w) ratio and the sample incubated for 6 hours at 25°C. Clarified supernatant was then loaded over a 1 mL MSS coupled in-line with a 1 mL HiTrap Benzamidine FF column (GE Healthcare, Bio-Sciences Corp, Piscataway, NJ). Flow-through and wash fractions were collected and analyzed for purity. The final pooled fractions were dialyzed overnight into 1 x PBS pH 7.4 at 4°C and subsequently stored at 4°C. Culture media containing wIL-12B-$(G4S)_3$-wIL12A was loaded onto a HiPrep Heparin column (GE Healthcare, Bio-Sciences Corp, Piscataway, NJ) pre-equilibrated with 20 mM Tris-HCl pH 7.5 (buffer B). The HiPrep Heparin column was washed with buffer B for 5 CV before elution with buffer B with 1 M NaCl using a linear gradient from 0-500 mM NaCl for 20 CV. The final pooled fractions were dialyzed overnight into 1 x PBS pH 7.4 at 4°C, and subsequently stored at 4°C. Culture media containing wIFN-α5-HIS-FLAG was loaded onto a 5 mL HisTrap excel column (GE Healthcare, Bio-Sciences Corp, Piscataway, NJ) pre-equilibrated with 1 x PBS pH 7.4 (buffer C). The HisTrap column was washed with 10 CV of buffer C before elution with 500 mM imidazole containing buffer C. The final pooled fractions were dialyzed into buffer C overnight at 4°C and subsequently stored at 4°C. All protein samples were tested to ensure >95% monomer, <0.5 EU/mL endotoxin level and >95% purity. The protein identity was also confirmed by mass spectrometry.

Woodchuck IL-12p40 antibody production

Mouse monoclonal anti-woodchuck IL-12p40 antibodies were generated by Antibody Solutions (Sunnyvale, CA). Briefly, the capture and detection antibodies were generated

by immunizing CD-1 mice biweekly with wIL-12B-$(G4S)_3$-wIL-12A in a non-denaturing adjuvant for four weeks. Mouse sera and subsequent hybridomas were evaluated for binding to wIL-12p40 by ELISA. Monoclonal antibodies (mAb) were developed from two of the hybridoma clones (M38 and M41). Of note, the M38-M41 antibody pair detects woodchuck IL-12p70 as well as IL-12p40 (IL-12p70 is a heterodimer of p35 and p40). However, the detection sensitivity of these mAbs for IL-12p70 is approximately 2-fold lower than for IL-12p40. Using an antibody pair (M38-M15) which is highly specific for woodchuck IL-12p70, we determined that GS-9688 treatment of WHV-infected woodchucks induces only low levels (typically < 4 pg/mL) of serum IL-12p70 (data not shown). Therefore, the signal detected by the M38-M41 antibody pair in the serum of GS-9688-treated woodchucks is likely to be primarily due to IL-12p40.

Woodchuck IL-12p40 assay

Woodchuck IL-12p40 levels in culture media or serum samples were measured by electrochemiluminescence assay (Meso Scale Diagnostics [MSD], Rockville, MD) using the M38-M41 antibody pair. The IL-12p40 concentration in each sample was calculated by interpolation from a standard curve with purified recombinant woodchuck IL-12p40. The assay was performed according to the manufacturer's instructions (MSD, Rockville, MD). Briefly, 96 well plates were coated with 0.5 μg/mL of the capture antibody (M38) per well overnight in PBS at 4°C. The plates were then washed with Wash Buffer (1 x PBS with 0.05% Tween), incubated for 1 hour with Blocking Buffer (1 x TBS with 1% casein) and washed again. Woodchuck serum or culture media samples were then transferred into each well. Following a 2 hour incubation, the plates were washed three times with Wash

Buffer, and then incubated with 0.2 μg/mL of the biotinylated M41 detection antibody (in Blocking Buffer diluted 1:3 in Wash Buffer) together with 0.1% IgG blocker D-M (MSD, Rockville, MD) for 1 hour. Plates were then washed three times with Wash Buffer and incubated with 1 μg/mL of secondary sulfo-tag streptavidin antibody (MSD, Rockville, MD) for 1 hour. The plates were washed three times with Wash Buffer and 1 x MSD Read Buffer T (MSD, Rockville, MD) was then added. The plates were subsequently analyzed on a Quickplex SQ120 instrument (MSD, Rockville, MD). The LLOD of the woodchuck IL-12p40 assay is 4 pg/mL.

Woodchuck interferon (IFN) bioassay

Due to the challenges of developing a woodchuck IFN-α detection assay (35), woodchuck IFN levels in serum were measured using a bioassay. Briefly, the woodchuck hepatoma cell line-17 cell line WCH-17 (CRL-2082; ATCC) was transfected with 100 ng plasmid encoding a luciferase reporter gene under the control of the human ISG56 minimal promoter (InvivoGen, San Diego, CA) using Lipofectamine 2000 (Life Technologies, Carlsbad, CA). One day post-transfection, the cells were incubated with woodchuck serum at 37°C for 16 hours. The cells were then lysed and luciferase activity (RLU) was quantified using ONE-Glo™ Luciferase assay system (Promega, Madison, WI) with a Victor3 Luminescent Device (PerkinElmer, Waltham, MA). The IFN concentration in each sample was calculated by interpolation from a standard curve with purified recombinant woodchuck IFN-α5. The LLOD of the assay is 100 pg/mL. When IFN levels were below the LLOD, the LLOD value was plotted. The upper limit of detection (ULOD) of the assay is 5000 pg/mL. When IFN levels were greater than the ULOD, the ULOD value was

plotted. Of note, the assay detects woodchuck IFN-α and IFN-γ with similar sensitivity. However, since vesatolimod is a potent inducer of IFN-α - but not IFN-β or IFN-γ - the signal detected by the IFN bioassay in the serum of vesatolimod-treated woodchucks is likely to be predominantly due to IFN-α.

Statistical analysis

Data is expressed as mean ± standard error of the mean (SEM) unless otherwise stated. For differential gene expression, statistical significance was tested using the Limma R package. For xCell analyses, statistical significance was tested using a Wilcoxon rank-sum test with or without multiple testing correction by the Benjamini and Hochberg method. Adjusted *p*-values (i.e. FDR) and unadjusted *p*-values are indicated in the text. For IPA, pathway enrichment of DEGs was calculated with the Fisher's exact test with multiple testing correction by the Benjamini and Hochberg method. For all other analyses, statistical significance was tested using a two-tailed *t*-test (for two sample comparisons) or either one-way or two-way ANOVA with multiple testing correction (for multiple comparisons). Correlation analysis was performed using a two-tailed Pearson correlation. For all analyses, a value of $p<0.05$ was considered significant.

Supplementary Results

GS-9688 is a selective agonist of woodchuck TLR8

The woodchuck model of CHB has been used to evaluate the antiviral efficacy of various immunomodulatory agents. However, the translational relevance of this model for evaluating TLR8 agonists has not been established. This is important because various other members of the order *Rodentia* (e.g. mouse and rat) have a five amino-acid deletion in TLR8 which prevents activation by ssRNA and small molecule agonists (36). As a first step, we cloned and sequenced woodchuck TLR8. Alignment of the woodchuck TLR8 amino acid sequence with human, cynomolgus monkey, cow, pig, horse, cat, mouse and rat orthologs revealed that woodchuck TLR8 retains the five amino-acid residues that are essential for activity of human TLR8 and which are absent in mouse and rat TLR8 (Supplementary Fig. 1A). Thus, sequence analysis suggests that woodchuck TLR8 is functional. Using a HEK293 reporter system transiently transfected with woodchuck TLR7 or TLR8, we confirmed that GS-9688 activated woodchuck TLR8 but not woodchuck TLR7 (Supplementary Fig. 1B). In line with previous studies, we determined that mouse TLR8 is non-responsive to GS-9688 (data not shown).

Stimulation of human PBMCs with TLR8 agonists induces a variety of cytokines, including IL-12p40, IL-12p70 (a heterodimer of p35 and p40), IL-18, TNF-α and IFN-γ (3, 6). Commercially available immunoassays typically fail to detect woodchuck cytokines, and so we developed a quantitative woodchuck IL-12p40 assay to evaluate the woodchuck PBMC response to GS-9688. Using this new assay, we demonstrated that GS-9688 induced

a dose-dependent increase in IL-12p40 in woodchuck PBMCs (Supplementary Fig. 1C). To better characterize the response of woodchuck immune cells to relevant TLR agonists, we next performed RNA-Seq analysis of woodchuck PBMCs stimulated with a tool TLR7 agonist or a tool TLR8 agonist (Supplementary Fig. 1D). This revealed that TLR8 activation significantly induced the expression of various cytokines, including *IL12B* (which encodes IL-12p40) and *IFNG*, as well as the expression of interferon-stimulated genes (ISG). TLR7 activation also significantly induced ISG mRNA expression (presumably via induction of IFN-α rather than IFN-γ), but lower levels of proinflammatory cytokine gene expression. Importantly, the transcriptional response of woodchuck PBMCs to these tool TLR7 and TLR8 agonists was similar to that of human PBMCs (Supplementary Fig. 2). Collectively, these data demonstrate that woodchuck TLR8 is functional and is selectively activated by GS-9688, and that the response of woodchuck and human PBMCs to TLR8 activation is comparable.

Evaluating the translational value of the woodchuck model of CHB with the TLR7 agonist vesatolimod

The translational value of the woodchuck model of CHB has recently been questioned because weekly oral dosing with the TLR7 agonist vesatolimod induced a comparable blood ISG mRNA response in CHB patients (4 mg dose, 12 weeks treatment) and WHV-infected woodchucks (5 mg/kg dose, 8 weeks treatment), but only had antiviral efficacy in the latter (10)(37, 38). We performed studies to address this discrepancy in preclinical vs. clinical response to better understand the relevance of the woodchuck model for predicting GS-9688 antiviral efficacy in CHB patients.

We first characterized the PD response of uninfected woodchucks to single ascending doses of vesatolimod using the new woodchuck IFN and IL-12p40 assays. In addition, whole blood *ISG15* mRNA levels were measured to enable effective comparison with the PD response observed in previous studies. Doses ≥0.3 mg/kg vesatolimod induced whole blood *ISG15* expression, and ≥2.5 mg/kg vesatolimod induced very high levels of serum IFN in uninfected woodchucks (Supplementary Fig. 12). Surprisingly, ≥1 mg/kg vesatolimod also induced substantial levels of serum IL-12p40 (Supplementary Fig. 12). It was previously determined that vesatolimod can activate human TLR8 at high concentrations (26), and therefore we next compared the selectivity of vesatolimod for woodchuck and human TLRs using the aforementioned HEK293 reporter system. Using this assay, vesatolimod had 15-fold selectivity for human TLR7 over TLR8 but only 4-fold selectivity for woodchuck TLR7 over TLR8 (Supplementary Fig. 13). Finally, we performed an efficacy study in woodchucks chronically infected with WHV to evaluate the antiviral response to a range of vesatolimod doses (Supplementary Fig. 14). In this study, WHV-infected woodchucks were dosed orally with vehicle or 0.3, 1, 2.5 or 5 mg/kg vesatolimod once per week for 4-14 weeks and then followed for an additional 4-11 weeks. Administration of 0.3 mg/kg vesatolimod for 6 weeks had no antiviral activity in WHV-infected woodchucks (n=7 total), and 14 weeks of 1 mg/kg induced an antiviral response in only 14% animals (n=7 total) (Supplementary Fig. 15). Treatment with 2.5 mg/kg vesatolimod for 8 weeks induced an antiviral response in 28% of animals (n=7 total), and short duration treatment (4 weeks) with 5 mg/kg induced an antiviral response in 33% of animals (n=3 total) (Supplementary Fig. 15).

