Mouse models of acute and chronic hepatitis virus infection

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An estimated 71 million people worldwide are infected with hepatitis C virus (HCV). The lack of small-animal models has impeded studies of antiviral immune mechanisms. Here we show that an HCV-related hepacivirus discovered in Norway rats can establish high-titer hepatotropic infections in laboratory mice with immunological features resembling those seen in human viral hepatitis. Whereas immune-compromised mice developed persistent infection, immune-competent mice cleared the virus within 3 to 5 weeks. Acute clearance was T cell dependent and associated with liver injury. Transient depletion of CD4+ T cells before infection resulted in chronic infection, characterized by high levels of intrahepatic regulatory T cells and expression of inhibitory molecules on intrahepatic CD8+ T cells. Natural killer cells controlled early infection but were not essential for viral clearance. This model may provide mechanistic insights into hepatic antiviral immunity, a prerequisite for the development of HCV vaccines.

We first explored whether NrHV could establish infection in the immune-compromised mouse strains NRG (NOD-Rag1−/− IL2Rγ−/−), A129 (IFNReβ−/−), and AG129 (IFNReβ−/− IFNRγ−/−) that lack adaptive immunity, type I, and type I/II interferon (IFN) signaling, respectively. We infected 4-week-old mice intravenously with 10^6 genome equivalents (GE) of NrHV derived from the serum of an infected laboratory rat. NrHV established a high-titer (10^9 to 10^10 GE per milliliter of serum) chronic infection in these mice (Fig. 1A). Mice lacking MAVS (mitochondrial antiviral signaling protein) cleared the virus within 3 weeks postinfection (p.i.) (Fig. 1A).

Intrahepatic infection of the immune-competent mouse strains C57BL/6J and BALB/c with 10^4 GE resulted in a high-titer (10^6 to 10^7 GE/ml serum) acute resolving infection (Fig. 1B). NrHV derived from rat serum was cleared significantly faster than NrHV passages one time through NRG mice (Fig. 1B), indicating that NrHV can adapt to the mouse host. To test the extent of NrHV adaptation in NRG mice, we performed either 12-week long-term adaptation in one mouse or serial passaging adaptation through five mice (4-week infection of each mouse) (fig. S1A). We then challenged naïve NRG and C57BL/6J mice with 9 × 10^4 GE of either the pooled adapted (long-term pool or serial pool) or the parental virus. Adapted viruses showed 0.5 to 1 log higher viral titers at week 1 p.i. and persisted longer in C57BL/6J mice than did the parental virus (fig. S1B), suggesting increased viral fitness in the mouse host.

Comparing the consensus NrHV genome open reading frame (ORF) sequences of the rat inoculum with those of the adapted viruses revealed changes in 2 and 59 nucleotide positions in the long-term and serial passaging pool, respectively (fig. SIC). Phylogenetic analysis of full-ORF clones revealed the presence of two subpopulations in the inoculum; the minor one was selected during the serial passage (Fig. 1C and fig. S1D). Single coding mutations in viral envelope proteins E1 and E2 occurred in both pools and in individual NRG mice from passage 5 of the serial adaptation (Fig. 1C, fig. S1E, and tables S1 and S2). The E1 mutation V353L (Val353→Leu) was combined with either T1908 (Thr190→Ser) (serial pool) or T1952 (Thr1952→Asn) (long-term pool), represent putative mouse adaptive mutations, as they were maintained in challenged C57BL/6J mice. Mutations at amino acid position 550, combined with at least one mutation in the cluster 361/369/370/371, were selected in NRG mice, but were immediately lost in C57BL/6J mice. Changing position 550 would disrupt a predicted Nxs(S/T) glycosylation site (where x is any amino acid except proline), possibly destabilizing neutralization epitopes as observed for HCV (6). For subsequent experiments we used serial pool virus as our source of NrHV.

