LETTERS

Subinfectious hepatitis C virus exposures suppress T cell responses against subsequent acute infection

Su-Hyang Park1, Naga Suresh Veerapu1,4, Eui-Cheol Shin1,4, Angélique Biancotto2, J Philip McCoy2, Stefania Capone3, Antonella Folgori3 & Barbara Rehermann1

Hepatitis C virus (HCV) is endemic in many countries due to its high propensity for establishing persistence1. The presence of HCV-specific T cells in subjects repeatedly exposed to HCV who test negative for HCV RNA and antibodies and who do not have any history of HCV infection has been interpreted as T cell–mediated protection2–5. Here, we show in nonhuman primates that repeated exposure to human plasma with trace amounts of HCV induced HCV-specific T cells without seroconversion and systemic viremia but did not protect upon subsequent HCV challenge. Rather, HCV-specific recall and de novo T cell responses, as well as intrahepatic T cell recruitment and interferon-γ (IFN-γ) production, were suppressed upon HCV challenge, concomitant with quantitative and qualitative changes in regulatory T cells (Treg cells) that occurred after subinfectious HCV exposure and increased after HCV challenge. In vitro Treg cell depletion restored HCV-specific T cell responses. Thus, T cells primed by trace amounts of HCV do not generate effective recall responses upon subsequent HCV infection. Subinfectious HCV exposure predisposes to Treg cell expansion, which suppresses effector T cells during subsequent infection. Strategies to reverse this exposure-induced immune suppression should be examined to aid in the development of T cell–based vaccines against HCV and other endemic pathogens.

At least 170 million people worldwide are persistently infected with HCV, a leading cause of chronic liver inflammation, cirrhosis and cancer1. Spontaneous HCV clearance occurs in less than 25% of acute infections and is typically associated with T cell rather than antibody responses1,6–9. HCV-specific T cell responses have also been described in subpopulations of injection drug users who test negative for HCV RNA and antibodies to HCV and do not have past HCV infection despite frequent exposure2–5, as well as in aviremic seronegative family members of HCV-infected subjects10,11. Based on these findings, it has been proposed that repeated subinfectious (low-dose) exposure primes and maintains HCV-specific T cells that confer protective immunity3–5,10,11.

We set out to test this hypothesis using chimpanzees that had participated in a study to assess the infectivity of plasma and peripheral blood mononuclear cell (PBMC) samples from patients positive for antibody to HCV who had trace amounts of HCV detected by nested RT-PCR below the detection limit of the standard clinical assay at the NIH (qualitative COBAS Amplicor HCV Test 2.0)12. The sporadic reappearance of HCV RNA in these patients coincided with HCV-specific T cell responses and did not result in high-level viremia12.

When chimpanzees A3A015 and A3A017 were infused at 9-week intervals with these plasma (infusions 1, 2 and 3) or PBMC (infusion 4) samples (Supplementary Table 1), they remained negative for HCV RNA in blood and liver and did not mount antibody responses, but they generated HCV-specific T cell responses, as evidenced by IFN-γ secretion upon in vitro stimulation of PBMCs with HCV peptides (Fig. 1). A third chimpanzee, A3A020, transiently tested positive for HCV RNA in the blood by nested RT-PCR 10 and 12 weeks after plasma infusion, concomitant with increased HCV-specific T cell responses (Fig. 1). Such responses were not observed in the control chimpanzees, A3A025, after repeated exposure to blood products from donors who were negative for HCV RNA and antibody to HCV (Fig. 1). Further characterization of the HCV-exposed chimpanzees revealed that both CD8+ and CD4+ T cells produced IFN-γ, tumor necrosis factor-α (TNF-α) or macrophage inflammatory protein-1β (MIP-1β) in response to multiple HCV antigens (Supplementary Fig. 1a–c), but only a minority were polyfunctional, i.e., secreted multiple cytokines (≤17% CD8+ T cells and ≤12% CD4+ T cells, Supplementary Fig. 1d). The majority of IFN-γ-producing CD8+ T cells were CD28− effector (61–88%) or effector memory (12–32%) cells, and none were central memory cells (Supplementary Fig. 1e).

