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

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Abstract

Globally, about 1% of pregnant women are persistently infected with the hepatitis C virus (HCV)[1]. Vertical transmission occurs in 3–5% of cases[2] and accounts for most new childhood HCV infections[1,3]. HCV-specific $CD8^+$ cytotoxic T-lymphocytes (CTLs) play a vital role in the clearance of acute infections[4–6], but in the 60–80% of infections that persist these cells become functionally exhausted or select mutant viruses that escape T-cell recognition[7–9]. Increased HCV replication during pregnancy[10,11] suggests that maternofetal immune tolerance mechanisms[12] may further impair HCV-specific CTLs, limiting their selection pressure on persistent viruses. To assess this possibility, we characterized the circulating viral quasispecies during and after

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consecutive pregnancies. This revealed a loss of some escape mutations in class I epitopes in pregnancy associated with emergence of more fit viruses[13]. CTL selection pressure was reimposed after childbirth, when escape mutations in these epitopes again predominated in the quasispecies and viral load dropped sharply[14]. Importantly, viruses transmitted perinatally were those with enhanced fitness due to reversion of escape mutations. Our findings indicate that immunoregulatory changes of pregnancy reduce CTL selection pressure on HCV class I epitopes, thereby facilitating vertical transmission of viruses with optimized replicative fitness.

We first studied a woman (subject M001) with a chronic genotype 2b HCV infection (additional patient details are available in online Methods). Viremia was nearly 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 (less than 10^4 IU ml^{-1}) through a 26 month inter-pregnancy period (Fig. 1a). Viral levels rebounded to 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. 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 non-synonymous evolution of the HCV genome through consecutive pregnancies could be indicative of changes in CTL selection pressure against epitopes, and perhaps viral fitness for replication[7,13,15]. An arginine to threonine substitution at position 495 (R495T) that arose after the first pregnancy was located in a known HLA B*5101 restricted epitope[16] within the envelope glycoprotein E2 (Fig. 1b, c). A CTL line expanded from the blood of this patient recognized the wild-type 492/9 (YPPRPCGIV) epitope but not the postpartum R495T variant (YPPTPCGIV) (Fig. 1d, left panel). Surprisingly, the 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). The three variants observed at position 495 before, during, and after the second pregnancy were tested for their impact on replication of the cell culture-adapted genotype 2a virus (JFHxJ6)[17]. The R495T substitution did not affect RNA replication when compared with the R495 wild-type sequence (Fig. 1e), but it considerably impaired production of infectious virus (Fig. 1f). Position 495 (492 in the reference H77 strain[18]) is highly conserved (arginine or lysine) in genotypes 1–6 and appears critical for viral entry based on alanine substitutions in HCV pseudotyped lentiviruses[19]. The conservative R495K substitution that emerged after the second pregnancy had no effect on infectious virus production (Fig. 1f) but provided for escape from the CTL response (Fig. 1d, left panel). 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, center and right panels).

A second subject (M003) presented with jaundice at week 26 of pregnancy with apparent acute genotype 1a HCV infection. Jaundice and alanine aminotransferase (ALT) elevations resolved in the final weeks of the first pregnancy, while viremia climbed to greater than 10^7

