

# Immune protection against reinfection with nonprimate hepacivirus

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**Hepatitis C virus (HCV) displays a restricted host species tropism and only humans and chimpanzees are susceptible to infection. A robust immunocompetent animal model is still lacking, hampering mechanistic analysis of virus pathogenesis, immune control, and prophylactic vaccine development. The closest homolog of HCV is the equine nonprimate hepacivirus (NPHV), which shares similar features with HCV and thus represents an animal model to study hepacivirus infections in their natural hosts. We aimed to dissect equine immune responses after experimental NPHV infection and conducted challenge experiments to investigate immune protection against secondary NPHV infections. Horses were i.v. injected with NPHV containing plasma. Flow cytometric analysis was used to monitor immune cell frequencies and activation status. All infected horses became viremic after 1 or 2 wk and viremia could be detected in two horses for several weeks followed by a delayed seroconversion and viral clearance. Histopathological examinations of liver biopsies revealed mild, periportal accentuated infiltrations of lymphocytes, macrophages, and plasma cells with some horses displaying subclinical signs of hepatitis. Following viral challenge, an activation of equine immune responses was observed. Importantly, after a primary NPHV infection, horses were protected against rechallenge with the homologous as well as a distinct isolate with only minute amounts of circulating virus being detectable.**

hepatitis C virus | nonprimate hepacivirus | infection | immune protection | rechallenge

Hepatitis C virus (HCV) infections constitute a major global health problem with ~130 million people being chronically infected (1). Once infected, 70–90% of individuals progress to chronicity, rendering HCV the leading cause of liver diseases, including liver fibrosis, cirrhosis, and hepatocellular carcinoma (2). HCV is a blood-borne, positive-stranded RNA virus classified to the family of Flaviviridae within the genus *Hepacivirus*. Seven different HCV genotypes and numerous subtypes have been described, which differ on the nucleotide level by ~30–35% between different genotypes and maximally 30% between subtypes (3). Furthermore, within infected individuals the virus circulates as a population of closely related but distinct genomes (4). Recently, significant progress has been made regarding the treatment of HCV with the implementation of direct-acting antiviral (DAA) drugs, which can be administered as all-oral IFN-free therapeutics and should help to reduce the global burden of HCV infections (5). However, there are still many caveats, including high treatment costs and the risk of reinfection after successful therapy, which is a problem especially in patient populations with frequent exposure to HCV. Furthermore, the inadequacy of current HCV

screening programs often results in late diagnosis of chronic HCV infection and subsequent proceeding to end-stage liver diseases (6). All of these challenges highlight the necessity for a prophylactic vaccine against HCV, which despite extensive research remains elusive. This fact is partly due to the extensive heterogeneity of the virus, contributing to its ability to effectively escape immunity, facilitating viral persistence (7). Furthermore, the narrow host tropism of HCV for humans and the associated lack of an immunocompetent small animal model hamper vaccine development. Previous studies used chimpanzees (*Pan troglodytes*), which were the only fully immunocompetent animal model experimentally susceptible to HCV infection. These studies allowed analysis of viral pathogenesis and immune control and

## Significance

**Hepatitis C virus (HCV) displays a narrow species tropism severely hampering development of small animal models that are required for vaccine and pathogenesis studies in vivo. The recent discoveries of HCV-related hepaciviruses in diverse hosts offer new opportunities with respect to the development of an immunocompetent animal model for HCV research. Among the hepaciviruses, the equine nonprimate hepacivirus (NPHV) represents the closest homolog of HCV discovered to date. We defined key aspects of natural immunity to NPHV challenge in the cognate host and provide evidence for natural protection from NPHV infection. Further characterization of the immune signatures that confer protection against NPHV could provide important information that may facilitate the development of new prophylactic strategies including protective vaccines against HCV.**

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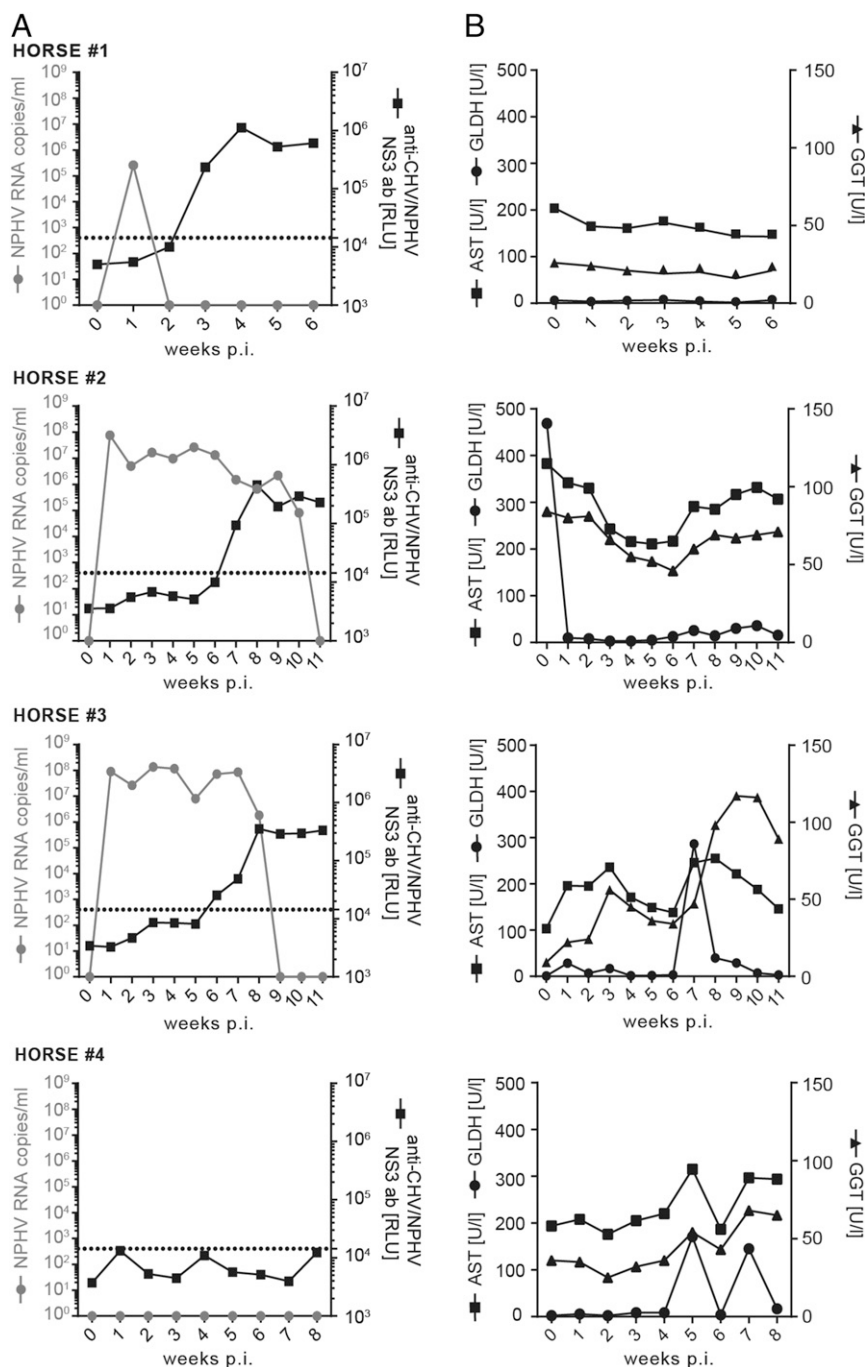
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contributed to vaccine development (8). Recently, multiple novel animal hepaciviruses naturally infecting various species have been identified (9, 10). Viruses classified within the genus *Hepacivirus* have been discovered in dogs (11) and subsequently in horses (12), rodents (13–15), bats (16), Old World primates (17), cattle (18), and recently catsharks (19). These viruses display different homologies to HCV and offer new insights into the origin and