Our results indicate that NrHV is both highly infectious and hepatotropic in mice. Even a low-dose infection with 10^4 GE resulted in high-titer viremia. The dose did not influence the outcome of infection, as mice infected with 10^6, 10^5, 10^4, and 10 GE cleared the virus with similar kinetics. In contrast, age influenced clearance: 4-week-old mice typically cleared the virus by week 5 p.i., whereas 2- to 6-month-old mice cleared the virus by week 3 p.i. (fig. S2, A to C). We consistently detected high viral titers in liver tissue (10^6 to 10^8 NrHV GE per gram of tissue) but not in spleen, kidney, and lung (Fig. 1D). NrHV replication was dependent on mir-122, a liver-specific microRNA required for HCV replication (7, 8) (Fig. 1E).

Like HCV in humans, NrHV infection was not associated with signs of acute disease or mortality. Chronically infected NRG and AG129 mice showed minimal to mild liver inflammation at week 35 p.i. (fig. S3).

To identify the cellular mediators of NrHV clearance, we characterized the immune response during acute resolving NrHV infection in 8-week-old C57BL/6J mice. High-titer viremia was detectable as early as 24 hours p.i.; titers started to decline at day 15 p.i. and were undetectable at day 21 p.i. (Fig. 2A). Early acute infection (days 3 to 9 p.i.) was associated with an expansion of intrahepatic Ly6C+ monocytes and NKp46+ NK cells (Fig. 2B). Starting at day 9 p.i., we observed a substantial increase in proliferating (Ki67+) intrahepatic CD4+ and CD8+ T cells. These cells were characterized by a CD44+ effector phenotype with an antiviral type 1 differentiation signature (9) as indicated by high T-bet expression and IFN-γ production (Fig. 2, B to E, and fig. S4, A and B). CD8+ T cells also showed a significant up-regulation of granzyme B (fig. S4C). The T cell response was predominant in the liver and less pronounced in peripheral blood and spleen (Fig. 2 and fig. S4). High levels of intrahepatic effector T cells coincided with a decline in viremia starting at day 15 p.i. and were associated with elevated alanine transaminase (ALT) levels (Fig. 2F, indicating that cell-mediated liver injury. Hepatic leukocyte infiltration was confirmed by histology (Fig. 2G). We detected NrHV-specific IFN-γ production by CD4+ and CD8+ T cells in response to peptide
Transient CD4+ T cell depletion (4 days before infection) in C57BL/6J mice initiated before infection resulted in chronic infection (analyzed until day 210 p.i.) in C57BL/6J and BALB/c mice even after the CD4+ cell population recovered. Transient CD8+ T cell depletion led to delayed viral clearance as compared to controls (Fig. 3A and fig. S7, A and B). Mice that were constantly (every 10 days p.i.) depleted of CD8+ T cells failed to clear the virus (Fig. 3B).

By contrast, in CD4+ T cell depleted mice, intrahepatic T cell recovery (fig. S8A) was associated with the emergence of CD4+ IFN-γ+ effector CD8+ T cells, elevated ALT levels, and viral clearance. This was preceded by the induction of intrahepatic CD4+ IFN-γ+ CD4+ T cells (fig. S8C). In CD8+ T cell depleted mice, intrahepatic T cell recovery (fig. S8B) yielded minimal induction of CD4+ IFN-γ+ CD4+ T cells. The presence of CD8+ effector cells induced in the absence of CD4 help was not associated with elevated ALT levels or viral clearance but resulted in chronicity (fig. S8, E and F).

These results indicate that CD4+ T cells are crucial for the initiation of a successful antiviral adaptive immune response, and that CD8+ T cells are main effectors in NrHV clearance.

In NrHV infection, memory CD8+ T cells also limited secondary infection (Fig. 3C). Mice that had cleared NrHV infection 4 to 7 months earlier became reinfected upon secondary challenge with the same inoculum but rapidly cleared the virus within 7 days (Fig. 3C). This was accompanied by a strong NrHV-specific CD8+ T cell recall response at day 5 p.i. (Fig. 3D). When we depleted CD8+ T cells 4 days before secondary infection, mice showed higher viral titer at day 5 p.i. compared to controls and could not clear the secondary infection by day 7 p.i. (Fig. 3C).