IU ml^{-1} (Fig. 2a). A transient 1,000-fold decline in viremia and spikes in ALT followed the first delivery. Viremia remained elevated through the second pregnancy and again transiently fell 1,000-fold after delivery. The viral quasispecies was stable during the last 6 weeks of the first pregnancy, but rapidly evolved after delivery of the first child (Fig. 2b). Of 13 amino acid substitutions observed at week 25 post-partum, eight occurred within described or predicted HLA class I epitopes and diminished T-cell recognition by postpartum PBMCs and/or CTL lines (data not shown). Two of these substitutions, leucine to phenylalanine at position 1403 (L1403F) and lysine to arginine at position 2471 (K2471R), transitioned through the same "gain, loss, gain" pattern of mutation observed for the 492/9 epitope in subject M001 and were therefore evaluated 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 NS3 protein (1395/9 HSKKKCDEL and 1402/9 ELAAGKLVAL, the common L1403 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 supplied as exogenous peptide, indicating that L1403F likely disrupted antigen processing[20] (Fig. 3b). L1403F was dominant in the viral quasispecies from 25 weeks after the first delivery until the start of the second pregnancy (Fig. 3a). Nevertheless, a transition to wild-type sequence began by the second trimester, reaching more than 95% of sequenced clones by the third trimester. Interestingly, the wild-type L1403 that arose midway through the second pregnancy was encoded by a novel codon, TTG, rather than the conventional CTC codon found at other time points, suggesting de novo selection of the wild-type leucine variant in pregnancy (Supplementary Fig. 2). Later synonymous replacement of the TTG codon by CTC suggests selective codon usage bias, possibly owing to constraints on secondary RNA structure or to the abundance of complementary wAG tRNA anti-codon in the human liver[21]. Twelve weeks after delivery, the L1403F substitution began to reappear in association with detectable 1395/9 and 1402/9 IFN-γ T-cell responses in the blood (Fig. 3c), but was soon replaced by unique escape substitutions (K1398R and A1409T) within the separate HLA B*0801 epitopes that eliminated peptide recognition (Fig. 3a, b). Incorporation of L1403F in the genotype 1a H77S.3[22] cell culture-adapted virus had little impact on viral RNA replication. Infectious virus yields were only 20% relative to wild-type however (Fig. 3d, e), suggesting that L1403F may interfere with NS3/4A function during virus assembly[23]. The later K1398R/A1409T substitutions similarly reduced infectious virus production but also appeared somewhat detrimental for RNA replication (Fig 3d, e), perhaps because K1398 is located within the helicase RNA-binding motif IV[13].

The second reverting substitution in M003, K2471R, was located at position 6 of the HLA-B*1501 restricted epitope 2466/9 (SQRQKKVTF)[24] in the NS5B protein and 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 (SKRQKRVTF). This variant demonstrated complete escape from an M003 2466/9 CTL line (Fig 4b, right panel). Late in the second pregnancy the K2471R substitution at position 6 reverted to wild-type while position 2 switched to a common Q2467L polymorphism found in 16% of curated genotype

1a viruses[25]. Reversion of K2471R in 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, right panel). The 2466/9 epitope was in close proximity to another HLA-B*1501 restricted epitope designated 2450/9 that acquired a stable T-cell escape substitution at position 3 (H2453Y) after the first delivery (Fig. 4a and 4b, left panel). Incorporation of the postpartum escape mutations from both B*1501 epitopes (H2453Y / Q2467K / K2471R in Fig. 4c) into the H77S.3/GLuc2A system impaired viral replication as determined by secreted luciferase activity[22]. The delivery sequence (H2453Y / Q2467L in Fig. 4c) replicated as well as the original wild-type sequence despite the residual H2453Y escape mutation in the 2450/9 epitope. These data indicate first that in 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 H2453 (wild-type) vs. Y2453 (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 well-balanced by compensatory mutations[26]. Nevertheless, it is important to note that few HCV strains have been adapted to replicate in cell culture. Because HCV replication and the capacity for mutation in cell culture is so finely balanced, we did not examine the simultaneous impact of all substitutions on virus production. 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.

Both infants (C003 and D003) born to M003 were HCV-RNA negative at birth, but HCV-RNA positive upon subsequent testing at 19 and 12 weeks respectively, indicating perinatal or late intrauterine viral transmission[27] (Fig. 2a). 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 NS3 or the NS5B mutations that had transiently reverted to wild-type during the second pregnancy (Supplementary Figs. 2 and 3). C003 inherited the maternal B*0801 class I allele and D003 inherited the B*1501 allele, but in both children the 1395/9, 1402/8 and 2466/9 epitopes remained intact through more than a year of follow-up (data not shown). Transmission of more fit wild-type/revertant viruses could conceivably favor a persistent course of infection in infants, particularly if they lack the restricting maternal class I allele[28] 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 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[24,29,30]. 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 indicates, first, that intrahepatic CTLs exert a degree of ongoing selection pressure on epitopes prior to pregnancy and, second, that these CTLs may be silenced by the immunoregulatory changes of pregnancy. Collectively, our findings strongly suggest that the increase in viremia often observed during pregnancy[10,11] 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 > 10^7 IU ml^{-1} as seen in subject M003, with an increased risk of vertical transmission[2,31]. 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[32] and enhanced expression of immune-suppressive molecules including negative co-stimulatory ligands and receptors[33,34], HLA-G [35,36] and indoleamine 2,3-dioxygenase (IDO)[37,38].