Chimpanzees that clear an acute HCV infection typically exhibit lower peak viremia levels and faster clearance of a secondary HCV challenge due to the presence of protective memory T cells8,9,13–16. However, when the HCV-preexposed chimpanzees (A3A015, A3A017 and A3A020) with HCV-specific T cell responses were challenged with 100 50% chimpanzee infectious doses (CID50) of HCV, they did not control viremia as rapidly as chimpanzee 1605, which had received the same HCV challenge after previous spontaneous clearance of acute

1Immunology Section, Liver Diseases Branch, NIDDK, National Institutes of Health (NIH), Department of Health and Human Services, Bethesda, Maryland, USA. 2Flow Cytometry Core Facility, NHLBI, NIH, Department of Health and Human Services, Bethesda, Maryland, USA. 3Okairos, Rome, Italy. 4Present addresses: Department of Life Sciences, Shiv Nadar University, Village Chitera, District Gautam Budh Nagar, Uttar Pradesh, India (N.S.V.) and Laboratory of Immunology and Infectious Diseases, Graduate School of Medical Science and Engineering, Korea Advanced Institute of Science and Technology, Daejeon, Republic of Korea (E.-C.S.). Correspondence should be addressed to B.R. (rehermann@nih.gov).

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HCV infection with high-titer viremia (Fig. 2a). Rather, they experienced the same prolonged high-titer viremia as the four HCV-naive control chimpanzees A3A025, 98A005, 97A009 and 97A015, which were also challenged with 100 ClID$_{50}$ HCV (ref. 17) (Fig. 2a). Two of three HCV-preexposed chimpanzees developed chronic infection (Supplementary Table 2).

Next, we investigated the reasons for the lack of immune protection in the three HCV-preexposed chimpanzees. At the time of HCV challenge (week 0), they displayed no HCV-E2–specific antibodies but had a higher frequency of HCV-specific IFN-$\gamma$–secreting CD8$^+$ and CD4$^+$ T cells than the HCV-recovered chimpanzee 1605 (Fig. 2b,c), along with higher frequencies of TNF-$\alpha$– and MIP-1$\beta$–secreting CD8$^+$ and CD4$^+$ T cells (Supplementary Fig. 2a,b). However, the T cell responses of the HCV-preexposed chimpanzees were not boosted but rather decreased to minimal levels by week 4 after challenge, the time point at which HCV-specific CD8$^+$ and CD4$^+$ T cell recall responses of chimpanzee 1605 peaked.
Figure 3  Repeated exposure to blood samples from patients positive for antibody to HCV and trace amounts of HCV suppresses intrahepatic T cell recruitment and IFN-γ production upon subsequent HCV challenge. (a–c) Intrahepatic CD8β (a), IFN-γ (b) and CXCR3 (c) mRNA levels as determined by RT-PCR, normalized to endogenous references (GAPDH and β2) and expressed as relative increase over week 0 expression, which was set to a value of 1. For chimpanzees 98A005, 97A009, 97A015 and 1605, the increase in CD8β and IFN-γ mRNA levels was calculated from previously reported data40. The vertical arrows indicate the time point (week 0) at which all chimpanzees were challenged with 100 CID50 HCV genotype 1a. Right graphs represent the peak mRNA levels during acute HCV infection for each chimpanzee. The horizontal lines indicate the median. P values were calculated with the Mann-Whitney U-test.

(FIG. 2b.c for IFN-γ and Supplementary Fig. 2a,b for TNF-α and MIP-1β), consistent with other recovered and rechallenged chimpanzees8,16. Moreover, ≤2% of the HCV-specific CD8+ T cells from HCV-preexposed chimpanzees were polyfunctional, compared to 42% of those from chimpanzee 1605 (Supplementary Fig. 2c).

Notably, new T cell responses were significantly suppressed in the three HCV-preexposed chimpanzees, as compared to the peak IFN-γ response of HCV-specific CD8+ and CD4+ T cells in the four control chimpanzees (P < 0.05, Fig. 2b,c and Supplementary Fig. 2d). This was consistent with suppressed TNF-α+ and MIP-1β+ responses of HCV-specific T cells (Supplementary Fig. 2a,b), whereas cytomegalovirus (CMV)-specific T cell responses remained unchanged (Supplementary Fig. 3).

