Loss of immune escape mutations during persistent HCV infection in pregnancy enhances replication of vertically transmitted viruses

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Globally, about 1% of pregnant women are persistently infected with the hepatitis C virus (HCV)1. Mother-to-child transmission of HCV occurs in 3–5% of pregnancies2 and accounts for most new childhood infections1,3. HCV-specific CD8+ cytotoxic T lymphocytes (CTLs) are vital in the clearance of acute HCV infections4–6, but in the 60–80% of infections that persist, these cells become functionally exhausted or select for mutant viruses that escape T cell recognition7–9. Increased HCV replication during pregnancy10,11 suggests that maternofetal immune tolerance mechanisms12 may further impair HCV-specific CTLs, limiting their selective pressure on persistent viruses. To assess this possibility, we characterized circulating viral quasispecies during and after consecutive pregnancies in two women. This revealed a loss of some escape mutations in HLA class I epitopes during pregnancy that was associated with emergence of more fit viruses13. CTL selective pressure was reimposed after childbirth, at which point escape mutations in these epitopes again predominated in the quasispecies and viral load dropped sharply14. Importantly, the viruses transmitted perinatally were those with enhanced fitness due to reversion of escape mutations. Our findings indicate that the immunoregulatory changes of pregnancy reduce CTL selective pressure on HCV class I epitopes, thereby facilitating vertical transmission of viruses with optimized replicative fitness.

The first woman (subject M001) whom we tracked through two pregnancies had a history of chronic genotype 2b HCV infection (additional patient details are available in the Online Methods). Viremia was nearly 1 × 10^6 IU ml^−1 at study enrollment during the third trimester of the initial pregnancy, fell 10,000-fold after delivery and remained low (<1 × 10^4 IU ml^−1) through a 26-month interpregnancy period (Fig. 1a). Viral levels rebounded to 1 × 10^6 IU ml^−1 during the second pregnancy before again dropping 10,000-fold following delivery and becoming undetectable 17 months later. Neither pregnancy resulted in vertical transmission of HCV. Clonal sequencing of circulating viral genomes revealed a total of 13 amino acid substitutions after the first pregnancy and additional substitutions during the second pregnancy and postpartum period (Fig. 1b). A close phylogenetic relationship of the HCV genomes over time was consistent with viral evolution within this subject rather than superinfection (Supplementary Fig. 1).

Large swings in viremia and nonsynonymous evolution of the HCV genome through consecutive pregnancies could be indicators of changing CTL selective pressure against epitopes and perhaps altered viral fitness for replication7,13,15. Of the nonsynonymous mutations detected after the first pregnancy, only R495T was situated in a previously described HLA class I epitope16 (Fig. 1b,c). Expansion of a peripheral blood CTL line specific for the B*5101-restricted epitope spanning amino acids 492–500 (YPPRPCGIV, designated 492/9) confirmed recognition of this epitope in M001 (Fig 1d). The viral R495T substitution at position 4 of the epitope (YPPTPCGIV) conferred escape from the CTL response, but, surprisingly, this R495T immune escape substitution reverted to wild-type sequence during the second pregnancy and was then replaced by a unique CTL escape substitution, R495K, after delivery (Fig. 1b–d). This gain-loss gain pattern of substitution was not seen elsewhere in the viral genome. We tested the three variants observed at position 495 before, during and after the second pregnancy for their impact on replication of the cell culture–adapted genotype 2a virus JFHx617. The R495T substitution did not affect RNA replication when compared with the Arg495 wild-type sequence (Fig. 1e), but it considerably impaired production of infectious virus (Fig. 1f). Position 495 is highly conserved (arginine or lysine) in genotypes 1–6 and appears critical for viral entry on the basis of alanine substitutions in HCV-pseudotyped lentiviruses18,19. The conservative R495K substitution that emerged after the second pregnancy had no effect on infectious virus production (Fig. 1f) but...
enabled escape from the CTL response (Fig. 1d). Clearance of viremia 17 months after the second delivery was associated with emergence of a 492/9 CTL response cross-reactive to this R495K mutant (Fig. 1d).

A second subject (M003) presented with jaundice at week 26 of pregnancy with apparent acute HCV genotype 1a infection. Jaundice of the B*0801 1402/9 and D003 (purple) relative to M003 week −6 sequence. Black arrows and vertical shading highlight substitutions arising within predicted HLA class I epitopes. See Supplementary Figures 2 and 3 for detailed viral sequence alignments of the B*0801 1395/9, B*0801 1402/9 and B*1501 2466/9 epitopes.

