GS-9620, an Oral Agonist of Toll-Like Receptor-7, Induces Prolonged Suppression of Hepatitis B Virus in Chronically Infected Chimpanzees

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BACKGROUND & AIMS: Direct-acting antiviral agents suppress hepatitis B virus (HBV) load, but they require life-long use. Stimulation of the innate immune system could increase its ability to control the virus and have long-lasting effects after a finite regimen. We investigated the effects of immune activation with GS-9620—a potent and selective orally active small molecule agonist of Toll-like receptor 7—in chimpanzees with chronic HBV infection. METHODS: GS-9620 was administered to chimpanzees every other day (3 times each week) for 4 weeks at 1 mg/kg and, after a 1-week rest, for 4 weeks at 2 mg/kg. We measured viral load in plasma and liver samples, the pharmacokinetics of GS-9620, and the following pharmacodynamics parameters: interferon-stimulated gene expression, cytokine and chemokine levels, lymphocyte and natural killer cell activation, and viral antigen expression. Clinical pathology parameters were monitored to determine the safety and tolerability of GS-9620. RESULTS: Short-term oral administration of GS-9620 provided long-term suppression of serum and liver HBV DNA. The mean maximum reduction of viral DNA was 2.2 logs, which occurred within 1 week of the end of GS-9620 administration; reductions of >1 log persisted for months. Serum levels of HBV surface antigen and HBV e antigen, and numbers of HBV antigen–positive hepatocytes, were reduced as hepatocyte apoptosis increased. GS-9620 administration induced production of interferon-α and other cytokines and chemokines, and activated interferon-stimulated genes, natural killer cells, and lymphocyte subsets. CONCLUSIONS: The small molecule GS-9620 activates Toll-like receptor 7 signaling in immune cells of chimpanzees to induce clearance of HBV-infected cells. This reagent might be developed for treatment of patients with chronic HBV infection.

Keywords: Innate Immunity; Interferon-α; Antiviral; Pathogen Recognition; TLR-7.

Therapeutic treatment of chronic hepatitis B virus (HBV) infection is currently limited to nucleos(t)ide analogues and pegylated interferon-(IFN)-α.1,2 First-line therapy for HBV is limited to the 2 nucleos(t)ide analogues tenofovir and entecavir, which are highly effective at suppressing viral replication and can reduce serum viral load to undetectable levels. However, these agents do not lead to viral eradication, potentially requiring life-long use and possible emergence of resistance.3 The potential for therapeutic immune modulation to treat HBV chronic infection is illustrated by durable responses, normalization of alanine aminotransferase (ALT), and sustained reduction in viremia attained in a small percentage (<20%) of patients treated for 1 year with pegylated IFN-α.4–6 A key observation is that the apparent cure rate after long-term high-dose IFN-α treatment increases for several years after treatment, based on loss of HBV surface antigen (HBsAg) and seroconversion for anti-HBsAg antibody. This supports the hypothesis that viral control is due to immune modulation and slow induction of a protective antiviral immune response. The low rate of HBsAg loss and seroconversion with current therapies illustrates the need for new approaches to induce a protective antiviral immune response and durable cure in patients with chronic HBV.

Toll-like receptor (TLR) 7 is a pathogen recognition receptor predominantly expressed in lysosomal/endosomal compartments of plasmacytoid dendritic cells (pDCs) and B lymphocytes that recognizes a pathogen-associated molecular pattern in viral single-stranded
RNA. Upon stimulation of TLR-7, pDCs produce IFN-α and other cytokines/chemokines and cause activation of natural killer (NK) cells and cross-priming of cytotoxic lymphocytes, thereby orchestrating both innate and adaptive immune responses. For these reasons, TLR-7 has been pursued as a therapeutic target for cancer, viral infections, and other diseases. GS-9620 is a potent, orally active TLR-7 agonist with selectivity for induction of IFN-α over proinflammatory cytokines. Here, we demonstrate that a TLR-7 agonist provides therapeutic efficacy for treatment of HBV chronic infection in chimpanzees, the only primate model of persistent HBV infection. The immune modulation induced by activation of TLR-7 resulted in rapid reduction of viremia, reduction in serum HBsAg and HBV e antigen (HBeAg) levels, and an apparent reduction of the numbers of infected hepatocytes with short-term therapy, and provided prolonged suppression of viremia after termination of therapy.

Methods

Animals and Treatment

Chimpanzees were housed at the Southwest National Primate Research Center at Texas Biomedical Research Institute. The animals were cared for in accordance with the Guide for the Care and Use of Laboratory Animals. Details on animal care and animal histories are provided in the Supplementary Materials. The trial design included 4 weeks of prestudy evaluation (days −28, −13, and just before first dose) and 2 cycles of oral GS-9620 treatment every other day. The animals were also intensely monitored for 14 weeks after treatment to assess tolerability and durability of response.

Assays for HBV DNA and Viral Antigens

HBV DNA levels were determined for the serum and liver biopsy samples in multiple assays during the study period. Serum levels were measured by AmpliPrep/COBAS TaqMan HBV Test, v2.0 and by an in-house TaqMan assay, see Supplementary Materials for details. Serum levels of HBsAg and HBeAg were determined by enzyme-linked immunosorbent assay (ETI-MAK-2 PLUS and ETI-EBK PLUS, respectively; DiaSorin, Saluggia, Italy). Immunohistochemical staining was performed on formalin-fixed liver tissue after antigen retrieval as described previously and is further described in the Supplementary Materials.

Quantitation of IFN-Stimulated Gene Transcript Levels by Reverse Transcription Polymerase Chain Reaction

Transcript levels for 2′,5′-oligoadenylate synthetase (OAS-1), MX1, interferon-stimulated gene (ISG) 15, interferon induced T-cell alpha chemoattractant (I-TAC), IFN-inducible protein 10 (IP-10), TLR-7, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were determined by quantitative TaqMan reverse transcription polymerase chain reaction as described previously. Briefly, 200 ng total cell RNA from liver or peripheral blood mononuclear cells (PBMCs) was analyzed by quantitative reverse transcription polymerase chain reaction assay using primers and probe from AB Gene Expression Assays and an ABI 7500 sequence analyzer (Applied Biosystems/Ambion, Austin, TX).

Flow Cytometry

Evaluations of lymphocyte subpopulations were performed using an 11-parameter CyAn ADP Flow Cytometer (Beckman-Coulter Inc, Fullerton, CA). All data were expressed as percentage of lymphocytes that have the specified surface markers. Detailed methods are provided in Supplementary Materials.

Cytokine and Chemokine Analysis

Monitoring of cytokines and chemokines was performed by Luminex 100 with the xMAP (multi-analyte platform) system using a 39-plex human cytokine/chemokine kit (Millipore, Billerica, MA). Dilutions of standards for each cytokine were evaluated in each assay. Cytokines were evaluated in serum samples at 0 and 8 hours post dose.

Results

Pharmacokinetics and Pharmacodynamics of GS-9620 in Uninfected Chimpanzees: Induction of IFN Response and Cytokines-Chemokines by TLR-7 Agonist GS-9620

GS-9620, a potent selective TLR-7 agonist, was designed to have rapid clearance and low-level systemic exposure after oral administration to allow for transient TLR-7 stimulation. Consistent with the selectivity of GS-9620 and the biology of TLR-7, chimpanzee PBMCs stimulated in vitro with GS-9620 displayed a lower minimum effective concentration for IFN-α, chemokines CXCL10 (IP-10), CCL7 (monocyte chemotactic protein [MCP]-3), and CCL4 (macrophage inflammatory protein [MIP]-1β), interleukin (IL)-1 receptor antagonist, and IFN-γ in comparison to proinflammatory cytokines (Supplementary Table 1). In vivo, single, oral doses of GS-9620 at 0.3 and 1 mg/kg in uninfected chimpanzees demonstrated a dose- and exposure-related induction of serum IFN-α, select cytokines/chemokines, and ISG in the peripheral blood and liver. After oral administration at 0.3 mg/kg (n = 3) and 1 mg/kg (n = 3 and n = 4), GS-9620 Cmax was 3.6 ± 3.5 nM, 36.8 ± 34.5 nM, and 55.4 ± 81.0 nM, respectively. Peak serum interferon responses occurred at 8 hours post dose and are shown in Figure 1. Mean peak levels of induced serum IFN-α were 66 pg/mL and 479 pg/mL at doses of 0.3 mg/kg and 1 mg/kg, respectively (Figure 1). GS-9620 treatment induced ISG transcripts, including ISG15, OAS1 MX1, IP-10, and I-TAC in PBMC (Supplementary Table 2) at 0.3 mg/kg and in both PBMC and the liver at 1 mg/kg (Figure 2 and Supplementary Table 2). Serum levels of 42 different cytokines were evaluated. The magnitude and breadth of cytokine induction correlated with GS-9620 dose (Figure 2 and Supplementary Table 3). The 0.3-mg/kg dose induced 3-fold increases in serum IL-7, IL-10, IP-10, fractalkine, IL-1α, IL-1 receptor antagonist, and granulocyte colony-stimulating factor, whereas the 1-mg/kg dose induced 3-fold increases in the same cytokines (except IL-7) and serum IL-12p40, IL-12p70, MCP-1, MCP-3, MIP-1α, MIP-1β, IL-8, IL-1β, IL-6, tumor
necrosis factor–β, and neopterin. GS-9620 was well tolerated in uninfected chimpanzees; the only drug-related changes were transient increases in peripheral blood neutrophils and decreases in lymphocytes, consistent with cell trafficking induced by the cytokines and chemokines mentioned. Based on these data, 1 mg/kg was selected as the starting dose for treatment of HBV-infected animals.