Postpartum reacquisition of escape mutations coincident with detectable IFN-γ responses in the peripheral blood in M003 indicates that CTLs targeting these epitopes regain function and likely contribute to the coincident declines in viremia that often follow delivery[14]. While 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[39] and may be more profoundly exhausted than those targeting escaped epitopes[8]. 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 human immunodeficiency virus and particularly hepatitis B virus, where viral load dynamics during and after pregnancy parallel those of HCV[40]. Finally, and perhaps most importantly, 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 sharply enhanced fitness for replication. Together these findings highlight the fine balance between replicative fitness and evasion of host immunity that shapes HCV quasispecies, and suggest that a systemic reduction of cellular immunity during pregnancy provides a unique niche for re-emergence of fit virus variants with consequences for both mother and child.

Methods

Subjects

HCV-infected subjects M001 and M003 were recruited from The Ohio State University Substance Treatment, Education, and Prevention in Pregnancy (STEPP) 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 35th 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 26th 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 prior, 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 Transcriptor Reverse Transcriptase (Roche). M001 (genotype 2b, low viral loads) viral genomes were amplified in seven overlapping fragments spanning nucleotides 1 to 8577 using Phusion Hot Start DNA polymerase (New England BioLabs). M003 (genotype 1a) viral genomes were amplified in five overlapping fragments spanning nucleotides 22 to 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, semi-nested, 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 re-amplified with alternative primer pairs using 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 products were sequenced by the Laboratory for Genomics & Bioinformatics at the University of Oklahoma Health Sciences Center.

Phylogenetic analysis

Neighbor-Joining[41] 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 while those of M003, C003, and D003 were assembled with archived genotype 1a sequences. Amino acid sequences were aligned with MUSCLE[42], and evolutionary distances were calculated with the p-distance model[43]. Bootstrap consensus trees were inferred from 1000 replicates, and branches reproduced in at least 80% of bootstrap replicates are displayed. Evolutionary analyses were completed using MEGA v5[44].

Identification of potential class I escape mutations

To identify the CTL escape mutations that revert 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). Predicted epitopes for both subjects included experimentally confirmed epitopes listed in Los Alamos[25] (http://

hcv.lanl.gov) and IEDB[45] (http://www.immuneepitope.org) databases. For the genotype 1a virus we also included epitopes or adaption sites predicted from HLA-associated sequence polymorphism studies[30,46]. 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 predicted smm IC_{50} binding score[47] of the maternal sequence exceeded 5,000 nM. With this approach eight substitutions arising after the first pregnancy in M003 fell within predicted epitopes while 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 (YPPRPCGIV) in subject M001 and 1395/9 (HSKKKCDEL) and 1402/9 (ELAGKLVAL) in subject M003 were derived by antigen stimulation of cryopreserved postpartum peripheral blood mononuclear cell (PBMC) samples. Briefly, $2–6\times10^6$ thawed PBMCs were re-suspended in 2 ml RP10-IL2 media (RPMI 1640, 10% fetal calf serum (FCS), and penicillin/streptomycin, with recombinant IL2 (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-IL2 media was added every 3–4 days. After 11–16 days, $CD4^+$ cells were depleted (Dynabeads; Invitrogen) and remaining cells were stimulated with anti-CD3 antibodies and irradiated heterologous feeder PBMCs. After 3–4 weeks in culture, the epitope specificity of each cell line was determined by IFN-γ 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 hour stimulation with peptide (wild-type or variant) and autologous irradiated BLCLs in duplicate as previously described[15]. 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 anti-CD28 and anti-CD49d co-stimulation as previously described[15]. GolgiPlug (BD Biosciences) was added after 1 hour of incubation. After 16 hours cells were stained for CD8, CD4, CD3, intracellular IFN-γ and vitality and were analyzed on a Becton Dickinson LSRII flow cytometer as previously described[15].