Suppression of immune responses occurred also in the liver, the site of HCV replication. Because liver biopsies yielded too few lymphocytes for ex vivo analysis, we used quantitative real-time PCR to monitor intrahepatic gene expression. There was almost no increase in the intrahepatic mRNA levels of CD8β, IFN-γ and CXCR3 during the first 6 weeks after HCV challenge in the three HCV-preexposed chimpanzees, whereas they peaked early (week 2) in chimpanzee 1605, consistent with a rapid recall response in this animal (Fig. 3). The intrahepatic responses of the HCV-preexposed chimpanzees occurred at the same time (weeks 12–14) but with significantly lower magnitude than those of the four control chimpanzees (Fig. 3). We also observed differences in CXCL11 mRNA levels, but these did not reach statistical significance (P = 0.114, data not shown). Thus, prior exposure to trace amounts of HCV impede HCV-specific recall responses, induction of new T cells and intrahepatic recruitment of CD8+ T cells and IFN-γ production after subsequent HCV challenge.

We next studied suppression by Treg cells as a candidate mechanism for the dampened response in the HCV-preexposed chimpanzees. Whereas the frequency of circulating Treg cells expressing CD4, CD25 and forkhead box P3 (Foxp3) did not differ among chimpanzees at the start of the study (week −42), it increased after repeated exposure to trace amounts of HCV (P < 0.05, week 0, Fig. 4a,b). This expansion may have been driven by interleukin-2 (IL-2) from HCV-specific effector T cells (Fig. 4c), as Treg cells require exogenous IL-2 for their maintenance and proliferation18. Challenge with 100 CID50 HCV further increased the Treg cell frequency in the three HCV-preexposed chimpanzees as compared to the previously HCV-recovered chimpanzee 1605 (Fig. 4a) and the four control chimpanzees (P = 0.0002, repeated-measures analysis of variance (ANOVA), Fig. 4b). Furthermore, the Treg cell subset composition changed with an expansion of CD38+ activated, CD39+ functional19 and CD27−CD197−CD45RA− effector20 Treg cells (Fig. 4d).

The three HCV-preexposed chimpanzees also displayed a higher intrahepatic Treg frequency (Fig. 4e) and higher peak intrahepatic Foxp3, IL-10 (P < 0.05, respectively) and TGF-β (P = 0.058) mRNA levels than chimpanzee 1605 and the four control chimpanzees after HCV challenge (Fig. 4f). In vitro depletion of Treg cells from PBMCs at 8 weeks and 19–37 weeks after HCV challenge enhanced HCV-specific T cell responses by a greater percentage in the HCV-preexposed chimpanzees than in the control chimpanzees (P < 0.05, Fig. 4g,h) with preserved immune hierarchy (Supplementary Fig. 4a). As IL-10 secreted by Treg cells can directly suppress antigen-specific T cell responses, we confirmed the suppressive function of Treg cells by in vitro IL-10 neutralization combined with IL-10 receptor blockade, which significantly enhanced HCV-specific T cell responses from HCV-preexposed but not control chimpanzees (P < 0.05, Fig. 4i and Supplementary Fig. 4b). The three HCV-preexposed chimpanzees also displayed a higher frequency of B7–H4+ circulating macrophages and higher intrahepatic B7–H4 mRNA levels than the four control chimpanzees after HCV challenge (Supplementary Fig. 5). Treg cells have been shown to suppress effector T cell responses by IL-10–mediated upregulation of B7–H4 on antigen-presenting cells21,22, and the timing of the intrahepatic B7–H4 mRNA peak coincided with the absence of HCV-specific T cell responses 8–12 weeks after HCV challenge in the HCV-exposed chimpanzees. The control chimpanzees mounted strong T cell responses at that time (Fig. 2b,c).

These results suggest that HCV-specific T cells were induced after HCV challenge in all chimpanzees but functionally suppressed in the HCV-preexposed chimpanzees. Of note, the sole HCV-preexposed chimpanzee that cleared the challenge inoculum (A3A020) did so after a decrease in Treg cell frequency (Fig. 4a) and a concomitant increase in HCV-specific CD8+ and CD4+ T cells between weeks 10 and 16 (Fig. 2b,c). In summary, we demonstrate that T cells primed by trace amounts of HCV do not generate effective recall responses upon subsequent HCV infection. Furthermore, exposure to trace amounts of HCV predisposes to Treg cell expansion, which suppresses HCV-specific effector T cells during subsequent acute infection. This scenario differs


from allergen desensitization, where exposure to increasing allergen doses suppresses IL-4 and IL-5 production through Treg cell induction and, contrary to our findings, increases IFN-γ responses. Likewise, it differs from IL-10–dependent immunotolerance in beekeepers, which is induced by repeated high-dose antigen exposure.