Antiviral Efficacy of TLR-7 Agonist GS-9620 in HBV-Infected Chimpanzees

Therapeutic evaluation was performed in 3 chimpanzees that had chronic HBV infections for >24 years. One chimpanzee (4x0139) had high baseline serum HBV DNA, while the other 2 chimpanzees (4x0328 and 4x0506) had lower HBV DNA levels at baseline (Figure 3 and Supplementary Table 4). Serum levels of HBV DNA declined gradually in all 3 animals during the first treatment cycle, with a 1-log reduction in the high-titer animal (Figure 3A). The second treatment cycle caused a continued but more rapid decline of viral DNA in all 3 animals (Figure 3A–C), with a maximum viral reduction of 2.8 logs and a mean reduction of 2.2 logs (Figure 3 and Supplementary Table 5). Suppression of serum viral DNA levels by >1 log persisted for a minimum of 64 days. The viral load in the high-titer animal (animal 4x0139) was 1.8 logs below baseline at the end of the study, day 121, and remained >1 log below baseline for 280 days after initiation of dosing. The 2 low-viral–load animals returned to within 1 log of baseline within 100 and 71 days of the initiation of dosing, but continued to be suppressed by approximately 1 log for 1 to 2 years after this study.

Treatment also caused a decline in HBV viral DNA in the liver of the high-titer animal (animal 4x0139). The decline in liver HBV DNA paralleled the decline in serum DNA, 1.0 and 2.1 logs at the end of the first and second treatment cycles, respectively. The 2 low HBV DNA titer animals (animals 4x0328 and 4x0506) had very low levels of hepatic HBV DNA at baseline and did not exhibit a significant decline in viral DNA in the liver during therapy (data not shown). The apparent lack of decline in hepatic viral DNA might have been due to limitations in the assay and background in the assay imposed by the presence of integrated viral DNA.

HBsAg and HBeAg are secreted from HBV-infected hepatocytes independent of viral particles and are important clinical markers of infection independent of viral DNA levels. In the high-titer animal (4x0139), GS-9620 treatment reduced HBsAg and HBeAg serum levels by 61% and 93% from baseline, respectively (Figure 3C and D), and levels remained suppressed through post-treatment follow-up. Although the low-titer animals (4x0328 and 4x0506) had low HBsAg levels at baseline, declines of 48% to 60% in HBsAg were observed in both animals during therapy (Figure 3C). One of the low-titer animals (4x0328) was HBeAg positive at baseline and had a decline of 55% in HBeAg, while the other low-titer animal, 4x0506, was anti-HBe–positive at baseline (Supplementary Table 4). The rapid declines in liver viral DNA and secreted viral antigens in the high-titer animal are consistent with an elimination of infected cells, thus we directly examined the elimination of infected cells by immunohistochemical staining of liver sections for HBV core antigen (HBcAg). In the high-titer animal, approximately 30% of hepatocytes were positive for HBcAg staining before
therapy (Figure 3E), and on the last day of dosing, when HBV DNA levels were reduced by >100-fold, few core-positive cells were detected (Figure 3F). These results are in stark contrast to those observed in patients during therapy with nucleos(t)ide analogues, which can reduce serum HBV DNA by ≥4 logs, yet no significant reduction occurs in serum HBsAg or HBcAg-positive hepatocytes over 48 weeks of therapy.20 Unfortunately, the number of HBcAg-positive cells was too low in the low-titer animals to accurately determine the degree of elimination.

**Induction of Cytokines and Chemokines and ISGs by TLR-7 Agonist GS-9620 in HBV-Infected Chimpanzees**

Levels of serum IFN-α and 38 other serum cytokines and chemokines were evaluated at pretreatment and at regular intervals during each treatment cycle. Prestudy IFN-α levels were below the limit of detection in animals 4x0139 and 4x0328, and these animals had dose-dependent increases in IFN-α after administration of GS-9620 at 1 mg/kg (mean, 119 pg/mL) and 2 mg/kg (mean, 700 pg/mL), although increases above baseline were not observed at every time point (Supplementary Table 6). The highest serum levels of IFN-α were induced at the 2-mg/kg dose were 1396 pg/mL and 1545 pg/mL for animals 4x0139 and 4x0328, respectively (Supplementary Tables 7 and 8). The pretreatment baseline level of serum IFN-α was high in animal 4x0506 (1160 pg/mL) and was not further induced by GS-9620 treatment (Supplementary Table 9). This animal also had an elevated pretreatment baseline level of serum IFN-γ, yet GS-9620 treatment induced up to a 53-fold increase in serum levels of IP-10, a chemokine induced by IFN-α and IFN-γ. Of the other 38 cytokines and chemokines examined, during the first treatment cycle (1 mg/kg) only IL-10, MCP-3, and IL-1α were increased 5-fold above baseline, and during the second treatment cycle (2 mg/kg), 13 cytokines and chemokines were induced ≥5-fold; with IL-7, MIP-1β, TNF-α, and granulocyte colony-stimulating factor being induced <10-fold; and IFN-α, IL-10, IP-10, MCP-1, MCP-3, IL-8, IL-1α, IL-1 receptor antagonist, and IL-6 being increased >10-fold (Supplementary Table 6).

Induction of ISG transcripts (ISG15, OAS1, MX1, IP-10, and I-TAC) was evaluated in PBMC and liver biopsy samples, and each was increased in both compartments in response to GS-9620 at both the 1- and 2-mg/kg dose levels; however, induction was not consistently present at the 1-mg/kg dose level for all days evaluated. ISG transcripts are rapidly up-regulated and down-regulated within a few hours of stimulation.21 Variability in the level of response may be technical and related to the use of a single time point to measure a response that may be increasing or decreasing from the maximum value at the time of sampling (8 hours post dose), although exposure to GS-9620 may have varied to some extent after individual doses. Induction of ISGs was both more consistently present and the fold increases were greater at the 2-mg/kg dose (Figure 4 and Supplementary Tables 7, 8, 9, and 10). The group mean increase in transcript levels of the chemokine IP-10 in PBMC was 49.6- and 194-fold during the first (1 mg/kg) and second (2 mg/kg) treatment cycles, respectively (Figure 4 and Supplementary Tables 7, 8, 9, and 10). Interestingly, despite high pretreatment levels of serum IFN-α in animal 4x0506, and no apparent increase in IFN-α levels after GS-9620 administration, GS-9620 administration caused increases in ISGs in both PBMCs and the liver during both treatment cycles in this animal (Supplementary Table 10). Because TLR-7 induction in PBMCs by IFN-α was previously observed in chimpanzees,21 the induction of TLR-7 transcript was measured in this study. At pretreatment, the relative expression of TLR-7 in these chronically infected animals was 30-fold higher in PBMC than liver. TLR-7 levels were increased at multiple time points in the liver during treatment, with a mean maximum induction of 11.9-fold, while increases in PBMC were minimal at most time points, with a mean...
maximum induction 4.4-fold compared with pretreatment levels (Figure 5).

**Activation of T Cells and NK Cells by TLR-7 GS-9620 Agonist in HBV-Infected Chimpanzees**

Because the stimulation of TLR-7 in pDCs can result in the subsequent activation of immune effector cells, we evaluated the activation status of peripheral blood lymphoid and NK cell subsets using cell surface CD69 expression as a biomarker. During the second treatment cycle (2 mg/kg), an increase in the percentage of CD69-expressing CD8-positive T lymphocytes, NK, and NKT cells occurred, which was maximal after the first dose (Supplementary Figure 1). Mean fold increases in the percentage of CD69-positive cells ranged from 3.6 to 5.8 (Supplementary Table 11). No significant increases occurred during the first treatment cycle at 1 mg/kg (Supplementary Table 11).

**Histological Changes in the Liver**

In general, the severity of hepatic inflammation in chimpanzees associated with chronic HBV infection is less than that described in humans. A minimal to mild primarily lymphocytic inflammatory infiltrate in the portal tracts was present in all 3 animals before treatment. Changes noted during treatment included an increased mononuclear cell periportal infiltrate during the first treatment cycle, which, during the second treatment cycle, extended into adjacent hepatic parenchyma and sinusoids. Additionally, there was increased single-cell hepatocyte apoptosis, which was often associated with minimal clusters of mononuclear cells. Histological changes noted during treatment fully reversed within 3 to 5 weeks after treatment. Immunohistochemistry of biopsies at pretreatment and on the last day of therapy demonstrated a marked increase in hepatocellular expression of ISG15 protein, a marker of IFN-α induction (Figure 6), and an in-
creased number of hepatocytes expressing activated caspase 3, a marker for apoptosis. The latter was associated with a correlative increase in hepatocellular regeneration and proliferation as determined by expression of Ki67.