Figure 2. Viremia and viral evolution through consecutive episodes of vertical transmission. (a) Course of hepatitis C viremia and ALT levels in mother M003 through consecutive pregnancies and in her infants. (b) Viral amino acid substitutions in subject M003 (black) and infants C003 (brown) and D003 (purple).
or both (data not shown). Two of these substitutions, L1403F and K2471R, transitioned through the same gain-loss-gain pattern of mutation observed for the 492/9 epitope in subject M001, and we therefore evaluated them for their impact on T cell recognition and HCV replication.

The L1403F substitution was located in the overlap of two well-described HLA-B*0801–restricted epitopes in the viral NS3 protein (1395/9 HSKKKDEL and 1402/9 ELAAGKLVAL, the common Leu1403 residue is underlined)\(^{20}\) (Fig. 3a). L1403F allowed more efficient escape from postpartum 1395/9 and 1402/9 CTL lines when antigen was delivered to target cells by mRNA transfection than when antigen was delivered to target cells by peptide transfection. Results were normalized for transfection efficiency.\(^{20}\) The L1403F substitution was located in the overlap of two well-described HLA-B*0801–restricted epitopes in the viral NS3 protein (1395/9 HSKKKDEL and 1402/9 ELAAGKLVAL, the common Leu1403 residue is underlined)\(^{20}\) (Fig. 3a). L1403F allowed more efficient escape from postpartum 1395/9 and 1402/9 CTL lines when antigen was delivered to target cells by mRNA transfection than when antigen was delivered to target cells by peptide transfection. Results were normalized for transfection efficiency.\(^{20}\)

Figure 3 Evolution and relative fitness of M003 NS3 CTL escape variants. (a) Time course of B*0801 1395/9 and 1402/9 escape variant frequencies. (b) IFN-\(\gamma\) production of T cell lines specific for wild-type 1395/9 (left) and 1402/9 (right) epitopes upon incubation with autologous B lymphoblastic cell lines and exogenous peptides at 0.5 \(\mu\)g ml\(^{-1}\) or upon incubation with autologous B lymphoblastic cell lines transfected with wild-type or mutant viral mRNA. Results were normalized for transfection efficiency. (c) Direct IFN-\(\gamma\)-enzyme-linked immunoslot (ELISOT) responses to the B*0801 1395/9 and 1402/9 minimal epitope peptides (cryopreserved PBMCs) and an overlapping NS3 peptide pool (ex vivo). (d,e) In vitro RNA replication (d) and infectious virus production (e) of viral variants in an H77S.3 backbone that was modified with an A1405G substitution to match the ‘wild-type’ M003 1395–1410 sequence (designated H77S.3/wt-NS3). Data in c and e represent means ± s.e.m.

The second reverting substitution in M003, K2471R, was located at position 6 of the HLA-B*1501–restricted epitope 2466/9 (SQRQKVRTF)\(^{24}\) in the NS5B protein and was accompanied by substitutions at position 2 within the same epitope (Fig. 4a and Supplementary Fig. 3). The predominant 2466/9 variant to emerge after the first pregnancy (and 12 weeks after the second pregnancy) was the double mutant Q2467K/K2471R (SKRQKVRTF). This variant demonstrated complete escape from an M003-derived 2466/9 specific CTL line (Fig. 4b). Late in the second pregnancy, the K2471R substitution at position 6 reverted to wild type, whereas position 2 switched to a common Q2467L polymorphism found in 16% of curated genotypes\(^{25}\). Reversion of K2471R in the context of Q2467L (SLRQKKVTF) substantially restored recognition by the 2466/9 CTL line, although not to the degree of the original wild-type epitope (Fig. 4b). The 2466/9 epitope was in close proximity to another well-described HLA-B*1501–restricted epitope, designated 2450/9, that acquired a stable escape mutation in the 2450/9 epitope. These data indicate first that in culture–adapted virus (genotype 1a) had little impact on viral RNA replication. Infectious virus yields were only 20% those of wild-type virus, however (Fig. 3d,e), suggesting that L1403F may interfere with NS3/4A function during virus assembly\(^{23}\). The later K1398R plus A1409T substitutions similarly reduced infectious virus production but also appeared somewhat detrimental for RNA replication (Fig. 3d,e), perhaps because Lys1398 is located within the helicase RNA-binding motif IV\(^{13}\).