evolution of *Hepaciviruses*. Furthermore, given the relationship of these animal viruses to HCV, they could facilitate the generation of immunocompetent surrogate animal models for HCV vaccine research. Among these newly discovered animal hepaciviruses, the equine nonprimate hepacivirus (NPHV) represents the closest homolog of HCV discovered to date. The virus was initially discovered in dogs (11); however, it was later shown to primarily



**Fig. 1.** Course of infection after experimental inoculation with NPHV<sup>+</sup> plasma. Horse 1 was i.v. transfused with 500 mL NPHV containing plasma, whereas horses 2 and 3 were i.v. transfused with 100 mL NPHV containing plasma (GenBank accession no. KY124246, viral load  $7.78 \times 10^6$  RNA copies per milliliter). Horse 4 was i.v. transfused with 100 mL NPHV<sup>-</sup> plasma. Serum samples were taken on a weekly basis postinoculation (weeks p.i.). (A) The viral load of NPHV RNA was determined by qRT-PCR and is depicted as gray dots (limit of quantification 50 RNA copies per serum sample). Anti-CHV (canine hepacivirus)/NPHV NS3 antibodies were measured by the luciferase immunoprecipitation (LIPS) assay and are depicted in black squares as relative light units (RLUs). A cutoff was calculated by the mean value of samples containing only buffer A, the renilla luciferase-NS3 (Ruc-NS3) fusion protein and A/G beads plus three SDs and is indicated by a dotted line. (B) Liver-specific enzymes in the serum were measured. Reference values are as follows: GLDH < 6 U/L, GGT < 20 U/L, and AST < 170 U/L.

infect horses with a high seroprevalence of ~35% (12). NPHV shares a sequence homology of ~50% to HCV (11). Several similarities to HCV could be observed upon equine hepacivirus infection. Similar to HCV, NPHV causes both acute and chronic infections in horses; although unlike HCV, the majority of animals are able to clear the infection (20–22). Experimental blood transmission has been shown, indicating that NPHV might be a blood-borne pathogen (23). Importantly, hepatotropism has been demonstrated for NPHV with viral replication occurring exclusively within the liver (20, 22, 23). Moreover, key viral evasion strategies are conserved between HCV and NPHV, as for instance, both viral proteases cleave their cognate innate immune adaptor protein MAVS (24, 25). Given the close similarities between HCV and NPHV, we aimed to investigate NPHV infection in the natural host to determine the course of infection and equine immune responses after experimental NPHV infection. Furthermore, we conducted challenge infections to investigate immune protection against secondary NPHV infections, elucidate hepaciviral biology, and the suitability of horses as surrogate models for HCV research.

## Results

**Horses Can Be Experimentally Infected with NPHV and Display Similar Hepatic Membrane Rearrangements to HCV.** To study NPHV infections *in vivo*, we experimentally challenged three NPHV RNA<sup>-</sup> horses that were devoid of serum markers of a previous NPHV infection with different doses of NPHV-containing plasma (Fig. 1A). Horse 1 was *i.v.* challenged with 500 mL of donor plasma (GenBank accession no. KY124246, viral load  $7.78 \times 10^6$  RNA copies per milliliter), whereas horses 2 and 3 received 100 mL of the same donor plasma each. Horse 4 received 100 mL of NPHV<sup>-</sup> plasma as control. Horses were monitored on a weekly basis for viral loads, seroconversion, and indications of liver injury. All NPHV-infected horses became viremic after 1 wk as determined by NPHV-specific quantitative RT-PCR (qRT-PCR) (Fig. 1A). Viral titers ranged between  $10^2$  copies per milliliter and  $10^5$  copies per milliliter. Horse 1 rapidly cleared the infection within 1 wk, whereas horses 2 and 3 stayed viremic for 8–10 wk before virus was cleared. A delayed seroconversion was observed in all horses with the appearance of NPHV-specific antibodies 3–7 wk postchallenge. The liver-specific enzymes  $\gamma$ -glutamyltransferase (GGT) and glutamate dehydrogenase (GLDH), which are specific markers of acute hepatocellular damage, and aspartate aminotransferase (AST), which complements the parameters in the screening enzymes, were monitored over the course of infection. Serum levels of liver enzymes stayed mainly within reference range with a mild elevation in horse 2 and horse 3 at seroconversion (Fig. 1B). Histopathological examinations revealed mild, multifocal, lymph–plasma–histiocytic infiltrates in periportal areas (Fig. S1A). Furthermore an intracytoplasmic, coarsely granular, yellow–brown pigment (hemosiderin) was multifocally demonstrated in hepatocytes, macrophages, and Kupffer cells at varying intensities. In addition, a mild, diffuse, hepatocellular vacuolation (most likely lipid storage) was found in one case (horse 3). Moreover, one biopsy exhibited single, multifocal, degenerating hepatocytes with an associated mild, lymphohistiocytic inflammation and a mild bile duct hyperplasia 1 (horse 2). In one biopsy, mild, multifocal, necrosuppurative hepatitis was present (horse 3). Similarly, a NPHV negative control horse displayed mild, multifocal, lymphohistiocytic infiltrates with single eosinophils in periportal areas and low amounts of intracytoplasmic deposits of a coarsely granular, yellow–brown pigment in hepatocytes, macrophages, and Kupffer cells. Furthermore, single, multifocal, degenerating hepatocytes with an associated mild neutrophilic inflammation were seen in the control animal. Immunohistochemical characterization of periportal infiltrates of NPHV-infected horses revealed 88.66–97.18% CD3<sup>+</sup> T cells, 0.00–0.59% Pax-5<sup>+</sup> B cells, and 2.23–11.34% MAC387<sup>+</sup> macrophages (Fig. S1B–D). Comparable results were obtained for periportal infiltrates of the control horse, where 92.1% CD3<sup>+</sup> T cells, 0.85% Pax-5<sup>+</sup> B cells, and 7.05% MAC387<sup>+</sup> macrophages were found (Fig. S1B–D).