Collectively these studies demonstrated that vesatolimod has no antiviral activity in woodchucks at a dose (0.3 mg/kg) that induced comparable pharmacodynamic responses in uninfected animals to those observed at a safe and tolerated dose (4 mg) in healthy human volunteers and CHB patients (37-39) (Supplementary Table 7). Higher doses of vesatolimod induced an antiviral response in a subset of woodchucks, but substantially increased serum IFN and IL-12p40 levels in uninfected animals (Supplementary Fig. 12 and 15). Taken together, these data suggest that the antiviral response to vesatolimod in the woodchuck model may be due to stronger activation of TLR7 than was achieved in CHB patients, and potentially also activation of TLR8.

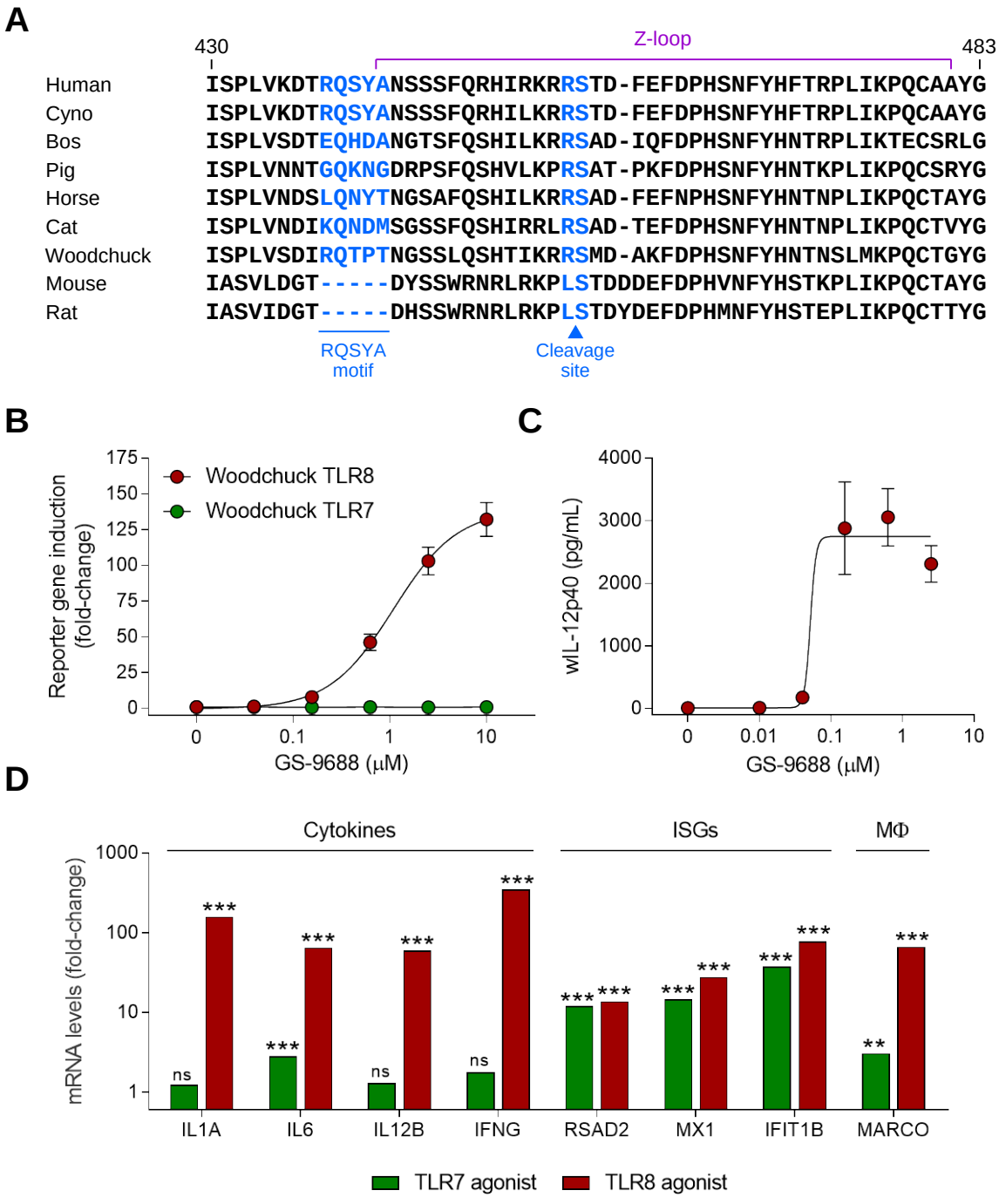
Supplementary References

26. Roethle PA, McFadden RM, Yang H, Hrvatin P, Hui H, Graupe M, Gallagher B, et al. Identification and optimization of pteridinone Toll-like receptor 7 (TLR7) agonists for the oral treatment of viral hepatitis. J Med Chem 2013;56:7324-7333.
27. Borducchi EN, Cabral C, Stephenson KE, Liu J, Abbink P, Ng'ang'a D, Nkolola JP, et al. Ad26/MVA therapeutic vaccination with TLR7 stimulation in SIV-infected rhesus monkeys. Nature 2016;540:284-287.
28. Abe K, Kurata T, Shikata T. Localization of woodchuck hepatitis virus in the liver. Hepatology 1988;8:88-92.
29. Menne S, Butler SD, George AL, Tochkov IA, Zhu Y, Xiong S, Gerin JL, et al. Antiviral effects of lamivudine, emtricitabine, adefovir dipivoxil, and tenofovir disoproxil fumarate administered orally alone and in combination to woodchucks with chronic woodchuck hepatitis virus infection. Antimicrob Agents Chemother 2008;52:3617-3632.
30. Cote PJ, Roneker C, Cass K, Schodel F, Peterson D, Tennant B, De Noronha F, et al. New enzyme immunoassays for the serologic detection of woodchuck hepatitis virus infection. Viral Immunol 1993;6:161-169.
31. Peek SF, Cote PJ, Jacob JR, Toshkov IA, Hornbuckle WE, Baldwin BH, Wells FV, et al. Antiviral activity of clevudine [L-FMAU, (1-(2-fluoro-5-methyl-beta, L-arabinofuranosyl) uracil)] against woodchuck hepatitis virus replication and gene expression in chronically infected woodchucks (Marmota monax). Hepatology 2001;33:254-266.
32. Jacob JR, Korba BE, Cote PJ, Toshkov I, Delaney WEt, Gerin JL, Tennant BC. Suppression of lamivudine-resistant B-domain mutants by adefovir dipivoxil in the woodchuck hepatitis virus model. Antiviral Res 2004;63:115-121.
33. Aran D, Hu Z, Butte AJ. xCell: digitally portraying the tissue cellular heterogeneity landscape. Genome Biol 2017;18:220.
34. Menne S, Cote PJ. Measurement of cell-mediated immune response in woodchucks. Methods Mol Med 2004;96:27-36.
35. Fletcher SP, Chin DJ, Gruenbaum L, Bitter H, Rasmussen E, Ravindran P, Swinney DC, et al. Intrahepatic Transcriptional Signature Associated with Response to Interferon-alpha Treatment in the Woodchuck Model of Chronic Hepatitis B. PLoS Pathog 2015;11:e1005103.
36. **Liu J**, **Xu C**, Hsu LC, Luo Y, Xiang R, Chuang TH. A five-amino-acid motif in the undefined region of the TLR8 ectodomain is required for species-specific ligand recognition. Mol Immunol 2010;47:1083-1090.
37. Agarwal K, Ahn SH, Elkhashab M, Lau AH, Gaggar A, Bulusu A, Tian X, et al. Safety and efficacy of vesatolimod (GS-9620) in patients with chronic hepatitis B who are not currently on antiviral treatment. J Viral Hepat 2018;25:1331-1340.
38. Janssen HLA, Brunetto MR, Kim YJ, Ferrari C, Massetto B, Nguyen AH, Joshi A, et al. Safety, efficacy and pharmacodynamics of vesatolimod (GS-9620) in virally suppressed patients with chronic hepatitis B. J Hepatol 2018;68:431-440.
39. Lopatin U, Wolfgang G, Tumas D, Frey CR, Ohmstede C, Hesselgesser J, Kearney B, et al. Safety, pharmacokinetics and pharmacodynamics of GS-9620, an oral Toll-like receptor 7 agonist. Antivir Ther 2013;18:409-418.

40. Schurich A, Pallett LJ, Lubowiecki M, Singh HD, Gill US, Kennedy PT, Nastouli E, et al. The third signal cytokine IL-12 rescues the anti-viral function of exhausted HBV-specific CD8 T cells. PLoS Pathog 2013;9:e1003208.
41. Pasare C, Medzhitov R. Toll pathway-dependent blockade of CD4+CD25+ T cell-mediated suppression by dendritic cells. Science 2003;299:1033-1036.
42. Stoop JN, Woltman AM, Biesta PJ, Kusters JG, Kuipers EJ, Janssen HL, van der Molen RG. Tumor necrosis factor alpha inhibits the suppressive effect of regulatory T cells on the hepatitis B virus-specific immune response. Hepatology 2007;46:699-705.
43. Elsaesser H, Sauer K, Brooks DG. IL-21 is required to control chronic viral infection. Science 2009;324:1569-1572.
44. Frohlich A, Kisielow J, Schmitz I, Freigang S, Shamshiev AT, Weber J, Marsland BJ, et al. IL-21R on T cells is critical for sustained functionality and control of chronic viral infection. Science 2009;324:1576-1580.
45. Yi JS, Du M, Zajac AJ. A vital role for interleukin-21 in the control of a chronic viral infection. Science 2009;324:1572-1576.
46. Sutherland AP, Joller N, Michaud M, Liu SM, Kuchroo VK, Grusby MJ. IL-21 promotes CD8+ CTL activity via the transcription factor T-bet. J Immunol 2013;190:3977-3984.
47. Tang L, Chen C, Gao X, Zhang W, Yan X, Zhou Y, Guo L, et al. Interleukin 21 Reinvigorates the Antiviral Activity of Hepatitis B Virus (HBV)-Specific CD8+ T Cells in Chronic HBV Infection. J Infect Dis 2019;219:750-759.
48. Fridlender ZG, Sun J, Mishalian I, Singhal S, Cheng G, Kapoor V, Horng W, et al. Transcriptomic analysis comparing tumor-associated neutrophils with granulocytic myeloid-derived suppressor cells and normal neutrophils. PLoS One 2012;7:e31524.
49. Condamine T, Dominguez GA, Youn JI, Kossenkov AV, Mony S, Alicea-Torres K, Tcyganov E, et al. Lectin-type oxidized LDL receptor-1 distinguishes population of human polymorphonuclear myeloid-derived suppressor cells in cancer patients. Sci Immunol 2016;1.