Neutralizing antibodies (nAbs) might also play a role in limiting secondary infection. We observed significantly lower viral titers 24 hours after reinfeciton as compared to primary infection (Fig. 3C). During primary infection, there was an increase in the number of hepatic B cells and captured frequencies of splenic and hepatic follicular T helper cells (fig. S9, A and B). Antibodies against NrHV NS3 were detected, starting at day 21 p.i. (fig. S9C). Preincubation of 10^4, 10^5,
Fig. 2. NrHV clearance in immune-competent mice is associated with a strong intrahepatic antiviral immune response. Eight-week-old C57BL/6J mice were infected i.v. with 10⁴ GE of NrHV. (A) Viremia during acute resolving infection. (B) Flow cytometric analysis of total numbers of hepatic Ly6C⁺ monocytes, NKp46⁺ NK cells, CD3⁺CD4⁺ T cells, and CD3⁺CD8⁺ T cells during acute infection. Dotted lines indicate baseline levels. (C to E) Flow cytometric analysis of peripheral and hepatic T cells during acute NrHV infection. Frequencies of CD44⁺ effector cells (C), T-bet⁺ cells (D), and IFN-γ⁺ producing cells (PMA/ionomycin stimulation) (E) within the CD4⁺ (upper panels) and CD8⁺ (lower panels) T cell subsets are shown. (F) ALT levels in serum of mice. Dotted line indicates baseline level. (G) Representative hematoxylin and eosin (H&E) histology at day −1 and day 9 p.i. Scale bars, 100 µm. Representative data from two independent experiments with four mice per group (mean ±SEM) are shown.

Fig. 3. Clearance of NrHV infection is T cell dependent. (A) Eight-week-old C57BL/6J mice were transiently depleted of CD4⁺ or CD8⁺ T cells with antibodies (day 4 before infection; day 7 and 28 p.i.) and infected with 10⁴ GE of NrHV. Viremia was analyzed until day 210 p.i. IgG, immunoglobulin G. (B) Mice were either transiently or constantly (day 4 before infection; every 10 days p.i.) depleted of CD8⁺ T cells and infected with 10⁴ GE NrHV. Viremia was analyzed until day 56 p.i. (C) Mice that cleared NrHV infection more than 4 months before were reinfected with 10⁴ GE of the same inoculum. One group of mice was depleted of CD8⁺ T cells 4 days before reinfection. Primary infection in age-matched mice served as a control. Viremia at days 1, 5 and 7 p.i. is shown. (D) NrHV-specific IFN-γ production of CD8⁺ T cells at day 5 after reinfection. Cells were stimulated ex vivo with NrHV NS3 or NS4 peptide pools. Representative data from two to five independent experiments with four or five mice per group (mean ±SEM) are shown. ***P ≤ 0.0001 (unpaired Student’s t test); ns, not significant.
or $10^2$ GE NrHV with serum from mice that previously cleared infection mostly prevented infection of naïve mice with $10^3$ GE but not with $10^4$ or $10^5$ GE (fig. S9D). This suggests that ntAbs are produced but at a frequency too low to completely prevent NrHV reinfection. That is similar to HCV infection, where previously infected humans and chimpanzees can be reinfected with the same HCV strain (14).

We also investigated the potential role of NK cells in viral clearance. During NrHV infection, we observed increased numbers, activation (CD69 up-regulation), and IFN-γ production of hepatic NK cells, starting at day 3 p.i. (Fig. 2B and fig. S10, A to C). This was attributed to an expansion in the number of conventional CD49b− NK cells while the number of liver-resident CD49a+ NK cells (15) remained stable, leading to a substantial change in the hepatic CD49b− to CD49a+ NK cell ratio over the course of infection (fig. S10, D and E). NK cell depletion resulted in significantly elevated viremia at day 3 p.i.. However, the kinetics of viral clearance and the extent of liver injury were similar in NK cell-depleted mice and controls (fig. S10, F and G). Thus, NK cells may contribute to the control of NrHV early in infection, but they are not required for NrHV clearance.