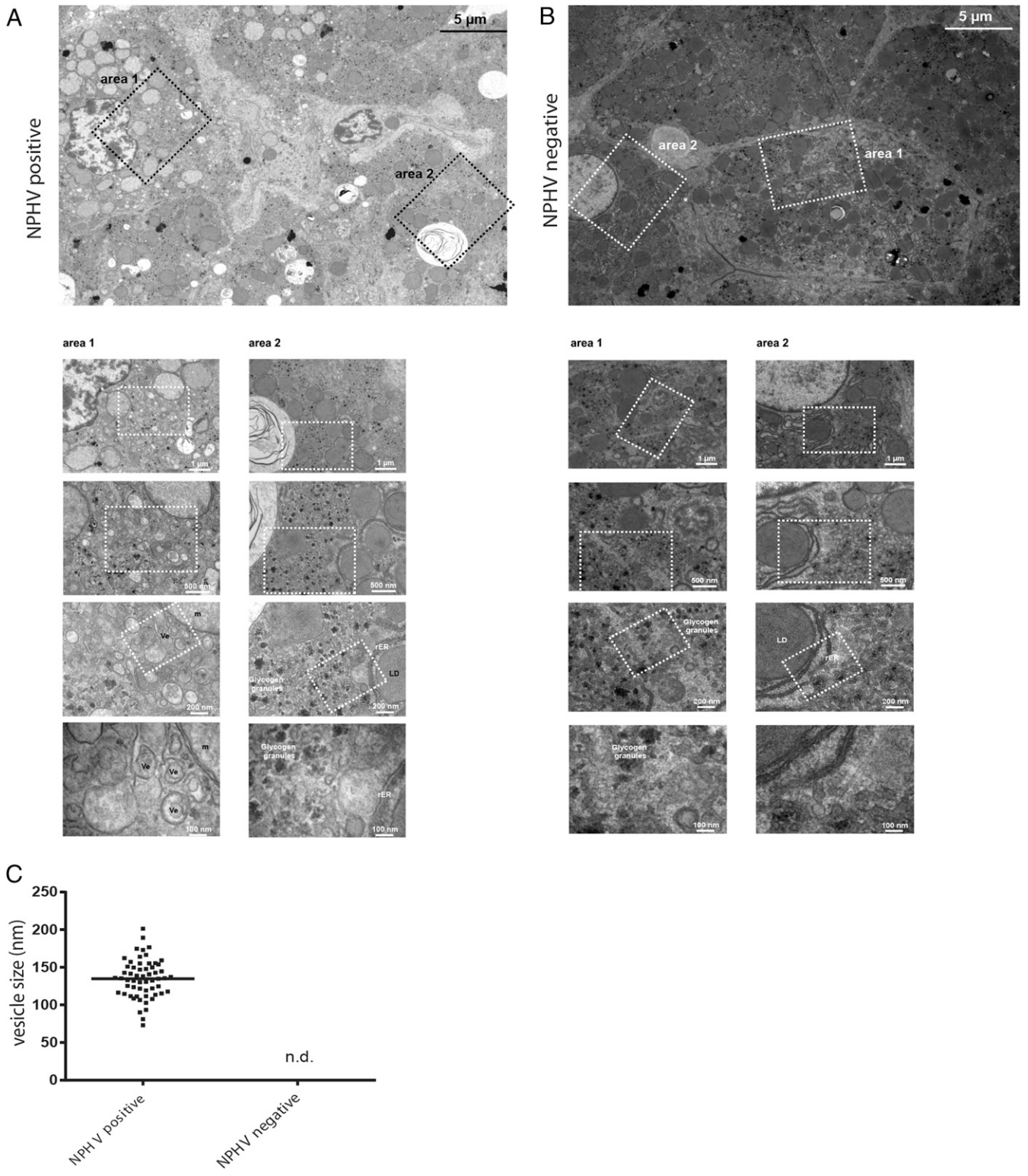
Hepatitis C virus is known to induce accumulations of vesicles forming a membranous web (MW) (26), consisting of mainly double membrane vesicles (DMVs) that are thought to be the site of viral RNA replication within the infected liver (27, 28). To examine whether MWs are formed *in vivo* within the liver of NPHV<sup>+</sup> horses, we performed electron microscopy (EM)-based studies on liver biopsies of an infected horse, and in various cells, we observed numerous vesicles (Ve), which could not be detected in an uninfected horse (Fig. 2A–C). These vesicles had an average diameter of about 120 nm and in some cases a double lipid bilayer could be observed, reminiscent of DMVs in HCV-infected cells (Fig. 2A). Furthermore, liver cells from the uninfected horse depicted a much more homogeneous appearance (Fig. 2B), in comparison with NPHV-infected liver cells (Fig. 2A). Importantly, these vesicles were also visible during a natural NPHV infection (Fig. S2A and B), indicating that NPHV and HCV induce similar intracellular membrane rearrangements.

**Experimentally Infected Horses Mount Immune Responses Against NPHV.** To better understand immune responses to NPHV infections, which could contribute to viral clearance, we established flow cytometric assays to monitor immune cell frequencies and activation status. We isolated peripheral blood mononuclear cells (PBMCs) from horses on a weekly basis and established a gating strategy to distinguish between CD3<sup>+</sup>CD4<sup>+</sup> T-helper cells, CD3<sup>+</sup>CD8<sup>+</sup> cytotoxic T cells, dendritic cells (DCs), B cells, and monocytes/granulocytes (Fig. 3A). Using this approach, we could analyze the peripheral immune cell frequency of individual subtypes; however, we did not observe any striking differences between individual horses (Fig. S3). Intracellular IFN- $\gamma$  expression was monitored in individual immune cell subtypes to analyze the activation status. Interestingly, all NPHV-infected horses (horses 1–3) displayed an elevated frequency of IFN- $\gamma$ -expressing monocytes/granulocytes following infection, indicating an activation of the innate immune response after experimental infection, which could not be observed to a similar extent in the negative control horse (Fig. 3B). To further analyze equine immune responses, we measured serum cytokine levels using a fluorescent bead-based multiplex assay. Serum cytokine levels varied between the individual horses. In two horses (horses 2 and 3) we observed a transient elevation of serum IL-4 and IFN- $\gamma$  3 wk *p.i.*; however, similar levels could also be observed in the negative control horse arguing for an NPHV-independent immune reaction (Fig. S4).