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pregnancy the quasispecies at 2466/9 shifted from an effective CTL escape sequence to one optimized for viral replication. Second, the equivalent replication of virus with His2453 (wild-type) as compared to that with Tyr2453 (2450/9 escape mutant) (Fig. 4c) is consistent with the notion that the CTL escape mutations that were stable through pregnancy were less detrimental to viral replication than reverting substitutions or were balanced by compensatory mutations. Nevertheless, we cannot exclude the possibility that enhanced virus replication during pregnancy due to loss of some escape mutations is at least partially offset by the other more stable substitutions in the HCV genome. Until now, very few HCV strains have been adapted for replication in cell culture. Assessing the collective impact of all mutations present during and after pregnancy in a native viral backbone must await development of a more robust cell culture model that facilitates replication of primary HCV strains such as those isolated from subjects M001 and M003.

Both infants (C003 and D003) born to M003 were negative for HCV RNA at birth but positive for HCV RNA upon subsequent testing at 19 and 12 weeks of age, respectively, indicating perinatal or late intrauterine viral transmission. Viruses sequenced from C003 at week 25 and D003 at week 12 closely approximated those present in maternal plasma at delivery (Fig. 2b). Viral sequences from D003 contained each of the class I escape mutations that were stable in M003, but not the NS5 or the NS5B mutations that had transiently reverted to wild type during the second pregnancy (Supplementary Figs. 2 and 3). C003 inherited the maternal HLA-B*0801 class I allele and D003 inherited the HLA-B*1501 allele, but in both children the 1395/9, 1402/8 and 2466/9 epitopes remained unchanged through more than a year of follow-up (data not shown). Transmission of more fit wild-type or revertant viruses could conceivably favor a persistent course of infection in infants, particularly if they lack the transiently restricted maternal class I allele or if they fail to exert effective and timely CTL pressure on targetable HCV epitopes, as suggested by the absence of evolution of the B*0801 epitopes in C003 and the B*1501 epitope in D003. Additional studies that address the kinetics and effectiveness of acute-phase CTL responses of perinatally infected infants and consider inheritance patterns of maternal and paternal class I alleles are needed to understand the role of infant CTL responses in HCV evolution and protection from persistence.

The phenomenon of ‘reversion’ of unfit CTL escape mutations has been previously described after viral transmission to recipients lacking the restricting HLA allele and has been inferred from HLA–virus sequence polymorphism studies. Observed reversion of three escape mutations in four epitopes targeted by subjects M001 and M003 provides, to the best of our knowledge, the first examples of this phenomenon within individuals with established chronic hepatitis C. As a surrogate readout of in vivo intrahepatic HCV-specific CTL activity, this evolution of viral class I epitopes suggests first, that intrahepatic CTLs exert a degree of ongoing selective pressure on epitopes before pregnancy and, second, that these CTLs may be inhibited by the immunoregulatory changes of pregnancy. Collectively, our findings suggest that the increase in viremia often observed during pregnancy is related to relaxed HCV-specific CTL immunity, coupled perhaps with increased replicative fitness of the virus. Increases in replication during pregnancy are important, as some studies have linked high maternal viral loads, particularly >1 × 10^7 IU ml^-1 as seen in subject M003, with an increased risk of vertical transmission. It is noteworthy that numerous known immunoregulatory changes of pregnancy parallel those found in chronic HCV infection and could conceivably exacerbate HCV-specific CTL dysfunction in pregnancy, including expansion of T regulatory cells and enhanced expression of immunosuppressive molecules such as negative co-stimulatory ligands and receptors, HLA-G and indoleamine 2,3-dioxygenase.

Postpartum reacquisition of escape mutations coincident with detectable IFN-γ responses in the peripheral blood in M003 indicates that CTLs targeting these epitopes regain efficacy and probably contribute to the coincident declines in viremia that often follow delivery. Although function may be restored to CTLs targeting revertant epitopes in the postpartum period, it remains unknown whether the same is true of T cells that target persistently intact epitopes. Outside of pregnancy, chronic-phase CTLs targeting intact epitopes are phenotypically distinct from and may be more profoundly exhausted than those targeting escaped epitopes. Study of this highly unusual postpartum revival of HCV-specific cellular immunity could provide unique insight into mechanisms of T cell silencing and interventions to restore function in the chronic phase of infection. Such mechanisms are likely to be relevant to other persistent viruses, such as HIV and, particularly, hepatitis B virus, where viral load dynamics during and after pregnancy parallel those of HCV. Finally, our data indicate that relaxation of cellular immunity and emergence of viruses that lack key escape mutations in the pregnant mother may result in vertical transmission of HCV variants with enhanced fitness for replication. Together, these findings highlight the fine balance between replicative...
fitness and evasion of host immunity that shapes HCV quasispecies, and they suggest that a systemic reduction of cellular immunity during pregnancy provides a unique niche for reemergence of fit virus variants with consequences for both mother and child.