The absence of substantial inflammation after subinfectious exposure to HCV may be key to the observed Treg cell induction, as suggested by the increased expression of Foxp3 in CD4+ T cells in mice that received small amounts of peptide without adjuvants. Here, we demonstrate that similar effects can be achieved with very small doses of live virus rather than inert antigen. A possible lack of signal 2 (co-stimulation) or, without sufficient viral replication, signal 3 (type I IFN) may impede the induction of optimal effector CD8+ T cells and instead favor Treg cell induction. The accelerated Treg cell expansion in the presence of high antigen levels after HCV challenge may imply HCV specificity, as suggested by in vitro studies. IL-2 consumption by the expanding Treg cell population after HCV challenge may suppress new effector T cells, which depend on prolonged IL-2 signals.

Our results were generated in the in vivo model that most closely represents HCV infection in humans, and they warrant confirmation in human populations. At present, limited data are available to distinguish between subinfectious and full-dose HCV exposure in humans. Published studies on T cell responses in HCV RNA–negative and seronegative individuals are of cross-sectional design and thus do not exclude the possibility that T cell responses resulted from past acute HCV infection with systemic viremia and subsequent antibody loss. However, we have recently shown in a prospective study that subinfectious HCV exposure of healthcare workers through accidental needlestick can indeed induce HCV-specific T cell responses in the absence of quantifiable viremia and HCV-specific antibodies. Based on this study in humans and the HCV challenge data in the nonhuman primate model, we refute the notion that individuals frequently exposed to subinfectious HCV who remain negative for antibody have T cell–mediated protective immunity upon HCV infection. We propose that T cell–based protective immunity, as described in rechallenged chimpanzees and suggested in some re-infected injection drug users, requires clearance of a previous acute infection with systemic viremia.

The number of experimental chimpanzees in this study was too small to allow a statistical comparison of the outcomes of HCV challenge in HCV-preexposed and naïve chimpanzees. However, it is...
conceivable that the observed Treg cell–mediated immune suppression also occurs in individuals who live in endemic areas, rendering them less resistant upon subsequent HCV infection than individuals in nonendemic areas. This may extend to T cell responses to other endemic pathogens and would be consistent with the finding that the percentage and the number of Treg cells are higher among healthy individuals in a rural village with seasonal malaria than among individuals in an urban area where malaria is rare.

Our findings may be relevant for vaccine research and for epidemiological studies because an increased Treg cell frequency reduces the response to vaccines. They may, for example, offer a potential explanation for geographical variation in the efficacy of Mycobacterium bovis bacillus Calmette-Guérin vaccination against tuberculosis, which is less effective in endemic areas. Along this line, it would be of interest to examine whether an increased Treg cell frequency in nonendemic areas may extend to T cell responses to other endemic pathogens and would be consistent with the finding that in nonendemic areas, HCV, HIV, Plasmodium species.

METHODS
Methods and any associated references are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS
S.-H.P., N.S.V. and B.R. designed the study; analyzed the data and wrote the manuscript; S.-H.P., N.S.V. and E.-C.S. processed blood and liver biopsy samples; S.-H.P. and E.-C.S. characterized T cell responses; A.B. and J.P.M. characterized Treg cell subsets by flow cytometry; N.S.V. performed virological assays and some RT-PCR assays and A.F. and S.C. challenged three control chimpanzees and performed virological analyses. All authors discussed the results and commented on the manuscript.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

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Online Methods

Chimpanzees. Chimpanzees A3A015, A3A017, A3A020 and A3A025 were studied at New Iberia Research Center (NIRC), New Iberia, LA under protocols approved by the University of Louisiana Lafayette Animal Care and Use Committee (ACUC). Chimpanzees A3A015, A3A017 and A3A020 were intravenously infused with 17–31 ml plasma from patients positive for antibody to HCV who, after completion of interferon-based therapy, had tested negative for HCV RNA by qualitative COBAS AmpliCeq HCV Test 2.0 (Roche) but positive by nested RT-PCR32. Chimpanzees A3A015 and A3A017 were subsequently infused at 9-week intervals with two additional plasma samples and one PBMC sample (3.5 x 10^9) from additional patients with trace amounts of HCV RNA. The study was conducted to assess whether these samples were infectious. The control chimpanzee A3A025 was infused, at the same time intervals, with plasma and PBMCs from blood donors without past HCV infection (Supplementary Table 1). Subjects gave informed consent and were studied under protocols approved by the NIDDK Institutional Review Board.