In humans and chimpanzees, a strong peripheral and intrahepatic T-cell immune response appears to be associated with HCV clearance (29). To analyze the impact of T cells on NPHV clearance, we established a NPHV-specific T-cell proliferation assay. We stimulated *ex vivo* isolated PBMCs with eight overlapping peptide pools spanning the whole nonstructural 3 (NS3) region of NPHV (Fig. 4A) and measured cell proliferation upon decrease of the fluorescence dye eFluor 670 (Fig. 4B). PBMCs were stimulated with PHA (phytohemagglutinin-M) as positive control (Fig. 4B). We gated on CD3<sup>+</sup>CD4<sup>+</sup> T cells (Fig. 4C) and CD3<sup>+</sup>CD8<sup>+</sup> T cells (Fig. 4D) and determined the respective proliferation as stimulation index (SI). No distinct NPHV-specific peripheral T-cell proliferation could be observed in horses 1 and 2 and the negative control horse 4; however, a weak CD3<sup>+</sup>CD8<sup>+</sup> T-cell proliferation could be detected in horse 3 (Fig. 4D). Taken together, we established assays to analyze peripheral immune responses in horses and could show a moderate immune activation following experimental NPHV infection in horses, although differences between individuals were observed.

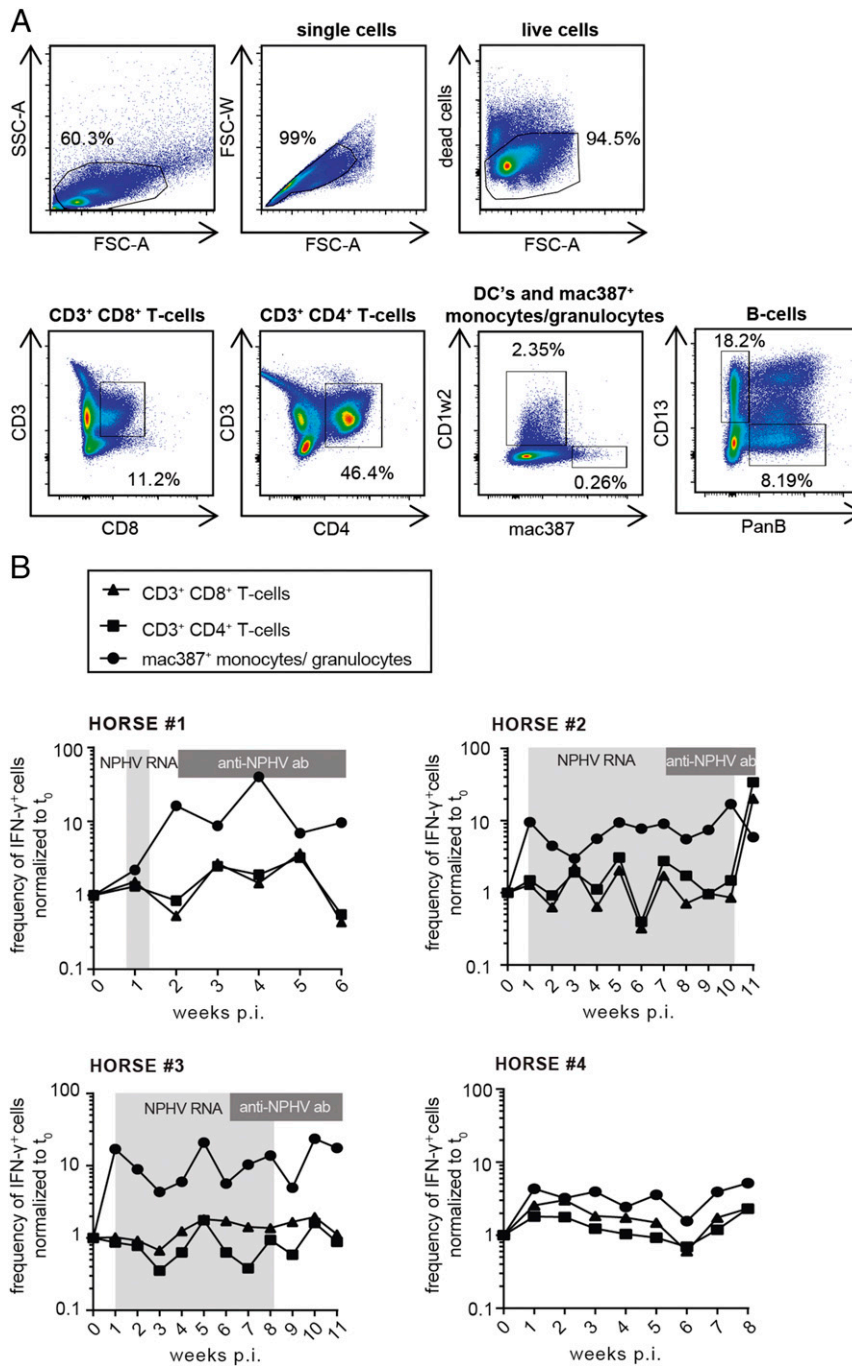
**Horses Are Protected Against Rechallenge with Homologous or Distinct NPHV Isolates.** Previous studies in chimpanzees and also reports from infected patients have shown that HCV reinfection is possible after resolving a primary HCV infection (30–32). To analyze whether horses are protected against a secondary NPHV reinfection, we





**Fig. 2.** Ultrastructural analysis of horse liver biopsies. Biopsy samples taken from the liver of one NPHV<sup>-</sup> horse as well as from a positive control horse experimentally infected with NPHV (Fig. 6) were immediately fixed with glutaraldehyde. Samples were sectioned in small pieces and, subsequently, processed and analyzed by transmission electron microscopy (TEM) as described in *SI Materials and Methods*. EM micrographs of (A) a liver biopsy of an experimentally NPHV-infected horse, 7 wk postinfection, and (B) a NPHV<sup>-</sup> horse. An overview of several liver cells is depicted at *Top*. Higher magnification images of two different areas are shown at *Bottom*. The white boxed areas highlight the areas that are shown at *Bottom* as magnified views. (C) Quantification of vesicle sizes (in nanometers) from an experimentally NPHV-infected horse ( $n = 55$ ) compared with a NPHV<sup>-</sup> horse. Ve, vesicle; LD, lipid droplet; m, mitochondria; rER, rough endoplasmic reticulum; n.d., not detected; nm, nanometer.