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

Accession numbers: Viral genomic sequence data from subjects M001, M003, C003 and D003 are available in GenBank via accession codes JQ061323 through JQ663389.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

ACKNOWLEDGMENTS
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ONLINE METHODS

Subjects. HCV-infected subjects M001 and M003 were recruited from The Ohio State University Substance Treatment, Education and Prevention in Pregnancy (STEPPE) program, a clinic that provides prenatal care and addiction treatment services for pregnant women with substance abuse histories. Follow-up visits for mothers and their infants were accomplished at Nationwide Children’s Hospital. Approval for this study was provided by institutional review boards at the Ohio State University and Nationwide Children’s Hospital, and informed consent was obtained for all subjects. Subject M001 was enrolled at the age of 26 years in the thirty-fifth gestational week of pregnancy. She had HCV genotype 2b viremia and a reported history of persistent HCV infection for more than 5 years. Both of her pregnancies were delivered at term by cesarean section due to prior cesarean section. Subject M003 presented at the age of 34 years in the twenty-sixth gestational week of pregnancy with jaundice, elevated alanine aminotransferase (ALT) levels and HCV genotype 1a viremia. Her symptoms were attributed to acute HCV infection on the basis of a reported needle stick in the first trimester of pregnancy, a negative HCV serologic test 18 months before she presented and exclusion of other infectious and non-infectious causes of acute hepatitis. She received two doses of betamethasone at 31 weeks’ gestation to promote fetal lung maturity and delivered infant C003 vaginally at 34 weeks’ gestation. No corticosteroids were given during her second pregnancy, and she delivered infant D003 vaginally at 38 weeks’ gestation.

Viral sequence analysis. Clonal sequencing of the HCV open reading frame was performed on EDTA plasma samples collected from M001 and M003 through two consecutive pregnancies and postpartum periods. Viral RNA was purified with the QIAamp Viral RNA Mini Kit (Qiagen), and cDNA was synthesized with Transcripter Reverse Transcriptase (Roche). M001 (genotype 2b, low viral loads) viral genomes were amplified in seven overlapping fragments spanning nucleotides 1–8577 using Phusion Hot Start DNA polymerase (New England BioLabs). M003 (genotype 1a) viral genomes were amplified in five overlapping fragments spanning nucleotides 22–9018 of the open reading frame using the Expand Long Template PCR System (Roche). Fragments that did not amplify with standard primer pairs were subdivided into smaller fragments for amplification. Second-round PCR was performed with nested, seminested or identical primer pairs. To confirm that the M003 L1403F escape mutation had indeed reverted to wild type during the second pregnancy, the NS3 sequence was reamplified with alternative primer pairs using a cDNA template derived from an independent RNA extraction. Specific primer pairs and sequencing conditions are available upon request. Second-round PCR products were cloned into TOPO XL vector (Invitrogen) or Zero Blunt TOPO vector (Invitrogen). All plasmid sequences were sequenced by the Laboratory for Genomics & Bioinformatics at the University of Oklahoma Health Sciences Center.

Phylogenetic analysis. Neighbor-joining41 trees of near full-length viral protein sequences from M001 were assembled with archived genotype 2a and 2b viral protein sequences from the Los Alamos database, whereas those of M003, C003 and D003 were assembled with archived genotype 1a sequences. Amino acid sequences were aligned with MUSCLE32, and evolutionary distances were calculated with the p-distance model43. Bootstrap consensus trees were inferred from 1,000 replicates, and branches reproduced in at least 80% of bootstrap replicates are displayed. Evolutionary analyses were completed using MEGA version 5 (ref. 44).