**Correlation of Viral Clearance and Elevation in Liver Enzymes**

GS-9620 therapy was generally well tolerated, and no serious adverse events occurred during therapy. Clinical signs, body temperature, body weight, hematology, and blood chemistries were monitored throughout the study. Body weights were mildly decreased in all 3 animals during the study and recovered during the post-treatment period. Adverse events in the study were limited to anemia

![Figure 4. Induction of ISG transcripts in liver and PBMC during GS-9620 therapy in HBV infected chimpanzees. The levels of transcripts for the ISGs; IP-10, ISG15, and I-TAC were determined by TaqMan quantitative reverse transcription polymerase chain reaction in total cell RNA from liver and PBMC during GS-9620 therapy. Animals were dosed orally 3 times per week for 4 weeks with 1 mg/kg or 2 mg/kg as described in the legend for Figure 3. Transcripts for the housekeeping gene GAPDH were determined as a control for nonspecific stimulation. Levels of transcripts are expressed as relative copy number per microgram of total cell RNA and were determined at 8 hours post dose.](image)

![Figure 5. Induction of TLR-7 transcripts in liver and PBMC of HBV-infected chimpanzees during therapy with GS-9620. Three chimpanzees chronically infected with HBV (4x0139, 4x0328, and 4x0506) were dosed orally 3 times per week for 4 weeks with 1 mg/kg or 2 mg/kg as described in the legend for Figure 3. Increases in TLR-7 transcripts were quantified by TaqMan quantitative reverse transcription polymerase chain reaction in PBMC and liver and are expressed as copy number per microgram of total cell RNA. Values for GAPDH are shown as a control housekeeping gene.](image)
and transient increases in serum liver enzymes. Anemia was mild to moderate in all 3 animals, maximal reductions in red blood cell counts were 11% to 18%, and fully or partially recovered by the end of the study (day 121). Increases in serum levels of the liver enzymes ALT, aspartate aminotransferase, and \( \gamma \)-glutamyl transpeptidase (GGT) occurred during the second treatment cycle (2 mg/kg) (Figure 7). In the HBV high-titer animal (animal 4x0139), a sharp increase in the level of serum ALT and GGT occurred after the first week of treatment at 2 mg/kg, dosing was suspended for this animal for 1 week during which both ALT and GGT rapidly decreased, and then treatment resumed. No further increases in ALT were noted in this animal, however, GGT increases were noted (Figure 4). Liver enzyme elevations fully reversed after treatment; ALT and aspartate aminotransferase returned to pretreatment baseline levels within 3 weeks and GGT by the end of the study. Mild, transient 2- to 3-fold increases in serum total bilirubin occurred at single time points in 2 animals (day 43 in animal 4x0139 and day 57 in animal 4x0506) during treatment at 2 mg/kg and were concurrent with liver enzymes increases.

The transient and low-level single-incidence bilirubin increases were not considered adverse, but warrant monitoring in future clinical trials.

**Discussion**

The ultimate goal of therapy for HBV chronic infection is viral eradication and cure of the underlying liver disease. The greatest advances in therapy have been made with nucleos(t)ide analogues that are chain terminators of the reverse transcription process. Although nucleos(t)ide therapies reduce circulating virus to unde-
tectable levels, they fail to eliminate infected hepatocytes, primarily due to the inability of this approach to eradicate the nonreplicating and stable form of viral DNA in the nucleus, covalently closed circular DNA. Although viral resistance was a major issue with first-generation nucleos(t)ide reverse transcriptase inhibitors, newer analogues such as tenofovir appear to have little if any potential for the development of resistance during long-term therapy. Nonetheless, the percentage of treated patients that develop loss of HBsAg is small, only 10% over 5 years with tenofovir and only 5% in 2 years with entecavir. Pegylated IFN-α therapy suppresses viremia to undetectable levels in only a small percentage of patients during 48 weeks of therapy, yet some patients exhibit apparent cure (no rebound of viremia off of therapy, loss of HBsAg, and seroconversion with detectable antibodies to HBsAg), and the percentage of patients experiencing cure increases for several years after cessation of therapy. These data suggest that the use of improved immunomodulators, such as the TLR-7 agonist GS-9620, can lead to a clinically relevant improvement in therapy, with a significantly higher incidence of viral eradication in patients with chronic HBV infection.

The mechanisms involved in viral clearance during acute and chronic HBV infection have been intensely examined, and although only partially understood, they are believed to be associated with antiviral CD8 T cells trafficking to the liver, the production of IFN-γ, and the induction of immune inflammatory liver disease. The most challenging aspect of viral clearance with HBV is the elimination of nuclear covalently closed circular DNA, which is a nonreplicating DNA that can exhibit stability equal to the lifespan of the hepatocyte. Because 40%-100% of hepatocytes can be infected, a neutralizing antibody response to HBsAg is required to prevent infection of new hepatocytes that arise due to proliferation induced by hepatocyte death and/or uninfected cells arising from cytokine-induced noncytolytic elimination of the infection. Studies in transgenic mice have demonstrated that the innate immune response is capable of noncytolytic elimination of HBV replicative intermediates, and supportive studies in chimpanzees have observed a decrease in viral DNA before lymphocytic infiltration in the liver, suggestive of noncytolytic mechanisms. However, studies in woodchucks during acute infection with woodchuck HBV have concluded that sufficient cell death occurs to account for viral clearance by cytolytic mechanisms, including loss of nuclear covalently closed circular DNA due to cell death and/or cell division. Collectively, these data suggest that both the innate and adaptive immune responses are critical for eradication of HBV infection.

This study demonstrated a long-term benefit from a short duration (8 weeks) of therapy with the oral TLR-7 agonist GS-9620. The mean maximal reduction in serum viral load was 2.2 logs, with a continued suppression of viral DNA by a minimum of 1 log for 2-4 months after dosing. Viral DNA remained suppressed in the high-titer animal for a period of 1 year, while both low-titer animals remained suppressed by approximately 1 log for at least 1 year. Consistent with stimulation of TLR-7 and activation of pDCs, the treatment induced select cytokines, including IFN-α, chemokines, and ISGs in PBMCs and liver. This response led to activation of specific populations of immune effector cells with increased expression of CD69 on CD8+ T and NK cells. Activation of pDCs is known to provide licensing for cross-priming of cytotoxic lymphocytes by classical licensing and NKT cell-dependent alternative licensing, and TLR-activated pDC recruit and activate NK cells to become more cytotoxic in vivo. GS-9620 induced a hepatic infiltration of mononuclear cells, including CD3+ and CD20+ lymphocytes, which, during the second treatment cycle, was also associated with single-cell hepatocellular apoptosis. The reduction in serum and liver HBV DNA was associated with a reduction in serum HBcAg, HBsAg, and HBcAg-positive hepatocytes and a concomitant increase in liver enzymes, hepatic immune cell infiltration, and hepatocellular apoptosis. Thus, the events occurring during therapy with GS-9620 are consistent with induction of antigen-specific T cell responses and NK cell responses with resultant selective killing of HBV-infected cells. The direct suppression of HBV replication by transient induction of type I IFNs and ISGs associated with TLR-7 activation might have contributed to the response, but would not account for the prolonged suppression of viral levels that was durable after cessation of treatment. The decline of serum HBsAg observed in all 3 animals and the apparent loss of core antigen–positive hepatocytes in the high-titer animal are not hallmarks of antiviral therapy with nucleos(t)ide therapy, and suggest a GS-9620 mechanism beyond suppression of viral replication. The results attained in this study are consistent with results we have reported in woodchucks chronically infected with woodchuck hepatitis virus, in which oral treatment with GS-9620 caused a sustained reduction in viral load, loss of woodchuck hepatitis virus surface antigen and seroconversion for antibodies to surface antigen. Collectively, the data support the hypothesis that TLR-7 agonism can induce immune-mediated clearance of infected cells and is a potential therapeutic approach for control or elimination of HBV infection with therapy of finite duration.

In this study, only 3 HBV chronically infected animals were available for the project; all were chronically infected for more than 2 decades. This limited the scope of the study to proof-of-concept that TLR-7 stimulation could impact HBV chronic infection. We were not able to evaluate regimen optimization or combination with direct antiviral therapy or to determine whether extended dosing at the low dose or combination with antiviral agents could result in the same reduction in viral DNA and antigen-positive hepatocytes without an increase in liver enzymes. In this context, it is also important to note that the results of this study presented evidence for a “presystemic” TLR-7 response: low doses of GS-9620 were capable of inducing an antiviral immune response at the level
of the liver, PBMC, and possibly gut-associated lymphoid tissue without induction of systemic IFN-α or its side effects. At the low dose, in both uninfected and infected animals, GS-9620 induced ISGs in the liver and/or in PBMC with no detectable increase in serum levels of IFN-α. Ongoing clinical trials are evaluating whether administration of GS-9620 at presystemic dose levels provides similar antiviral benefits alone, as recognized in this study, or in combination with direct-acting antivirals, without the side effects often associated with systemic administration of IFN-α. Identification of a well-tolerated, finite therapeutic regimen, including the possible combination of immune modulators, such as GS-9620, with direct-acting antiviral agents for the treatment and cure of chronic HBV infection in a significant and clinically relevant percentage of treated patients would be transformative for this disease.