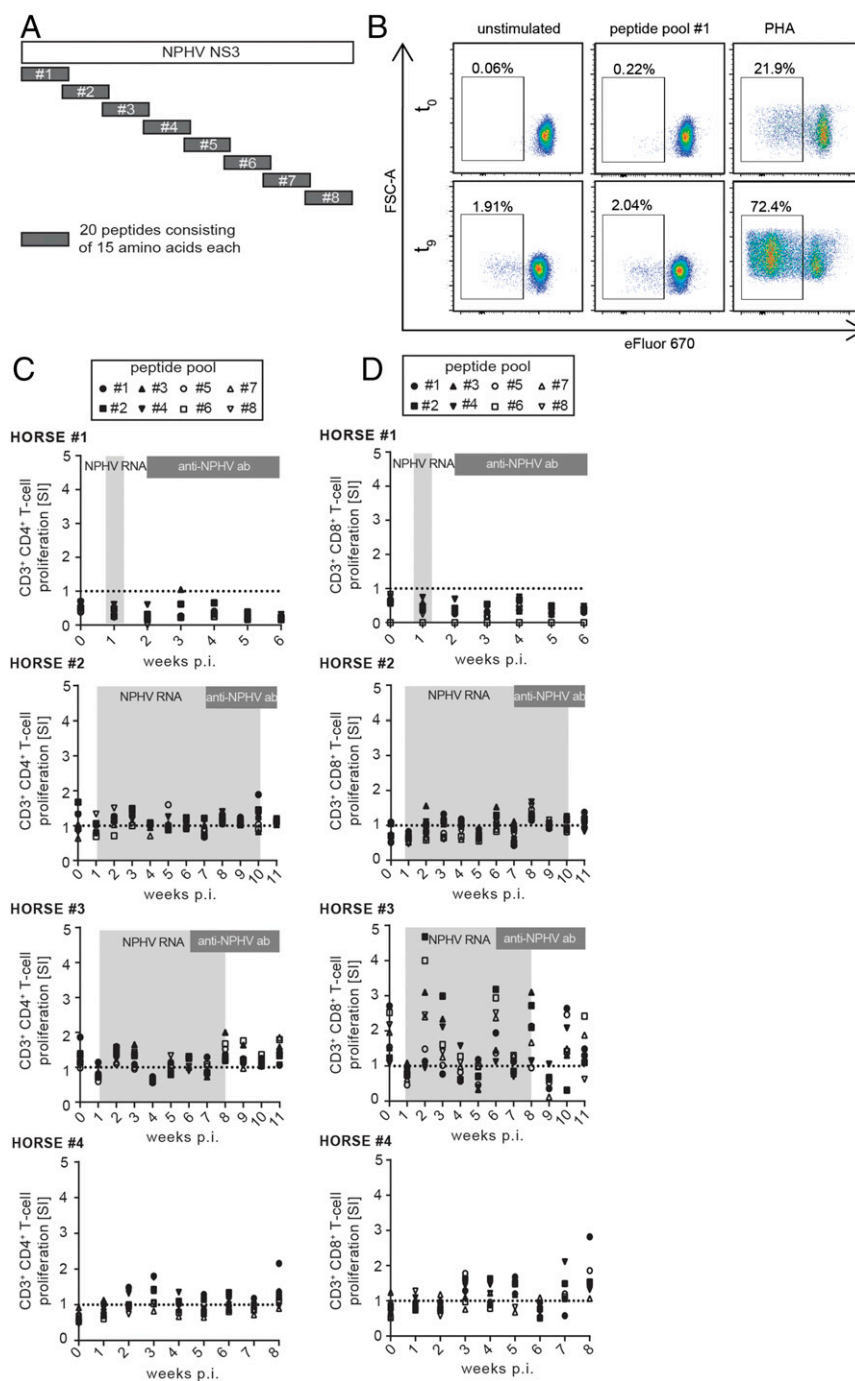
experimentally rechallenge two of the horses, which had previously resolved a NPHV infection (horses 2 and 3) with 100 mL of the homologous virus isolate (GenBank accession no. KY124246, viral load  $7.78 \times 10^6$  RNA copies per milliliter). Inclusion of a previously



**Fig. 3.** Development of a flow cytometry-based method to measure immune cell frequencies and intracellular IFN- $\gamma$  expression. PBMCs were isolated on a weekly basis postinoculation by centrifugation on a Ficoll-Hypaque density gradient and stored at  $-150^{\circ}\text{C}$  before flow cytometry staining. PBMCs were stained for the surface markers CD8, CD4, CD3, PanB, CD13, CD1w2, and Mac387 using equine-specific antibodies to detect T lymphocytes (CD3 $^{+}$ CD4 $^{+}$  T-helper cells, CD3 $^{+}$ CD8 $^{+}$  cytotoxic T cells), B lymphocytes (PanB $^{+}$ ), CD13 $^{+}$  cells (expressed on blood neutrophils, basophils, monocytes, but not B or T cells), dendritic cells (CD1w2 $^{+}$ ), and monocytes/granulocytes (Mac387 $^{+}$ ), respectively. Additionally, intracellular IFN- $\gamma$  staining was performed on all cells. Samples were analyzed by flow cytometry. (A) Exemplary depiction of the gating strategy. Dead cells as well as cell duplets were excluded before subsequent gating on the respective immune cell subset. (B) The frequency of IFN- $\gamma$ -expressing immune cells (CD3 $^{+}$ CD4 $^{+}$  T cells, CD3 $^{+}$ CD8 $^{+}$  T cells, and mac387 $^{+}$  monocytes/granulocytes) of each time point postinoculation normalized to  $t_0$  is depicted for all four horses. Time points that tested positive for NPHV RNA and anti-CHV/NPHV NS3 antibodies are indicated in light- and dark-gray boxes, respectively.

naïve horse (positive control horse) confirmed that the used plasma was still infectious (Fig. 5A, Lower graph). Both rechallenged horses retained a high NPHV-specific antibody titer before secondary challenge (naïve vs.  $t = 0$ ; Fig. 5A). Interestingly, no productive infection could be established in the rechallenged animals with only trace amounts of viral RNA being detectable (gray boxes and agarose gel) (Fig. 5A). Detected viral genomes were sequenced to

confirm homologous genome identity. NPHV-specific antibody titers further increased in the rechallenged horses 3–6 wk postinfection (Fig. 5A). Liver-specific enzymes mainly stayed within the reference range (Fig. 5B) and histopathological examination revealed mild, multifocal, lymph-plasma-histiocytic infiltrates in periportal areas. Furthermore, an intracytoplasmic, coarsely granular, yellow-brown pigment (hemosiderin) was multifocally demon-