Identification of potential class I escape mutations. To identify the CTL escape mutations that reverted to wild type during the second pregnancy, we first attempted to identify substitutions arising in predicted class I epitopes after the first pregnancy (see Supplementary Table 1 for maternal class I haplotypes). Epitopes for both subjects included predicted and experimentally confirmed epitopes listed in Los Alamos42 (http://hcv.lanl.gov) and IEDB43 (http://www.iedb.org) databases. IEDB epitope accession numbers included 75376 for M001 and 59020, 24221, 24762, 12926, 144403, 144480 and 68694 for M003. For the genotype 1a virus, we also included epitopes or adaptation sites predicted from HLA-associated sequence polymorphism studies40,46. Where the maternal viral sequence in the first pregnancy did not match the epitope sequence found in the database, the epitope was excluded if the half-maximal inhibitory concentration of the maternal sequence matrix method (ref. 47) exceeded 5,000 nM. With this approach, eight substitutions arising after the first pregnancy in M003 fell within predicted epitopes, whereas only the R495T substitution fell within a predicted class I epitope for M001.

Generation of HCV-specific CD8+ T cell lines from peripheral blood. T cell lines specific for class I epitopes 492/9 (YPPRPCCGG) in subject M001 and 1395/9 (HSKKKCCDEL) and 1402/9 (ELAGKLVAL) in subject M003 were derived by antigen stimulation of cryopreserved postpartum peripheral blood mononuclear cell (PBMC) samples. Briefly, 2 × 10^6–6 × 10^7 thawed PBMCs were resuspended in 2 ml RP10–IL2 medium (RP10 1640, 10% FCS and penicillin/streptomycin) with recombinant interleukin-2 (IL-2, 50 U ml⁻¹) in a 24-well plate and stimulated with the respective peptide at a final concentration of 10 μg ml⁻¹. Fresh RP10–IL-2 medium was added every 3–4 d. After 11–16 d, CD4+ cells were depleted (Dynabeads; Invitrogen), and remaining cells were stimulated with mouse anti-human CD3 antibody (Beckman Coulter, clone X3, final concentration 0.015 μg ml⁻¹) and irradiated heterologous feeder PBMCs. After 3–4 weeks in culture, the epitope specificity of each cell line was determined by IFN-γ intracellular cytokine staining (ICS) or ELISPOT assay. In some cases, CTL lines were subcloned to derive lines with greater epitope specificity. Class I HLA restriction was confirmed using peptide-pulsed partially HLA-matched heterologous B lymphoblastic cell lines (BLCLs) in the IFN-γ ICS assay.

IFN-γ ELISPOT assay. The affinity of CTL lines for wild-type versus mutant epitopes was tested by a titration of the respective peptides in the IFN-γ ELISPOT assay. IFN-γ-producing CD8+ T cells were enumerated with the IFN-γ ELISPOT (U-CyTech) after a 42-h stimulation with peptide (wild-type or variant) and autologous irradiated BLCLs in duplicate as previously described15. Direct IFN-γ ELISPOT assays of fresh or frozen PBMCs using peptide pools or individual peptides were performed without additional antigen-presenting cells.

Intracellular cytokine stain assay. CD8+ T cell lines were stimulated with either peptide-pulsed or HCV mRNA-transfected autologous BLCLs with mouse anti-human CD28 (BD Pharmingen, clone CD82.2, final concentration 1 μg ml⁻¹) and mouse anti-human CD49d (BD Pharmingen, clone 9F10, final concentration 1 μg ml⁻¹) co-stimulation as previously described15. GolgiPlug (BD Biosciences) was added after 1 h of incubation. After 16 h, cells were stained for CD8, CD4, CD3, intracellular IFN-γ and vitality and were analyzed on a Becton Dickinson LSRII flow cytometer as previously described15. Antibodies used were as follows: mouse anti-human CD3 (peridinin chlorophyll protein conjugated; BioLegend, clone UCHT1, final concentration 4 μg ml⁻¹), mouse anti-human CD4 (Pacific Blue conjugated; BioLegend, clone OKT4, final concentration 1.25 μg ml⁻¹), mouse anti-human CD8 (allophycocyanin conjugated; BioLegend, clone RPA-T8, final concentration 1 μg ml⁻¹) mouse anti-human IFN-γ (phycoerythrin conjugated; BD Pharmingen, clone 45.B3, final concentration 1 μg ml⁻¹) and Live/Dead Green (Molecular Probes, 1:1,000).