**Supplementary Material**

Note: To access the supplementary material accompanying this article, visit the online version of Gastroenterology at www.gastrojournal.org, and at http://dx.doi.org/10.1053/j.gastro.2013.02.003.

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**Reprint requests**

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Conflicts of interest
These authors disclose the following: Abigail Fosdick, Christian R. Frey, Jim Zheng, Grushenka Wolfgang, Randall L. Halcomb, and Daniel B. Tumas are employees of Gilead Sciences. Robert E. Lanford was funded by Gilead Sciences to conduct this research. The remaining authors disclose no conflicts.

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Supplementary Methods

Animals and Treatment

The care of chimpanzees housed at the Southwest National Primate Research Center at Texas Biomedical Research Institute is detailed in the text. Animals were sedated for all procedures. Some aspects of the HBV infections with animals 4x0139, 4x0328, and 4x0506 have been described previously. The HBV genotype infecting these animals is unknown. However, a portion of the HBV genome isolated from 4x0328 and 4x0506 have been previously sequenced and clustered with other chimpanzee isolates and were most closely related to human genotype F. Animal 4x0139 was infected with HBV in 1979, HCV in 1986, and human immunodeficiency virus (HIV) in 1986. At the time of enrollment, the status of the animal was chronic HBV positive, HCV negative, and HIV negative. This animal was enrolled in other investigational studies in the past and has received treatments, including recombinant plasmid IL-12 in 1999, short-term lamivudine treatment in 2002, and a single dose of pegylated IFN-α treatment in 2006. This animal was also exposed to investigational HBV vaccines between 1999 and 2001. Animal 4x0328 was infected with an unknown source of HBV infection before initial testing for HBsAg in 1986 and is a chronic HBV carrier. This animal has had no prior exposures to HCV or HIV. This animal has in the past been enrolled in other investigational studies and has received treatments including antibodies to HBsAg in 1990, 1994, and 2001, and an experimental antiviral for HBV in 2005, which did not reduce viral load. Animal 4x0506 was infected with an unknown source of HBV before the initial testing for HBsAg in 1983 and is a chronic HBV carrier. This animal has had no prior exposures to HCV or HIV. This animal has in the past been enrolled in other investigational studies and has received treatments, including an HBV vaccine in 1995 and a human monoclonal antibody to HBsAg in 1998.

Clinical Pathology and Urinalysis

At each sedation, an extensive safety profile was performed on each animal, including complete blood count with differential, whole panel blood chemistries, and urinalysis. Coagulation profiles with prothrombin time and activated partial thromboplastin time were performed at selected times during high- and low-dose periods. Blood chemistries were determined with a Unicel DxC 600 Analyzer (Beckman Coulter, Inc, and Diagnostic Chemicals Ltd, Oxford, CT). Coagulation parameters were determined with an ACL 8000 Analyzer using PT-Fibrinogen Kit for prothrombin time, and Simplastin for activated partial thromboplastin time determinations. Values from uninfected animals from the same colony were used to establish normal ranges. Urinalysis was by manual techniques using common dip-stick technology. No drug-related alteration in urinalysis was observed.

Liver Biopsies

Needle biopsies from the liver were taken in anesthetized animals by a standard procedure. Biopsy material was divided immediately into a fraction for histopathology and fractions snap frozen for RNA and DNA extraction. The sections for histopathology were processed for fixation in 10% formalin in phosphate-buffered saline, paraffin embedded, and stained with H&E. Histopathological evaluations were performed by a board-certified veterinarian pathologist (Dr Edward Dick) at Southwest National Primate Research Center under code.

Drug Formulation and Dosing

Final dose formulations for each animal were prepared based on individual animal body weight and the target dose. GS-9620 was diluted to 100 mL in water and administered to a sedated animal by oral gavage. Two dose levels were utilized in the study, 1 and 2 mg/kg. The 1 mg/kg dose was administered 3 times per week for 4 weeks (12 doses; days 1–25), and after a 6-day rest, 2 mg/kg was administered 3 times per week for 4 weeks (12 doses; days 31–57). The exception was animal 4x0139 which, due to increases in serum levels of ALT, was not administered GS-9620 on days 43, 45, and 47; dosing was reinitiated on day 50 in this animal.

Determination of GS-9620 Concentrations in Chimpanzee Serum

An aliquot of 50 μL of each serum sample was treated with 100 μL of acetonitrile containing internal standard. After the protein precipitation, 100 μL of the supernatant was mixed with 100 μL water, and 20 μL of the solution was injected into a C18 HPLC column (30x2.1 mm; ThermoHypersil; #22105-032130) using a TSQ Ultra Quantum LC/MS/MS system. Mobile phases A and B contained 1% and 80% acetonitrile, respectively, in 10 mM ammonium formate aqueous solution containing 1% formic acid.

Assays for Serum and Liver HBV DNA

HBV DNA levels were determined for the serum and liver biopsy samples in multiple assays during the study period. Serum levels were measure by AmpliPrep/COBAS TaqMan HBV Test, v2.0 by the Scott & White Hospital Molecular Pathology Laboratory (Temple, TX) and by an in-house TaqMan assay.1 Liver DNA could not be determined by COBAS and was measured using the in-house TaqMan assay. The Roche COBAS assay computes HBV DNA levels based on the World Health Organization reference standard in international units per milliliter of serum with a lower limit of detection of 20 IU/mL. The in-house TaqMan assay expresses HBV DNA as genome equivalents (GE) per microgram of liver DNA.
For comparison of serum and liver DNA values, 1 IU in the Roche Assay is approximately 5 GE for the in-house TaqMan assay. For the in-house TaqMan assay, DNA was purified from liver biopsies by homogenization in 20 mM Tris pH 8/20 mM EDTA/20 mM NaCl/1% sodium dodecyl sulfate and digestion with Proteinase K for 2 hours at 65°C. Digested samples were sequentially extracted with phenol/chloroform/amy1-alcohol followed by chloroform, and then precipitated with 100% ethanol. DNA samples were analyzed by real-time polymerase chain reaction using TaqMan technology as described previously, with primers and probe designed against the HBV core gene (forward primer, HBV core F-CGAGGCAGGYGATTG; probe, HBV core R-TGC-60°C. Assays using an ABI 7500 sequence detector using the standard curve for each TaqMan assay ranging from 10 to 1 million GE. Samples were analyzed in TaqMan duplicate extractions were performed for liver, and DNA purification. If sufficient liver biopsy sample was available, duplicate extractions were performed for liver, and DNA from each extraction was run in duplicate. A plasmid containing an HBV DNA insert was used to generate a standard curve for each TaqMan assay ranging from 10 GE to 1 million GE. Samples were analyzed in TaqMan assays using an ABI 7500 sequence detector using the following cycle parameters: 2 minutes at 50°C/10 minutes at 95°C/45 cycles of 15 seconds at 95°C/1 min at 60°C.

Quantitation of ISG Transcript Levels by Reverse Transcription Polymerase Chain Reaction

The transcript levels for OAS, MX1, ISG15, I-TAC, IP-10, TLR-7, and GAPDH were determined by quantitative reverse transcription polymerase chain reaction (qRT-PCR) as described previously. Briefly, 200 ng of total cell RNA from liver or PBMC was analyzed by qRT-PCR assay using primers and probe from AB Gene (forward primer, HBV core F-CGAGGCAGGTCCCTAGAAG; reverse primer, HBV core R-TGC-36-TAMSP/3’). Each serum sample was extracted in duplicate for DNA purification. If sufficient liver biopsy sample was available, duplicate extractions were performed for liver, and DNA from each extraction was run in duplicate. A plasmid containing an HBV DNA insert was used to generate a standard curve for each TaqMan assay ranging from 10 GE to 1 million GE. Samples were analyzed in TaqMan assays using an ABI 7500 sequence detector using the following cycle parameters: 2 minutes at 50°C/10 minutes at 95°C/45 cycles of 15 seconds at 95°C/1 min at 60°C.