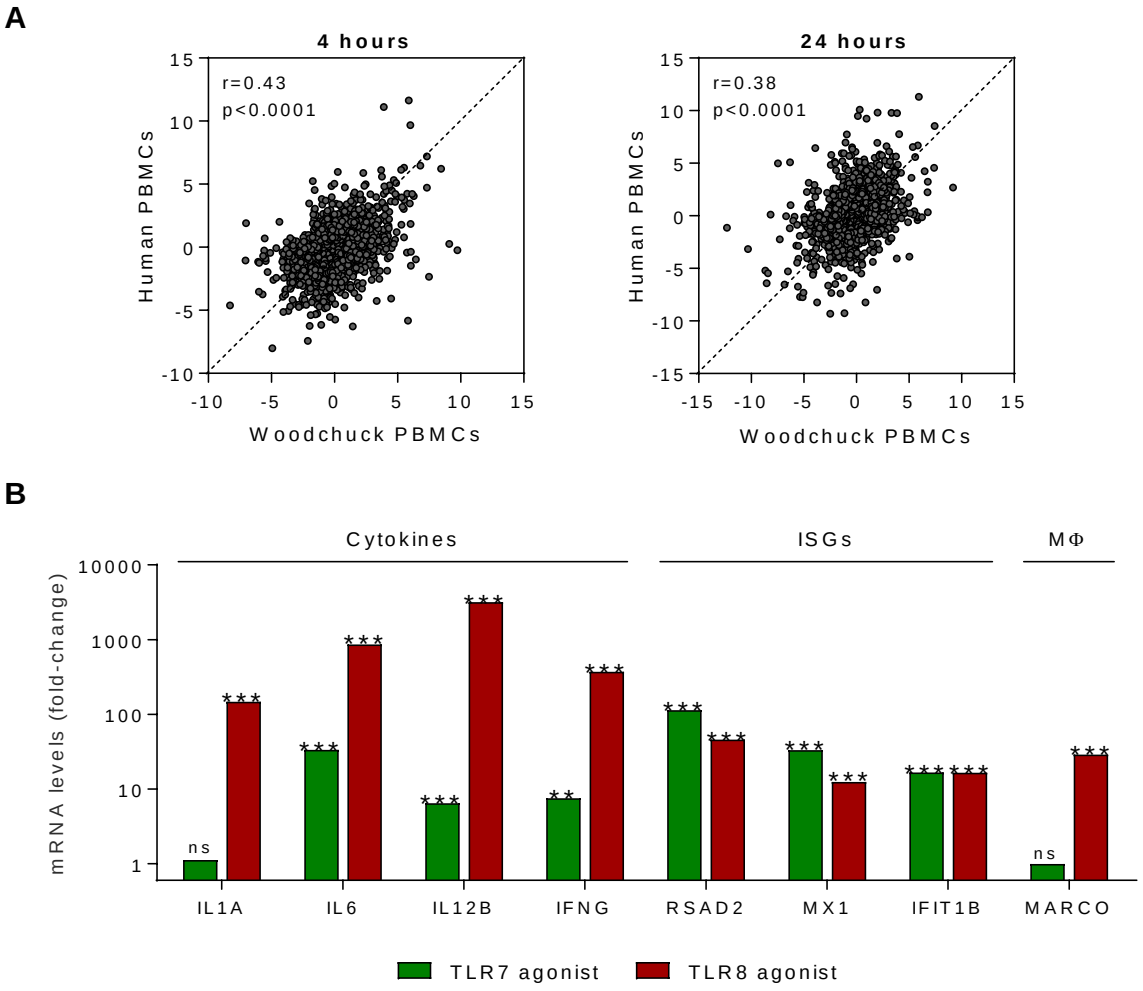
Author names in bold designate shared co-first authorship.

Supplementary Figures

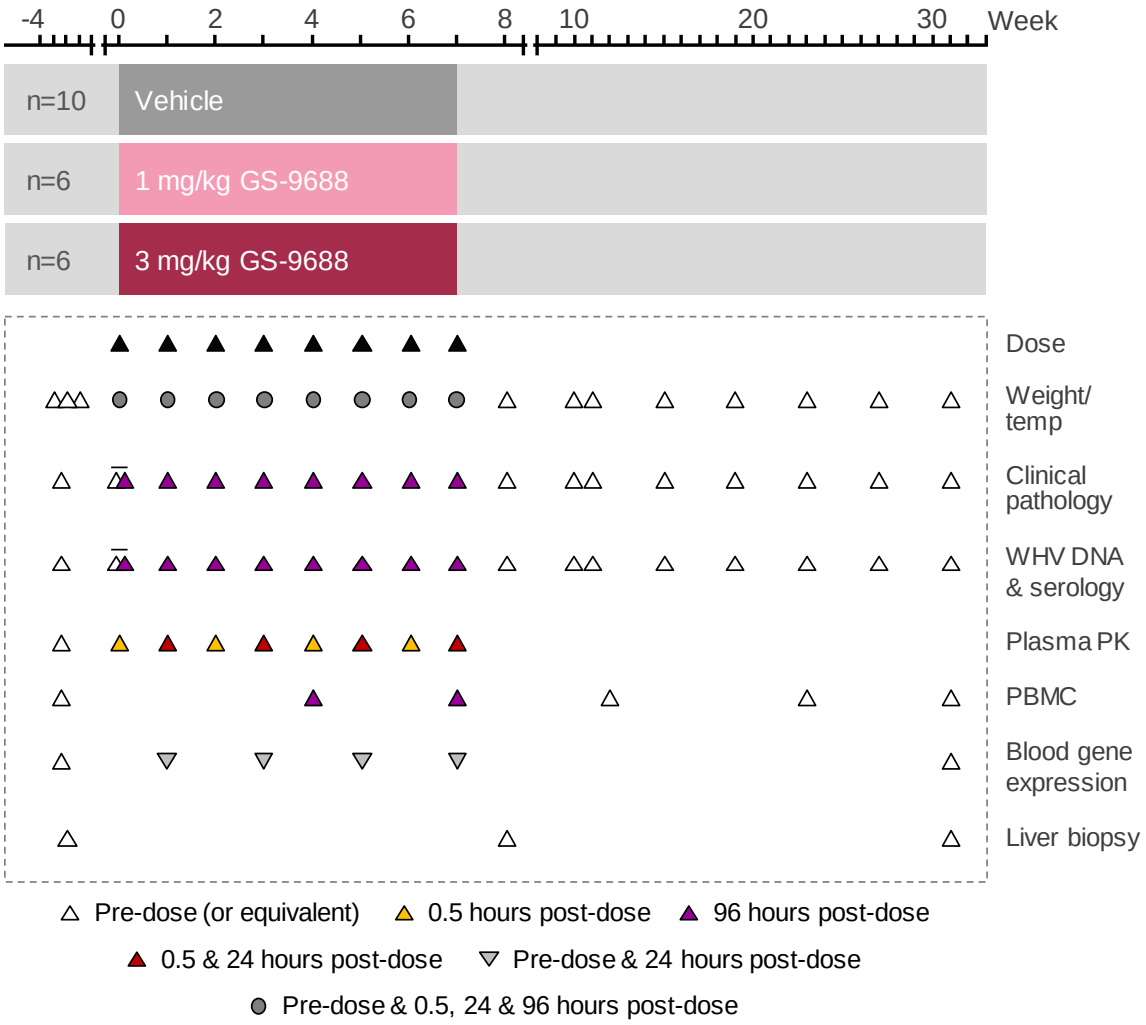


Supplementary Fig. 1. Legend on next page.

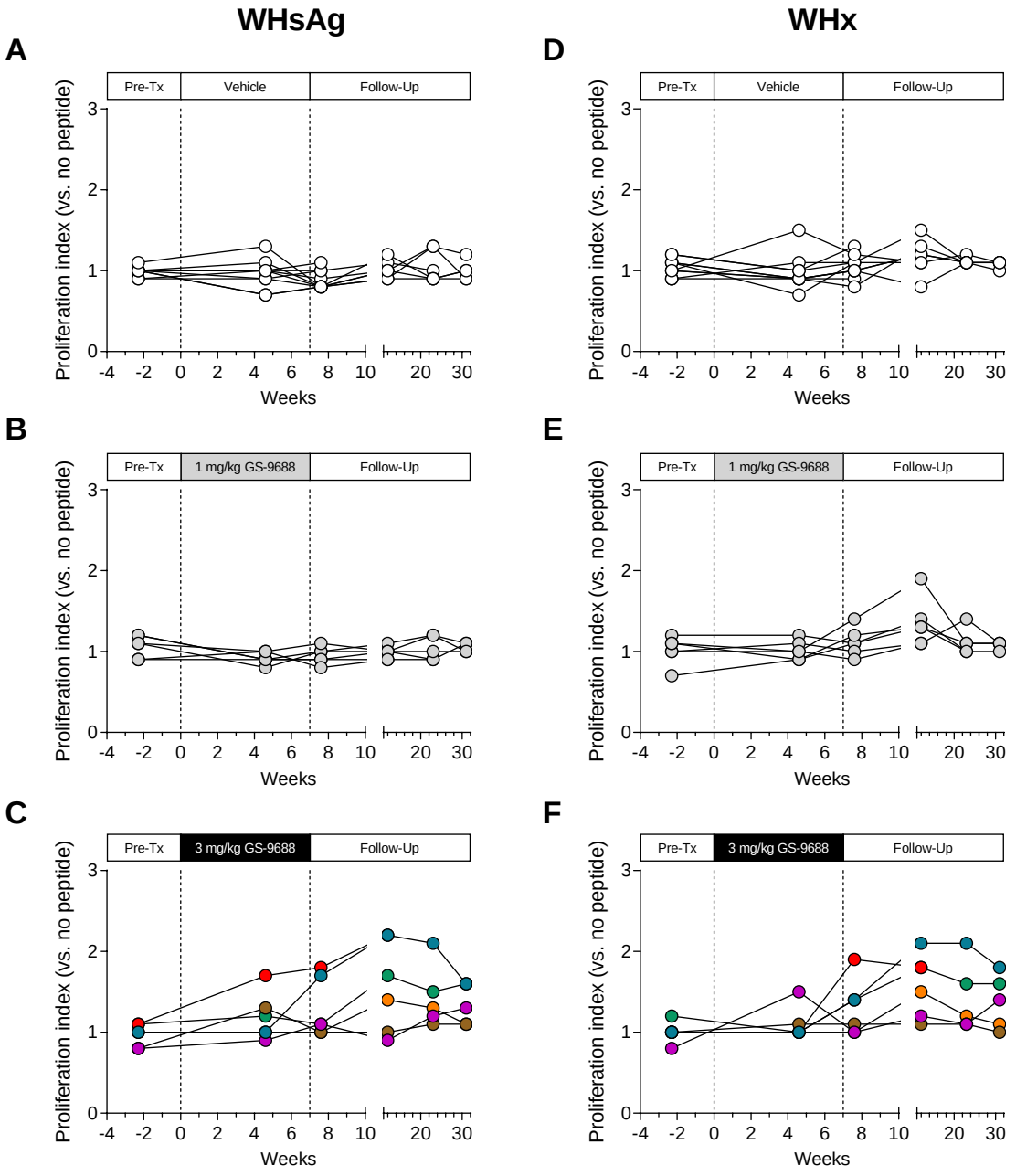
Supplementary Fig. 1. Woodchuck TLR8 is functional and is selectively activated by GS-9688. (A) Alignment of woodchuck, human, cynomolgus monkey (cyno), cow (bos), pig, horse, cat, mouse and rat TLR8 proteins. The long-inserted loop region (Z-loop) between leucine-rich repeats (LRR) 14 and 15, the proteolytic cleavage site and RQSYA five amino acid motif are highlighted. (B) HEK293 cells were transiently transfected with a luciferase reporter gene under the control of the transcription factor NF-κB together with woodchuck TLR8 or woodchuck TLR7. Cells were treated with GS-9688 for 16 hours and then luciferase activity was measured. Data is expressed as fold-change in luciferase activity relative to the no compound (DMSO) control. (C) IL-12p40 levels secreted by woodchuck PBMCs (from n=3 uninfected animals) treated with GS-9688 for 16 hours. For (B) and (C), circles indicate the mean and the error bars represent the SEM. (D) Woodchuck PBMCs (from n=4 uninfected animals) were treated with DMSO, 2.4 nM tool TLR7 agonist (GS-986) or 625 nM tool TLR8 agonist for 4 or 24 hours, and the transcriptional response was then analyzed by RNA-Seq. The bar height indicates the maximal average fold-change (at either 4 or 24 hours post-treatment) for select genes relative to the time-matched DMSO control. All genes passed a low expression filter. The FDR relative to the DMSO control is displayed for each gene. **FDR<0.01, ***FDR<0.0001, ns: not significant (FDR>0.05). ISG, interferon-stimulated gene. Mϕ, macrophage gene. FDR, false discovery rate.