HCV mRNA transfection of BLCLs. Peptides bearing the initial L1403F substitution did not escape recognition by M001 1395/9 or 1402/9 T cell lines as efficiently as peptides bearing the separate K1398R/A1409T substitution. With this approach, eight substitutions arising after the first pregnancy in M003 fell within predicted epitopes, whereas only the R495T substitution fell within a predicted class I epitope for M001.
a positive control wild-type A*3101–restricted NS5B epitope (VGIYLLPNR), and M003 nucleotides 4458–4441 (5’-TCATCGGTGGGGAGGAATTGAGTTAGTGGTCGGACGGATGTTAGTGGTCGGAGACGC-3’). PCR product sequences were confirmed and used to generate mRNA with a poly(A) tail using the mMESSAGE mMACHINE T7 Ultra Kit (Ambion). mRNA was cleaned with the RNaseasy Micro Kit (Qiagen). 1 × 10⁶ autologous BLCs were electroporated with 14 µg viral mRNA as described51 and incubated in RPMI medium for 24 h before being mixed with CD8⁺ T cells at an 8:1 E:T ratio in an IFN-γ ICS assay as described above.

**Plasmids.** To assess the relative fitness of the E2 mutants in mother M001 (genotype 2b) and NS3 and NS5B escape mutants found in mother M003 (genotype 1a), we used the genotype 2a JFHxJ6 (Cp7) chimera17,49, genotype 1a H77S.3 (ref. 22) and genotype 1a H77S.3/GLuc2A22 infectious virus systems. Mutant plasmids were constructed in the JFHxJ6 and H77S.3 backgrounds by QuikChange and QuikChange Lightning Multi site-directed mutagenesis kits (Stratagene). For M001, the unmodified J6xJFH plasmid contained an arginine at position 495 (position 492 in H77 numbering system) that matched the week −4 ‘wild-type’ M001 sequence and was used to construct the R495T and R495K variants. For M003 NS3, the pH77S.3 was first modified with an alanine to glycine substitution at position 1405 in order to match the M003 week −6 ‘wild-type’ 1395–1410 sequence. This modified backbone, designated H77S.3/wt-NS3, was then used for generation of the M003 escape mutants. For M003 NS5B, mutants were generated on an altered pH77S.3/GLuc2A backbone that contained a cysteine to serine substitution at 2466 to match the M003 wild-type 2450–2474 sequence (H77S.3/wt-NS5B). All mutations were verified by DNA sequencing. Negative controls included replication-defective NS5B RNA-polymerase mutants GND49 and H77S.3/AAG22 for the RT-PCR RNA-replication assays and a JFHxJ6 plasmid lacking envelope glycoproteins (ΔE1E2) for testing the infectivity of the M001 E2 mutants.

**Cells.** Huh-7.5 cells50 were used for all fitness assays and maintained in DMEM high-glucose medium containing 10% FBS and 1× penicillin/streptomycin at 37 °C in a 5% CO₂ environment.

**RNA transcription and transfection.** Plasmid DNA was linearized by XbaI restriction digestion before the transcription reaction. RNA was then synthesized from the linearized DNA using a MEGAscript kit (Ambion). The transcribed RNA was confirmed by spectrophotometry and electrophoresis. RNA was transfected by electroporation as previously described51.

**RNA replication.** HCV-RNA per µg of total RNA was quantified every 24 h by quantitative reverse-transcription PCR as described51 for the M001 E2 and M003 NS3 experiments. For M003 NS5B, culture supernatant from the H77S.3/GLuc2A RNA-transfected cells was collected every 24 h and replaced with fresh medium as described22. Daily secreted Gaussia luciferase activity (minus background), an indicator of RNA replication within the cells, was plotted as a fold change compared to that of 6 h after transfection. Averages and standard errors were calculated from at least two independent electroporations.

**Virus titration.** Culture supernatant collected from cells at day 3 after RNA transfection was inoculated onto naïve Huh-7.5 cells, and the cells were fixed and stained 3 d later for HCV NS5A protein for determination of the 50% tissue culture infective dose (JFHxJ6) or stained for HCV core protein to quantify the number of fluorescent focus-forming units of virus (H77S.3) as described23,51. Means and standard errors were calculated from at least duplicate assays.

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Corrigendum: Loss of immune escape mutations during persistent HCV infection in pregnancy enhances replication of vertically transmitted viruses

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In the version of this article initially published, the K1398R/A1409T variant of the 1395–1410 amino acid sequence was erroneously depicted with an arginine in position 1397 in Figure 3a. The correct depiction of the K1398R/A1409T variant should have arginine in position 1398. The error has been corrected in the HTML and PDF versions of the article.