Fifteen weeks (for A3A015, A3A017 and A3A025) and 26 weeks (for A3A020) after the final infusion, all chimpanzees were challenged with 100 C_{50} HCV genotype 1a. At the indicated study time points, serum was isolated and cryopreserved as described41. PBMCs and liver-infiltrating lymphocytes were shipped overnight to NIH. PBMCs and liver-infiltrating lymphocytes were isolated as described.

Cryopreserved PBMCs, serum and liver biopsies from (i) chimpanzees A3A015, A3A017, A3A020 and A3A025, which had been challenged with 100 C_{50} HCV genotype 1a in a previous study at NIRC33, and (ii) chimpanzee 1D65, which had resolved an acute HCV infection before challenge with 100 C_{50} HCV genotype 1a at the Food and Drug Administration (FDA), Rockville, Maryland, in a previous study34, were studied for comparison (Supplementary Table 1) under protocols approved by the University of Louisiana Lafayette ACUC and the FDA ACUC, respectively.

Multicolor flow cytometry. Analysis of HCV-specific T cell responses by cytokometric bead array. Fresh PBMCs (2 x 10^6 per well) were stimulated in RPMI1640 containing 5% FBS, 100 IU ml\(^{-1}\) penicillin, 100 µg ml\(^{-1}\) streptomycin and 2 mM l-glutamine (Mediatech) with 18 pools of overlapping 15-mer HCV genotype 1a (H77 peptides) of the identical sequence as the challenge virus (1 µg ml\(^{-1}\) per peptide, 600 peptides total)32 or DMSO in 96-well plates. Culture supernatants were harvested after 42 h to quantify IFN-γ, TNF-α, MIP-1β and IL-2 by cytokometric bead array (BD Biosciences). The HCV-specific response to each peptide pool was calculated by subtracting the background response (in the absence of peptides from the mean number of spots to each HCV peptide pool. Cumulative responses to structural and nonstructural HCV antigens are shown (Fig. 1).

Analysis of HCV-specific T cell responses by intracellular cytokine staining. Cryopreserved and thawed PBMCs (2 x 10^6 per tube) were stimulated with or without six pools of 15-mer HCV genotype 1a peptides with an 11-amino-acid overlap that spanned the HCV NS3, NS4, NS5A and NS5B sequences (4 µg ml\(^{-1}\) per peptide). Antibodies to CD28 at 1 µg ml\(^{-1}\) (clone 9F10, BD Biosciences), IFN-γ at 1 µg ml\(^{-1}\) (clone 1D1K, 1:33, BD Biosciences), IL-2 at 10 µg ml\(^{-1}\) (clone Mabtech), allophycocyanin-Cy7–conjugated antibody to CD25 (clone M-A251, 1:10), phycoerythrin–Cy7–conjugated antibody to CD3 (clone UCHT1, 1:100), phycoerythrin-Cy5.5–conjugated antibody to HLA-DR (clone Tü36, 1:20), phycoerythrin-isothiocyanate–conjugated antibody to CD3 (clone A1, 1:10), phycoerythrin-Cy5–conjugated antibody to CD103 (clone LF61, 1:20) from Invitrogen, phycoerythrin–Texas Red–conjugated antibody to CD45RA (clone HI100, 1:33, Mabtech) and analyzed as described in Supplementary Fig. 6a.