HCV mRNA transfection of BLCLs

Peptides bearing the initial L1403F substitution did not escape recognition by M003 1395/9 or 1402/9 T-cell lines as efficiently as peptides bearing the separate K1398R and A1409T substitutions when tested by IFN-γ ICS (Fig. 3b), leading us to hypothesize that the L1403F mutation might escape the M003 CTL responses more by disrupting intracellular epitope processing than by impairing T-cell receptor recognition[20,48]. To test the effect of

intracellular antigen processing we adapted the method described by Timm et al[20] to transfect HCV mRNA variants bearing these epitopes into autologous antigen presenting cells. Briefly, representative NS3 viral sequence clones from week –6 (wild-type), week 40 (L1403F), and week 152 (K1398R/A1409T) were used as template for a third round PCR that would be used to generate the mRNA. The forward primer included a T7 promoter with the Kozak consensus sequence and M003 nucleotides 3947–3963 (5′-TAATACGACTCACTATAGGGAGAGCCACCATGGACGAGTGCCACTCCACG-3′). The reverse primer included a stop codon, coding sequence for a positive control wild-type A*3101 restricted NS5B epitope (VGIYLLPNR), and M003 nucleotides 4458–4441 (5′-TCATCGGTTGGGGAGGAGGTAGATGCCTACTTGAGTGCGGGAGACAGC-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 RNeasy Micro Kit (Qiagen). 10^6 autologous BLCLs were electroporated with 14 μg viral mRNA as described[15] and incubated in RP10 media for 24 hours before being mixed with $CD8^+$ T-cells at an 8:1 E:T ratio in an IFN-γ ICS assay as described above.

Viral replicative fitness assays

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 utilized the genotype 2a JFHxJ6 (Cp7) chimera[17,49], 1a H77S.3[22], and 1a H77S.3/GLuc2A[22] infectious virus systems. Mutant plasmids were constructed in the JFHxJ6 and H77S.3 backgrounds by QuickChange 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 GND[49] and H77S.3/AAG[22] 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 cells[50] were used for all fitness assays and maintained in DMEM high glucose medium containing 10% fetal bovine serum and 1X penicillin/streptomycin at 37 °C in a 5% CO_2 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 described[51].

RNA replication—HCV-RNA per μg of total RNA was quantified every 24 hours by quantitative reverse transcription PCR as described[51] 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 hours and replaced with fresh medium as described[22]. 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 hours 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 days 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 described[23,51]. Means and standard errors were calculated from at least duplicate assays.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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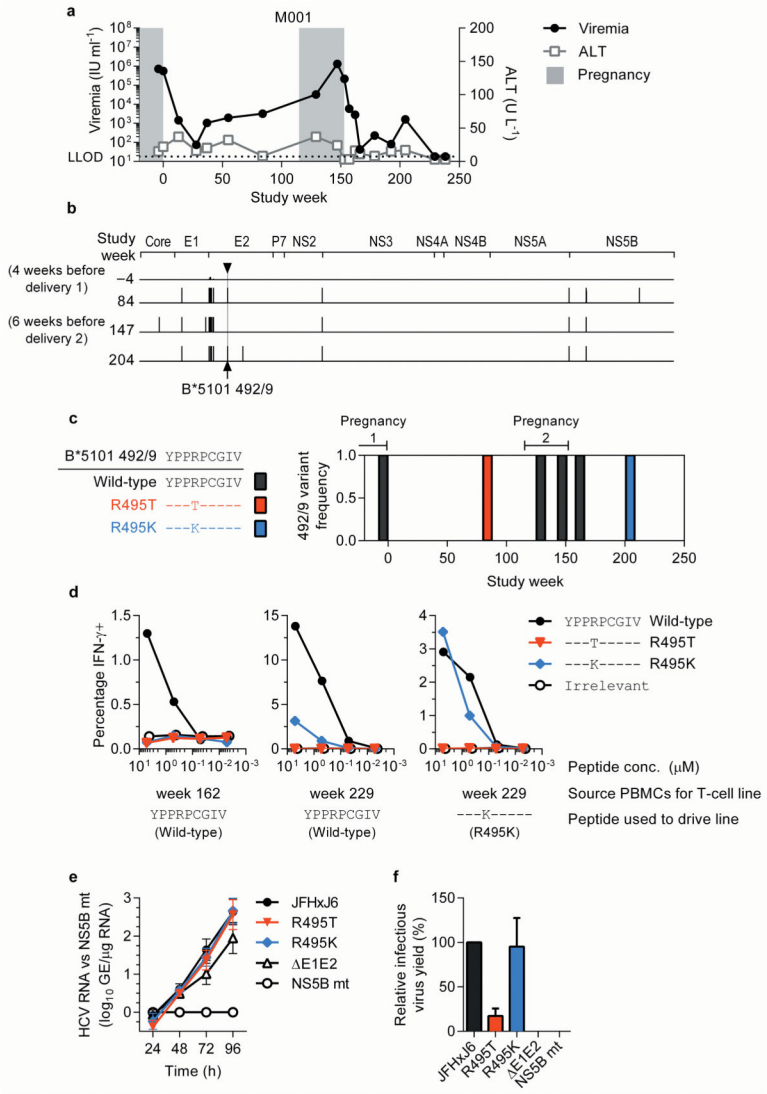

Figure 1.