Chronic viral hepatitis in humans and chronic LCMV infection in mice are characterized by antiviral immune responses by Tregs (Tregs) (70, 71). We found that chronic infection, in contrast to acute clearance, coincided with the emergence of intrahepatic Tregs that remained at high levels throughout infection (Fig. 4, A and B). Thus, suppression of antiviral immune responses by Tregs might play a role in the establishment of chronic NrHV infection.

Chronic NrHV infection also displayed elevated frequencies of intrahepatic CD8+ T cells with an exhausted phenotype characterized by PD-1highCD44low surface expression (Fig. 4, C and D), coexpression of the inhibitory receptors 2B4 and Tim-3, and high expression levels of the transcription factors Foxp3+CD4+ T cells (Tregs) (70, 71). After transient CD4+ T cell depletion, NrHV established a long-term chronic infection in immune-competent mice (Fig. 3A) that was associated with mild liver inflammation (fig. S11). We found that chronic infection, in contrast to acute clearance, coincided with the emergence of intrahepatic Tregs that remained at high levels throughout infection (Fig. 4, A and B). Thus, suppression of antiviral immune responses by Tregs might play a role in the establishment of chronic NrHV infection.

**Fig. 4.** Chronic NrHV infection is associated with hepatic CD8+ T cell exhaustion. Flow cytometric analysis of C57BL/6J mice that developed chronic NrHV infection after transient CD4+ T cell depletion. (A) Foxp3+CD4+ Treg frequencies within the CD4+ T cell subset in blood (left) and liver (right) during acute resolving and chronic NrHV infection. (B) Representative flow cytometry plots showing hepatic Tregs at day 210 p.i. (C) Frequencies of PD-1highCD44low CD8+ T cells at day 42 and 210 p.i. in blood (left) and liver (right). (D) Representative flow cytometry plots showing hepatic PD-1highCD44low CD8+ T cells at day 210 p.i. (E to G) Chronically NrHV infected C57BL/6J mice were treated with a PD1-L blocking antibody or appropriate IgG control starting at day 42 (E), day 84 (F), or day 140 (G) p.i. Viremia was analyzed at day 1 before the start of treatment and compared to viremia at day 14 (E) or day 21 [(F) and (G)] after treatment. Representative or combined data from two to five independent experiments with four or five mice per group (mean ±SEM) are shown. *P ≤ 0.01, **P ≤ 0.001, ***P ≤ 0.0001 (unpaired and paired Student’s t test).
factor eomesodermin (fig. S12A) (16, 17). The frequencies of these cells were lower in acute resolving mice, suggesting that chronic NrHV infection may lead to T cell exhaustion (Fig. 4C).

Checkpoint inhibitor blockade [e.g., the inhibition of PD-1/PD-1 ligand (PD-1L) interactions] is a promising immunotherapy that can invigorate exhausted T cells (18). PD-1/PD-1L blockade showed mixed results when tested in HCV-infected chimpanzees and patients, so its efficacy in the future for the development and testing of HCV vaccines.

In this study, we have developed an immune-competent inbred mouse model of an HCV-related hepativirus. Because NrHV can adapt to infect mice with diverse genetic backgrounds, this model can potentially help unravel mechanisms of hepatitis virus host adaptation, immune evasion, and the development of liver disease. It can also be used to select for viral variants that can establish chronic infection in immune-competent mice. Given the similarities between NrHV infection in mice and HCV infection in humans, this model might prove valuable in the future for the development and testing of HCV vaccines.

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SUPPLEMENTARY MATERIALS
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References (11, 22)
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New York City rats provide a gift to virologists

Despite the development of curative drugs for hepatitis C virus (HCV) infection, global eradication of HCV will likely require a prophylactic vaccine. Progress toward a vaccine has been impeded by the absence of mouse models suitable for studying the immune response to HCV. Billerbeck et al. found that a HCV-related virus isolated from New York City rats produces an infection in laboratory mice that shares several immunological features with human infections (see the Perspective by Klenerman and Barnes). Their initial analyses of the infected mice revealed that acute clearance of the virus was dependent on T cells but not on natural killer cells.

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