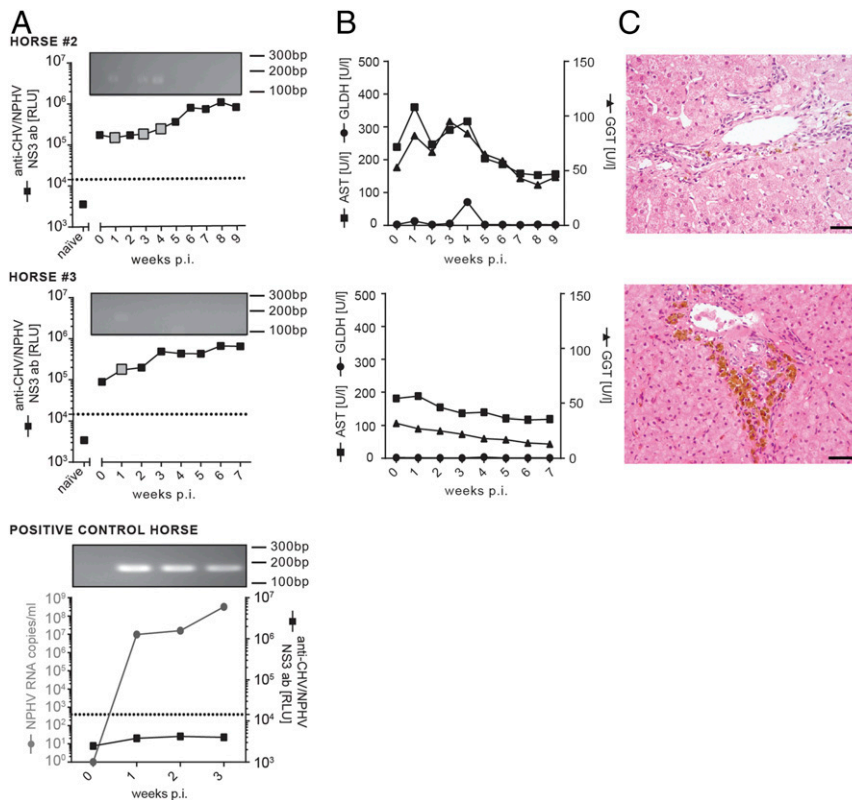
**Fig. 4.** Analysis of NPHV-specific T-cell proliferation. (A) Eight distinct but overlapping peptide pools originating from the NS3 region of NPHV were synthesized based on the NS3 reference sequence of NPHV isolate H10 (GenBank accession no. KP640276). Individual peptides were pooled with each pool containing 20 peptides consisting of 10 overlapping amino acids each. Isolated PBMCs were stimulated in duplicates with the peptide pools and incubated for 7 d at 37 °C before flow cytometry analyses. As positive control, PBMCs were stimulated with PHA. (B) Gating strategy after flow cytometry-based analysis of T-cell proliferation. Seven days postinfection, PBMCs were stained for CD3, CD4, and CD8 surface markers to gate on the respective cell population as described above. A decrease in the proliferation dye eFluor 670 indicates proliferation of the respective population. Representative plots of horse 2 at  $t_0$  and  $t_9$  are shown. The amount of proliferating CD3<sup>+</sup>CD4<sup>+</sup> (C) and CD3<sup>+</sup>CD8<sup>+</sup> (D) T cells was calculated by dividing the stimulated samples by the unstimulated control of each time point depicted as stimulation index (SI). The respective values of each peptide pool are indicated with a single symbol for all of the respective time points measured in duplicates. The dotted line indicates the unstimulated control. Time points that tested positive for NPHV RNA and anti-CHV/NPHV NS3 antibodies are indicated in light- and dark-gray boxes, respectively.

strated in hepatocytes, macrophages, and Kupffer cells in varying intensities (Fig. 5C).

To examine the breadth of immune protection, we rechallenge the same horses (horses 2 and 3) with 100 mL of a distinct NPHV isolate (GenBank accession no. KY124248,

viral load  $6.0 \times 10^5$  RNA copies per milliliter) that differs from the primary inoculum at 11.54% of nucleotide sites of the near-completely sequenced E1E2 glycoproteins, thus mimicking a rechallenge with a NPHV subtype (Fig. 6A). Similar to the first rechallenge, previously infected horses were protected against