Flow Cytometry

Evaluations of lymphocyte subpopulations were performed on days −28, 7, 14, 25, 45, and 57 by the Southwest National Primate Research Center Immunology Core Laboratory using an 11-parameter CyAn ADP Flow Cytometer (Beckman–Coulter Inc, Fullerton, CA). All data were expressed as percentage of lymphocytes that have the specified surface markers. For example, T cells were expressed as the percentage of lymphocytes that were CD3+. The lymphocyte population was determined based on forward and side scatter and this gated population was used for all subsequent analysis. The following subpopulations were examined for activation based on reactivity to a panel of monoclonal antibodies: B lymphocytes: CD20+ cells for increased expression of CD69, CD86, and HLA-DR (MFI); NK cells: CD16+CD56+ cells for increased expression of CD69; NKT cells: CD16+CD56+CD3+ cells for increased expression of CD69 and CD25; NK8 cells: CD16+CD56+CD3−CD8− cells for increased expression of CD69; T lymphocytes: CD3+CD4+CD8− for increased expression of CD69, CD25, and FoxP3. The following antibody reagents were utilized for fluorescence-activated cell sorting: CD25-APC (BC96; Biolegend, San Diego, CA), CD3-Alx700 (Sp34-2; BD Biosciences), CD4-PerCP-Cy5.5 (SK3; BD Biosciences), CD69-PacBlue (FN50; Biolegend), CD8-FITC (SK1; BD Biosciences), CD8-PerCP-Cy5.5 (SK1; BD Biosciences), CD56-Alx488 (B159; BD Biosciences), CD69-PacBlue (FN50; Biolegend), CD8-FITC (SK1; BD Biosciences), CD8-PerCP-Cy5.5 (SK1; BD Biosciences), CD56-PE (B159; BD Biosciences), CD86-PE (2331; FUN-1; BD Biosciences), CD16-FITC (3G8; BD Biosciences), HLA-DR-Alx700 (B159; BD Biosciences), CD86-9K (259D; Biolegend). The following antibody reagents were utilized for fluorescence-activated cell sorting: CD25-APC (BC96; Biolegend, San Diego, CA), CD3-Alx700 (Sp34-2; BD Biosciences), CD4-PerCP-Cy5.5 (SK3; BD Biosciences), CD69-PacBlue (FN50; Biolegend), CD8-FITC (SK1; BD Biosciences), CD8-PerCP-Cy5.5 (SK1; BD Biosciences), CD56-Alx488 (B159; BD Biosciences), CD69-PacBlue (FN50; Biolegend), CD8-FITC (SK1; BD Biosciences), CD8-PerCP-Cy5.5 (SK1; BD Biosciences), CD56-PE (B159; BD Biosciences), CD86-PE (2331; FUN-1; BD Biosciences), CD16-FITC (3G8; BD Biosciences), HLA-DR-Alx700 (L243; Biolegend), CD20-APC (2H7; BD Biosciences), and FOXp3-PE (259D; Biolegend).

Immunohistochemical Staining

Liver biopsies were fixed in buffered-formalin, paraffin embedded, and sectioned at 4 µm. Slides were de-paraffinized in EZ-DeWax (BioGenex; HK 585-5K) 2 times for 5 minutes and rinsed with water. Antigen retrieval was performed in a microwave pressure cooker for 15 minutes at 1000 watts and 15 minutes at 300 watts in citrate buffer (antigen retrieval solution; BioGenex; HK 086-9K) as described previously. Cooled slides were rinsed with water and phosphate-buffered saline and treated sequentially with peroxidase suppressor, universal block, and avidin (all reagents from Pierce 36000 Immunohisto Peroxidase Detection Kit; Pierce, Rockford, IL). Slides were incubated sequentially for 1 hour at room temperature with primary antibody diluted in universal block containing a biotin block, for 0.5 hour with biotinylated goat anti-mouse IgG, and for 0.5 hour with avidin-biotin complex. Slides were developed with ImmPact Nova Red peroxidase substrate (SK-4805; Vector, Burlingame, CA), counterstained with Mayers (Lillie’s) hematoxy-
lin (DAKO, Glostrup, Denmark; S3309), dehydrated, and mounted in nonaqueous mounting media (VectaMount, H-5000; Vector). Rabbit anti-HBV core was prepared from purified core particles expressed in baculovirus. Rabbit monoclonal antibody 5A1E (Cell Signaling, Danvers, MA; 9964) was used at 1/50 dilution to detect activated caspase 3. Mouse monoclonal M1B-1(DAKO; #7240) was used at 1/100 dilution to detect Ki67. Rabbit polyclonal H-150 (Santa Cruz Biotechnology, Santa Cruz, CA; sc-50366) was used at 1/400 dilution to detect ISG15.

**Supplementary References**


### Supplementary Table 1.

**Individual Animal and Mean GS-9620 In Vitro MEC Data for Cytokine Induction in Chimpanzee PBMC Cultures**

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>GS-9620 MEC (nM) of individual chimpanzees</th>
<th>GS-9620 MEC Mean ± SD (nM)</th>
<th>Range of GS-9620 MEC (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4x0427</td>
<td>4x0428</td>
<td>4x0437</td>
</tr>
<tr>
<td>IFN-α</td>
<td>370</td>
<td>370</td>
<td>370</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>1100</td>
<td>1100</td>
<td>370</td>
</tr>
<tr>
<td>IL-12p40</td>
<td>10,000</td>
<td>10,000</td>
<td>370</td>
</tr>
<tr>
<td>IL-12p70</td>
<td>&gt;30,000</td>
<td>1100</td>
<td>370</td>
</tr>
<tr>
<td>sIL-2 RA</td>
<td>10,000</td>
<td>10,000</td>
<td>3300</td>
</tr>
<tr>
<td>IL-10</td>
<td>3300</td>
<td>3300</td>
<td>3300</td>
</tr>
<tr>
<td>IP-10 (CXCL10)</td>
<td>370</td>
<td>370</td>
<td>370</td>
</tr>
<tr>
<td>MCP-3 (CCL7)</td>
<td>370</td>
<td>370</td>
<td>370</td>
</tr>
<tr>
<td>MIP-1α (CCL3)</td>
<td>3300</td>
<td>&gt;30,000</td>
<td>370</td>
</tr>
<tr>
<td>MIP-1β (CCL4)</td>
<td>370</td>
<td>1100</td>
<td>1100</td>
</tr>
<tr>
<td>IL-4</td>
<td>&gt;30,000</td>
<td>&gt;30,000</td>
<td>10,000</td>
</tr>
<tr>
<td>IL-5</td>
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<td>&gt;30,000</td>
</tr>
<tr>
<td>IL-6</td>
<td>10,000</td>
<td>10,000</td>
<td>3300</td>
</tr>
<tr>
<td>IL-7</td>
<td>10,000</td>
<td>10,000</td>
<td>3300</td>
</tr>
<tr>
<td>IL-13</td>
<td>&gt;30,000</td>
<td>&gt;30,000</td>
<td>1100</td>
</tr>
<tr>
<td>IL-1α</td>
<td>10,000</td>
<td>10,000</td>
<td>370</td>
</tr>
<tr>
<td>IL-1β</td>
<td>3300</td>
<td>10,000</td>
<td>3300</td>
</tr>
<tr>
<td>IL-1 RA</td>
<td>370</td>
<td>1100</td>
<td>370</td>
</tr>
<tr>
<td>TNF-α</td>
<td>370</td>
<td>3300</td>
<td>1100</td>
</tr>
<tr>
<td>TNF-β</td>
<td>3300</td>
<td>10,000</td>
<td>3300</td>
</tr>
</tbody>
</table>

**NOTE.** Assay was done in duplicate. The following cytokines were omitted due to minimal or no response, IL-2, -3, -8, -9, -15, -17, sCD40L, fractalkine, MCP-1 (monocyte chemoattractant protein-1), MDC (macrophage derived chemokine), Flt-3 ligand (fms-related tyrosine kinase), eotaxin, granulocyte colony-stimulating factor, granulocyte-macrophage colony-stimulating factor, epidermal growth factor, fibroblast growth factor 2, transforming growth factor 2, and GRO (CXCL1).

MEC, minimum effective concentration; RA, receptor antagonist; SD, standard deviation; TNF, tumor necrosis factor.

### Supplementary Table 2.

**Increase in ISG Transcripts in PBMC and Liver in Uninfected Chimpanzees After a Single Oral Dose of GS-9620**

<table>
<thead>
<tr>
<th>RNA</th>
<th>Dose (mg/kg)</th>
<th>PMBCs</th>
<th>Liver</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Group Mean ± SD</td>
<td>Range of fold increase</td>
<td>Group Mean ± SD</td>
</tr>
<tr>
<td>ISG15</td>
<td>0.3</td>
<td>23 ± 14</td>
<td>8.4-36</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>77 ± 74</td>
<td>4.3-166</td>
</tr>
<tr>
<td>OAS-1</td>
<td>0.3</td>
<td>4.4 ± 2.0</td>
<td>2.5-6.5</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>6.7 ± 4.3</td>
<td>2.1-14</td>
</tr>
<tr>
<td>MX1</td>
<td>0.3</td>
<td>8.6 ± 3.8</td>
<td>2.5-6.5</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>13 ± 9.3</td>
<td>2.7-18</td>
</tr>
<tr>
<td>IP-10</td>
<td>0.3</td>
<td>45 ± 50</td>
<td>3.5-101</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>50 ± 40</td>
<td>1.7-116</td>
</tr>
<tr>
<td>I-TAC</td>
<td>0.3</td>
<td>9.8 ± 7.2</td>
<td>4.2-18</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>33 ± 40</td>
<td>3.0-102</td>
</tr>
</tbody>
</table>

**NOTE.** Assay was done in duplicate. The following cytokines were omitted due to minimal or no response, IL-2, -3, -8, -9, -15, -17, sCD40L, fractalkine, MCP-1 (monocyte chemoattractant protein-1), MDC (macrophage derived chemokine), Flt-3 ligand (fms-related tyrosine kinase), eotaxin, granulocyte colony-stimulating factor, granulocyte-macrophage colony-stimulating factor, epidermal growth factor, fibroblast growth factor 2, transforming growth factor 2, and GRO (CXCL1).