Multicolor flow cytometry. Analysis of HCV-specific T cell responses by cytokometric bead array. Fresh PBMCs (2 x 10^6 per well) were stimulated in RPMI1640 containing 5% FBS, 100 IU ml\(^{-1}\) penicillin, 100 µg ml\(^{-1}\) streptomycin and 2 mM l-glutamine (Mediatech) with 18 pools of overlapping 15-mer HCV genotype 1a (H77 peptides) of the identical sequence as the challenge virus (1 µg ml\(^{-1}\) per peptide, 600 peptides total)32 or DMSO in 96-well plates. Culture supernatants were harvested after 42 h to quantify IFN-γ, TNF-α, MIP-1β and IL-2 by cytokometric bead array (BD Biosciences). The HCV-specific response to each peptide pool was calculated by subtracting the background response (in the absence of peptides from the mean number of spots to each HCV peptide per well). Cumulative responses to structural and nonstructural HCV antigens are shown (Fig. 1).

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All stained cells were analyzed on an LSRII flow cytometer (BD Biosciences) using FACS Diva version 6.1.2 (BD Biosciences) and FlowJo version 8.8.6 (Tree Star) software.

Enzyme-linked immunosorbent spot assays. Three different ELISPOT assays were performed: (i) Treg cells were isolated from PBMCs with magnetic beads using the CD4\(^{+}\)CD25\(^{+}\)CD127\(^{−}\) regulatory T cell isolation kit II (Miltenyi) according to the manufacturer’s protocol. PBMCs that had been depleted of Treg cells (300,000 cells per well) were stimulated with 18 pools of overlapping HCV genotype 1a peptides (1 µg ml\(^{-1}\) per peptide, 600 peptides total), (ii) 300,000 PBMCs per well were stimulated with HCV peptide pools with or without IL-10–neutralizing (clone JES5-9D7, 10 µg ml\(^{-1}\) eBioscience) and IL-10Rβ-blocking (clone 90220, 10 µg ml\(^{-1}\), R&D Systems) antibodies, (iii) 300,000 PBMCs per well were stimulated with pools of influenza A virus, CMV and Epstein-Barr virus T cell epitopes (1 µg ml\(^{-1}\) per peptide, Supplementary Methods). All IFN-γ ELISPOT assays were set up in duplicates and processed as described32. The specific response was calculated by subtracting the mean number of spots in the absence of peptides from the mean number of spots to each HCV peptide pool.

Quantitation of intrahepatic gene expression by RT-PCR. RNA was isolated from snap-frozen liver biopsies using Pico Pure RNA isolation kit (Arcturus) or RNeasy Mini Kit (Qiagen) according to the manufacturer’s instructions. Complementary DNA was synthesized using the MonsterScript 1st-Strand cDNA Synthesis Kit (Epicentre Biotechnologies). TaqMan Gene Expression Assays (Applied Biosystems) were performed in duplicate to determine C_{50} HCV, IFN-γ, Foxp3, IL-10, TGF-β, CXCR3, CXCL11 and B7-1 mRNA levels. The amount of specific mRNA was calculated using comparative cycle threshold values and standard curves normalized to internal controls (GAPDH and B7).
and shown as increase over week 0 expression, which was set to 1. For chimpanzees 98A005, 97A009, 97A015 and 1605, previously reported data were used to calculate the increase in CD8β and IFN-γ mRNA levels over baseline, with baseline (week 0) expression set to 1.

Quantitative and qualitative detection of hepatitis C virus RNA. Plasma HCV RNA was quantitated by COBAS Ampliprep–COBAS Taqman HCV test (Roche). The limit of quantitation was 116 copies per ml, and the limit of detection was 28 copies per ml (95% probability rate). Viral titers for chimpanzees 98A005, 97A009 and 97A015 and chimpanzee 1605 were previously reported and are shown for reference purposes only. For qualitative RT-PCR, RNA was extracted from 1 ml blood using the Roche High Pure Viral Nucleic Acid Large Volume Kit (Roche). Reverse transcription (RT) and qualitative nested RT-PCR were performed as previously described except that Superscript III (Invitrogen) and a primer amount of 2.5 pmol were used in the RT reaction. The RT-PCR sensitivity was two copies per ml as determined by testing serial dilutions of serum with known HCV titer (as assessed by COBAS Ampliprep–COBAS Taqman HCV test) in preinfection serum samples from the same chimpanzees.

Statistical analyses. Nonparametric Mann-Whitney U-test and repeated-measures ANOVA were performed with GraphPad Prism Version 5.0 (GraphPad Software) and JMP (SAS). A P value < 0.05 was considered significant.

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