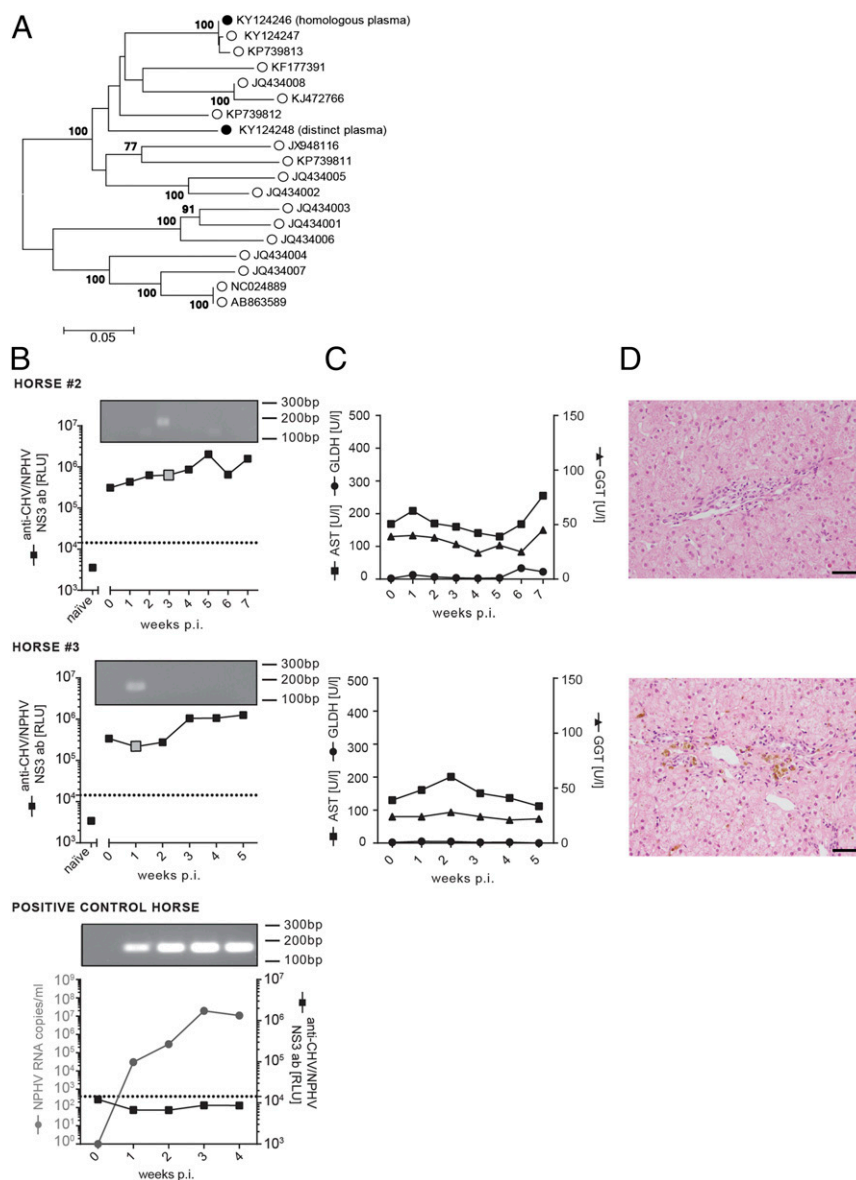
**Fig. 5.** Homologous NPHV reinfection of horses. Horses 2 and 3 were rechallenged ~5 mo post initial viral clearance with 100 mL homologous inoculum (GenBank accession no. KY124246, viral load  $7.78 \times 10^6$  RNA copies per milliliter). Serum and PBMC samples were taken on a weekly basis and stored at  $-20^\circ\text{C}$ – $150^\circ\text{C}$  before analysis. (A) Course of the experimental, homologous reinfection. All serum samples were analyzed for the presence of NPHV RNA and anti-CHV/NPHV NS3 antibodies, respectively. Anti-CHV/NPHV NS3 antibodies were measured by the LIPS assay in duplicates and the mean values are depicted as black squares as relative light units (RLUs). A cutoff was determined as described above and is depicted as dotted line. The value of the naïve sample represents the first serum sample taken before the initial inoculation in the first part. NPHV RNA values of horses 2 and 3 were below the detection limit at all time points (limit of quantification 50 RNA copies per serum sample). To detect trace amounts of NPHV RNA, all qRT-PCR samples were loaded on an agarose gel as shown at the Top of each graph. Positive signals in the agarose gel are highlighted in each graph by a big gray square. As control, one naïve horse was inoculated with 100 mL homologous plasma. (B) Monitoring of liver enzyme values within sera. AST, GLDH, and GGT were determined in all serum samples of horses 2 and 3. Reference values are as follows: GLDH < 6 U/l, GGT < 20 U/l, and AST < 170 U/l. (C) Three weeks postinoculation, liver biopsies were taken and stained with hematoxylin and eosin (H&E). Periportally, biopsies revealed a low number of lymphocytes and macrophages. In addition, a variable degree of coarsely granular, brown pigment within the cytoplasm of hepatocytes and macrophages was present. (Scale bars, 50  $\mu\text{m}$ .)

reinfection compared with a naïve horse (positive control horse) (Fig. 6A). Only trace amounts of viral RNA could be detected 3 wk and 1 wk postinfection, respectively. The naïve positive control horse rapidly developed robust viremia 1 wk postinfection with high viral titers (Fig. 6B). Sequencing of viral trace amounts confirmed the correct viral sequence of the inoculum. Rechallenged horses (horses 2 and 3) displayed a slight increase in NPHV-specific antibody titers (Fig. 6B); however, no infection-associated clinical signs for hepatitis could be observed upon biochemical and histopathological examination (Fig. 6C and D). In chimpanzees, after reinfection a rapid virological control has been connected to HCV-specific T-cell responses (31, 33, 34). We therefore analyzed peripheral T-cell responses against NPHV after rechallenge; however, we could detect only weak T-cell proliferation in both horses after homologous or distinct rechallenge (Fig. S5 A and B). In conclusion, we could show that horses are protected against homologous and distinct viral rechallenge, although no sterilizing immunity is induced as trace amounts of viral RNA can be detected.

## Discussion

Persistent HCV infections account for ~500,000 deaths annually and are the leading indication for liver transplantation (35). With the development of robust *in vitro* cell culture systems the viral

replication cycle could be studied in detail and ultimately contributed to the development and approval of efficient treatment options in the form of DAAs (36). Nevertheless, a protective vaccine is still lacking, which prevents effective global control and eradication of this virus. The lack of a traceable immunocompetent animal model for HCV hampers vaccine development, as cell culture systems do not permit detailed studies of virus–host interactions and cannot completely elucidate mechanisms that contribute to viral clearance versus persistence. So far, chimpanzees are the only fully immunocompetent host supporting a HCV infection, but due to ethical concerns, use of chimpanzees for research has been banned in the United States and the European Union. Therefore, the development of a new tractable immunocompetent animal model is of utmost importance. The recent discovery of animal hepaciviruses provides new opportunities to broaden our knowledge about hepaciviral virus–host interactions and could lead to the development of new surrogate experimental models for HCV. To estimate the significance of these novel viruses as surrogate models for HCV, a detailed understanding and biological characterization is crucial. This study provides insights into the closest homolog of HCV discovered to date, the equine NPHV. Previous studies already demonstrated similarities between HCV and NPHV, including molecular features as well as the mode of transmission and hepatotropism (20, 22, 23, 37, 38).



**Fig. 6.** Nonhomologous NPHV reinfection of horses. Horses 2 and 3 were rechallenged ~5 mo after viral clearance of the homologous reinfection (1 y post initial infection) with 100 mL of a distinct inoculum (GenBank accession no. KY124248, viral load  $6.0 \times 10^5$  RNA copies per milliliter). Serum and PBMC samples were taken on a weekly basis and stored at  $-20^\circ\text{C}/-150^\circ\text{C}$  before analysis. (A) Phylogenetic analysis of NPHV isolates. The consensus sequence of inoculum used for the homologous infection (GenBank accession no. KY124246) and the distinct rechallenge (GenBank accession no. KY124248) were sequenced in the near complete E1E2 region and are depicted in relation to reference NPHV isolates. The evolutionary history was inferred by using the maximum likelihood method based on the general time reversible model. Depicted is the tree with the highest log likelihood; bootstrap values are indicated at tree nodes; and scale bar refers to branch lengths measured in the number of substitutions per site. (B) Course of the experimental, nonhomologous reinfection. Anti-CHV/NPHV NS3 antibodies were determined by the LIPS assay and the mean values of the duplicate samples are presented as black squares as relative light units (RLUs). The naïve sample corresponds to the  $t_0$  value before the first infection. NPHV RNA values were below the detection limit (limit of quantification 50 RNA copies per serum sample) and qRT-PCR products are visualized by separation on an agarose gel. Positive signals are indicated with a gray big square. A naïve (NPHV seronegative and RNA negative) horse was inoculated with the distinct plasma as positive control. (C) Determination of the AST, GGT, and GLDH level in the serum. Reference values are as follows: GLDH  $< 6$  U/L, GGT  $< 20$  U/L, and AST  $< 170$  U/L. (D) Three weeks postinoculation, liver biopsies were taken and stained with hematoxylin and eosin. Periportally, biopsies revealed a low number of lymphocytes and macrophages. In addition, a variable degree of coarsely granular, brown pigment within the cytoplasm of hepatocytes and macrophages was present. (Scale bars, 50  $\mu\text{m}$ .)