MEC, minimum effective concentration; RA, receptor antagonist; SD, standard deviation; TNF, tumor necrosis factor.

**NOTE.** Assay was done in duplicate. The following cytokines were omitted due to minimal or no response, IL-2, -3, -8, -9, -15, -17, sCD40L, fractalkine, MCP-1 (monocyte chemoattractant protein-1), MDC (macrophage derived chemokine), Flt-3 ligand (fms-related tyrosine kinase), eotaxin, granulocyte colony-stimulating factor, granulocyte-macrophage colony-stimulating factor, epidermal growth factor, fibroblast growth factor 2, transforming growth factor 2, and GRO (CXCL1).

MEC, minimum effective concentration; RA, receptor antagonist; SD, standard deviation; TNF, tumor necrosis factor.

**NOTE.** Assay was done in duplicate. The following cytokines were omitted due to minimal or no response, IL-2, -3, -8, -9, -15, -17, sCD40L, fractalkine, MCP-1 (monocyte chemoattractant protein-1), MDC (macrophage derived chemokine), Flt-3 ligand (fms-related tyrosine kinase), eotaxin, granulocyte colony-stimulating factor, granulocyte-macrophage colony-stimulating factor, epidermal growth factor, fibroblast growth factor 2, transforming growth factor 2, and GRO (CXCL1).

MEC, minimum effective concentration; RA, receptor antagonist; SD, standard deviation; TNF, tumor necrosis factor.

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MEC, minimum effective concentration; RA, receptor antagonist; SD, standard deviation; TNF, tumor necrosis factor.
### Supplementary Table 3. Increase in Serum Cytokine and Chemokine Levels in Uninfected Chimpanzees After a Single Oral Dose of GS-9620

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>0.3 mg/kg dose group&lt;sup&gt;a&lt;/sup&gt;</th>
<th>1 mg/kg dose group&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean cytokine Cmax ± SD (pg/mL)</td>
<td>Fold increase (Mean Cmax Compared to Pre-dose Mean)</td>
</tr>
<tr>
<td><strong>Antiviral Cytokines</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFN-α</td>
<td>66 ± 69</td>
<td>4</td>
</tr>
<tr>
<td><strong>Immunomodulatory Cytokines</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFN-γ</td>
<td>0 ± 0</td>
<td>&lt;3</td>
</tr>
<tr>
<td>IL-7</td>
<td>6.0 ± 10</td>
<td>4</td>
</tr>
<tr>
<td>IL-10</td>
<td>12 ± 8.3</td>
<td>4</td>
</tr>
<tr>
<td>IL-12p40</td>
<td>4.5 ± 7.3</td>
<td>&lt;3</td>
</tr>
<tr>
<td>IL-12p70</td>
<td>0.3 ± 0.5</td>
<td>&lt;3</td>
</tr>
<tr>
<td><strong>Chemokines</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IP-10</td>
<td>1003 ± 831</td>
<td>8</td>
</tr>
<tr>
<td>Fractalkine (CX3CL1)</td>
<td>92 ± 110</td>
<td>5</td>
</tr>
<tr>
<td>MCP-1 (CCL2)</td>
<td>452 ± 268</td>
<td>&lt;3</td>
</tr>
<tr>
<td>MCP-3 (CCL7)</td>
<td>3.7 ± 1.7</td>
<td>&lt;3</td>
</tr>
<tr>
<td>MIP-1α (CCL3)</td>
<td>0 ± 0</td>
<td>&lt;3</td>
</tr>
<tr>
<td>MIP-1β (CCL4)</td>
<td>44 ± 8.1</td>
<td>&lt;3</td>
</tr>
<tr>
<td>IL-8 (CXCL8)</td>
<td>14 ± 5.9</td>
<td>&lt;3</td>
</tr>
<tr>
<td><strong>Acute Phase Response Cytokines</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-1α</td>
<td>140 ± 91</td>
<td>4</td>
</tr>
<tr>
<td>IL-1 Receptor Antagonist</td>
<td>20 ± 11</td>
<td>4</td>
</tr>
<tr>
<td>IL-1β</td>
<td>0.1 ± 0.1</td>
<td>&lt;3</td>
</tr>
<tr>
<td>IL-6</td>
<td>9.2 ± 5.9</td>
<td>NC</td>
</tr>
<tr>
<td>TNF-β</td>
<td>0.1 ± 0.2</td>
<td>NC</td>
</tr>
<tr>
<td><strong>Hematopoietic Cytokines</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G-CSF</td>
<td>44 ± 18</td>
<td>3</td>
</tr>
<tr>
<td><strong>Other</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neopterin</td>
<td>2.2 ± 0.2</td>
<td>&lt;3</td>
</tr>
</tbody>
</table>

BLD, below limit of detection; G-CSF, granulocyte colony-stimulating factor; NC, not calculated due to division by 0; baseline sample below limit of detection; TNF, tumor necrosis factor.

<sup>a</sup>Mean and standard deviation from n = 3 animals.

<sup>b</sup>Mean and standard deviation from n = 7 animals for combined experiments A and B. The same 3 animals were part of the 0.3-mg/kg dose group and both A and B 1-mg/kg dose groups. The 1-mg/kg B dose group included 1 additional animal, for a total of 7 animals in the combined 1-mg/kg dose groups.

### Supplementary Table 4. Baseline Characteristics of HBV-Infected Chimpanzees

<table>
<thead>
<tr>
<th>Animal ID</th>
<th>HBV DNA (GE/mL)</th>
<th>HBeAg</th>
<th>Anti-HBeAg</th>
<th>Anti-HBcAg</th>
<th>Sex</th>
<th>HBV infection (years)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4x0139</td>
<td>6.5x10&lt;sup&gt;7&lt;/sup&gt;</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>F</td>
<td>30</td>
</tr>
<tr>
<td>4x0328</td>
<td>2.5x10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>M</td>
<td>&gt;24</td>
</tr>
<tr>
<td>4x0506</td>
<td>1.6x10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>F</td>
<td>&gt;27</td>
</tr>
</tbody>
</table>

NOTE. HBV DNA levels were determined using TaqMan quantitative polymerase chain reaction as described in Methods and are expressed as genome equivalents per mL (GE/mL). HBeAg levels and antibody to HBeAg and HBcAg were determined by enzyme-linked immunosorbent assay. The exact times of infection of 4x0328 and 4x0506 are unknown and are stated as before the first time the animals were tested in an HBsAg assay.
### Supplementary Table 5. Viral Load Reduction in HBV-Infected Chimpanzees During GS-9620 Therapy

<table>
<thead>
<tr>
<th>Animal ID</th>
<th>Maximal viral load reduction (Log)</th>
<th>Day of maximal viral load reduction (Study Day)</th>
<th>Duration of viral load reduction(a) (Days)</th>
<th>Mean maximal log viral load reduction</th>
<th>Mean duration of viral load reduction(a) (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4x0139</td>
<td>2.8</td>
<td>54</td>
<td>&gt;121</td>
<td>2.2 ± 0.6</td>
<td>&gt;95 ± 29(b)</td>
</tr>
<tr>
<td>4x0328</td>
<td>1.9</td>
<td>64</td>
<td>100</td>
<td>1.4 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>4x0506</td>
<td>1.9</td>
<td>54</td>
<td>64</td>
<td>1.9 ± 0.3</td>
<td></td>
</tr>
</tbody>
</table>

\(a\)Viral DNA levels were determined using the Roche COBAS AmpliPrep/COBAS TaqMan HBV Test, v2.0. Viral load reduction and standard deviation were based on comparison to pretreatment levels on day 13. The duration of viral load reduction was determined as the number of days with viral load reduction of at least one log.

\(b\)Animal 4x0139 had a >1 log reduction in serum viral load at the last day of evaluation.