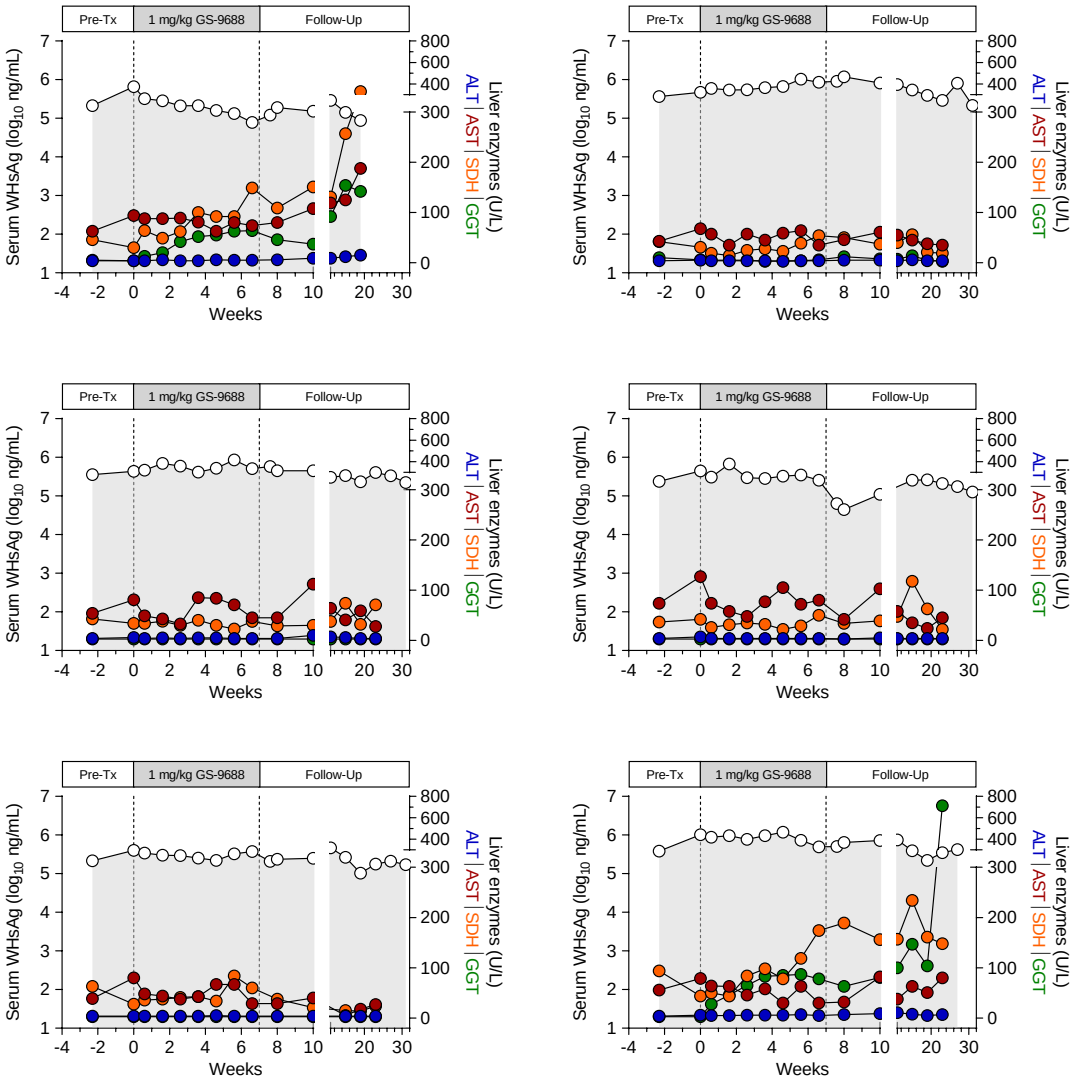
Supplementary Fig. 2. Tool TLR8 agonist induces a comparable transcriptional profile in human and woodchuck PBMCs. Woodchuck PBMCs (from n=4 uninfected animals) and human PBMCs (from n=4 healthy donors) were treated with DMSO, 2.4 nM tool TLR7 agonist (GS-986) or 625 nM tool TLR8 agonist for 4 or 24 hours, and the transcriptional response was then analyzed by RNA-Seq. (A) Correlation analyses of the transcriptional response to the tool TLR8 agonist in human and woodchuck PBMCs by Pearson correlation. The plots display $\log_2$-fold change values at 4 hours (left) or 24 hours (right) post-treatment with the tool TLR8 agonist relative to time-matched DMSO-treated PBMCs. The analysis was restricted to genes that had been identified in the woodchuck transcriptome assembly and assigned to human-woodchuck ortholog pairs based on sequence similarity (n=11,176 genes). (B) Induction of select mRNAs by the tool TLR7 and TLR8 agonists in human PBMCs. The bar height indicates the maximal average fold-change (at either 4 or 24 hours post-treatment) relative to the time-matched DMSO control. All genes passed a low expression filter. The FDR relative to the DMSO control is displayed for each gene. **FDR<0.01, ***FDR<0.0001, ns: not significant (FDR>0.05). ISG, interferon-stimulated gene. Mϕ, macrophage gene. FDR, false discovery rate.



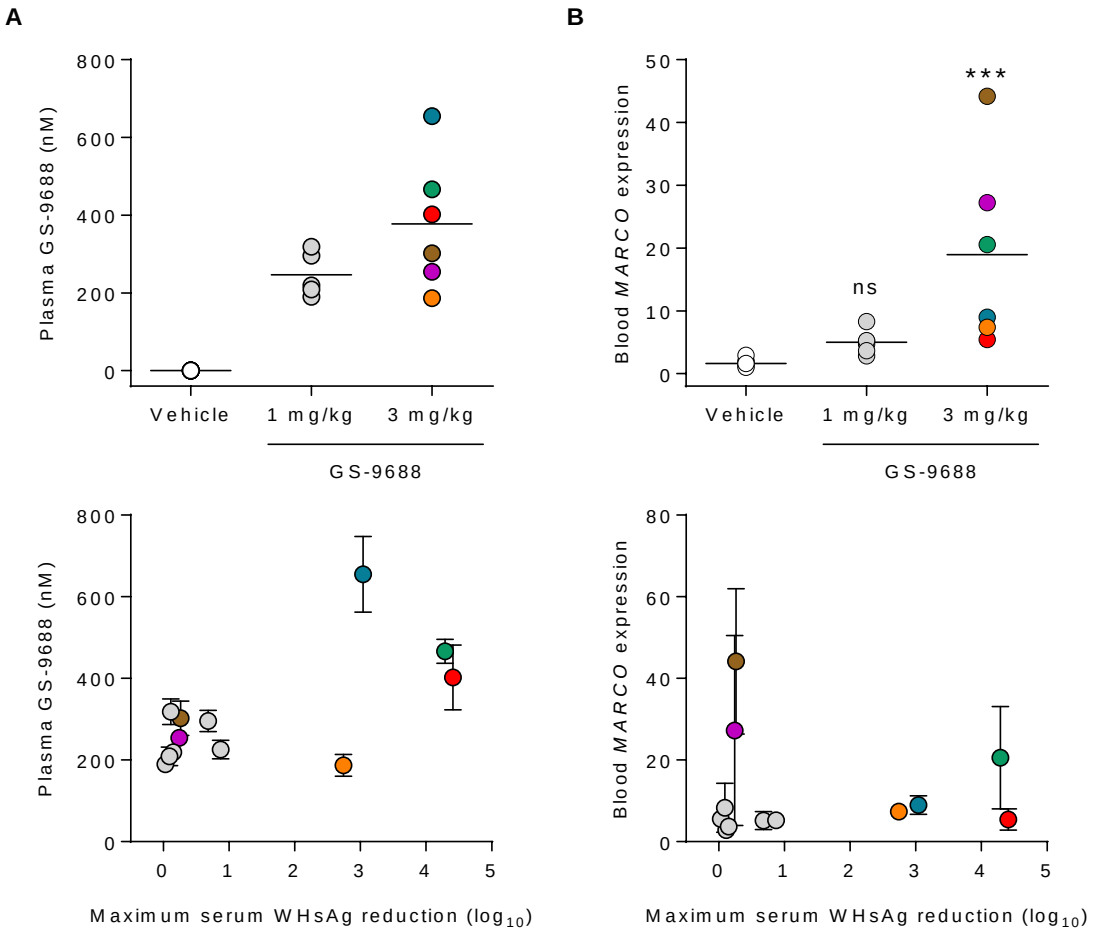
Supplementary Fig. 3. Design of the first GS-9688 efficacy study in WHV-infected woodchucks. Woodchucks chronically infected with WHV were dosed orally once per week for 8 weeks with vehicle or GS-9688. Animals were followed for additional 24 weeks after the treatment period (follow-up). One animal in the 3 mg/kg group did not receive the 6th dose of GS-9688 (see text for details). The horizontal line in the WHV DNA and serology sampling schema indicates that samples were taken at both pre-dose and 96 hours post-dose in week 0. The flash-frozen liver biopsy tissue was analyzed for intrahepatic WHV parameters, but was not of sufficient quality to perform RNA-Seq. Of note, the 24-hour time-point was selected for whole blood gene expression sampling because it represented the peak *MARCO* mRNA response after a single dose of 3 mg/kg GS-9688 in uninfected adult woodchucks (Fig. 1B). Eight animals died during the study: five from the vehicle group, two from the 1 mg/kg GS-9688 group and one from the 3 mg/kg GS-9688 group. All of these animals had elevated GGT levels (>3 U/L) at the beginning of the study and/or at the time of death. Most of the animals that survived until end-of-study and had elevated GGT (either at the beginning and/or at end-of-study) also exhibited signs of advanced HCC (tumors) or liver disease (abnormal liver morphology) at necropsy. Of note, one of the animals in the vehicle group was excluded from the analysis because it had very low WHsAg levels at the start of treatment.