However, only a few studies have addressed detailed analysis of NPHV infection in vivo. We could confirm previously published data that horses can be experimentally infected by i.v. inoculation (23). All inoculated horses became viremic 1 wk postinfection and with the exception of one horse (horse 1) stayed viremic for several weeks. Reasons for the different clearance rates between the animals need to be further investigated, but one could imagine that undetected previous natural infections with NPHV might

contribute to the observed differences. Ultrastructural studies of liver biopsies from infected versus naïve horses revealed that NPHV infection induces the formation of vesicles within the liver, similar to the vesicles that have been previously observed in liver biopsies of an HCV-infected chimpanzee (26) and comparable to the structures that are induced by HCV upon infection of culture cells (27). These findings indicate that HCV and NPHV might share common strategies for viral replication, resulting in



the formation of similar replication structures, also known as replication factories. It is believed that formation of such vesicles not only facilitates the coordination of the different steps of the viral replication cycle, but could also contribute to immune evasion by shielding viral RNA from recognition by innate sensors such as retinoic acid-inducible gene I (RIG-I) or melanoma differentiation-associated gene 5 (MDA5) (27). However, it is not completely understood how host immune responses contribute to viral clearance compared with the establishment of chronic infection (39). Studies of early immune events during a HCV infection in humans are difficult due to various reasons, including the late diagnosis in the majority of cases rendering animal models valuable surrogate models. Furthermore, several studies indicated that HCV-specific CD4 and CD8 T-cell responses in both humans as well as chimpanzees are weak after HCV infection and that spontaneous clearance is associated with a stronger cellular response. The latter is typically broad, polyfunctional, and sustained, indicating that cellular immunity is an important factor (40). To analyze the impact of the equine immune system on viral clearance, we established a variety of immunological assays to evaluate equine immune responses. However, given the fact that the horse is not a commonly used animal model, immunological tools are limited compared with murine model systems. Nevertheless, we explored innate and adaptive immune responses and despite viral clearance in our horses, only weak immune responses following viral challenge were detected. An earlier study using immune-deficient foals indicated that disease outcome varies based on the development of the immune system. Normal immunocompetent foals and immune-deficient foals were not able to clear NPHV infection, whereas young adult horses did clear the virus (23). Future studies directly addressing intrahepatic immune responses could further clarify the roles of specific immune cells in viral clearance. A previously resolved HCV infection does not necessarily confer sterilizing immunity against reinfection, which impedes global control of HCV infections (32). Reinfection following sustained virological response (SVR) has been reported in several studies among high-risk populations, including people who inject drugs (PWIDs) and prisoners. However, these studies are rare and sometimes show inconclusive results (32, 41). Important insights regarding reinfection and protection have been gained from earlier experiments in chimpanzees. Challenge experiments have demonstrated that despite apparently efficient immune responses in primary infection resulting in viral clearance, reinfection was possible in chimpanzees with both homologous and heterologous viruses. However, reinfection seems to be associated with improved control of viral replication, a short course of infection, and an increased viral clearance rate compared with primary infections (30, 31, 33, 42). To analyze immune protection upon previous NPHV infection, we rechallenged previously infected horses with homologous as well as with nonhomologous NPHV isolates. As NPHV exhibits low sequence diversity between isolates, classical heterologous rechallenge could not be conducted. However, homologous or distinct rechallenge experiments revealed that horses were partially protected with only trace amounts of viral RNA being detectable. This finding indicates the existence of immune mechanisms that contribute to protection. In chimpan-

zees, rapid virological control after reinfection has been connected to HCV-specific T-cell responses (31, 33, 34). However, first analyses of NPHV specific T-cell responses did not indicate a strong peripheral T-cell response. One limitation of the used assay is that T-cell responses were only determined with NS3-specific peptides and other immunodominant epitopes outside NS3 were not tested. Further analyses of equine hepatic T-cell responses might provide additional insights into protective immune-mediated mechanisms. In addition, the role of virus-specific antibodies needs to be further addressed. In humans, HCV strain-specific neutralizing antibodies have been found to be associated with spontaneous recovery (43, 44). Our reinfected horses displayed a higher titer of NPHV-specific antibodies before rechallenge, which even further increased after a new exposure. Although it will be interesting to analyze the presence and importance of neutralizing antibodies, these studies are currently not feasible as no cell culture system is available for NPHV.

Given the close genetic relatedness between HCV and NPHV, our study contributes to the biological characterization and to the understanding of hepaciviral pathogenesis and associated immunity. Detailed characterization of hepaciviral infections in their natural hosts will aid our understanding of viral and host determinants, which in the end, contributes to elucidate the mechanisms of HCV persistence and immunity.

## Materials and Methods

**Animals.** Animal experiments were first examined by the animal welfare representatives of the University of Hannover Foundation, and then approved by the Lower Saxony's official authorities (LAVES 13/1262).

**Determination of Liver-Specific Enzymes.** To monitor occurrence of hepatitis, heparinized blood samples were taken at weekly time points and analyzed for the liver-specific enzymes GLDH, GGT, and AST in the laboratory of the small animal clinic at the University of Veterinary Medicine Hannover Foundation.

**Extraction of Viral RNA and qRT-PCR.** Viral RNA was purified from serum samples by the High Pure Viral RNA Kit (Roche) as described in the manufacturer's instructions. Purified RNA was subsequently transcribed into cDNA by using the Prime Script RT Master Mix Kit (Takara) with random hexamer primers. cDNA was stored at  $-20^{\circ}\text{C}$  until further analysis. A SYBR Green-based qRT-PCR was performed with SYBR Premix Ex Taq II (Takara) and previously described NPHV-specific primers targeting the 5'-untranslated region (5'-UTR) (12). Absolute quantification of NPHV RNA copies was conducted. Serial dilution of a plasmid containing the NPHV 5'-UTR sequence was used to generate a standard curve (limit of quantification 50 RNA copies per serum sample) and to calculate viremia in individual samples.

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