### Supplementary Table 6. Induction of Serum Cytokines and Chemokines in HBV-Infected Chimpanzees During GS-9620 Therapy

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>1 mg/kg dose level</th>
<th>2 mg/kg dose level</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean cytokine Cmax ± SD (pg/mL)(a)</td>
<td>Fold increase (Mean Cmax)</td>
</tr>
<tr>
<td>Antiviral Cytokines</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IFN-(\alpha)</td>
<td>119</td>
<td>12</td>
</tr>
<tr>
<td>Immunomodulatory Cytokines</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IL-7</td>
<td>14.4 ± 10.5</td>
<td>3</td>
</tr>
<tr>
<td>IL-10</td>
<td>12.4 ± 6.3</td>
<td>7</td>
</tr>
<tr>
<td>Chemokines</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IP-10</td>
<td>1280 ± 369</td>
<td>3</td>
</tr>
<tr>
<td>MCP-1 (CCL2)</td>
<td>2040 ± 1770</td>
<td>4</td>
</tr>
<tr>
<td>MCP-3 (CCL7)(b)</td>
<td>12.1</td>
<td>6</td>
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<tr>
<td>MIP-1(\beta) (CCL4)</td>
<td>364 ± 251</td>
<td>2</td>
</tr>
<tr>
<td>IL-8 (CXCL8)(b)</td>
<td>52.3</td>
<td>2</td>
</tr>
<tr>
<td>Acute Phase Response Cytokines</td>
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<td></td>
</tr>
<tr>
<td>IL-1(\alpha)</td>
<td>23.2</td>
<td>7</td>
</tr>
<tr>
<td>IL-1 Receptor Antagonist</td>
<td>26.4 ± 30</td>
<td>2</td>
</tr>
<tr>
<td>IL-6(b)</td>
<td>4.5</td>
<td>2</td>
</tr>
<tr>
<td>TNF-(\alpha)</td>
<td>16.6 ± 8.4</td>
<td>2</td>
</tr>
<tr>
<td>Hematopoietic Cytokines</td>
<td></td>
<td></td>
</tr>
<tr>
<td>G-CSF</td>
<td>105 ± 59.4</td>
<td>2</td>
</tr>
</tbody>
</table>

\(a\)Mean and standard deviations of the mean values attained for each animal at 6 doses; days 7, 14, and 25 for 1 mg/kg doses and days 31, 45, and 57 for 2 mg/kg doses. Day 45 was not included for animal 4x0139 because GS-9620 was not administered to this animal on days 43, 45, and 47.

\(b\)Animal 4x0506 was not included in calculations of IFN-\(\alpha\), IL-1\(\alpha\), IL-6, MCP-3, and IL-8, as it had very high levels of these cytokines on days −28 and −13 before GS-9620 treatment.
### Supplementary Table 7. Fold Increase of ISGs in PBMC and Liver in 4x0139 During GS-9620 Therapy

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Tissue</th>
<th>Dose (mg/kg)</th>
<th>Day 7</th>
<th>Day 14</th>
<th>Day 25</th>
<th>Day 31</th>
<th>Day 45</th>
<th>Day 57</th>
</tr>
</thead>
<tbody>
<tr>
<td>GS-9620 Cmax (nM)</td>
<td>Serum</td>
<td>1</td>
<td>2.1</td>
<td>2.9</td>
<td>3.7</td>
<td>45.5</td>
<td>ND</td>
<td>70.6</td>
</tr>
<tr>
<td>IFN-α (pg/ml)/a</td>
<td>Serum</td>
<td>25</td>
<td>BLD</td>
<td>BLD</td>
<td>1396</td>
<td>ND</td>
<td>487</td>
<td></td>
</tr>
<tr>
<td>ISG15/b</td>
<td>PBMC</td>
<td>3.5</td>
<td>2.2</td>
<td>1.2</td>
<td>73</td>
<td>&lt;1</td>
<td>65.1</td>
<td></td>
</tr>
<tr>
<td>OAS1</td>
<td>Liver</td>
<td>1</td>
<td>5.3</td>
<td>5.7</td>
<td>39</td>
<td>4.5</td>
<td>30.6</td>
<td></td>
</tr>
<tr>
<td>MX1</td>
<td>PBMC</td>
<td>1.5</td>
<td>1.3</td>
<td>&lt;1</td>
<td>9.1</td>
<td>&lt;1</td>
<td>8.7</td>
<td></td>
</tr>
<tr>
<td>IP-10</td>
<td>Liver</td>
<td>1.3</td>
<td>2.5</td>
<td>1.2</td>
<td>13</td>
<td>2.9</td>
<td>19</td>
<td></td>
</tr>
<tr>
<td>I-TAC</td>
<td>PBMC</td>
<td>3.8</td>
<td>2.7</td>
<td>&lt;1</td>
<td>23</td>
<td>1</td>
<td>26.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Liver</td>
<td>1.4</td>
<td>2.7</td>
<td>2.3</td>
<td>51.9</td>
<td>2.6</td>
<td>34.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PBMC</td>
<td>1.5</td>
<td>3.5</td>
<td>1</td>
<td>118</td>
<td>1</td>
<td>232</td>
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<tr>
<td></td>
<td>Liver</td>
<td>1.3</td>
<td>1.7</td>
<td>1</td>
<td>15.8</td>
<td>1</td>
<td>183</td>
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<tr>
<td></td>
<td>PBMC</td>
<td>&lt;1</td>
<td>1.2</td>
<td>90</td>
<td>ND</td>
<td>183</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Liver</td>
<td>&lt;1</td>
<td>1.1</td>
<td>41</td>
<td>ND</td>
<td>70</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

BLD, below the limit of detection; ND, not done; OAS-1 2′,5′-oligoadenylate synthetase.
aIFN-α was measured in serum at 2 and 8 hours post dose and the peak level is reported.
bRNA was isolated from liver at 8 hours post dose and from PBMCs at 0 and 8 hours post dose on indicated days. The fold increase of ISGs was determined by comparison to a day −28 prestudy sample for liver and an average of the day −28 and day −13 prestudy samples for PBMC.
cAnimal 4x0139 was not administered GS-9620 on days 43, 45, and 47.

### Supplementary Table 8. Fold Increase of ISGs in PBMC and Liver in 4x0328 During GS-9620 Therapy

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Tissue</th>
<th>Dose (mg/kg)</th>
<th>Day 7</th>
<th>Day 14</th>
<th>Day 25</th>
<th>Day 31</th>
<th>Day 45</th>
<th>Day 57</th>
</tr>
</thead>
<tbody>
<tr>
<td>GS-9620 Cmax (nM)</td>
<td>Serum</td>
<td>1</td>
<td>11.7</td>
<td>6.2</td>
<td>0.8</td>
<td>39.7</td>
<td>31.8</td>
<td>57.2</td>
</tr>
<tr>
<td>IFN-α (pg/ml)/a</td>
<td>Serum</td>
<td>635</td>
<td>26/b</td>
<td>30/b</td>
<td>1545</td>
<td>34/b</td>
<td>38</td>
<td></td>
</tr>
<tr>
<td>ISG15/c</td>
<td>PBMC</td>
<td>105</td>
<td>1.8</td>
<td>2.1</td>
<td>187</td>
<td>5.9</td>
<td>6.2</td>
<td></td>
</tr>
<tr>
<td>OAS1</td>
<td>Liver</td>
<td>34.2</td>
<td>5.2</td>
<td>2.3</td>
<td>80</td>
<td>9.1</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>MX1</td>
<td>PBMC</td>
<td>9</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>13</td>
<td>1.5</td>
<td>2.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Liver</td>
<td>12</td>
<td>1.6</td>
<td>&lt;1</td>
<td>13</td>
<td>2.9</td>
<td>5.3</td>
<td></td>
</tr>
<tr>
<td>IP-10</td>
<td>PBMC</td>
<td>22.7</td>
<td>1.5</td>
<td>1.3</td>
<td>28</td>
<td>3</td>
<td>5.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Liver</td>
<td>31.6</td>
<td>2.3</td>
<td>2.2</td>
<td>50.4</td>
<td>4.9</td>
<td>6.8</td>
<td></td>
</tr>
<tr>
<td>I-TAC</td>
<td>PBMC</td>
<td>304</td>
<td>1.7</td>
<td>&lt;1</td>
<td>536</td>
<td>1</td>
<td>56.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Liver</td>
<td>29</td>
<td>1.6</td>
<td>&lt;1</td>
<td>42.2</td>
<td>2.2</td>
<td>44.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>PBMC</td>
<td>67</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>245</td>
<td>2.7</td>
<td>8.2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Liver</td>
<td>5.2</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>14</td>
<td>2.5</td>
<td>17</td>
<td></td>
</tr>
</tbody>
</table>

/aIFN-α was measured in serum at 2 and 8 hours post dose and the peak level is reported.
bIFN-α was not increased at least 3-fold from predose for that day.
cRNA was isolated from liver at 8 hours post dose and from PBMCs at 0 and 8 hours post dose on indicated days. The fold increase of ISGs was determined by comparison to a day −28 prestudy sample for liver and an average of the day −28 and day −13 prestudy samples for PBMC.
### Supplementary Table 9. Fold Increase of ISGs in PBMC and Liver in 4x0506 During GS-9620 Therapy