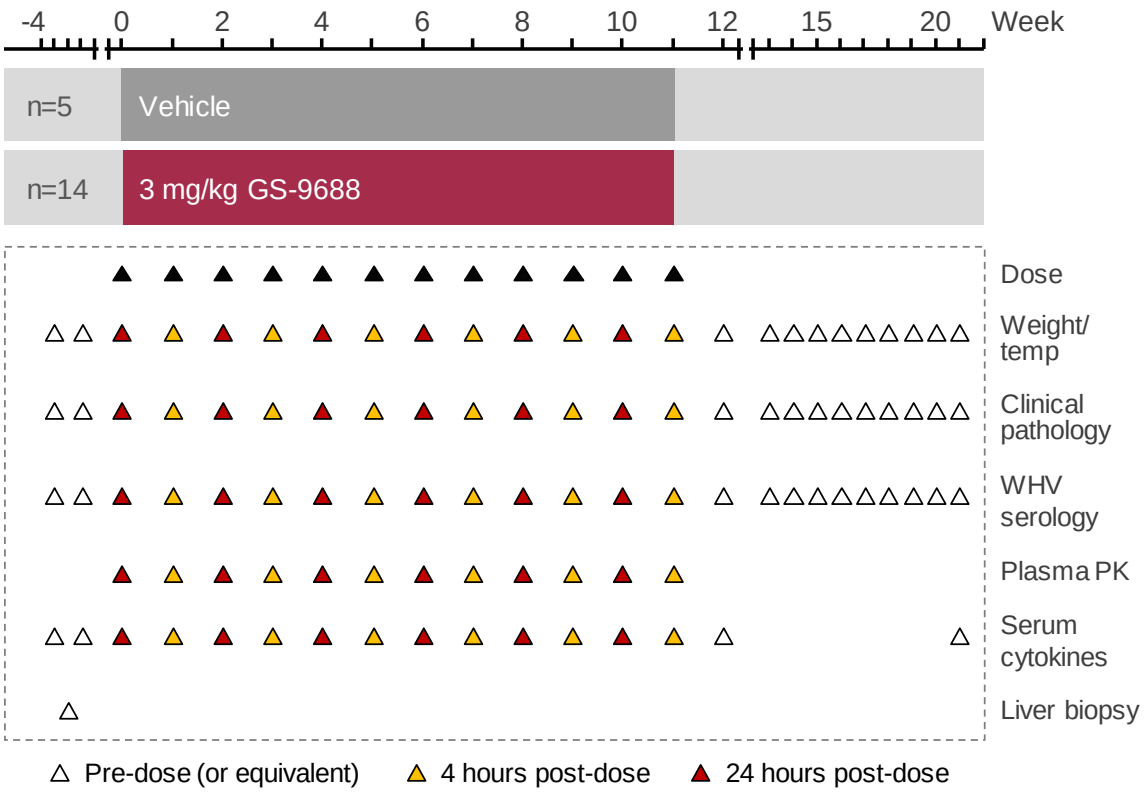
Supplementary Fig. 4. PBMC proliferative responses in WHV-infected woodchucks treated with GS-9688. Woodchucks chronically infected with WHV were dosed orally once per week for 8 weeks with vehicle or GS-9688 as described in Supplementary Fig. 3. Vertical dotted lines denote the dosing period. (A-C) Proliferative response of PBMCs to WHsAg peptides by dose group. (D-F) Proliferative response of PBMCs to WHx peptides by dose group. A proliferation index of ≥ 2.0 is considered positive in this assay. Each line represents an individual animal; the circle colors match those in Fig. 2. Pre-Tx: pre-treatment.



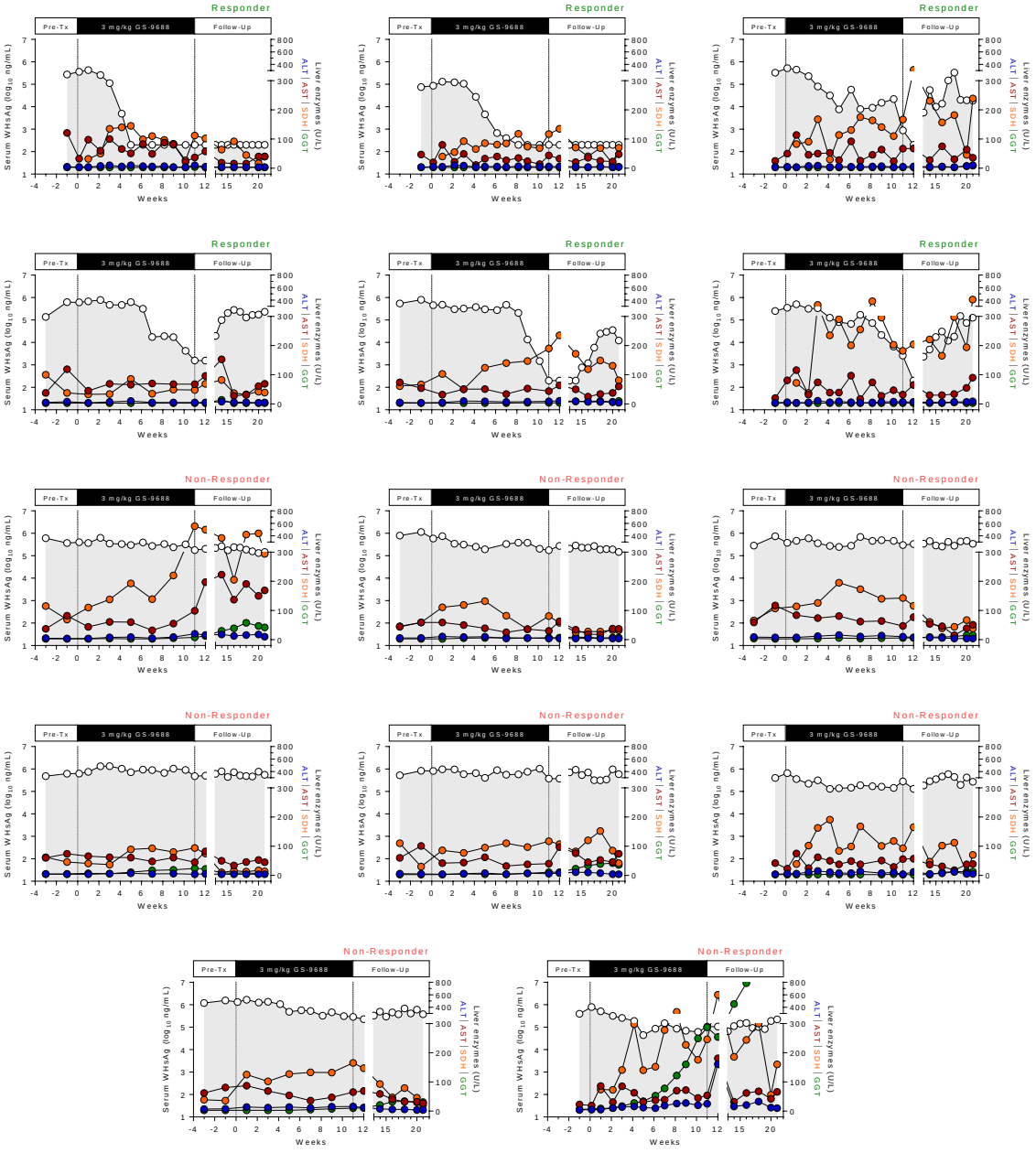
Supplementary Fig. 5. Serum WHsAg and liver enzyme levels in WHV-infected woodchucks treated with 1 mg/kg GS-9688. Woodchucks chronically infected with WHV were dosed orally once per week for 8 weeks with 1 mg/kg GS-9688 as described in Supplementary Fig. 3. Serum WHsAg (open circles) is plotted on the left y-axis. Serum ALT (blue circles), AST (red circles), SDH (orange circles) and GGT (green circles) are all plotted on the right y-axis. Liver enzyme data at week 31 (24 weeks after last dose) was not plotted because some surviving animals had developed HCC by this time. Indeed, liver enzyme elevations earlier in the follow-up period in the top left and bottom right animals were likely coincident with the development of HCC. Pre-Tx: pre-treatment.



Supplementary Fig. 6. Relationship between GS-9688 PK, PD and antiviral response. Woodchucks chronically infected with WHV were dosed orally once per week for 8 weeks with vehicle or GS-9688 as described in Supplementary Fig. 3. (A) Top: mean plasma GS-9688 levels (at 0.5 hours post-dose) during the treatment period in each dose group. Bottom: relationship between plasma GS-9688 levels (mean ± SEM) and the maximum reduction in WHsAg during treatment. (B) Top: mean blood *MARCO* mRNA levels during the treatment period in each dose group. Data is expressed as fold-change at 24 hours post-dose relative to pre-dose. Statistical significance relative to vehicle was calculated by one-way ANOVA with Dunnett's multiple comparison correction; ***$p<0.001$, ns: not significant ($p>0.05$). Bottom: relationship between blood *MARCO* mRNA levels (mean ± SEM) and the maximum reduction in WHsAg during treatment. For all plots, each circle represents an individual animal. The circle colors match those in Fig. 2.



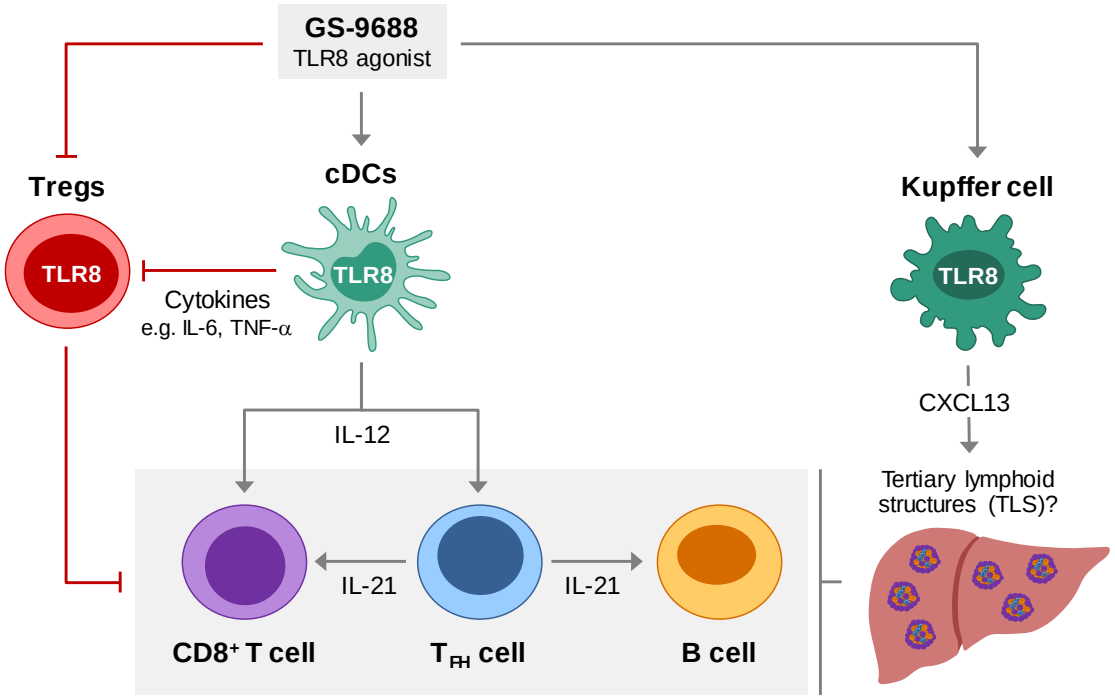
Supplementary Fig. 7. Design of the second GS-9688 efficacy study in WHV-infected woodchucks. Woodchucks chronically infected with WHV were dosed orally once per week for 12 weeks with vehicle or GS-9688. Animals were followed for an additional 10 weeks after the treatment period (follow-up). Consistent with previous studies, there were no antiviral responders in the vehicle group (data not shown). One animal from the 3 mg/kg GS-9688 group died during the study (week 4) from fibrinous pneumonia-associated complications that did not appear to be related to GS-9688 treatment. Of note, clinical pathology samples were only collected every other week during the dosing period (weeks 1, 3, 5, 7, 9 and 11) in a subset of GS-9688-treated animals (n=5).