Evolution and relative fitness of M001 E2 CTL escape variants. (**a**) Course of hepatitis C viremia and alanine aminotransferase (ALT) levels in mother M001 through consecutive pregnancies. (**b**) Viral amino acid substitutions relative to week –4 sequence. Significant "new" amino substitutions (those with initial frequencies of < 20% and subsequent frequency increases of ≥50%) are depicted by vertical lines, with height proportionate to frequency. Substitutions arising within predicted HLA class I epitopes after the first pregnancy are highlighted (black arrows and shading). (**c**) Time course of B*5101 492/9 escape variant frequencies. (**d**) IFN-γ production by CTL lines upon stimulation with titrated concentrations of wild-type and mutant 492/9 peptides. CTL lines were derived from PBMCs collected at early postpartum (left panel) or late postpartum time points (middle and right panels) by antigen specific expansion using the wild-type or R495K 492/9 peptides. (**e**) Viral RNA replication monitored by RT-PCR of cells transfected with wild-type (JFHxJ6) or mutant RNAs, or a ΔE1E2 control RNA lacking the envelope glycoproteins, plotted as

increase over a replication-defective NS5B mutant. (**f**) Infectious virus production. Viral particles produced by the RNA-transfected cells were inoculated onto naïve Huh-7.5 cells for titration of infectious virus. TCID50 was calculated and plotted as percent of JFHxJ6.