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Tissue</th>
<th>Day 7</th>
<th>Day 14</th>
<th>Day 25</th>
<th>Day 31</th>
<th>Day 45</th>
<th>Day 57</th>
</tr>
</thead>
<tbody>
<tr>
<td>GS-9620 Cmax (nM)</td>
<td>Serum</td>
<td>4.5</td>
<td>1.8</td>
<td>35.9</td>
<td>30</td>
<td>14</td>
<td>104</td>
</tr>
<tr>
<td>IFN-α (pg/ml)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Serum</td>
<td>1214&lt;sup&gt;b&lt;/sup&gt;</td>
<td>956&lt;sup&gt;b&lt;/sup&gt;</td>
<td>972&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1127&lt;sup&gt;c&lt;/sup&gt;</td>
<td>644&lt;sup&gt;b&lt;/sup&gt;</td>
<td>647&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>ISG15&lt;sup&gt;c&lt;/sup&gt;</td>
<td>PBMC</td>
<td>7.9</td>
<td>5.8</td>
<td>12</td>
<td>40</td>
<td>2.3</td>
<td>41.5</td>
</tr>
<tr>
<td></td>
<td>Liver</td>
<td>3.6</td>
<td>3.8</td>
<td>6.1</td>
<td>14</td>
<td>6.6</td>
<td>18.7</td>
</tr>
<tr>
<td>OAS1</td>
<td>PBMC</td>
<td>4.1</td>
<td>3.2</td>
<td>3.4</td>
<td>8.6</td>
<td>1.7</td>
<td>8.2</td>
</tr>
<tr>
<td></td>
<td>Liver</td>
<td>2.4</td>
<td>2.4</td>
<td>2.2</td>
<td>5.4</td>
<td>2.5</td>
<td>8.1</td>
</tr>
<tr>
<td>MX1</td>
<td>PBMC</td>
<td>6.4</td>
<td>5</td>
<td>6</td>
<td>15</td>
<td>2</td>
<td>24.2</td>
</tr>
<tr>
<td></td>
<td>Liver</td>
<td>3.3</td>
<td>3.1</td>
<td>3.4</td>
<td>13.6</td>
<td>4.1</td>
<td>26.2</td>
</tr>
<tr>
<td>IP-10</td>
<td>PBMC</td>
<td>19.9</td>
<td>22.8</td>
<td>87</td>
<td>80</td>
<td>3</td>
<td>548</td>
</tr>
<tr>
<td></td>
<td>Liver</td>
<td>&lt;1</td>
<td>1</td>
<td>1</td>
<td>7.9</td>
<td>4.4</td>
<td>47.6</td>
</tr>
<tr>
<td>I-TAC</td>
<td>PBMC</td>
<td>2.1</td>
<td>1.7</td>
<td>3.3</td>
<td>8.8</td>
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<td>&lt;1</td>
<td>&lt;1</td>
<td>6</td>
<td>6.3</td>
<td>58</td>
</tr>
</tbody>
</table>

<sup>a</sup>IFN-α was measured in serum at 2 and 8 hours post dose and the peak level is reported.

<sup>b</sup>IFN-α was 1160 pg/mL at baseline and was not increased by GS-9620 administration.

<sup>c</sup>RNA was isolated from liver at 8 hours post dose and from PBMCs at 0 and 8 hours post dose on indicated days. The fold increase of ISGs was determined by comparison to a day −28 prestudy sample for liver and an average of the day −28 and day −13 prestudy samples for PBMC.

### Supplementary Table 10. Mean Fold Increase in ISGs in PBMC and Liver During GS-9620 Therapy

<table>
<thead>
<tr>
<th>ISG</th>
<th>GS-9620 1 mg/kg&lt;sup&gt;a&lt;/sup&gt;</th>
<th>GS-9620 2 mg/kg&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ISG mean fold increase ± SD</td>
<td>ISG mean fold increase ± SD</td>
</tr>
<tr>
<td></td>
<td>PBMC</td>
<td>Liver</td>
</tr>
<tr>
<td>ISG15</td>
<td>16 ± 18</td>
<td>7.5 ± 6.8</td>
</tr>
<tr>
<td>OAS1</td>
<td>2.8 ± 1.4</td>
<td>3.0 ± 1.7</td>
</tr>
<tr>
<td>MX1</td>
<td>5.6 ± 3.0</td>
<td>5.8 ± 5.4</td>
</tr>
<tr>
<td>IP-10</td>
<td>49.6 ± 49.7</td>
<td>4.3 ± 5.4</td>
</tr>
<tr>
<td>I-TAC</td>
<td>8.9 ± 12.3</td>
<td>1.5 ± 0.8</td>
</tr>
</tbody>
</table>

<sup>a</sup>Days 7, 14, and 25.

<sup>b</sup>Days 31, 45, and 57. Values for animal 4x0139 from day 45 were not included in these calculations as the animal was not administered GS-9620 on days 43, 45, and 47.

For values in Supplementary Tables 6, 7, and 8 for which fold increases were <1, a value of 1 was used for the purpose of mean calculation.
Supplementary Figure 1. Induction of CD69 activation marker in lymphocyte subsets during GS-9620 therapy. PBMCs were examined by flow cytometry on day 31, the first dose at 2 mg/kg, at 0 and 8 hours post dose. Plots illustrate the level of fluorescence for the activation marker CD69 in T cells (CD3<sup>+</sup>/H11001<sup>+</sup>), NK cells (CD16<sup>+</sup>/H11001<sup>+</sup>, CD56<sup>+</sup>/H11001<sup>+</sup>, CD3<sup>+</sup>), and NKT cells (CD16<sup>+</sup>/H11001<sup>+</sup>, CD56<sup>+</sup>/H11001<sup>+</sup>, CD3<sup>+</sup>). The horizontal bar indicates the cutoff for positive response in comparison to the isotype control. The percentage of positive cells are shown for 0 and 8 hours in the upper quadrant of each plot.
**Supplementary Table 11.** Increase in CD69$^+$ Positive Lymphocytes in PBMC during GS-9620 Therapy

<table>
<thead>
<tr>
<th>Cell type and marker</th>
<th>GS-9620 Dose (mg/kg)</th>
<th>Animal</th>
<th>Day</th>
<th>0 hr Pre-Dose</th>
<th>8 hr Post-Dose</th>
<th>Fold Increase</th>
<th>Group mean fold Increase ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>T Lymphocytes CD3$^+$</td>
<td>1 4x0139 7 2.93 3.67 1.3</td>
<td>1.2 ± 0.2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>4x0328 7 3.66 4.60 1.3</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>4x0506 7 4.85 4.75 1.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2 4x0139 31 3.69 14.78 4.0</td>
<td>3.6 ± 2.2</td>
<td></td>
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<td>4x0328 31 5.63 31.49 5.6</td>
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<td></td>
</tr>
<tr>
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<td>4x0506 31 5.44 6.28 1.2</td>
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<td></td>
</tr>
<tr>
<td>T Lymphocytes CD4$^+$</td>
<td>1 4x0139 7 3.57 2.59 0.7</td>
<td>0.9 ± 0.2</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td></td>
<td>4x0328 7 3.44 3.82 1.1</td>
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</tr>
<tr>
<td></td>
<td>4x0506 7 4.17 3.63 0.9</td>
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</tr>
<tr>
<td></td>
<td>2 4x0139 31 3.20 1.83 0.6</td>
<td>2.7 ± 3.1</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>4x0328 31 4.55 28.25 6.2</td>
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</tr>
<tr>
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<td>4x0506 31 4.27 4.91 1.2</td>
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<tr>
<td>T Lymphocytes CD8$^+$</td>
<td>1 4x0139 7 3.61 3.39 0.9</td>
<td>1.1 ± 0.2</td>
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<tr>
<td></td>
<td>2 4x0139 31 3.75 13.24 3.5</td>
<td>3.1 ± 1.8</td>
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<tr>
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<td>4x0506 31 6.07 6.67 1.1</td>
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<tr>
<td>B Lymphocytes CD20$^+$</td>
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<td>3.4 ± 1.9</td>
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<td>4x0328 7 1.15 3.67 3.2</td>
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</tr>
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<td></td>
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<tr>
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<td>2 4x0139 31 3.71 9.09 2.5</td>
<td>1.8 ± 0.7</td>
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<td></td>
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<tr>
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<td></td>
</tr>
<tr>
<td></td>
<td>4x0506 31 6.03 9.55 1.6</td>
<td></td>
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</tr>
<tr>
<td>NK CD16$^+$ CD56$^+$ CD3$^-$</td>
<td>1 4x0139 7 9.15 7.87 0.9</td>
<td>1.4 ± 0.5</td>
<td></td>
<td></td>
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</tr>
<tr>
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<td>4x0328 7 12.72 22.37 1.8</td>
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</tr>
<tr>
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<td>4x0506 7 8.31 11.25 1.4</td>
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</tr>
<tr>
<td></td>
<td>2 4x0139 31 5.87 36.01 6.1</td>
<td>5.0 ± 1.0</td>
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<td>4x0328 31 14.03 63.44 4.5</td>
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</tr>
<tr>
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<td>4x0506 31 7.02 30.48 4.3</td>
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NOTE. Peripheral blood mononuclear cells were examined by flow cytometry at 0 and 8 hours post dose to determine the increase in percent CD69$^+$ cells in different lymphocyte subsets in comparison to an isotype control. SD, standard deviation; Treg, regulatory T cells.