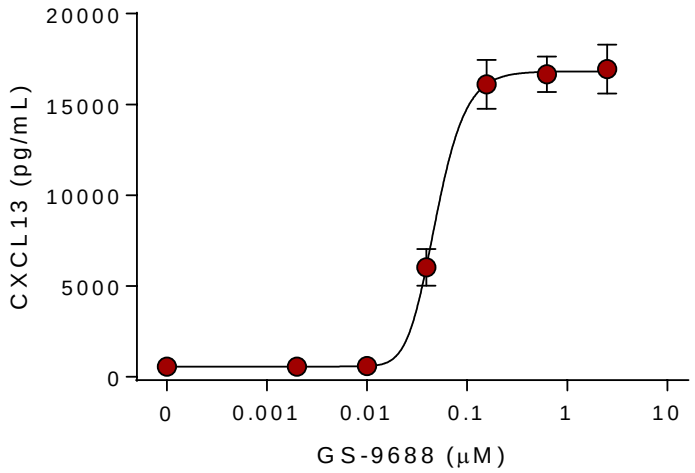
Supplementary Fig. 8. Serum WHsAg and liver enzyme levels in WHV-treated woodchucks treated with 3 mg/kg GS-9688. Woodchucks chronically infected with WHV were dosed orally once per week for 12 weeks with 3 mg/kg GS-9688 as described in Supplementary Fig. 7. Serum WHsAg (open circles) is plotted on the left y-axis. Serum ALT (blue circles), AST (red circles), SDH (orange circles) and GGT (green circles) are all plotted on the right y-axis. Of note, liver enzyme elevations in the follow-up period in the top left non-responder and bottom right non-responder animals were likely coincident with the development of HCC. Pre-Tx: pre-treatment.

Supplementary Fig. 9. Legend on next page.

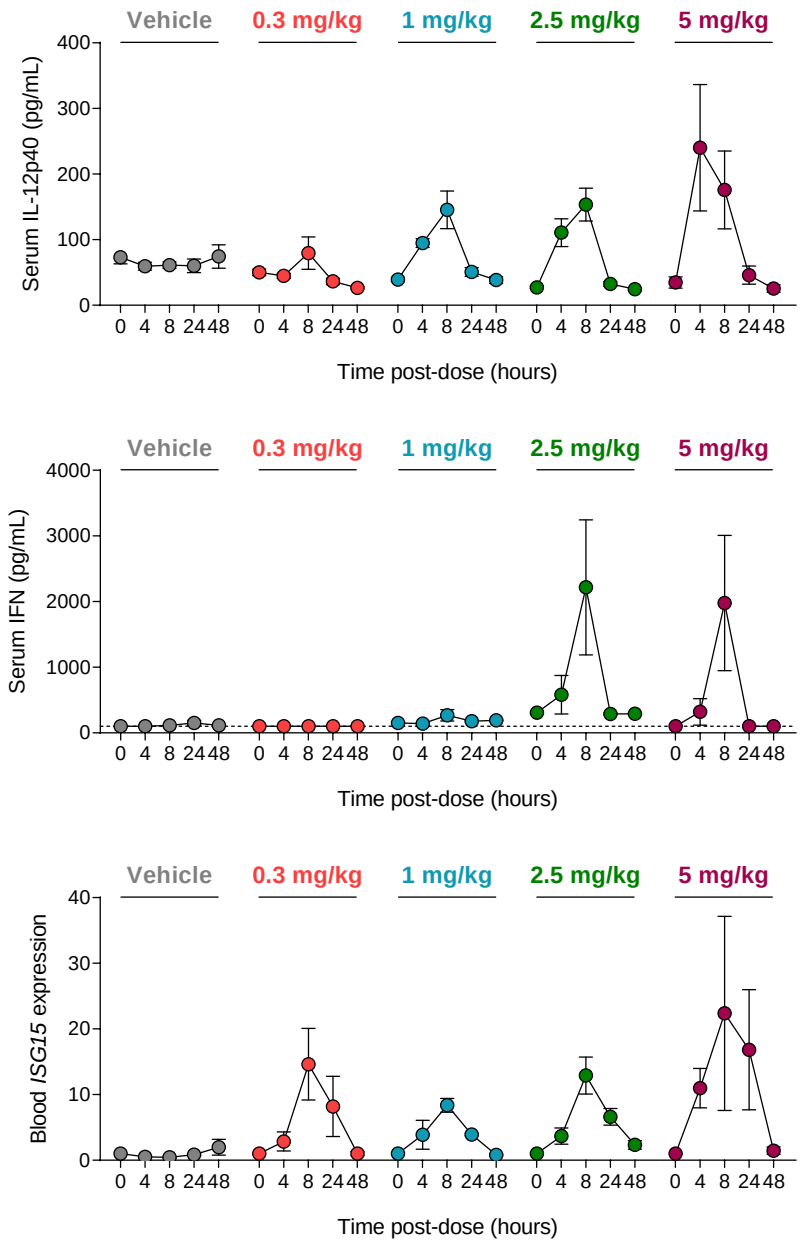
Supplementary Fig. 9. Cell type gene enrichment analysis of intrahepatic transcriptional signatures. Woodchucks chronically infected with WHV were dosed orally once per week for 12 weeks with vehicle or 3 mg/kg GS-9688 as described in Supplementary Fig. 7. Liver biopsies collected from uninfected woodchucks (U, n=9) and from WHV-infected woodchucks (C, n=19) prior to GS-9688 treatment (baseline) were analyzed by RNA-Seq. The WHV-infected animals were classified by treatment and response: vehicle-treated (n=5), antiviral responders to GS-9688 (C^R, n=6) and antiviral non-responders to GS-9688 (C^{NR}, n=8). Antiviral response was defined as described in Fig. 6. RNA-Seq data was analyzed by xCell. Each column represents a different animal and each row represents an individual cell type; over-expression (red) and under-expression (blue) are indicated by the scale bar for z-score values. Symbols adjacent to the cell type names indicate statistical significance: *FDR<0.05 for C vs. U, †unadjusted *p*-value < 0.05 for C^R vs. U, ‡unadjusted *p*-value < 0.05 for C^{NR} vs. U. For C^R vs. U, "activated dendritic cells" reached FDR<0.05. For C^{NR} vs. U, "activated dendritic cells", "macrophages" and "pro-B cells" were all FDR<0.05. For C^R vs. C^{NR}, no cell type reached FDR<0.05. MPP: multipotent progenitors, HSC: hematopoietic stem cells, T_{EM}: effector memory T cell, GMP: granulocyte-macrophage progenitor, T_{CM}: central memory T cell, CLP: common lymphoid progenitor, cDC: conventional dendritic cell, CMP: common myeloid progenitor. The xCell data for U, C^{NR} and C^R animals is presented in Fig. 7A for the cell types in bold.



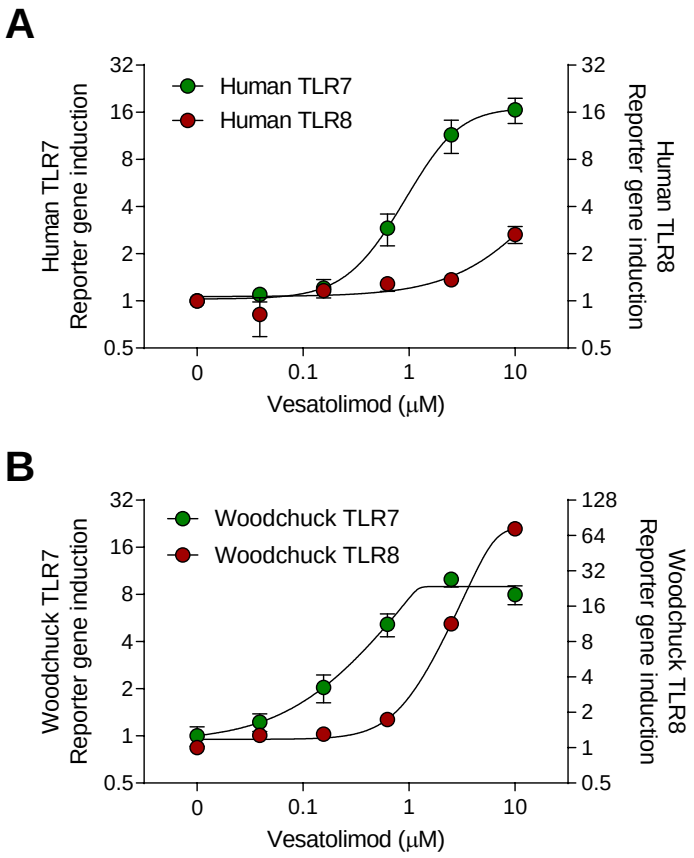
Supplementary Fig. 10. Model of the intrahepatic antiviral immune response induced by GS-9688 in WHV-infected woodchucks. Antiviral responders to GS-9688 were enriched for intrahepatic transcriptional signatures of Tregs, T_{FH}, plasma cells and M1 macrophages. This suggests a model in which (A) IL-12 from cDCs activated by GS-9688 rescues "exhausted" $CD8^+$ T cells (40) and/or augments the T_{FH} response (9); (B) Suppression of T_{FH}, $CD8^+$ T cells and/or B cells is alleviated by inhibition of Tregs by GS-9688, either directly (4) or via cytokine production (41, 42); (C) IL-21 produced from T_{FH} enhances $CD8^+$ T cell activity and drives differentiation of B cells into antibody secreting plasma cells (14, 17)(43-47); (D) Kupffer cell activation by GS-9688 facilitates development of an effective intrahepatic immune response, e.g. by promoting CXCL13-dependent lymphoid organization (22). Cell types that express TLR8 are indicated.



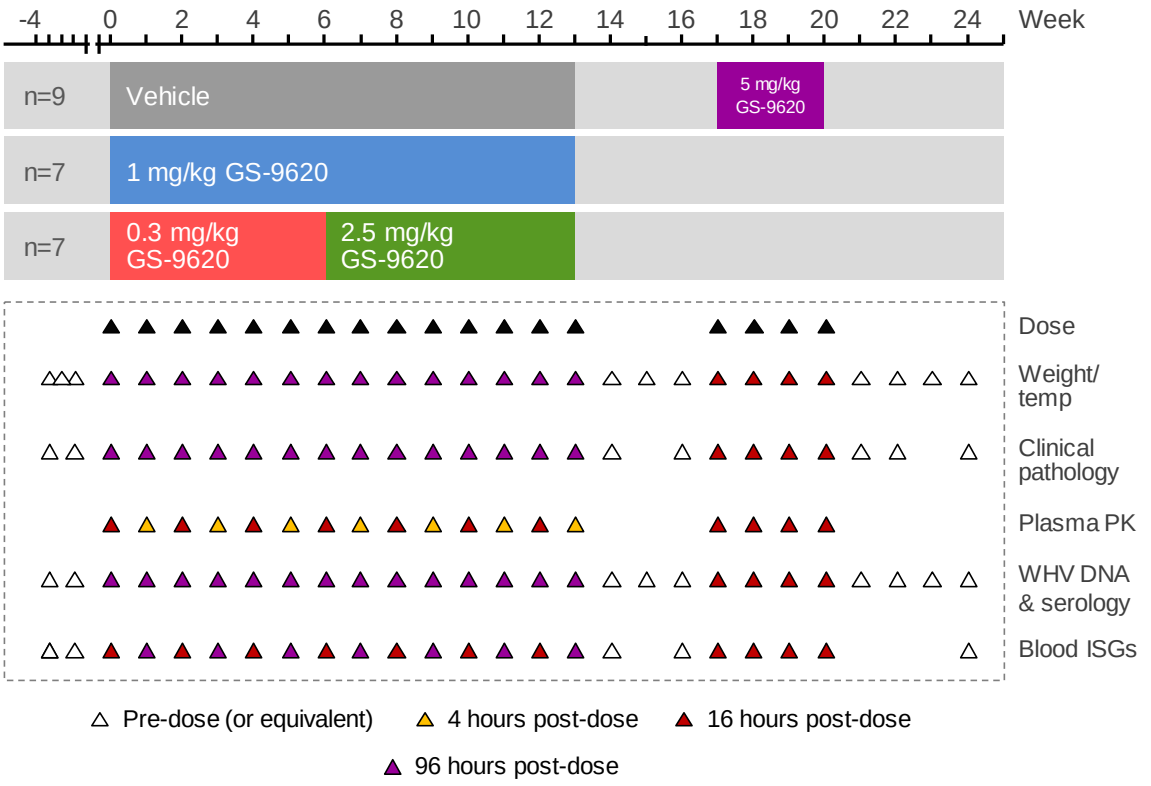
Supplementary Fig. 11. GS-9688 induces CXCL13 in human PBMCs. CXCL13 levels secreted by human PBMCs (from n=3 healthy donors) treated with GS-9688 for 16 hours. Circles indicate the mean and the errors bars represent the SEM. Of note, CXCL13 is not present in the woodchuck transcriptome assembly, and so we were unable to determine whether GS-9688 induces *CXCL13* expression in woodchuck PBMCs.