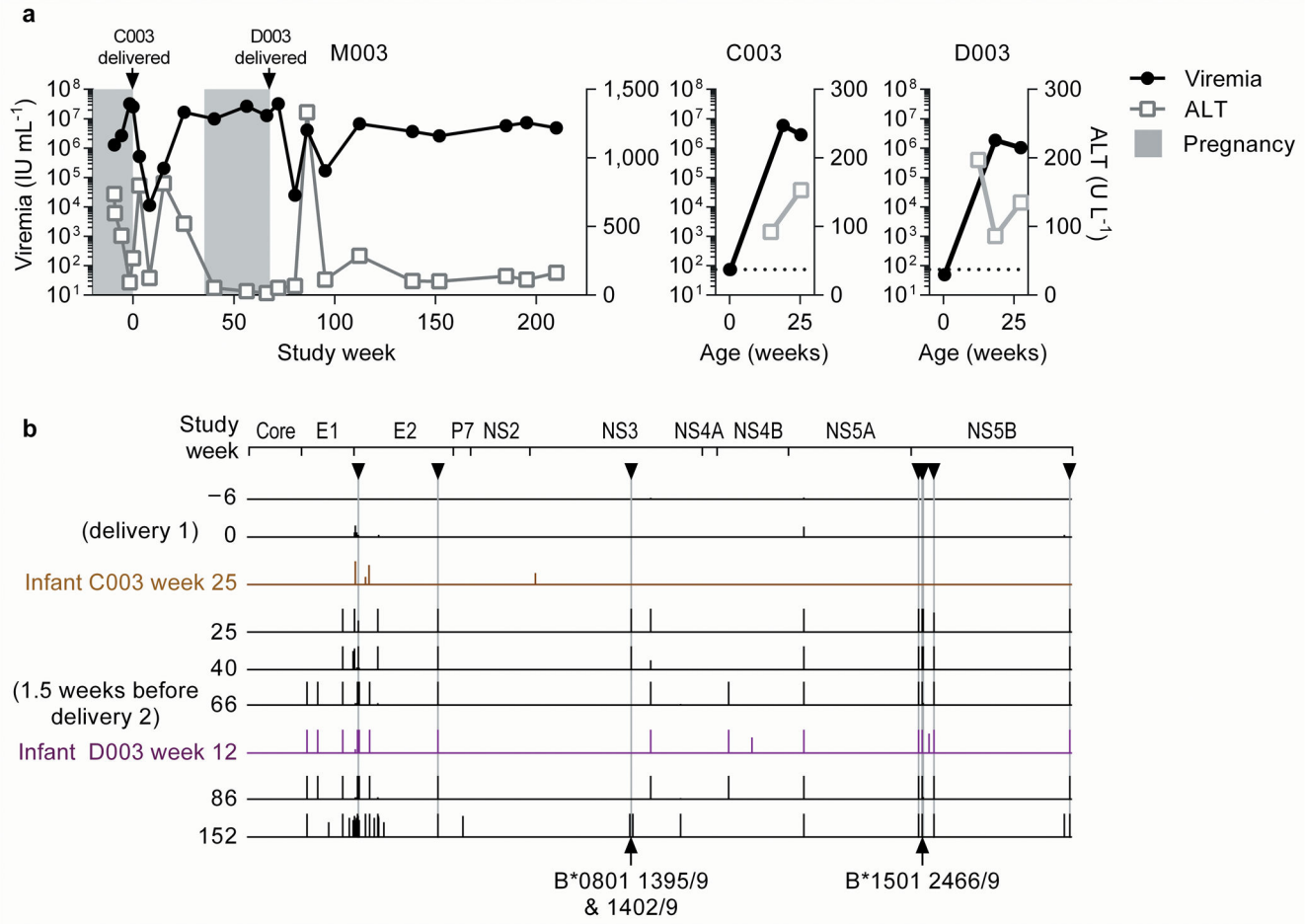

Figure 2.

Viremia and viral evolution through consecutive episodes of vertical transmission. (**a**) Course of hepatitis C viremia and alanine aminotransferase 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) 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.

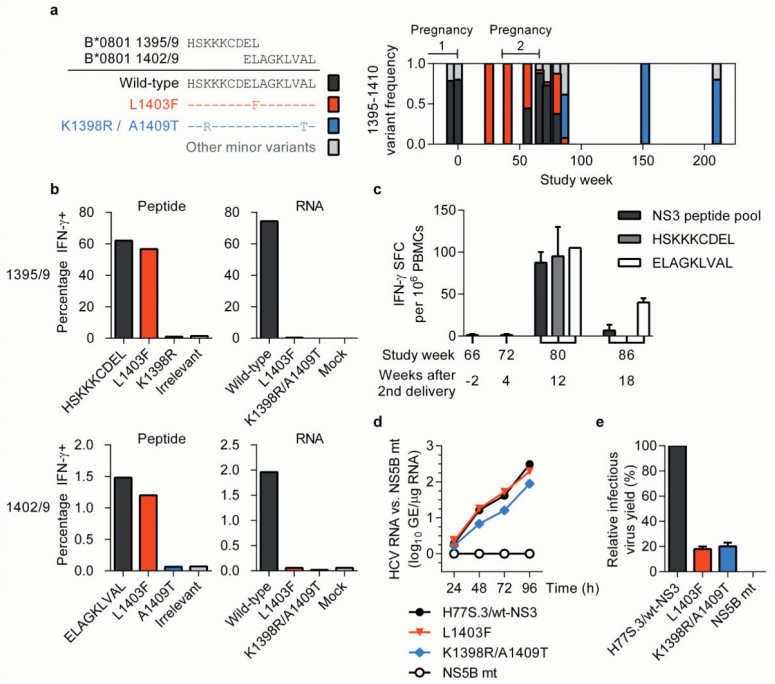

Figure 3.

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-γ production of T-cell lines specific for wild-type 1395/9 (top panels) and 1402/9 (bottom panels) epitopes upon incubation with autologous B-lymphoblastic cell lines and exogenous peptides at 0.5 μg ml^{-1} (left panels) or upon incubation with autologous B-lymphoblastic cell lines transfected with wild-type or mutant viral mRNA (right panels). Results were normalized for transfection efficiency. (**c**) Direct IFN-γ ELISpot responses to the B*0801 1395/9 and 1402/9 minimal epitope peptides and an overlapping NS3 peptide pool. Ex-vivo IFN-γ responses to the NS3 peptide pool were not detected late in the second pregnancy but transiently surged 12 weeks after delivery, concomitant with IFN-γ responses to both minimal B*0801 epitopes using cryopreserved PBMCs. (**d, e**) *In vitro* RNA replication and infectious virus production 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).