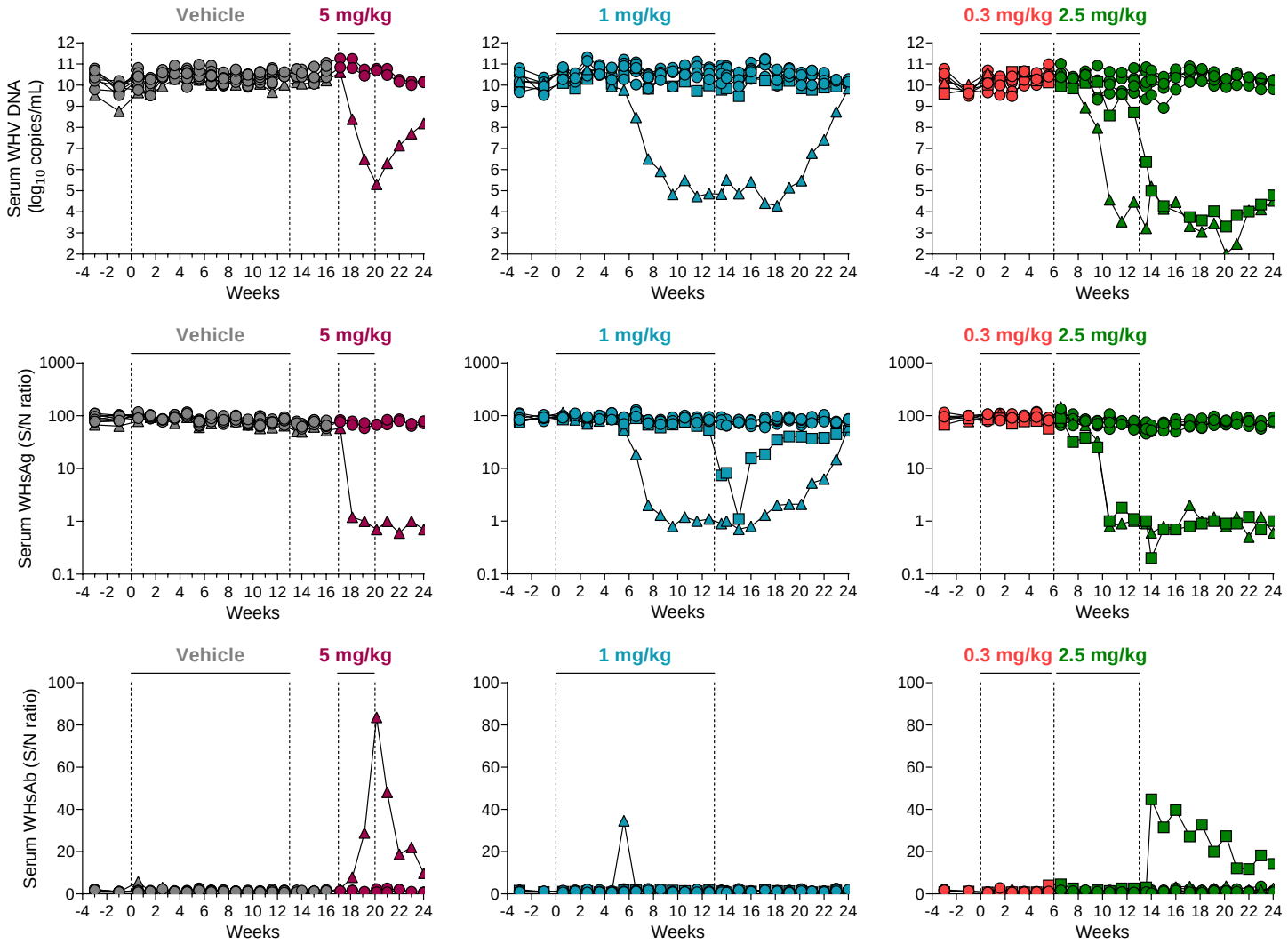
Supplementary Fig. 12. Pharmacodynamic response of uninfected woodchucks to oral vesatolimod. Uninfected woodchucks (n=4/group) were orally administered a single dose of vehicle, 0.3 mg/kg vesatolimod, 1 mg/kg vesatolimod, 2.5 mg/kg vesatolimod or 5 mg/kg vesatolimod. Serum IL-12p40, serum IFN and whole blood *ISG15* mRNA levels were measured by electro-chemiluminescence assay, bioassay and qRT-PCR, respectively. The LLOD of the IL-12p40 assay is 4 pg/mL. The LLOD of the IFN assay is 100 pg/mL and is indicated by a dotted line. The ULOD of the IFN assay is 5000 pg/mL; the 8-hour time-point was >ULOD for one animal in the 2.5 mg/kg vesatolimod group and one animal in the 5 mg/kg vesatolimod group. These values were plotted as the ULOD. The qRT-PCR data is expressed as fold-change relative to pre-dose. For all plots, circles indicate the mean and the errors bars represent the SEM. LLOD, lower limit of detection. ULOD, upper limit of detection.



Supplementary Fig. 13. Vesatolimod has reduced selectivity for woodchuck TLR7 over TLR8 compared with the selectivity for human TLR7 over TLR8. HEK293 cells were transiently transfected with an expression vector for human TLR7, human TLR8, woodchuck TLR7 or woodchuck TLR8 together with an NF-κB luciferase reporter vector and stimulated with various concentrations of vesatolimod. Luciferase activity was measured and expressed as fold-change relative to DMSO-treated cells. Circles indicate the mean of n=3 independent experiments and the error bars represent the SEM. (A) Selectivity of vesatolimod for human TLR7 over TLR8. Human TLR7 MEC 0.4 μM, human TLR8 MEC 6 μM; TLR7-TLR8 selectivity index = 15. (B) Selectivity of vesatolimod for woodchuck TLR7 over TLR8. Woodchuck TLR7 MEC 0.2 μM, woodchuck TLR8 MEC 0.7 μM; TLR7-TLR8 selectivity index = 4. Minimal effective concentration (MEC) was defined as a 2-fold increase relative to the DMSO control.



Supplementary Fig. 14. Design of the vesatolimod (GS-9620) efficacy study in WHV-infected woodchucks. Woodchucks chronically infected with WHV were dosed orally once per week for 14 weeks with either vehicle or vesatolimod. Animals in the vesatolimod dosing groups were followed for an additional 11 weeks after the treatment period (follow-up). In the vehicle group, after 3 weeks of follow-up, a subset of animals (n=3) were dosed with 5 mg/kg vesatolimod for 4 weeks and then followed for additional 4 weeks. One animal in the vehicle group died during the study (week 11) due to a blood clot. Vesatolimod plasma PK and whole blood ISG expression was comparable to previous studies in uninfected and WHV-infected woodchucks (data not shown). Only qualitative assessment of serum WHsAg and WHsAb (expressed as signal/noise, S/N) was performed for this study. ISG, interferon-stimulated gene.



Supplementary Fig. 15. Serum WHV DNA, WHsAg and WHsAb levels in WHV-infected woodchucks treated with vesatolimod. Woodchucks chronically infected with WHV were dosed orally once per week with vehicle or vesatolimod as described in Supplementary Fig. 14. Top: serum WHV DNA. Middle: qualitative serum WHsAg. Bottom: qualitative serum WHsAb. Each line represents an individual animal. S/N, signal/noise. In the absence of quantitative WHsAg data, antiviral response was defined as >2 $\log_{10}$ reduction in serum WHV DNA.

Supplementary Tables

Treatment group	Animal ID	Antiviral Response	Liver histology scores: PH/BDP/SH		
			Week -2	Week 8	Week 31
Vehicle	C101	NA	0/0/1	2/1/1	-
	C102	NA	2/0/0	2/3/1	-
	C103	NA	0/0/0	1/1/0	2/2/0
	C104	NA	0/0/0	1/1/0	1/2/0
	C105	NA	1/2/0	-	-
	C106	NA	0/0/0	0/0/0	1/0/0
	C107	NA	0/0/0	0/0/0	1/0/0
	C108	NA	0/0/0	2/2/0	-
	C110	NA	0/0/0	0/0/0	0/0/0
	C111	NA	2/0/1	1/1/0	-
1 mg/kg GS-9688	C201	Non-responder	0/0/0	1/1/0	-
	C202	Non-responder	0/0/0	0/0/0	0/0/0
	C203	Non-responder	2/3/1	0/0/0	0/0/0
	C204	Non-responder	0/0/0	1/0/0	0/0/0
	C205	Non-responder	0/0/0	0/0/0	0/0/0
	C206	Non-responder	0/0/0	0/0/0	-
3 mg/kg GS-9688	C301	Sustained responder	0/3/1	1/0/0	0/0/0
	C302	Non-responder	0/0/0	0/0/0	2/1/0
	C303	Non-responder	0/0/0	0/0/0	0/0/0
	C304	Sustained responder	3/0/3	3/2/1	-
	C305	Transient responder	0/0/0	1/0/0	0/0/0
	C306	Sustained responder	0/0/0	2/1/1	0/0/0

Supplementary Table 1. Histologic response to vehicle and GS-9688 treatment. Woodchucks chronically infected with WHV were dosed orally once per week for 8 weeks with vehicle or GS-9688 as described in Supplementary Fig. 3. Liver biopsies were obtained before treatment (week -2), one week after treatment (week 8) and at the end-of-study (week 31). The liver tissue was fixed in phosphate-buffered formalin, embedded in paraffin, and then sectioned and stained with H&E for histopathological analysis by a board-certified pathologist. Liver histology scores were determined for both portal parameters (n>4 portal tracts examined) and lobular parameters. The former was assessed by evaluating portal hepatitis (PH) (0: none, 1: slight inflammation, 2: additional inflammation with or without slight portal tract distension, 3: further inflammation with occasional piecemeal necrosis of the limiting plate with some spread of inflammation into the parenchyma, 4: more significant portal inflammation with widespread piecemeal necrosis and spread of inflammation into the parenchyma) and bile duct proliferation (BDP) (0: none, 1: mild; 2: moderate; 3: marked; 4: severe). The latter was assessed by evaluating sinusoidal hepatitis (SH) (0: none, 1: mild; 2: moderate; 3: marked; 4: severe). Dashed lines indicate no sample available. NA, not applicable. For the 3 mg/kg GS-9688 group, the animal ID colors match the circle colors in Fig. 2.

Canonical pathway	Adjusted p-value
Th1 and Th2 activation pathway	<0.0001
Th2 pathway	<0.0001
Th1 pathway	<0.0001
Dendritic cell maturation	<0.0001
ICOS/ICOSL signaling in T helper cells	<0.0001
Antigen presentation pathway	<0.0001
PD-1/PD-L1 cancer immunotherapy pathway	<0.0001
T helper cell differentiation	<0.0001
Immune cell interactions[1]	<0.0001
Crosstalk between dendritic cells and NK cells	<0.0001
Type I diabetes mellitus signaling	<0.0001
T cell and B cell signaling	<0.0001
Primary immunodeficiency signaling	<0.0001
B cell development	<0.0001
CD28 signaling in T helper cells	<0.0001
Role of NFAT in immune response	<0.0001
Graft-versus-host disease signaling	<0.0001
PKCθ signaling in T cells	<0.0001
Autoimmune thyroid disease signaling	<0.0001
T cell exhaustion signaling pathway	<0.0001

Supplementary Table 2. Characterization of the intrahepatic transcriptional signature of WHV-infected woodchucks by pathway analysis. Liver biopsies collected from uninfected woodchucks (n=9) and from WHV-infected woodchucks (n=19) prior to treatment (baseline) were analyzed by RNA-Seq. The table displays the top canonical pathways up-regulated in WHV-infected vs. uninfected woodchucks as identified by Ingenuity Pathway Analysis (IPA). [1]Full name: communication between innate & adaptive immune cells.

Pathway	Adjusted p-value
Innate immune system	<0.0001
Adaptive immune system	<0.0001
Cytokine signaling	<0.0001
Interleukin signaling	<0.0001
Neutrophil degranulation	<0.0001
Interferon signaling	<0.0001
FcεR signaling	<0.0001
Immune cell interactions[1]	<0.0001
BCR signaling	<0.0001
FcγR dependent phagocytosis	<0.0001
FcεR mediated NF-κB activation	<0.0001
TCR signaling	<0.0001
Downstream TCR signaling	<0.0001
Complement cascade	<0.0001
Antigen processing cross-presentation	<0.0001
TNFR2 non-canonical NF-κB pathway	<0.0001
IFN-γ signaling	<0.0001
Downstream BCR signaling	<0.0001

Supplementary Table 3. Characterization of the intrahepatic transcriptional signature of WHV-infected woodchucks by GSEA. Liver biopsies collected from uninfected woodchucks (n=9) and from WHV-infected woodchucks (n=19) prior to treatment (baseline) were analyzed by RNA-Seq. The table displays the top immunomodulatory pathways up-regulated in WHV-infected vs. uninfected woodchucks as identified by GSEA. The criteria for pathway selection were canonical reactomes with immunomodulatory function. [1]Full name: immune-regulatory interactions between a lymphoid and a non-lymphoid cell.

Pathway	Adjusted p-value
CD22 mediated BCR regulation	0.0028
FcγR activation	0.0028
Creation of complement C4 and C2 activators	0.0028
Initial triggering of complement	0.0028
BCR activation	0.0028
FcεR mediated Ca^{2+} mobilization	0.0028
FcεR mediated MAPK activation	0.0028
Complement cascade	0.0028
FcεR mediated NF-κB activation	0.0028
FcγR dependent phagocytosis	0.0028
BCR signaling	0.0028
Immune cell interactions[1]	0.0028
FcεR signaling	0.0028
Neutrophil degranulation	0.0038
Innate immune system	0.0079
IL-4 and IL-13 signaling	0.0354

Supplementary Table 4. Antiviral response is associated with baseline intrahepatic transcriptional signature in WHV-infected woodchucks treated with GS-9688. Woodchucks chronically infected with WHV were dosed orally once per week for 12 weeks with vehicle or 3 mg/kg GS-9688 as described in Supplementary Fig. 7. Liver biopsies collected prior to treatment (baseline) were analyzed by RNA-Seq. The animals treated with GS-9688 were classified as antiviral responders (C^{R}, n=6) or antiviral non-responders (C^{NR}, n=8) as defined in Fig. 6. The table displays the top immunomodulatory pathways down-regulated in antiviral responders vs. non-responder animals as identified by GSEA. The criteria for pathway selection were canonical reactomes with immunomodulatory function. [1]Full name: immunoregulatory interactions between a lymphoid and a non-lymphoid cell. There was no significantly enriched up-regulated pathways in antiviral responders.

Gene	Associated cell type‡	C vs. U	C^R vs. U	C^{NR} vs. U	C^R vs. C^{NR}
IGKV2-40	B cell	2.84	5.59	1.83	3.06
HERC6	NA	3.40	3.86	1.88	2.05*
IGHV4-30-4	B cell	4.40	2.92	5.27	0.55*
IGLV3-9	B cell	2.54	1.81	3.40	0.53
IGHV4-61	B cell	6.02	4.03	7.97	0.51*
IGHV4-34	B cell	2.76	1.73	3.49	0.50
IGHV4-31	B cell	5.13	3.30	6.74	0.49*
CSF3R	Neutrophil	2.87	1.74	3.74	0.46*
TNFRSF17	B cell	2.82	1.84	4.02	0.46
S100A9	Neutrophil	2.95	1.58	3.52	0.45*
IGLL1	B cell	4.87	2.60	7.15	0.36
IGLL5	B cell	4.68	2.55	7.19	0.35
CCL2	NA	3.45	1.81	5.18	0.35
IGHA2	B cell	24.09	15.14	45.82	0.33
IGLV2-18	B cell	8.63	4.41	13.65	0.32
IGLV2-11	B cell	5.35	2.44	8.63	0.28
IGLV1-44	B cell	7.36	3.17	12.62	0.25*
IGKV1D-43	B cell	5.61	2.60	11.03	0.24
IGLV2-8	B cell	12.13†	2.46	26.50	0.09

Supplementary Table 5. Differential baseline intrahepatic expression of select genes in antiviral responders and non-responders to GS-9688 treatment. Woodchucks chronically infected with WHV were dosed orally once per week for 12 weeks with vehicle or 3 mg/kg GS-9688 as described in Supplementary Fig. 7. Liver biopsies collected from uninfected woodchucks (U, n=9) and from WHV-infected woodchucks (C, n=19) prior to treatment (baseline) were analyzed by RNA-Seq. The WHV-infected animals treated with GS-9688 (n=14) were classified as antiviral responders (C^R, n=6) or antiviral non-responders (C^{NR}, n=8) as defined in Fig. 6. The table displays RNA-Seq data for genes differentially induced (above line) or reduced (below line) in C^R vs. C^{NR}. Fold-change values relative to uninfected animals (C vs. U, C^R vs. U and C^{NR} vs. U) or relative to C^{NR} (C^R vs. C^{NR}) are displayed. Underlining denotes statistical significance, either FDR<0.05 (C vs. U, C^R vs. U and C^{NR} vs. U) or unadjusted *p*-value <0.05 (C^R vs. C^{NR}). †*Fold-change ratios were close to statistical significance; †FDR = 0.06, *unadjusted *p*-value ≤ 0.06. No gene reached FDR<0.05 for the C^R vs. C^{NR} comparison. ‡Indicates the cell type which characteristically express each gene. NA, no assignment. Of note, it is challenging to deconvolute neutrophils from granulocytic myeloid-derived suppressor cells (gMDSCs, also known as PMN-MDSCs) based on gene expression patterns from whole tissue. However, upregulation of *MPO* but not *OLR1* (*LOX1*) (data not shown) suggests that neutrophils (and not gMDSCs) are the predominant source of the granulocyte gene signature in C^{NR} (48, 49). Hugo symbols are used for gene names. Alternative gene names; *CSF3R*: *GCSFR*, *TNFRSF17*: *BCMA*, *CCL2*: *MCP1*.

Gene	C vs. U	C^R vs. U	C^{NR} vs. U
CXCR5	2.83	5.04†	1.82
ICOS	3.53	4.01†	3.21
CD4	1.81	1.90	1.63
TNFRSF4	2.71	2.71	2.11
CD8A	3.18	2.50	3.46
CD8B	2.38	1.76	2.69

Supplementary Table 6. Baseline intrahepatic expression of T_{FH}-associated genes in WHV-infected woodchucks treated with GS-9688. Woodchucks chronically infected with WHV were dosed orally once per week for 12 weeks with vehicle or 3 mg/kg GS-9688 as described in Supplementary Fig. 7. Liver biopsies collected from uninfected woodchucks (U, n=9) and from WHV-infected woodchucks (C, n=19) prior to treatment (baseline) were analyzed by RNA-Seq. The WHV-infected animals treated with GS-9688 (n=14) were classified as antiviral responders (C^R, n=6) or antiviral non-responders (C^{NR}, n=8) as defined in Fig. 6. The table displays RNA-Seq data for select genes induced in C^R vs. U and/or C^{NR} vs. U. Fold-change values relative to uninfected animals are displayed. Underlining denotes statistical significance (FDR<0.05). †Fold-change ratios were close to statistical significance (FDR = 0.08). C^R vs. C^{NR} data is not included because no gene reached FDR<0.05 or unadjusted *p*-value < 0.05. Hugo symbols are used for gene names. Alternative gene name; *TNFRSF4*: *OX40*. Of note, CXCL13 (chemokine for CXCR5-expressing cells, e.g. T_{FH}) was not present in the woodchuck transcriptome assembly, and so we were unable to determine whether antiviral responders had increased intrahepatic *CXCL13* levels.

Parameter	Woodchucks[a,b]				Humans[c,d]
	0.3 mg/kg	1 mg/kg	2.5 mg/kg	5 mg/kg	4 mg
Mean ∆serum IFN[e]	No induction (<LLOD)	2-fold (286 pg/mL)	>8-fold (>2366 pg/mL)	>20-fold (>1978 pg/mL)	No induction (<LLOQ)
Mean ∆serum IL-12p40[f]	No induction (80 pg/mL)	4-fold (149 pg/mL)	6-fold (166 pg/mL)	5-fold (241 pg/mL)	No induction (<12 pg/mL)
Mean blood *ISG15* mRNA[g]	15-fold	6-fold	13-fold	20-fold	26-fold
Antiviral response	0%	14%	28%	33%	0%

Supplementary Table 7. Comparable PD and antiviral response to 0.3 mg/kg vesatolimod in woodchucks and 4 mg dose in humans.

a. Woodchuck serum IFN, serum IL-12p40 and blood *ISG15* mRNA data is from uninfected woodchucks in this study (Supplementary Fig. 12).
b. WHV-infected woodchuck response data is from this study (Supplementary Fig. 15). Response was defined as >2 $\log_{10}$ reduction in serum WHV DNA.
c. Human serum IFN-α, serum IL-12p40 and blood *ISG15* mRNA data is from healthy human volunteers [reference (39)].
d. CHB patient response data is from references (37) and (38). Response was defined as >1 $\log_{10}$ reduction in serum HBsAg.
e. Mean maximal fold-change between 4-24 hours post-dose, with absolute levels provided in parentheses. The woodchuck IFN bioassay lower limit of detection (LLOD) was 100 pg/mL and the upper limit of detection (ULOD) was 5000 pg/mL. The human IFN-α immunoassay lower limit of quantitation (LLOQ) was 15.6 pg/mL. The majority of baseline (pre-dose) woodchuck samples were <LLOD, and all human samples were <LLOQ. Two post-dose woodchuck samples were >ULOD, and the ULOD was used in the calculations.
f. Mean maximal fold-change between 4-24 hours post-dose, with absolute levels provided in parentheses. The woodchuck IL-12p40 immunoassay LLOD was 4 pg/mL, and the human IL-12p40 immunoassay LLOQ was 2.4 pg/mL. No woodchuck samples were <LLOD, but some human samples were <LLOQ.
g. Mean maximal fold-change between 4-24 hours post-dose.