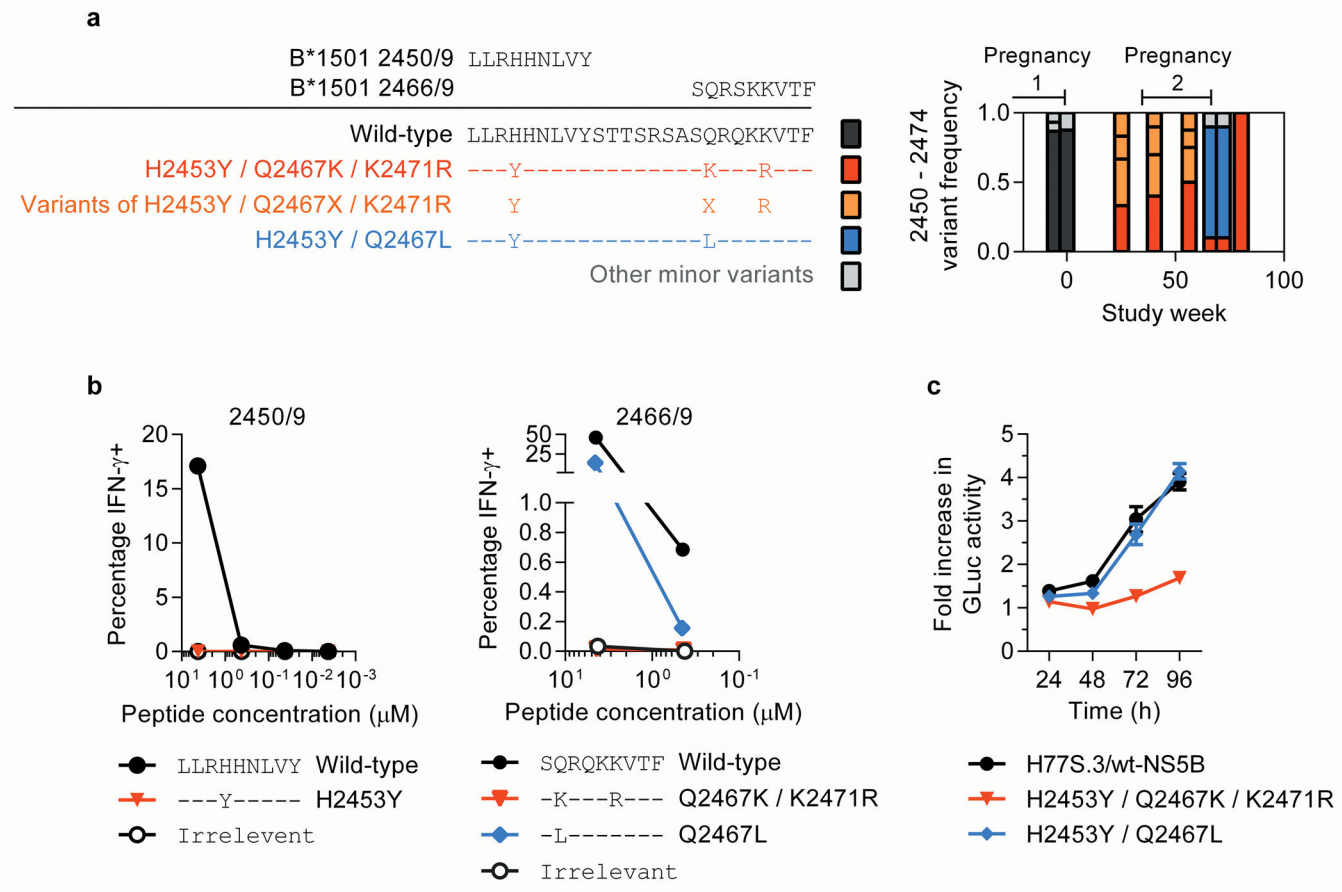

Figure 4.

Evolution and relative fitness of M003 NS5B CTL escape variants. (**a**) Time course of B*1501 2450/9 and 2466/9 escape variant frequencies. (**b**) IFN-γ production by CTL lines specific for wild-type 2450/9 (left panel) and 2466/9 (right panel) upon stimulation with titrated concentrations of wild-type and mutant peptides. (**c**) *In vitro* RNA replication of H77S.3/GLuc2A mutants as indicated by daily secreted Gaussia luciferase activity plotted as fold-change of light units over values found 6 hours post-transfection. Mutants were constructed in a modified H77S.3/GLuc2A backbone that bore a C2466S substitution to match the “wild- type” M003 2450–2474 viral sequence (designated H77S.3/wt-NS5B).